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EFFECTS OF GALACTOOLIGOSACCHARIDE AND *PEDIOCOCCUS ACIDILACTICI* ON ANTIOXIDANT DEFENCE AND DISEASE RESISTANCE OF RAINBOW TROUT, *ONCORHYNCHUS MYKISS*

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Abstract

The present study investigated the effects of dietary prebiotic galactooligosaccharide [(GOS), 1%], probiotic (*Pediococcus acidilactici*) [7.57 log CFU g^{-1}] and synbiotic (GOS + *P. acidilactici*) on antioxidant enzymes activity and disease resistance of rainbow trout (15.04±0.52 g). After 8 weeks feeding on the experimental diets, liver catalase (CAT), glutathione S-transferases (GST), glutathione reductase (GR) activities, and malondialdehyde (MDA) levels were measured. Thereafter, all fish were challenged by *Streptococcus iniae*. Probiotic, prebiotic and synbiotic had no significant effect on liver MDA level compared to the control group (P>0.05). However, CAT, GST and GR activities were significantly higher in the fish fed probiotic, prebiotic and synbiotic, compared to the control group (P<0.05). The highest CAT and GST activities were observed in the fish fed diet supplemented with synbiotic. There were no significant differences in GR activity among different groups (P>0.05). Bacterial challenge showed that feeding on probiotic, prebiotic and synbiotic remarkably increased resistance against *S. iniae* (P<0.05), with the highest resistance observed in the synbiotic group. The results indicated that although both GOS and *P. acidilactici* significantly increased antioxidant defence and improved disease resistance, combination of GOS and *P. acidilactici* had an additive effect. Combination of GOS and *P. acidilactici* is recommended to increase trout antioxidant capacity and streptococcosis.

Key words: rainbow trout, galactooligosaccharide, *Pediococcus acidilactici*, antioxidant defence, disease resistance

Antibiotics have been used to increase fish growth and prevent diseases; however, they also have adverse effects on fish and environment (Esiobu et al., 2002).

Immunostimulants could be used as the alternative for antibiotics in the fish diet. Probiotic, prebiotic and synbiotic (combination of prebiotic and probiotic) have been used to increase fish growth, innate immunity and disease resistance (Merrifield et al., 2010; Nayak, 2010; Ringø et al., 2010; Hoseinifar et al., 2013).

Despite the attempts for elevation of beneficial bacterial groups in the gut microbiota of fish through dietary administration of probiotics, the probiotic strains could not remain as dominant species in the gut after treatment cessation (Robertson et al., 2000; Panigrahi et al., 2005). To resolve this issue, an innovative strategy was suggested to help probiotic bacteria having sustainable domination in the gut microbiota of fish, which is synbiotic idea: co-administration of probiotic with appropriate prebiotic as substrate (Rurangwa et al., 2009). Recently, the application of synbiotics strategy has been increased in aquaculture for their additive advantages (Zhang et al., 2013).

Living organisms are under constant attack of free oxidant compounds, which can lead to serious cellular damage if produced in excess. Reactive oxygen species (ROS) are naturally produced in the organism in a variety of physiological mechanisms. Antioxidants, including enzymatics [e.g. superoxide dismutase (SOD), CAT, GR and GST] and non-enzymatics (e.g. ascorbic acid and alpha-tocopherol), are responsible for counterattacking ROS in an organism. Environmental stress like hypoxia, hyperoxia and xenobiotic exposure etc. cause oxidative stress (Winston and Di Giulio, 1991; Halliwell and Gutteridge, 1999; Livingstone, 2001; Manduzio et al., 2005; Zenteno-Savín et al., 2006). Therefore, increase in antioxidant capacity of an organism helps to have a better performance when aforementioned problems are encountered. Studies on the antioxidant-improving effects of prebiotics and probiotics are limited. Some recent studies have shown that prebiotics, probiotics and synbiotics are capable of modulating the antioxidant capacity in shrimp, *Litopenaeus stylirostris* (Castex et al., 2009, 2010) and fish, *Megalobrama terminalis* (Zhang et al., 2013) and *Lutjanus peru* (Guzman-Villanueva et al., 2013). Study on this issue could result in a better understanding of antioxidant- and immune-modulating effect of prebiotics, probiotics and synbiotics, as immune system has a close relationship with antioxidant system (Adema et al., 1991). GOS is produced through the enzymatic conversion of lactose and mainly consists of galactose and glucose molecules and has been shown to a prebiotic in fish (Sako et al., 1999; Burr et al., 2010; Zhou et al., 2010; Hoseinifar et al., 2013). *P. acidilactici* has been found to be useful probiotic for fish (Merrifield et al., 2011; Hoseinifar et al. 2015 a, b) and augments antioxidant capacity in shellfish (Castex et al., 2009, 2010). However, there are no studies on antioxidant effects of GOS and/or *P. acidilactici* on rainbow trout, *O. mykiss*, despite the fact that it is an important aquaculture species throughout the world, especially in Iran with annual production of more than 50000 tons in 2011 (Tahmasebi-Kohyani et al., 2011). Therefore, the aim of the present study was to investigate the effect of prebiotic (GOS), probiotic (*P. acidilactici*) and synbiotic (GOS + *P. acidilactici*) on antioxidant status and resistance to streptococcosis in rainbow trout juveniles, *O. mykiss*.

