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Original Article:

Evaluation of the Antioxidant Potential of *Lactobacillus Fermentum* as a Nutritional Supplement in Alleviating Pb2+-induced Oxidative Stress in Poisoned Adult Zebrafish

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Abstract

Introduction: The present study aims to evaluate the possible amelioration of the toxic effect of Pb2+ on the liver and kidney organs of zebrafish (Danio rerio) by the probiotic strain *Lactobacillus fermentum* as a dietary supplement.

Materials and Methods: Zebrafish that had beedn treated with probiotics for 60 days were exposed to 0-1500 µg/L Pb^{2+} for 72h. The LC50 for the control group was determined, and biometric, histological, and antioxidant biomarkers were assessed. This included lipid peroxidation, SOD (superoxide dismutase) activity, reactive oxygen species (ROS) levels, and inflammatory response, focusing on interleukin-8 (IL-8) and interferon-gamma (IFN-γ) gene expression.

Results: Based on morphological analysis, the probiotic-enriched diet had no significant effect on the weight and length of zebrafish as biometric parameters. However, it reduced tissue heavy metal accumulation and mortality by 83% at 1500 µg/L Pb2+ after 72 hours of T2 treatment. Tissue damage, including hepatocyte necrosis, renal and tubular degeneration, decreased significantly in treated groups. Oxidative stress analysis revealed that probiotic treatment alleviated free radical production, SOD activity, and malondialdehyde (MDA) levels in zebrafish, allowing them to tolerate more than 300 µg/L Pb2+. In addition, probiotic-treated zebrafish showed a significant recovery in SOD2, IL-8, and IFN-γ mRNA expression, indicating an improved immune system.

Conclusion: In conclusion, a diet enriched with *Lactobacillus fermentum* reduced the oxidative stress and improved the resistance of zebrafish to the sub-lethal dose of Pb^{2+}. Overall, *Lactobacillus fermentum* shows potential as a dietary supplement for aquaculture in Pb2+-contaminated areas.

Keywords: Zebrafish, Probiotic, Oxidative stress, Immunology, Aquaculture

1. Introduction

Lead (Pb^{2+}), one of the most hazardous environmental pollutants, may be lethal to animals, particularly aquatic species, as it accumulates in human and animal tissues via the food chain and through inhalation (Paul, 2014 #1). Chronic exposure to Pb^{2+}, even at low concentrations, may affect fish health through disruption of cellular and molecular mechanisms. This ion inhibits the activity

of alkaline phosphatase and methylesterase while increasing transaminase levels and lipid peroxidation [1]. Previous research has shown that certain strains of Lactobacillus, including *L.rhamnosus*, *L.plantarum* and *L.brevis*, can protect cells from heavy metal toxicity. These strains are capable of binding to heavy metals through various mechanisms, including biosorption, or converting them into less toxic forms [2]. In addition, as a microbial dietary supplement (probiotic), they exhibit antioxidant properties in animal tissues.

A probiotic-enriched diet balances the animal's intestinal bacteria and improves the immune system. Thus, probiotics contribute to health management strategies that protect humans and aquatic species against environmental pollutants [3]. Lactobacillus strains, including *L.fermentum*, *L.plantarum* and *L.acidophilus*, are among the most beneficial probiotics for health-enhancing functional foods that improve growth, survival and feed efficiency as well as prevent gut disorders in fish or humans [4].There are many factors involved in regulating probiotic supplements, such as treatment duration, dose, and the source of bacteria. According to previous studies, long-term probiotic treatment maintains intestinal homeostasis and enhances bacterial adherence to the intestinal mucosa in aquatic species [5].

Toxicological studies on aquatic animals with biochemical mechanisms similar to those of humans are essential for understanding the effects of environmental pollutants on wildlife and, subsequently, on humans. The zebrafish (Danio rerio) is at the forefront of basic physiological and toxicological studies and is recognised as an excellent model of human disease. It also has large offspring and is easy to culture [6].

This study was designed to investigate whether a probiotic diet could ameliorate cytotoxic effects of lead acetate ($Pb(C_2H_3O_2)_2$) in zebrafish. To this end, we studied the survival, immune responses, and histopathological characteristics of *Lactobacillus fermentum*-treated zebrafish during exposure to a sub-lethal dose of Pb^{2+}. In addition, heavy metal accumulation and antioxidant responses during exposure to $Pb(C_2H_3O_2)_2$ were investigated at both the cellular and molecular levels. This analysis aimed to elucidate the cellular mechanisms responsible for the protective effect of the probiotic diet against Pb^{2+}.

