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EFFECTS OF DIETARY *LACTOBACILLUS HELVETICUS* ATCC 15009 ON GROWTH PERFORMANCE, HEMATOLOGY PARAMETERS, INNATE IMMUNE RESPONSES, AND THE ANTIOXIDANT STATUS OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS)* UNDER HIGH REARING DENSITY

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Abstract

This study aimed to evaluate the effects of dietary probiotic supplementation of *Lactobacillus helveticus* on growth, digestive enzymes, and hematological, biochemical, immune, and antioxidant parameters, as well as intestinal microbiota of rainbow trout (*Oncorhynchus mykiss*). Fish (35.46±0.9 g) were fed with different levels of dietary *L. helveticus*: control, 1×10^6, 1×10^7, 1×10^8, and 1×10^9 at high stocking density (80 kg m^{-3}) for 60 days. Results indicated that growth performance significantly improved in probiotic supplemented fish (P<0.05). Digestive enzyme parameters revealed that supplementation could significantly increase amylase, protease, and lipase (P<0.05). The treated groups showed significant improvements in serum immune parameters including lysozyme (LYZ), alternative complement (ACH_{50}), respiratory burst activity (RBA), and myeloperoxidase (MPO) (P<0.05). Total protein (TP), albumin (ALB), and globulin (GLO) increased in fish fed experimental diets (P<0.05). Lactate dehydrogenase (LDH) activity was significantly lower in fish fed dietary additives (P<0.05) while white blood cells (WBC), lymphocytes, neutrophils, hematocrit (Hct), red blood cells (RBC) were significantly enhanced (P<0.05). Fish fed with supplemented diets showed significantly enhanced antioxidant status, catalase (CAT) and superoxide dismutase (SOD). Malondialdehyde (MDA) content was significantly lower in fish fed dietary additives (P<0.05). Lactic acid bacteria (LAB) in the treatment groups were significantly increased (P<0.05). In conclusion, dietary supplementation of *L. helveticus* reduced detrimental effects of high stocking density on growth performance and immune response. It appears that *L. helveticus* can be recommended as a beneficial probiotic feed additive for rainbow trout.

Key words: immunity, fish, probiotic, antioxidant, intensive culture

The contribution of aquaculture has increased with the global demand for world food production over the last few decades. This has led to the intensification of farming production systems and emphasis on the need to reduce economic losses when aquatic animals are exposed to stressful situations (Raissy et al., 2022). Long-term crowding stress in intensive culture is a risk factor for pathogen infections, immunosuppression, oxidative stress, and high mortality rates (Ramesh and Souissi, 2018).

Rainbow trout (*Oncorhynchus mykiss*) is an important commercial fish species farmed under intensive or semi-intensive systems (Ghafarifarsani et al., 2021 a). Excessive physiological and behavioral responses to high rearing density can negatively affect growth performance and humoral and immune responses of rainbow trout (Naderi et al., 2019). Identifying beneficial production strategies can improve fish health and welfare in such systems and potentially decrease the adverse effects of stress, while diminishing the need for therapeutic agents like antibiotics (Gonçalves et al., 2011).

Biotherapeutics or immunostimulants such as probiotics and prebiotics have been successfully incorporated in feed formulations to improve feed efficiency, prevent or minimize diseases, and mitigate fish stress (Paray et al., 2020). Probiotics are living microorganisms, including Gram-positive bacteria, Gram-negative bacteria, and

also bacteriophages and unicellular algae (Irianto and Austin, 2002 a). The most common probiotics that have been used in aquaculture include *Bacillus* spp., *Lactobacillus* spp., *Lactococcus* spp., and *Saccharomyces cerevisiae* (Harikrishnan et al., 2010 a). Probiotic supplementation can also stimulate the immune responses of the target organism and act as a growth promoting factor (Das et al., 2017). Additionally, some probiotics elevate antibodies and phagocytic activity against pathogenic microorganisms (Martínez Cruz et al., 2012; Brown et al., 2021). Previous studies have reported that oral administration of probiotics modulates the immune, hematological, and serum biochemical parameters of aquatic animals (Irianto and Austin, 2002 b; Cerezuela et al., 2013; Hoseinifar et al., 2015 b; Iorizzo et al., 2022).

Lactic acid bacteria (LAB) mainly consists of *Lactobacilli* species. These have been widely used in aquaculture and are considered safe as feed-supplementing probiotics (Harikrishnan et al., 2010 b; Abou-El-Atta et al., 2019). LAB are host-derived probiotics that induce distinct mucosal cytokine profiles, exert a strong intrinsic adjuvant activity, and enhance host immune responses by preventing edwardsiellosis, furunculosis, and vibriosis diseases (Perdigón et al., 1988; Gatesoupe et al., 1999; Van Doan et al., 2021). *L. helveticus,* which belongs to the LAB group, has the potential to adhere to epithelial cells, produce bioactive peptides or bacteriocins, prevent gastrointestinal infections, and affect the composition of the intestinal microbiota (Slattery et al., 2010). Therefore, *L. helveticus* may stimulate host immunity through various health-promoting properties (Taverniti et al., 2012; Yousefi et al., 2023).

Although a variety of studies have investigated the effect of dietary probiotic supplementation as a potential stress management tool to improve growth performance and immunity of fish (de Carla Dias et al., 2020; Iorizzo et al., 2022), there is only limited information regarding effects on fish exposed to long-term high stocking density. In the present study, the effects of *L. helveticus* administration on antioxidant, immunological, and biochemical responses of rainbow trout during long-term crowding stress were investigated.

