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COMBINED EFFECTS OF *SPIRULINA PLATENSIS* AND *PEDIOCOCCUS ACIDILACTICI* ON THE GROWTH PERFORMANCE, DIGESTIVE ENZYME ACTIVITY, ANTIOXIDATIVE STATUS, AND IMMUNE GENES IN ZEBRAFISH

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Abstract

The combined effects of *Spirulina platensis* (SP) and *Pediococcus acidilactici* (PA) on growth performance, digestive enzyme activity, antioxidative status, and immune genes in zebrafish were investigated in the present study. Four experimental diets were designed: control and three test diets mixed with SP at 2.5%, PA at 10^7 CFU/g, or a combination of 2.5% SP and 10^7 CFU/g PA. After 56 days, fish treated with PA and SP mixture had higher final weight, weight gain, SGR, and lower FCR than fish fed the control and SP diets (P<0.05). The results also illustrated that fish fed PA, SP, and their mixture had higher (P<0.05) protease and amylase activities than the control. The lipase activity was significantly higher in fish treated with PA or the mixture of PA and SP than in the control (P<0.05). The alternative complement pathway (ACH50) and lysozyme activity in the mucus samples of fish treated with PA or both PA and SP were significantly higher (P<0.05) than in fish treated with the control and SP diets. The total immunoglobulin level in the skin mucus was significantly higher (P<0.05) in fish fed PA than in control. In the body homogenates samples, the lysozyme activity and immunoglobulin levels were markedly higher (P<0.05) in fish treated with the mixture of PA and SP than in the control. The dietary PA and SP diet mixture improved the glutathione peroxidase, superoxide dismutase, and total antioxidative capacity. The expression of IL-1β and IL-8 genes in fish treated with PA or the mixture of PA and SP was significantly higher (P<0.05) than in fish treated with the control or SP diets. Fish treated with PA, SP, or both PA and SP had marked (P<0.05) upregulation of the lysozyme gene expression. In conclusion, the mixture of *S. platensis* and *P. acidilactici* is more effective than using each individually for improving the growth performance, digestive enzyme activity, and the immune and antioxidative capacity of zebrafish.

Key words: aquaculture, antioxidative capacity, immunity, medicinal plants, probiotics

The aquaculture sector has been rapidly developed compared to all other animal food-production sectors in recent decades. Thus, intensive farming systems are applied to overcome the high demand for aquaculture. However, intensive fish culture has led to immunosuppression and, eventually, infectious diseases (Dawood et al., 2018). Applying different growth promotors and immunostimulants, such as probiotics, is a preventive practice that stimulates feed efficiency, and specific and innate immune systems. Probiotics are effective viable bacterial cells increasingly used to improve fish growth, digestion capacity, immunity, and tolerance against various diseases (Akhter et al., 2015; Hoseinifar et al., 2018; Melo-Bolívar et al., 2020; Yilmaz et al., 2022). Lactic acid bacteria (LAB) are the most promising bacteria with marked beneficial effects in aquaculture (Ringo et al., 2019). *Pediococcus acidilactici* MA 18/5M (PA) is one of the most applicable LAB in aquaculture with numerous positive effects. Improved growth rate, activated intestinal microbiota, immune system, and disease resistance are well reported in different fish species treated with *P. acidilactici* (Ashouri et al., 2018; Ringo et al., 2019; Al-Hisnawi et al., 2019; Ahmadifar et al., 2020).

Spirulina platensis (SP) is a filamentous, planktonic algae with 60–70% protein, 13.6% carbohydrate, vitamins, and minerals (Gupta et al., 2017). Previous studies have shown the nutritional values of SP on growth performance, immune and antioxidant systems, and gut microbiota in several fish species (Hamed et al., 2019; Sheikhzadeh et al., 2019 a, b; Teimouri et al., 2019; El-Araby et al., 2022). Besides, the beneficial effects of SP had stimulatory increased the resistance of aquatic animals to infection with pathogens. Interestingly, SP inclusion has synergistically affected the activity of LAB, namely *Lactococcus lactis*, *Bifidobacterium longum*, and *Lactobacillus bulgaricus*, showing promising prebiotic properties (Gupta et al., 2017). Therefore, a combination of SP with a LAB-like PA may present higher efficacy than the individual administration of the PA or SP. Thus, the current study was designed to evaluate the potential

of an individual or combined administration of dietary SP and PA on the growth rate, digestive enzyme activities, and immunological and antioxidant indices in zebrafish.