2. Materials and Methods

Chemical solutions

Lead acetate ($Pb(C_2H_3O_2)_2$) and dichlorofluorescein diacetate (DCF-DA) were purchased from Sigma-Aldrich, USA. Aqueous solutions of 1% w/v $Pb(C_2H_3O_2)_2$ were prepared and diluted to the required concentrations in zebrafish tanks. All the chemical reagents were of analytical grade, and the concentration of Pb^{2+} in the experimental tanks was measured using atomic absorption spectroscopy.

Fish samples

The inclusion criteria for the fish were their weight and age. Accordingly, adult zebrafish (Danio rerio) weighing approximately 1.06±0.23 g (at around 10 weeks of age), were obtained from the Zebra Fishy Center, Tehran, Iran. The fish were acclimated to laboratory conditions (20 ± 0.5 °C, pH=6.5-7) in 80 L tanks containing dechlorinated tap water under controlled environmental conditions (12/12h light/dark photoperiod) for two weeks prior to the experiment. During the acclimation and experimental periods, the fish were fed Tetra Rubib Granules commercial food twice daily (1% body weight/day).

Bacterial strains and growth conditions

Lactobacillus fermentum (ATCC 14931) was obtained from the Iranian Biological Resource Centre, Tehran, Iran. The bacteria were cultured anaerobically in MRS broth (Sigma-Aldrich, USA) at 37°C for 48 hours. The bacterial medium was centrifuged at 3000 rpm for 10 min at 4 °C. The pellets were collected [7] and were then washed three times in phosphate-buffered saline (PBS) (pH=7.4, Solarbio, Beijing, China) and resuspended to a final concentration of 1.5×10^8 CFU/mL. By comparing the turbidity of the culture media with that of a 0.5 McFarland standard, the cell density of the cultures was estimated.

Probiotic-enriched diets were prepared weekly by fortifying commercial diets with different concentrations of *Lactobacillus fermentum*, drying for 10 h at room temperature and storing at 4°C. Control groups received commercial diets combined with an equivalent volume of sterile PBS (modified commercial diet). The quality of the probiotic feed was monitored by means of MRS plate culture with a criterion of a minimum survival rate of 95% for the lactobacilli in the experimental diet [8].

Experimental design

This experimental research was conducted in the Razaf laboratory complex at the Islamic Azad University, Rudehen in 2021. The research design followed the guidelines of the Organisation for Economic Co-operation and Development (OECD). A total of 540 adult zebrafish were randomly divided into four experimental groups: Negative Control

(N.C), Positive Control (P.C), T1 and T2 groups. Zebrafish were transferred to 10 L semi-static rotating water flow tanks. A feeding regimen of 60 days was established for each group as follows: the N.C and P.C groups received the modified commercial diet; the T1 group received the commercial diet enriched with 3.5 $\times 10^4$ CFU/g probiotic, and the T2 group received the commercial diet enriched with 7.0 $\times 10^4$ CFU/g probiotic.The water in the tanks for all groups was changed on a daily basis to prevent any unwanted bacterial contamination [9].

P.C, T1 and T2 were added to 0-1500 µg/L Pb^{2+} contaminated water for 72 h after 60 days of probiotic treatment. The median lethal concentration for acute toxicity of Pb^{2+} to zebrafish was estimated as LC_{50} [10]. During the toxicity test, the fish were not fed. Fish behavior was observed daily, with mortality recorded every 12 hours; deceased fish were promptly removed. The remaining zebrafish were euthanatized by placing them on ice for 15 min. Kidneys and livers were collected and syored in 2 ml Eppendorf tubes on ice for biomarker analysis, with 10 fish sampled in each group.

