EFFECTS OF DIETARY ADMINISTRATION OF *BACILLUS* PROBIOTICS ON THE NON-SPECIFIC IMMUNE RESPONSES OF TINFOIL BARB, *BARBONYMUS SCHWANENFELDII* (ACTINOPTERYGII: CYPRINIFORMES: CYPRINIDAE)

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Background. Probiotics are known as immunostimulants as well as growth and survival promoters in fish, but despite the plethora of studies on the effect of probiotics on the fish non-specific immune responses, very few information is available for ornemental fish. Thus, this study was carried out to investigate the effects of short-term dietary administration of *Bacillus* probiotics (*B. subtilis* and *B. licheniformis*) on the immune responses of tinfoil barb, *Barbonymus schwanenfeldii* (Bleeker, 1854).

Materials and methods. Three experimental diets were prepared by supplementing the diets with different concentrations of probiotics including 0 (Control), 1×10^4 (T1) and 1×1330^6 (T2) CFU $\cdot$ g^{-1} diet. Tinfoil barb weighing 16.20 ± 0.13 g were fed different diets for 15 days. At the end of the experiment, blood samples were collected in order to estimate the hematological (total protein, albumin, and globulin) and immune (total antiprotease, plasma peroxidase, and bactericidal activities) parameters.

Results. The plasma total protein, albumin, and globulin of fish were not affected by dietary probiotics. A six-fold increase in the plasma total antiprotease activity of fish fed 1×10^6 CFU $\cdot$ g^{-1} diet was observed. Moreover, the plasma peroxidase and bactericidal activities of fish significantly increased in T2 treatment compared to control. On the other hand, plasma natural haemolytic complement and lysozyme activities did not significantly vary between diets although the highest level of these parameters was observed in the probiotic treatments.

Conclusion. This study showed the ability of dietary probiotics to enhance some innate immune responses of tinfoil barb especially after a 2-week administration of 1×10^6 CFU $\cdot$ g^{-1} diet (T2). Further study should focus on the effect of this dietary bacterial concentration on the fish response to bacterial and parasitical challenges in order to test its potential protective effect against the common pathogens of tinfoil barb.

Keywords: Ornamental fish, immunostimulant, innate immunity, *Bacillus subtilis*, *Bacillus licheniformis*

INTRODUCTION

The aquaculture- and aquarium fishes are sensitive to stress and diseases. Preventive methods to avoid diseases and loss of fish include vaccination, but vaccines are not available against all pathogens and especially against parasites. This lack of vaccines together with the environmental impact of extensive use of therapeutic agents such as antibiotics justify the increasing demand for efficient non-specific immunostimulants for aquaculture (Sakai 1999, Bricknell and Dalmo 2005). Immunostimulants, which are biological and synthetic compounds, can modulate the immune responses and disease resistance of fish by enhancing the fish non-specific immunity (Sakai 1999). According to FAO definition, probiotics are "live microorganisms, which when administered in adequate amounts confer a health benefit on the host" (Anonymous 2002). Dead cells are also known as probiotic when the immunomodulation feature of probiotics are concerned (Adams 2010); thus, probiotics are alive or dead beneficial bacteria, which can control pathogens through improving the immune responses of hosts (Irianto and Austin 2002, Aly et al. 2008, Qi et al. 2009, Adams 2010,

