The effect of Pediococcus acidilactici *on mucosal immune responses, growth, and reproductive performance in zebrafish* (Danio rerio)

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The effect of *Pediococcus acidilactici* on mucosal immune responses, growth, and reproductive performance in zebrafish (*Danio rerio*)

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Abstract A completely randomized experimental design carried out to investigate the effects of different levels of *Pediococcus acidilactici* (PA) including 0 (basal diet as a control diet), 1×10^6, 2×10^6, 4×10^6, and 8×10^6 colony-forming unit (CFU) per gram of the diet for 60 days on the mucosal immunity responses, growth, and reproductive performance, in zebrafish, *Danio rerio* (with mean weigh ± SE: 120 ± 10 mg). The obtained results revealed that the best growth and reproduction indices were related to the concentration of 4×10^6 CFU PA g^{-1} diet ($P < 0.05$). The maximum activities of mucosal immune responses including total protein, alternative complement system, IgM, and lysozyme were observed in the fish fed with 4×10^6 CFU PA g^{-1} diet ($P < 0.05$). Furthermore, the maximum alkaline phosphatase activity of skin mucus was recorded in the fish fed with 8×10^6 CFU PA g^{-1} diet ($P < 0.05$). Fish fed with 4×10^6 CFU PA g^{-1} diet had the highest villus length and width of the intestine ($P < 0.05$). Supplementing the diet with 4×10^6 CFU PA g^{-1} diet more significantly enhanced *Cyp19a* gene expression in comparison with this in other groups. Hence, PA with a concentration of 4×10^6 CFU g^{-1} diet can be considered as a proper level of probiotic for improving the health, growth, and reproductive performance of the *D. rerio*.

Keywords Reproduction · Mucosal immunity · *Pediococcus acidilactici* · Zebrafish

Introduction

Fuller (1989) described probiotics as living microorganisms that increase growth and promote health and immunity of the host by adjusting the microbial biota of the digestive system. For fish farming, the most beneficial effects of probiotics reside in its ability to enhance the immune system and growth of fish. Probiotic bacteria have the ability to vitamin synthesis, production of short-chain fatty acids via fermentation as an energy resource, and the increase of enzymatic activity for fish larvae and spawners that may improve feed digestibility, growth, and reproduction performance (Elumalaia et al. 2020; Van Doan et al. 2019; Ashouri et al. 2019; Ringø 1998). It has been reported that probiotics also improve reproduction, survival rate, and final weight of the larva (Ghosh et al. 2007; Carnevali et al. 2016) and immunity (Castex et al. 2010).

One of the most important probiotic bacteria in the aquaculture is Bactocell®, *Pediococcus acidilactici*

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(PA). PA is a positive Gram coccus that could live in a vast range of temperature, pH, and osmotic pressure (Ringø 1998). It can adhere to the villi of the intestine and clone there (Merrifield et al. 2011). The effect of PA on the growth indices, reproduction, and immune responses of the aquatic organisms has been reported in some species (*Litopenaeus stylirostris*, Castex et al. 2010; *Oreochromis niloticus*, Ferguson et al. 2010; *Oncorhynchus mykiss*, Abedian Amiri et al. 2017; *Lates calcarifer*, Ashouri et al. 2018). It had been showed that probiotics can upregulate the expression of the genes involved in reproduction including *Cyp19a* in the ovary (Gioacchini et al. 2010). *Cyp19a* is an important gene during female maturation. This gene mostly expressed in the ovary and causes the conversion of the testosterone to the estradiol in the ovarian tissues by the construction of the aromatase enzyme (Carnevali et al. 2016).

Zebrafish (*Danio rerio*) is a laboratory model fish. This fish belongs to the Cyprinidae family and Danioninae subfamily. Maximum length and age of this fish is 5 cm and 3 years, respectively (Fishman 2001). *D. rerio* is an important candidate species for many research areas because of its unique characteristics such as high growth rate and reproduction in captivity (Blahova et al. 2020; Ahmadifar et al. 2019; Fiorino et al. 2018; Hoseinifar et al. 2018; Nath et al. 2018; Aragona et al. 2017).