Oxidative stress analysis

The liver homogenates were analyzed for malondialdehyde (MDA) levels to assess lipid peroxidation and tissue oxidative stress. The zebrafish were euthanized after 72 hours of exposure to Pb^{2+} toxicity. Hepatic tissue was extracted and homogenized in ice-cold Tris-HCl buffer (50mM, 1mM EDTA, 0.15KCl, pH 7.4). The homogenate was centrifuged at 8,000 rpm for 10 minutes at 4 °C. The supernatant was collected and analyzed for MDA and SOD. For MDA measurement, 500 µL of the reaction mixture (0.4% thiobarbituric acid (TBA), 15% trichloroacetic acid) was supplemented with 250 µL of the heat-treated supernatant. MDA reacted with TBA to form thiobarbituric acid reactive species (TBARS). TBARS levels were monitored spectrophotometrically at 535 nm. The concentration of MDA was determined using an extinction coefficient of 155 mM^{-1} cm^{-1} [9]. Superoxide dismutase (SOD) activity was determined spectrophotometrically according to the manufacturer's commercial kit (Zellbio) at 450 nm [10]. Typically, SOD reduces the superoxide concentration, leading to the formation of a yellow-colored product produced by xanthine oxidase in the test mix. Reactive oxygen species (ROS) levels were measured using the ROS test kit (Teb Pazhouhan Razi, Tehran, Iran). Briefly, the homogenized sample was centrifuged at 4000 rpm for 2 min to pelletize the tissue. After removal of the supernatant, the pellet was washed with the kit's assay buffer and stained with DSF. Finally, ROS levels were measured by flow cytometry using DCFH-DA [11]. All oxidative stress assays were carried out in triplicate.

RNA isolation and quantitative RT-PCR

In order to investigate the molecular mechanisms behind the protective properties of probiotics against heavy metal toxicity, changes in RNA expression of SOD, interleukin-8 (Il-8) and interferon-γ (IFN-γ) were analyzed according to Yin et al. [12]. Briefly, total RNA was isolated from the liver of each sample using TRIzol® extraction reagent (Ambion). RNA was promptly used as a template for cDNA synthesis. The absorbance ratio (>1.9) at 260/280 nm was used to verify RNA purity spectroscopically. Zebrafish cDNA was reverse transcribed from 2 µg total RNA using oligo-d(T)18 primers (Table 1). The SSU rRNA, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and ACTB genes served as reference housekeeping gene to evaluate expression levels of SOD, Il-8 and IFN-γ genes. Real-time PCR was performed in accordance with the protocol of the Ambion qRT-PCR kit (Thermo Fisher Scientific) using SYBR Green PCR (Table 2).

Table 1. Biometric analysis of negative control group (CTRL) and probiotic treatment groups (T1 and T2).

	Weight (g)	Whole length (cm)	LC50 (µg/L)
CTRL	1.2 ± 0.3	4.16 ±0.31	**300 ±6**
T1	1.02 ±0.21	3.75 ±0.11	**430 ±3.4**
T2	1.27 ±0.28	3.95 ±0.26	**670 ±5.2**

SOD_2, Il-8 and INF-γ primers used in RT-PCR.

Bioconcentration and bioaccumulation factors measurement

For the calculation of the bioconcentration factor (BCF) and the bioaccumulation factor (BAF), the liver, gills and kidneys of the zebrafish were collected. Tissues were homogenised and centrifuged at 8000 rpm for 10 min at 4°C in cold physiological saline (0.86% NaCl) [12]. The supernatant was used for the determination of metal concentrations in the tissues through atomic absorption spectroscopy. The BCF and BAF of the studied heavy metal in zebrafish at steady-

state conditions were calculated according to equations 1 and 2, respectively:

$$BCF_{ss} = C_{B\ liver}/C_w \quad [1]$$

$$BAF_{ss} = C_{B\ all\ tissues}/C_w \quad [2]$$

In these equations, *CB* represents the concentration of Pb^{2+} in liver tissue or all tissues, and *Cw* is the heavy metal concentration in the exposure solution [13]. The total chemical concentration in the bulk water phase (CW) was measured using atomic absorption spectroscopy.

Histopathological analysis

The liver and kidney of some euthanized specimens were excised and immediately fixed in Bouin's solution for 24 hours. Fixed tissues were dehydrated through graded ethanol, embedded in paraffin, sectioned at 4 µm and stained with haematoxylin-eosin solution. The histological sections were then stained with haematoxylin and eosin (H&E) and examined under a light microscope [9].

Statistical analysis

Data related to different treatment groups were analyzed by one-way analysis of variance (ANOVA). The confidence level was set at 0.05, and the statistical analyses were performed using SPSS 19 (IBM Corp.). All data are presented as mean ± SD. Gene expression levels were reported as fold change according to the formula fold change=2-ΔΔCt [12]. Fold change of the SOD2, Il-8 and IFN-γ gene expression was compared between subgroups using a two-sample t test.

