Interaction of dietary *Pediococcus acidilactici* and folic acid on growth performance, haematological parameters and non-specific immune response of finger barbel, *Acipenser nudiventris*

Delsoz N.[1]; Khara H.[1*]; Shenavar A.R.[2]; Mohseni M.[2]

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Abstract

This study was conducted to investigate the effects of dietary *Pediococcus acidilactici* and folic acid (FA) and their combinations on growth performance, haematological parameters and non-specific immune response of *Acipenser nudiventris*. 210 individuals (initial body weight: 12.84 ±1.53g) were fed seven practical diets: the basal diet as the control diet was supplemented with two levels of *P.acidilactici* (2 and 3 g per kg diet), FA (2 and 4 mg per kg diet) and their combinations. The results showed that the group fed a combined diet of *P. acidilactici* and FA (4 mg FA+3 g *P. acidilactici* per kg diet) showed significant increase in body weight increase (BWI), specific growth rate (SGR), feed conversion ratio (FCR) and condition factor (CF). However, experimental diets had no significant effects on survival rate. Both haematological indices and leucocyte counts were significantly (p<0.05) influenced by dietary *P. acidilactici* and FA and their combinations. In the group fed 4 mg FA+3 g *P. acidilactici*, lysozyme activity and total immunoglobulin (Ig) levels significantly increased compared to those fed other diets (p<0.05). Moreover, the fish fed diets supplemented with 2 mg FA+ 3 g *P. acidilactici* per kg diet had considerably higher immunoglobulin (IgM) level (p<0.05). Under the experimental conditions, dietary *P.acidilactici* and FA had a synergistic effect on enhancing growth performance and immunity of *A. nudiventris*.

Keywords: *Acipenser nudiventris*, Probiotic, Physiological measurements, Immune response

1- Department of Fishery, Lahijan Branch, Islamic Azad University, Lahijan, Iran, P.O. Box: 1616

2- International Sturgeon Research Institute, Iranian Fisheries Science Research Institute (IFSRI), Agricultural Research Education and Extension Organization (AREEO), Rasht, Iran, P.O. Box: 41635-3464

*****Corresponding author's Email: h.khara1974@yahoo.com

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Introduction

The demand for animal protein in the world is expected to increase progressively with each increase in human population. Aquaculture is one of the most important options for animal protein production and requires high quality feeds with high protein content as well as some complementary additives to keep organisms healthy and to favor growth. The supplementation of probiotics through feeds is the best method to reduce feed costs (Lara - Flores *et al.,* 2003). The use of probiotics as farm animal feed supplements dates back to the 1970.

Probiotics are recently used in aquaculture to promote growth rate, inhibit growth of pathogens (Rengpipat *et al.,* 1998) and improve feed efficiency, reproduction and health (Gatesoupe, 1999; Irianto and Austin, 2002; Marzouk*et al.,* 2008; Kesarcodi-Watson *et al.,* 2008).

The commonly used probiotics in fish culture practices belong to *Saccharomyces*, *Clostridium*, *Bacillus*, *Enteroccus*, *Lact obacillus*, *actococcus*,*Aeromonas* and several other genera (Ghosh *et al.,* 2008; Capkin and Altinok 2009; Boonthai *et al.,* 2011). *P. acidilactici* is a species of probiotic bacteria which belonged to the genus *Lactobacillus*. *P. acidilactici* has emerged as a potential probiotic that has improved growth efficiency and enhanced immune response (Perdigo´n *et al.,* 1988). In green terror, *Aequidens rivulatus*, use of dietary *P. acidilactici* improved growth and innate immunity parameters including lysozyme activity, total immunoglobulin and alternative complement activity (Neissi *et al.,* 2013). In tilapia, *Oreochromis niloticus*, dietary *P. acidilactici* has had a protective action on the intestinal mucosal cells, stimulating the innate immune response after feeding for a period of six weeks (Standen *et al.,* 2013).