Given the simultaneous beneficial effects related to PA on physiological processes including growth, immunity, and reproduction and importance of zebrafish as a model, research on different aspects of probiotics increase biological science and could improve the culture of this species. The present study aimed to investigate the effects of dietary supplementation of *P. acidilactici* on the mucosal immune responses, growth, reproductive indices, intestinal histoarchitecture and changes in *Cyp19a* gene expression in the zebrafish, *D. rerio*, as a model species to provide a comprehensive view of the effect of PA on zebrafish.

Materials and methods

Diet preparation

The present study was conducted in a completely randomized design including 5 treatments each in 3 replicates for 60 days. 0, 1×10^6, 2×10^6, 4×10^6, and 8×10^6 CFU *P. acidilactici* (PA) (Bactocell®, CNCM-MA 18/5 M, Lallemand, France) g^{-1} diet was incorporated into a basal diet (Kimiagaran-e-Taghziye, Iran). The ingredients and chemical components of the diet (AOAC 1990) are described in Table 1. Each gram of the probiotic was containing 10^{10} cells. The weight of the needful probiotic was computed with an accuracy of 0.001 g and mixed manually with the basal diet (Ashouri et al. 2018). The experimental diets were prepared weekly and kept in the refrigerator at 4 °C. The activity of the bacteria during the experiments was confirmed by culturing samples of diets in the MRS broth media, as described by Ashouri et al. (2018).

Experimental design

Six hundred healthy zebrafish were purchased from a private ornamental fish company in Isfahan, Iran, and transferred to the laboratory by oxygenated plastic bags. After 2 weeks acclimation to the experimental condi-

Table 1 Ingredient and chemical components of the using diet for the experiments

Ingredient	g/kg
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^{-1}) containing Mg, 100; Zn, 60; Fe, 40; Cu, 5; Co, 0.1; and I, 0.1

[b] Vitamin premix (mg kg^{-1}) containing BHT, 100; 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

tion, the fishes (120 ± 10 mg and 15 ± 2 mm) randomly divided to the 15 aquariums (50 L) which were supplied with 30 L water ($n = 40$). Under normal circumstances, after the reproduction of zebrafish, the male to female sex ratio among the larvae of this fish is considered 1:1. Fish were fed 10% of body weight 3 times per day (8:00, 13:00, and 18:00) for 60 days. Experimental diets were evaluated as triplicate. All procedures were done regarding the ethics guides for animals in vivo experiments, presented by Khorramshahr University of Marine Science and Technology, Iran. Aquarium water was exchanged about 50%, daily. Water quality parameters including temperature, pH, total hardness, nitrite, and nitrate were controlled daily by a 340i Multimeter (WTW, Weilheim, Germany) and maintained at 28 ± 2 °C, 8 ± 0.5, 310 ± 10 mg/L, 0.08 ± 0.02 mg/L, and 3 ± 1 mg/L. The light period in the laboratory was 14 L:10 D. After 60 days, all fish in each aquarium, whether male or female, were weighed for determination of following indices.

$$\text{Weight gain (WG, \%)} = (\text{Final weight} - \text{Initial weight}) \times 100$$
$$\text{Specific growth rate (SGR)} = [(\ln \text{final body weight} - \ln \text{initial body weight})/60 \text{ (days)}] \times 100$$
$$\text{Food consumption efficiency (FCE)} = \text{Weight gain (mg)}/\text{Feed intake (mg)}$$
$$\text{Condition factor (CF)} = \text{Final weight (mg)} \times 100/\text{Final length (mm)}3$$
$$\text{Survival rate (SR, \%)} = (\text{Initial fish number} - \text{Dead fish number}) \times 100/\text{Initial fish number}$$