3. Results

Biometric results

In order to investigate the effects of a probiotic-enriched diet on the growth parameters of zebrafish, the samples were fasted for a minimum of 18 hours. The samples in all the experimental groups were weighed and analysed prior to the toxicity test. The probiotic-enriched diet did not significantly affect the weight and length of zebrafish in the probiotic-treated groups (p-value > 0.05) (Figure 1, Table 1). Despite no significant change in fish growth parameters, the LC_{50} increased by 51% and 100%, for T1 and T2 groups compared to the P.C group, respectively (Figure 2, Table 1). In the P.C. group, the 72-hour LC_{50} of Pb^{2+} was 300 ±6 µg/L. This is in agreement with the results of previous studies (14). The 72-hour LC_{50} for T1 and T2 treatments was 430 ±3.4 and 670 ±5.2 µg/L, respectively. In view of the LC_{50} and to evaluate the concentration-mortality time response of the probiotics by Pb^{2+} toxicity, P.C, T1 and T2 groups were exposed to 200 µg/L Pb^{2+} for 72 h in a static manner (Figure 3). The results indicated that the survival rate of the probiotic-treated samples under Pb^{2+} toxicity was significantly higher than that of P.C. group. However, there were no significant differences in the zebrafish mortality rates of the T1 and T2 groups.

Table 2. SOD_2, Il-8 and INF-γ primers used in RT-PCR

Gene name	Sequence of primer	Accession number
SOD_2	F:GCGTGACTTTGGCTCATTTC R:ATTCTCCCAGTTTACAACATTCC	NM_199976.1
Il-8	F:TTTTCCTGGCATTTCTGACC R:CCTATCAATGATCTTCTTAACCC	XM_001342570
IFN-γ	F:CCATCTTCCTGCGAATCCTG R:ATGCTTTAGCCTGCCGTCTC	NM_212864

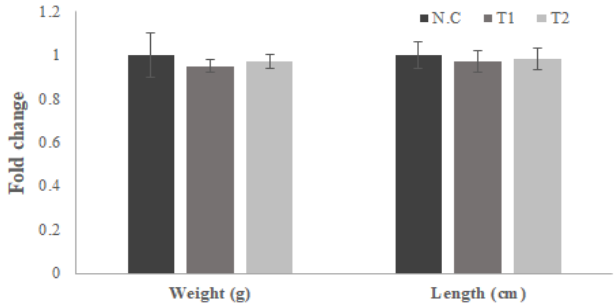

Figure 1. Biometric analysis of the experimental specimens; negative control group:N.C., 3.5 ×10^4 CFU/ml probiotic-trated group:T1 and 7 ×10^4 CFU/ml probiotics-treated group: T2.

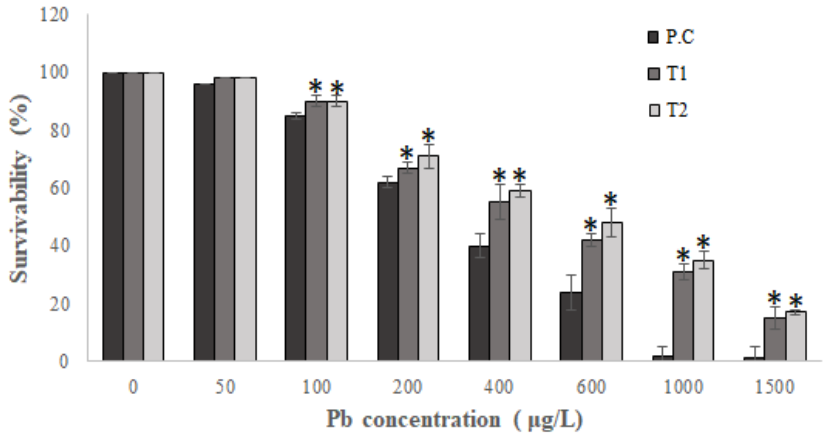

Figure 2. Effect of probiotic-enriched diet on Pb^{2+} toxicity tolerance in zebrafish. T1 and T2 are 3.5 ×10^4 and 7 ×10^4 CFU/ml probiotics-treated zebrafish, respectively. * Denotes $p<0.05$ compared to positive control (P.C).