Material and methods

Experimental diet preparation

Powdered *Spirulina platensis* (SP) (60.14% crude protein, produced by C.B.N. Bio-engineering Company, China) was used in this study. Lyophilized *Pediococcus acidilactici* MA 18/5M (PA) (produced by Lallemand Inc., Canada), as the probiotic source, was also used. Probiotic preparation was performed according to a previous study (Ahmadifar et al., 2020). Four experimental diets were designed: control (CTR) and three test diets mixed with SP at 2.5%, PA at 10^7 CFU/g, or a combination of 2.5% SP and 10^7 CFU/g PA, which were named SP, PA, and SP+PA, respectively. All ingredients in different experimental diets (shown in Table 1) were ground and mixed thoroughly. Lipid ingredients and SP or PA in different diets were added to other dry components, and then water was added to prepare a consistent dough. The final mixture was air-dried at room temperature and then stored at 4ºC for weekly use. Fresh batches of diets were ready every week to ensure the viability of the probiotic.

Fish and experimental design

Healthy Tuebingen strain zebrafish (mean weight 69.57±0.11) reared in Royan Research Center (Tehran, Iran) were used in this study. Two hundred and forty fish were distributed into 12 glass tanks (40 L) at a density of 20 zebrafish per tank. The glass tanks were supplied with filtered freshwater aerated using air stones. During the study, water quality indices were checked to maintain within the normal ranges for temperature (24.5±0.7), pH (7.3±0.1), and dissolved oxygen (7.25±0.16) by the portable analyzer (Aquacombo, China). During the 10-day adaptation period, fish were administered with CTR diet at a rate of 3% daily. Following this time, fish were fed with different experimental diets, including control (CTR), *Spirulina platensis* (SP), *Pediococcus acidilactici* (PA), or SP+PA diets at a rate of 3% a day for 56 consecutive days.

Ethics statement

The procedures of this study involving animals and their care were approved by the Animal Care and Use Committee of the University of Kharazmi.

Growth performance

After the feeding period, all fish were fasted for 24 hours and then euthanized with clove oil (50 ml/L). Different growth indices, including final weight, weight gain, feed conversion ratio (FCR), specific growth rate (SGR), as well as survival rate during the 56-day feeding trial, were calculated based on the previous study (Sheikhzadeh et al., 2017).

Sampling procedure

After the biometric calculations, nine fish in each tank were randomly selected. Different tissues, including gut, kidney, and liver, from each three fish were pool-sampled and frozen in liquid nitrogen. After homogenizing with tris buffer (25 mM), samples were centrifuged at 3000 g for 10 min, and collected supernatants were frozen at –80°C. Three fish from each tank were randomly selected, and after removing the head and tail, the remaining parts were homogenized as mentioned above for immunological parameters. Eight fish from each tank were also pooled to calculate mucus secretions' immunological and antioxidant parameters. Fish were transferred into the polyethylene bags with 50 mM NaCl (10 ml) and gently rubbed to collect the skin mucus, according to the previous study (Hoseinifar et al., 2020). Collected samples were centrifuged (1500 *g*) for 10 min, and final supernatants were gathered and kept at –70ºC for further analysis.

Table 1. Formulation and composition of different diets used in this experiment

Ingredients	CTR	S.P.	P.A.	S.P.+P.A.
Fish meal[1]	430	405	430	405
Wheat flour	50	50	50	50
Soybean meal[2]	300	300	300	300
Corn flour	130	130	130	130
Microcrystalline cellulose	10	10	10	10
Vitamin premix	30	30	30	30
Mineral premix	30	30	30	30
Spirulina[3]	0	25	0	25
P. acidilactici MA 18/5M	0	0	10^7 CFU g^{-1}	10^7 CFU g^{-1}
Proximate analysis (%)				
crude protein	41.04	41.00	41.03	41.01
crude lipid	7.35	6.99	7.35	7.02
dry matter	85.06	82.06	85.10	85.10
ash	7.47	7.33	7.42	7.35
gross energy (kcal kg^{-1})	4213	4209	4210	4212

[1]crude protein: 61.60.
[2]crude protein: 44.20.
[3]crude protein: 60.14.
c, d – premix formulation detailed by Mousavi et al. (2020).