Material and methods

Diet preparation

A commercial trout feed, BioMar (BioMar SAS, 60, Rue Pierre-Georges Debouchaud, Zone Industrielle, FR-16440 Nersac, France; Table 1) was used as control diet. In order to produce diets containing probiotic, prebiotic and synbiotic, the control diet was supplemented with 1% GOS, 7.57 log CFU g^{-1} *P. acidilactici and* 1% GOS + 7.57 log CFU g^{-1} *P. acidilactici,* respectively according to Merrifield et al. (2011). Selection of the doses and preparation of the experimental diets was done based on suggestion of previously published works (Grisdale-Helland et al., 2008; Merrifield et al., 2011; Hoseinifar et al., 2014).

Table 1. Proximate composition of the basal commercial diet (%)

Proximate composition (% dry matter basis)	
Dry matter	92.8
Crude protein	42.5
Crude lipid	24.2
Ash	5.4
Energy (MJ kg^{-1})	22.80

Fish and maintenance conditions

A total number of 240 rainbow trout (15.04 ± 0.52 g) were purchased from a local farm and transferred to the laboratory. The fish were randomly distributed into 12 tanks (300 L) and remained about two weeks to acclimatize to the experimental conditions. The fish were fed with control diet during the acclimation period. After acclimation, the fish were assigned to four groups: control (fish were fed the control diet), prebiotic (fish were fed the control diet supplemented with 1% GOS), probiotic (fish were fed the control diet supplemented with 7.57 log CFU g^{-1} *P. acidilactici*) and synbiotic (fish were fed the control diet supplemented with 1% GOS and 7.57 log CFU g^{-1} *P. acidilactici*). Three tanks were assigned for each treatment. The fish were fed with their corresponding diet for eight weeks. During the trial, all fish were fed 3% of biomass day^{-1} (Yarahmadi et al., 2015) and were batch-weighed every other week, after 24 h starvation. All tanks were continuously aerated during the experiment period, and tanks' water was daily exchanged at the rate of 70%.

Sampling and analyses

At the end of the experiment, three fish were caught from each tank and killed by decapitation after a sharp blow on head. The fish livers were quickly dissected and washed by phosphate buffer (pH=7.4). Livers were divided in two parts to determine MDA level and antioxidant enzymes' activity. Individual samples for enzymatic determinations were prepared as follows: one part of samples was homogenized in 5 parts of phosphate buffer (0.1 M, pH = 7.4). The homogenate was centrifuged at 5000 g for 20 min at 4ºC to obtain the supernatant, which was used for biochemical analyses. The supernatant was divided into 100-microliter aliquots and stored in –80ºC, until further analysis. CAT activity was measured according to the method previously described by Cohen et al. (1996). GST assay was done based upon the method of Habig and Jakoby (1981) with slight modification for a 96-well format and using 1-chloro-2, 4-dinitrobenzene (CDNB) as a conjugation substrate (McFarland et al., 1999). GR activity was measured according to Cohen and Duvel (1988). MDA level was measured according to Ringwood et al. (2003) and expressed as nano-moles of malondialdehyde per gram wet weight.

Disease resistance test

Fifteen fish per tank were selected for disease resistance test and fish were intraperitoneally injected by predetermined pathogenicity dose (2×10^7 cells mL^{-1}) of *S. iniae* (Brunt et al., 2007). *S. iniae* culture was kindly supplied by Department of Veterinary Science of University of Tehran. Dead fish were daily removed from the challenge tanks; *S. iniae* was reisolated. The mortalities were monitored over an 18-day period and the mortalities (%) of rainbow trout in different treatment were calculated at the end of the period.

