



EFFECT OF DIETARY SUPPLEMENTATION OF *LACTOBACILLUS SAKEI* SUBSP. *SAKEI* ON THE GROWTH PERFORMANCE, IMMUNE RESPONSES, AND ANTIOXIDANT DEFENSE OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) EXPOSED TO THE HERBICIDE BUTACHLOR*

Xu Wang^{1,2*}, Yi-Tong Han¹, Jia-Yi Zhang¹, Sudabe Ramezani³, Hamed Ghafarifarsani⁴

¹College of Agriculture, Liaodong University, Dandong 118003, Liaoning, China

²Institute of Advanced Characteristic Agriculture Studies, Liaodong University, Dandong 118003, Liaoning, China

³Department of Aquatic Animal Health, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

⁴Department of Animal Science, Chaharmahal and Bakhtiari Agricultural and Natural Resources Research and Education Center, Agricultural Research, Education and Extension Organization (AREEO), Shahrekord, Iran

*Corresponding authors: liaodonguwx@126.com

Abstract

The aim of this work was to determine the benefits of dietary supplementation with the probiotic *Lactobacillus sakei* subsp. *sakei* (LS) on the growth, intestinal microbiota, digestive enzymes, immunological responses, humoral antioxidant markers and liver enzymes of rainbow trout (*Oncorhynchus mykiss*) exposed to the herbicide butachlor (BU). Six experimental groups including C1: non-supplemented and non-exposed fish, LS1: 1×10^6 (cfu/g) *L. sakei*, LS2: 1×10^8 (cfu/g) *L. sakei*, BU: 12.5% LC_{50} butachlor, LS1 + BU: 1×10^6 (cfu/g) *L. sakei* + 12.5% LC_{50} butachlor, LS2 + BU: 1×10^8 (cfu/g) *L. sakei* + 12.5% LC_{50} butachlor were designed. After the 30-day experimental period, a significant increase was observed in the survival rate (SR), final weight (FW), and weight gain (WG) of the fish fed with LS ($P < 0.05$). The total intestine bacteria (TBC) were elevated in the groups supplemented with probiotic, while a significant decrease was observed in the fish exposed to butachlor ($P < 0.05$). Fish fed with probiotic showed a higher value of protease activity, while a significant decrease was observed in protease activity in the butachlor group ($P < 0.05$). The fish of probiotic group showed a significant increase in immunity parameters components including lysozyme and total Ig. Furthermore, an improvement was observed in hormonal antioxidant markers [superoxide dismutase (SOD) and catalase (CAT)] (and liver enzymes in the probiotic groups) ($P < 0.05$). Overall, results suggested a protective role for LS against the harmful effects of butachlor through restoring gut health, the digestive enzyme activities, immune and antioxidant responses in the rainbow trout.

Key words: probiotics, farmed fish, immune response, growth promoter, herbicide

The aquaculture industry has globally and dramatically developed in recent decades and reduced the increasing rate of fishing pressure on commercial fish species in nature and contributed to meet global needs for protein sources (Verdegem et al., 2023). However, diseases and toxic substances such as pesticides are a sustainability serious threat to aquaculture and affect all subsectors of aquaculture (DeWeerd, 2020). Probiotics are a group of bacteria that are used for various purposes such as increasing the level of immunity, growth, and resistance to diseases and environmental stressors in aquaculture (Pandiyan et al., 2013). However, their effects on the resistance of fishes against pesticides have been studied very little (Mohapatra et al., 2012; Kole et al., 2022).

Lactobacillus species are considered to improve the health and growth of the aquatic animals (Panigrahi et al., 2011). *L. sakei* is a famous facultative heterofermentative bacterium that has probiotic characteristics (Lin et al.,

2007; Mikelsaar and Zilmer, 2009; Ramos et al., 2013). Although researchers reported the positive potentials of *Lactobacillus* genus on immune responses, growth performance, intestinal morphology and disease confrontation of aquatic species such as *Oncorhynchus mykiss* (Dang et al., 2022), *Litopenaeus vannamei* (Minh, 2021) and *Salvelinus alpinus* (Knobloch et al., 2022), information about the effect of *Lactobacillus* genus especially the *Lactobacillus sakei* subsp. *sakei* on the rainbow trout after exposure to herbicides is limited and needs further investigation. Herbicides are commonly produced in different parts of the worlds and are widely used in different amounts, and therefore they have been reported to co-occur in environmental water samples collected from different watersheds (Gilliom, 2007; Faggiano et al., 2010).

