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LACTOBACILLUS CASEI (IBRC-M 10,711) AMELIORATES THE GROWTH RETARDATION, OXIDATIVE STRESS, AND IMMUNOSUPPRESSION INDUCED BY MALATHION TOXICITY IN GOLDFISH (*CARASSIUS AURATUS*)

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Abstract

Probiotics can functionally improve fish wellbeing and are suggested as antioxidative agents to protect fish from xenobiotics toxicity. Herein, dietary *Lactobacillus casei* (IBRC-M 10,711) was included in the diets of goldfish (*Carassius auratus*) to protect against malathion toxicity. Fish (12.47 ± 0.06 g) were randomly allocated to six groups (triplicates), as follows: T1) control; T2) fish exposed to 50% of malathion 96 h LC_{50}; T3) *L. casei* at 10^6 CFU/g diet; T4) *L. casei* at 10^7 CFU/g diet; T5) fish exposed to 50% of malathion 96 h LC_{50} + *L. casei* at 10^6 CFU/g diet; T6) fish exposed to 50% of malathion 96 h LC_{50} + *L. casei* at 10^7 CFU/g diet. After 60 days, goldfish fed T4 had the highest final body weight (FBW), weight gain (WG), and specific growth rate (SGR), and the lowest feed conversion ratio (FCR) among the groups (P<0.05). However, the T2 group showed lower FBW, WG, and SGR and higher FCR than fish in T1 (P<0.05). Fish in the T4 group had the highest blood total proteins, albumin, and globulin, while fish in T2 had the lowest levels (P<0.05). Fish in the group T2 had the highest triglycerides, cholesterol, cortisol, lactate dehydrogenase (LDH), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) levels in the blood, while fish fed T4 had the lowest values (P<0.05). The superoxide dismutase (SOD) and catalase (CAT) showed the highest activities in T3 and T4 groups, and the lowest SOD was seen in the T2 group, whereas the lowest CAT was seen in the T2, T5, and T6 groups (P<0.05). Fish in the T5 and T6 groups had higher glutathione peroxidase (GSH-Px) activities than fish in T1 and T2 groups but T3 and T4 groups showed the highest values (P<0.05). T2 group had the highest malondialdehyde (MDA) level, while T3 and T4 groups had the lowest MDA level (P<0.05). Blood immunoglobulin (Ig) and lysozyme activity were significantly higher in T3 and T4 groups and lower in the T2 group than in the control (P<0.05). The alternative complement pathway (ACH_{50}) was significantly higher in T2, T3, T4, T5, and T6 groups than in the T1 group (P<0.05). Skin mucus Ig was significantly higher in T3 and T4 groups and lower in the T2 group than in the control (P<0.05). The highest lysozyme activity, protease, and ACH_{50} in the skin mucus samples were in the T4 group, while the lowest values were in the T2 group (P<0.05). In conclusion, dietary *L. casei* protects goldfish from malathion-induced growth retardation, oxidative stress, and immunosuppression.

Key words: aquaculture, pesticides, probiotics, mucus immunity, antioxidative capacity, goldfish

The aquatic ecosystem is threatened with several challenges involved in the reduction of aquatic animals' health and productivity (FAO, 2020). Water-borne insecticides are toxic compounds used to fight against harmful insects in the agriculture sector (Zheng et al., 2021). However, the remaining derivatives can reach the eco-system leading to toxicity and adverse effects on the living organisms (Bharti and Rasool, 2021). Organophosphorus compounds such as malathion have been widely used in agriculture activities to eliminate harmful insects (Chang et al., 2020). The continuous application, especially in developing countries, increases the residuals in the water bodies and thereby the fish and aquatic ecosystem (Ma et al., 2019). Adversely, high accumulation levels of malathion caused cellular DNA damage, oxidative stress, and hepatic failure (Poorbagher et al., 2018; Bautista-Covarrubias et al., 2020; Rahbar et al., 2020). The lipid peroxidation of cellular membranes is also another negative feature attributed to malathion toxicity (Chorehi et al., 2013; Olakkaran et al., 2020; Ullah et al., 2018). Consequently, an imbalance in the physiological function and immune capacity results from

malathion toxicity (Silva de Souza et al., 2020). The toxicity of malathion induced oxidative stress and liver failure in rohu (*Labeo rohita*, Hamilton) (Ullah et al., 2018), goldfish (*Carassius auratus gibelio*) (Huculeci et al., 2009), and *Channa punctatus* (Bloch) (Bharti and Rasool, 2021). Further, tambaqui (*Colossoma macropomum*) exposed to malathion showed neurotoxicity and homeostasis (Souza et al., 2021). Ortiz-Delgado et al. (2021) also reported that malathion toxicity induced failure of gills, intestines, liver, and kidney tissues and inhibition of cholinesterase activities in Senegalese sole (*Solea senegalensis*). In white shrimp (*Litopenaeus vannamei*), malathion toxicity caused oxidative stress and immunosuppression, as Bautista-Covarrubias et al. (2020) reported.

Beneficial bacterial cells known as probiotics, such as lactic acid bacteria (LAB), are increasingly used in aquaculture for their potential roles (Mugwanya et al., 2021). Markedly, LAB possesses several pharmaceutical properties associated with antioxidative and immunomodulation roles (Saide and Gilliland, 2005). More specifically, *Lactobacillus* strains showed several powerful effects in aquatic animals. The prohibition of lipid peroxidation and the scavenging effect against excessive free radicals were recently proved for *Lactobacillus* strains (Gao et al., 2011; Zhai et al., 2013). Interestingly, *Lactobacillus casei* alleviated the toxic effects of malathion in *Caenorhabditis elegans* nematodes via the reduction of oxidative stress (Kamaladevi et al., 2013).