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Ringø et al. 2012). The dietary application of probiotics has numerous beneficial effects on fish including growth promotion through the modulation of the host's metabolic functions possibly enhancing the fish digestive activity and synthesis of vitamins (Gatesoupe 2008, Kesarcodi-Watson et al. 2008, Ringø et al. 2012), pathogen exclusion through the modification of the gut microflora and disease resistance through the enhancement of fish immunity (Aly et al. 2008, Gatesoupe 2008, Nayak 2010, Ringø et al. 2012). Such exogenous microbes antagonistic to pathogens are termed biocontrol agents (Merrifield et al. 2010, Maeda et al. 1997). There is therefore a growing use of probiotics in aquaculture to control fish diseases (Gatesoupe 1999, Kumar et al. 2008). Both alive and dead cells can be used as probiotic in order to improve fish welfare, albeit viable live probionts seems better than the non-viable cells (Ringø 2012), for they can safely and more strongly enhance immunity and do not cause any infection or disease in fish. Fish immunomodulation by probiotics depends however on the type of probiotic strain, the fish species, and their genetic makeup and the feeding duration and dosage (Nikoskelainen et al. 2003, Salinas et al. 2005, Kim and Austin 2006, Son et al. 2009, Rodríguez et al. 2003). During the last 10 years, many investigations conducted on the dietary application of probiotics in aquaculture have focused on *Bacillus* spp. and *Lactobacillus* spp. (Wang et al. 2008, Qi et al. 2009, Nayak 2010, Merrifield et al. 2010, Kiron 2012) and one of the most popular probiotics used in aquaculture are the *Bacillus* strains (Qi et al. 2009, Nayak 2010). In particular, *Bacillus* probiotics have been shown to possess antimicrobial and immunomodulatory activities in host (Cutting 2011). In fish, *B. subtilis* and *B. licheniformis* have been reported to elevate the immune responses and disease resistance of rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792), and Indian major carp—rohu, *Labeo rohita* (Hamilton, 1822) (see Kim and Austin 2006, Kumar et al. 2008).

Although a large number of studies have been conducted to investigate the effects of probiotics on the immune responses of aquaculture fish, few studies have been carried out on the influence of the dietary administration of probiotics on the immunity of ornamental fish. The culture of ornamental fish is an important activity in several Asian countries. Since there is a globally rising trend towards aquarium fish, ornamental fish culture industry is steadily growing worldwide (Yanar et al. 2008). Cyprinidae is a dominant family in this industry and tinfoil barb, *Barbonymus schwanenfeldii* (Bleeker, 1854), is one of the most important cyprinid species, widely reared in South East Asia. However, this species is commercially used in warm-water aquaculture industry for integrated farming with other cyprinids (Bailey and Cole 1999). This species can be carrier of protozoan and parasitic nematodes (Gante et al. 2008) and is also sensitive to the white spot disease or other parasitic infections, ulcerative rhabdovirus syndrome, and various bacterial diseases (Bassleer 1997). Immunostimulation of this fish may prevent disease occurrences, thus reducing the economic cost

and environmental impact of the extensive use of therapeutics. In the majority of previous studies, *Bacillus* probiotics and particularly *B. subtilis* have been well established to enhance the immune responses of cyprinids and other fish with similar digestive tract (Nayak et al. 2007, Aly et al. 2008, Kumar et al. 2008, Kiron 2012). Interestingly, a study in gilthead seabream, *Sparus aurata* L., showed that the use of multispecies probiotics had better immunomodulating capacity than dietary administration of mono-species probiotics (Salinas et al. 2005). Several fish studies showed that a 10 to 14 day administration period of dietary probiotics was optimal for fish immunostimulation (Rodríguez et al. 2003, Salinas et al. 2005, Sharifuzzaman and Austin 2009, Son et al. 2009). It seems that the influence of probiotics can be evidenced even after short-term administration. However, various studies showed that efficient probiotic species and dietary dosage vary between fish species. Therefore even commercially available probiotics should be checked in order to determine optimal probiotic species, dosage, and duration of administration for each fish species of interest. Hence, the presently reported study was aimed to investigate the effects of short-term (2 weeks) dietary administration of a mixture of *Bacillus subtilis* and *B. licheniformis* on the non-specific immune responses including antiproteases, peroxidase, lysozyme, natural haemolytic complement, and bactericidal activities as well as plasma protein, albumin, and glubolin of tinfoil barb.

MATERIALS AND METHODS

Experimental design and test diets. The probiotic containing spores of *Bacillus subtilis* and *B. licheniformis* were obtained from Protexin Co. (Iran-Nikotak). The colony forming units (CFU) of *Bacillus* probiotics were checked by culturing probiotics on Tryptic Soy Agar (TSA) (Rengpipat et al. 1998). Three experimental diets with different levels of probiotics concentration including 0 (control), 1×10^4 (T1), and 1×10^6 (T2) CFU $\cdot$ g^{-1} diet were prepared.