Reproduction assessment

For reproduction assessment, after biometry, female fish were sacrificed with an overdose of the same anesthetic. At the end of the experimental period, the age of the fish was 3 months (1 month before the study and 2 months during the study), so female fish were distinguishable from males based on their phenotypic characteristics. Then the ovary of fish was removed completely and weighed with 1 mg precision and its ovule enumerated under binocular. Reproduction performance was assessed according to the standard formulas:

$$\text{Gonadosomatic index (GSI)} = \text{Gonad weight (mg)} \times 100/\text{Final weight (mg)}$$
$$\text{Absolute fecundity (AF)} = (\text{Number of ovule in ovary sample} \times \text{Weight of ovary (mg)})/\text{Weight of ovary sample (mg)}$$
$$\text{Relative fecundity (RF)} = \text{Number of total ovules in the ovary} \times \text{Weight of fish (mg)}$$
$$\text{Working fecundity (WF)} = \text{Number of hatched eggs}$$
$$\text{Hatching percentage (HR, \%)} = \text{Number of hatched eggs} \times 100/\text{Total number of eggs}$$

Intestine villi

According to Fischer et al. (2008), histological sections were prepared from whole cross-section of the fish. For this purpose, after euthanasia, 3 fish from each group were completely put in 10% buffered formalin for 24 h. Subsequently, samples were rinsed with PBS, dehydrated in ascending series of alcohol and embedded in paraffin. Transversal dewaxed serial sections (7 μm) were prepared and stained by hematoxylin–eosin. The sections were examined with an Olympus BX60 microscope and visualized through the Color-View Camera. The images were acquired and analyzed through the software ImageJ. The length and width of the villi were measured in 3 scopes for each fish.

Mucus immunity

For assessment of the mucosal immune parameters of the zebrafish fed with different levels of dietary PA for 60 days, total protein, complement activity, alkaline phosphatase, immunoglobulin M (IgM), and lysozyme were examined. According to Subramanian et al. (2007), at the end of the trial, fish were fasted for 24 h before sampling and the mucosa samples were collected from 3 fish per replicate (9 fish per treatment). Fish were

put in small plastic zip bags with 5 ml sodium salt buffer (50 mM NaCl) and rub for 2 min. Then the dissolved mucus was removed from the zip bag and transferred into a 5-ml centrifuge sterile tube and centrifuged (1500×*g*, 10 min, 4 °C). Subsequently, the supernatant was separated and kept at − 80 °C until the time of analysis. The total protein was measured using bovine serum albumin (Sigma-Aldrich) as standard at 750 nm (Lowry et al. 1951). Complement activity was measured according to Yano (1992) by determining 50% hemolysis of the rabbit red blood cells. Alkaline phosphatase activity was spectrophotometrically measured using Pars Azmoon kit (Karaj, Iran). IgM activity was measured according to the Siwicki and Anderson (1993) method. Briefly, the total protein of the mucus was measured before and after the sedimentation of immunoglobulin molecules using polyethylene glycol 12%. Then the difference was considered as the immunoglobulin content. Mucus lysozyme activity was assayed as described by Demers and Bayne (1997), based on the lysis of the lysozyme-sensitive Gram-positive bacterium *Micrococcus lysodeikticus* (Sigma-Aldrich, USA).

Cyp19a expression

DNase-treated total RNA was extracted from ovary tissues (30 mg) using RNA Isolation Kit (Yekta Tajhiz, Iran) according to the manufacturer's instructions. The total concentration of RNA was measured at OD 260 nm and impurities were assessed by the OD 260/280 nm ratio using the Picodrop P200 system (Alpha Biotech Ltd., UK). The RNA quality was tested by confirming the presence of 28S:18S rRNA ratio fractions after staining by loading buffer (bromophenol blue + sucrose + H_2O) in a 1% agarose gel and running for 30 min (85 V). cDNA was made from 1 μg of total RNA using a reverse transcriptase kit (Thermo Scientific, GmBH, Germany) in which the total volume of reaction was 20 μL.

Amplification and detection of specific products were performed using StepOne Real-Time PCR System (Life Technologies, Carlsbad, CA, USA), qPCR Master Mix containing SYBR® Green (Life Technologies, Carlsbad, CA, USA), and *Cyp19a* specific primers (GenBank accession number AF183906). *β-Actin* was used as internal control for gene expression normalization. PCR primers are listed in Table 2. The samples were amplified in 25 μL reaction mixtures containing 0.3 μM forward and reverse primers (*Cyp19a* or *β-actin*), 12.5 μL Maxima SYBR Green/ROX qPCR Master Mix (2×), DNA template ≤ 500 ng/reaction, and nuclease-free water. The thermal cycle protocol was denaturation, annealing, and extension at 94 °C for 20 s, 60 °C for 30 s, and 72 °C for 40 s, respectively. The present sizes of all PCR products were verified by inspection of the dissociation curve and gel electrophoresis. The relative quantification of gene expression was calculated using the following equation (Livak and Schmittgen 2001):

$$\text{Relative gene expression} = 2^{-(\Delta Ct\ \text{sample} - \Delta Ct\ \text{control})}$$

