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DIETARY ADMINISTRATION OF *LACTOBACILLUS REUTERI* AND *LACTOBACILLUS PLANTARUM* REDUCES WHOLE BODY OXIDATIVE STRESS AND INCREASES IMMUNE RESPONSE, DIGESTIVE ENZYME, GROWTH PERFORMANCE AND RESISTANCE OF ZEBRAFISH (*DANIO RERIO*) AGAINST *AEROMONAS HYDROPHILA* INFECTION

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Abstract

This study evaluated the individual and combined effects of *L. plantarum* and *L. reuteri* on the growth performance, digestive enzymes, antioxidant, and immunity-related genes in zebrafish. Six hundred zebrafish (38.19±1.4 mg) were randomized into twelve tanks (50 fish per), and fed prepared diets comprising unsupplemented (T0), 1×10^8 CFU/g *L. plantarum* (T1), 1×10^{11} CFU/kg *L. reuteri* (T2), and 1×10^8 CFU/g *L. plantarum* + 1×10^{11} CFU/kg *L. reuteri* (T3) for 8 weeks. Experimental diets had meaningful effects on weight gain (WG), final weight (FW), and the highest WG and FW were seen in T3. Fish-fed supplemented diets had higher amylase and lipase activities. No significant differences were seen in protease, pepsin, chymotrypsin, and trypsin activities between treatments. The supplemented groups showed a significant increase in lysozyme activity and total immunoglobulin levels. Meaningful differences were noticed in ACH50, lysozyme activity, total immunoglobulin, and IgM levels. ALT levels were markedly higher in T2 and T3. Catalase (CAT), total antioxidant, and glutathione peroxidase (GPX) activity were significantly impacted by the experimental diets. The experimental diets showed no marked impact on superoxide dismutase (SOD) activity, and malondialdehyde (MDA) values. Relative expressions of *CAT*, *GPX*, and *SOD* genes were notably upper in T3. Moreover, *IL1*, *LYZ*, and *TNFα* gene expressions were significantly enhanced in T3 diets. The challenge test with *A. hydrophila* showed that zebrafish fed *L. plantarum* and *L. reuteri* had a lower mortality rate than the control. Consequently, the combination of *L. reuteri* and *L. plantarum* is proposed to improve growth efficiency, immunity, and reduce the negative effects of *A. hydrophila* infection in zebrafish.

Key words: *Lactobacillus reuteri*, *Lactobacillus plantarum*, aquaculture, *Danio rerio*, probiotics

The unconscious use of antibiotics causes negative effects on the health of people consuming processed seafood, the emergence of antibiotic-resistant strains, and various problems in the aquatic environment (Larsson et al., 2022; Hossain et al., 2022; Mello et al., 2022; Wang et al., 2023). Researchers have tried to find cost-effective, natural, and environmentally safe substitutions for replacing antibiotics in aquaculture. Probiotics have an important place among these alternative feed supplements, and their effectiveness was proven in many fish and shrimp species today (El-Saadony et al., 2021; Yilmaz et al., 2022; Abdel-Latif et al., 2022).

Zebrafish was used as a fish model organism for many years in feeding trials on the effects of feed additives on fish physiology. Probiotic applications appear to have positive effects on zebrafish's reproductive ability (Gioacchini et al., 2010; Carnevali et al., 2013), sperm quality (Valcarce et al., 2019), gastrointestinal tract viability, and motility (Lu et al., 2019), prevention of pathogenic bacterial colonization in intestines (Rendueles et al., 2012), immune status (Wang et al., 2018), health status (López Nadal et al., 2020), and correction of anxiety-related behavior (Valcarce et al., 2020).

Bacterial species belonging to *Bacillus*, *Lactobacillus*, and *Bifidobacterium* groups among probiotic feed supplements have an important position with their ability to settle in the intestinal flora, as well as their ability to produce vitamins, bacteriocins, organic acids, growth-

promoting metabolites, and digestive enzyme secretions. Previous studies showed that *L. plantarum* (Abumourad et al., 2013; Sagada et al., 2021; Li et al., 2022 a; Keereelang et al., 2022) and *L. reuteri* (Giri et al., 2018; Li et al., 2022 b) separately added into fish feeds have positive effects on fish health and development. However, research has shown that including many strains of probiotic bacteria into fish diets has a more pronounced synergistic impact on both fish development and health (Pillinger et al., 2022; Abdel-Latif et al., 2023). It is mentioned that adding *L. reuteri* and *L. plantarum* to the feed in weaned piglets can prevent the occurrence of diarrhea (Dell'Anno et al., 2021). Using multi-strain probiotics in aquaculture showed optimal effects compared to single-strain probiotics (Kwoji et al., 2021). Multi-strain probiotics utilization leads to increased effects of bowel immunity via increasing proinflammatory cytokines (TNF-α and IL-1β) in the intestine (Standen et al., 2016), enhances non-specific immunity and the abundance of intestinal microbiota (Xie et al., 2019), stimulates cellulolytic and amylolytic enzymes secretions with improved growth output (Bairagi et al., 2004). Nevertheless, based on our knowledge, no study was done on the effect of co-addition of *L. reuteri* and *L. plantarum* to the fish diets on their health and growth indices. The effects of separate and the co-addition of *L. reuteri* and *L. plantarum* into zebrafish feed on digestive enzymes, antioxidant status, growth performance, and immune parameters of fish were investigated for the first time.