Goldfish (*Carassius auratus*) is a highly valued commercial fish species mainly used as ornamental fish species (Chen et al., 2020; Romano et al., 2020). It can also be used as a bioindicator to test the negative impacts of insecticides on the aquatic ecosystem. In this study, possible protective roles of *L. casei* against malathion-induced oxidative stress and immunosuppression in goldfish were investigated.

Material and methods

Experimental animals and setup

Goldfish (*Carassius auratus* (fingerlings were purchased from a fish farm in Karaj, Iran, and shortly transported to the laboratory. Fingerlings were acclimatized to experimental conditions and diet in 1000 L tanks under controlled conditions. They were hand-fed a commercially available diet (Faradaneh Co., Shahrekord, Iran; containing 38% crude protein, 6% crude fat, 7% moisture, 8% ash, 3% crude fiber, and 1.25% phosphorus) thrice daily at 3% of body weight. Water was replaced every 24 h at a rate of 40% of tank volume. After two weeks, 360 healthy fish weighing 12.47±0.06 g (mean ± SE) were randomly allocated in 18 (150 L) fiberglass tanks (20 fish/tank), supplied with continuous aeration. Six experimental groups with triplicates were designed, as follows: T1) control; T2) fish exposed to 50% of malathion 96 h LC_{50}; T3) probiotic at 10^6 CFU/g diet; T4) probiotic at 10^7 CFU/g diet; T5) fish exposed to 50% of malathion 96 h LC_{50} + probiotic at 10^6 CFU/g diet; T6) fish exposed to 50% of malathion 96 h LC_{50} + probiotic at 10^7 CFU/g diet. The experiment lasted for 60 days. During experiment, the levels of temperature (24.5 ± 1.05°C); pH (7.29 ± 0.52); total ammonia nitrogen (<0.2 mg/L); dissolved oxygen (6.49 ± 0.41 mg/L); total hardness (188.24 ± 11.49 mg/L) were recorded.

Malathion

The commercial organophosphorus insecticide malathion (57% EC) was supplied from Kavosh Co., Iran. Malathion stock solution was generated using water and then it was further diluted to obtain the experimental concentration in the study tanks. Water in melatonin-treated tanks was exchanged (40%) every 24 h with water having the same malathion concentration. The water of control and malathion-free groups was replaced with normal chlorine-free tap water (Karmakar et al., 2016). The concentration of malathion was selected based on a previous study, where the 96 h LC_{50} value of malathion for goldfish was determined to be 4.71 mg/L (Shahbazi Naserabad et al., 2015).

Probiotic and diet preparation

The probiotic *Lactobacillus casei* (IBRC-M 10,711) used in this study was obtained from Persian Type Culture Collection, Iran. The initial bacterial stock was incubated under anaerobic conditions at 30°C in a de Man, Rogosa and Sharpe (MRS) broth medium (Merck, Germany). After 24 h, the medium containing probiotic was centrifuged (4000 × g, 10 min) and the precipitates were washed with sterile phosphate-buffered saline (PBS) three times. Then, bacterial cells were resuspended in PBS and serially diluted and probiotic density was determined using McFarland standards. Finally, probiotic solutions were separately sprayed into a well-grounded basal diet. The combinations were finely mixed and pelletized again (Hedayati et al., 2021). The concentration of probiotic *L.* casei in the supplemented diets (10^6 or 10^7 CFU/g) was assured by growing feed samples on MRS agar (Merck, Germany). The basal commercial diet (Faradaneh Co., Shahrekord, Iran) was without any prebiotic or probiotic additives. The probiotic supplemented diets were freshly prepared every 10 days. Fish were hand-fed thrice daily at 3% of body weight.

Growth assessment

At the end, experimental fish were not fed for 24 h and then all fish were accurately weighed and counted to determine the following growth-related parameters: Weight gain (WG) = [final weight (g) – initial weight (g)] ÷ initial weight (g); Specific growth rate (SGR; %) = Ln [final weight (g)] – Ln [initial weight (g)] ÷ test days × 100; Feed conversion ratio (FCR) = weight gain (g) ÷ feed consumed (g); Survival (%) = (fish harvested counts ÷ stocked counts) × 100 (Mani and Ebrahimi, 2021; Mohammadi et al., 2021 a).

Serum isolation

At the end of the trial, fish were anesthetized using clove powder (150 mg/L) and blood was sampled from the caudal vein of three fish (n=9), poured into sample tubes, and allowed to clot for 3 h at room temperature. Serum was isolated from freshly sampled blood by allowing it to clot for 3 h at room temperature then centrifugation at 3000 × g for 10 minutes at 4°C. Finally, the supernatant was transferred into new tubes and stored at –70°C for later analysis.

Serum biochemicals

Total protein (TP), albumin (ALB), glucose (Glu), alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), cholesterol (Chol), lactate dehydrogenase (LDH), and triglycerides (Tri) were measured in serum sample using commercial kits (Pars Azmun Co., Iran) on an automatic biochemical analyzer (LXTM20; Beckman Coulter, USA) (Mohammadi et al., 2020 b; Yousefi et al., 2021). The globulin (Glo) levels were obtained by subtracting the amount of albumin from the total protein in the same sample (Vali et al., 2020). An enzyme-linked immunosorbent assay kit (ZellBio Co., Germany) was used to detect cortisol (Cort) levels in serum samples at 450 nm, following the kit's manual (Hajirezaee et al., 2020).