Table 2 List of primers for real-time PCR analysis

Gene	Forward primer	Reverse primer
Cyp19a	CCGTTCTTATGGCA GGTGAT	TTGTGTGGTCGATG GTGTCT
β-actin	GGTACCCATCTCCT GCTCCAA	GAGCGTGGCTACTC CTTCACC

Statistical analysis

All data were presented as mean ± standard error (SE). The normality of data and homogeneity of variance were evaluated with the Shapiro–Wilk and Levene tests, respectively. A one-way analysis of variance (ANOVA) was used for determining the significant difference between treatments. The Duncan test was used for comparing the mean of data between experimental diets as $P < 0.05$. Statistical analyses were executed in IBM SPSS Statistics, version 23.

Results

Growth assessment

The maximum value of FW, FL, GW, SGR, FCE, and SR were observed in the 4 × 106 CFU g^{-1} diet while their minimum value was recorded in control group ($P < 0.05$). As seen in Table 3, growth indices showed an increasing trend from the control group to the 4 × 10^6 CFU g^{-1} group and a serene decrease in the 8 × 10^6 CFU g^{-1} group. Fish fed diet supplemented with

Table 3 Growth performance of zebrafish (*Danio rerio*) fed with different levels of *Pediococcus acidilactici* in the diet for 60 days

Growth indices	Control	1×10^6 CFU g^{-1}	2×10^6 CFU g^{-1}	4×10^6 CFU g^{-1}	8×10^6 CFU g^{-1}
WBi (mm)	120 ± 10	120 ± 10	120 ± 10	120 ± 10	120 ± 10
WBf (mg)	371 ± 12.58^{d}	413 ± 7.63^{c}	503 ± 14.04^{b}	642 ± 8.73^{a}	635 ± 1159^{a}
WG (%)	209 ± 10^{d}	244 ± 6^{c}	319 ± 11^{b}	435 ± 7^{a}	429 ± 9^{a}
FCE	26.63 ± 0.65^{d}	31.16 ± 0.78^{c}	37.27 ± 0.56^{b}	45.96 ± 1.16^{a}	44.58 ± 0.69^{a}
SR (%)	65.5 ± 2.5^{d}	74.0 ± 3.6^{c}	83.0 ± 2.0^{b}	92.3 ± 1.5^{a}	85.6 ± 1.5^{b}
CF	2.3 ± 0.32^{a}	0.69 ± 0.04^{b}	0.76 ± 0.06^{b}	0.67 ± 0.06^{b}	0.78 ± 0.10^{b}

Data are presented as mean ± SE ($n = 9$). Different letters display significant difference in each row ($P < 0.05$)

BW_i initial body weight, BW_f final body weight, *WG* weight gain, *FCE* food consumption efficiency, *SR* survival rate, *CF* condition factor

4×10^6 CFU g^{-1} diet PA and those fed control diet had the lowest (2.17 ± 0.05) and highest (3.75 ± 0.09) FCR, respectively. The highest value of CF was observed in control diet and then decrease until the 4×10^6 CFU g^{-1} diet PA and slightly increased in the 8×10^6 CFU g^{-1} diet PA ($P < 0.05$). CF in control group was about 3.4 times more than the 4×10^6 CFU g^{-1} diet PA group.

Reproductive indices

Table 4 represents the effects of supplementation of diets with PA on reproductive indices in zebrafish. Fish fed diets supplemented with PA had higher GSI, AF, WF, HR, and RF were compared to the control group ($P < 0.05$). The highest amount of GSI, AF, WF, and HR was observed in the 4×10^6 $CFUg^{-1}$ diet ($P < 0.05$). All parameters increased from control diet to the 4×10^6 $CFUg^{-1}$ group and then slightly decreased in the 8×10^6 $CFUg^{-1}$ group. RF was higher in fish fed diet supplemented with 1×10^6 CFU g^{-1} diet PA than other experimental groups ($P < 0.05$).