Briefly, according to manufacturer's procedure, probiotics were added to sterile water (10 g $\cdot$ L^{-1}) and allowed to incubate for 8 h at 37ºC. Then, the different levels of probiotics were sprayed on a commercial pelleted diet containing fishmeal, soybean mean, corn and wheat flour, shrimp, fish oil, soybean oil, carotenoids, monophosphate calcium, vitamin, and mineral premix (Energy 4EF3001, Thailand; moisture 12%, crude protein 41%, crude fat 6% and fiber 2%) and dried at 40°C, in an incubator for 6 h and kept at 4°C until use. The experimental diets were prepared every 3 days.

Fish and feeding trial. Prior to the beginning of the experiment, the fish were acclimatized to the control diet (same to aforementioned commercial diet: moisture 12%, crude protein 41%, crude fat 6% and fiber 2%).and rearing conditions for two weeks. Ninety juvenile tinfoil barb weighing 16.20 ± 0.13 g were randomly distributed into 9 rectangular glass aquaria (50 × 30 × 35 cm, 52 L) filled with dechlorinated tap water with adequate aeration and

filtration at 12L : 12D photoperiod. Twenty percent of the water contained in each tank was replaced with fresh, dechlorinated tap water every day. This study was performed in triplicate (10 fish per aquarium; 3 aquaria per dietary treatment). The fish in each treatment were fed experimental diets to apparent satiation twice a day at 0800 h and 1600 h for 15 days. The fish were starved for 24 h before sampling. Water quality parameters were monitoredduring the experiment (temperature 26.90 ± 0.14ºC, dissolved oxygen 6.3 ± 0.3 mg · L^{-1}, ammonia 0.140 ± 0.004 mg · L^{-1}, nitrites 0.059 ± 0.060 mg · L^{-1}, hardness 193.4 ± 13.2 mg · L^{-1}, and pH 7.3 ± 0.4). Oxygen and temperature were assayed using an oximeter (WTW oxi 33oi, Weilheim, Germany), whereas the measurement of other water quality parameters followed the methods of Tripathi and Govil (2001).

Sampling. After 15 days of feeding trial, blood samples were collected to estimate the immunological parameters of fish in various treatments. Six fish from each replicate were netted and anaesthetised by 400 mg · L^{-1} of clove powder. Then, 0.5–0.6 mL of blood samples were collected by caudal vein puncture using a 2-mL heparinized syringe. The blood samples were immediately placed in 2 mL tubes and the plasma was separated from blood by centrifugation at 3000 × g for 10 min. The plasma of two fish in each replicate waspooled, giving a total of three pooled plasma samples for each replicate aquarium; thus, 9 samples per dietary treatment were prepared. The plasma samples were stored at –70°C until analysed.

Total protein, albumin, and globulin. Plasma total protein quantity was determined using a kit (Bio-Rad Laboratories GmbH, Munich, Germany) with bovine serum albumin as a standard (Bradford 1976). Plasma albumin was detected at 620 nm (Quantichrom^{TM} BCG Albumin Assay Kit) (Nicholson et al. 2000). Additionally, the content of total globulin was calculated by subtracting albumin from total protein (Kumar et al. 2005).

Plasma antiproteases activity. Total antiprotease activity was assayed as an indicator of the plasma capacity for inhibiting trypsin activity (Magnadóttir et al. 1999). The antiprotease activity was expressed as the percentage of trypsin inhibition (Zuo and Woo 1997).

Peroxidase activity. Plasma Peroxidase activity was measured using 3,3′,5,5′-tetramethylbenzidine hydrochloride (TMB) and hydrogen peroxide as peroxidase enzyme substrate (Quade and Roth 1997). Briefly, 15 µL of plasma was diluted with 50 µL of Hank's buffered salt solution (HBSS) without Ca^{+2} or Mg^{+2} in flat-bottomed 96-well plates. Then, 100 µL of 0.1 mM TMB and 2.5 mM H$_2$O$_2$ were added and incubated for 2 min. The colour-developing reaction in plasma samples was stopped after 2 min by adding 50 µL of 2 M sulphuric acid and the optical density (OD) was read at 450 nm using a microplate reader (Stat Fax-2100, USA). Wells without plasma were used as blanks. The peroxidase activity was defined as one unit of peroxidase producing an absorbance change of 1 OD.

Plasma lysozyme activity. Plasma lysozyme activity was assayed based on the turbidimetric method (Cha et al. 2008).