Rainbow trout (*Oncorhynchus mykiss*) is the most abundant and widespread farmed fish in Iran and the world (Pauly and Zeller, 2017). The rainbow trout is an

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economically important cultured fish species and is considered a valuable animal protein source in Iran (Kalbasi et al., 2013). Due to the widespread use of herbicide compounds such as acetochlor, butachlor, metolachlor, alachlor, and pretilachlor in the modern intensive agriculture, they can be found in different environmental compartments, including aquatic ecosystems (Vonk and Kraak, 2020). Butachlor (N-(butoxymethyl)-2-chloro-N-(2,6-diethylphenyl) acetamide) is a widely used herbicide in the rice fields in Iran, in order to control grasses and weeds (Yaghoubi et al., 2020). This herbicide can cause disturbance of the endocrine system and prevents the synthesis of long chain fatty acids in animals (Kau-shik et al., 2006). Many researchers reported the adverse effects of butachlor on various life aspects of fish such as immune system, blood parameters and mucus immunity (Hedayati and Gerami, 2014; Anbumani and Mohankumar, 2015; Kumar et al., 2022). Although many studies have reported the positive effects of probiotics in aquaculture such as boosting the recovery rate, improving blood glucose, red blood cell, hematocrit, and hemoglobin; there were few studies on the possible ameliorating effects of them against the toxicity caused by pesticides. Therefore, the present study aims to test the effects of the probiotic, *Lactobacillus sakei* subsp. *sakei* on the growth and immunity of the rainbow trout after exposure to the pesticide butachlor.

Material and methods

Fish sample collection and experimental design

Prior to the experiment, 600 live specimens of the rainbow trout were obtained from a local hatchery. Fish were randomly transferred into 1000 L (water temperature: 15–16°C) tanks for 2 weeks to acclimate and fed standard food twice a day. After the acclimating process, 540 rainbow trout were randomly stocked in 18 tanks. Each tank contained 30 specimen juveniles (12.21 ± 0.25 g; mean $\pm$ SE) in three replicates. The experimental treatments are described in Table 1.

Table 1. Different experimental treatments in this study

Group name	Butachlor concentration (%)	Probiotic dosage (cfu/g)
C	–	–
LS1	–	1×10^6
LS2	–	1×10^8
BU	12.5	–
BU+LS1	12.5	1×10^6
BU+LS2	12.5	1×10^8

During the 30-day experiment, 80% of the water was exchanged every 12 hours and the concentration of butachlor was reset immediately. Single-air stones provided aeration in each tank and feces were collected from each replicate two times a day. During the experiment,

water quality parameters were maintained constant: pH: 7.56 ± 0.45 (TS1); temperature: 15–16°C; hardness: 293 ± 2.35 ppm and dissolved oxygen: 8.80 ± 0.06 mg/l (DO-5510). The feeding rate was adjusted 3 times a day (8:00, 13:00, and 19:00, based on *ad libitum*) (Giannenas et al., 2012).

Acute toxicity test of butachlor

In order to measure the lethal concentrations and toxicity levels of butachlor on *O. mykiss* and to choose the appropriate doses, the static acute toxicity test was performed prior to the main experiment. Toxicity test was performed based on OECD (1993; Organization for Economic Co-operation and Development). Animals were subjected to 6 different levels of BU including 0, 0.05, 0.1, 0.2, 0.4, and 0.8 mg/l (10 animals in each concentration with 3 replicates, totally 30 fish). Mortality rates (MR) were recorded at various times (24, 48, 72, and 96 hours) and then the dead fish were immediately removed. The nominal dose of toxin causing death (LC_{30} , LC_{50} , and LC_{70}) was calculated using a probit test (Finney, 1971) and shown in Table 2.

