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Dietary supplementation effects of *Pediococcus acidilactici* as probiotic on growth performance, digestive enzyme activities and immunity response in zebrafish (*Danio rerio*)

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Abstract

This study was carried out to assess the effects of *Pediococcus acidilactici* on zebrafish (*Danio rerio*). Different levels of *P. acidilactici* including 0, 1×10^6, 2×10^6, 4×10^6 and 8×10^6 colony-forming unit per g of the diet ($cfug^{-1}$) were examined in fish with 120 ± 10 mg weight for 60 days in a completely randomized design. The results showed that the best growth indices were recorded in group 4×10^6 $cfug^{-1}$ ($p < 0.05$). The highest number of total viable count and lactic acid bacteria of intestine were found in group 4×10^6 $cfug^{-1}$ ($p < 0.05$). The maximum activity of digestive enzymes including amylase, lipase, protease and alkaline phosphatase was observed in group 4×10^6 $cfug^{-1}$. The highest activity for superoxide dismutase was recorded in group 4×10^6 $cfug^{-1}$ while catalase, glutathione peroxidase and glutathione reductase showed the highest activity in group 8×10^6 $cfug^{-1}$. The most growth inhibition zone of *Aeromonas hydrophylla*, *Flavobacterium columnare*, *Vibrio anguillarum* and *Edwardsiella tarda* was found in group 4×10^6 $cfug^{-1}$ ($p < 0.05$). Therefore, *P. acidilactici* as a probiotic improved growth and immunity of the zebrafish and could be used by zebrafish farmers.

KEYWORDS

Danio rerio, dietary administration, digestive enzymes, growth, immunity, *Pediococcus acidilactici*

1 | INTRODUCTION

Probiotics are living microorganisms that can improve health condition of the host through amending microbial population (Fuller, 1989). Nowadays, it has been proved that probiotics decrease the need to antibiotics by improving microbiota of the digestive system, secretion of antibacterial substances such as bacteriocins and organic acids, competition with pathogenic agents for sticking to the digestive system and competence with pathogens for nutrients (Allameh, Ringø, Yusoff, Daud, & Ideris, 2015; Carnevali, Maradona, & Gioacchini, 2016; Eslamloo, Falahatkar, & Yokoyama, 2012). On the other hand, it has been shown that probiotics have positive effects on fecundity, gonadosomatic index (GSI), survival rate, (SR), weight gain (WG), length (L), reduction in dead and deformed larva (Ashouri et al., 2018; Gioacchini et al., 2014), and improvement in the larval ontogeny (Avella et al., 2011; Carnevali et al., 2006).

According to the current literature, many studies have been reported on the effects of different probiotic bacteria on fecundity (Gioacchini et al., 2010), backbone calcification and gonadal differentiation (Avella et al., 2012), immunity and stress responses (Gioacchini et al., 2014) on zebrafish. Also, lactic acid bacteria (LAB) of intestine (Rane & Markad, 2015) and offspring immunity (Qin et

al., 2014; Safari, Paolucci, & Motlagh, 2017) had been affected by dietary probiotics in this fish. But there are a few studies about the effects of *Pediococcus acidilactici* on zebrafish.

Pediococcus acidilactici is Gram-positive cocci which grow in an extensive range of pH, temperature and osmotic pressure, and can stick to the gastrointestinal tract of fish and colonize there (Hoseinifar, Mirvaghefi, & Merrfield, 2011). The efficiency of this bacterium in improving growth and immunity of the cultured species had been showed in previous studies. Neissi, Rafiee, Nematollahi, and Safari (2013) showed the effectiveness of *P. acidilactici* diet supplementation on the non-specific immune responses and growth of green terror, *Aequidens rivulatus*. Also, using *P. acidilactici* led to higher weight gain (WG) and less feed conversion ratio (FCR) in *Oscar astronautus* (Safari & Mehraban, 2013) and *Litopenaeus vannamei* (Ahmadi et al., 2014). Castex, Lamaire, Wabete, and Chim (2010) found the positive effects of *P. acidilactic* on antioxidant defence, reduction in oxidative stress and survival increment of *Litopenaeus stylirostris* challenged with *Vibrio nigripulchritudo*. Also, positive effects of *P. acidilactici* on digestive system of *Oreochromis niloticus* had been reported (Ferguson et al., 2010).