Natural haemolytic complement activity. The natural haemolytic complement activity was determined using sheep red blood cells (SRBC) as target cells (Ortuno et al. 1998) with slight modifications. SRBC were washed and resuspended at 3% (v/v) phenol red-free Hank's balanced salt solution (HBSS) containing Mg^{2+} and 10mM EGTA (EGTA-HBSS). Aliquots of 100 µL plasma were serially diluted in EGTA-HBSS and were added to 100 µL of SRBC After a 1-h incubation at 22°C and centrifugation,the concentration of haemoglobin in the supernatants was assessed by measuring the absorbance at 450 nm in a microplate reader (Stat Fax-2100, USA). Maximum and spontaneous haemolysis were obtained by adding 100 µL of distilled water or HBSS to 100 µL samples of SRBC, respectively. The lysis curve for was determined for each sample on a log$_{10}$–log$_{10}$ scaled graph. The amount of plasma complement producing 50% haemolysis (ACH50) was obtained and results were expressed as ACH50 units mL^{-1}.

Plasma bactericidal activity. Plasma bactericidal activity against *Aeromonas hydrophila*, which is a common fish pathogen, was measured following the method of Rao et al. (2006). Fish plasma was incubated with the bacteria then plated on agar. Colony forming units (CFU) were counted and bactericidal activity was deduced.

Statistical analyses. Statistical analyses were performed using the software SPSS 15.0 (Chicago, IL, USA). The normality of distribution of variables was tested by Kolmogorov–Smirnov test. The homogeneity of variances was tested using the Levene's F test. Differences between various treatments were analyzed by one-way ANOVA, followed by Tukey's *post hoc* test. Differences were considered statistically significant when $P \leq 0.05$. All experimental data was expressed as mean ± SE.

Ethical issues. Despite the fact that no ethical regulation exist in Irak, animals were handled with care and their welfare was taken into account during samplings when fish were properly anaesthetised.

RESULTS

After 15 days of feeding trial, plasma total protein ($P = 0.741$), albumin ($P = 0.649$), and total globulin ($P = 0.711$) of fish did not significantly vary between treatments (Fig. 1). However, the highest levels of plasma protein and albumin were seen in T1 and T2 groups ranging between 3.72 to 3.95 and 1.37 to 1.42 g · dL^{-1} for protein and albumin respectively.

At the end of the experiment, some non-specific immune responses were significantly altered by dietary probiotics. The amount of plasma trypsin inhibition was higher in fish fed *Bacillus* probiotics compared to control. Indeed, the amount of plasma trypsin inhibition in T2 group increased to 60% in comparison with control group where 10% inhibition was obtained ($P = 0.004$) (Fig. 2).

The amount of peroxidase activity significantly increasedin fish fed with 1×10^6 CFU · g^{-1} diet (T2) compared to control and T1 groups ($P < 0.001$; Fig. 3).

The lysozyme activity of fish fed *Bacillus* probiotics was higher than that in control group. It increased from

19.0 ± 2.1 U · mL^{-1} in control group to 28.3 ± 3.5 U · mL^{-1} in T1 and to 26.6 ± 4.7 U · mL^{-1} in T2. However, significant differences were not observed between treatments ($P = 0.086$; Fig. 4).
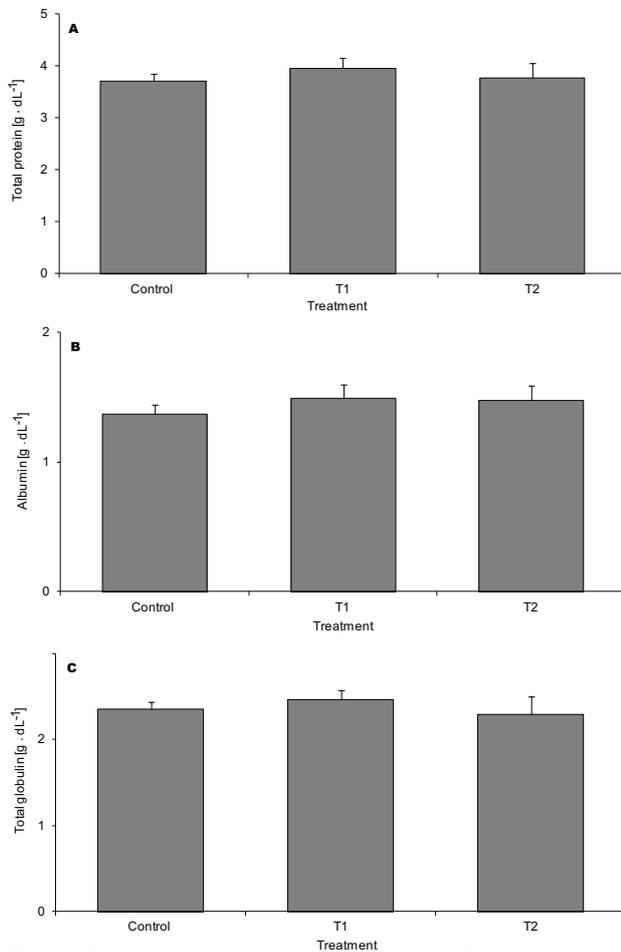
The amount of plasma natural haemolytic complement activity (ACH50), ranging between 30.5 and 39 U · mL^{-1}, did not change significantly between groups, in spite of the fact that the highest level of ACH50 was seen in T1 treatment ($P = 0.091$; Fig. 5).