Intestine villi

The study of the intestinal histomorphology (Table 5) showed that with increasing concentration of PA in the diet up to 4×10^6 $CFUg^{-1}$ diet the villus length and width increased significantly ($P < 0.05$) and then decreased slightly. Fish fed diet supplemented with 4×10^6 CFU g^{-1} diet PA and those fed control diet had highest (260 ± 10 μm) and lowest (150 ± 10 μm) villus length, respectively ($P < 0.05$). Also, the highest villus width (100 ± 10 μm) was observed in the fish fed diet including 4×10^6 CFU g^{-1} diet PA.

Mucus immunity

As illustrated in Table 6, dietary supplementation of PA significantly increased skin mucus total protein, complement activity, alkaline phosphatase, IgM, and lysozyme compared to the control group ($P < 0.05$). The amount of total protein, complement, and IgM were increased from control to the 4×10^6 $CFUg^{-1}$ diet and then decreased in the 8×10^6 $CFUg^{-1}$ diet, but the

Table 4 Reproduction performance of zebrafish (*Danio rerio*) fed with different levels of *Pediococcus acidilactici* in the diet for 60 days

Reproductive indices	Control	1×10^6 CFU g^{-1}	2×10^6 CFU g^{-1}	4×10^6 CFU g^{-1}	8×10^6 CFU g^{-1}
GSI (%)	8.15 ± 0.01^{e}	12.41 ± 0.51^{d}	15.76 ± 0.06^{c}	20.07 ± 0.20^{a}	18.61 ± 0.37^{b}
AF (number)	103 ± 7.63^{c}	174 ± 5.29^{b}	178 ± 3.60^{b}	249 ± 8.08^{a}	244 ± 6.11^{a}
RF	277 ± 12^{d}	420 ± 5^{a}	353 ± 3^{c}	388 ± 7^{b}	384 ± 3^{b}
WF (number)	46.6 ± 5.45^{d}	80.66 ± 4.92^{c}	145.4 ± 5.53^{b}	215.3 ± 10.81^{a}	205.5 ± 7.53^{a}
HR (%)	45 ± 2.0^{c}	46 ± 1.5^{c}	81 ± 1.5^{b}	86 ± 1.5^{a}	84 ± 1.0^{a}

Data are presented as mean ± SE ($n = 9$). Different letters display significant difference in each row ($P < 0.05$)

GSI gonadosomatic index, *AF* absolute fecundity, *RF* relative fecundity, *WF* working fecundity, *HR* hatching percentage.

Table 5 Length and width of intestine villi in zebrafish (*Danio rerio*) fed with diets containing different levels of *Pediococcus acidilactici* for 60 days

	Control	1×10^6 CFU g^{-1}	2×10^6 CFU g^{-1}	4×10^6 CFU g^{-1}	8×10^6 CFU g^{-1}
Villi length (μm)	150 ± 10^{d}	180 ± 10^{c}	213 ± 15^{b}	260 ± 10^{a}	220 ± 10^{b}
Villi width (μm)	40 ± 10^{d}	60 ± 10^{c}	81 ± 7^{b}	100 ± 10^{a}	95 ± 5^{ab}

Data are presented as mean ± SE ($n = 9$). Different letters display significant difference in each row ($P < 0.05$)

highest activity of alkaline phosphatase and lysozyme observed in the 8×10^6 CFU g^{-1} diet ($P < 0.05$).

Cyp19a expression

Expression of *Cyp19a* in zebrafish (*D. rerio*) significantly influenced by dietary administration of PA. The results showed that ovarian *Cyp19a* expression was increased until the 4×10^6 CFUg^{-1} diet ($P < 0.05$) and then declined. In the fish fed diet administrated with 4×10^6 CFU g^{-1} diet PA, the fold change was observed 6.01 times more than those fed control diet (Fig. 1).