Recently, ornamental fish production industry faces ongoing problems such as disease prevalence, antibiotic resistant, low quality of brood fish, egg and fry, and the lack of specific diet formulations. Zebrafish (*Danio rerio* Hamilton, 1822) which is also known as zebra danio belongs to the family of Cyprinidae and subfamily of Danioninae. Due to its fast growth, short period of reproduction, ease of keeping, feeding, producing and reproduction, this fish has become a desirable model for a number of biological investigations (Carnevali, 2014; Fishman, 2001; Gioacchini et al., 2014). The present study was aimed to assess the antioxidant enzymes and mucus bactericidal activity as important parts of immune system in zebrafish fed by different dietary levels of *P. acidilactici*. In addition, the effect of the *P. acidilactici* on the gastrointestinal microbiota, TVC and LAB in the intestine, digestive enzyme activity in fish intestine and growth performance was also examined.

2 | MATERIALS AND METHODS

2.1 | Diet preparation

A completely randomized design study including five treatments, with triplicates for each, was used during 60 days' trial using *P. acidilactici* (Bactocell, CNCM-MA 18/5 M, Lallemand, France). The EXS2 commercial feed (Kimiagaran-e-Taghziye, Iran) was used as basal diet. The size of the pelleted diet was 0.4–0.7 mm. The diet components (AOAC, 1990) are presented in Table 1. The experimental treatments were included *P. acidilactici* with various levels of 1×10^6, 2×10^6, 4×10^6 and 8×10^6 colony-forming unit in gram of feed ($cfug^{-1}$) and control group not included *P. acidilactici*. Based on the declaration of the probiotic manufacturing company, the weight of each 10^{10} cfu of the probiotic stock was one gram. So for each treatment, the necessary probiotic was calculated, precisely weighted, spread on the feed and mixed manually. The experimental diets were prepared weekly and kept in refrigerator at 4°C. Viability of the bacteria during the experiment was controlled by culturing random samples of the probiotic stock in the MRS broth media, during the study period (Ashouri et al., 2018).

TABLE 1 Composition and proximate analysis of diet (g/kg of dry matter basis)

	g/kg
Ingredient	
Fish meal	500
Meat powder	50
Corn gluten	150
Soybean meal	150
Vegetable oil	50
Fish oil	50
Mineral premix[a]	15
Vitamin premix[b]	15
Methionine	5
Lysine	5
Anti fungi	5
Antioxidant	5
Chemical composition (g/kg dry matter basis)	
Dry matter	930
Crude protein	500
Crude lipid	150
Ash	100
Carbohydrates	180

[a]Mineral premix (mg/kg) contains the following: Mg, 100; Zn, 60; Fe, 40; Cu, 5; Co, 0.1; I, 0.1; and BHT, 100. [b]Vitamin premix (mg/kg) contains the following: E, 30; K, 3; thiamine, 2; riboflavin, 7; pyridoxine, 3; pantothenic acid, 18; niacin, 40; folacin, 1.5; choline, 600; biotin, 0.7; and cyanocobalamin, 0.02.

2.2 | Experimental design

Six hundred healthy zebrafish with mean weight of 76 ± 5 mg and length of 12 ± 1 mm were supplied from Gholdasi aquarium centre in Isfahan, Iran. During the adaptation period, there was no mortality and fish health checked visually. After two weeks of adaptation, the fish were randomly assigned to 15 experimental aquariums with 50 L total volume and 30 L of water (n = 40).

The initial weight and length of the fish at the beginning of the experiment reached to 120 ± 10 mg and 15 ± 1.5 mm, respectively. The fish were fed for 60 days (Hoseinifar, Mirvaghefi, Amoozegar, Sharifian, & Esteban, 2015) by experimental diets at 10% of the body weight, three times per day, following the animal ethic for in vivo experiments developed by Khorramshahr University of Marine Science and Technology. The fish were kept under 14-hr lightness and 10-hr darkness. A weekly water exchange rate of 50% of the volume of the reservoirs was carried out. Temperature was 28 ± 2°C, dissolved oxygen 7.3–8.8 mg/L, pH 7.5–8.5, total

hardness 310 ± 10 mg/L, nitrite 0.03 ± 0.01 mg/L and nitrate 3 ± 1 mg/L.