Fig. 1. Plasma total protein (**A**), albumin (**B**) and globulin (**C**) levels of tinfoil barb, *Barbonymus schwanenfeldii*, fed different levels of dietary probiotics (control, 0 CFU · g^{-1} diet; T1, 1×10^4 CFU · g^{-1} diet; T2, 1×10^6 CFU · g^{-1} diet) for 15 days; Data are presented as mean ± SE ($n = 9$); No significant difference ($P > 0.05$) was observed between treatments
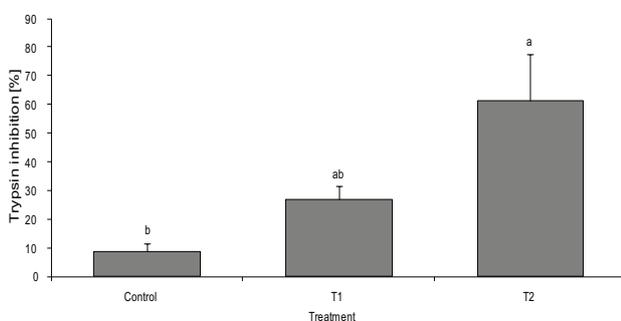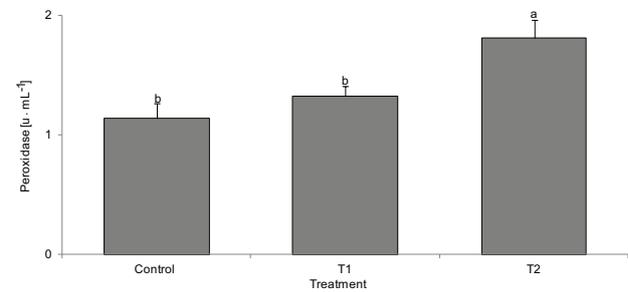

Fig. 2. Plasma trypsin inhibition activity of tinfoil barb, *Barbonymus schwanenfeldii*, fed different levels of dietary probiotics (control, 0 CFU · g^{-1} diet; T1, 1×10^4 CFU · g^{-1} diet; T2, 1×10^6 CFU · g^{-1} diet) for 15 days; Data are presented as mean ± SE ($n = 9$); Bars with different letters were significantly different ($P < 0.05$)

Fig. 3. Plasma peroxidase activity of tinfoil barb, *Barbonymus schwanenfeldii*, fed different levels of dietary probiotics (control, 0 CFU · g^{-1} diet; T1, 1×10^4 CFU · g^{-1} diet; T2, 1×10^6 CFU · g^{-1} diet) for 15 days. Data are presented as mean ± SE ($n = 9$): Bars with different letters were significantly different ($P < 0.05$)
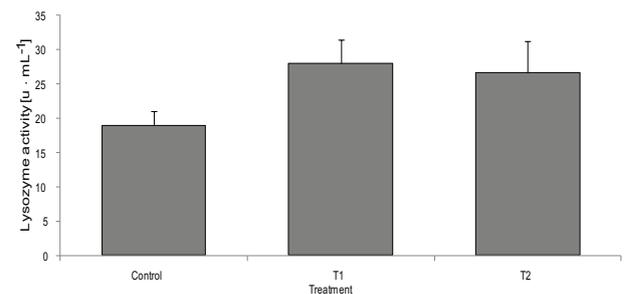