Material and methods

Fish and maintenance conditions

Seven hundred apparently healthy rainbow trout fingerlings were purchased from a private farm. The fish were acclimatized in a 1000-L tank under laboratory conditions for 14 days and fed a commercial diet. After the adaptation period, 5 groups of 120 fish each were stocked in tanks (300 L) in triplicates at a density of 80 kg m^{-3} (Mirghaed et al., 2018 a; Naderi et al., 2019). The basic physicochemical water parameters were maintained at 15 ± 0.5°C, 7.8 ± 0.4 mg L^{-1} dissolved oxygen, 7.3 ± 0.2 pH (Hach HQ40d, Loveland, Colorado, USA), and <0.07 mg L^{-1} ammonia nitrogen (Wagtech 7100, Berkshire, UK). Daily feed rations were adjusted according to the change in biomass, which was estimated every two weeks. Fish were hand-fed 3 times daily at 08:00, 13:00, and 18:00 to apparent satiety (Naderi Farsani et al., 2021).

Diet preparation and probiotic

Lactobacillus helveticus ATCC 15009 were prepared from the National Center for Genetic and Biological Resources (Tehran, Iran). The bacteria were grown in Man Rogosa Sharpe agar (MRS) and were incubated at 37°C for 24 h. After that, the plates were centrifuged at 4500 g for 30 min and washed in phosphate-buffered saline twice. Bacteria were re-suspended in salt buffer. Bacterial quantifications were made using spectrophotometers and serial dilution (Yang et al., 2021).

Table 1. Feedstuffs and compositions of the basal diet

Ingredients	(g/kg in dray basis)	Proximate composition	(g/kg)
Fish meal[1]	370	Crude protein	425
Soybean meal (defatted)[2]	163	Crude lipid	164
Wheat flour	218	Crude fiber	32
Poultry meat meal[3]	100	Ash	96
Fish oil	50	Moisture	90.1
Soybean oil	30	NFE[10]	224.6
Binder[4]	20		
Mineral mix[5]	16	Gross energy (cal/g)[11]	5016.16
Vitamin mix[5]	10		
Cellulose	10		
Antifungi[6]	5		
Phytase[7]	3		
DL-methionine[8]	3		
Antioxidant[9]	2		
Total (g)	1000		

[1]67% protein; 10% lipid, and 13% ash (Khazar Company, Mazandaean Iran).
[2]Gorgan Soya Co., Gorgan, Iran (46% crude protein).
[3]60% protein and 18% lipid.
[4]Ametbinder™, MehrTaban-e-Yazd, Iran.
[5]The premix provided following amounts per kg of feed: A: 1000 IU; D_3: 5000 IU; E: 20 mg; B_5: 100 mg; B_2: 20 mg; B_6: 20 mg; B_1: 20 mg; H: 1 mg; B_9: 6 mg; B_{12}: 1 mg; B_4: 600 mg; C: 50 mg; Mg: 350 mg; Fe: 13 mg; Co: 2.5 mg; Cu: 3 mg; Zn: 60 mg; Se: 0.3 mg; I: 1.5 mg; Mn: 10 mg.
[6]ToxiBan antifungal (Vet-A-Mix, Shenan-doah, IA).
[7]Golbid Co., Tehran, Iran (10000 IU).
[8]Mad Tiour Co., Sanandaj, Iran.
[9]Butylated hydroxytoluene (BHT) (Merck, Germany).
[10]Nitrogen-free extract (calculated by difference) = 100 – (protein % + lipid % + ash % + fiber %)
[11]Gross energy (GE) was calculated from NRC (2011) as 16.7, 37.4, and 16.7 kJ/g for protein, lipid, and carbohydrates, respectively.

Ingredients and proximate composition of the basal diet are shown in Table 1. To prepare the diet, enough distilled water was added to the feed ingredients and thoroughly mixed. The treatments consisted of the control (LH0); 1×10^6 (LH1); 1×10^7 (LH2); 1×10^8 (LH3); and 1×10^9 (LH4). Finally, the dough was passed through a meat grinder to obtain uniform particles. The pellets were dried at room temperature for 36 h. Dried pellets were kept in a refrigerator until use. The viability of the supplemented bacteria during storage was assessed by

the spread plate method (Nikoskelainen et al., 2003). Probiotic treatment concentrations were chosen based on a previous study (Ahire et al., 2019; Yang et al., 2021).

Growth performance

Growth performance and survival parameters were calculated using the following equations (Kari et al., 2021; Hamid et al., 2022):

Weight gain (WG; g) = final body weight − initial body weight

Specific growth rate (SGR; % day^{-1}) = [(ln (final body weight) -ln (initial body weight)) / trial period] ×100

Feed conversion rate (FCR) = feed intake (g) / weight gain (g)

Protein efficiency ratio (PER) = WG/(total protein intake (g)

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

Sampling

At the end of the experiment (day 60), the fish were fasted for 24 h and anesthetized with eugenol (100 mg L^{-1}, Yousefi et al., 2021). Blood was withdrawn from caudal veins using 2-mL syringes and each blood sample was divided into two parts, one with heparin for hematological studies, and the other without heparin for serum separation. The non-heparinized blood remained in a refrigerator for 3 h. Serum samples were separated at 3000 × g for 10 minutes in 4°C centrifugation and stored at –70°C for further analysis.

To measure the intestinal microbiota, anesthetized fish were killed. Then, fish skin surfaces were disinfected with ethanol (70%) and intestines were dissected using sterilized surgical scissors. To measure the activity of digestive enzymes, intestine samples were homogenized by adding Tris-HCL buffer (50 mM) and centrifuged for 10 min at 6000 × g at 4°C. Thereafter, supernatants were carefully stored at −80°C until analysis.

Digestive enzyme activity

Amylase activity was quantified using starch (2%) as substrate in 0.1 M phosphate buffer. Lipase activity was assessed. Crude enzyme extracts were incubated with p-nitrophenyl myristate and cholate buffer (0.25 mM Tris HCl + 0.25 mM 2-methoxyethanol + 5 mM sodium cholate, pH = 9.0) at 30°C for 15 min. The reaction was stopped by adding acetone/n-heptane (5:2, v/v). The absorbance value was recorded at 405 nm. Total protease activity was assayed using casein as the substrate in 0.5 ml Tris–HCl (0.1 M; pH= 8). The reaction was stopped in 5% trichloroacetic acid at 25°C for 1 h. Finally, the absorbance was measured at 440 nm (Kari et al., 2022).