Real-time PCR assay

Total RNA from kidney and liver homogenates was extracted using a GeneAll extraction kit according to the manufacturer's instruction (GeneAll Biotechnology, South Korea). Isolated RNA quality and quantity were assessed according to the previous study (Ahmadifar et al., 2019). The first-strand complementary DNA (cDNA) was synthesized using the GeneAll kit based on the manufacturer's instructions (GeneAll Biotechnology, South Korea). In the current study, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene to normalize the gene expressions. The expression of targeted genes (Table 2) was calculated by real-time PCR in 20 µl reaction mixture consisting of 10 µl SYBR green master mix (Ampliqon, Denmark), 0.5 µl of each primer, and 2 µl cDNA. The amplification and detection of different PCR products were performed. The relative quantification of targeted gene expressions was calculated using the $2^{-\Delta\Delta Ct}$ equation (Livak and Schmittgen, 2001).

Table 2. The list of primers and their nucleotide sequences used in this study for amplifying genes

Gene	Primer	Length (pb)
IL-1β	F: 5' CGGGCAATATGAAGTCACC 3'	19
	R: 5' GTCCACATCTCCAGCCTGA 3'	19
IL-8	F: 5' ACTTAGGCAAAATGACCAGC3'	20
	R: 5' TATGTGTTTTCCAATGCGTC 3'	20
TNF-α	F: 5' TAGAACAACCCAGCAAACTC 3'	20
	R: 5' TCTCCTTCTTCAACATCCAA 3'	20
Lysozyme	F: 5' AATATGAGGCTGGCAGTGG 3'	19
	R: 5' AGTCCTTCCCCGTATCAGC 3'	19
GAPDH	F: 5' TCACAAACGAGGACACAACC 3'	20
	R: 5' GCCACGATCTCCACTTTCTT 3'	20

Digestive enzyme activity

Before the assays, protein concentration in each fish intestinal homogenate was determined based on the Lowry method (Lowry et al., 1951). Activity related to trypsin and chymotrypsin enzymes in fish intestinal homogenates were measured using commercial Elisa kits (Eastbiopharma Company, China) and recorded as µmol/mg protein. The amylase activity was estimated based on the previous study (Bernfeld, 1995). Fish intestinal homogenates (50 µl) were mixed with soluble starch (250 µl) and incubated for 30 min at 37°C. After adding 1% dinitrosalicylic acid (0.5 ml), boiling and cooling of the mixture were performed, and the final absorbance was recorded at 540 nm.

The lipase activity was measured based on the previous study (Bulow and Mosbach, 1987). Reaction buffer (1 ml) consisted of Tris-HCl (50 mM), and ethanol (4%) was mixed with fish intestinal homogenate (20 µl) and 50 mM p-nitrophenyl butyrate (60 µl). Absorbance was continuously recorded at 405 nm for 5 min.

The protease activity was measured based on the previous study (Sheikhzadeh et al., 2012). The reaction buffer consisted of azocasein (0.7%), and ammonium bicarbonate (100 mM) was mixed with the same volume of fish intestinal homogenate. After adding trichloroacetic acid (4.6%), centrifugation (5000 *g*) was performed for 10 min. The final supernatant was mixed with NaOH (0.5 M), and absorbance was recorded at 450 nm.

Immunological parameters

Lysozyme activity in fish mucus and total body homogenate were determined based on the previous study (Sheikhzadeh et al., 2012). Suspension of *Micrococcus luteus* bacterium in phosphate citrate buffer (75 µl) was mixed with fish mucus or total body homogenate (25 µl), and absorbance was continuously recorded at 450 nm for 5 min.

Antibody levels in fish mucus and total body homogenate were determined based on the previous study (Siwicki et al., 1994). To calculate the antibody level in homogenates, total protein content in fish mucus or body homogenate was assessed before and after antibody molecules precipitation with polyethylene glycol (12%).

Alternative complement activity in fish mucus was determined based on the previous study (Andani et al., 2012). Rabbit red blood cells washed in gelatin veronal buffer were adjusted to 2×10^8 cells/ml. Rabbit cell suspension (100 µl) was mixed with fish mucus (250 µl) and incubated at 20°C for 90 min with continuous shaking. After adding NaCl (0.85%) and centrifugation (1600 g) for 10 min, absorbance was recorded at 414 nm.