Vitamins including folic acid are used widely in aquaculture as immunostimulants and are important in modulating the fish immune system. Folic acid deficiency has resulted in appropriate growth (Smith 1968); health (Smith 1968; Esmaeili and Khara 2014) and pathological symptoms (Smith 1968) in cultured fish. 2.5 mg kg^{-1} folic acid had positive effects on growth, feed efficiency and immunity (total immunoglobulin) of rainbow trout, *Oncorhynchus mykiss* (Esmaeili and Khara, 2014). Again in rainbow trout, dietary folic acid resulted in an improvement in immunological functions (Abbasian *et al.,* 2013).

To the best of our knowledge, there are no studies on the use of *P. acidilactici* as a dietary probiotic in sturgeons especially in barbel sturgeon, *A. nudiventris*. This is also the case with folic acid. The barbel sturgeon, *A. nudiventris* is a critically endangered anadromous species. The rearing of these species has seen considerable progress in the past years. Despite the economic importance of barbel sturgeon, there is little information on

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its nutrient requirements. Thus, in the present study, we decided to examine the effects of dietary *Pediococcus acidilactici* and folic acid (FA) and their combinations on growth performance, haematological parameters and non-specific immune response in *Acipenser nudiventris*.

Materials and methods

Experimental fish

The experiment was carried out in Shahid Beheshti Sturgeon Propagation and Rearing Center, Rasht, Iran. The experimental fish were acclimatized at ambient laboratory conditions for 15 days and starved for 24 h before the beginning of the feeding trial. A total of 210 fish were used during the experiment (30 fish per treatment) which was distributed randomly in 21 fiberglass tanks. Each treatment included 3 replicates (tank) with 10 fish stocked in each tank. Each tank was supplied with an air pump with air stones for aeration. Tap water was stored for 24 hours in fiberglass tanks for dechlorination. Tanks were filled after replacing 100% of water, daily. Water temperature (via a thermometer) was measured daily and the average was between 16-18 °C during the experimental period. Water quality parameters were measured weekly. The pH (using WTW, Model PH330i-pH-meter) and dissolved oxygen (using WTW, Model PH330i-dissolved oxygen meter) were also determined.

Food preparation

A commercial probiotic product entitled Bactocell containing *Pediococcus acidilactici* (Lallemand Company, Toulouse, France) and the FA (Dae Jung Chemical Company, Korea) [25 g FA ($C_{19}H_{19}N_7O_6$)] as FA donor were added to test diets. The proportion of each ingredient required was calculated precisely providing allowance for the premix (Table 1). The dough was steam cooked and cooled to room temperature. Then, different concentrations of *P. acidilactici*, FA and their combinations were mixed with the dough to provide seven treatments as follows:T1(4 mg FA per kg diet), T2 (3 g *P. acidilactici* per kg^{-1} diet), T3 (4 mg FA+2 g *P. acidilactici* per kg^{-1} diet), T4 (2 mg FA+ 3 g *P. acidilactici* per kg^{-1} diet), T5 (2 mg FA+ 2 g *P. acidilactici* per kg^{-1} diet), T6 (4 mg FA+3 g *P. acidilactici* per kg^{-1} diet) and T7 which served as the control (0% of probiotic and FA). The diets were pelletized by pressing through 2 mm diameter pelleting unit. The pellets were dried in a drying oven at 40^{o}C for 24 hours and stored at – 4^{o}C until use during the trial to avoid oxidation and rancidity. The proximate body composition (moisture, crude protein, crude lipid, and ash) was determined using the standard methods of the Association of Official Analytical Chemists (AOAC, 1995).

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Table 1: Composition of the experimental diet (dry weight).

Ingredients	**% kg^{-1} dry weight**
Fish meal	40
Wheat meal	20
Corn meal	5
Fish oil	6.5
Soybean meal	20
Beet molasses	2
Salt	1
Vitamin mixture [a]	2
Mineral mixture [b]	1
Lysine	1.5
Methionine	1.5
Proximate composition	%
Crude protein	40
Crude lipid	9
Moisture	5.5
Ash	55.2
Crude Energy (kcal kg^{-1})	1897

[a] Vitamin mixture was manually provided according to feed requirements of the fish and ingredients were obtained from Science Laboratories (Ghazvin, Iran); which each 1000 g vitamin mixture provides vitamin A, 1,600,000 I.U; vitamin D3, 400 000 I.U; thiamin, 6 g; riboflavin, 8 g; niacin, 12 g; pantothenic acid, 40 g; pyridoxine, 4 g; folic acid, 2 g; cyanocobalamin, 8 mg; vitamin C, 60 g; vitamin K3, 2 g; biotin, 240 mg and inositol, 20 g.