Material and methods

Diets

L. plantarum and *L. reuteri* bacteria were prepared from Iranian Biological Resource Center (Tehran, Iran). The strains were cultured in de Man, Rogosa, and Sharpe (MRS) broth for 24 h at 37°C. The grown bacteria were diluted using the phosphate buffer saline (PBS) (pH 7.4) to the desired concentration (10^8 CFU/g for *L. plantarum* and 10^{11} CFU/kg for *L. reuteri*) based on Giri et al. (2021) and Li et al. (2022 b). A basal diet (BioMar SAS, Nersac, France) was purchased and supplemented with 10^{11} CFU/kg *L. reuteri* and 10^8 CFU/g *L. plantarum*. The treatments were: unsupplemented (T0), 10^8 CFU/g *L. plantarum* (T1), 10^{11} CFU/kg *L. reuteri* (T2), and 10^8 CFU/g *L. plantarum* + 10^{11} CFU/kg *L. reuteri* (T3). The diet was spread on discrete trays and dried at room temperature for 24 hours. The meals were packaged and kept at –20°C, while the daily diets were kept at 4°C.

Fish and experimental conditions

A total number of 650 healthy zebrafish (38.19±1.4 mg) were purchased from Royan Institute (Tehran, Iran), and transferred to Kharazmi University (Iran). The fish were randomly assigned to twelve aquariums, with 50 fish per tank (four treatments with three replicates). They were then subjected to experimental conditions for fourteen days. Commercial diets were administered to fish on a twice-daily basis. Then, aquaria were randomized to 4 treatments (50 fish per aquarium), and the fish were fed three times for 56 days. Water was changed daily (70%) with fresh water. During the feeding trial, dissolved oxygen, water temperature, and pH were checked and reported as 7.11±0.16 mg L^{-1}, 25.1±0.6°C, and 7.53±0.2, respectively.

Growth performance

After 8 weeks (before challenging), the fish in all aquaria, which had been starved for 24 hours, were euthanized with 50 ml l^{-1} clove oil (Sigma Aldrich, Germany) (Ahmadifar, 2023). The fish were weighed, and their growth indexes were estimated as follows (NRC, 1993):

Feed conversion ratio (FCR) = dry weight of feed given (g)/WG (g),

Weight gain (WG) = final weight (g) – initial weight (g),

Specific growth rate (SGR) = Ln final weight − Ln initial weight/days × 100,

Survival rate (%) = (final number of fish/initial number of fish) × 100.

Tissue and mucus sampling

After 8 weeks, ten fish (30 fish for each treatment) per aquarium after 24 h starvation were euthanized with a clove oil bath (50 ml L^{-1}). The heads and fins of fish were removed, and then whole-body samples were prepared based on the Holbech et al. (2001) method. Samples were moved to sterile tubes, and kept at –80°C until use. In accordance with a previous experiment (Hoseinifar et al., 2020), five fish were selected from each aquarium (15 fish for each treatment). The fish were then placed in a polyethylene bag containing 10 mL of 50 mM NaCl and gently brushed to remove skin mucous. The samples were subjected to centrifugation (1500 g for 10 min), and the resulting supernatant was collected and kept at –80°C for analysis.

Digestive enzymes assay in tissue

Whole body of five fish (per tank) was homogenized in PBS (pH 7.4) by a homogenizer and then, the mixture was centrifuged (at 3000 rpm) for 20 minutes, and then supernatants were collected and kept at –80°C until analysis of enzyme activity or protein content. Bradford's method (1976) with bovine serum albumin solution as a standard solution was used to measure total soluble protein. ELISA kits from Eastbiopharm Company (China) were utilized for the quantification of trypsin (Cat. No: CK-E91540, Eastbiopharm Co., China) and chymotrypsin (Cat. No: CK-E92024, Eastbiopharm Co., China) activities. The method (Bernfeld et al., 1955) was used to detect the amylase activity. In short, the samples (50 µL) were combined with a starch solution (250 µL) and subjected to incubation at 37°C for 30 minutes. Afterwards, the mixture was enriched with 1% dinitro salicylic acid (0.5 mL) and then heated to boiling temperature. Follow-

ing the process of chilling, the absorbance was quantified at a wavelength of 540 nm. For the evaluation of lipase activity, fish homogenate (20 µL) was mixed with p-nitrophenyl butyrate (50 mM, 60 µL), and buffer (Tris-HCl 50 Mm and ethanol 4%, 1 mL). The absorbance was continuously monitored at 405 nm for a period of 5 minutes (Bulow and Mosbach, 1987). The protease activity was quantified using the method previously described (Worthington et al., 1991). At first, fish homogenate was combined with a reaction buffer (ammonium bicarbonate 100 mM and azocasein 0.7%). Subsequently, trichloroacetic acid (4.6%) was added to the mixture, which was then subjected to centrifugation at 5000 g for 10 minutes. The supernatant was removed, and blended with NaOH (0.5 M). Finally, the absorbance was measured at 450 nm (Worthington et al., 1991). Proteolytic activity was assessed by means of the azocasein hydrolysis assay as per the protocol established by Palaksha et al. (2008). An equivalent amount of fish homogenate was subjected to incubation with a 100 mM ammonium bicarbonate buffer containing 0.7% azocasein, pH 7.8, for a duration of 19 hours on a shaker at a temperature of 30°C. The reaction was terminated by introducing 4.6% trichloroacetic acid, followed by a centrifugation step of 10 minutes at a speed of 10,000 rpm. The supernatant obtained was then mixed with an equal volume of 0.5 M NaOH within a microplate well, and the resulting increase in optical density values was quantified at a wavelength of 405 nm.