Table 2. Lethal concentrations (LC_{30-70}) of butachlor depending on time (24–96 h) for rainbow trout

Point	Concentration (mg/l)			
	24 h	48 h	72 h	96 h
LC_{30}	0.47	0.43	0.39	0.23
LC_{50}	0.55	0.50	0.49	0.32
LC_{70}	0.63	0.58	0.58	0.41

Bacterial strains

L. sakei subsp. *sakei* (LS) 15521 was cultivated in MRS medium (de Man, Rogosa and Sharpe) at 38°C for 48 h. Afterward, the medium containing the grown bacteria was centrifuged for 4 min at 4°C ($\times 4000$ g) and the supernatant was discarded. Then, the pellets containing LS were washed thrice by using PBS (phosphate buffer saline). Again, the supernatant was removed by centrifugation and the pellets were resuspended in the PBS. Finally, the concentrations were adjusted at 1×10^{10} and 1×10^8 CFU/ml, spectrophotometrically (Rasouli et al., 2021). For this purpose, the optical density (OD) of the probiotic samples was measured at 600 nm, and bacterial cell concentration was subsequently determined by referencing a standard curve correlating OD readings with known cell concentrations. The LAB (LS)-supplemented diets were obtained by gently spraying the desired amount of LAB suspension on the basal diet (10 ml bacterial suspension per kg diet) and blending in a mixer to prepare a final LAB concentration of 1×10^8 and 1×10^6 CFU/g. In addition, the viability and count of LAB colonies in experimental treatments were confirmed using a MRS culture medium (Mohammadian et al., 2019; Yousefi et al., 2023 a). For this purpose, the LAB samples are serially diluted and spread on MRS plates, a selective medium designed to support LAB growth. After plating, the samples are incubated under anaerobic conditions

at 37°C for 48 hours. Colony-forming units (CFUs) are then counted on plates. The CFU count was used to calculate the viable LAB concentration in the original sample, typically expressed as CFU/g.

Nutrient composition in feed

Nutritional status and the components of the control pellets applied in this work are represented in Table 3. Primarily, the raw materials were initially shattered and mixed with water to create a base mixture. To produce the experimental pellets, the pellet components were mixed thoroughly in a mixer machine and kept for 36 hours in room temperature and transferred to refrigerator. In addition, the levels of LS strains (1×10^6 and 1×10^8 CFU/g) were selected based on positive data documented in former studies (Harikrishnan et al., 2010; Reyes-Becerril et al., 2014; Salih et al., 2023).

The proximate composition of the modified basal diet was assayed based on AOAC (2005) (Association of Official Analytical Chemists) protocol. Furthermore, LS survival in diets was confirmed using MRS medium.

Table 3. Dietary formulation and proximate composition of the basal diet (%) (Yousefi et al., 2021)

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

^aPeygir Co., Gorgan, Iran (crude protein 58.8%).

^bSoyabean Co., Gorgan, Iran (crude protein 45.2%).

^cPeygir Co., Gorgan, Iran (crude protein 51.0%).

^dShahdineh Aran Co (crude protein 78.3%).

^eGolbid Co., Tehran, Iran.

^fMad Tiour Co., Sanandaj, Iran.

^gThe premix provided following amounts per kg of feed: A: 1000 IU; D₃: 5000 IU; E: 20 mg; B₁: 100 mg; B₂: 20 mg; B₆: 20 mg; B₁₂: 20 mg; H: 1 mg; B₉: 6 mg; B₁₂: 1 mg; B₄: 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. Chinchin Co., Tehran, Iran.

Growth parameters

On the 30th day of the experiment, feeding was ended. After 24 hours, fish were anesthetized with 150 mg/l eugenol and the following biological characteristics were measured (Yousefi et al., 2022):

$$\text{Weight gain (WG, g)} = FW - IW$$

$$\text{Feed conversion ratio (FCR)} = FI / (FW - IW)$$

$$\text{Specific growth rate (SGR, \% / day)} = 100 \times [(\ln FW - \ln IW) / d]$$

$$\text{Survival rate (SR, \%)} = (Nt / Ni) \times 100$$

Final weight (FW); Initial weight (IW); Feed intake (FI); Day (d); Number of total (Nt); Number of individuals (Ni).