Immune parameters

Lysozyme (LYZ) activity was measured through turbidometric assay according to Ellis (1990). *Micrococcus luteus* was used as a target in phosphate buffer (0.2 mg ml^{-1} in a 0.05 M sodium phosphate buffer, pH 6.2). A suspension of 50 µl serum with 2 ml bacterial suspension was kept at room temperature and absorbance was measured at 550 nm for 5 min at 30°C.

Alternative complement activity (ACH_{50}) was determined based on Yano's procedures (1992). Sheep RBC was used as a target and the reaction medium was a veronal buffer containing EGTA, gelatin, and magnesium.

Total serum immunoglobulin (Ig) was calculated based on the amount of protein before and after the addition of 12% polyethylene glycol. Respiratory burst activity (RBA) was determined using chemiluminescent (LUMI Skan Ascent T392, Finland) according to the Binaii et al. (2014) method. Myeloperoxidase (MPO) activities were measured using a microplate reader at 450 nm based on Quade and Roth's (1997) method.

Biochemical parameters

Total protein (TP) was determined based on Bradford's method (1976). Albumin (ALB) (Pars Azmoun Co., Iran) was measured by the colorimetric method described by Nicholson et al. (2000) at 620 nm. Lactate dehydrogenase (LDH) activity was measured using commercial kits (Greiner Diagnostic Group, Bahlingen, Germany). Serum globulin (GLO) was expressed as the difference between serum total protein and albumin. The serum cortisol (CORT) activity was determined with commercial kits (IBL Co., Gesellschaft für Immunchemie und Immunbiologie, Germany) using an ELISA plate reader. Glucose (GLU) was measured with a commercial kit (Pars Azmoun Co., Tehran, Iran).

Hematological parameters

The red (RBC) and white blood cells (WBC) were manually counted by Neubauer hemocytometer according to Sarder et al. (2001) method. Blood hematocrit (Hct) levels were measured through the microhematocrit as explained by Brown (1980) and blood hemoglobin (Hb) was determined using the Blaxhall and Daisley (1973) method. For differential counts of WBC (lymphocyte, neutrophil, monocyte, eosinophil), blood smears were fixed in methanol and evaluated using Giemsa staining under an electron microscope (Borges, 2004).

Antioxidant status

Glutathione peroxidase (GPx) was measured by oxidized glutathione as a substrate, using a commercial kit (Zellbio, Hamburg, Germany, Hoseini et al., 2021). Superoxide dismutase (SOD) was determined by the rate of reduction of cytochrome C using another commercial kit (ZellBio GmbH, Veltinerweg, Hoseini et al., 2021). Catalase (CAT) was utilized to decrease H_2O_2 absorbance according to Goth (1991). Malondialdehyde (MDA) level was determined based on the thiobarbituric acid reaction at 95°C using a commercial kit (ZellBio GmbH, Veltinerweg).

Intestinal microbiota assay

At the end of the experiment, intestines were washed and homogenized in phosphate buffer. Homogenized liq-

uid was then diluted in phosphate buffer. Then, lactic acid bacteria (LAB) and total bacteria counts (TBC) were determined through spreading of 100 µL portion samples onto MRS plates (Merck, Germany) and tryptic soya agar (TSA, Merck, Germany), respectively. Plates were incubated at 30°C for 48 h. LAB and TBC colonies were counted and expressed as log CFU g^{-1} (Merrifield et al., 2010).

Statistical analysis

Before performing the analysis of variance tests, normality and homogeneity of variances were checked using the Kolmogorov–Smirnov test. A one-way variance test and Tukey's test were used to compare the differences between treatments. The minimum level of significance of the tests was considered to be $P<0.05$. The obtained data were presented as mean ± standard error. Data analyses were conducted by SPSS version 20 software and Excel software version 2013 was used to draw graphs.

Results

Growth performance

The results of the growth performance indices and survival rate after feeding for 60 days are summarized in Table 2. Significant increases in FW, WG, PER, and SGR were found in fish fed higher levels of *L. helveticus* supplemented diets (LH2, LH3, and LH4) compared with the control group ($P<0.05$). The highest FCR values were recorded in the non-supplemented (control) group versus all treatment groups ($P<0.05$). There were no significant differences in SR compared to the control group ($P>0.05$).

Digestive enzyme activity

As shown in Table 3, amylase levels in LH3 and LH4 were significantly higher than the control group ($P<0.05$). Protease assessment showed a significant increase in all dietary groups compared to the LH0 ($P<0.05$), however, no significant differences were observed among all dietary groups ($P>0.05$). Also, significantly higher lipase values were found in LH2, LH3, and LH4 treatments compared with the control ($P<0.05$).

Immune parameters

The results of the rainbow trout immune parameters are presented in Table 4. Fish treated with LH2 and LH4 had significantly higher LYZ activity than LH0 ($P<0.05$); however, other dietary groups showed no significant differences from LH0 ($P>0.05$). All experimental groups, except LH3, were not modified in terms of ACH_{50} and MPO levels ($P>0.05$). Moreover, there was no significant difference for Ig levels in dietary treatments ($P>0.05$). RBA values significantly increased in fish fed LH3 and LH4 diets ($P<0.05$), whereas the other treatments displayed no significant differences from the control ($P>0.05$).