Measurement of innate immune factors

Mucus and body homogenate lysozyme activity were assayed following the Sheikhzadeh et al. (2012) method. The total protein was quantified based on the biuret method using an assay kit from Pars Azmun Company (Pars Azmun, Karaj, Iran) following the protocols. Total immunoglobulin was quantified based on the method of Siwicki et al. (1994) using previously described protocol. Alternative complement activity (ACH_{50}) in fish mucus was measured according to the method proposed by Andani et al. (2012).

Antioxidant parameters and liver enzyme

A former method (Benzie and Strain, 1996) was used to measure the antioxidant potential of fish samples. First, the initial absorption of reaction buffer was reported at 593 nm, and then fish samples (50 µL), and distilled water (150 µL) were incorporated into the reaction buffer and incubated at 37°C for 5 minutes. Subsequently, the total antioxidant potential was determined by taking into account the disparity between the initial and final absorbance values. The catalase (CAT) activity in fish samples was measured using the procedure developed by Goth et al. (1991). Nishikimi et al. (1972) method was used to assay superoxide dismutase (SOD) activity in fish samples. The method of a previous study (Pagalia and Valentine, 1967) was used to determine glutathione peroxidase (GPX) activity in fish samples. Enzyme-linked immunosorbent assay (ELISA) commercial kit (Pars Azmun, Iran) was utilized to quantify alanine aminotransferase (ALT), alkaline phosphatase (ALP), and aspartate aminotransferase (AST).

Gene expression analysis

After 8 weeks, three fish were randomly selected from each aquarium and euthanized with overdose of clove oil bath (50 ml L^{-1}). The fins and heads were eliminated, and quickly taken in liquid nitrogen. The extraction of total RNA was done by GeneAll Kit. The Nanodrop spectrophotometer (260/280 nm), and 1% agarose gel were applied to verify the concentration and purity of the isolated RNA. Using GeneAll Kit (GeneAll Biotechnology, Seoul, Korea), complementary DNA (cDNA) was synthesized from the isolated RNA. To evaluate the selected gene expression (Table 1), primers were outlined by Oligo primer analysis software. SYBR Green method was employed to determine the expression of the selected gene, as previously described in the study by Ahmadifar et al. (2019). The fold change in the selected gene's mRNA expression was computed through $2^{-\Delta\Delta Ct}$ method. Gapdh gene was used as a reference gene.

Table 1. The list of primers and their nucleotide sequences used in this study for amplifying genes

Gene	Primer	Length (pb)	Tm (°C)	NCBI Reference
CAT	F: 5' ACAAGTTAAATGCGATGACG 3' R: 5' CGTCTTTCCAATATGCTCAA 3'	20 20	54.74 53.71	XM_021470442.1
GPX	F: 5' GACACGCCCACAAAAACTT 3' R: 5' CTTTGAACAGTCCTGCTCGT 3'	19 20	56.97 58.31	NM_200222.1
SOD	F: 5' GTCGGTTTCTTTCACTCTCTC 3' R: 5' ACTGGCTTCTTTTCACCCT 3'	21 19	56.32 56.44	NM_131294.1
IL-1	F: 5' CGGGCAATATGAAGTCACC 3' R: 5' GTCCACATCTCCAGCCTGA 3'	19 19	56.8 58.71	NM_212844.2
TNF-α	F: 5' TAGAACAACCCAGCAAACTC 3' R: 5' TCTCCTTCTTCAACATCCAA 3'	20 20	55.59 53.87	NM_212859.2
LYZ	F: 5' AATATGAGGCTGGCAGTGG 3' R: 5' AGTCCTTCCCCGTATCAGC 3'	19 19	59.92 60.67	NM_139180.1

CAT: Catalase; *GPX*: Glutathione peroxidase; *SOD*: Superoxide dismutase; *IL-1*: Interleukin-1; *TNF-α*: Tumor necrosis factor-alpha; *LYZ*: Lysozyme.

Challenge with *Aeromonas hydrophila*

A. hydrophila (ATCC 7966) was purchased from Iranian Biological Resource Center (Tehran, Iran) and cultured in TSB for 24 h. After 8 weeks, 30 fish were moved from each tank to new tanks, the water volume was minimized to half, and the fish were submerged in a bacterium of 1.7×10^6 CFU mL^{-1} concentration (Rashidian et al., 2021). These tanks were well-aerated and kept the bacteria suspended for maximum interaction. After an exposure of 24 h, the water was completely changed twice, and the dead fish were quickly taken out over 14 days.

Statistical analysis

This study employed a randomized design, and all the analyses were performed in three replicates. All data were statistically analyzed using SPSS version 22.00 for Windows. First, the Kolmogorov-Smirnov test was performed to check the data normality, and Levene's test was applied to check the homogeneity of variances. Moreover, a one-way analysis of variance (ANOVA) was utilized to compare among the meaningful differences in treatments. Differences among the treatments were evaluated by Tukey's test.

Results

Growth performance

Table 2 shows the growth performance of zebrafish after being given the test diets. Experimental diets had a considerable effect on FW and WG, and the highest FW and WG were observed in T3 ($P<0.05$). A meaningful difference in FCR was noticed among the treatments, and the lowest FCR was seen in T3 ($P<0.05$). No remarkable difference in SGR and survival rate was seen among the treatments ($P>0.05$).

Digestive enzyme activity in fish tissue

The digestive enzyme activity of zebrafish was reported in Table 3. The fish that were fed with supplemented diets exhibited a greater amylase activity compared to the fish that were fed with unsupplemented diets ($P<0.05$). Lipase activity was markedly higher in T2 and T3 than in the control ($P<0.05$). No meaningful difference was seen in protease, pepsin, chymotrypsin, and trypsin activity among the treatments ($P>0.05$).