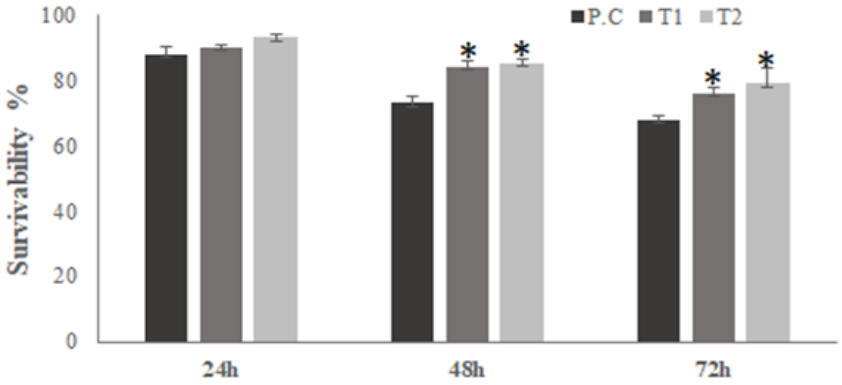

Figure 3. Survival of probiotic-treated zebrafish exposed to 200 µg/L Pb^{2+} for 72 h. T1 and T2 are 3.5 ×10^4 and 7 ×10^4 CFU/ml probiotics-treated zebrafish, respectively. * denotes significant differences between exposed and control groups ($p < 0.05$).

Oxidative stress results

An increase in MDA levels in the poisoned samples was expected, as Pb^{2+} ions have the redox potential to generate free radicals and cause oxidative damage to membrane phospholipids (Figure 4). There was a probiotic concentration-dependent decrease in MDA levels (p-value < 0.05), and in both probiotic groups, MDA levels in liver tissue decreased (up to 47%) after 72 hours of exposure to Pb^{2+} compared to P.C. group. To evaluate the effects of Pb^{2+} on the antioxidant system of probiotic-fed zebrafish liver, SOD activity and ROS production were measured (Fig. 4). The results showed that the probiotic diet significantly decreased 42% and 70% for SOD activity and ROS content, respectively, in the Pb^{2+} poisoned zebrafish compared to the P.C group. However, the higher dietary probiotic dose failed to induce a significant decrease in the enzyme compared to the T1 group.

Genes expression and RT-PCR results

The expression of SOD2, Il-8 and IFN-γ genes was examined to assess the antioxidant and immune system responses to Pb^{2+} toxicity in probiotic-treated zebrafish at the molecular level (Figure 5). There was an inverse relationship between probiotic concentration and SOD2 gene expression, and the relative up-regulation of SOD2 gene expression for the T1 and T2 groups was significantly lower than that for the P.C. group.

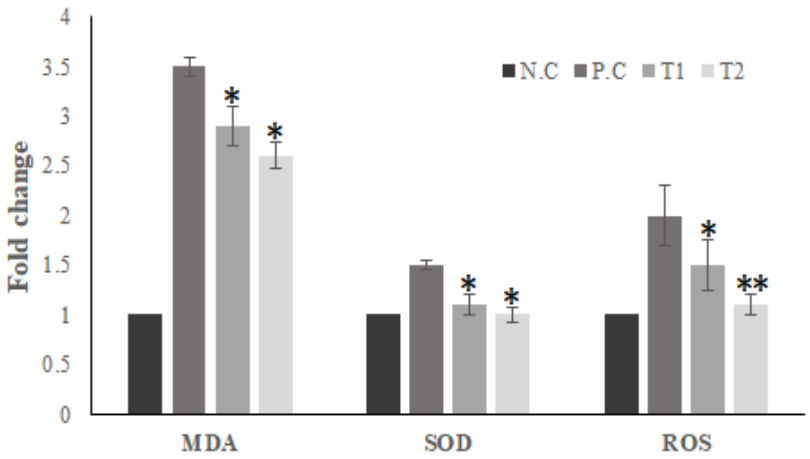

Figure 4. Effect of probiotic-enriched diet on liver MDA, SOD activity and ROS levels in samples exposed to Pb^{2+} for 72 h. T1 and T2 are 3.5 ×10^4 and 7 ×10^4 CFU/ml probiotics-treated zebrafish, respectively. * and ** denote significant differences between exposed and control groups ($p < 0.05$) and T1 and T2 groups, respectively.

