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IMMUNOLOGICAL, ANTIOXIDANT, GROWTH RESPONSES, AND DISEASE RESISTANCE OF RAINBOW TROUT, *ONCORHYNCHUS MYKISS*, WITH FEEDING DIETS SUPPLEMENTED WITH *LACTOBACILLUS SALIVARIUS* AND LUTEIN

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Abstract

The aim of the present study was to assess the effects of dietary supplementation with *Lactobacillus salivarius* (LS) ATCC 11741 and lutein (LU) on immunological, antioxidant, and growth responses, and resistance against *Yersinia ruckeri* infection in rainbow trout (*Oncorhynchus mykiss*). There were seven experimental diets containing un-supplemented diet (CTL), *L. salivarius* at 1 × 10^6 (LS6) and 1 × 10^8 (LS8) CFU/g, lutein at 50 (LU50) and 150 (LU150) mg/kg, *L. salivarius* at 1 × 10^6 CFU/g plus lutein at 50 g/kg (LS6+LU50), and *L. salivarius* at 1 × 10^8 CFU/g plus lutein at 150 g/kg (LS8+LU150). These diets were provided to the fish (N= 735; 14.0±0.38 g). After 60 days of feeding, all experimental treatments exhibited significantly higher growth performance, compared to the CTL treatment. The intestinal protease activity and the intestinal lactic acid bacteria population in the probiotic-treated fish were significantly higher than in the CTL fish. The intestinal lipase activity was only higher in the LS6+LU50 treatment, compared to the CTL treatment. Compared to the CTL treatment, the intestinal total bacterial count, and serum superoxide dismutase significantly increased in the LS6+LU50 and LS8+LU150 treatments. The serum catalase significantly increased in LU150, LS6+LU50, and LS8+LU150 treatments, and serum glutathione peroxidase significantly increased in the lutein-treated fish. All experimental treatments, particularly LS6+LU50 and LS8+LU150, had significantly lower serum malondialdehyde levels, compared to the CTL treatment. The levels of serum alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and lactate dehydrogenase in LU50, LS6+LU50, and LS8+LU150 treatments were significantly lower than the CTL. All experimental treatments showed significantly higher serum lysozyme (LYZ), complement, blood respiratory burst activity (RB), and skin mucus alkaline phosphatase (ALP), compared to the CTL treatment. The highest serum LYZ, C4, total immunoglobulin (Ig), blood RB activity, and skin mucus peroxidase, ALP, and Ig were observed in the LS6+LU50 and LS8+LU150 treatments. All the experimental treatments, especially LS6+LU50, exhibited significantly lower mortality after the bacterial challenge compared to the CTL. In conclusion, dietary supplementation with 1 × 10^6 CFU/g *L. salivarius* and 50 mg/kg lutein can maximally improve growth performance, digestive enzymes, antioxidant parameters, immune responses, intestinal lactic acid bacteria, and resistance against yersiniosis in rainbow trout.

Key words: yersiniosis, carotenoid, probiotic, disease, blood, fish

With the rapid growth of the aquaculture sector, the risk of disease outbreaks has increased, with nearly 6 billion USD in damages per annum (Stentiford et al., 2017). Opportunistic bacteria cause major diseases in aquaculture and a wide range of antibiotics has been used to control them (Lenchenko et al., 2022; Ferri et al., 2022). *Yersinia ruckeri* is one of the opportunistic bacteria, causing enteric red mouth disease in rainbow trout, *Oncorhynchus mykiss* (Pajdak-Czaus et al., 2019). This is a systematic disease, causing high mortality in trout farms (Rashidian et al., 2020). Similar to other bacterial diseases, antibiotics are used to cure *Y. ruckeri* infection in trout farms. However, antibiotic administration has been limited due to environmental concerns, the rise of resistant strains, and negative impacts on the host (Zargar et al., 2020; Yousefi et al., 2023). Besides, due to the less-developed adaptive immunity in fish compared to the higher vertebrate, vaccination has been less efficient in aquaculture (Robinson et al., 2023). Therefore, the main focus of the aquaculture industry has been on disease prevention. Besides management practices, improving innate immunity is an efficient way to prevent pathogen localization in the fish body (Taheri Mirghaed et al., 2018).

Dietary supplementation is a reliable approach to increase fish innate immunity (Lee et al., 2015; Rahman et al., 2022 c). Alongside immunostimulation, feed supplements may have other benefits in hosts, including growth

promotion, antioxidant effects, and improved nutrient digestibility (Mahboub et al., 2022). Probiotics are one of the feed supplements that improve intestinal health and development (Hoseinifar et al., 2018). Probiotics have antagonistic effects on harmful microbes in the intestine, by excreting antimicrobial compounds, decreasing the intestine pH, and competition for adhering space (Hoseinifar et al., 2018). Lactic acid bacteria (LAB) are well-known probiotics in aquaculture that have exhibited growth-promoting, immunostimulant, and antioxidant effects, increased disease resistance, and digestive enzymes' activity (Ringø and Gatesoupe, 1998). Certain LAB have been extensively studied in aquatic organisms and have been beneficial regarding growth rate, antioxidant, immuno-stimulation, and disease resistance (El-Saadony et al., 2021; Naiel et al., 2021; Chong et al., 2019; Pereira et al., 2022). However, there are certain LAB that have been less studied in fish. Numerous studies have demonstrated that *Lactobacillus salivarius* ATCC 11741, a type of beneficial bacteria, possesses probiotic properties. These properties include the production of bacteriocins, regulation of intestinal microbial flora, stimulation of immune responses, and generation of short-chain fatty acids. As such, it is considered a promising probiotic strain (Chen et al., 2013; Chaves et al., 2017; Wasfi et al., 2018). *L. salivarius* is one of the less-studied LAB in aquatic organisms, but seems to have great probiotic potentials. This species has been obtained from the breast milk and the cecum of animals (Chaves et al., 2017; Chen et al., 2022) and has shown great probiotic potentials in non-aquatic species, as it reduces dietary antinutrients (Aljuobori et al., 2014), produces phytase, and stimulates the immune system (Lee et al., 2013). In aquatic animals, *in vitro* assays have shown this species has antagonistic effects on a fish pathogen, *Edwardsiella tarda*, whereas *in vivo* examination has revealed that feeding Nile tilapia, *Oreochromis niloticus*, with a *L. salivarius*-supplemented diet for 14 d significantly decreases mortality caused by *E. tarda* infection. The treated fish have shown higher serum bactericidal activity against the pathogen and higher macrophage immune functions (Pimentel and Katagiri, 2008). *Artemia* nauplii encapsulation with a combination of *L. salivarius* and *Lactobacillus reuteri* have significantly improved growth and survival, but decreased *Flavobacterium* sp. load and body deformity in pike-perch, *Sander lucioperca*, larvae (Ljubobratovic et al., 2017). However, there is a need to find the effects of *L. salivarius* on other fish species.