Innate immune factors in fish tissue

The results of immune factors in fish tissue are shown in Table 4. The effects on lysozyme activity and total immunoglobulin among the treatments were notable, and they were markedly higher in the supplemented groups in contrast to the control (Table 4, $P<0.05$). In terms of total protein, no significant difference was noticed among the treatments (Table 4, $P>0.05$).

Innate immune factors in fish mucus

A significant difference was found in ACH50 among the treatments, and it was markedly larger in the fish fed with supplemented diets in contrast to the control group (Figure 1 A, $P<0.05$). Compared to the control group, the lysozyme activity (Figure 1 B) and total immunoglobulin (Figure 1 C) were notably enhanced in T2 and T3. However, no meaningful difference was noticed between T2 and T3 ($P<0.05$). The fish fed with supplemented diets showed markedly higher IgM levels (Figure 1 D) ($P<0.05$).

Table 2. Growth performance of zebrafish (*Danio rerio*) fed with *L. plantarum* and *L. reuteri* for 8 weeks. Different letters designate significant differences as determined by Tukey's post-hoc tests (Mean ± SE)

	Initial weight (mg)	Final weight (mg)	Weight gain (mg)	FCR	SGR (% day^{-1})	Survival rate %
T0	39.44±1.64	87.11± 3.20 a	47.67±2.03 a	2.74±0.12 b	1.41±0.05	100
T1	37.78±1.31	90.56±2.63 ab	52.78±2.47 ab	2.47±0.11 ab	1.57±0.06	100
T2	37.11±1.64	102.45±3.78 ab	65.33±4.99 ab	2.01±0.15 a	1.82±0.13	100
T3	38.44±1.96	106.55±4.75 b	68.11±6.28 b	1.94±0.18 a	1.81±0.15	100

Different letters designate significant differences as determined by Tukey's post-hoc tests (Mean ± SE).

Table 3. Digestive enzyme activity of zebrafish (*Danio rerio*) fed with *L. plantarum* and *L. reuteri* for 8 weeks

	Amylase U/mg protein	Lipase U/mg protein	Protease U/mg protein	Pepsin µmol/mg protein	Chymotrypsin µmol/mg protein	Trypsin µmol/mg protein
T0	39.85±1.81 a	3.12±0.35 a	6.40±0.47	0.12±0.01	0.12±0.01	0.48±0.03
T1	55.72±2.29 b	2.91±0.20 a	6.65±0.62	0.15±0.03	0.12±0.01	0.50±0.03
T2	62.53±2.79 b	3.82±0.27 ab	6.92±0.56	0.17±0.02	0.15±0.01	0.58±0.01
T3	57.80±2.95 b	4.32±0.21 b	8.47±0.69	0.18±0.02	0.18±0.02	0.50±0.04

Different letters designate significant differences as determined by Tukey's post-hoc tests (Mean ± SE).

Table 4. Lysozyme activity, total immunoglobulin and total protein in zebrafish (*Danio rerio*) fed with *L. plantarum* and *L. reuteri* for 8 weeks

	Lysozyme activity U/mg protein tissue	Total immunoglobulin mg/g tissue	Total protein mg/100 g tissue
T0	14.75±0.71 a	0.70±0.02 a	131.24±4.70
T1	17.28±0.78 ab	0.83±0.03 b	135.74±4.67
T2	17.37±1.14 ab	0.87±0.02 b	148.94±5.08
T3	19.83±1.13 b	0.85±0.03 b	142.61±4.52

Different letters designate significant differences as determined by Tukey's post-hoc tests (Mean ± SE).

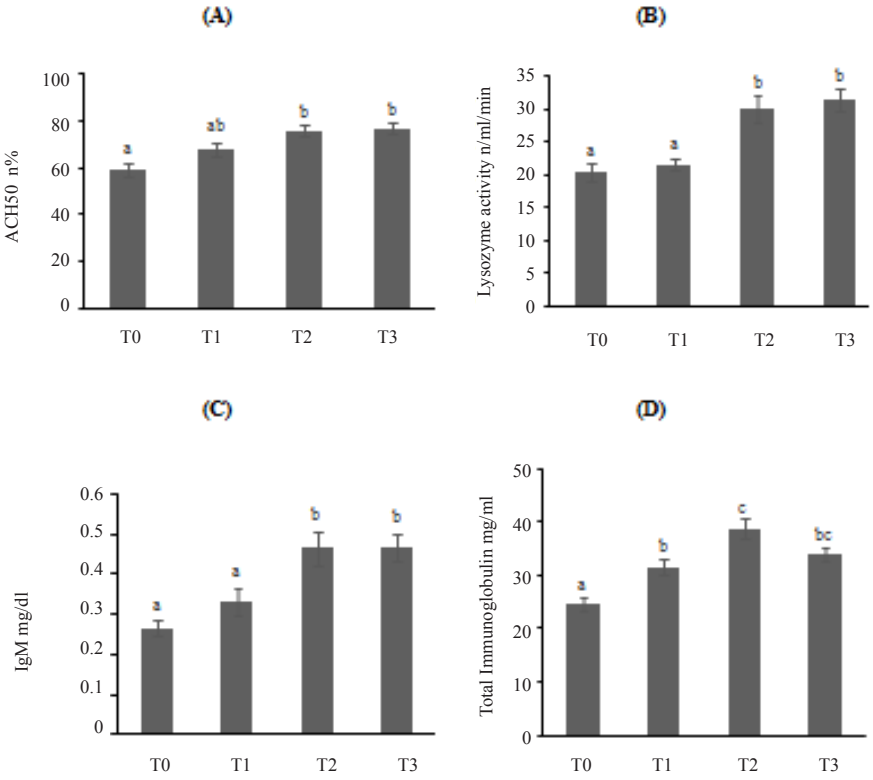


Figure 1. ACH50 (A), lysozyme activity (B), total immunoglobulin (C), and IgM (D) in the mucus of zebrafish fed experimental diets. Different letters designate significant differences as determined by Tukey's post-hoc tests (Mean ± SE)