In order to measure serum immunity and antioxidant parameters, fish were anesthetized and blood samples were collected from the caudal vein using 1-ml syringe and centrifuged ($3000 \times g$, 10 min, 4°C) and stored at -70°C until further use. In addition, three fish from each tank were sampled and kept in a bag containing 10 ml physiological serum (50 millimolar) for three minutes and mucous membranes were collected and centrifuged ($2500 \times g$, 10 min, 4°C) and stored at -70°C for further analysis (Du et al., 2015).

Fish intestinal microbiota analysis

After 30 days of the experiment, three euthanized fish were harvested from each replicate. The specimen's skin was washed with distilled water and then disinfected with 70% ethanol and the intestine was aseptically excised. The intestine was washed and homogenized using phosphate-buffered saline (PBS) in a macerator. Then, 1 g of this homogenate was added to 9 mL PBS, and vigorously vortexed for at least 2 minutes. In the next step, samples were serially diluted with PBS and 100 µL of diluted samples were transferred to tryptic soy agar (Merck, Germany) and MRS agar to measure total bacteria (TBC) and lactic acid bacteria (LAB), respectively. The plates containing TSA were stocked at 28°C for 24 h (Reda et al., 2016; Jasim et al., 2022) and plates containing MRS incubated at 37°C for 48 h (Prabawati et al., 2022; Yanez-Lemus et al., 2022). TBC and LAB colonies were enumerated according to the method described by Merrifield et al. (2010).

Digestive enzyme analysis

For enzyme activity assays, 3 fish were randomly collected at the end of the experiment, and their intestine was removed aseptically and washed with 50 mM Tris-buffered saline. The intestines were then mechanically homogenized. The crude content of the intestine was extracted and homogenized by 50 millimolar Tris-buffered saline and centrifuged ($6000 \times g$, 10 min, 4°C). The remaining supernatant was carefully separated and kept at -80°C for further analysis (Yousefi et al., 2023 a). The digestive enzyme activities (U/mg protein) were determined using the colorimetric assays and relevant assay kits (Nanjing Jiancheng, Bioengineering Institute, China, C number: (A054-2) and the optical density (OD) respectively read at 660, 540, and 440 nm wavelength by a spectrophotometer previously carried out by Rahimnejad et al. (2019) and Lu et al. (2020). A unit of protease activity (catalog number: A080-2-2) was defined as the amount of enzyme required to hydrolyze casein to produce 1 µg tyrosine per minute at 37°C in pH 7.5

solution, measured at 440 nm. A unit of lipase activity (catalog number: A054-2) was considered as the amount of enzyme required to produce 1 μ mol of butyric acid per minute at 37°C in pH 7.0 solution at 540 nm. A unit of amylase activity (catalog number: C016-1) was considered as the amount of enzyme required to hydrolyze 10 mg of starch during 30 min at 37°C in pH 6.9 at 660 nm.

Humoral antioxidant markers

In order to measure blood superoxide dismutase (SOD) and catalase (CAT) activities, commercial kits (Zellbio, Hamburg, Germany) were used based on manufacturer's instructions. SOD activity (catalog number: ZX-44108 – ZELLX) was determined based on rate of reduction of cytochrome C (Hoseini et al., 2021); whereas, CAT activity (catalog number: ZX-44102 – ZELLX) was measured by the decomposition of 1 μ M of hydrogen peroxide (Goth, 1991). One unit of SOD was defined as the amount of enzyme required to inhibit the rate of reduction of cytochrome C by 50%; whereas, one unit of CAT was defined as the amount of enzyme required to decompose 1 μ M of hydrogen peroxide. Malondialdehyde (MDA) (catalog number: ZX-44116 – ZELLX) was also determined based on its reaction with thiobarbituric acid using a commercial kit (Zellbio, Hamburg, Germany). An aliquot of the enzyme extract was pipetted to 0.67% TBA solution and incubated at 95°C for 0.5 h, then OD was recorded.