2.3 | Growth assessment

At the end of the experiment, the fish were randomly taken by using a net, anaesthetized by 200 mg/L clove powder, and then, biometry was done. Fish were weighed with 0.001 g precision, and their length was measured using a biometric ruler with 1 mm precision. Growth performance was assessed according to the following formulas (Abedian Amiri, Azari Takami, Afsharnasab, & Razavilar, 2017):

$$\text{Weight gain } (\text{WG, mg}) = W_2 - W_1;$$

$$\text{Weight gain per cent } (\text{WG, \%}) = (W_2 - W_1) \times 100/W_1;$$

$$\text{Specific weight growth rate } (\text{Weight, SGR, \%/day}) = (\text{Ln}\, W_2 - \text{Ln}\, W_1) \times 100/t;$$

$$\text{Specific length growth rate } (\text{Length, SGR, \%/day}) = (\text{Ln}\, L_2 - \text{Ln}\, L_1) \times 100/t;$$

$$\text{Food conversion ratio } (\text{FCR}) = \text{feed intake (mg)} / \text{Weight gain (mg)};$$

where W_2, W_1, t, L_2 and L_1 are final weight (mg), primary weight (mg), the experiment period (day), final length (mm) and primary length (mm), respectively.

2.4 | Bacteriological analysis

At the end of experiment, nine fish from each treatment (three individuals per aquarium) were randomly selected and transferred to the laboratory, euthanized with 500 mg/L clove powder, rinsed with sterile distilled water and dissected by a sterile scalpel under disinfection conditions. Then, the intestine was completely removed, weighed and manually homogenized with physiological serum (sodium chloride 0.9 w/v) using a sterile mortar (Hoseinifar, Mirvaghefi, Merrfield, & Ringø, 2017). One hundred microlitres from the prepared homogenate was spread onto plate count agar (PCA; Merck Co) and de Man, Rogosa and Sharpe agar (MRS; Merck Co) in three replicates to determine the total viable count (TVC) and the lactic acid bacteria (LAB) concentration in the alimentary tract, respectively. The plates were incubated for 24 (TVC) and 24-72 (LAB) hours, and colony-forming units were calculated in the plates contained 30-300 colonies (Hoseinifar et al., 2017).

2.5 | Digestive enzyme activities

The live fish (nine individuals per treatment) were transferred to the laboratory, euthanized with 500 mg/L clove powder and dissected with scalpel. The intestine was removed, rinsed using distilled water, dried with paper towels, homogenized with 30 g/70 ml distilled water using a homogenizer (DI 18 Disperser). Then, the samples were centrifuged at 10,000 *g* for 25 min at 4°C (Safari & Paolucci, 2017), and the supernatant was stored in liquid nitrogen. All intestine tissues were collected one by one without pooling.

The amylase activity was measured using starch as the substrate at 550 nm optical density (OD) with UV/Vis spectrophotometer (Pharmacia Biotech Ultrospec, 2000; Coccia, Varricchio, & Paolucci, 2011). Lipase activity was measured using α-naphthyl caprylate as substrate at 540 nm (López-López, Nolasco, & Vega-Villasante, 2003). Protease activity was evaluated using azocasein as substrate at 366 nm (Fernández Gimenez, García-Carreño, Navarrete del Toro, & Fenucci, 2001). Alkaline phosphatase activity was assayed using diethanolamine and p-nitrophenyl phosphate as substrate at 405 nm (Fernández Gimenez et al., 2001). In the present study, specific enzyme activity was defined as enzyme unit (U) per milligram of tissue protein.