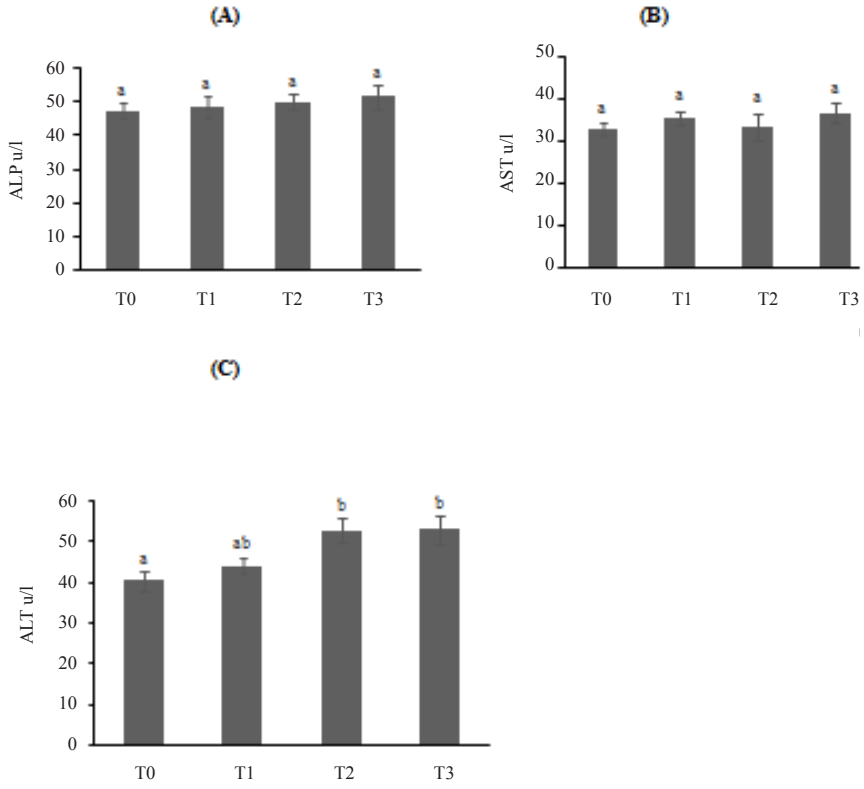


Figure 2. Alkaline phosphatase (ALP, A), aspartate aminotransferase (AST, B), and alanine aminotransferase (ALT, C) in the mucus of zebrafish fed experimental diets. Different letters designate significant differences as determined by Tukey's post-hoc tests (Mean ± SE)