[b] Aquatic mineral premix, for cold and warm water fish, is manufactured by Science Laboratories (Ghazvin, Iran); which each 1000 g contains mineral trace elements such as ferrous, 6000 mg; zinc, 10 000 mg; selenium, 20 mg; cobalt, 100 mg; copper, 600 mg; magnesium, 5000 mg; iodine, 600 mg. In addition, choline chloride (6000 mg), which is vital for fish and cannot be combined with other vitamins, is also included in mineral premix.

Protein by estimating the Kjeldahl nitrogen (6.25×), moisture by heating at 105ºC to constant weight, ash by incinerating in a crucible at 600ºC for 18 h and crude lipid was determined by using Soxhlet apparatus.

Feeding trial

During 60 days, *A. nudiventris* with an initial weight of 12.84 ±1.53g was used for the feeding trial. The fish were randomly distributed in twenty one 400 L fiberglass tanks (30 per tank in each triplicate) continuously aerated in order to maintain the optimal oxygen level. Then experimental fish were fed with experimental diets in each treatment. Feeding operation was done two times a day (9:00 am and 9:00 pm). The daily amount of feed was calculated based on 5% of body weight. Unfed feed and feces were collected daily after feeding.

Growth parameters

After the feeding trial, the growth parameters including survival rate, body weight gain (BWI), specific growth rate (SGR), feed conversion rate (FCR), condition factor and survival rate (%) was individually determined by the

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following equations (Tekinay and Davis, 2001):

FCR = dry feed intake (g)/wet WG (g)

SGR (% day^{-1}) = (Ln W_f - Ln W_i) × 100/t

CF = 100 × [wet weight (g)/TL $(cm)^3$]

Where W_f and W_i were final and initial fish weights, respectively; TL was total length and t is the experimental duration in day.

Blood analysis

The fish from each tank were quickly captured and sampled after being anesthetized. Then, blood samples were collected from the caudal vein of each fish using a 2 mL heparinized syringe. Blood samples were divided into two portions. Half of the blood was used for separating plasma from it and the remaining was used for hematological analysis. Plasma was separated by centrifugation (1500 × g for 10 min) and stored at -80 ºC until further analysis. Hemoglobin concentration was determined using the cyanmethemoglobin method. The numbers of white blood cells (WBC), red blood cells (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration were calculated according to Ranzani-Paiva *et al.* (2004). To determine differential count of leukocyte, that is, measure of lymphocyte, neutrophil, eosinophil and monocyte, the obtained smears were first air dried, fixed in 96% ethanol for 30 min, stained by Giemsa staining for 30 min and were examined for leukocyte differential count under light microscope (Klontz, 1994). Lysozyme level was determined by turbidometric assay according to the method of Sankaran and Gurnani (1972) with slight modifications. Aliquots (1.75 mL^{-1}) of *Micrococcus lysodeikticus* suspension (Sigma) (0.375 mg mL^{-1}, 0.05 M PBS, pH 6.2) were mixed with 250 mL^{-1} of each sample and the optical density was measured after 15 and 180s by spectrophotometer (Biophotometer Eppendorf) at 670 nm. PBS was used as the blank and results were expressed in amounts of lysozyme (mg) per 1 mg of sample calibrated using a standard curve determined with hens egg white lysozyme (Sigma) in sterile sodium phosphate buffer. The serum IgM was measured according to Fuda *et al.* (1991). Antisera for fish were prepared by immunizing rabbits as previously described by Fuda *et al.* (1991). The procedure for labeling antibody fragment with enzyme was performed. Plasma total immunoglobulin (Ig) content was determined following the method of Puangkaew *et al.* (2004) with slight modification.