Statistical analyses

After confirmation of the normality of data by Shapiro-Wilk's test, the data were subjected to one-way ANOVA and Duncan's test to detect significant difference between the treatments. Percentile data (mortality) were Arcsin transformed before analyses. Data are presented as mean ± SD. $P<0.05$ were considered as significance. All analyses were performed using SPSS v. 18.

Results

The results of CAT, GR, GST activity and MDA are presented in Figures 1 and 2. The experimental diets had significant effects on CAT activity ($P<0.05$). The highest CAT activity was observed in synbiotic fed fish while the lowest was observed in the control group. Furthermore, singular pro- and prebiotic fed fish showed significantly higher CAT activity when compared to control ($P<0.05$). Similar to the results obtained with CAT, dietary administration of pre-, pro- and synbiotic notably elevated GR activity ($P<0.05$). Although there were no significant differences between pre-, pro- and synbiotic in the case of GR activity, the activity of GR in fish fed supplemented diets was significantly higher than that of the control group ($P<0.05$). The activity of GST was significantly increased in rainbow trout fed pre-, pro- or synbiotic supplemented diets ($P<0.05$) and the highest activity was observed in synbiotic group. There was no notable difference in MDA levels among the treatments ($P>0.05$).

Figure 3 represents the cumulative mortality (%) of rainbow trout fed diets supplemented with pre-, pro and synbiotic during 18 days post challenge with *S. iniae*. The mortality (%) was significantly ($P<0.05$) lower in the fish fed diets supplemented with prebiotic, probiotic and synbiotic, compared with the control group, and the highest resistance was observed in the synbiotic fed fish (Table 2). At the end of the challenge period, the mortality (%) rates were 72.5±3.21, 42.0±5.30, 47.5±7.07 and 32.5±3.23, respectively in prebiotic, probiotic and synbiotic.