2.6 | Antioxidant enzyme activity

Antioxidant defence status was investigated in liver tissue. Catalase (CAT, E.C. 1.11.1.6) activity was determined in the homogenates by the decrease in absorbance at 240 nm (e = 40 M^{-1} cm^{-1}) using 50 mM H_2O_2 as substrate (Aebi, 1984). Superoxide dismutase (SOD, E.C. 1.15.1.1) activity was measured at 550 nm as the degree of inhibition of cytochrome c reduction by O_2^- generated by the xanthine oxidase/hypoxanthine system, according to Mc Cord and Fridovich (1969). The glutathione peroxidase (GPx, E. C. 1.11.1.9) activity was measured using Ransel kit (Randox, Ireland). Glutathione reductase (GR, E.C. 1.6.4.2) was measured using Stepien and Grazyna's (2005) method.

2.7 | Mucus bactericidal activity

The bactericidal activity of fish mucus samples was measured using diffusion disc plates on agar media (Bauer, Kirby, Sherris, & Turck, 1996). In order to evaluate bactericidal activity, four fish pathogenic bacteria, including *Aermonas hydrophila* (ATCC 7966), *Flavobacterium columnare* (ATCC 49512) *Vibrio anguillarum* (ATCC 19264) and *Edwardsiella tarda* (ATCC 15947), were used based on previous reports (Roberts, Palmeiro, & Weber, 2009; Trust & Bartlett, 1974). After culturing bacteria (24 hr at 37°C) in the nutrient broth medium (Merck, Germany), aliquots (0.1 ml) of each broth culture medium (1.5×10^8 $cfuml^{-1}$; OD600) were cultured on nutrient agar (Merck, Germany). Paper discs (6 mm diameter) were inoculated with 150 ml of the mucus sample and kept for 20 min to absorb the mucus, placed in the medium and incubated (37°C for 24 hr). Finally, the discs were checked and the diameter of the growth inhibition zone was measured with a calliper. A clear zone enveloping the discs was considered as bactericidal activity (Hoseinifar et al., 2015).

TABLE 2 Growth performance of zebrafish fed with diets containing different levels of *Pediococcus acidilactici* for 60 days (n = 3). Data are presented as mean ± *SE*

Growth indices	Control	1 × 10^9/CFU/kg	2 × 10^9/CFU/kg	4 × 10^9/CFU/kg	8 × 10^9/CFU/kg
Initial weight (mg)	120 ± 10^a	120 ± 10^a	120 ± 10^a	120 ± 10^a	120 ± 10^a
Final weight (mg)	371 ± 12^d	413 ± 7^c	503 ± 14^b	642 ± 8^a	635 ± 11^a
Weight gain (WG, mg)	251 ± 12^d	293 ± 7^c	383 ± 14^b	522 ± 8^a	515 ± 11^a
Weight gain (WG, %)	209 ± 10^d	244 ± 6^c	319 ± 11^b	435 ± 7^a	429 ± 9^a
Initial length (mm)	15 ± 1.5^a	15 ± 1.5^a	15 ± 1.5^a	15 ± 1.5^a	15 ± 1.5^a
Final length (mm)	25 ± 1.5^c	39 ± 1.0^b	40 ± 1.5^b	45 ± 1.5^a	43 ± 2.1^a
Special growth rate (Weight, SGR, %/day)	1.88 ± 0.05^d	2.06 ± 0.03^c	2.38 ± 0.04^b	2.79 ± 0.02^a	2.77 ± 0.03^a
Special growth rate (Length, SGR, %/day)	0.87 ± 0.09^d	1.59 ± 0.04^c	1.64 ± 0.06^{bc}	1.85 ± 0.05^a	1.76 ± 0.08^{ab}
Food conversion ratio (FCR)	3.75 ± 0.09^a	3.21 ± 0.07^b	2.68 ± 0.04^c	2.17 ± 0.05^d	2.24 ± 0.03^d

Note. Different letters in each row show significant difference (p < 0.05)

2.8 | Statistical analysis

All data have been expressed as mean ± *SE*. Normality and homogeneity of data were investigated using Shapiro–Wilk and Levene test, respectively. One-way analysis of variance (ANOVA) followed by Duncan post hoc was used to characterize significant difference among various groups as p < 0.05. All statistical analyses were carried out using IBM SPSS Statistics, version 23 (Safari & Paolucci, 2017).