Statistical analyses

One way analysis of variance (ANOVA; SPSS, 13.0) was used to determine whether significant variation between the treatments existed. Differences between means were determined and compared by post hoc multiple comparison tests. All the tests used a significance level of $p<0.05$.

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Data are reported as means ± standard deviations.

Results

Data presented in Table 2 indicated that the highest final body weight was obtained in T6 group (60± 2.66 g) which received the diet supplemented with 4 mg FA+3 g *P. acidilactici* followed by T4 (58.1±3.3 g), T2 (56.4±4.14 g) and T1 (54.5±8.49 g) groups. The lowest values were obtained in the control. The results showed that FCR, SGR, BWI and CF were significantly affected by *P. acidilactici* and FA supplementation (Tables 2 and 3). During the whole experimental period, T6 fish produced the lowest FCR (1.08±0.011) and also had highest SGR (3.18±0.04) and BWI (347.2±9.83%).The values of survival rate and condition factor were higher in fish supplemented with *P. acidilactici* and FA compared with the control (Table 3, $p<0.05$). Data in Table 4 showed significant ($p<0.05$) increase in white blood cell (WBC), red blood cell (RBC), hemoglobin (Hb) and hematocrit (Ht) in fish supplemented with a *P. acidilactici* and FA compared with the control. All immune parameters showed significant ($p>0.05$) difference among dietary treatments. Lysozyme activity and Ig level were higher in T6 which received diet supplemented with 4 mg FA+3 g *P. acidilactici* compared with the other treatments (Figs. 1 and 2), whereas IgM content of fish fed 2 mg FA+ 3 g *P. acidilactici* per kg^{-1} diet (T4) was significantly ($p<0.05$) higher than that of the other groups.

Table 2: Combined effects of dietary *Pediococcus acidilactici* and folic acid on the growth performance of *Acipenser nudiventris*.

Treatments	Initial body weight	Final body weight	BWI (%)	FCR	SGR
T 1	12.48±1.53	54.5±8.49	327.9±9.98 ^{c}	1.13±0.017 ^{a}	2.97±0.047 ^{c}
T 2	12.38±1.41	56.4±4.14 ^{de}	340.5±6.93 ^{c}	1.11±0.015 ^{a}	3.03±0.03^{cd}
T 3	12.39±1.24	50.7±7.4 ^{bc}	288.3±19.08 ^{b}	1.21±0.036 ^{b}	2.77±0.099^{b}
T 4	12.36±1.19	58.1±3.3 ^{de}	358.6±26.23 ^{cd}	1.09±0.006 ^{a}	3.11±0.11 ^{cd}
T 5	12.40±1.62	48.9±7.39 ^{ab}	278.3±27.53 ^{ab}	1.26±0.074 ^{b}	2.71±0.15 ^{ab}
T 6	12.43±1.58	60±2.66 ^{e}	347.2±9.83^{d}	1.08±0.011 ^{a}	3.18±0.04 ^{d}
Control	12.49±1.73	45.5±5.06 ^{a}	255.2±7.16 ^{a}	1.34±0.019 ^{c}	2.59±0.041^{a}

The values with different letters in the figure are significantly different ($p<0.05$).

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Table 3: Combined effects of dietary *Pediococcus acidilactici* and folic acid on CF and survival rate.

Treatments	Condition factor (CF)	Survival rate (%)
T 1	0.52 ± 0.01^{bc}	82.5 ± 5.77^{ab}
T 2	0.53 ± 0.005^{c}	84 ± 1.67^{a}
T 3	0.53 ± 0.03^{c}	86 ± 1.67^{b}
T 4	0.48 ± 0.02^{abc}	87 ± 3.33^{ab}
T 5	0.49 ± 0.03^{abc}	81 ± 3.33^{a}
T 6	0.44 ± 0.02^{a}	87 ± 1.67^{ab}
Control	0.47 ± 0.06^{ab}	78 ± 2.54^{c}

The values with different letters in the figure are significantly different ($p<0.05$).

Table 4: Combined effects of dietary *Pediococcus acidilactici* and folic acid on haematological indices of *Acipenser nudiventris*.