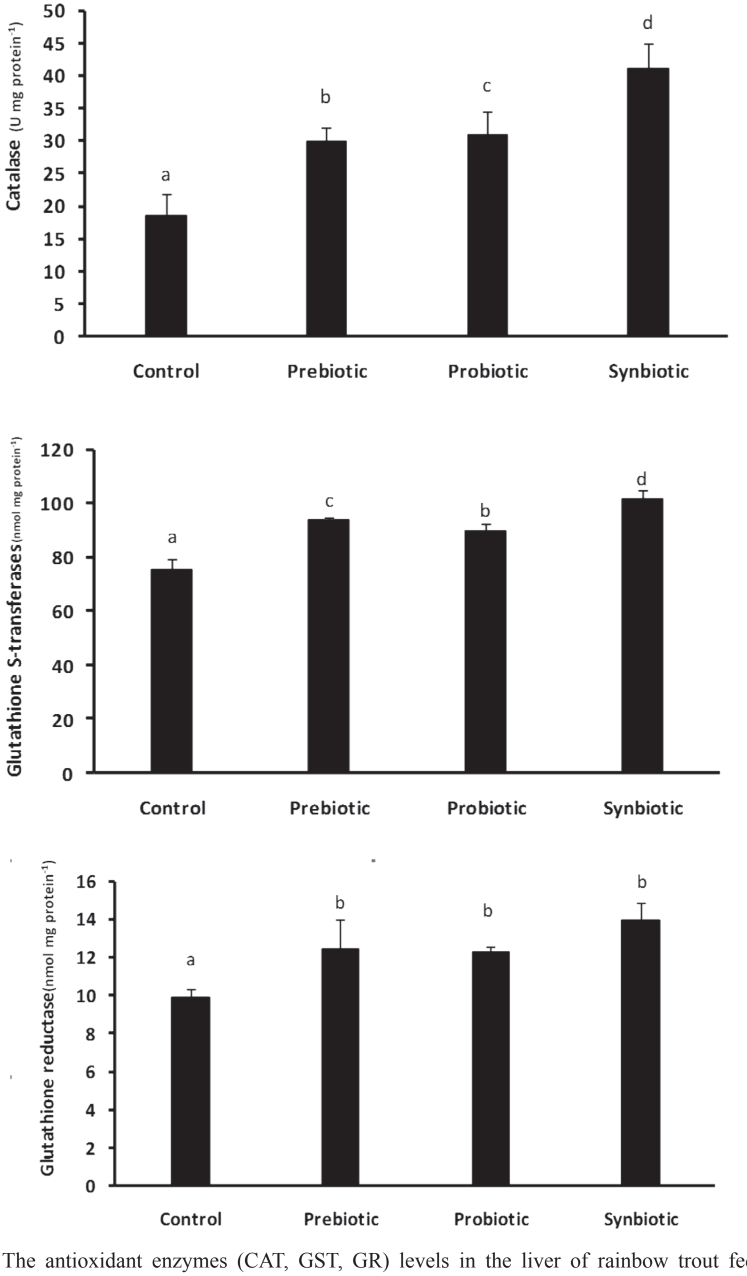

Figure 1. The antioxidant enzymes (CAT, GST, GR) levels in the liver of rainbow trout fed diets supplemented with pre-, pro- and synbiotic. The bars assigned with different letters denote significant difference ($P<0.05$)

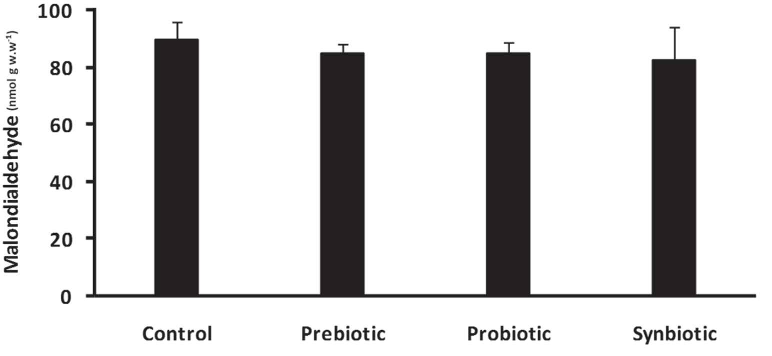

Figure 2. The malondialdehyde level in the liver of rainbow trout fed diets supplemented with pre-, pro- and synbiotic

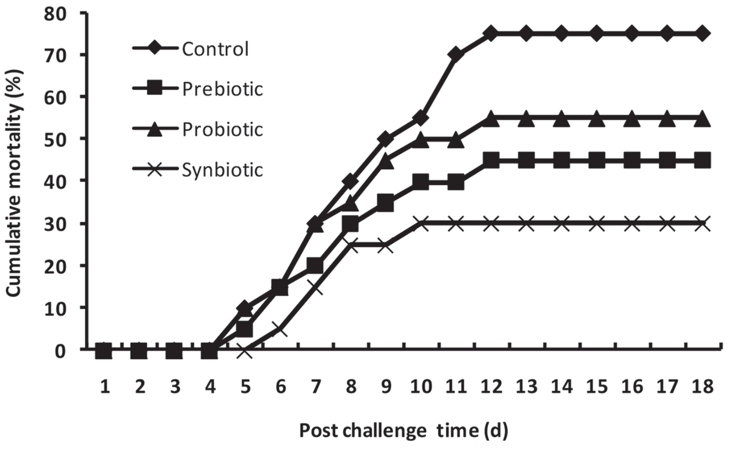

Figure 3. The cumulative mortality (%) of rainbow trout fed diets supplemented with pre-, pro- and synbiotic during 18 days post challenge with *S. iniae*

Table 2. Average mortality (%) in different treatments on day 18 post challenge

	Control	Prebiotic	Probiotic	Synbiotic
Mortality	72.5±3.21 a	42.0±5.30 b	47.5±7.07 b	32.5±3.23 c

a, b, c – means within a row with different superscripts differ (P<0.05).

Discussion

Synbiotic, nutritional supplements that contain both probiotics and prebiotics that work together and beneficially affect the health of the host, are an alternative to probiotic or prebiotic administration in aquaculture (Hoseinifar et al., 2014). Lactic acid bacteria (LAB) are commonly used as probiotics in aquaculture, and they are nutritionally fastidious but need carbohydrates and other nutrients to grow (Llewellyn et al., 2014). In this context, recent *in vitro* and *in vivo* experiments on determination of the best prebiotic as substrate for *P. acidilactici* revealed that galactooligosaccharide is the best prebiotic to be used as synbiotic with *P. acidilactici* (Hoseinifar et al., 2015 a). Therefore, in the present study, we have evaluated the *in vivo* efficiency of *P. acidilactici* + GOS (synbiotic) in comparison to singular administration of *P. acidilactici* and GOS in the case of effects on antioxidant enzymes activity and disease resistance of rainbow trout.