3 | RESULTS

3.1 | Growth assessment

During the experiments, the fish ate the feed voraciously. The highest amount of WG, L and SGR was observed in the group 4 × 10^6 $cfug^{-1}$ (p < 0.05). These indices have an increasing trend until group 4 × 10^6 $cfug^{-1}$ and then have a slight decrease in group 8 × 10^6 $cfug^{-1}$. In the group 4 × 10^6 $cfug^{-1}$, these indices are about 2 times more than the control group at the end of the experiments. Also, the lowest FCR (2.17 ± 0.05) was obtained in this group (p < 0.05). This index has a decreasing trend from control until group 4 × 10^6 $cfug^{-1}$ and then has a serene increase in group 8 × 10^6 $cfug^{-1}$. This index in the group 4 × 10^6 $cfug^{-1}$ is about 1.6 less than the control group (Table 2).

3.2 | Bacteriological analysis

With the increase in *P. acidilactici* content, an increasing trend in TVC was recorded in all groups, and a serene decrease was observed in group 8 × 10^6 $cfug^{-1}$ again. The maximum value of TVC bacteria was in the group 4 × 10^6 $cfug^{-1}$ (2.88 ± 0.10 × 10^7 $cfug^{-1}$) while its least value was observed in the control group (2.20 ± 0.02 × 10^7 $cfug^{-1}$).

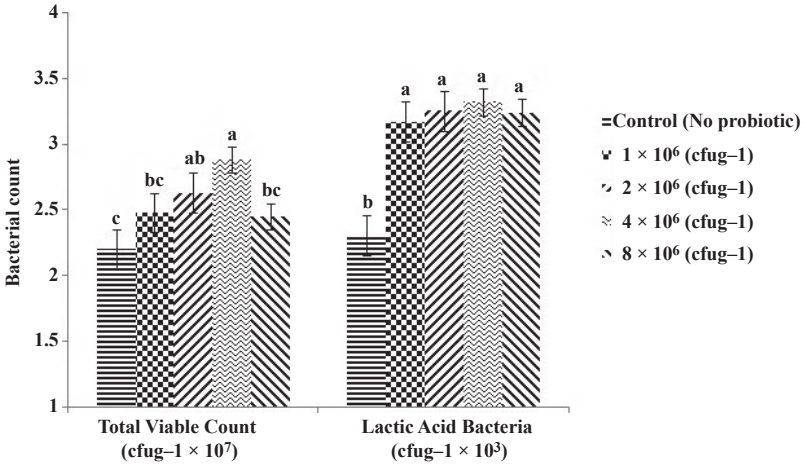

FIGURE 1 Total viable count (TVC) ($cfug^{-1}$ × 10^7) and acid lactic bacteria (LAB) ($cfug^{-1}$ × 10^3) in zebrafish intestine fed with diets containing different levels of *Pediococcus acidilactici* for 60 days in three replicates. Different letters in each column show significant difference between experimental groups (p < 0.05)

The highest content of LAB ($3.32 \pm 0.01 \times 10^3$ cfug^{-1}) was enumerated in the intestine of fish fed diets containing 4×10^6 cfug^{-1}, and the least content of LAB ($2.31 \pm 0.06 \times 10^3$ cfug^{-1}) was seen in the control group. In all of experimental groups, an increase in the LAB number was recorded in comparison with the control group ($p < 0.05$; Figure 1).

3.3 | Digestive enzyme activity

The highest activity of digestive enzymes including amylase (1.22 ± 0.01 U/mg protein), lipase (0.35 ± 0.01 U/mg protein), protease (1.30 ± 0.01 U/mg protein) and alkaline phosphatase (1.29 ± 0.01 U/mg protein) was observed in the group 4×10^6 cfug^{-1} ($p < 0.05$). The lowest activity of these enzymes was found in the control group ($p < 0.05$). The activity of the enzymes had an increasing trend until group 4×10^6 cfug^{-1} and then decreased at group 8×10^6 cfug^{-1} (Figure 2).