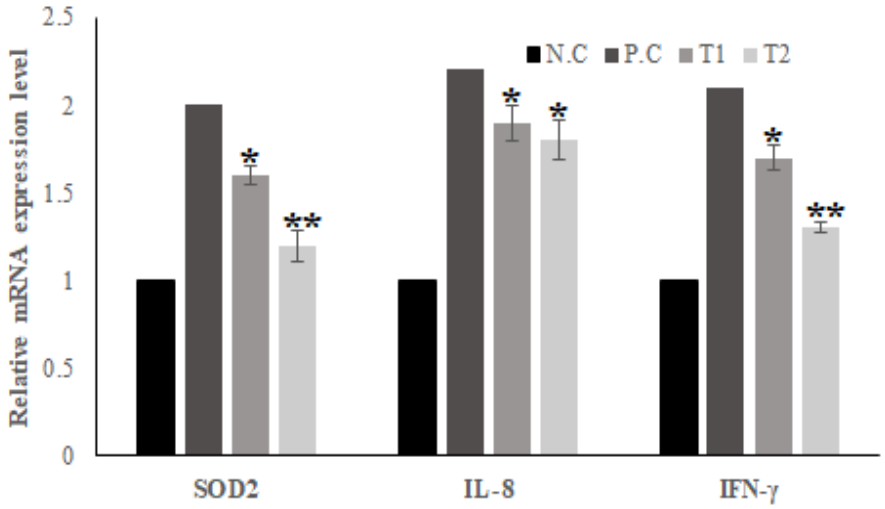

Figure 5. Effect of probiotic-enriched diet on the expression of SOD2, Il-8 and IFN-γ genes in Pb^{2+}-exposed zebrafish. T1 and T2 are 3.5 ×10^4 and 7 ×10^4 CFU/ml probiotics-treated zebrafish, respectively. * and ** denote significant differences between exposed and control groups ($p < 0.05$) and T1 and T2 groups, respectively.

To analyze the immune response in probiotic-fed zebrafish during Pb^{2+} toxicity, zebrafish Il-8 and IFN-γ gene expression were examined. Pb^{2+} activated the main genes involved in the inflammatory response in all the experimental groups (Figure 5). However, Il-8 and IFN-γ gene expression reduced by 14% and 19%, respectively, in the T1 group, indicating that the probiotic diet reduced the inflammatory response to heavy metal toxicity. The T2 group's immune response is significantly more controlled than the positive control, with Il-8 and IFN-γ gene expression reduction by 19% and 40%, respectively.

BCF and BAF results

Metal uptake potential in zebrafish was represented by BCF and BAF (Figure 6). The BCF of Pb^{2+} in the probiotic treated zebrafish was significantly lower than that of P.C. The BCF of the T2 treatment was 49% lower than that of P.C. Both probiotic treated groups showed a significantly lower BAF than that of P.C.

Histopathological results

The histological results of the P.C and T2 groups exposed to Pb^{2+} are shown in (Figure 7). As a positive

control, 200µg/L Pb^{2+} induced hepatocellular necrosis associated with inflammatory cell infiltration (Figure 7). The results indicated that in P.C group, Pb^{2+} accumulated in zebrafish liver soon after Pb(C2H3O2)2 exposure, whereas in T2 group, Pb^{2+} accumulation in liver was significantly reduced by probiotic diet. Renal and tubular degeneration was also observed in the kidneys of the P.C. group (Figure 7). Severe histological damage to the liver and kidneys was not observed in any of the probiotic-treated groups exposed to the toxic doses.

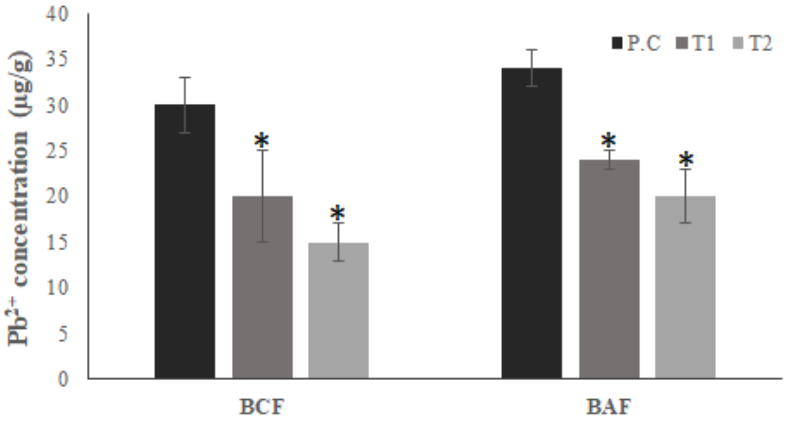

Figure 6. Bioconcentration factor (BCF) and bioaccumulation factor (BAF) levels in zebrafish exposed to Pb^{2+} for 72 h. * and ** indicate significant differences between exposed and control ($p < 0.05$) and T1 and T2 groups. T1 and T2 are 3.5 ×10^4 and 7 ×10^4 CFU/ml probiotics-treated zebrafish, respectively.