Antioxidant parameters

Antioxidant power in fish mucus was determined based on the previous study (Benzie and Strain, 1996). Reaction buffer consisted of 2,4,6-tripyridyl-s-triazine (10 mM/l), acetate buffer (300 mM/l), ferric chloride (20 mM/l), as well as hydrochloride acid (40 mM/l) was prepared. After initial absorbance recording at 593 nm, fish mucus homogenates (50 µl) were mixed with distilled water (150 µl) and reaction buffer (1.5 ml). After incubation at 37°C for 5 min, final absorbance was again recorded, and the difference between initial and final absorbance was considered as the total antioxidant power of each mucus sample.

Lipid peroxidation products in fish mucus were determined based on the previous study (Satoh, 1978). Briefly, trichloroacetic acid (20%) (1.25 ml) was mixed with fish mucus homogenates (0.25 ml) and centrifuged (2000 g) for 10 min. Then, 1.25 ml sulfuric acid (0.05 M) and 1 ml thiobarbituric acid (0.2%) were added to the collected sediment and boiled for 30 min. After adding n-butanol (2 ml), centrifugation (2000 g) was performed for 10 min. Final absorbance was recorded at 532 nm.

Catalase activity in fish mucus was determined based on the previous study (Goth, 1991). Reaction buffer (1 ml) consisted of hydrogen peroxide (65 mM/l) and sodium–potassium phosphate buffer (60 mM/l) was mixed with fish mucus (0.05 ml) for 1 min at 37°C. After terminating the enzymatic reaction with 1 ml ammonium

molybdate (32.4 mM/l), absorbance was recorded at 405 nm.

Superoxide dismutase activity in fish mucus was determined based on the previous study (Nishiimi et al., 1972). The reaction buffer consisted of 2.6 ml phosphate buffer (0.017 mM), 0.1 ml phenazine methosulfate, and 0.1 ml nitro blue tetrazolium mixed with fish mucus (0.05 ml). After adding 0.1 ml NADH (2.34 mM), the absorbance was recorded at 560 nm for 3.5 min.

Glutathione peroxidase activity in fish mucus was determined based on the previous study (Pagalia and Valentine, 1967). Reaction buffer (0.88 ml) consisted of GSH (1 mM), NADPH (150 mM), and sodium azide (100 mM) in potassium phosphate buffer was mixed with fish mucus (20 µl). The absorbance was recorded at 340 nm for 1 min.

Statistical analysis

The results were shown as mean ± standard error (SE). Shapiro–Wilk and Levene tests were run for data normality and variance homogeneity. Data were then subjected to a one-way analysis of variance (ANOVA) followed by the Tukey test in the Statistical Package for Social Sciences (SPSS) version 22. P values < 0.05 were considered significant.

Results

Growth performance

The growth-related traits are presented in Table 3. The results showed non-significant differences in the groups that received the control, PA, and SP diets regarding final weight, weight gain, SGR, and FCR (P>0.05). However, fish treated with PA and SP mixture had higher final weight, weight gain, SGR, and lower FCR than fish fed the control and SP diets (P<0.05). Further, no meaningful differences were seen between fish-fed PA and the mixture of PA and SP (P>0.05).

Digestive enzyme activity

Table 4 shows the digestive enzyme activity of zebrafish treated with PA, SP, and their mixture. The results illustrated that fish fed PA, SP, and their mixture had higher (P<0.05) protease and amylase activities than the control. The amylase activity showed the highest value (P<0.05) of amylase observed in fish treated with PA followed by those treated with the mixture of PA and SP and then those treated with SP. The lipase activity was significantly higher in fish treated with PA or the mixture of PA and SP than in control (P<0.05), with no differences with SP treatment (P>0.05). Chymotrypsin showed higher activity (P<0.05) by the mixture of PA and SP than the control and SP groups without differences from the PA group. At the same time, pepsin was markedly higher (P<0.05) in fish treated with PA than in control without significant differences with SP and the mixture of PA and SP. The trypsin activity was not significantly affected by A, SP, and their mixture (P>0.05).