Treatments	WBC ($\times10^3$mm)	RBC ($\times10^6$)	Hb (g/dL)	Ht (%)	MCV (fl)
T 1	65.25 ± 6.7^{ab}	8.03 ± 2.6^{b}	6.7 ± 0.17^{b}	33 ± 0.82^{b}	441.25 ± 5.91^{a}
T 2	89.2 ± 17.08^{cd}	8.6 ± 4.02^{c}	7.2 ± 0.26^{c}	35.7 ± 1.71^{c}	412.75 ± 5.56^{a}
T 3	81 ± 18.2^{bc}	7.9 ± 3.4^{b}	6.6 ± 0.30^{b}	33.5 ± 1.29^{b}	419.75 ± 3.4^{ab}
T 4	105.7 ± 6.7^{d}	8.7 ± 4.2^{c}	7.3 ± 0.36^{c}	37.2 ± 1.71^{c}	424.75 ± 3.9^{b}
T 5	130.2 ± 19.3^{e}	8.05 ± 1.9^{b}	6.7 ± 0.17^{b}	33.2 ± 0.96^{b}	412.5 ± 6.35^{a}
T 6	131.7 ± 20.3^{e}	8.9 ± 4.3^{c}	7.4 ± 0.3^{c}	37.2 ± 1.26^{c}	423.75 ± 10.44^{b}
Control	62.2 ± 8.5^{a}	6.5 ± 3.4^{a}	5.4 ± 0.26^{a}	27.5 ± 1.36^{a}	417.25 ± 0.06^{ab}

The values with different letters in the figure are significantly different ($p<0.05$).

Table 5: Combined effects of dietary *Pediococcus acidilactici* and folic acid on differential count of leukocyte of *Acipenser nudiventris*.

Treatments	MCH (pg)	MCHC (g/dL)	Lymphocyte (%)	Neutrophil (%)	Monocyte (%)
T 1	84 ± 0.82^{e}	8.9±4.3	131.7 ± 20.3^{b}	8.9 ± 4.3^{a}	131.7 ± 20.3^{a}
T 2	83.2 ± 0.96^{a}	8.9 ± 4.3^{a}	131.7 ± 20.3^{a}	8.9 ± 4.3^{bc}	131.7 ± 20.3^{a}

The values with different letters in the figure are significantly different ($p<0.05$).

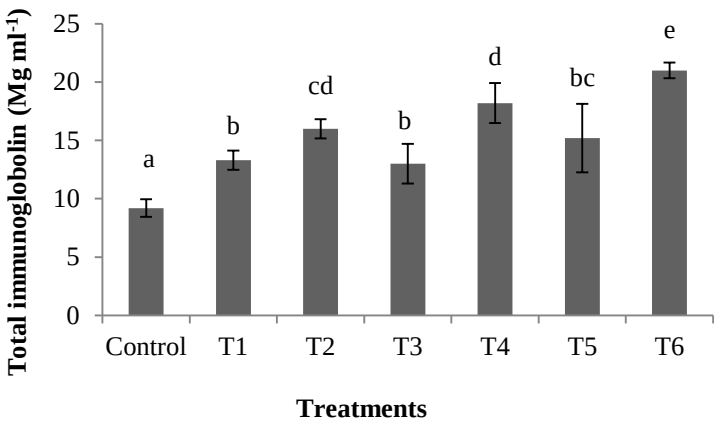


Figure 1: Plasma Ig level of *Acipenser nudiventris* fed the experimental diets for 60 days.

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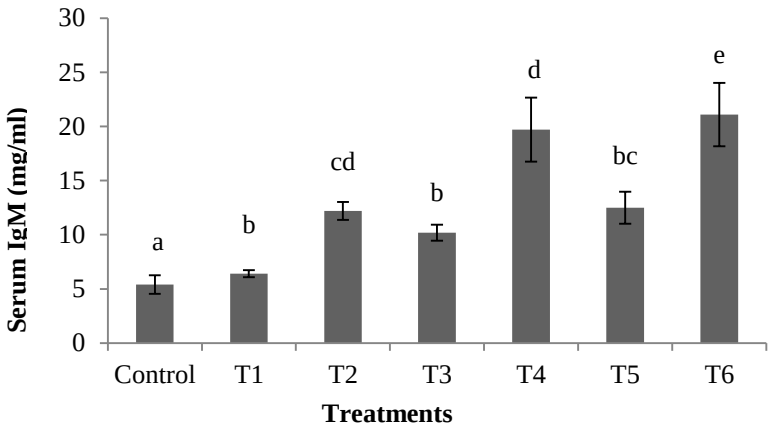


Figure 2: Plasma IgMlevel of *Acipenser nudiventris* fed the experimental diets for 60 days.