Discussion

According to the previous findings, probiotics play an important role in enhancing the immunity and growth of aquatic animals or improving water quality (Nayak 2010; Abedian Amiri et al. 2017). The beneficial bacteria improved the gut microbial flora and digestion and also prevented the attachment and cloning of pathogenic bacteria to the intestinal wall of fish (Balcazar et al. 2006; Ashouri et al. 2019). The beneficial role of probiotics in growth is primarily due to their ability to produce enzymes, vitamins, and other beneficial substances for the host (Ringø et al. 2007). They may also stimulate the host to secrete these substances. As a result, probiotic bacteria improved digestion and uptake, reduced feed conversion ratio, increased feed efficiency, and ultimately increased aquatic growth (Valipour et al. 2018). So, the aquatic animals fed diet supplemented with probiotics had higher growth and showed less mortality or disease than the probiotic-deficient groups (Carnevali et al. 2006; Carnevali 2014, Carnevali et al. 2016). In the present study, the use of probiotic PA also increased the final weight and length of fish in experimental groups compared to the control group. Also, the efficiency of feed consumption improved as in the 4×10^6 CFU g^{-1} diet PA increased 1.72 times compared to the control diet. Also, the least amount of FCR was observed in the fish fed diet administrated with 4×10^6 CFU g^{-1} diet PA that showed the proper efficiency of food consumption in this group. In line with findings of this study, growth improvement of ornamental fishes includes swordtail (*Xiphophorus helleri*, *X. maculatus*) and guppy (*Poecilia reticulate*, *P. sphenops*), increased by adding *Bacillus subtilis* and *Streptomyces* to their diet (Ghosh et al. 2008).

In larviculture of ornamental fish, one of the most important factors is survival at the end of the reproductive period. Reproduction as an energy-based process can be successful when sufficient sources of energy are available. Since there is an obvious relation between fish metabolism status and reproduction, like other animals, some central mediators, hypothalamic neuropeptides, and several peripheral molecular mediators influence the regulation of the interaction between those both

Table 6 Mucus immunity in zebrafish (*Danio rerio*) fed with diets containing different levels of *Pediococcus acidilactici* for 60 days

Immunity agent	Control	1×10^6 CFU g^{-1}	2×10^6 CFU g^{-1}	4×10^6 CFU g^{-1}	8×10^6 CFU g^{-1}
Total protein (mg/ml)	0.87 ± 0.01^{d}	0.92 ± 0.04^{c}	0.97 ± 0.01^{b}	1.02 ± 0.01^{a}	0.99 ± 0.01^{ab}
Complement (μg/ml)	0.12 ± 0.01^{e}	0.25 ± 0.01^{d}	0.33 ± 0.01^{c}	0.57 ± 0.01^{a}	0.43 ± 0.01^{b}
Alkaline phosphatase (U/mg protein^{-1})	0.87 ± 0.01^{d}	0.98 ± 0.02^{c}	1.26 ± 0.01^{b}	1.33 ± 0.02^{a}	1.35 ± 0.01^{a}
Immunoglobulin M (IgM, μg/ml)	0.25 ± 0.01^{e}	0.38 ± 0.01^{d}	0.47 ± 0.02^{c}	0.77 ± 0.01^{a}	0.69 ± 0.01^{b}
Lysozyme (μg/ml)	0.57 ± 0.01^{e}	0.69 ± 0.01^{d}	0.73 ± 0.01^{c}	0.88 ± 0.01^{b}	0.91 ± 0.01^{a}

Data are presented as mean ± SE ($n = 9$). Different letters display significant difference in each row ($P < 0.05$)