Another group of feed additives is carotenoids; pigments with variety roles such as coloration, anti-stress effects, and stimulation of the antioxidant and immune systems (Pereira da Costa and Campos Miranda-Filho, 2020). Lutein is one of the xanthophylls which is a group of carotenoid compounds and found in various plants such as marigold, alfalfa, and yellow clove. Several studies have shown that lutein is a coloring agent, but not a growth-promoter in different yellow croaker, *Larimichthys croceus* (Yi et al., 2016), Lake Kurumoi rainbowfish, *Melanotaenia parva* (Meilisza et al., 2017), fancy carp, *Cyprinus carpio* (Yuangsoi et al., 2011), and channel catfish, *Ictalurus punctatus* (Li et al., 2007). However, dietary lutein supplementation has been found to augment survival in goldfish, *Carassius auratus* (Besen et al., 2019), increase growth rate, survival, antioxidant and immune responses, improve digestive enzymes' activity, and decrease intestinal total bacterial count in oriental river prawn, *Macrobrachium nipponense* (Ettefaghdoost and Haghighi, 2021), and increase growth performance and antioxidant parameters in whiteleg shrimp, *Litopenaeus vannamei* (Fang et al., 2021). Moreover, dietary supplementation with lutein-rich plants, such as marigold and *Barranca yajiagengensis*, has been found to increase resistance to a viral disease in rainbow trout, *Oncorhynchus mykiss* (Amar et al., 2012), and improve antioxidant capacity, immune responses, and hepatic and intestinal health in pompano, *Trachinotus ovatus* (Zhao et al., 2022). Previous findings showed that the combination of probiotics and herbal metabolites due to possible synergistic properties between them can exert extensive benefits on the host. For example, Asadi et al. (2023) reported that the mixture of procyanidin and commercial probiotic Bio-Aqua® provided better effects on growth performance, and immune responses in rainbow trout compared to their single consumption. Similar findings were also reported by Naderi Farsani et al. (2021) in rainbow trout fed with resveratrol and two-strain probiotics (*Lactobacillus acidophilus* and *Bifidobacterium bifidum*). Probiotics in the gut can improve the absorption of various plant metabolites and enhance the properties of this nutrient after absorption (Pegah et al., 2021). On the other hand, it has been reported that these bioactive compounds can play a useful role in the adhesion of bacteria to the intestinal epithelium cells through biosynthesis or secretion of multifunctional proteins (moonlighting proteins; e.g., glycolytic enzyme pyruvate kinase) (Celebioglu et al., 2018).

Due to the different properties of *L. salivarius* and lutein as well as the possible synergistic effects between them, the addition of these two biostimulants to the diet can exert a wide range of biological effects on the rainbow trout. Therefore, this study aimed to assess the single and combined effects of *L. salivarius* and lutein on growth performance, digestive enzymes' activity, intestinal microflora, antioxidant, immune, and serum biochemical responses, and resistance against yersiniosis in rainbow trout.

Material and methods

L. salivarius and lutein sources

L. salivarius ATCC 11741 isolated from human saliva was purchased from the Iranian Biological Resource Center (Tehran, Iran) in lyophilized form. The bacterium was cultured on Man, Rogosa and Sharpe (MRS) medium at 37°C for 24 h. Then, the culture was centrifuged for

30 min at 4500 × *g*. The supernatant was discarded, and the bacterial pellet was washed three times with phosphate buffer and re-suspended to have densities of 1×10^8 and 1×10^{10} CFU/ml. Lutein (purity >97%) was purchased from Sigma-Aldrich Co. (MO, USA).

Preparation of diets

There were a total of seven experimental diets used in the study, which included a control group (lacking *L. salivarius* and lutein), *L. salivarius* at concentrations of 1×10^6 (LS6) and 1×10^8 (LS8) CFU/g, lutein at concentrations of 50 (LU50) and 150 (LU150) mg/kg, *L. salivarius* at a concentration of 1×10^6 cfu/g along with lutein at 50 mg/kg (LS6+LU50), and *L. salivarius* at a concentration of 1×10^8 cfu/g along with lutein at 150 mg/kg (LS8+LU150). Lutein in treated diets was replaced by wheat meal (Taheri Mirghaed et al., 2018). The concentrations of lutein were chosen based on the study of Ettefaghdoost and Haghighi (2021). The *L. salivarius* levels used in this work were selected based on the positive effects of other LAB on the growth and immunity responses of rainbow trout and other commercial species (Nikoskelainen et al., 2003; Topic Popovic et al., 2017; Mohammadian et al., 2020; Ali et al., 2022; Omidi et al., 2022). A diet without supplementation of *L. salivarius* and lutein served as the control diet (CTL). To make the diets (Table 1), feedstuffs were mixed thoroughly and added with 300 mL/kg of water to form a paste. The paste was pelleted using a meat grinder. The produced feeds were maintained at room temperature for 24 h. Then the pellets were kept in a refrigerator until use, and the viability of *L. salivarius* was confirmed using MRS culture medium (Mohammadian et al., 2019).