3.4 | Antioxidant enzymes activity

The highest activity of catalase (3.30 ± 0.01 U/mg protein), glutathione reductase (3.73 ± 0.13 U/mg protein) and glutathione peroxidase (3.20 ± 0.02 U/mg protein) was found in the group 8×10^6 cfug^{-1}. There was an increasing trend for these enzymes by increase in prebiotic dosage in diet. The superoxide dismutase (SOD) activity was significantly higher in the group 4×10^6 cfug^{-1} (1.03 ± 0.01 U/mg protein) in comparison with other experimental groups ($p < 0.05$). This enzyme increased to the group 4×10^6 cfug^{-1} and then decreased at the group 8×10^6 cfug^{-1} (Figure 3).

3.5 | Mucus bactericidal activity

The highest inhibition zone diameter of *Aeromonas hydrophylla* (5.08 ± 0.01 mm), *F. columnare* (2.23 ± 0.01 mm), *V. anguillarum* (1.97 ± 0.01 mm) and *E. tarda* (3.18 ± 0.03 mm) was measured in group 4×10^6 cfug^{-1}, and the lowest growth inhibition zone for all of the mentioned bacteria was found in the control group (0.93 ± 0.03, 0.87 ± 0.01, 0.43 ± 0.01 and 1.24 ± 0.11 mm, respectively), which were significantly different with the other groups ($p < 0.05$). For all of these bacteria, the inhibition zone increased until group 4×10^6 cfug^{-1} and then decreased in group 8×10^6 cfug^{-1} (Figure 4).

4 | DISCUSSION

The results showed that the diets including *P. acidilactici* led to an increase in the weight gain (WG), final length (FL) and specific growth rate (SGR), but it reduced the feed conversion ratio (FCR) in zebrafish. This finding is beneficial to ornamental fish producers; it can help them to reduce the costs and expense involved in reaching a desirable weight and size for marketing. These findings are similar to the previous findings in other species studied such as *L. vannamei* (Ahmadi et al., 2014) and *Oncorhynchus mykiss* (Abedian Amiri et al., 2017). As one of the main goals of the ornamental fish production industry is to increase growth performance, the inclusion of *P. acidilactici* in the diet is an effective way not only to reduce both the production period and costs, but also to improve fish health. This ability could be attributed to specific features of probiotics which either produce different enzymes, such as amylase, lipase, protease and alkaline phosphatase, in the digestive system, or stimulate the digestive system of the host to produce these enzymes (Eslamloo et al., 2012), resulting in better growth performance of fish (Carnevali et al., 2006) due to the enhanced absorption of different nutrients such as protein and lipid (Haroun, Goda, & Kabir, 2006). In the present study, the digestive enzyme activities were enhanced as reported by other authors as

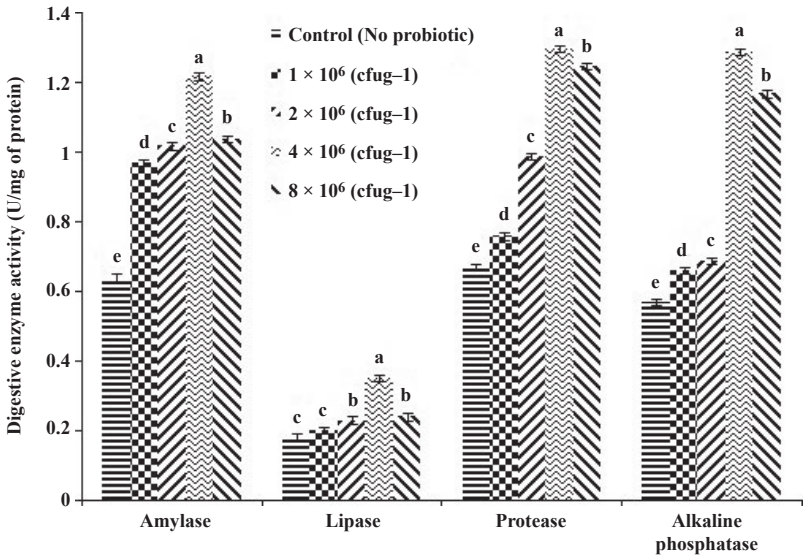

FIGURE 2 Activity of digestive enzymes (U/mg protein) in intestines of zebrafish fed with diets containing different levels of *Pediococcus acidilactici* for 60 days in three replicates. Different letters in each column show significant difference between experimental groups ($p < 0.05$)