Serum antioxidants

Serum samples were checked for the enzymatic activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and malondialdehyde (MDA) using corresponding commercial diagnostic kits purchased from ZellBio Co., Germany (Mohammadi et al., 2021 b). In brief, MDA levels were measured using thiobarbituric acid assay at 535 nm (Dawn-Linsley et al., 2005). GSH-Px was measured using 2-nitro-5-thiobenzoic acid formation method at 412 nm (Beutler, 1963). CAT was measured by monitoring the rate of H_2O_2 disintegration at 405 nm (Beutler, 1963). Nitro-blue-tetrazolium dye was used to measure SOD levels by reading the absorbance at 420 nm (Marklund and Marklund, 1974).

Serum immune responses

Serum total immunoglobulin (Ig) concentrations were quantified based on the descriptions of Siwicki and Anderson (1993). In brief, total protein levels of serum samples were determined using a commercial kit (Pars Azmun Co., Iran), and then samples were treated with 12% polyethylene glycol (Sigma) and checked again for total protein concentrations. The difference between the two measurements is the serum total Ig content.

Serum alternative complement activity (ACH50) was measured using the hemolysis of rabbit red blood cells (RaRBC) and recording the absorbance at 414 nm. An amount of samples causing 50% hemolysis was used to compute ACH50 activity following Yano (1992).

Serum samples were checked for lysozyme (LYZ) activity as described by Ellis (1990). In summary, samples were mixed with *Micrococcus lysodeikticus* suspension (75 µg/mL; Sigma) in wells of a 96-well plate and incubated at room temperature while continuously shaken. The absorbance was monitored at 450 and one unit of LYZ activity was defined as the concentration that declines 0.001 of absorbance per minute. Lysozyme obtained from hen's egg (Sigma) was used to plot the standard curve (Vali et al., 2020).

Mucus separation and analysis

Skin mucus was collected from previously sedated fish (4 fish/tank) following the method of Ross et al. (2000). In short, fish were individually rinsed with sterile NaCl solution (50 mM), and skin mucosal excretions were sampled by gentle hand-rubbing of individuals in polyethylene bags filled with 10 mL of sterile NaCl solution (50 mM). The collected mucus samples were kept in sterile test tubes, debris was precipitated by centrifugation at 6000 × g for 8 min at 4°C, and the supernatant was stored (–70°C) until later use in the analysis of mucus immune-related parameters.

Skin mucus was evaluated in terms of ALP, total Ig, LYZ, and ACH50 levels based on the same methods outlined above for serum samples (Mohammadi et al., 2020 a). However, skin mucus protease activity was determined through azocasein hydrolysis assay detailed by Ross et al. (2000).

Statistical analysis

The statistical analysis of data was accomplished using SPSS version 26 (SPSS Inc., USA). The data were confirmed in terms of normal distribution and homogeneity of variances by the Shapiro-Wilk and Levene's tests, respectively. The results are presented as mean ± S.E. (standard error) and significant differences were detected with the significance level set at $P<0.05$ using one-way ANOVA followed by Tukey HSD.

Results

Growth performance and survival rate

Goldfish fed *L. casei* at 10^7 cfu/g diet (T4) had markedly the highest final body weight (FBW), weight gain (WG), and specific growth rate (SGR), and the lowest feed conversion ratio (FCR) among the groups ($P<0.05$) (Table 1). However, fish exposed to malathion (T2) showed lower FBW, WG, and SGR and higher FCR than fish in the control group (T1) ($P<0.05$). Fish fed *L. casei* at 10^6 cfu/g diet (T3) had higher FBW than T1 and T2 groups but lower than T4 group ($P<0.05$) while no significant differences were shown with fish fed *L. casei* and exposed to malathion (T5 and T6) ($P>0.05$). Further, the groups of fish fed *L. casei* and exposed to malathion (T5 and T6) showed non-significant differences with fish in control (T1) and T3 groups ($P>0.05$) in terms of WG, SGR, and FCR. The survival rate showed the highest values in T3 and T4 groups (100%) and the lowest value in the T2 group (88.33%)

(P<0.05), while fish in the T1, T5, and T6 groups had no significant differences (P>0.05) (Table 1). Also, no significant differences were seen between T3, T4, T5, and T6 groups regarding the survival rate at the end of the feeding trial (P>0.05).

Blood biochemical traits

Table 2 presents the blood biochemical profile of goldfish fed *L. casei* and exposed to malathion for 60 days. Fish in the T4 group had the highest blood total proteins (TP), albumin (ALB), and globulin (GLO), while fish in T2 had the lowest TP, ALB, and GLO (P<0.05). Further fish in T3 and T4 groups treated with *L. casei* without malathion toxicity had similar TP, ALB, and GLO without significant differences (P>0.05). Fish in T1, T3, and T6 groups had similar TP levels without significant differences (P>0.05). Fish in the T3 and T4 groups had the lowest blood cholesterol, triglycerides, glucose, and cortisol, while fish in T2 presented the highest values (P<0.05). Probiotic administration in T5 and T6 groups significantly reduced serum cholesterol, triglycerides, glucose, cortisol, and LDH levels as compared to T2 group (P>0.05). The lowest levels of LDH were noticed in the T5 group (P<0.05). Further, T6 group showed statistically similar levels of cholesterol, triglycerides, glucose, and LDH levels as compared to T1 (P<0.05). Fish in the group T2 exposed to malathion without *L. casei* feeding had the highest ALT, AST, and ALP levels in the blood, while fish fed *L. casei* at 10^7 cfu/g diet (T4) had the lowest values (P<0.05). Besides, fish in the T3 group had higher ALT than the T4 group and lower than the remaining groups (P<0.05). Fish in T1, T5, and T6 groups showed similar AST levels (P>0.05). Fish in T5 and T6 groups had higher ALT than fish in T1, T3, and T4 groups but lower than the T2 group (P<0.05).