Biochemical parameters

As illustrated in Table 5, fish fed the LH2 and LH3 diets exhibited significantly higher TP levels compared to the control group ($P<0.05$). ALB contents in fish fed LH3, and GLO in fish fed LH2, were significantly higher than those in fish fed the control diet ($P<0.05$). However, there were no significant differences between the treatment groups ($P>0.05$). Fish fed the LH2, LH3, and LH4 diets exhibited significantly higher LDH levels compared to the control group ($P<0.05$). As shown in Figure 2, the GLU contents of fish fed LH3 and LH4 were significantly lower than those in the control group ($P<0.05$). Also, CORT level significantly decreased in fish fed the LH2, LH3, and LH4 diets compared to the control group ($P<0.05$).

Table 2. Growth parameters of rainbow trout (*Oncorhynchus mykiss*) fed different levels of dietary *Lactobacillus helveticus*

Parameters	LH0	LH1	LH2	LH3	LH4
IW (g)	35.46±0.77	35.20±0.51	35.76±0.64	35.66±0.46	35.30±0.43
FW (g)	75.83±1.09 c	80.23±1.24 bc	84.33±1.16 ab	88.33±0.88 a	85.83±1.64 ab
WG (g)	40.36±1.24 c	45.03±0.73 bc	48.56±1.56 ab	52.66±1.33 a	50.53±1.53 ab
FCR	1.62±0.04 a	1.45±0.03 b	1.35±0.04 bc	1.25±0.02 c	1.33±0.02 bc
PER	1.31±0.03 c	1.46±0.03 bc	1.57±0.05 ab	1.69±0.03 a	1.59±0.02 ab
SGR (% d^{-1})	3.66±0.04 c	3.74±0.04 bc	3.87±0.02 ab	3.96±0.01 a	3.86±0.02 ab
SR (%)	95.00±1.00	96.33±2.02	97.33±1.33	98.66±1.33	97.66±2.33

Data are expressed as the mean ± SE (n=3). Different letters (a–c) in the same row indicate significant differences among the treatments ($P<0.05$). LH0 (0, control); LH1 (1×10^6); LH2 (1×10^7); LH3 (1×10^8); LH4 (1×10^9). IW: Initial weight; FW: Final weight; WG: Weight gain; FCR: Food conversion ratio; PER: Protein efficiency ratio; SGR: Specific growth rate; SR: Survival rate.

Table 3. Digestive enzyme of rainbow trout (*Oncorhynchus mykiss*) fed different levels of *Lactobacillus helveticus*

Parameters	LH0	LH1	LH2	LH3	LH4
Amylase (U/mg protein)	0.56±0.06 b	0.78±0.05 ab	0.69±0.06 ab	0.94±0.03 a	0.89±0.05 a
Protease (U/mg protein)	3.22±0.12 b	4.88±0.31 a	4.59±0.25 a	4.35±0.09 a	4.28±0.24 a
Lipase (U/mg protein)	1.07±0.10 b	1.39±0.09 ab	1.45±0.07 a	1.44±0.06 a	1.46±0.04 a

Data are expressed as the mean ± SE (n=3). Different letters (a–e) in the same row indicate significant differences among the treatments ($P<0.05$). LH0 (0, control); LH1 (1×10^6); LH2 (1×10^7); LH3 (1×10^8); LH4 (1×10^9).

Table 4. Serum immune parameters of rainbow trout (*Oncorhynchus mykiss*) fed different levels of dietary *Lactobacillus helveticus*

Parameters	LH0	LH1	LH2	LH3	LH4
LYZ (U/ml)	17.71±1.29 b	18.02±1.29 b	25.82±2.30 a	24.71±1.33 ab	27.01±1.60 a
ACH_{50} (U/ml)	123.83±10.87 b	129.33±10.97 ab	151.90±10.24 ab	177.43±11.56 a	168.85±9.92 ab
Total Ig (mg/dl)	10.08±1.32 a	11.01±0.59 a	12.75±1.05 a	14.89±1.47 a	14.46±0.94 a
RBA (RLU/S)	842.66±24.25 c	841.33±25.82 c	918.66±30.90 bc	1034.66±35.04 ab	1141.66±22.04 a
MPO (OD 450)	1.25±0.15 b	1.73±0.12 ab	1.83±0.14 ab	2.13±0.18 a	1.90±0.17 ab

Data are expressed as the mean ± SE (n=3). Different letters (a–e) in the same row indicate significant differences among the treatments (P<0.05). LH0 (0, control); LH1 (1×10^6); LH2 (1×10^7); LH3 (1×10^8); LH4 (1×10^9). LYZ: Lysozyme; ACH_{50}: Alternative complement; Total Ig: Total immunoglobulin; RBA; Respiratory burst; MPO: Myeloperoxidase.

Table 5. Biochemical parameters of rainbow trout (*Oncorhynchus mykiss*) fed different levels of dietary *Lactobacillus helveticus*

Parameters	LH0	LH1	LH2	LH3	LH4
TP (g/dL)	2.83±0.17 c	3.10±0.15 bc	3.80±0.15 ab	4.16±0.20 a	3.43±0.29 abc
ALB (g/dL)	1.70±0.15 b	1.86±0.21 ab	2.26±0.14 ab	2.66±0.18 a	2.03±0.20 ab
GLO (g/dL)	1.13±0.03 b	1.23±0.14 ab	1.53±0.03 a	1.50±0.05 ab	1.40±0.10 ab
LDH (mg/dL)	53.00±2.88 a	48.66±2.72 ab	40.33±2.02 bc	35.66±2.33 c	40.00±2.30 bc

Data are expressed as the mean ± SE (n=6). Different letters (a–c) in the same row indicate significant differences among the treatments (P<0.05). LH0 (0, control); LH1 (1×10^6); LH2 (1×10^7); LH3 (1×10^8); LH4 (1×10^9). TP: Total protein; ALB: Albumin; GLO: Globulin; LDH: Lactate dehydrogenase.