Fig. 4. Plasma lysozyme activity of tinfoil barb, *Barbonymus schwanenfeldii*, fed different levels of dietary probiotics (control, 0 CFU · g^{-1} diet; T1, 1×10^4 CFU · g^{-1} diet; T2, 1×10^6 CFU · g^{-1} diet) for 15 days; Data are presented as mean ± SE ($n = 9$); No significant difference ($P > 0.05$) was observed between treatments
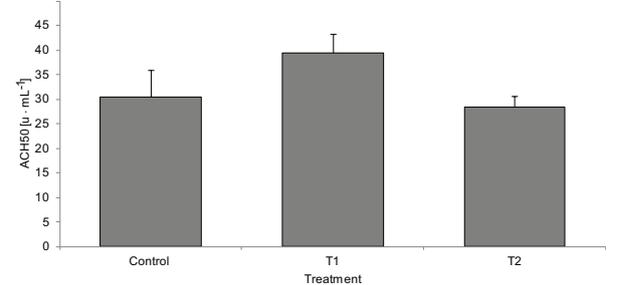

Fig. 5. Plasma natural haemolytic complement activity (ACH50) of tinfoil barb, *Barbonymus schwanenfeldii*, fed different levels of dietary probiotics (control, 0 CFU · g^{-1} diet; T1, 1×10^4 CFU · g^{-1} diet; T2, 1×10^6 CFU · g^{-1} diet) for 15 days; Data are presented as mean ± SE ($n = 9$); No significant difference ($P > 0.05$) was observed between treatments

The plasma bactericidal activity of fish in T2 group significantly increased compared to control group with significantly less viable bacteria in contact with the sera of fish fed *Bacillus* at 1×10^6 CFU $\cdot$ g^{-1} diet, 180 CFU compared to 207 CFU $\cdot$ g^{-1} in control fish ($P = 0.041$; Fig. 6). Although the same tendency was observed in T1 group, the difference with the control group was not significant (Fig. 6). The number of CFU $\cdot$ g^{-1} in negative control samples (using PBS instead of plasma) was 335 ± 16.

DISCUSSION

Proteins having a key role in physiological and immunological systems are the main serum components (Kumar et al. 2005). In this study, plasma total protein, albumin, and total globulin did not significantly vary between treatments. However, the highest levels of these parameters were observed in probiotic treatments. Similarly, the plasma proteins of Nile tilapia, *Oreochromis niloticus* (L.), did not significantly change after rearing in water containing *Enteroccus faecium* 1×10^7 CFU $\cdot$ mL^{-1} (Wang et al. 2008). Adding *Bacillus* probiotics to rearing water of tilapia led to an increase in plasma total protein, yet this variation was not seen for plasma albumin and total globulin (Zhou et al. 2010). On the other hand, dietary administration of *B. subtilis* appreciably increased serum total protein, albumin, and total globulin in Indian major carp—rohu, *Labeo rohita*, and serum total protein in rainbow trout, *Oncorhynchus mykiss* (see Nayak et al. 2007, Newaj-Fyzul et al. 2007). Therefore, it seems that the influence of probiotics on the elevation of serum proteins varied between species and bacterial strains and also depends on the duration of administration. The variations of the total protein concentration may reflect variations of acute phase response (APR) with an equilibrum between production of acute phase protein (positive APP) and decline of other acute phase proteins (negative APP) (Brunt et al. 2008).