Table 1. Feedstuffs and compositions of the basal diet (Yousefi et al., 2022)

Ingredients	g/kg	Proximate composition	g/kg as dry matter basis
Fish meal[a]	310	Crude protein	425
Soybean meal[b]	200	Crude lipid	163
Wheat meal (lutein)	166	Crude ash	79.5
Poultry by-product[c]	130	Moisture	88.2
Wheat gluten[d]	100		
Phytase[e]	8		
Fish oil	40		
Soybean oil	30		
Lysine[f]	7		
Methionine[f]	4		
Mineral mix[g]	2.5		
Vitamin mix[g]	2.5		
Total	1000		

[a]Peygir Co., Gorgan, Iran (crude protein 58.8%).
[b]Soyabean Co., Gorgan, Iran (crude protein 45.2%).
[c]Peygir Co., Gorgan, Iran (crude protein 51.0%).[d]Shahdineh Aran Co (crude protein 78.3%).
[e]Golbid Co., Tehran, Iran. [f]Mad Tiour Co., Sanandaj, Iran.
[g]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: 20mg; B_1: 20 mg; H: 1 mg; B9: 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: 60mg; Se: 0.3 mg; I: 1.5 mg; Mn: 10 mg). Chinechin Co., Tehran, Iran.

Experimental protocol

Rainbow trout juveniles were purchased from a local farm and transported to the laboratory. The fish were acclimatized to the laboratory conditions and the CTL diet over 2 weeks; after that, 735 fish were randomly distributed into 21 tanks. Each tank was stocked with 35 fish (14.0±0.38 g) and was fed with each of the seven diets mentioned above. Each tank was fed a specific diet three times a day until based on *ad libitum*, for a total of 60 days. To determine the amount of uneaten food, the method used was based on Gu et al. (2017). The uneaten pellets were collected from the bottom of each tank approximately one hour after each feeding, dried at 70°C, and subtracted from the original amount of food provided. The water temperature, dissolved oxygen, pH, and unionized ammonia were 15.1±0.20°C, 8.10±0.18 mg/L, 7.20±0.30, and 0.02±0.005 mg/L, respectively. At the end of the rearing period, growth performance was calculated according to the following formulas:

Specific growth rate (SGR, %/d) = [(Ln final weight – Ln initial weight) / day] × 100
Feed conversion ratio (FCR) = feed consumed (g) / [final weight (g) – initial weight (g)]
Survival rate (SR, %) = final number of fish / initial number of fish × 100

Blood sampling and analysis

At the end of the rearing period, three fish were blood-sampled per tank. The fish were anesthetized by clove oil (100 mg/L) and the blood samples were collected from the caudal blood vessels using 2-mL syringes. An aliquot of each blood sample was poured into a heparinized tube and used for determination of respiratory burst (RB) activity. The remaining blood samples were centrifuged at 3000 × *g* (10 min at 4°C). The sera were collected in new tubes and kept at –70°C until analysis.

Serum antioxidant parameters were measured using commercial kits supplied by ZellBio GmbH (Lonsee, Germany). Superoxide dismutase (SOD) activity was determined based on the reduction of the cytochrome C. Catalase (CAT) activity was determined based on the decomposition of hydrogen peroxide. Glutathione peroxidase (GPx) activity was determined based on the conversion of reduced glutathione to glutathione disulfide. Malondialdehyde (MDA) content was determined based on the reaction with thiobarbituric acid. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), complement protein (C3 and C4) were determined using commercial kits supplied by Pars Azmun Co. (Tehran, Iran).

Serum lysozyme (LYZ) activity was measured according to Ellis (1990), based on the lysis of *Micrococcus luteus* in phosphate buffer (pH 6.2) using the turbidity measurement protocol at 405 nm. Serum total immunoglobulin (Ig) level was measured after precipitation by 12% polyethylene glycol (Siwicki and Anderson, 1993).

Serum alternative complement (ACH50) activity was determined based on the hemolysis rate of sheep erythrocytes as a hemolytic agent in buffer solution (pH = 7.0) and reading OD at 412 nm (Yano, 1992). The blood respiratory burst (RB) activity was determined using fresh heparinized blood, based on the method suggested by Siwicki et al. (1996). For this, an equal volume of blood sample and 0.2 nitroblue tetrazolium (NBT) were kept at a room temperature for 30 min. In the next step, 50 µl of the mixture was transferred to glass tubes and blended with 1 ml of dimethylformamide. Finally, the supernatant was obtained by centrifuging at 2,000 g for 5 min, and OD recorded at 540 nm.

Skin mucus sampling and analysis

To collect the skin mucus samples, three fish were sampled from each tank. The fish were anesthetized in a clove oil (100 mg/L) bath, then placed in polyethylene bags, containing 10 mL physiological saline. After 3 min, the mucus samples were collected in plastic tubes, centrifuged (2500 × *g*, 10 min, 4°C), the supernatants were collected in new tubes, and kept at –70°C until analysis (Ross et al., 2000). Mucosal peroxidase activity was determined using hydrogen peroxide and tetramethylbenzidine (Van Doan et al., 2020). Mucus alkaline phosphatase activity was detected by a commercial kit (Pars Azmun Co., Tehran, Iran) and the protocol provided by the company. Mucosal LYZ, and Ig levels were determined according to the methods used for serum analysis (see above).

Intestinal sampling and analysis

To investigate the intestinal microbial communities, the fish body surface was disinfected with 70% ethanol. Then, the abdominal cavity was opened, the intestine was dissected, and homogenized in phosphate buffer. Total bacterial count (TBC) and lactic acid bacteria (LAB) count were determined by culturing 100 µL of the homogenates on Tryptic Soy Agar (TSA) and MRS media (Merck Co., Germany), respectively. The plates were incubated at 28°C over 24 h and the bacterial counts were reported as CFU/g (Merrifield et al., 2010).