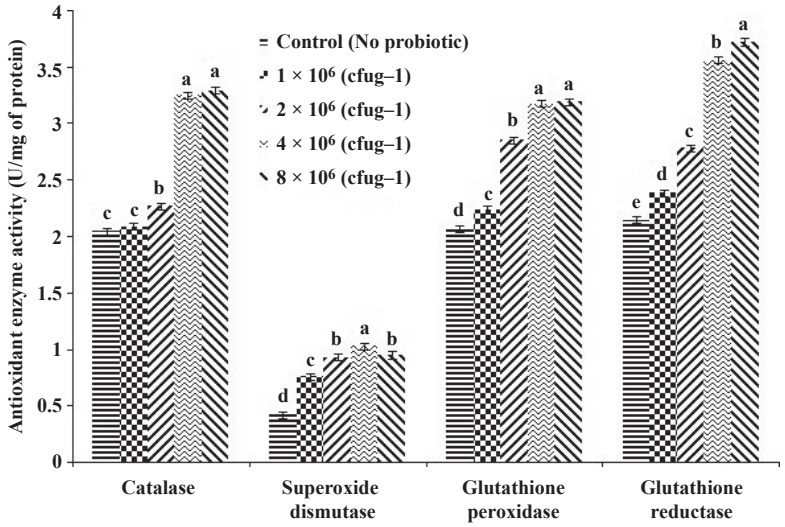

FIGURE 3 Activity of antioxidant enzymes (U/mg protein) in zebrafish liver fed with diets containing different levels of *Pediococcus acidilactici* for 60 days in three replicates. Different letters in each column show significant difference between experimental groups ($p < 0.05$)

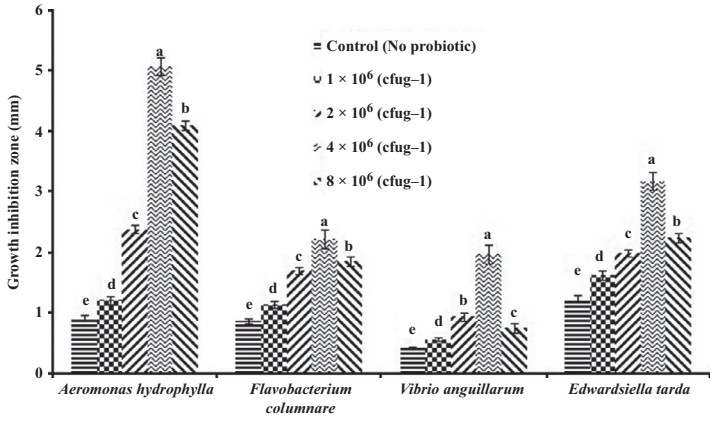

FIGURE 4 The mean (±*SD*) of growth inhibition zone (mm) of bacteria *Aeromonas hydrophylla*, *Flavobacterium columnare*, *Vibrio anguillarum* and *Edwardsiella tarda* in zebrafish mucus fed with diets containing different levels of *Pediococcus acidilactici* for 60 days in three replicates. Different letters in each column show significant difference between experimental groups ($p < 0.05$)

well (Carnevali et al., 2016; Ferguson et al., 2010). Thus, it can be concluded that the enhancement of digestive enzyme activity via improving the potential of digestion exerted a positive effect on the growth indices.