Liver enzyme analysis

Serum biochemical parameters were measured (Pars Azmun Co., Tehran, Iran) using a UV-visible spectrophotometer and commercial kits (Pars Azmun Co., Tehran, Iran). Total protein (TP) (catalog number: 1500028) concentration in plasma was quantified using the Bradford (1976) method. Albumin content (catalog number: 101500) was determined using the colorimetric method described by Nicholson et al. (2000) with a spectrophotometer. Albumin content was calculated by the dye-binding features of serum albumin using bromocresol green. An enhancement in the blue-green color was determined at 620 nm wavelength. Finally, globulin was determined by subtraction of total protein and albumin. Serum lactate dehydrogenase (LDH) (catalog number: 1050022) was measured by converting pyruvate to lactate at 340 nm, and alkaline phosphatase (ALP) (catalog number: 102400) was measured by converting nitrophenol phosphate into nitrophenol and phosphate at 405 nm. Serum aspartate aminotransferase (AST) (catalog number: 118400) and alanine aminotransferase (ALT) (catalog number: 119400) activities were calculated by measuring NADH consumption and its conversion to NAD⁺ at 340 nm (Banaee et al., 2022).

Glucose and cortisol

Serum cortisol was measured by ELISA method using commercial assay kits (IBL Co., Gesellschaft für Immunchemie und Immunbiologie, Germany, catalog number: RE52061) based on manufacturer's instructions

at wavelength of 450 nm, recently validated for rainbow trout. Glucose was also determined using a commercial kit (Pars Azmun Co., Tehran, Iran, catalog number: 117500) at 546 nm at 37°C based on glucose oxidase protocol use with reagents provided in the commercial kit (Akbari and Jahanbakhshi, 2018).

Immunological responses

In order to measure serum and skin mucus lysozyme activity, an equal volume (0.05 ml) of suspension of Gram-positive bacterium *Micrococcus luteus* and the mucus/serum sample was incubated at 30°C and the reduction in absorbance was measured at 450 nm after 0 and 15 min in a microplate reader (Sigma-Aldrich, catalog number: LY0100). The total immunoglobulin (total Ig) in skin mucus and serum (mg/dL) was determined by the method described by Anderson and Siwicki (1995) using polyethylene glycol (Sigma-Aldrich, catalog number: SE120068). In this method, initially protein value of samples was determined based on Bradford (1976). Then 12% polyethylene glycol (Sigma-Aldrich) was added to them. The mixture was kept at 22°C for 2 h, centrifuged (5000 $\times$ g for 5 minutes), then the protein value of the upper solution was calculated again by the method of Bradford (1976). The Ig concentration was computed by calculating the difference in protein value before and after the addition of polyethylene glycol. Serum complement concentration (C3 and C4, mg/dL) was determined using ELISA reader (ELX800, BioTek, Vermont, USA) by commercial kit (Pars Azmun Co., Tehran, Iran). Serum alternative complement activity (ACH50) was determined using the sheep red blood cells (RaRBC) as substrates in veronal buffer (Yano, 1992). In this method, various concentrations (10, 5, 2.5, 1.25, 0.625, and 0.312%) were obtained from desired samples, and 25 μ l of each sample were blended with 0.125 ml buffer solution and 0.050 ml of SRBC. After 2 hours of storage at 22°C, the cocktail was centrifuged (1600 $\times$ g; 10 min) and the OD was determined at 412 nm. Furthermore, the mucus protease was determined by reaction of the enzyme with azocasein as substrate (Hoseinifar et al. 2016). For this purpose, the reaction cocktail containing an equal volume of ammonium bicarbonate solution and mucus supernatant was kept at 30°C for 20 hours. Then the reaction was terminated by pipetting trichloroacetic acid (4.6%) and the cocktail was subsequently centrifuged at 10000 $\times$ g for 10 minutes. 0.5 N hydroxide was added to the supernatants. Finally, the OD was measured at 450 nm. Serum myeloperoxidase (MPO) activity (Sigma-Aldrich, catalog number: MAK068) was also measured at 450 nm using Hank's buffer (HBSS) according to Quade and Roth (1997). In addition, serum respiratory activity (RB) was estimated according to the method of Anderson and Siwicki (1995) using N-dimethylformamide.

Statistical analysis

In this work, Excel (version 13) and SPSS (version 20) softwares were used for drawing graphs and statis-

tical analysis, respectively. Determining the significant difference between different treatments was done by one-way ANOVA and Tukey's tests at $P < 0.05$. Data normality was analyzed by Shapiro-Wilk and Levene tests. Results were represented as mean $\pm$ standard error.