The intestinal digestive enzymes' activity was measured after dissecting the intestinal samples from three fish per tank. The fish were euthanized by exposure to an overdose of clove oil and the posterior part of the intestine was dissected. The samples were emptied, washed by 50 mM Tris buffer, and homogenized by the same buffer. The homogenates were then centrifuged at 4°C (6000 *x*g; 10 min) and the supernatants were frozen at –80°C until analysis. The intestinal amylase activity was determined using starch as the substrate according to Bernfeld (1955). The intestinal lipase activity was measured using p-nitrophenyl myristate as the substrate (Iijima et al., 1998). The protease activity was determined based on the AZO-casein method (García-Carreño and Haard, 1993).

Challenge test

After the completion of the feeding trial (61st day), the fish were challenged by *Yersinia ruckeri* (KC291153; obtained from infected rainbow trout and maintained at the Faculty of Veterinary Science, University of Tehran, Iran). Ten fish from each tank (30 fish per treatment) were caught and anesthetized in clove oil (100 mg/L). Then, they were injected intraperitoneally by 0.1 ml of the bacterium suspension (1×10^7 cells ml^{-1}), lethal dose (LD_{50}) for this strain was previously determined by Darvishi et al. (2022). The fish mortality was recorded until 14 days post-infection. The relative percentage survival (RPS) of samples was computed based on the following equation:

$$RPS\ (\%) = (1 - \%\ of\ mortality\ in\ treated\ groups / \%\ of\ mortality\ in\ control\ group) \times 100.$$

Statistical analysis

Normality and homoscedasticity of the data were confirmed by Shapiro-Wilk and Levene tests, respectively. The data were then analyzed by one-way ANOVA and Tukey tests. Significant differences among the treatments were checked at $P<0.05$. All analyses were performed in SPSS v.22.

Results

Growth performance

All experimental diets significantly improved the growth performance of the fish, compared to the CTL diet ($P<0.05$). Different concentrations of dietary *L. salivarius* or lutein induced no significant changes in growth performance ($P<0.05$). FW in LS6+LU50 and LS8+LU100 were similar and significantly higher than those in the other experimental treatments ($P<0.05$). Lutein supplementation in the probiotic-containing diets induced no improvement in SGR and FCR. There was no significant difference in fish survival among the treatments (Table 2) ($P<0.05$).

Biochemical indices

Serum AST activities of the experimental group, except LS8, were significantly lower than that of the CTL group (Table 3) ($P<0.05$). Serum ALT activity significantly decreased in the fish fed with LU50, LS6+LU50, and LS8+LU150, compared to the fish fed the CTL diet (Table 3) ($P<0.05$). Lutein alone and in combination with the probiotic significantly decreased serum ALP activity ($P<0.05$). Combination of lutein and probiotic significantly decreased serum ALP activity, compared to the lutein alone (Table 3) ($P<0.05$). Compared to the CTL group, serum LDH activity significantly decreased in the fish fed diets supplemented with lutein alone and in combination with the probiotic (Table 3) ($P<0.05$).

Immunological parameters

Dietary supplementation with the probiotic and/or lutein significantly increased serum LYZ, ACH50, and

RB activities, compared to the CTL (Table 4) (P<0.05). The LS6+LU50 exhibited significantly higher LYZ activity, compared to LS8, LU50, and LU150 (P<0.05). All fish fed with probiotic-and/or lutein-supplemented diets showed similar ACH50 activity (P<0.05). Simultaneous supplementation of the probiotic and lutein significantly increased RB activity, compared to the probiotic and lutein alone (P<0.05). Dietary supplementation with the probiotic and/or lutein significantly increased serum Ig, C3, and C4, compared to the CTL (Table 4) (P<0.05). Moreover, LS6 exhibited significantly higher serum C3, compared to the CTL (P<0.05).

Skin mucus immune parameters

Simultaneous supplementation of the diets with the probiotic and lutein significantly increased the mucosal LYZ, peroxidase, and Ig, compared to the CTL (Table 5) (P<0.05). Moreover, the LS6 treatment exhibited significantly higher mucosal LYZ, compared to the CTL (P<0.05). All fish fed with probiotic- and/or lutein-supplemented diets showed similar mucosal ALP activities (P>0.05) and were significantly higher than that of the CTL (P<0.05). Simultaneous supplementation of the diets with the probiotic and lutein significantly increased mucosal ALP, compared to the probiotic and lutein alone (Table 5) (P<0.05).

Digestive enzymes

Dietary supplementation with the probiotic and probiotic+lutein significantly increased the intestinal protease activity, compared to the CTL (P<0.05). The intestinal amylase exhibited no significant differences among the treatments (P>0.05). The fish in LS6+LU50 exhibited significantly higher intestinal lipase activity, compared to the CTL (Figure 1) (P<0.05).

Table 2. Growth performance and survival of the fish fed diets supplemented with *L. salivarius* and/or lutein for 60 days

	Different experimental diets						
	CTL	LS6	LS8	LU50	LU150	LS6+LU50	LS8+LU150
IW (g)	14.10±0.20	13.70±0.15	13.83±0.38	14.16±0.28	14.23±0.12	13.86±0.1	14.10±0.25
FW (g)	65.50±1.04 e	76.13±0.98 b	75.00±0.57 bc	71.93±0.63 cd	70.83±0.72 d	82.66±1.01 a	80.83±0.60 a
SGR (%/d)	2.55±0.04 d	2.85±0.03 abc	2.81±0.05 abc	2.70±0.04 bc	2.67±0.03 c	2.97±0.02 a	2.91±0.04 ab
FCR	1.35±0.03 a	1.12±0.02 cd	1.14±0.02 bcd	1.21±0.02 bc	1.23±0.02 b	1.03±0.01 d	1.08±0.01 d
SR (%)	93.00±1.73	95.33±2.90	95.33±2.33	94.00±1.00	94.00±1.00	97.33±1.33	95.00±1.00

Initial weight (IW), final weight (FW), specific growth rate (SGR), food conversion ratio (FCR), survival rate (SR). Different letters in a row show significant differences among the treatments (n = 3; Tukey).