Immune responses

The alternative complement pathway (ACH50) in the mucus samples of fish treated with PA or both PA and SP was significantly higher (P<0.05) than in fish treated with the control and SP diets (Figure 1). The mucus lysozyme activity was markedly higher (P<0.05) in fish treated with the mixture of PA and SP than in the control without significant differences with PA or SP (Figure 1). Total immunoglobulin level in the skin mucus was significantly higher (P<0.05) in fish fed PA than in control without significant differences with SP and PA and SP mixture (Figure 1).

Table 3. Growth performance of zebrafish fed with the control (T0), *Spirulina platensis* (T1), *Pediococcus acidilactici* (T2) or their mixture (T3)

	Initial weight	Final weight	Weight gain	Specific growth rate (SGR) (%)	Feed conversion ratio (FCR)	Survival rate (SR)
T0	69.53±0.63 a	167.77±1.26 a	97.22±1.25 a	1.70±0.08 a	2.14±0.21 b	100
T1	69.69±0.51 a	167.12±1.22 a	97.43±1.3 a	1.82±0.04 a	2.15±0.19 b	100
T2	69.43±0.61 a	168.28±1.60 ab	98.85±1.42 ab	1.89±0.06 ab	2.10±0.18 ab	100
T3	69.65±0.56 a	170.04±0.46 b	109.39±1.20 b	1.96±0.11 b	1.91±0.14 a	100

Different letters in each row indicate a significant difference (P<0.05).

Table 4. Digestive enzyme activity of zebrafish fed with the control (T0), *Spirulina platensis* (T1), *Pediococcus acidilactici* (T2) or their mixture (T3)

	Protease u/mg protein	Lipase u/mg protein	Amylase u/mg protein	Trypsin µmol/mg protein	Chymotrypsin µmol/mg protein	Pepsin µmol/mg protein
T0	7.60±0.34 a	2.88±0.22 a	38.13±1.99 a	0.58±0.05 a	0.10±0.01 a	0.14±0.01 a
T1	9.57±0.38 b	3.86±0.17 ab	50.72±1.88 b	0.46±0.04 a	0.10±0.01 a	0.23±0.02 ab
T2	9.33±0.40 b	4.08±0.26 b	78.32±2.82 d	0.67±0.07 a	0.12±0.01 ab	0.25±0.02 b
T3	9.36±0.36 b	4.12±0.38 b	66.12±2.09 c	0.68±0.08 a	0.14±0.01 b	0.22±0.03 ab

Different letters designate significant differences as determined by Tukey's post-hoc tests (P<0.05).

In the body homogenates samples, the lysozyme activity and immunoglobulin levels were markedly higher ($P<0.05$) in fish treated with the mixture of PA and SP than in control without significant differences with PA or SP (Figure 2). However, the total protein was not significantly affected by A, SP, and their mixture ($P>0.05$) (Figure 2).

Antioxidative responses

The catalase activity was not significantly affected by A, SP, and their mixture ($P>0.05$) (Figure 3). The glutathione peroxidase in fish treated with PA was significantly higher ($P<0.05$) than in fish treated with the control, SP, and the mixture of PA and SP diets (Figure 3). Superoxide dismutase in fish treated with the mixture of PA and SP was significantly higher ($P<0.05$) than in fish treated with PA or SP (Figure 3). The total antioxidative capacity of fish treated with PA or both PA and SP was significantly higher ($P<0.05$) than in fish treated with the control and SP diets (Figure 4).

Immune-related genes

The expression of IL1B and IL8 genes in fish treated with PA or the mixture of PA and SP was significantly higher ($P<0.05$) than in fish treated with the control or SP diets (Figure 4). There are no differences between fish fed PA and SP in the expression of IL1B and IL8. Fish treated with PA, SP, or both PA and SP had marked ($P<0.05$) upregulation of the lysozyme gene expression (Figure 4). The TNF expression was not significantly different among the groups (Figure 4).