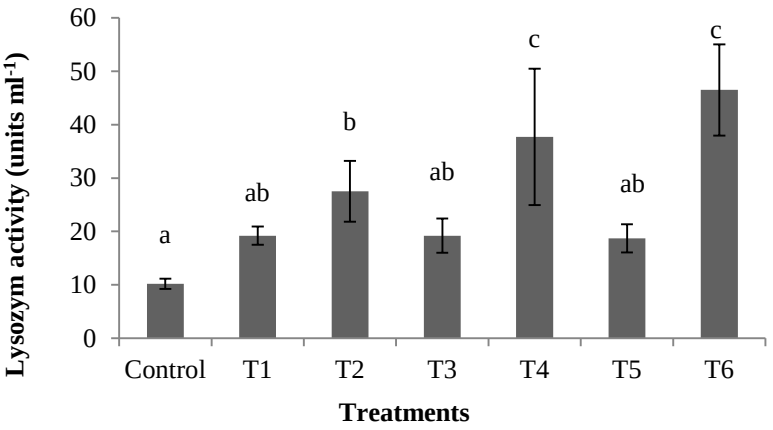


Figure 3: Lysozyme activity of *Acipenser nudiventris* fed the experimental diets for 60 days.

Discussion

Several studies on probiotics have been published in recent years which suggest that probiotics provide nutritional benefits (Balca´zar*et al.,* 2006; Kesarcodi-Watson *et al.,* 2008; Merrifield *et al.,* 2010; Naseri *et al.,* 2013; Zare *et al*., 2017). In the present study for first time, the effects of probiotic, *P. acidilactici*, folic acid (FA) and their combinations were investigated on three biological aspects of barbel sturgeon including Growth performance and survival, haematological and immunological parameters:

Growth performance and survival

In this study, we observed a tendency in fish fed a probiotic-supplemented diet towards better growth performance which may be due to the several mechanisms stimulated by *P. acidilactici* including increasing nutrients availability for absorption and activity of digestive enzymes in gastrointestinal tract (Doeschate and Coyne, 2008). As a result, it increases

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availability of nutrients and digestives. Better digestibility and absorption capacity could cause an increasing trend in growth indices (and survival) or a decreasing trend in FCR (Goldin 1998; Doeschate and Coyne 2008; Ghosh *et al.,* 2008). Previous studies also support this fact for *P. acidilactici* in larval Pollock, *Pollachius pollachius* when fed with artemia feed enriched with *P. acidilactici* (Gatesoupe, 2002) and carp fed with the probiotic *Streptococcus faecium* (Noh *et al.,* 1994). However, no influences have been observed (Gatesoupe, 2002; Shelby *et al.,* 2007; Ferguson *et al.,* 2010; Zhou *et al.,* 2010) in some studies that might be attributed to the differences in concentration of probiotics added to the diet, the bacterial strains, experimental design and the species of organisms examined. In our study, higher survival rate and growth were observed in fish fed diets supplemented with FA or FA+*P. acidilactici*. Similar results were found in grouper, *Epinephelus malabaricus* (Lin *et al.,* 2011). Stimulated growth by FA is attributed usually to the role of this vitamin in enhancing oxygen transporting capacity in blood through participating in formation of red blood cells and thus maintaining the metabolism in normal state (Hosokawa 1989). In addition to this, numerous studies have shown that microorganisms are a significant source of folic acid for some species including common carp, *Cyprinus carpio* (Kashiwada *et al.,* 1971) and channel catfish (Duncan *et al.,* 1993). It seems that probiotic in combination with FA in diets may improve nutritional and physiological function, but further studies are needed to prove this synergic effect.