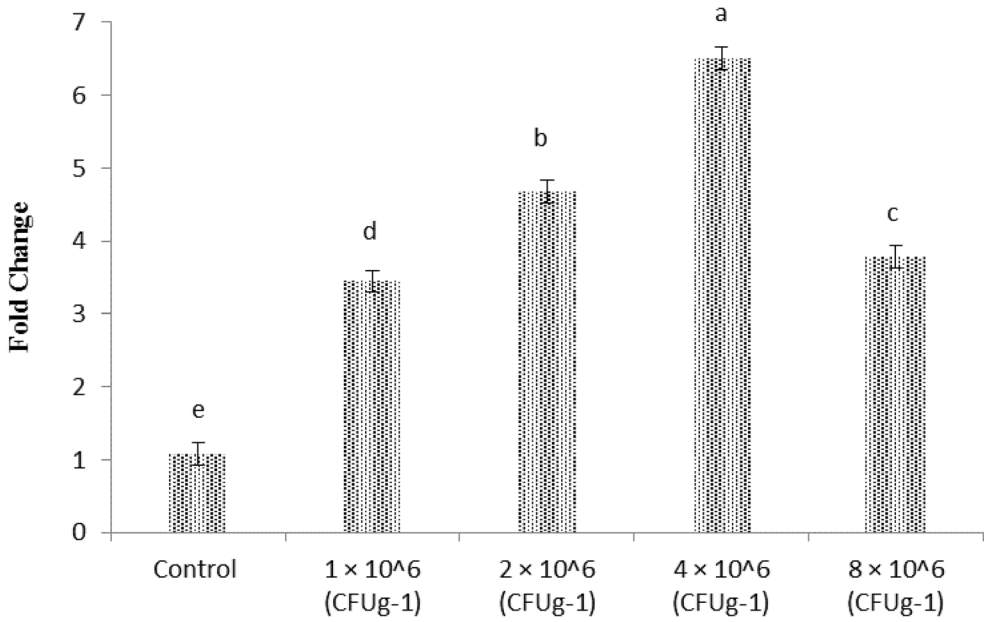

Fig. 1 The mean of relative expression (fold change) of *Cyp19a* in the ovary of zebrafish, *Danio rerio* fed with diets containing different levels of *Pediococcus acidilactici* for 60 days compared with *β-actin* as reference gene. Different letters display significant difference in each column ($P < 0.05$). Data are presented as mean ± SE and analyzed by a one-way ANOVA ($n = 3$)

processes (Rashidian et al. 2020; Vazirzadeh et al. 2020; Allameh et al. 2015; Carnevali et al. 2016). Ghosh et al. (2007) found that *Bacillus subtilis* (10^6 to 10^8 g^{-1} diet) was improved reproductive performance but using higher doses did not lead to better results in livebearing ornamental fish including *Poecilia reticulata*, *Poecilia sphenops*, *Xiphophorus helleri*, and *Xiphophorus maculatus.* In this context, the result of the current study revealed that dietary supplementation of 4×10^6 CFU g^{-1} diet PA in zebrafish (*D. rerio*) had the highest reproductive performance compared to fish fed 8×10^6 CFU g^{-1} diet PA. Therefore, the use of a higher dosage of the probiotic in diet did not lead to the maximum enhanced reproductive performance of the spawners.

On the other hand, probiotics can be defined as live microorganisms that when administered in adequate amounts confer beneficial effects on the host by improving its intestinal microbial balance (Mohammadi Arani et al. 2019). It had been reported that supplementation diet with PA improved health and growth through the modulated intestinal microflora in zebrafish *D. rerio* (Abedian Amiri et al. 2017).

Previous studies have been also shown the positive effects of dietary administration of probiotics on reproductive indices such as GSI, fecundity, and hatching percentage in different fish species (Carnevali et al. 2016; Gioacchini et al. 2012; Qin et al. 2014). Applications of *Lactobacillus rhamnosus* showed a significant decrease in apoptosis and a significant increase in the number of vitellogenic and mature follicles compared to the control in zebrafish *D. rerio* (Gioacchini et al. 2011a, 2011b, 2012). These fish showed higher levels of GVBD and GSI index, indicating a probiotic-stimulating role of *L. rhamnosus* on the maturation of the follicles. Also, the higher levels of germinal vesicle breakdown (GVBD) and GSI index could be attributed to *L. rhamnosus* which is a probiotic stimulating the maturation of the follicles.