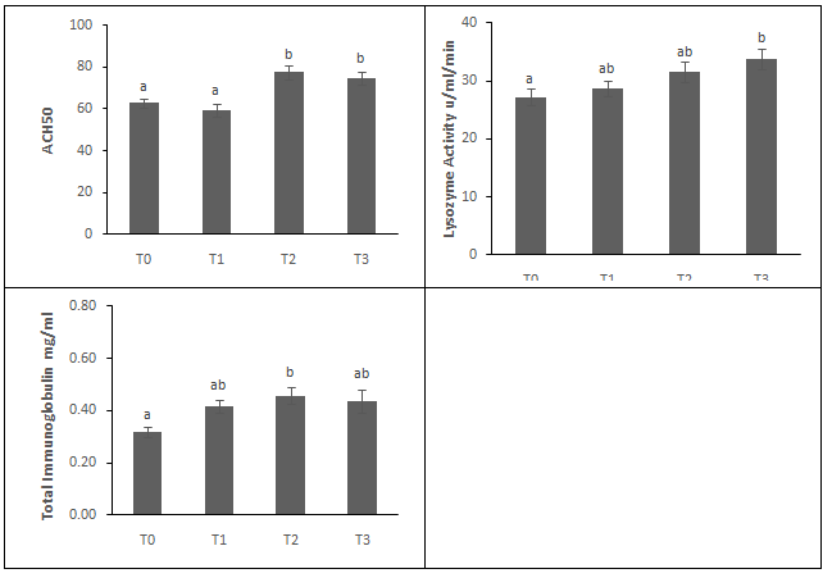


Figure 1. Mucus immune responses of zebrafish fed the control (T0), *Spirulina platensis* (T1), *Pediococcus acidilactici* (T2) or their mixture (T3). Different letters designate significant differences as determined by Tukey's post-hoc tests ($P<0.05$)

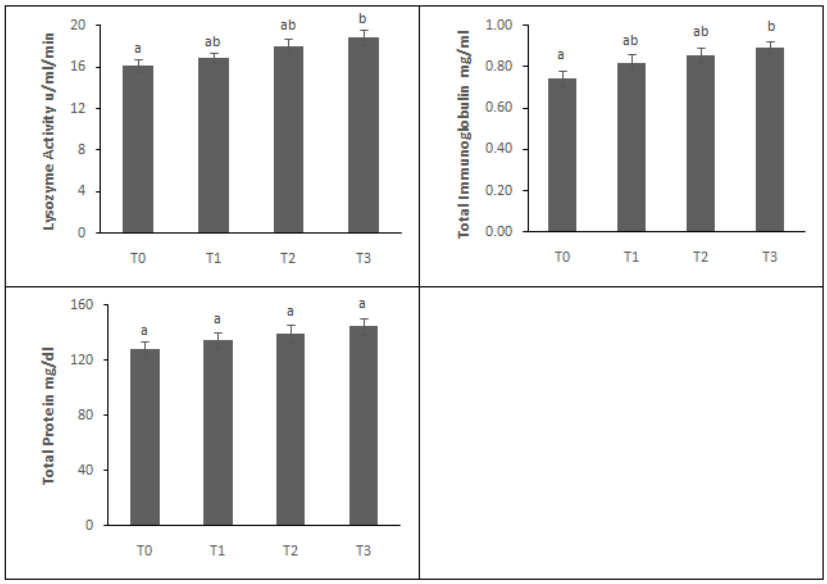


Figure 2. Whole body immune responses of zebrafish fed with the control (T0), *Spirulina platensis* (T1), *Pediococcus acidilactici* (T2) or their mixture (T3). Different letters designate significant differences as determined by Tukey's post-hoc tests ($P<0.05$)

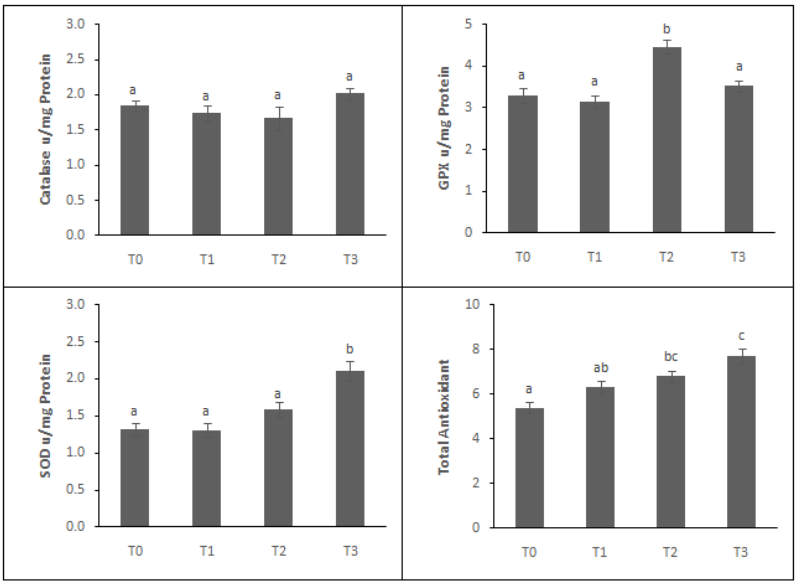


Figure 3. Antioxidative capacity of zebrafish fed with the control (T0), *Spirulina platensis* (T1), *Pediococcus acidilactici* (T2) or their mixture (T3). Different letters designate significant differences as determined by Tukey's post-hoc tests (P<0.05)