Table 1. Growth parameters of goldfish (*C. auratus*) fed *L. casei* and exposed to malathion

Parameter	T1	T2	T3	T4	T5	T6
IBW (g)	12.51±0.18	12.56±0.10	12.59±0.19	12.33±0.20	12.39±0.14	12.44±0.20
FBW (g)	19.27±0.09 c	16.70±0.10 d	20.61±0.38 b	22.18±0.25 a	19.83±0.10 bc	20.18±0.16 bc
WG (g)	6.75±0.13 b	4.14±0.19 c	8.02±0.57 b	9.85±0.44 a	7.44±0.24 b	7.74±0.30 b
SGR (% day^{-1})	0.72±0.02 b	0.47±0.02 c	0.82±0.06 ab	0.98±0.04 a	0.78±0.03 b	0.81±0.03 b
Survival rate (%)	93.33±1.67 b	88.33±1.67 c	100.00±0.00 a	100.00±0.00 a	96.67±1.67 ab	98.33±1.67 ab
FCR	4.47±0.09 b	7.32±0.34 a	3.78±0.25 bc	3.06±0.14 d	4.07±0.13 b	3.91±0.13 bc

IBW: Initial body weight, FBW: Final body weight, BWI: Body weight increment, SGR: Specific growth rate, FCR: Feed conversion ratio. T1: Control; T2: 50% of malathion LC_{50}; T3: *L. casei* at 10^6 cfu/g diet; T4: *L. casei* at 10^7 cfu/g diet; T5: 50% of malathion LC_{50} + *L. casei* at 10^6 cfu/g diet; T6: 50% of malathion LC_{50} + *L. casei* at 10^7 cfu/g diet. Values are expressed as means ± S.E. (*n =3*). Bars bearing different letters are significantly different at P≤0.05.

Table 2. Blood biochemical parameters of goldfish (*C. auratus*) fed *L. casei* and exposed to malathion

Parameter	T1	T2	T3	T4	T5	T6
TP (g/dL)	4.72±0.06 bc	4.35±0.06 d	4.86±0.06 ab	5.02±0.05 a	4.43±0.06 d	4.57±0.03 cd
ALB (g/dL)	3.23±0.06 ab	3.12±0.06 b	3.40±0.03 a	3.39±0.03 a	3.14±0.03 b	3.16±0.03 b
GLO (g/dL)	1.49±0.11 ab	1.23±0.00 b	1.46±0.03 ab	1.63±0.08 a	1.30±0.03 b	1.41±0.01 ab
Triglycerides (mg/dL)	209.35±1.01 c	227.35±2.64 a	196.02±2.19 d	194.30±2.14 d	219.75±1.87 ab	216.42±1.61 bc
Cholesterol (mg/dL)	98.63±1.12 b	114.29±1.50 a	81.78±1.06 c	76.56±0.99 c	104.08±1.73 b	102.39±1.68 b
Glucose (g/dL)	56.92±0.97 c	65.20±0.99 a	51.75±0.94 d	48.62±0.76 d	61.41±0.61 ab	58.93±0.74 bc
Cortisol (ng/mL)	76.73±1.07 c	86.73±1.10 a	66.41±1.12 d	69.19±0.55 d	78.83±0.71 bc	82.11±0.87 b
LDH (U/mL)	112.03±1.32 b	120.29±1.21 a	114.97±1.83 ab	96.76±1.70 c	83.37±1.60 d	108.75±0.79 b
ALT (U/mL)	70.60±0.78 c	81.32±0.96 a	66.33±0.60 d	62.69±0.71 e	75.34±0.45 b	73.49±0.55 bc
AST (U/mL)	102.28±1.10 c	113.87±1.74 a	94.64±1.29 b	91.70±0.97 b	106.66±1.15 c	105.15±1.50 c
ALP (U/mL)	80.06±1.00 c	94.46±1.25 a	80.30±0.62 c	77.73±0.78 c	91.25±0.50 b	88.07±1.06 b

TP: total protein; ALB: albumin; GLO: globulin; LDH: lactate dehydrogenase; ALT: alanine aminotransferase; AST: aspartate transaminase; ALP: alkaline phosphatase; T1: Control; T2: 50% of malathion LC_{50}; T3: *L. casei* at 10^6 cfu/g diet; T4: *L. casei* at 10^7 cfu/g diet; T5: 50% of malathion LC_{50} + *L. casei* at 10^6 cfu/g diet; T6: 50% of malathion LC_{50} + *L. casei* at 10^7 cfu/g diet. Values are expressed as means ± S.E. (*n =3*). Bars bearing different letters are significantly different at (P≤0.05).

Oxidative status

Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and malondialdehyde level (MDA) are shown in Figure 1. The SOD showed the highest activities in T3 and T4 groups, and the lowest SOD was seen in the T2 group (P<0.05). T1 had higher SOD than T2, T5, and T6 groups and lower than T3 and T4 groups (P<0.05). Markedly, the T5 group had higher SOD than the T2 group (P<0.05). The CAT showed the highest activities in T4 groups, and the lowest CAT was recorded in the T2, T5, and T6 groups (P<0.05). T3 group also showed higher CAT than T1, T2, T5, and T6 groups and lower than T4 group (P<0.05). Fish in the T5 and T6 groups had higher GSH-Px activities than fish in T1 and T2 groups but were lower than fish in T3 and T4 groups (P<0.05). Fish in T2 group recorded the highest serum MDA concentrations (P<0.05). In contrast, the lowest serum MDA levels were observed in fish of T3 and T4 groups (P<0.05). The MDA level was higher in the T1 group than T3 and T4 groups and lower than T5 and T6 groups (P<0.05).