Since the fish have direct interaction with the immediate environment, this makes the study of fish mucosal immunity of particular attention. Mucosal immunity plays an important role in combating pathogens and enhancing immunity in fish (Hoseinifar et al. 2019). A variety of enzymes, proteases, antibacterial peptides, lectins, and immunoglobulins play important roles in ornamental aquatic mucosa against pathogens (Ghiasi et al. 2018). Some studies have represented the effect of probiotics on fish mucosal immunity. Newaj-Fyzul et al. (2007) showed that diet supplemented with *Bacillus subtilis* in rainbow trout (*Oncorhynchus mykiss*) had significant effects on mucosal lysozyme activity. It is reported that using dietary *B. amyloliquefaciens* increased mucus total protein in Indian major carp, *Catla*

catla (Das et al. 2013). In another study, the dietary inclusion of *Lactobacillus delbrueckii* significantly increased T cells and acidophilic granulocytes in European seabass, *Dicentrarchus labrax* (Picchietti et al. 2009). In the current study, the mucus immunity is enhanced by increasing some immunity agents, such as total protein, complement, alkaline phosphatase, IgM, and lysozyme activities, 1.17–4.75 times in the 4×10^6 CFU g^{-1} diet compared to control diet. Based on the obtained results, the administration of PA in the diet can be considered as an immunostimulant for increasing the mucus immunity in zebrafish (*D. rerio*). In line with the results of this study, dietary supplementation of probiotic including *Lactobacillus acidophilus* in black swordtail (*Xiphophorus helleri*) showed beneficial effects on growth performance (WG, SGR, FCR), skin mucosal immune responses (total protein level, bactericidal and alkaline phosphatase activities), and intestinal microbiota (total and lactic acid bacteria counts) with significant elevation (Hoseinifar et al. 2015).

In the present study, supplementing PA in the diet of zebrafish (*D. rerio*) increased the intestinal villus length and width. The intestinal villus length increased from 150 μm in the control diet to 260 μm in the 4×10^6 CFU PA g^{-1} diet and the intestinal villus width increased from 40 μm in the control diet to 100 μm in the 4×10^6 CFU g^{-1} PA diet. This significant increase in intestinal villus and/or absorption surface absorption may explain the improvements in growth observed in fish fed by PA-supplemented diets. The increase in the intestinal villus length also had been reported in Atlantic salmon, *Salmo salar*, after feeding by PA-added diet (Abid et al. 2013). Furthermore, adding *Bacillus cereus* and *L. rhamnosus* to diets increased intestinal villi length in rainbow trout (*O. mykiss*) and Nile tilapia (*Oreochromis niloticus*), respectively (Gisbert et al. 2013).

One of the most important effects of probiotics is their effects on the expression of genes involved in reproduction that have been studied in ornamental fish (Ghosh et al. 2007). It had been shown that probiotics increase the expression of genes promoting maturation like *Cyp19a*, *vtg*, and *erα* and reduce the expression of inhibitory genes including *Tgfb1*, *Gdf9*, and *Bmp15* (Carnevali et al. 2016). In the current study, gene expression of *Cyp19a* that is an important gene for the creation of the female genus in the larvae increased in fish fed diet supplemented with PA, which shows the potential ability of PA for upregulating this gene. In agreement with our findings, supplementing the diet with *L. rhamnosus* increased expression of *Cyp19a* in zebrafish, *D. rerio* (Qin et al. 2014; Gioacchini et al. 2010). Also, *L. rhamnosus* inhibited follicular apoptosis and increased their survival (Gioacchini et al. 2013) that leads to increased fecundity and progeny production in fish.

In conclusion, the positive effects of *Pediococcus acidilactici* as a dietary supplementation were observed in zebrafish. Growth and reproduction indices improved in the experimental groups compared to the control group. On the other hand, the administration of PA enhanced the mucosal immune responses that can increase survival in this species. Upregulation of *CYP19a* that accelerates the gonad maturation was another beneficial effect of PA. Based on the results obtained, it can be suggested supplementing diet with PA as a functional feed additive in rearing zebrafish.

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Author contributions Mojtaba Mohammadi Arani: carrying out the study, preparation of early draft

Amir Parviz Salati: biochemical analysis, experimental design, finalizing manuscript

Saeed Keyvanshokooh: gene expression analysis, statistical analysis

Omid safari: Histological analysis

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The authors confirm that this project was approved by the ethical committee of Khorramshahr University of Marine Science and Technology.

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