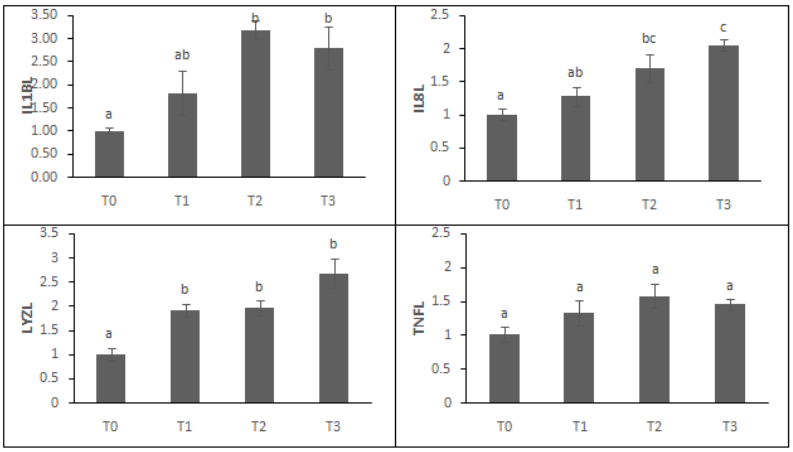


Figure 4. Immune related genes of zebrafish fed with the control (T0), *Spirulina platensis* (T1), *Pediococcus acidilactici* (T2) or their mixture (T3). Different letters designate significant differences as determined by Tukey's post-hoc tests (P<0.05)

Discussion

Successful aquaculture practices consider the high productivity and well-being of aquatic animals. In this regard, feed additives have gained a broad interest in aquaculture, devoted to their beneficial functional roles (Dawood, 2021). They can enhance growth performance, digestion ability, immunity, and stress resistance. Individual supplementation is mainly applied in aquaculture, including probiotics, prebiotics, medicinal plants, algae, and immunostimulants. However, recently, the combinations of different additives have been encouraged, especially if there are synergistic effects and potential roles on aquatic animals' performances (Shehata et al., 2022). In this study, probiotics (*Pediococcus acidilactici*) or/and *Spirulina platensis* were evaluated on zebrafish's growth and digestion performance, immune response, and antioxidative capacity.

The results indicated that the growth traits (final weight, weight gain, and SGR) were markedly increased while the FCR decreased in the fish fed both PA and SP group. The results illustrated that PA effectively enhanced the growth performance, which agrees with Merrifield et al. (2011) rainbow trout (*Oncorhynchus mykiss*), Rahimnejad et al. (2018) rockfish (*Sebastes schlegeli*), Ashouri et al. (2020) Asian sea bass (*Lates calcarifer*), Ahmadifar et al. (2020) zebrafish (*Danio rerio*), and Modanloo et al. (2017) common carp (*Cyprinus carpio*). Further, the inclusion of SP in the diets of zebrafish increased the growth performance, which is also in line with Siringi et al. (2021) Nile tilapia (*Oreochromis niloticus*), El-Araby et al. (2022), and Mohammadiazarm et al. (2020) Oscar

fish (*Astronotus ocellatus*). The inclusion of probiotics may enhance the growth performance via the protection of intestines and regulating their microbial content leading to efficient digestion and absorption of nutrients (Assan et al., 2022). Further, beneficial bacterial cells secret digestive enzymes that facilitate the digestion of nutrients eventually enhance digestion ability (Mugwanya et al., 2021). On the other hand, SP has balanced nutritional value, such as essential amino acids, fatty acids, minerals, and vitamins that can be digested and passed through the bloodstream leading to regulated physiological and metabolic functions (Alagawany et al., 2021). Besides, spirulina has no fibers, which makes it digestible and nutritious (Zhang et al., 2020). Synergistically, the inclusion of SP may act as prebiotics resulting in activated PA cells in the intestines of zebrafish. The activation of PA by SP is probably responsible for stimulating local intestinal digestion and immunity capacities (Dawood, 2021). Concisely, the results indicated enhanced digestive enzyme activities (protease, lipase, amylase, trypsin, and chymotrypsin), which may explain the enhanced feed efficiency and eventual growth performance of zebrafish received PA and SP.