Blood immunity

Blood immunoglobulin (Ig) and lysozyme activity were significantly higher in T3 and T4 groups and lower in the T2 group than in the control (P<0.05) (Figure 2). Serum Ig and lysozyme activity were higher in T5 and T6 groups than in the T2 group and lower than in the control group. Interestingly, fish in the T1, T5, and T6 showed non-significant differences in lysozyme activity (P>0.05). The alternative complement pathway (ACH50) was significantly higher in T2, T3, T4, T5, and T6 groups than in the T1 group (P<0.05) (Figure 2). T2 group had higher blood ACH50 than the T1 group and lower than T3, T4, T5, and T6 groups (P<0.05).

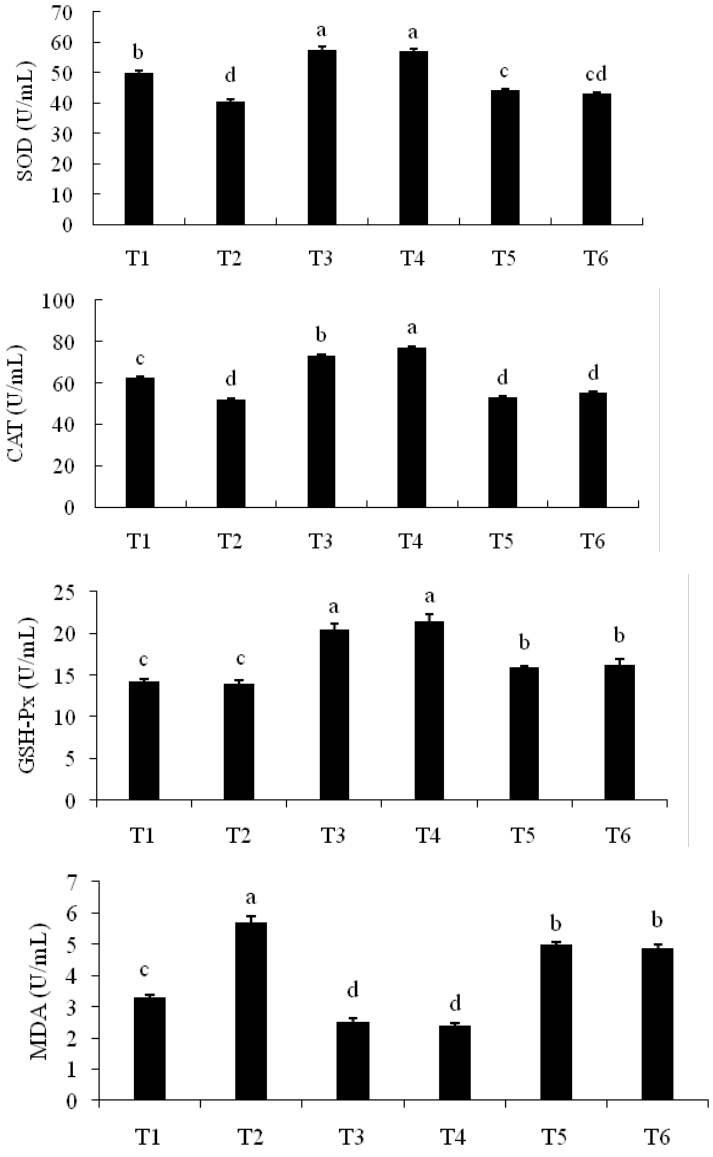

Figure 1. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and malondialdehyde (MDA) of goldfish (*C. auratus*) fed *L. casei* and exposed to malathion. T1: Control; T2: 50% of malathion LC50; T3: *L. casei* at 10^6 cfu/g diet; T4: *L. casei* at 10^7 cfu/g diet; T5: 50% of malathion LC50 + *L. casei* at 10^6 cfu/g diet; T6: 50% of malathion LC_{50} + *L. casei* at 10^7 cfu/g diet. Values are expressed as means ± S.E. (*n =3*). Bars bearing different letters are significantly different at (P≤0.05)