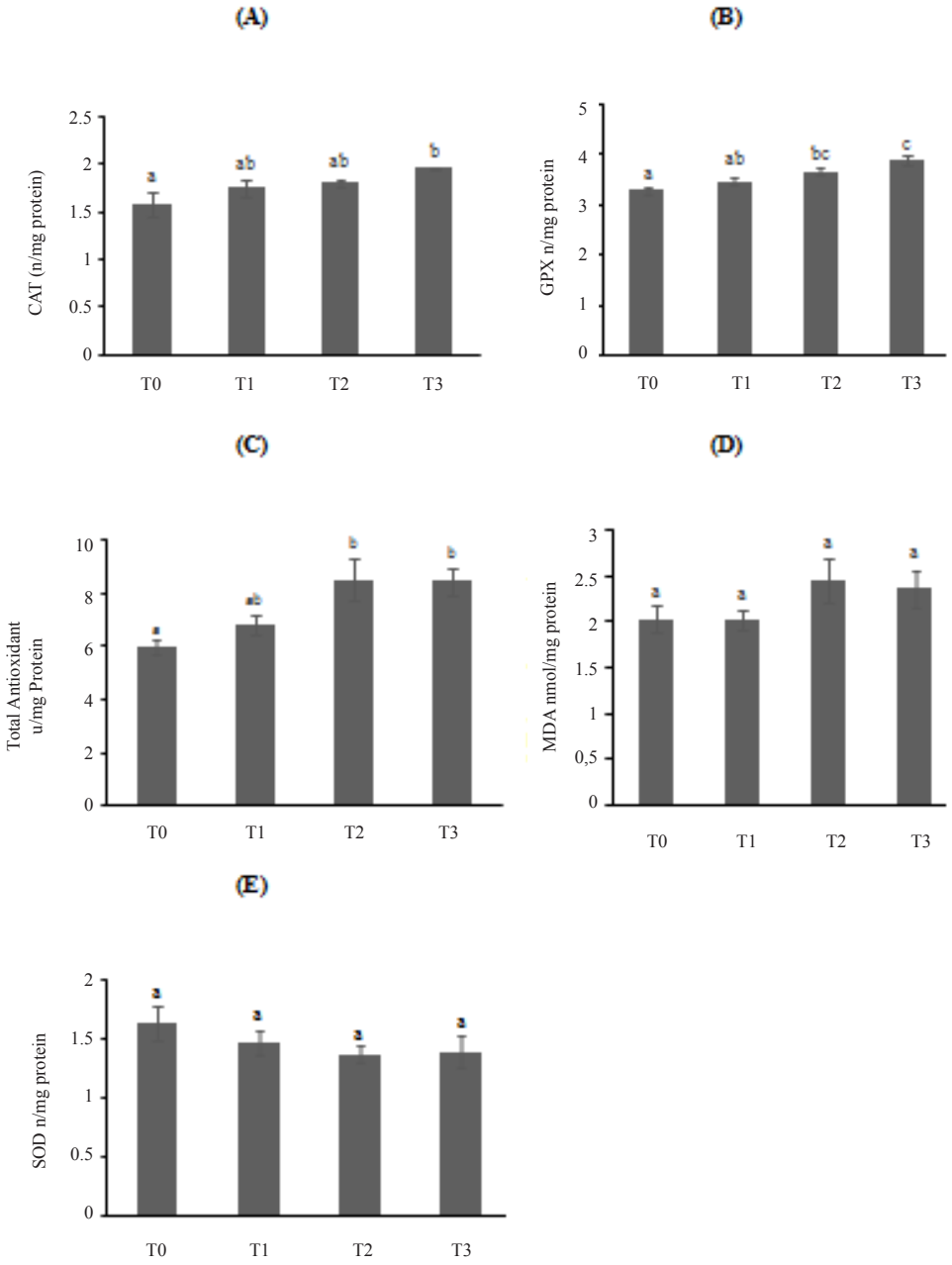


Figure 3. Catalase (CAT, A), Glutathione peroxidase activity (GPX, B), Total antioxidant (C), Malondialdehyde (MDA, D), and Superoxide dismutase (SOD, E) in the mucus of zebrafish fed experimental diets. Different letters designate significant differences as determined by Tukey's post-hoc tests (Mean ± SE)

Liver enzyme activity and antioxidant parameters in fish mucus

Supplemented diets did not affect ALP and AST activity (Figure 2 A, and B, P>0.05). Contrary to the control group, ALT activity was markedly higher in T2 and T3 (Figure 2 C, P<0.05). Fish fed with LR, LP, or a combination of LR and LP exhibited greater levels of CAT activity (Figure 3 A), GPX (Figure 3 B), and total antioxidant (Figure 3 C), which were all substantially impacted by excremental diets (P<0.05). The excremental diets did not significantly affect the MDA (Figure 3 D) and SOD (Figure 3 E) activities (P>0.05).

Antioxidant and immunity-related genes

Relative expression detected in *CAT* gene (Figure 4 A), *GPX* (Figure 4 B), and *SOD* (Figure 4 C) was remarkably enhanced in T1, T2, and T3 in contrast to T0 (P< 0.05). Contrary to the control group, *IL1* gene expression (Figure 4 D) was markedly increased in T3 diets (P<0.05). Lysozyme gene expression (Figure 4 E) was meaningfully upregulated in T1, T2, and T3 (P<0.05). Compared to the base group, the expression of *TNFα* (Figure 4 F) was notably enhanced in all the treatments (P<0.05).

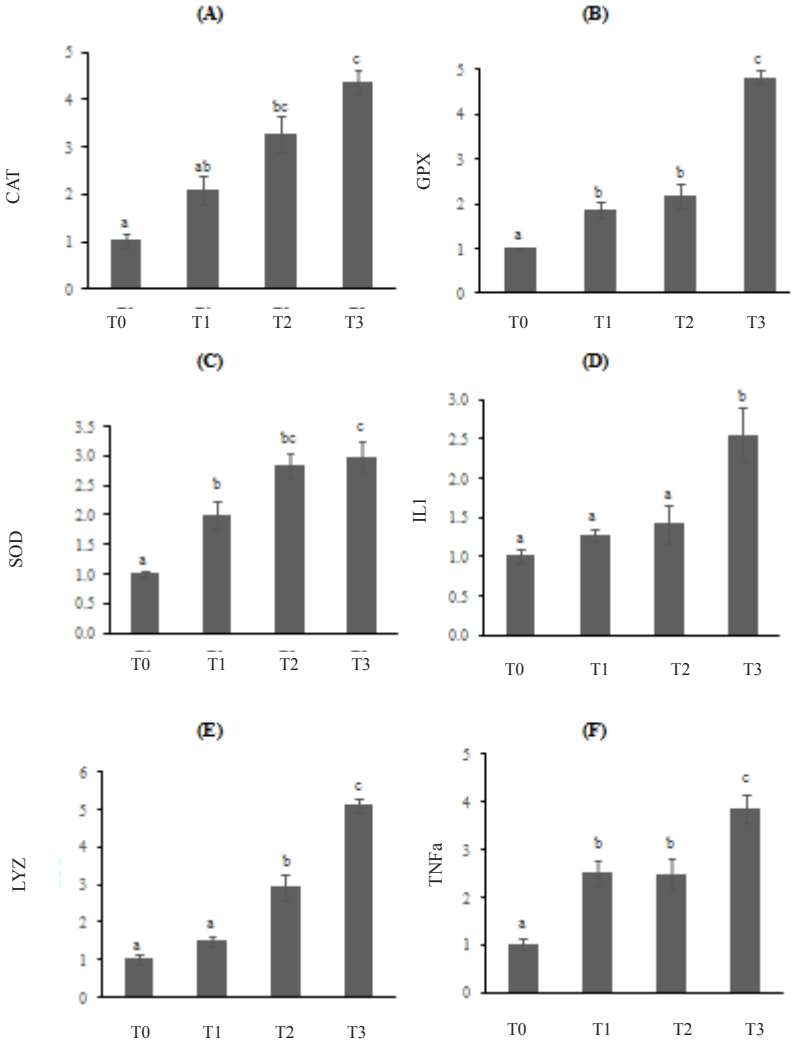


Figure 4. Relative expression of catalase (A, *CAT*), glutathione peroxidase (B, *GPX*), superoxide dismutase (C, *SOD*), interleukin 1 (D, *IL1*), and lysozyme (*LYZ*, E), and *TNFα* (F) of zebrafish fed experimental diets. Different letters designate significant differences as determined by Tukey's post-hoc tests (Mean ± SE)