The serum antiprotease activities of hosts, which mainly include α_1-antiprotease, α_2-antiplasmin, and α_2-macroglobulin, are identified as anti-enzymes that possess a capacity for delaying or inhibiting pathogens attacking the body through proteolytic enzyme secretion (Magnadóttir 2006). The antiprotease activity is known to be highly influenced by immunization or infection (Magnadóttir 2006). In the present study, the plasma antiprotease activity markedly increased in fish fed *Bacillus* probiotics; in other words, the amount of this anti-enzyme in T2 treatment was six-fold greater than that of the control group. Similarly, the activity of serum antiprotease in rainbow trout doubled after two weeks of oral administration of commercial probiotics, but these significant differences were not reported for either shorter (one week) or longer (3 and 4 weeks) times of administration (Sharifuzzaman and Austin 2009). Also similar to our study, total antiprotease activity in rainbow trout significantly increased after two weeks of oral administration of *B. subtilis* (see Newaj-Fyzul et al. 2007). The results of this study showed that the oral administration of
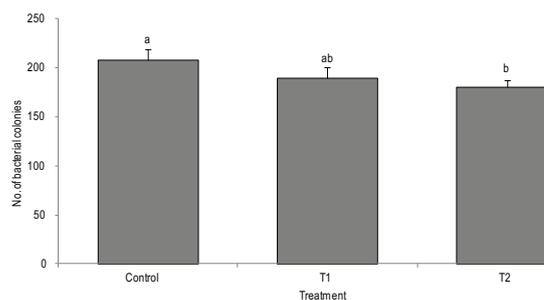

Fig. 6. Plasma bactericidal activity of tinfoil barb, *Barbonymus schwanenfeldii*, fed different levels of dietary probiotics (control, 0 CFU $\cdot$ g^{-1} diet; T1, 1×10^4 CFU $\cdot$ g^{-1} diet; T2, 1×10^6 CFU $\cdot$ g^{-1} diet) for 15 days; Data are presented as mean $\pm$ SE ($n = 9$); Bars with different letters were significantly different ($P < 0.05$)

Bacillus probiotics could significantly increase the antiprotease activity of this species, thus protecting the fish against the proteolytic activity of potential pathogens.

In the presently reported research, the amount of plasma peroxidase activity significantly increasedin fish fed 1×10^6 CFU $\cdot$ g^{-1} probiotics (T2), yet the differences between T1 group and control was negligible. Similarly, Wang et al. (2008) have reported that the myeloperoxidase (MPO) activity of tilapia increased after 40 days of rearing in water containing the *Enteroccus faecium* probiotic. In contrast, serum peroxidase activity in rainbow trout fed with *B. subtilis* remained unchanged (Newaj-Fyzul et al. 2007). Additionally, peroxidase contents in the serum and head kidney leucocytes of gilthead seabream, *Sparus aurata*, did not considerably vary during four weeks of dietary administration of probiotics (Díaz-Rosales et al. 2006). The plasma peroxidase activity is usually measured as indicator of leucocytes activity (Quade and Roth 1997). It has been shown that dietary administration of probiotics can remarkably enhance the phagocytosis activity of fish head kidney leucocytes (Panigrahi et al. 2004). Accordingly, the increased peroxidase activity recorded in this study could be due to higher phagocytic activity of leucocytes accompanied by an increased respiratory burst activity, and thus an increase need to detoxify the peroxide produced.

Lysozyme as a bactericidal enzyme is an important component of the non-specific humoral defense system of fish. Lysozyme is released by leucocytes and reacts against both gram-positive and gram-negative bacteria (Saurabh and Sahoo 2008). The variation of lysozyme activity depends on several factors such as nutritional status, seasonal variation, sex and sexual maturation, salinity, pH, water temperature, stress, and infections (Saurabh and Sahoo 2008). In the presently reported study, the plasma lysozyme activity of fish in the various treatments was not significantly affected by dietary probiotics, albeit the amount of plasma lysozyme activity in probiotic treatments was noticeably higher than that of the control group. Similarly, administration in the rearing water of either

Enteroccus faecium or *Bacillus* spp. probiotics did not significantly change the level of lysozyme activity in tilapia (Wang et al. 2008, Zhou et al. 2010). On the contrary, the dietary administration of *Bacillus* strains markedly elevated the serum lysozyme activity of rainbow trout (Brunt et al. 2007, Newaj-Fyzul et al. 2007, Sharifuzzaman and Austin 2009); furthermore, an increase in the lysozyme activity of tilapia (Aly et al. 2008) and olive flounder, *Paralichthys olivaceus* (Temminck et Schlegel, 1846) (see Harikrishnan et al. 2010), has been reported after the dietary administration of *Bacillus* and *Lactobacillus* probiotics. Sun et al. (2010) have demonstrated that the serum lysozyme activity of orange-spotted grouper, *Epinephelus coioides* (Hamilton, 1822), was enhanced after 60 days of feeding with probiotics, whereas no significant difference between the treatments was observed after 30 days of dietary administration. Apparently, the influence of probiotics on fish serum lysozyme activity could depend on the duration and dose of administration, bacterial strains and experimental conditions and the differences observed here may become significant after prolongedadministration periods.