The current study also showed that fortified diet with *P. acidilactici* increased the TVC in the digestive system of zebrafish (especially in the group 4×10^6 cfug^{-1}), resulting in better digestion and absorption of feed as well as an increase in the growth. Similar to our findings, *P. acidilactici* has been reported to be able to improve microbial flora of alimentary tract of *O. niloticus* (Ferguson et al., 2010) and *O. mykiss* (Abedian Amiri et al., 2017). As shown in Figure 1, the increase in *P. acidilactici* dose resulted in TVC increase in the intestine of fish, showing an increment trend until 4×10^6 cfug^{-1}, but the TVC declined in group 8×10^6 cfug^{-1}. Thus, it seems that high doses of *P. acidilactici* have deleterious effects on the number of TVC. *Pediococcus acidilactici* increased the LAB content significantly in the experimental groups. The LAB produce lactic acid, stimulate the growth of other useful bacteria, outcompete with harmful bacteria and strengthen the natural defence system of the animals. The use of LAB as a feed supplement has been proposed as an alternative for improving health (Panigrahi et al., 2004). Rane and Markad (2015) showed the positive effects of lactic acid bacteria (LAB) on the growth and survival of zebrafish, a finding which is similar to what was found in this study.

Another positive aspect of probiotics is the reduction in oxidative stress. Generally, reactive oxygen species (ROS) are generated in aerobic metabolism. ROS destroy cell wall and tissues of the creatures and lead them to weakness or death (Najafpour, Salati, Keyvanshokooh, Yavari, & Pasha-Zanoosi, 2017). Probiotics are shown to be capable of increasing the activity of antioxidant enzymes (Castex et al., 2010). In the present study, the supplementation of *P. acidilactici* to the diet of zebrafish increased the activity of the antioxidant enzymes which, in turn, can increase the immunity of the fish and decrease the oxidative stress in them. Similar to our findings, Gioacchini et al. (2014) reported that dietary *Lactobacillus rhamnosus* (IMC 501) enhanced immunity and reduced the hepatic stress in the zebrafish. Probiotics may increase the activity of phagocytic. The production of reactive oxygen metabolites by macrophages resulted in an increase in the CAT activity (Panigrahi et al., 2004; Reyes et al.., 2008). SOD activity first increased until group 4×10^6 cfug^{-1} and then decreased in group 8×10^6 cfug^{-1}. Son et al. (2009) reported that SOD activity decreased in grouper, *Epinephelus coioides*, fed by *Lactobacillus plantarum*-supplemented diets.

They hypothesized that the decrease in SOD activity may have occurred because the diets supplemented by *L. plantarum* must have retained the superoxide anion level or converted it into the singlet oxygen ($^1O_2$) and/or hydroxyl radicals (OH·) via a metal-catalysed interaction as to enhance the microbial-killing capacity of phagocytes (Son et al., 2009).

According to Fuller (1989), probiotics could reduce the need to antibiotics through the production of antibacterial materials. The growth inhibition zone of *A. hydrophylla*, *F. columnare*, *V. anguillarum*, and *E. tarda* increased when fed with *P. acidilactic*-supplemented diet (Figure 4). Therefore, it can be claimed that using *P. acidilactic* provides mucus immunity against bacteria and decreases the risk of diseases. For *A. hydrophylla*, the growth inhibition zone was 5 mm. Thus, our findings indicate a high capability of *P. acidilactici* for tackling the pathogenic species of *A. hydrophylla*. Also, Lalloo, Ramchuran, Ramduth, Gorgens, and Gardiner (2007) found that the pathogenicity of *A. hydrophylla* in ornamental fish reduced when fed with *Bacillus* strains. For other bacteria used in this study, there was also an increase in the growth inhibition zone compared to the control group, which indicates the ability of *P. acidilactic* to kill these pathogenic bacteria. Contrary to the findings of the present study, Merrifield et al. (2011) found that the use of *P. acidilactic* had no effect on the growth of rainbow trout, Nile tilapia (Shelby, Lim, Yildirim-Aksoy, & Delaney, 2006) and Channel catfish (Shelby, Lim, Yildirim-Aksoy, & Klesius, 2007). These differences could be related to the difference in size, weight and species of the fish, dose of the probiotic, experimental conditions and physicochemical parameters of the water.

Considering the positive effects of the probiotic *P. acidilactic* on the growth indices, TVC and LAB population in the alimentary tract, digestive system enzymes and the immunity of mucus in killing pathogens in zebrafish, it is advisable to use 4×10^6 cfu of the probiotic *P. acidilactici* in each gram of the zebrafish diet. Although these findings can be generalized to other ornamental and edible fish, to obtain the desired density of probiotic, this research has to be carried out on other species of fish.

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