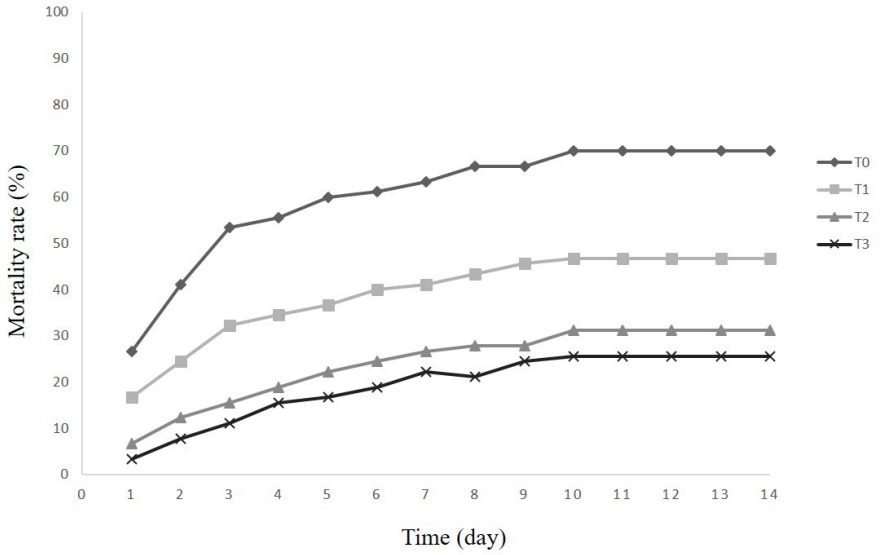


Figure 5. Cumulative mortality (%) of zebrafish fed experimental diets and subsequently challenged with *A. hydrophila*. Data are presented as mean ± SE

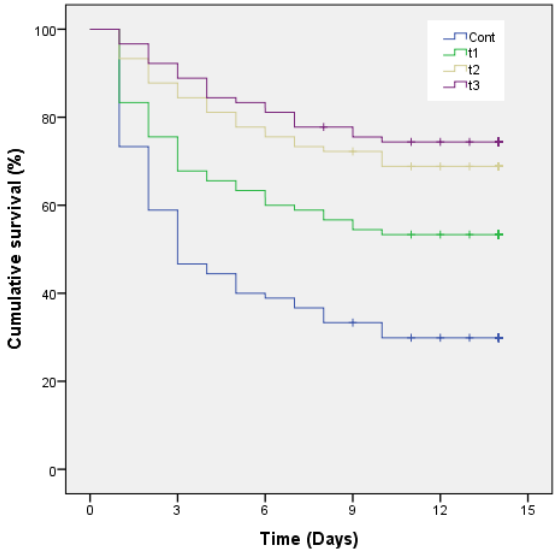


Figure 6. Kaplan–Meier survival curve for zebrafish fed experimental diets for 8 weeks and challenged with *A. hydrophila* for 14 days

Challenge with *A. hydrophila*

The challenge test with *A. hydrophila* showed that zebrafish fed *L. plantarum* and *L. reuteri* had a lower mortality rate than the control (Figures 5, 6).

Discussion

Dietary supplementation separately with *L. reuteri* and *L. plantarum* had no marked effects on zebrafish growth after a feeding experiment for 56 days. Nevertheless, the combined use of dietary *L. reuteri* and *L. plantarum* had a tangible effect on digestive enzymes and growth performance in the fish. Considering the results, the fish fed with a mixture of *L. reuteri* and *L. plantarum* showed the highest growth performance. Therefore, the increased activity of digestive enzymes like lipase, amylase, and protease, which were specifically found in the T3 group, may help the zebrafish perform better and better utilize their nutrients. Moreover, the growing effects of the amalgamation of *L. reuteri* and *L. plantarum* on the growth parameters of fish could be potentially connected to their enhancement of the gut morphology and structure, as well as the activation of antioxidant enzymes. Previous reports showed that *L. reuteri* (Li et al., 2022 b) and *L. plantarum* (Abumourad et al., 2013; Keereelang et al., 2022; Sagada et al., 2021) supplementations enhanced digestive enzyme activities and growth performance in several fish species. Nevertheless, to date, there are no reports regarding the usage of *L. reuteri* and *L. plantarum* as a growth promoter supplement in zebrafish feed. A few reports showed that single-strain probiotic supplements could enhance the digestive enzyme activities, growth performance, and/or antioxidant status of zebrafish (Mohammadi Arani et al., 2019; Arani et al., 2021). Probiotics aggressively prevent the growth of possible pathogens in the gastrointestinal system by altering microbial metabolism, promoting antibiosis, and competing with them for resources and space. Furthermore, they enhance nutritional quality by neutralizing potentially harmful substances in food, modifying the structure of potentially indigestible components via the hydrolytic enzymes like amylases and proteases, synthesizing essential vitamins, such as biotin and vitamin B_{12} (Hoshino et al., 1997). Furthermore, they produce inhibitory compounds and boost the host's immune response (Nguyen et al., 2017).