Results

Growth performance

The effects of LS and the different BU contents on growth performance and feed conversion are shown in Table 4. The diets containing LS1 and LS2 significantly improved growth performance and feed conversion ratio including FW, WG, FCR and SGR compared to the control and BU groups ($P < 0.05$). In the BU group, a significant decrease in FW (24.33 g), WG (12.13 g) and SGR (2.30%/d) and an increase in FCR (1.46) were observed ($P < 0.05$). In addition, survival rates were similar across treatments. No interaction was observed between the effects of LS and BU on the growth and nutritional parameters FW ($P = 0.937$), WG ($P = 0.954$), FCR ($P = 0.506$), SGR ($P = 0.247$) and SR ($P = 0.289$).

Intestinal microbiota analysis

The effects of different LS levels and BU on the gut microbiota of *O. mykiss* are depicted in Figure 1. Administration of LS1 significantly elevated LAB count (5.10 log CFU/g) and TBC count (7.46 log CFU/g) compared to other groups ($P < 0.05$). The lowest LAB and TBC counts were observed in the BU group (1.90 and 4.53 log CFU/g) ($P < 0.05$). Besides, no interaction was found between the effects of LS and BU on gut microbiota LAB ($P = 0.995$) and TBC ($P = 0.685$).

Digestive enzymes

The digestive enzyme analysis in different treatments is shown in Figure 2. According to the results, protease activity in fish fed with diet LS1 (3.98 U/mg protein) and

LS2 (3.75 U/mg protein) illustrated a higher level compared to other groups ($P < 0.05$). Furthermore, the lowest protease activity was observed in the BU group (1.74 U/mg protein) ($P < 0.05$). In addition, all dietary LS supplementation significantly increased amylase activity compared to BU group (0.34 U/mg protein) ($P < 0.05$). Dietary inclusion of LS1 (0.45 U/mg protein) significantly increased lipase activity compared to the BU treatment. No interaction was found between the effects of LS and BU on gut protease activity ($P = 0.765$) and lipase activity ($P = 0.653$); however, there was an interaction between the effects of LS and BU on gut amylase activity ($P = 0.029$).

Immunity responses

In this study, the serum and mucus immune parameters in response to different treatments are represented in Table 5 and Table 6, respectively. The results revealed that serum immunity parameters including lysozyme (22.3 U/ml), total Ig (17.2 mg/ml), C4 (10.8 g/dl), C3 (4.4 g/dl), ACH50 (94.0 U/ml), RB (0.3 OD at 540 nm), and MPO (0.9 OD at 450 nm) in BU group significantly decreased compared to other treatments ($P < 0.05$). In addition, the highest values of these parameters were recorded in LS1 and LS2 groups. No interaction was found between the effects of LS and BU on serum immunity parameters including lysozyme ($P = 0.237$), total Ig ($P = 0.458$), C4 ($P = 0.917$), C3 ($P = 0.485$), ACH50 ($P = 0.608$), NBT ($P = 0.082$) and MPO activity ($P = 0.500$). Furthermore, mucus immunity responses including lysozyme (9.18 U/mg protein), protease (6.26 U/mg protein), and ALP (21.36 U/mg protein) in fish of BU were significantly lower than other groups ($P < 0.05$). Besides, the highest values of these parameters were found in the LS1 and LS2 groups. No interaction was found between the effects of LS and BU on mucus immunity parameters including lysozyme ($P = 0.632$), peroxidase ($P = 0.937$), protease ($P = 0.247$), total Ig ($P = 0.954$), and ALP activity ($P = 0.506$).

Table 4. Effect of different supplementation level of BC and exposure of BU on growth performance of *O. mykiss* in this study (mean $\pm$ SE)