Table 3. Serum enzymatic activities of the fish fed diets supplemented with *L. salivarius* and/or lutein for 60 days

	Different experimental diets						
	CTL	LS6	LS8	LU50	LU150	LS6+LU50	LS8+LU150
AST (U/L)	154.33±2.60 a	125.16±2.89 bc	141.33±2.40 ab	115.33±2.33 c	116.66±4.05 c	109.00±4.16 c	111.33±4.48 c
ALT (U/L)	55.00±2.64 a	46.85±1.87 abc	49.16±1.74 ab	44.00±2.30 bcd	46.00±2.03 abcd	37.33±1.45 d	39.00±1.15 cd
ALP (U/L)	300.00±5.77 a	281.33±4.08 ab	283.00±3.21 ab	267.66±4.33 b	273.83±3.21 b	240.66±5.77 c	244.33±5.80 c
LDH (U/L)	760.00±5.77 a	743.00±7.50 abc	756.10±5.85 ab	721.66±4.40 cd	729.33±5.80 bcd	712.66±4.80 d	716.00±5.13 cd

Aspartate aminotransferase (AST), alanine aminotransaminase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH). Different letters in a row show significant differences among the treatments (n = 6; Tukey).

Table 4. Serum immunological characteristics of the fish fed diets supplemented with *L. salivarius* and/or lutein for 60 days

	Different experimental diets						
	CTL	LS6	LS8	LU50	LU150	LS6+LU50	LS8+LU150
LYZ (U/mL)	24.26±0.57 d	30.67±0.64 abc	29.76±0.70 bc	28.46±0.51 c	30.03±0.32 bc	33.13±0.59 a	32.36±0.75 ab
ACH50 (U/mL)	99.66±4.05 b	117.66±2.33 a	119.66±2.92 a	121.00±2.88 a	119.66±2.62 a	129.66±2.33 a	127.33±1.98 a
C3 (g/dL)	6.43±0.66 c	10.16±0.66 ab	9.28±0.63 bc	6.70±0.64 c	7.20±0.47 c	13.06±0.56 a	11.86±0.50 ab
C4 (g/dL)	13.65±0.39 c	15.26±0.37 bc	14.66±0.35 c	14.33±0.60 c	14.36±0.56 c	18.53±0.67 a	17.76±0.72 ab
RB (540 nm)	0.31±0.05 c	0.60±0.03 b	0.55±0.03 b	0.66±0.02 b	0.64±0.01 b	0.88±0.04 a	0.91±0.05 a
Ig (mg/mL)	17.40±0.66 c	20.03±0.57 bc	19.53±0.58 bc	18.53±0.50 c	18.36±0.46 c	23.33±0.88 a	22.56±0.80 ab

Lysozyme (LYZ), alternative complement (ACH50), complement protein (C3 and C4), respiratory burst (RB), total immunoglobulin (Ig). Different letters in a row show significant differences among the treatments (n = 6; Tukey).

Table 5. Mucosal immunological characteristics of the fish fed diets supplemented with *L. salivarius* and/or lutein for 60 days

	Different experimental diets						
	CTL	LS6	LS8	LU50	LU150	LS6+LU50	LS8+LU150
LYZ (U/mL)	13.44±0.63 c	16.48±0.44 ab	15.90±0.37 bc	15.36±0.76 bc	15.60±0.62 bc	19.25±0.60 a	18.06±0.63 ab
ALP (U/L)	18.91±0.75 d	24.36±1.02 c	25.90±0.51 c	27.10±0.48 bc	26.33±0.60 c	31.26±0.72 a	30.43±0.80 ab
Peroxidase (U/mL)	8.33±0.72 b	11.43±0.80 ab	10.93±0.80 ab	9.86±0.54 b	9.60±0.60 b	13.43±0.53 a	13.23±0.50 a
Ig (mg/mL)	9.30±0.68 b	11.83±0.72 ab	10.40±0.60 b	10.06±0.58 b	9.73±0.66 b	13.83±0.52 a	13.36±0.44 a

Lysozyme (LYZ), alkaline phosphatase (ALP), total immunoglobulin (Ig). Different letters in a row show significant differences among the treatments (n = 6; Tukey).

Table 6. Relative percentage survival (RPS) of rainbow trout fed diets supplemented with *L. salivarius* and/or lutein for 60 days and 14 days infected with *Yersinia ruckeri*

Different experimental diets	RPS (%)
CTL	
LS6	41.57
LS8	34.73
LU50	31.95
LU150	25.25
LS6+LU50	58.48
LS8+LU150	51.92

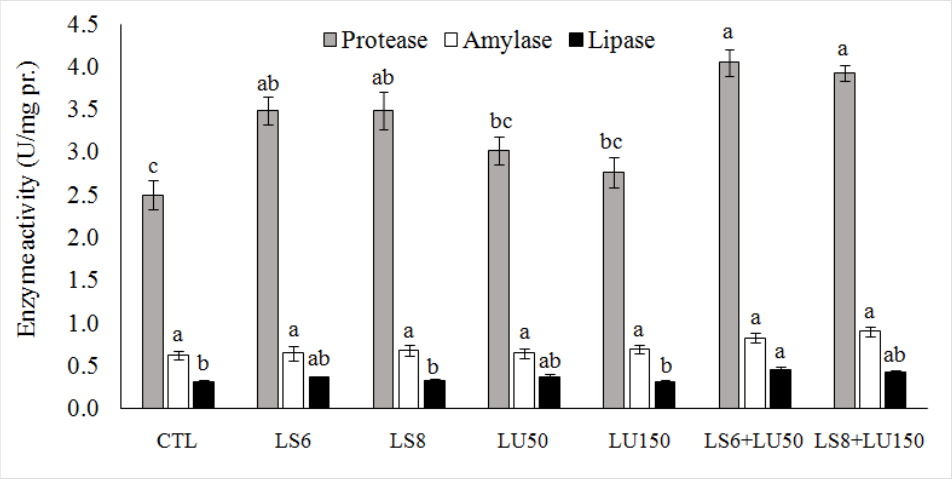


Figure 1. Intestinal digestive enzymes' activities of the fish fed diets supplemented with *L. salivarius* and/or lutein for 60 days. Different letters above the bars show significant differences among the treatments (n = 6; Tukey)