Haematological parameters

Haematological indices are considered as valuable tools for assessing fish health and are reported to be effected by dietary probiotic (Irianto and Austin, 2002). In this study, significant (p<0.05) increase in WBC, RBC, Hb and Ht in fish fed the probiotic and FA-supplemented diet compared to the control. On the effects of probiotics on haematology different results have been reported. Dias *et al*. (2012) found no differences in RBC, MCV and Hb concentration in *Brycon amazonicus* breeders in relation to dietary supplementation with B. subtilis, while Faramazi *et al*. (2011) found higher RBC, Hb and Ht. This suggests that fish fed probiotic-supplemented diets are healthier than controls, possibly because of decreased blood cortisol levels, as reported by Carnevali *et al*. (2006) and Rollo *et al*. (2006) in sea bream (*Sparus aurata*). In our study, increases in WBC in fish fed probiotic-supplemented diets may be due to the stimulation of immunity by bacteria, *P. acidilactici*as reported in relation to other dietary probiotic bacteria (Borchers *et al.,* 2009). Ferguson *et al.,* (2010) showed that significant increases in the total number of leucocytes were observed in tilapia fed *P. acidilactici* supplemented diets. However, contrary

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to these findings, other probiotic studies observed either no differences (Aly *et al.,* 2008a, El-Rhman *et al.,* 2009) or elevated haematocrit levels (Aly *et al.,* 2008b), but the reason for this is not clear. Anemia was observed in relation to dietary FA deficiency in some examined fish (Smith 1968; Hosokawa 1989). This fact was reinforced when we observed more RBC, Hb and Ht in FA- supplemented fish compared to the control group. It was confirmed that FA has an important role in production of blood cells of fish species (Hosokawa, 1989; Abbasian *et al.,* 2013).

Immunological parameters

Probiotics act as the stimulators of innate immune response and in this regard, the primary effect of *P. acidilactici* on the immune system of barbel sturgeon was evaluated based on two indices including total immunoglobulin and lysozyme activity (Rengpipat *et al.,* 2000; Heyman and Me´nard, 2002; Taoka *et al.,* 2006; Nayak, 2010). In the present study, the IgM and lysozyme levels showed significantly higher values in fish fed with combined probiotic and FA groups compared to the control. This confirms that the probiotic bacteria can stimulate the antibody production and lysozyme activity in fish (Panigrahi *et al.,* 2004). Our results were in accordance with those reported with *L. rhamnosus*, *Carnobacterium maltaromaticum*, *Carnobacterium divergens* in rainbow trout (Panigrahi*et al.,* 2004; Kim and Austin, 2006), *P.acididlactici* in red tilapia, *Oreochromis niloticus* (Ferguson *et al.,* 2010), *B. subtilis* in *Labeo rohita* (Nayak *et al*., 2007) and yeast cells in seabream, *Sparusaurata* (Cuesta *et al.,* 2004).

In contrast, diets containing *Aeromonas sobria* fed to *O. mykiss* (Brunt *et al*., 2007) and to tilapia, *Oreochromis niloticus* (Wang *et al.,* 2008) failed to improve the level of lysozyme. These differences in results can be caused by various factors like source, type, dose and duration of supplementation of probiotics which can significantly affect the immunomodulatory activity of probiotics (Nayak, 2010).

In general, in the probiotic and FA combined group the level of various immune parameters was significantly enhanced compared to the individual probiotic and FA treated groups. This might be due to the establishment of the *P.acididlactici* in the gastrointestinal tract of *A. nudiventis* because the co administration of FA along with probiotic bacterium might have promoted the growth of other gut bacteria, which either utilized or reduced the FA.

Generally, the results of the present study indicated clearly that the supplementation of *P. acididlactici* and FA enhanced not only the growth performance of *A.nudiventis*, but also the non-specific immune responses. Further studies regarding the application of *P. acididlactici* and FA in aquatic animals, especially their appropriate inclusion levels in specific

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species and specific rearing conditions, are needed.

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