Treatment	IW (g)	FW (g)	WG (g)	FCR	SGR (%/d)	SR (%)
C1	12.37 $\pm$ 0.32 a	28.17 $\pm$ 1.25 b	15.80 $\pm$ 1.31 c	1.26 $\pm$ 0.09 b	2.74 $\pm$ 0.18 b	95.33 $\pm$ 4.04 a
LS1	12.17 $\pm$ 0.29 a	33.00 $\pm$ 1 a	20.83 $\pm$ 0.76 a	1.01 $\pm$ 0.05 c	3.33 $\pm$ 0.05 a	97.33 $\pm$ 2.31 a
LS2	12.11 $\pm$ 0.27 a	32.17 $\pm$ 1.04 a	20.06 $\pm$ 0.80 a	1.04 $\pm$ 0.07 c	3.26 $\pm$ 0.05 a	92.33 $\pm$ 1.73 a
BU	12.20 $\pm$ 0.35 a	24.33 $\pm$ 0.76 c	12.13 $\pm$ 0.55 d	1.46 $\pm$ 0.10 a	2.30 $\pm$ 0.07 c	92.00 $\pm$ 1.73 a
LS1+ BU	12.20 $\pm$ 0.26 a	29.63 $\pm$ 1.30 ab	17.43 $\pm$ 1.10 b	1.15 $\pm$ 0.07 b	2.96 $\pm$ 0.10 b	97.33 $\pm$ 2.31 a
LS2+ BU	12.27 $\pm$ 0.25 a	28.60 $\pm$ 1.27 ab	16.33 $\pm$ 1.15 bc	1.23 $\pm$ 0.09 b	2.82 $\pm$ 0.12 b	96.33 $\pm$ 3.51 a

Note: IW: initial weight, FW: final weight, WG: weight gain, FCR: feed conversion ratio, SGR: specific growth rate, SR: survival rate. Means in the same column with different letters differ significantly among groups ($P < 0.05$) on the basis of Tukey's HSD test.

Two-way ANOVA (P-value)						
	IW (g)	FW (g)	WG (g)	FCR	SGR (%/d)	SR (%)
LS	0.800	0.000	0.000	0.000	0.000	0.348
BU	0.950	0.000	0.000	0.000	0.000	0.341
LS $\times$ BU	0.632	0.937	0.954	0.506	0.247	0.289

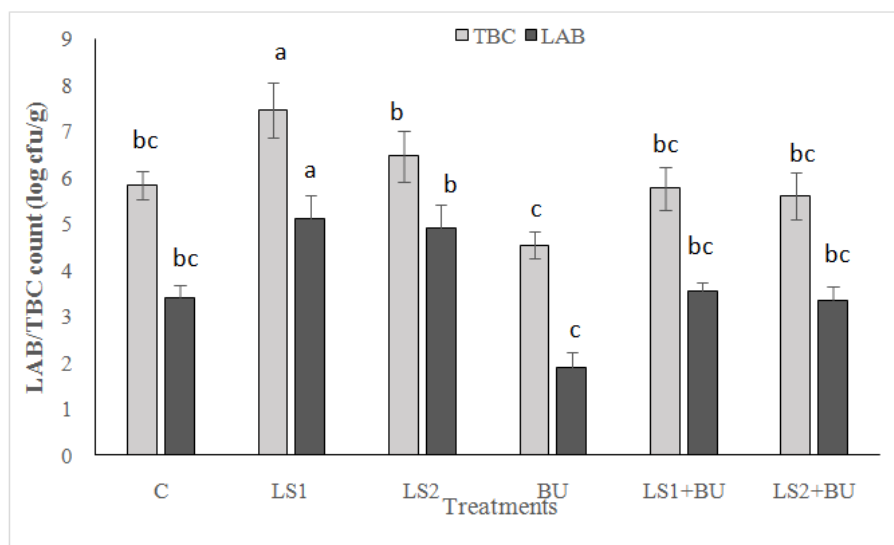


Figure 1. Results of lactic acid bacteria (LAB) and total intestine bacteria (TBC) for the intestinal microbiota of *O. mykiss* in different treatments (mean±SE). Dissimilar letters above the bars with same color, represent significant differences ($P<0.05$) based on Tukey's HSD test

Two-way ANOVA (P-value)		
	LAB (log cfu/g)	TBC (log cfu/g)
LS	0.002	0.000
BU	0.000	0.005
LS × BU	0.995	0.685