The biochemical parameters analysis can be considered a fundamental biomarker for evaluating the health status of fish (Yilmaz and Ergün, 2018). Serum and/or tissue enzyme activities are accepted as important indicators of tissue damage, for example, ALT, AST, and ALP are indicators of liver damage (Yilmaz et al., 2019). Therefore, increases in these enzymes in blood could be attributed to liver tissue damage. It was shown that tissue total protein and liver function enzymes (AST, ALT, and ALP) did not markedly differ among the experimental groups. These findings also demonstrated that the liver functions of zebrafish were not adversely affected by dietary *L. reuteri*, *L. plantarum*, or their combination. Moreover, the protective effect of *L. reuteri* and *L. plantarum* against heavy metals-induced damage of the liver in fish was reported (Zhai et al., 2017; Giri et al., 2018).

Enzymatic antioxidant protectors, including CAT, SOD, and GPX play a significant role in the detoxification and impede the negative effects of free radicals and reactive oxygen species (ROS) (Oberley and Oberley, 1988). MDA is frequently used as a biomarker of lipid peroxidation. It was found that total antioxidant and GPX enzyme activities considerably increased in the T3 group, and the expression of antioxidant-encoding genes (CAT, SOD, and GPX) significantly increased especially in T2 and T3 groups. Parallel with our study, this probiotic could enhance the antioxidant capacities of black sea

bream, *Acanthopagrus schlegelii* (Sagada et al., 2021), common carp, *Cyprinus carpio*, (Giri et al., 2021), and grass carp, *Ctenopharyngodon idellus* (Xue et al., 2020). The findings of the current study can be associated with the antioxidant abilities of probiotics. Recent research showed a novel Nrf2 control mechanism that functions without the assistance of Keap1 (Li et al., 2012; Lo et al., 2017; Perez-Leal et al., 2016). Thus, *L. reuteri* and *L. plantarum* might trigger Nrf2 activation via a Keap1-independent pathway to enhance the intestinal antioxidant capacity, while further research is required to elucidate its underlying mechanism. Moreover, our findings suggest that the inclusion of *L. reuteri* and *L. plantarum* in the diet can boost cellular antioxidant responses by elevating CAT levels, and GPX activity. Dawood et al. (2016) illustrated the beneficial effects of probiotic *Lactobacillus* strains on the antioxidant capability of red sea bream, *Pagrus major* (Dawood et al., 2016). Hoseinifar et al. (2020) reported that probiotics could produce some metabolites, such as glutathione, exopolysaccharides, folate, and butyrate which have enhanced the antioxidant capacity in fish.

According to Yilmaz and Ergün (2018), it is often believed that higher levels of serum total protein, lysozyme activity, alternative complement proteins, and immunoglobulins are associated with stronger immune responses in fish. The present study, therefore, indicates a combination of *L. plantarum* and *L. reuteri* has immunostimulant effects in zebrafish, characterized by higher tissue-soluble immune components. The dietary application of probiotics increased the expression of immune-related genes, including *TNF-α, LYZ,* and *IL-1*. These results confirm the immunomodulatory effects of probiotic supplements in zebrafish. The findings were in line with the results of some published works which implied that dietary supplementation with probiotic supplements could enhance the innate immune response (Dawood et al., 2019; Abdel-Latif et al., 2023), and the expression of immune-related genes (Won et al., 2020; Krishnaveni et al., 2021) in fish.

It was indicated that dietary *L. reuteri*, *L. plantarum*, and their combination significantly enhanced the resistance of zebrafish after being experimentally infected with *A. hydrophila*. Results showed a significant decrease in cumulative fish mortalities after an observation period of 14 days. Additionally, they were supported by the increased expression of immune and antioxidant-related genes, antioxidant parameters, and immune responses in fish that were fed a probiotic-supplemented diet. Some similar studies reported the advantages of dietary probiotic supplements as increasing the resistance of zebrafish against the challenging bacterial pathogens including *L. plantarum* 08·923 and *B. coagulans* 09·712 (Wang et al., 2016), B. amyloliquefaciens (Lin et al., 2019), and *Paenibacillus ehimensis* NPUST1 (Lin et al., 2022). The benefits of dietary *L. reuteri, L. plantarum*, and their combination to enhance the fish resistance to diseases might be in terms of their activating immunity by releasing cytokines from macrophages and lymphocytes (Lin et al., 2019; Krishnaveni et al., 2021), stimulation of humoral immune functions (Lee et al., 2017), and secrete antimicrobial metabolites that serve as a protective shield in intestine against pathogenic bacteria. Further research may be required to have a better understanding of the probiotic components' effects on fish immunity and disease resistance.

Conclusions

In conclusion, our research indicated that supplementation of a combination of *L. reuteri* and *L. plantarum* successfully improved the growth rate, and health of zebrafish after feeding for 8 weeks. Besides, zebrafish resistance against *A. hydrophila* infection increased in fish supplemented with a combination of *L. reuteri* and *L. plantarum*. Therefore, for better efficiency, the combination of *L. reuteri* and *L. plantarum* is suggested to increase the health and growth of fish. This study only examined growth and health in zebrafish. Therefore, it is advised that future research examine how these two bacterial strains interact with other genes and histology, as well as determine the ideal degree of inclusion for each of these strains.

Author statement

Delaram Eslimi Esfahani: project administration, supervision, funding; Mehdi Ahmadifar: data curation and funding; Pouya Ebrahimi: methodology; Ehsan Ahmadifar: writing and editing; Poulin Shohreh: writing original draft; Hossein Adineh: data duration and editing; Mohsen Shahriari Moghadam: formal analysis, software; Sevdan Yilmaz: revising and editing; Nika Mashhadizadeh: formal analysis and conceptualization; Khalid Ali Khan: editing. All the authors have read and approved the manuscript and all are aware of its submission to the journal.

Data availability statement

All data are available in this manuscript.

Conflicts of authors

The authors declare no conflicts of interest.

Ethical approval

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