Complement is an efficient mechanism of the fish immune system, which results in the killing of microorganisms through lysis of their cellular membranes (Ellis 1999). In the presently reported research project, the amount of alternative complement (ACH50) did not significantly vary between treatments. However, the highest value of ACH50 was observed in T1 treatment. Similarly, the variations of ACH50 in rainbow trout and gilthead seabream fed different levels of probiotics were not statistically significant (Díaz-Rosales et al. 2006, Newaj-Fyzul et al. 2007). On the other hand, the value of ACH50 in Nile tilapia and olive flounder considerably increasedby either immersion or dietary administration of probiotics (Aly et al. 2008, Wang et al. 2008, Harikrishnan et al. 2010). Moreover, the dietary administration of *B. pumilus* or *B. clausii* containing diets could activate the complement system (C_3 and C_4) of grouper, *Epinephelus coioides*, after 30 days of feeding, but no significant difference in serum complement C_3 and C_4 levels were observed between the treatments and the control after 60 days of feeding (Sun et al. 2010). The duration of probiotic administration must therefore be determined for each fish species for the dietary treatment to be an efficient immunostimulant.

In this study, the plasma bactericidal activity against a Gram-negative bacterial strain of fish fed with 1×10^6 CFU $\cdot$ g^{-1} diet significantly increased compared to that of control fish. Similarly, the serum bactericidal activity of Indian major carp and rainbow trout fed *B. subtilis* noticeably improved after 2 weeks of feeding (Newaj-Fyzul et al. 2007, Kumar et al. 2008). Aly et al. (2008) have reported that both bactericidal activity and resistance to the bacterial challenge of *Aeromonas hydrophila* were enhanced in Nile tilapia, *Oreochromis niloticus*, fed *B. subtilis* and *Lactobacillus acidophilus*. An improvement in the resistance of fish to bacterial infection by the dietary administration of probiotics has been reportedin several studies (Kesarcodi-

Watson et al. 2008, Nayak 2010). In the the effective large antimicrobial arsenal in the serum of tinfoil barb potentially enhancing the fish resistance to bacterial infections.

Probiotics are reported to modulate fish immune responses and resistance to pathogens through stimulation of antibody secreting cell response, induction of phagocytosis activity, modification of cytokine production, and natural complement activity (Panigrahi et al. 2004, 2007, Ringø et al. 2012). The improved immune parameters (plasma antiprotease and peroxidase activities) of fish fed graded levels of dietary probiotics in this study could be due to higher secretion of antibodies and activation of leucocytes phagocytic activity in fish fed dietary probiotics. Correspondingly, the enhancement of natural immunity in the fish serum (significantly higher level of bactericidal activity in T2 and slight enhancement of other antibacterial components of serum such as lysozyme and complement activities) can correspond to an improvement of fish resistance to pathogens. Hence, this stronger bactericidal activity in T2 can be caused by higher stimulation of the antibacterial arsenal of fish by dietary bacterial probiotics.

It can be concluded from the the presently reported study that all non-specific immune parameters tested in tinfoil barb, *Barbonymus schwanenfeldii*, were enhanced to some extent, by the dietary administration of *Bacillus* probiotics, although the observed changes were not always significant. The strongest effect of dietary probiotics was observed in fish fed the 1×10^6 CFU g^{-1} diet (T2). This dosage of *Bacillus* probiotics can enhance the immune responses of this species after 2 weeks of dietary administration. Further study should focus on the effect of this dietary bacterial concentration on the fish response to bacterial and parasitical challenges in order to test its potential protective effect against the common pathogens of tinfoil barb. Furthermore, longer administration periods should be studied and the expression of immune-related genes be included in order to check if the immune system of tinfoil barb is not burdened by a long-term dietary administration of probiotics and to elucidate the mechanisms of immunomodulation. Additionally, further study should be conducted on the gastrointestinal tract and digestive system of tinfoil barb in response to dietary probiotics.

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