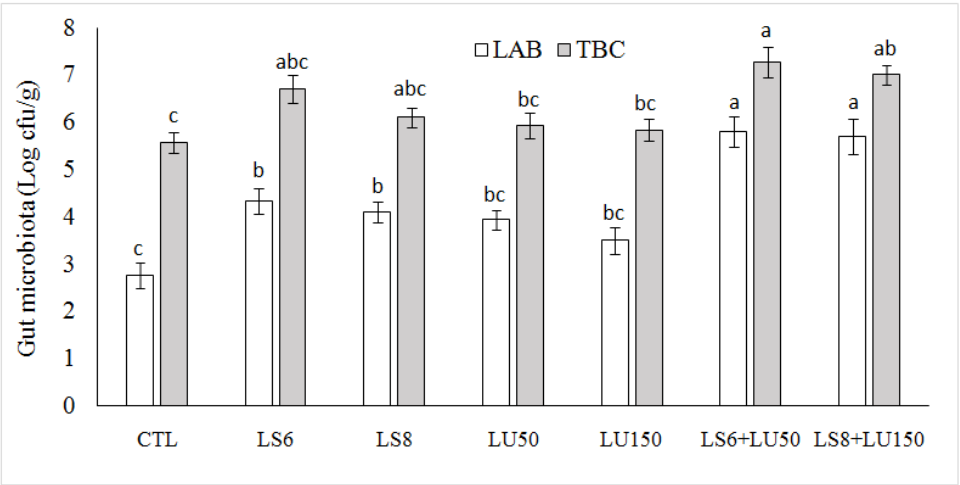


Figure 2. Intestinal total bacterial count (TBC) and lactic acid bacteria (LAB) of the fish fed diets supplemented with *L. salivarius* and/or lutein for 60 days. Different letters above the bars show significant differences among the treatments (n = 6; Tukey)

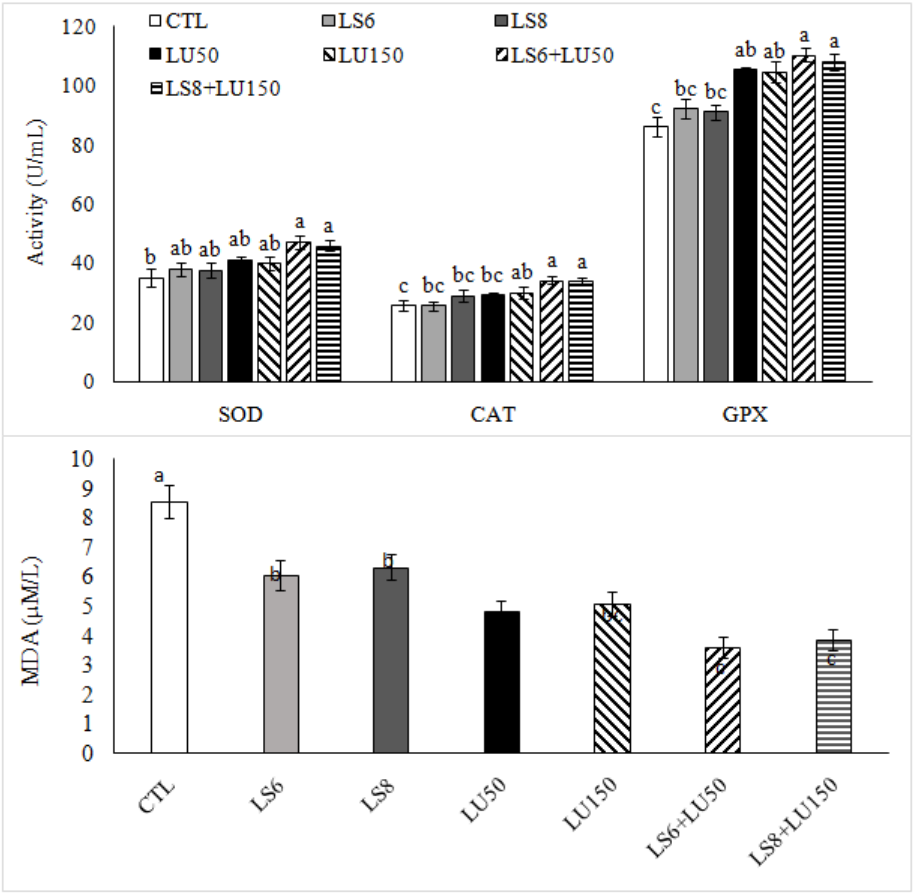


Figure 3. Serum antioxidant parameters of the fish fed diets supplemented with *L. salivarius* and/or lutein for 60 days. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), malondialdehyde (MDA). Different letters above the bars show significant differences among the treatments (n = 6; Tukey)