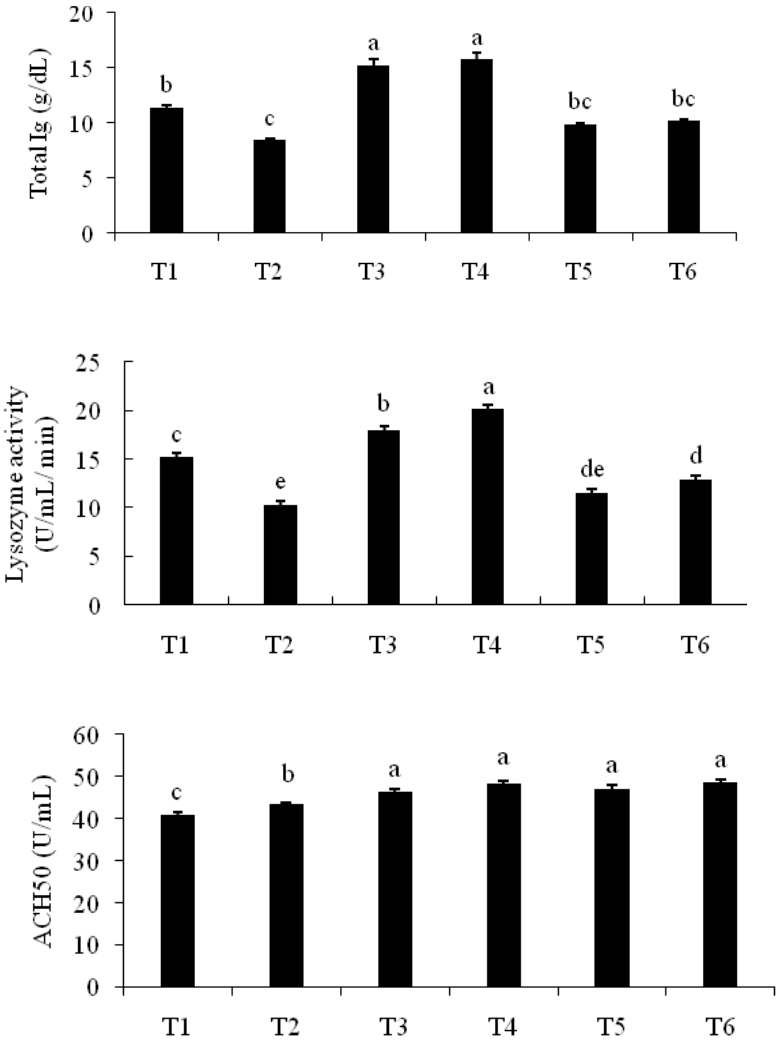

Figure 2. Serum total immunoglobulin (Ig), lysozyme activity, and alternative complement pathway (ACH_{50}) of goldfish (*C. auratus*) fed *L. casei* and exposed to malathion for 60 days. T1: Control; T2: 50% of malathion LC_{50}; T3: *L. casei* at 10^6 cfu/g diet; T4: *L. casei* at 10^7 cfu/g diet; T5: 50% of malathion LC_{50} + *L. casei* at 10^6 cfu/g diet; T6: 50% of malathion LC_{50} + *L. casei* at 10^7 cfu/g diet. Values are expressed as means ± S.E. (*n =3*). Bars bearing different letters are significantly different at (P≤0.05)

Skin mucus immunity

Skin mucus total Ig was significantly higher in T3 and T4 groups and lower in the T2 group than in control (P<0.05) (Figure 3). Fish in T1, T5, and T6 showed non-significant differences in skin mucus total Ig (P>0.05). The highest lysozyme activity, protease, and ACH50 in the skin mucus samples was in the T4 group, while the lowest lysozyme activity, protease, and ACH50 was in the T2 group (P<0.05) (Figure 3). Further, fish in the T3 group had lower lysozyme activity than T4 and higher than the remaining groups (P<0.05). Fish in T1 had lower lysozyme activity than T3 and T4 groups and higher than T2, T5, and T6 groups (P<0.05). Markedly, fish in the T6 group had higher lysozyme activity than in the T2 group (P<0.05). Non-significant differences were seen between fish in T1, T3, and T6 groups in terms of protease and ACH50 (P>0.05).

Discussion

Toxicological studies are needed to detect the direct and indirect impacts of pesticides and insecticides on the health status of humans (Abdel-Warith et al., 2021). Fish are recognized as bioindicators in toxicological studies due to their sensitivity to contamination, pollution, and toxicity (Khabbazi et al., 2015; Hedayati et al., 2021). Malathion is a highly toxic pesticide that abundantly exists in the water bodies, sediments, and ecosystems (Ortiz-Delgado et al., 2021). The studies showed that toxicity with malathion is involved in many environmental hazards and severe impacts on aquatic animals (Souza et al., 2021). On the other hand, probiotics are known for their beneficial role in performance and health status (Romano, 2021). Hence, in this study, we hypothesized that dietary *L. casei* could relieve the impacts of malathion toxicity in goldfish (*C. auratus*).