Table 6. Hematological parameters of rainbow trout (*Oncorhynchus mykiss*) fed different levels of dietary *Lactobacillus helveticus*

Parameters	LH0	LH1	LH2	LH3	LH4
WBC ($10^3/mm^3$)	14.20±0.78 b	14.78±1.04 b	16.05±1.06 ab	19.73±0.75 a	18.21±1.05 ab
Lymphocyte (%)	78.62±1.58 a	78.37±1.14 a	79.00±1.35 a	72.13±0.88 b	75.61±0.81 ab
Neutrophil (%)	15.25±1.10 b	15.79±1.11 ab	14.94±0.87 b	20.29±0.89 a	17.69±1.27 ab
Monocyte (%)	4.34±0.28 ab	3.52±0.27 b	4.69±0.54 ab	5.94±0.29 a	5.09±0.65 ab
Eosinophil (%)	1.78±0.21	2.31±0.27	1.36±0.30	1.63±0.28	1.59±0.38
RBC ($10^6/mm^3$)	1.26±0.07 c	1.42±0.06 bc	1.60±0.08 abc	1.80±0.06 a	1.64±0.06 ab
Hb (g/dl)	7.12±0.60	7.82±0.73	8.05±0.62	9.39±0.65	8.33±0.60
Hct (%)	29.50±1.60 c	32.66±1.45 bc	38.93±1.48 ab	42.10±0.83 a	39.06±1.61 ab

Data are expressed as the mean ± SE (n= 6). Different letters (a-c) in the same row indicate significant differences among the treatments (P<0.05). LH0 (0, control); LH1 (1×10^6); LH2 (1×10^7); LH3 (1×10^8); LH4 (1×10^9). WBC: White blood cell; RBC: Red blood cell; Hb: Hemoglobin; Hct: Hematocrit.

Table 7. Serum antioxidant parameters of rainbow trout (*Oncorhynchus mykiss*) fed different levels of dietary *Lactobacillus helveticus*

Parameters	LH0	LH1	LH2	LH3	LH4
MDA (μM/L)	7.93±0.88 a	5.63±0.71 ab	4.97±0.53 ab	3.82±0.93 b	5.55±1.02 ab
SOD (U/ml)	26.00±2.08 b	31.00±2.02 ab	35.52±2.80 ab	37.57±2.19 a	35.37±3.06 ab
CAT (U/ml)	12.31±1.92 b	12.61±1.35 b	15.56±1.45 ab	20.36±0.80 a	15.75±1.76 ab
GPx (U/ml)	48.33±3.48	53.33±4.40	61.33±4.66	67.66±4.33	61.33±5.78

Data are expressed as the mean ± SE (n=3). Different letters (a–c) in the same row indicate significant differences among the treatments (P<0.05). LH0 (0, control); LH1 (1×10^6); LH2 (1×10^7); LH3 (1×10^8); LH4 (1×10^9). MDA: Malondialdehyde; SOD: Superoxide dismutase CAT: Catalase; GPx: Glutathione peroxidase.

Hematological parameters

Fish fed LH3 exhibited a significant increase in WBC, lymphocyte and neutrophil activity compared to the control (P<0.05); whereas the fish fed other supplemented diets showed no significant differences (P>0.05) (Table 6). The monocyte, eosinophil and Hb content of fish fed the *L. helveticus* diet showed no significant difference than that of the control (P>0.05). Except for fish fed the LH1 diet, Hct significantly increased in all experimental groups, compared to the values obtained from the control group (P<0.05).

Antioxidant status

Serum antioxidant values of rainbow trout are shown in Table 7. MDA, SOD, and CAT values significantly increased in fish fed the LH3 diet compared to the control (P<0.05), whereas the other treatments displayed no significant differences (P>0.05). No statistically significant variations were recorded for GPx value among the treatments (P>0.05).

Intestinal microbiota assay

The lactic acid bacteria (LAB) and total bacterial counts (TBC) are presented in Figure 1. The LH3 and LH4 treatments had significantly higher LAB counts compared to the control treatment (P<0.05). TBC values of the treatment groups were not statistically different compared to the control group (P>0.05).

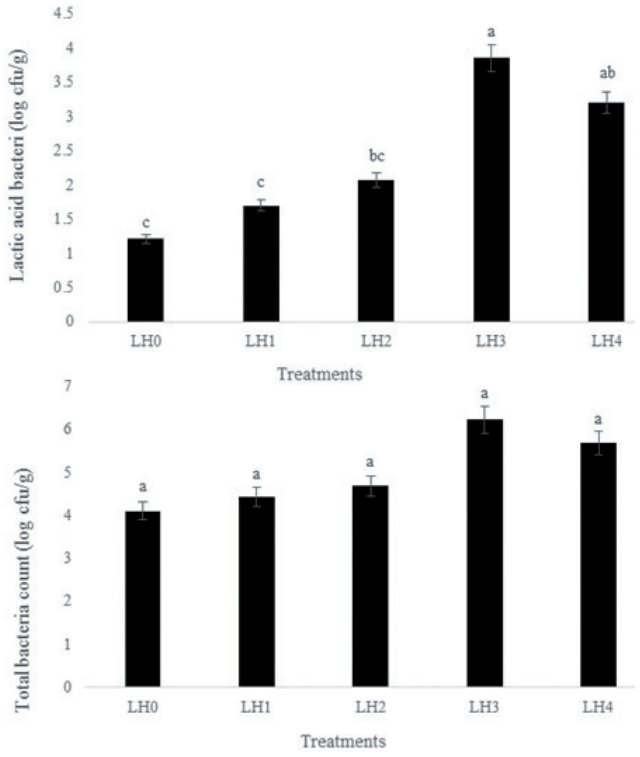

Figure 1. Lactic acid bacteria (LAB) and total bacterial count (TBC) levels of rainbow trout (*Oncorhynchus mykiss*) fed different levels of dietary *Lactobacillus helveticus*. LH0 (0, control); LH1 (1×10^6); LH2 (1×10^7); LH3 (1×10^8); LH4 (1×10^9)