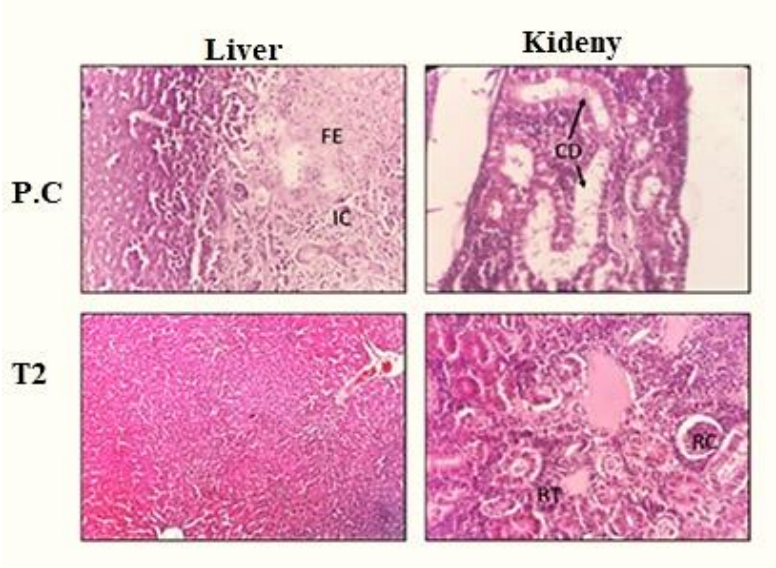

Figure 7. Histopathology of the liver and kidney in the control and treatment groups; FE: fibrosis of asbestosis, IC: infiltration of inflammatory cells, CD: degeneration of renal tubular cells, RT: Renal tubule, RC: Renal capsule (Bomen), Hematoxylin-Eosin X100 (liver) and X200 (kidney).

4. Discussion

Environmental contamination of Pb^{2+} ions has received much attention in recent years due to their destructive effects on biochemical and physiological mechanisms in aquatic animals. In this research study, the proper functioning of the probiotic-enriched diet in the aquaculture immune system improvement against heavy metal pollution is well studied. Jhamtani (2017) has reported on the adverse haematological, physiological and neurodegenerative effects of Pb^{2+} toxicity in the zebrafish [15]. In addition, food

contaminated with Pb^{2+} reduces the nerve conduction velocity and impairs spermatogenesis in humans [16]. In view of the worldwide distribution of Pb^{2+} in industry, scientists have developed various remediation approaches for the removal of Pb^{2+} from aquatic systems, such as membrane filtration, nanomaterials and bioremediation [17]. However, it could be an important process for removing heavy metals [18]. The aim of this article was to evaluate long-term probiotic feeding to alleviate chronic Pb^{2+} toxicity in aquatic animals, considering the dual ability of probiotics such as Lactobacillus strains to remediate heavy metals and improve the gut microbiota and immune system of aquatic animals. Previous studies suggested that 10^4-10^8 CFU/g of probiotic as a dietary supplement be effective in improving gut microbiota [19]. To prevent bacterial contamination from long-term feeding, the commercial diet used in this study was fortified with 10^4 CFU/g probiotic.

Different amounts of heavy metals such as Pb^{2+} are tolerated by aquatic animals. Previous studies indicated that physiological responses to heavy metal ions, chemical properties of metal compounds, and experimental conditions (water temperature, salinity, oxygen, and pH) may result in different heavy metal LC_{50} [14]. Depending on the stage of development and the physicochemical properties of the water, 18-1559 µg/L Pb^{2+} is acutely toxic to zebrafish [20]. Because of the multiple effects of Pb^{2+} on fish physiology and biochemistry, the LC_{50} of Pb^{2+} range from low (63 µg/L in Rasbora sumatrana) to high (34 to 77 mg/L in L. rohita, B. sharpeyi, C. carpio and P. hypophthalmus) [21]. According to Yin (2018), the 96-h LC_{50} for Pb^{2+} in zebrafish is approximately 375 µg/L, and the 72-hour LC_{50} for Pb^{2+} obtained for zebrafish in the present study is in agreement with the results reported in other studies [12]. The probiotic-enriched diet improved survivability and resistance to various doses of Pb^{2+}, increasing the LC_{50} ~1.5-2-fold compared to the control group. However, growth factors such as weight and length were not significantly affected by the probiotic supplements.