ROS are normally produced during physiological processes which are coped by several antioxidant defence mechanisms (Zhang et al., 2013). The antioxidant enzymes include SOD, GPX and CAT considered as the first line of defence of the animal body against free radicals (Yang et al., 2010). It has been reported that the defensive abilities of reared fish are insufficient and hence elevation of the antioxidant system capacity of fish is of high importance in commercial aquaculture (Santacroce et al., 2012). In general, measurement of the antioxidant enzyme activity together with resistance of cultured fish provides valuable information about antioxidant and health status (Zhang et al., 2013).

The results of present study revealed that activity of CAT, GR and GST was significantly increased in prebiotic, probiotic and synbiotic groups compared with the control. There are contradictory results regarding the effect of prebiotic, probiotic and synbiotic on the activity of antioxidant enzymes and oxidative status of aquatic organisms. Castex et al. (2010) reported an increase in total antioxidant status (TAS) of *L. stylirostris* hemolymph as a result of dietary *P. acidilactici* supplementation. However, they reported no change in TAS, SOD, MDA, carbonyl protein and glutathione peroxidase (GPx), and significant decrease in CAT activity of *L. stylirostris* digestive gland. Such results showed that the activity of different antioxidant enzymes varies in the different body parts. Guzman-Villanueva et al. (2013) showed an increase in SOD and no change in CAT activity in *L. peru* liver fed diet supplemented with 0.1, but not 0.2% prebiotic. Zhang et al. (2013) showed an increase in liver SOD, CAT and GPx as well as plasma SOD as a result of fructooligosaccharide and *B. licheniformis* supplementation in *M. terminalis* diet. The elevation of antioxidant activity of the rainbow trout fed pre-, pro- and synbiotic might have occurred due to the effects of these dietary supplements on the translation process and/or post-translational process of these antioxidant enzymes (Zhang et al., 2013). Nevertheless, determination of the mode of action of dietary synbiotic on antioxidant enzymes activity merits further research.

MDA formation has been used as a common assay for determination of lipid peroxidation level (Del Rio et al., 2005). Decrease of MDA level can be considered as an increase of antioxidants of defence mechanisms (Shen et al., 2010). However, in the present study despite the remarkable increase of antioxidant enzymes activity of fish fed pre-, pro- and synbiotic, no notable difference was noticed in the case of MDA. Likewise, in a research with *L. stylirostris*, dietary administration of *P. acidilactici* as probiotic had no significant effects on MDA levels (Castex et al., 2010). The findings of the present study regarding antioxidant enzymes activity together with the results obtained for disease resistance (discussed below), showed an improvement in the health status of rainbow trout fed *P. acidilactici* + GOS (synbiotic).

Nutritional manipulation is a useful way to increase fish resistance to pathogens (Kiron, 2012; Hoseinifar et al., 2015 c). The present results clearly showed the increase in resistance to streptococcosis of rainbow trout as a result of prebiotic, probiotic and synbiotic supplementation. Similarly, Castex et al. (2010) reported increased resistance to vibriosis of *L. stylirostris* as a result of dietary *P. acidilactici* supplementation. Furthermore, Neissi et al. (2013) determined immunomodulatory effects of *P. acidilactici* on green terror (*Aequidens rivulatus*). Likewise, administration of dietary GOS improved resistance of Caspian roach (*Rutilus rutilus*) fry (Hoseinifar et al., 2013). Beneficial effect of prebiotics may be related to stimulation of the growth of beneficial bacteria such as lactic acid bacteria (Zhang et al., 2011). These bacteria cell walls consist of components such as lipopolysaccharide, which stimulate immune system (Bricknell and Dalmo, 2005). Besides this, increase of the population of these beneficial bacteria leaves little adherence sites for pathogenic bacteria (Kaneko et al., 1995). When prebiotic and probiotic are used simultaneously (synbiotic), both bacteria and its feed are transferred to the fish gut. Therefore, it is supposed that synbiotic should have more pronounced effect than prebiotic and probiotic alone, as observed in the present study (Cerezuela et al., 2011). Although other factors such as the type of prebiotic and probiotic, administration method, fish species, life stage and rearing condition may affect the results (Hoseinifar et al., 2014).

In conclusion, the results of the present study revealed that administration of GOS and *P. acidilactici* elevated antioxidant enzyme activity and resistance of rainbow trout. Furthermore, our findings showed that there is a synergism between GOS and *P. acidilactici* in the case of effects on fish antioxidant enzymes activity and disease resistance. Therefore, GOS + *P. acidilactici* can be considered as a synbiotic for rainbow trout.

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