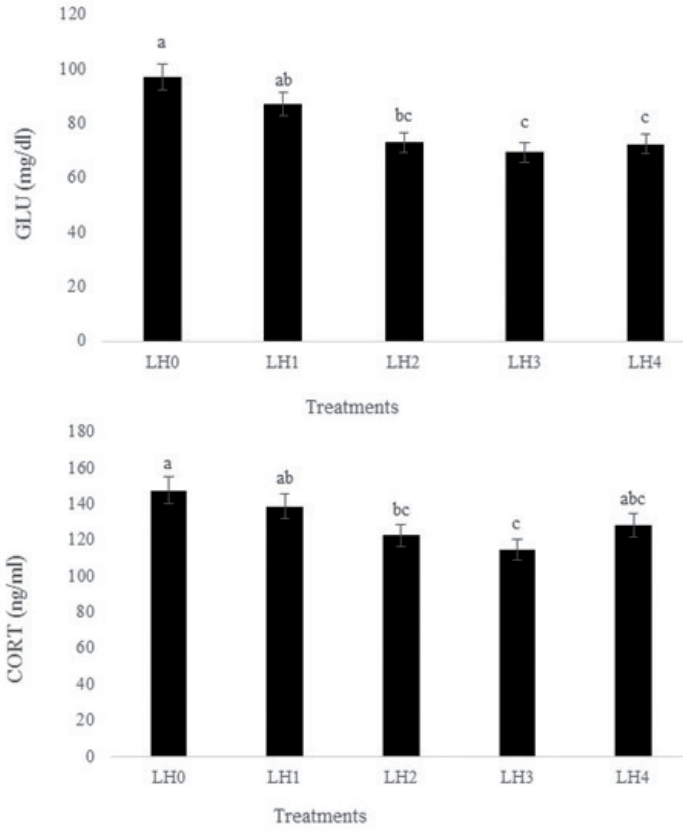

Figure 2. Cortisol and glucose levels of rainbow trout (*Oncorhynchus mykiss*) fed different levels of dietary *Lactobacillus helveticus*. LH0 (0, control); LH1 (1×10^6); LH2 (1×10^7); LH3 (1×10^8); LH4 (1×10^9). CORT: Cortisol; GLU: Glucose

Discussion

With the constant growth of the global population, consumer demand for foods of both plant and animal origins has increased in recent years (Markowiak and Śliżewska, 2018). Immunostimulants such as probiotics, prebiotics, and synbiotics are useful alternatives to chemical agents in a number of cultured species (Akhter et al., 2015). Probiotics can stimulate specific and nonspecific immunity, enhance health and well-being and improve resistance to viral and bacterial pathogens (Ganguly et al., 2010, Martínez Cruz et al., 2012). Among previously studied probiotics, LAB has received greater attention as an additive of fish feed because of their occurrence as indigenous components of fish gut microbiota in many species, and for their beneficial capacities (Dimitroglou et al., 2011; Lee et al., 2016). No prior data are available about the effects of *L. helveticus* supplementation on rainbow trout immunity in intensive culture. Our results revealed that this additive could influence the growth performance, serum immune and biochemical factors, intestinal enzymes and microbiota, and antioxidant capacity, as well as promote the innate immune responses and improve stress resistance of rainbow trout in crowded conditions.

The present results revealed that dietary supplementation of probiotic *L. helveticus* enhanced growth indices like FW, WG, PER, and SGR in rainbow trout. Fish fed supplemented diets also had lower FCR levels. These results may have been because LAB increased fish growth performance and feed efficiency due to the improvement of the microbial flora balance and colonization on the intestinal epithelial cells (Carr et al., 2002). Through these means, LAB can stimulate fish appetite, digestive enzyme secretion (as shown in the Table 3), and nutrient digestibility (Anuradha et al., 2005; Bernardeau et al., 2006). Rainbow trout microbial profiles in this study also indicated that *L. helveticus* supplementation influences LAB populations (Figure 2). Similar improvements in growth performance have also been observed in Nile tilapia (*Oreochromis niloticus*) fed *Lactobacillus plantarum* (Abou-El-Atta et al., 2019), European lobster (*Homarus gammarus* L.) fed *Bacillus* spp. (Daniels et al., 2010), Caspian roach (*Rutilus rutilus*) fed PrimaLac (Imanpoor and Roohi, 2015), common carp (*Cyprinus carpio*) fed *Lactobacillus fermentum* (Krishnaveni et al., 2021), and pond loach (*Misgurnus anguillicaudatus*) fed *L. helveticus* (Yang et al., 2021).

L. helveticus supplementation in the present study generally resulted in the elevation of intestinal amylase, lipase, and protease levels. Increased digestive enzyme activity might be due to the secretion of digestive enzymes by intestinal microbiota due to intestinal morphology improvements which in turn can improve appetite, digestibility and growth (Moriarty, 1996, 1998; Dobrianska et al., 2021). The higher level of enzyme activity obtained with probiotics suggests enhanced digestion of proteins, starches, fats, and cellulose (Lara-Flores et al., 2003). Similar effects have been reported, induced by Gram-positive bacteria, particularly members of the genus *Lactobacillus* that secrete a wide range of exo-enzymes (Moriarty, 1998; Suzer et al., 2008). Examples include *Penaeus vannamei* fed *Bacillus* (Wang, 2007) and PrimaLac (Miandare et al., 2016), as well as Pengze crucian carp (*Carassius auratus*) fed *Bacillus cereus* (Yang et al., 2019). Furthermore, *L. helveticus* HML037 diets improved intestinal digestive capacity by elevating intestinal protease, amylase, and lipase in pond loach culture (Yang et al., 2021).