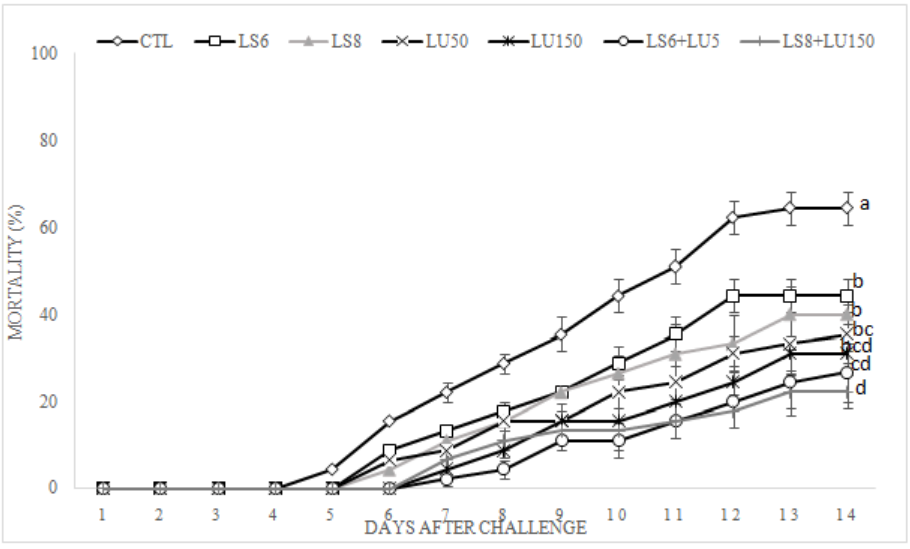


Figure 4. Mortality of the fish fed diets supplemented with *L. salivarius* and/or lutein and challenged by *Yersinia ruckeri*. Different letters show significant differences among the treatments (n = 6; Tukey

Intestinal microbiota

Simultaneous supplementation of the diets with the probiotic and lutein significantly increased TBC in the fish intestine, compared to the CTL ($P<0.05$). The fish fed the probiotic- and probiotic+lutein-supplemented diets exhibited significantly higher intestinal LAB, compared to the CTL (Figure 2) ($P<0.05$).

Antioxidant parameters

Simultaneous supplementation of the diets with the probiotic and lutein significantly increased the serum SOD activity, compared to the CTL. The LU150, LS6+LU50, and LS8+LU150 treatments exhibited significantly higher serum CAT activity, compared to the CTL ($P<0.05$). Dietary lutein and probiotic+lutein supplementation significantly increased the serum GPx, compared to the CTL ($P<0.05$). Dietary probiotic and/or lutein supplementation significantly decreased the serum MDA, compared to the CTL ($P<0.05$) and the lowest values were related to the LS6+LU50, and LS8+LU150 treatments (Figure 3).

Challenging test

Dietary probiotic and/or lutein supplementation significantly decreased fish mortality after the bacterial challenge, compared to the CTL and the lowest mortality was related to the LS6+LU50, which showed no significant differences, compared to the LS8+LU150 and LS6 treatments (Figure 4). In addition, the lowest and highest RPS% was obtained in LU50 (25.25%) and LS6+LU150 (58.48%), respectively.

Discussion

Intestinal microbiota, digestive enzymes, and growth performance

LAB are beneficial microorganisms found in the fish intestine, but they are not a dominant microbial group. Dietary supplementation is a reliable method to increase LAB populations in the fish intestine (Hoseini et al., 2022 b). The present results are in line with previous studies that found dietary LAB supplementation significantly increases the intestinal LAB populations in rainbow trout and other species (Hoseinifar et al., 2015, 2017). Besides, the intestinal TBC increased in the probiotic-treated fish in the present study, which is in line with previous studies using LAB- or probiotic-supplemented diets (Merrifield et al., 2010; Hoseinifar et al., 2015, 2017). An increase in the number and/or height of the intestinal villi has been reported in different fish species fed LAB-supplemented diets (Foysal et al., 2021; Yang et al., 2021), which may explain the increase in the intestinal TBC: an increase in the intestinal folding increases more surfaces for adherent microbes. This is a potential reason for increased growth performance of the fish fed the probiotic-supplemented diets in the present study, as the increase in the intestinal surface provides a wider space for nutrient absorption. On the other hand, *L. salivarius* has been found to decrease dietary antinutrients (Aljuobori et al., 2014) and produce phytase, which may contribute to higher nutrient utilization (Lee et al., 2013), but this topic needs evaluation in aquatic animals.

The present study demonstrated that dietary probiotic supplementation improves intestinal protease activity, which may be partly responsible for higher growth performance in these groups. Likewise, previous studies have found that dietary probiotics increase digestive enzyme activity and fish growth performance (Askarian et al., 2011; Akter et al., 2019). However, unlike other studies, the intestinal lipase and amylase did not follow a protease pattern (Ashouri et al., 2020; Kong et al., 2021). It is hard to explain the reasons of this inconsistency based on the parameters measured in this study, but the domination of protease-producing microorganisms in the probiotic-treated fish may contribute. However, this topic needs further evaluation.

Lutein supplementation improved growth performance and feed efficiency in the present study. Lutein effects on the growth performance of aquatic animals have been controversial. Lutein seems to stimulate growth performance in crustaceans by shortening the molting cycle, modulating carbohydrate and fat metabolism, and energy balance (Ettefaghdoost and Haghighi, 2021; Fang et al., 2021), but has no growth promoting effects in fish (Li et al., 2007; Yuangsoi et al., 2011; Yi et al., 2016; Meilisza et al., 2017; Besen et al., 2019). Conversely, the present study found growth promotion, without an increase in digestive enzymes and improving intestinal health, when rainbow trout were fed lutein-supplemented diets. The exact mechanisms behind these results are not known and need further evaluation.

Antioxidant parameters

Proper functioning of the antioxidant system guarantees maximum fish health (Hoseini et al., 2022 a; Rahman et al., 2022 b). Fish in aquaculture facilities are under constant threats of oxidative stress, due to dietary shortages and stressors (Hoseini et al., 2022 c; Rahman et al., 2022 a). Under these conditions, SOD, CAT, and GPx are in the first line of the antioxidant defense, which neutralize superoxide ions, hydrogen peroxide, and organic hydro-peroxides. If the antioxidant system works improperly, pro-oxidants attack biological molecules particularly fatty acids that lead lipid peroxidation (Fazelan et al., 2020). One of the features of probiotics is improving the antioxidant capacity of the host. In particular, LAB has been found to improve antioxidant capacity in different fish (Hoseinifar et al., 2021); however, no study has assessed the antioxidant responses of fish to dietary *L. salivarius* supplementation. The present results show, for the first time, that this bacterium can suppress lipid peroxidation without stimulating SOD, CAT, and GPx activities; however, the bacterium effects on other antioxidant enzymes/molecules should not be neglected. Carotenoids are famous antioxidant compounds (Pereira da Costa and Campos Miranda-Filho, 2020) and lutein is a well-known antioxidant agent, improving antioxidant capacity in crustaceans (Ettefaghdoost and Haghighi, 2021; Fang et al., 2021) and pompano (Zhao et al., 2022). Here again, lutein has stimulated GPx activity and decreased lipid peroxidation. A previous study suggests that lutein can modulate antioxidant signaling at the transcriptional level, which leads to higher antioxidant activity (Zhao et