Cellular antioxidant defence is activated by heavy metal-induced oxidative stress to protect cells from membrane transport perturbations and maintain redox homeostasis by increasing MDA levels and SOD activity in aquatic gill, liver and kidney [22]. As expected, 72-hour Pb^{2+} toxicity in zebrafish increased ROS levels, MDA levels and SOD activity. However, the application of probiotic supplements contributed to the reduction of oxidative stress and cellular damage, as the MDA level, SOD activity and SOD2 mRNA expressions significantly reduced in both probiotic-treated groups, and the probiotic concentration-ROS level was reversed in the presence of Pb^{2+}. In addition, the antioxidant potential of the probiotic-enriched diet was directly related to the concentration of probiotics in the diet. RNA expression levels of inflammatory response biomarkers were examined to assess the protective effect of the probiotic diet against Pb^{2+} toxicity. The validity of IL-8 and IFN-γ as biomarkers of heavy metal toxicity in zebrafish was reported by Yin and co-workers (2018) [12]. The analysis of the biomarkers of inflammatory response showed that the mRNA expression levels of Il-8 and IFN-γ in the probiotic-treated zebrafish were lower than those in the positive control group. In addition, IFN-γ mRNA expression levels and inflammatory responses significantly reduced in the commercial diet supplemented with 7×10^4 CFU/g probiotic. These results were confirmed by the results of the BCF and BAF. BCF and BAF are indicators of the intensity of the immune response in fish. Lethal toxicity and tissue damage occurred at BCF and BAF values close to 100 [23]. According to the results, the BCF and BAF values for all experimental groups were less than 100, indicating the absence of acute toxicity and fatal inflammatory reaction in the sample. In the T1 and T2 experimental groups, the probiotic-enriched diet decreased BCF and BAF. However, there was no significant correlation between probiotic concentration and BCF reduction. It was assumed that these positive changes in the biochemistry of the cells would be responsible for the increased survival rate of the zebrafish exposed to a sublethal dose of Pb^{2+}. In order to confirm this assumption, a histological analysis of the damage to the liver and kidney of the P.C. and T2 groups was carried out. Accumulating heavy metals in most samples caused liver and kidney damage [24]. General screening of tissues showed various degrees of cellular damage to the tissues of P.C. after 72 h exposure to Pb^{2+}. The observed hepatic porosity and vacuolations in the kidney could be due to carbohydrate deposition and metabolic disorders as a consequence of exposure to heavy metals [25]. Moreover, inflammatory responses were characterised by hepatic cellular infiltration and liver and kidney haemorrhage [26], and Liver, renal and gill lesions may contribute to mortality [27]. The results confirmed that the amount of tissue damage caused by Pb^{2+} toxicity was significantly reduced by the probiotic-enriched diet. The probiotic-enriched diet dramatically reduced the susceptibility of the zebrafish compared to the P.C. group. It also increased the resistance of the fish to Pb^{2+} toxicity. Overall, probiotics showed promising growth in the fish gut [27] and their ability to colonize the fish external surface and adhere to the mucus layer may contribute to their antitoxic property [28, 29].

Due to the limitations of the histopathological and

biochemical study of zebrafish, it is recommended to investigate the effects of probiotic-enriched diet on different animal model (in vivo and in vitro study).

5. Conclusion

This study aimed to investigate the protective potential of a long-term probiotic diet on the survival of lead-poisoned zebrafish. Histological analysis of Pb2+-poisoned zebrafish revealed that due to the reduction of Pb^{2+} accumulation in the liver and kidney, the survivability of zebrafish fed with *Lactobacillus fermentum* significantly improved. By reducing MDA activity and ROS content as oxidative stress and inflammatory responses (SODII, IL-8 and IFN-γ genes expression) in liver tissue, dietary *Lactobacillus fermentum* alleviated Pb^{2+}-induced toxicity in zebrafish. Based on the findings, *Lactobacillus fermentum* as a probiotic diet was a suitable supplement for aquatic organisms living in Pb^{2+}-polluted water. It also proved to be a valuable agent for improving aquatic health and thus human food safety.

Ethical Considerations

Compliance with ethical guidelines

Animal handling and tissue sampling procedures were carried out under the standard principles of laboratory animal care to reduce animal suffering; the study was approved by the local ethics committee of the Faculty of veterinary Medicine, Islamic Azad University (reference number IR.IAU.SRB.REC.2949448). For euthanasia, the fish were first anesthetized with clove powder.

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Author's contributions

Najmeh Hadizadeh Shirazi designed and performed experiments, analyzed the data and wrote the paper. Reza Kazempoor rendered the data analysis. Faranak Tehrani performed the experiments.

Conflict of interest

The authors have no relevant financial or non-financial interests to disclose.

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