The innate immune system in aquatic animals has been considered an essential component of resistance against opportunistic pathogens (Magnadóttir, 2006). Probiotics affect some hydrolytic enzymes such as LYZ that have bacteriostatic properties when present in serum, mucus, and the lymphoid system (Magnadottir et al., 2005; Miandare et al., 2016; Torki et al., 2018). In the present work, dietary LH2 and LH4 significantly increased the serum LYZ level. Previous studies have reported that shrimps fed with multi-strain probiotics showed an increased level of LYZ gene expression (Miandare et al., 2016). Abarike et al. (2018) pointed out that diets supplemented with *Bacillus subtilis* and *Bacillus licheniformis* led to LYZ enhancement in Nile tilapia. Moreover, Yang et al. (2021) reported that *L. helveticus* metabolites can regulate the immune system response by affecting the secretion of immune related enzymes like LYZ in Nile tilapia.

The current work revealed that the LH3 diet used in this study remarkably increased serum ACH_{50} and MPO. ACH_{50} defends the fish from infection, and influences phagocytic activity and inflammation (Zhou et al., 2001). Different works have reported an increase in serum ACH_{50} in common carp fed pre-, pro-, and synbiotic supplemented diets (Modanloo et al., 2017). Additionally, dietary *Lactobacillus paracasei* significantly increased ACH_{50} in Nile tilapia (Van Doan et al., 2021). Moreover, MPO enhances neutrophils and macrophages in fish blood and utilizes oxidative radicals (Holmblad and Söderhäll, 1999; Güllü et al., 2016). In this regard, previous studies demonstrated an increase in serum MPO in Nile tilapia after feeding with *Bacillus subtilis* and *Bacillus licheniformis* (Abarike et al., 2018).

Total Ig, as a glycoprotein, defends fish against pathogens (Ghafarifarsani et al., 2021 b). Our results indicate that the total Ig was independent of dietary *L. helveticus*. In contrast, previous studies revealed that the serum Ig level was found to increase after probiotic mixture supplementation in *Labeo rohita* (Mohapatra et al., 2014). Also, *Bacillus licheniformis* significantly affected the total Ig level in triangular bream (*Megalobrama terminalis*) (Zhang et al., 2013).

RBA participates in the degradation of internalized bacteria or foreign agents during phagocytosis (Cerezuela et al., 2012). In the present study, a significant increase in RBA activity of rainbow trout fed high doses of the experimental diet were observed. These results suggest

that *L. helveticus* can enhance fish immunity and disease resistance under high-density conditions. RBA enhancement may be due to the increase in macrophages and granulocytes numbers (Dawood et al., 2017). Following these results, in a study by Hoseinifar et al. (2015 a), all the supplemented diets under evaluation (e.g. pro-, pre-, and synbiotic diets) significantly increased the RBA level of rainbow trout. A similar response was reported in white shrimp, *Litopenaeus vannamei* fed *Lactobacillus plantarum* (Chiu et al., 2007).

Biochemical parameters are considered a tool to evaluate general health conditions (Dawood et al., 2015). TP is associated with the influence of serum proteins as an indicator for the enhanced immune system of fish (Magnadottir, 2010). The results of biochemical analyses in the present investigation revealed that the values of TP, ALB, and GLO were significantly increased in fish fed *L. helveticus,* suggesting immunomodulatory effects. This effect may be associated with the health of the liver, the main source of protein syntheses (Adineh et al., 2021). The findings are in accordance with previous studies' conclusions that TP, ALB, and GLO significantly increase with probiotic supplementation. For example, Nile tilapia fed *Lactobacillus plantarum* (Abou-El-Atta et al., 2019) and *Aspergillus oryzae* (Dawood et al., 2020). Additionally, LDH may be influenced by metabolic demands as a result of starvation or nutritional manipulation (Dawood et al., 2020). In this study, fish fed with *L. helveticus* demonstrated a significantly decreased LDH level. Similarly, rainbow trout fed dietary PrimaLac showed a significant decrease in LDH level (Naderi Farsani et al., 2020).

Blood GLU concentration is an indicator used to assess stress (Yin et al., 1995). Nutritional treatments could influence GLU levels (Yang and Chen, 2003). In this trial, the values of serum GLU content in fish fed *L. helveticus* were significantly lower in long-term rearing at high density. This may have been due to the capability of probiotics to enhance glycaemic control, GLU tolerance, and insulin secretion, all of which reduce the effects of stressors and elevate immune status (Yun et al., 2009). According to Mohapatra et al. (2014) *Labeo rohita* fed with a probiotic supplemented diet (*Bacillus subtilis*, *Lactococcus lactis,* and *Saccharomyces cerevisiae*) had lower blood GLU levels in comparison to the non-supplemented group. In addition, Dawood et al. (2020) reported that *Aspergillus oryzae* as a probiotic additive reduced GLU levels in Nile tilapia. Despite that, GLU was positively associated with the concentration of *Bacillus cereus* in Pengze crucian carp (Yang et al., 2019).

Through glucogenesis in the liver, the neuro-endocrine system releases catecholamine and CORT that increase available energy to cope with adverse internal and external conditions (Urbinati and Carneiro, 2001; Khosravi-Katuli et al., 2021). In this study, supplementing the diet at all probiotic levels, except LH2, led to a significant decrease in CORT level. CORT elevation could be linked either to hypoglycemic hormone (insulin) stimulation or a reduction in GLU absorption (El Basuini et al., 2021). Additionally the hypothalamus–pituitary–interrenal axis controls the secretion of CORT and may activate to mobilize energy during feed deprivation (McCormick et al., 1998). In line with our results, plasma CORT of red sea bream (*Pagrus major*) fed *Lactobacillus rhamnosus* diets were significantly lower than those fed control diets after exposure to a stress test (Dawood et al., 2017). In addition, lower plasma CORT levels were noticed in whiteleg shrimp (*Litopenaeus vannamei*) postlarvae fed 1×10^4 and 1×10^8 CFU/g probiotic *Bacillus subtilis* (Sadat Hoseini Madani et al., 2018). This trend indicated higher resistance against common stress conditions and mitigation of negative effects of crowding density. While probiotic supplementation increased CORT levels in Nile tilapia, CORT activity showed no significant differences in rainbow trout fed PrimaLac diets (Iwashita et al., 2015).