The supplementation of PA and SP enhanced the growth performance of zebrafish and the immune response of the whole body and the skin mucus. In this study, the ACH50, lysozyme activity, and total immunoglobulin (Ig) were markedly improved in the skin mucus samples of zebrafish fed both PA and SP. Furthermore, the lysozyme activity and total Ig were enhanced in the homogenates of the whole zebrafish treated with PA and SP. The results are in the line of earlier studies that indicated the improved ACH50, lysozyme activity, and total Ig by dietary PA in zebrafish (Ahmadifar et al., 2020), common carp (Modanloo et al., 2017), and angelfish (*Pterophyllum scalare*) (Azimirad et al., 2016) fed dietary PA. Further, the inclusion of SP in the diets of rainbow trout (*Oncorhynchus mykiss*) (Sheikhzadeh et al., 2019 a) and Nile tilapia (El-Araby et al., 2022) led to improved ACH50, lysozyme activity, and total Ig. The activated immunity of zebrafish may result from PA and SP supplements as a synbiotic mixture (Al-Deriny et al., 2020). The high nutritional value of SP is attributed to its content of proteins, lipids, vitamins, and minerals, and the low content of fibers makes SP more digestible by bacteria (Alagawany et al., 2021). Consequently, the PA bacteria show high viability and activity, resulting in high immunity (Ghiasi et al., 2018). It is well documented that probiotics can compete with harmful microorganisms and relieve their impact on local intestinal immunity leading to activated entire body immunity (Yilmaz et al., 2022).

The enhancement of the immune response and the growth performance reflects the activation of the antioxidation status (Chen et al., 2020). In stressful conditions, the entire body shows less resistance to the overproduction of free radicals (ROS), which end in oxidative stress. Excessive ROS induces adverse effects on the body, including the immune cells, and eventually, a high possibility of infection with invaders and mortality (Birnie-Gauvin et al., 2017). In this study, zebrafish treated with PA and SP showed high antioxidative status as expressed by the total antioxidative activity and superoxide dismutase activity (SOD). Further, fish treated with PA had higher glutathione peroxidase (GPx) than the remaining groups. In the same line, zebrafish fed dietary PA had increased antioxidative status (Ahmadifar et al., 2020), while Nile tilapia (El-Araby et al., 2022) and blunt snout bream (*Megalobrama amblycephala*) (Jiang et al., 2022) provided SP showed improved antioxidative capacity. The inclusion of both PA and SP suggests their potential role as natural antioxidants in aquafeed (Alagawany et al., 2021). However, future studies are needed to understand their mode of action as antioxidant supplements.

The immune-related cytokines (IL-1β and IL-8) are regulated by TNF-α control through the activation of lymphoid cells leading to high resistance to pathogenic invaders (Modanloo et al., 2017; Olmos and Lladó, 2014). The study showed upregulated IL-1β and IL-8 genes in the liver of zebrafish treated with PA and SP. Similarly, zebrafish (Ahmadifar et al., 2020), and common carp (Chi et al., 2014) treated with PA, had improved IL-1β and IL-8 gene expression. Interestingly, dietary PA and SP also upregulated the lysozyme gene expression, indicating enhanced immunity in zebrafish. The activation of the lysozyme gene may be responsible for the enhancement of the lysozyme activity in the skin mucus and homogenates of zebrafish treated with PA and SP. The activated immune-related genes also confirm the immunomodulation roles of the PA and SP mixture.

Conclusion

All in all, the results indicated that using *Spirulina platensis* and *Pediococcus acidilactici* individually or combined is important for enhancing the growth performance, digestive enzyme activity, and zebrafish's immune and antioxidative capacity. Markedly, the mixture of *S. platensis* and *P. acidilactici* is more effective than using each of them individually. The obtained results recommend using a mixture of feed additives in the aquafeed industry for their potential roles as growth-promoting, immunomodulation, and antioxidation effects.

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