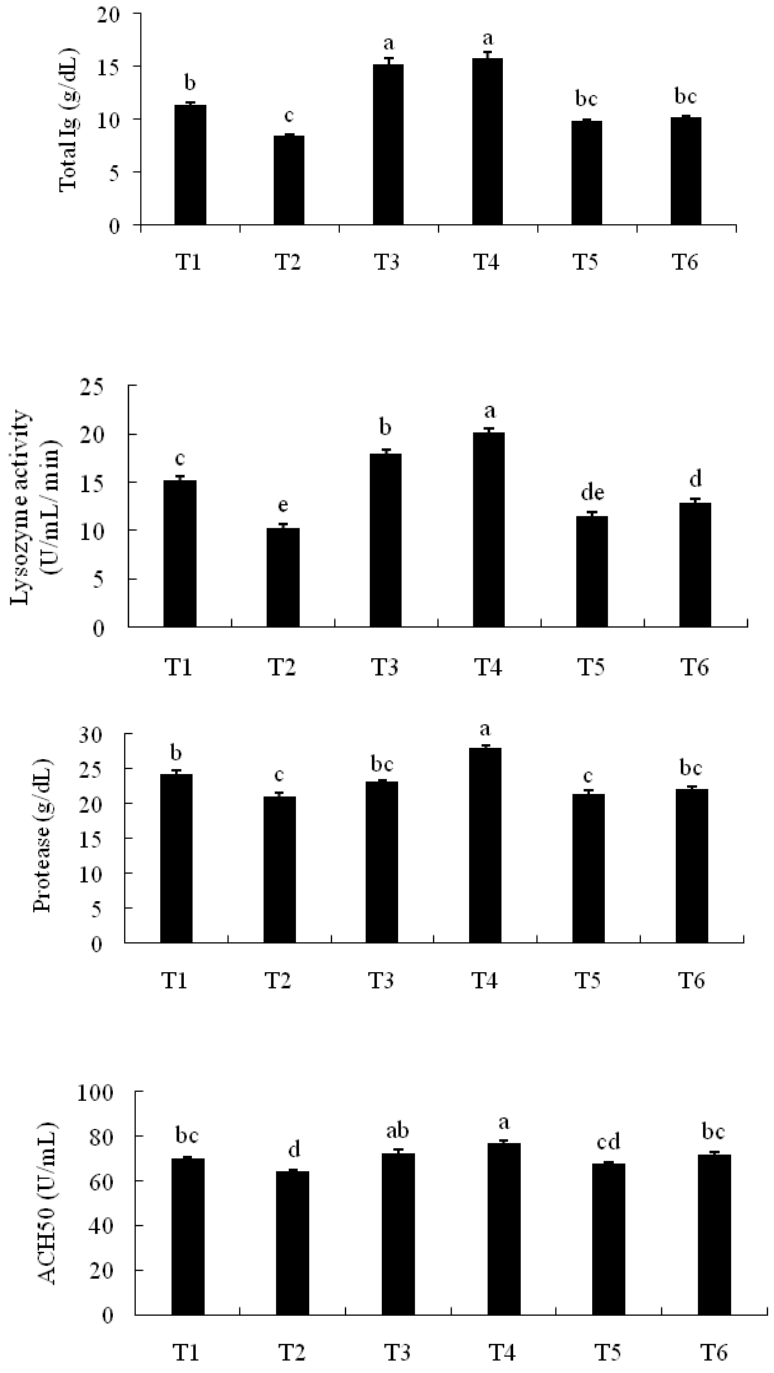

Figure 3. Mucus total immunoglobulin (Ig), lysozyme activity, protease activity, and alternative complement pathway (ACH_{50}) of goldfish (*C. auratus*) fed *L. casei* and exposed to malathion for 60 days. T1: Control; T2: 50% of malathion LC_{50}; T3: *L. casei* at 10^6 cfu/g diet; T4: *L. casei* at 10^7 cfu/g diet; T5: 50% of malathion LC_{50} + *L. casei* at 10^6 cfu/g diet; T6: 50% of malathion LC_{50} + *L. casei* at 10^7 cfu/g diet. Values are expressed as means ± S.E. (*n =3*). Bars bearing different letters are significantly different at ($P \leq 0.05$)

The growth performance of goldfish improved by *L. casei* but deteriorated by malathion toxicity. Further dietary *L. casei* relieved the impacts of malathion toxicity and restored the growth performance similar to the control and higher than malathion exposed group. In line with this study, convict cichlid Fish (*Amatitlania nigrofasciata*) (Mohammadiazarm and Maniat, 2021), goldfish (*C. auratus*) (Kong et al., 2020 b), shabot fish (*Tor grypus*) (Mohammadian et al., 2020), and channel catfish (Zhang et al., 2019) fed dietary *L. casei* showed improved growth performance. Enhanced growth performance is probably attributed to the potential role of *L. casei* on the intestinal microbiota (Mohammadiazarm and Maniat, 2021). Probiotics can colonize in the GIT and protect from pathogenic invaders leading to improved digestion and good absorption of nutrients (Brown et al.,

2021; Dawood, 2021). In this context, dietary *L. casei* reduced FCR value indicating enhanced feed digestibility and utilization in goldfish. However, goldfish exposed to malathion had impaired growth performance, and FCR compared with *L. casei* fed to fish. The deterioration of growth performance and FCR can be attributed to the negative impact of malathion on the GIT microbial population (Gao et al., 2018; Huculeci et al., 2009). Waterborne malathion reaches the fish intestines and disrupts the microbial balance, thereby feed digestion and absorption (Huculeci et al., 2009). Furthermore, continuous toxicity led to intestinal damage, cellular oxidative stress, and lipid peroxidation (Olakkaran et al., 2020; Ullah et al., 2018). Pesticide toxicity can initially pass through the gills and deteriorate their function via inflammatory and oxidative stress features (Cengiz and Unlu, 2006). Consequently, fish suffer from low respiration capacity, metabolic function, and general health weakness (Abdo et al., 2021). Thus, reduced growth performance and feed digestion can be related to the negative impact of malathion on the physiological function of fish (Abarghoei et al., 2015). In this regard, the survival rate of goldfish exposed to malathion is higher than fish fed *L. casei* either with or without malathion. The high mortality rate in the group treated with malathion is also related to oxidative stress and the impaired health status of goldfish (Hedayati et al., 2015).

Blood biochemical traits are commonly detected to reveal the impact of toxicity, feeding strategies, and environmental effects on the physio-chemical status of fish (Coz-Rakovac et al., 2008; Khodadadi et al., 2018). In this study, goldfish fed *L. casei* and exposed to malathion showed effects on blood biochemical traits. In terms of blood protein profile, including total protein, albumin, and globulin, fish fed *L. casei* had higher values than fish exposed to malathion. The enhancement in blood proteins refers to regulated metabolic function and available proteins for physiological processes as well as enhanced immunity status (Yousefi et al., 2022). Indeed, *L. casei* feeding was earlier proved to be a functional supplement involved in fortifying blood proteins in barramundi (*Lates calcarifer*) (Siddik et al., 2022). However, reduction of blood protein profile in goldfish is probably related to the malnutrition, oxidative stress, and immunosuppression caused by malathion exposure (Ullah et al., 2018). Similarly, toxicity with malathion reduced the blood protein and globulin in Persian sturgeon (*Acipenser persicus*) (Rahbar et al., 2020). In the present study, the cholesterol level was higher in malathion exposed fish than *L. casei* fed to fish. These results are similar to previous investigations that indicated reduced cholesterol in fish fed dietary probiotics (Kong et al., 2020 a). Regulated cholesterol levels refer to the balance of metabolic function in fish fed dietary *L. casei*, while increased levels refer to lipid vacuolation and accumulation of lipids associated with malathion toxicity. However, the authors suggest further investigation in this regard.