Hematological parameters are valuable indicators employed to monitor fish health, immunological functions, stress, and disease conditions (Adel et al., 2017). In the current study, administration of *L. helveticus* increased WBC, RBC, Hct, lymphocyte, and neutrophil levels but had no effect on monocyte, eosinophil, and Hb in rainbow trout. WBC counts (comprising neutrophils, eosinophils, lymphocytes, and monocytes) produce antibodies and perform macrophage activities, phagocytosis, inflammation, and antibody production (Jalali et al., 2009; Deng and Huttenlocher, 2012; Devi et al., 2019). WBC increases after exposure to stressors (Talpur and Ikhwanuddin, 2012). Hct is the volumetric percentage of red cells in blood circulation and depends on their number and size (Ghafarifarsani et al., 2021 b). An increase in RBC, Hct, and Hb facilitates tissue oxygenation and elimination of carbon dioxide and toxic metabolites (Abdel-Tawwab et al., 2006). *L. helveticus* may protect the RBC against hemolysis by free radicals and oxidative injury through immune-stimulatory and health properties. This statement is supported by higher activities of antioxidant enzymes and reduced peroxidation of lipids in rainbow trout. Studies show the incorporation of probiotics stimulates hematopoiesis. Erythrocyte and leukocyte counts increased in Indian magur (*Clarius batrachus* L.) fed a diet supplemented with the probiotic *Lactobacillus sporogens* (Dahiya et al., 2012). The RBC content of probiotic supplemented *Labeo rohita* was found to be higher than its non-supplemented counterparts (Mohapatra et al., 2014). Also, the results are in line with Devi et al. (2019) who showed increases in *Labeo rohita* blood WBC counts following dietary administration of probiotic *Bacillus subtilis* in healthy and infected fish after the 6th week. On the contrary, supplementation with probiotics had no reported effect on Hb, MCV, and MCHC and the total leukocyte count of Nile tilapia (Iwashita et al., 2015). Hematological indices of fish can be attributed to differences in the properties of supplements, doses, and fish species.

The antioxidant defense system protects fish against oxidative damages caused by ROS (Hoseinifar et al., 2020). MDA level is a measure of oxidative stress occur-

rence (Yousefi et al., 2022). SOD is a cytosolic enzyme that scavenges superoxide radicals and involves tissue injury (Syed Raffic Ali et al., 2018). H_2O_2 can be eliminated by CAT, active both in mitochondria and peroxisomes (Hoseinifar et al., 2020). In the present investigation, the antioxidant levels (MDA, SOD, and CAT) significantly increased in the LH3 supplemented fish compared to the control group. However, GPx did not exhibit any influence by treatment. Following these results, in the pond loach *L. helveticus* significantly improved both activity and mRNA expression levels of SOD and CAT, while the MDA levels decreased. *L. helveticus* and its metabolites can regulate the immune system response by affecting the secretion of immune related enzymes and the expression of related genes, improving the disease resistance (Yang et al., 2021). In a different study, the intestine samples of goldfish (*Carassius auratus*) fed *L. helveticus* diets showed increased DPPH (*α*,*α*-diphenyl-β-picrylhydrazyl) radical scavenging activity compared to control fish, which suggests this probiotic increases intestinal antioxidants (Ahire et al., 2019). Furthermore, Dawood et al. (2020) demonstrated that probiotics resulted in protective effects against oxidative stress by increasing SOD and GPx activities.

Probiotics positively affect the intestinal microbiota, protecting the intestinal mucosal cells, and secreting antibacterial substances, competing with pathogens to prevent their adhesion to the intestine of the host (Martínez Cruz et al., 2012). LAB are generally considered as a health promoting bacterial group, commonly found in the gastrointestinal tract of fish (Hoseinifar et al., 2015 b). The results of the present study revealed a remarkable enhancement of LAB number in LH3 and LH4 treatments. It seems that *L. helveticus* had positive effects on the numbers of LAB to prevent the colonization of pathogens and compete for nutrients and receptors on the gut wall (Akrami et al., 2009). Meanwhile, *L. helveticus* had no effect on TBC. Likewise, *L. helveticus* CD6 (10^7 cells per day) showed successful colonization into the intestinal track of goldfish (Ahire et al., 2019). The elevation of LAB and TBC levels has also been reported in angelfish (*Pterophyllum scalare*) fed with synbiotic (*Pediococcus acidilactici* and fructooligosaccharide) enriched adult Artemia. Caspian white fish (*Rutilus fresii kutum*) fed with PrimaLac exhibited significantly increased total autochthonous intestinal microbiota and autochthonous LAB levels (Mirghaed et al., 2018 b).

Conclusion

The present study explored dietary supplementation of *L. helveticus* as a dietary probiotic strain leading to improve growth performance, enhanced hematological and biochemical parameters, and stimulated immune system of rainbow trout. Based on our result *L. helveticus* as a feed supplement is recommended when fish are exposed to high-density intensive culture. However, further investigations are required to fully confirm the results obtained here.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All experiments were performed following the protocol approved by the committee of ethics of the Baharavaran Nastaran Agricultural Applied Scientific Training Center, Applied Scientific University, Qom, Iran (1053; 2021).

Authors' contributions

All the authors of this article have made important contributions to testing, collecting data, analyzing results, and writing the article.

Conflict of interest

The authors have no conflict of interest.

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