al., 2022). The present results also show that *L. salivarius* and lutein can synergistically improve antioxidant capacity and mitigate lipid peroxidation. The antioxidant results may explain the changes in serum ALT, AST, ALP, and LDH activities. These enzymes are not functional in serum but are indicators of damage to different tissues (Hoseini et al., 2014; Ghelichpour et al., 2020). Higher antioxidant capacity and lower lipid peroxidation suggest higher cell integrity and health; thus, less ALT, AST, ALP, and LDH leakage into the circulation. Here again, the lowest activities of these enzymes were observed in the treatment with the highest antioxidant activity and lowest lipid peroxidation.

Immune responses and disease resistance

The innate immune system plays important role in early responses to pathogens and is crucial in disease prevention (Shoemaker et al., 2015). Improvement in the innate immune system has been found to decrease fish mortality against a subsequent bacterial challenge. LYS is an important bactericidal enzyme found in mucus and circulation, involved in bacterial disease combating (Song et al., 2021). Ig is produced by B cells and lymphoid tissues, responsible for the adaptive immune response by recognizing antigens; however, basal Ig has been found responsive to immunostimulant (Cuesta et al., 2004). Complement proteins are produced in the liver that kill foreign cells and increase macrophage phagocytosis efficiency by opsonization (Yousefi et al., 2016). RB activity is an indicator of neutrophil phagocytosis and reactive oxygen species release (Biller and Takahashi, 2018). Overall, the present results suggest dietary lutein and *L. salivarius* improved several humoral soluble and cellular immune parameters with different magnitudes. In septicemic diseases, humoral immune strength may be important in disease outbreak and mortality. Studies have shown that *Y. ruckeri* infection leads to activation of LYZ (Costa et al., 2011) and complement proteins (Raida and Buchmann, 2009), an increase in neutrophil percentage and functions (Anderson et al., 1992; Tobback et al., 2007), and RB (Tobback et al., 2007). Moreover, studies have shown that an increase in basal serum Ig levels by immuno-stimulants results in higher survival after *Y. ruckeri* infection (Adel et al., 2016; Naderi Farsani et al., 2021). The present results are partially in line with previous studies. *L. salivarius* has been found to increase the resistance of Nile tilapia against *E. tarda* infection, the property that seems to be associated to antagonistic effects against the pathogen, secreting bactericidal products, and stimulation of macrophages (Pimentel and Katagiri, 2008). Moreover, studies on mammalians have revealed that *L. salivarius* administration modulates cytokines' secretion, suppressing inflammation, and intestinal damage (Lee et al., 2013; Sun et al., 2020). Lutein has been found to stimulate immune responses in oriental river prawn (Ettefaghdoost and Haghighi, 2021), with unknown mechanisms and not under pathogenic conditions. On the other hand, no studies have assessed the effects of lutein on immune responses and disease resistance in fish. But, lutein-rich plants have been found to stimulate the immune system in Nile tilapia (Zhao et al., 2022) and to increase resistance against a viral disease in rainbow trout (Amar et al., 2012). It is necessary to conduct further studies, illustrating the underlying mechanisms by which lutein affects the immune system and disease resistance in fish. Based on the present study, it is speculated that the increase in fish survival after *Y. ruckeri* infection may be attributed to enhancement in humoral soluble and cellular immune components. As an antioxidant, lutein can interact with free radicals and protect cells from oxidative damage. This means that in the presence of lutein in the fish's body, the chances of cell damage due to oxidative stress can be reduced, which in turn can help the immune system remain efficient in fighting disease.

In the present study, the bacterial challenge was conducted by intraperitoneal injection that overlooks the role of skin mucosal immunity; however, *L. salivarius* and, with less extent, lutein improved the fish skin mucosal immunity. As mentioned above, LYS and Ig participate in bactericidal and antigen recognition in the skin mucus. Moreover, peroxidase has important role in the skin mucosal immunity as it participates in the formation of the peroxidase–H_2O_2–halide complex that is a very strong microbicidal and cytotoxic agent (Guardiola et al., 2014). The skin mucosal ALP has been found to change during the activation of the immune system. Although the exact roles of the skin mucosal ALP have not been yet understood, it is believed that the enzyme participates in the dephosphorylation and detoxification of pro-inflammatory microbial compounds (Lallès, 2019). The present results suggest that *L. salivarius* and, with less extent, lutein may improve rainbow trout resistance against yersiniosis in the field, where the main route of the bacterium infection and transmission is by direct contact (Costa et al., 2011). Similar to the present results, improvement in the skin mucosal immunity has been found in association with higher survival of rainbow trout challenged by *Y. ruckeri* (Adel et al., 2016, 2020; Naderi Farsani et al., 2021).

Conclusion

In conclusion, dietary *L. salivarius* and lutein can improve growth performance, antioxidant biomarkers, liver function, immune responses, and resistance against yersiniosis in rainbow trout. *L. salivarius* has higher effects on growth, immune responses, and disease resistance, and lutein is more potent as an antioxidant agent; however, the best results are obtained when the diet is supplemented with 1×10^6 cfu/g *L. salivarius* and 50 mg/kg lutein.

Ethics statement

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

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Data availability statements

The datasets generated during and/or analyed during the current study are available from the corresponding author on reasonable request.

Conflict of interest

The authors declare that they have no conflict of interest.

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