Cortisol and glucose axis are involved in regulating organism response towards abiotic and biotic stressors (Rotllant and Tort, 1997). In fish, stressful conditions, including low feed value and toxicity with waterborne insecticides, led to a high release of cortisol that induces high production of glucose as a primary source of energy required to cope with the stress (Brun et al., 2019; Wendelaar Bonga, 1997). Concisely, goldfish fed *L. casei* had lower glucose and cortisol levels than fish exposed to malathion, indicating a lack of stress in fish treated with *L. casei*. Similar to this study, common carp (*Cyprinus carpio*) fed *L. casei* had reduced glucose and cortisol levels (Hedayati et al., 2021) while *A. persicus* exposed to malathion had increased glucose and cortisol levels (Rahbar et al., 2020). Markedly, fish fed *L. casei* and exposed to malathion had similar blood protein and lipid profiles and the glucose and cortisol levels that confirm the functional role of *L. casei* in regulating the physiological function of goldfish.

Liver function-related biomarkers (e.g., ALT, AST, and ALP) are vital indicators for liver function, especially when fish are exposed to pesticides and insecticides (Dawood et al., 2020; Oyeniran et al., 2021). The liver's function is to detoxify the toxins and reduce their impact on the internal body (De Anna et al., 2021). However, high toxicity levels led to high production of free radicals, which induce lipid peroxidation and damage of cellular membranes in the liver tissue (Lackner, 1998; Qu et al., 2014). Hence, the liver secretes high amounts of ALT, AST, and ALP, referring to damaged liver function and less detoxification role (Dawood et al., 2020; Oyeniran et al., 2021). The obtained results showed high ALT, AST, and ALP levels in goldfish exposed to malathion while reduced by dietary *L. casei*. The results are similar to Ullah et al. (2018), who stated elevated ALT, AST, and ALP levels in rohu exposed to malathion. However, dietary *L. casei* regulated the levels of ALT, AST, and ALP, which can be associated with the liver protective role of *L. casei*. Similarly, feeding *L. casei* reduced ALT, AST, and ALP levels in common carp (Hedayati et al., 2021) and *L. calcarifer* (Siddik et al., 2022).

Oxidative stress is the main feature of malathion toxicity that can explain the impairment of aquatic animals' growth performance and health status (Huculeci et al., 2009). The high production of free radicals and reactive oxygen species (ROS) associated with severe toxicity with pesticides is the primary inducer for lipid peroxidation in cellular membranes (Mohammadi et al., 2022). The lipid peroxidation is evaluated by detecting the amount of malondialdehyde (MDA) involved in apoptosis and DNA damage (Üner et al., 2006; Ghafarifarsani et al., 2021 a, b, c). Antioxidative defenses including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) can overcome the high production of MDA in case of acute short term malathion exposure as indicated by Ullah et al. (2018). However, in this study, goldfish were exposed to malathion for 60 days which may explain the increased MDA levels and reduced SOD, CAT, and GSH-Px activities. Similarly, toxicity with malathion reduced the antioxidative capac-

ity in tambaqui (*Colossoma macropomum*) (Souza et al., 2021). Interestingly, *L. casei* feeding regulated the antioxidation capacity of goldfish exposed to malathion through the activation of SOD, CAT, and GSH-Px and the reduction of MDA concentration. Similarly, the incorporation of *L. casei* enhanced the antioxidation capacity in common carp (Hedayati et al., 2021) and largemouth bass (*Micropterus salmoides*) (Wang et al., 2021).

Oxidative stress induced by malathion toxicity is also associated with impaired immunity in fish (Lee et al., 2019). The serum and skin mucus immune responses are vital tools to protect fish against infection with pathogenic microorganisms (Xu et al., 2013). The results revealed lowered total immunoglobulin (total Ig), lysozyme, and complement pathway (ACH50) activities in serum and skin mucus samples of goldfish exposed to malathion. Nevertheless, dietary *L. casei* enhanced the serum and skin mucus total Ig, lysozyme, and ACH50. In the same line, Hedayati et al. (2021) stated that common carp fed dietary *L. casei* had enhanced serum and skin mucus immune responses. Further, Hedayati et al. (2021) related increased resistance of common carp to iron oxide nanoparticles toxicity and enhanced antioxidative and immunity resulting from *L. casei* feeding. Also, Mohammadiazarm and Maniat (2021) reported that *A. nigrofasciata* fed dietary *L. casei* displayed enhanced serum and skin mucus immune responses. Total proteins including lysozyme, total Ig, globulins, protease, and complement play pivotal roles in the fish immune system through bactericidal activity and antigen neutralization (Magnadóttir, 2006; Whyte, 2007; Sadat Hoseini Madani et al., 2018; Adorian et al., 2019; Ghafarifarsani et al., 2021 d). The enhancement of blood total proteins, antioxidation capacity, serum, and skin mucus immune responses of goldfish fed dietary *L. casei* may explain the high protection to malathion toxicity.

Conclusion

In summary, goldfish exposed to 50% of malathion 96 h LC_{50} showed poor growth performance, blood biochemical traits, antioxidative capacity, and immune responses. However, *L. casei* protects goldfish from alterations induced by malathion toxicity through modifying the growth performance, blood biochemistry, antioxidative capacity, serum, and skin mucus immunity.

Compliance with ethical standards

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Author contributions

Marwan Mahmood Saleh: Writing – original draft preparation; Saif Y. Hasan: Conceptualization, Methodology; Sarmad Ghazi Al-Shawi: Supervision, Writing – review and editing; Muneam Hussein Ali: Writing – review and editing; Thulfeqar Ahmed Hamza: Writing – review and editing; Mazin A.A. Najm: Supervision; Rustem Adamovich Shichiyakh: Resources; Abduladheem Turki Jalil: Formal analysis; Fariborz Narimanizad: Methodology.

Conflict of interest

The authors declare that they have no conflict of interest.

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