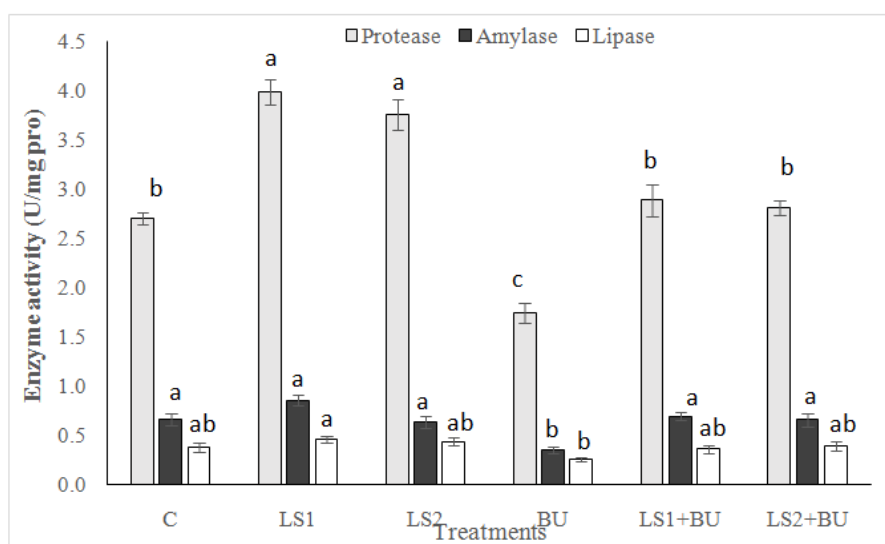


Figure 2. Effects of dietary BC and BU exposure on digestive enzyme activity, protease, lipase and amylase in *O. mykiss* during this study (Mean±SE). Dissimilar letters above the bars with same color, represent significant differences ($P<0.05$) based on Tukey's HSD test

Two-way ANOVA (P-value)			
	Protease (U/mg pro)	Lipase (U/mg pro)	Amylase (U/mg pro)
LS	0.000	0.055	0.001
BU	0.000	0.021	0.004
LS × BU	0.765	0.653	0.029

Table 5. Results of serum immunity parameters measured in this study (mean±SE)

Treatment	Lysozyme (U/ml)	Total Ig (mg/ml)	C4 (g/dL)	C3 (g/dL)	ACH50 (U/ml)	RB (OD at 540 nm)	MPO (OD at 450)
C1	27.3±1.9 b	21.6±1.2 b	14.6±1.4 b	7.4±1.2 b	105.3±5.5 b	0.6±0.1 b	1.2±0.1 b
LS1	35±1.4 a	24.6±1.4 a	17.1±2.1 a	9.5±0.6 a	117.3±4.5 a	0.9±0.1 a	1.8±0.1 a
LS2	31.4±1.8 a	22.3±1.2 b	15.9±1.4 a	10.3±1.1 a	115.7±4 a	0.9±0.1 a	1.6±0.1 a
BU	22.3±0.8 c	17.2±1.1 c	10.8±1.3 c	4.4±0.6 c	94.0±4 c	0.3±0.1 c	0.9±0.1 c
LS1+ BU	29.1±1.5 ab	21.5±0.9 b	14.3±1.2 b	7.6±1.3 b	108.0±5 b	0.6±0.1 b	1.2±0.1 b
LS2+ BU	27.6±1.3 b	21.2±0.8 b	14.2±1.2 b	7.2±1.2 b	106.7±7.5 b	0.5±0.1 b	1.3±0.1 b

Note: Total Ig: total immunoglobulin, C3, C4: complement concentration, ACH50: alternative complement activity, RB: respiratory burst activity, MPO: myeloperoxidase. Means in the same column with different letters differ significantly among groups ($P<0.05$) on the basis of Tukey's HSD test.

Two-way ANOVA (P-value)

	Lysozyme (U/ml)	Total Ig (mg/ml)	C4 (g/dL)	C3 (g/dL)	ACH50 (U/ml)	NBT (540)	MPO (450)
LS	0.000	0.000	0.002	0.000	0.001	0.000	0.000
BU	0.000	0.000	0.002	0.002	0.000	0.000	0.000
LS × BU	0.237	0.458	0.917	0.485	0.608	0.082	0.500

Table 6. Results of mucus immunity parameters measured in this study (mean±SE)

Treatments	Mucus immunity responses				
	Lysozyme (U/mg pro)	Peroxidase (U/mg pro)	Protease (U/mg pro)	Total Ig (mg/ml)	ALP (U/mg pro)
C1	13.40±0.60 b	9.33±0.60 bc	10.20±0.47 b	10.43±0.97 b	27.17±1.06 b
LS1	17.48±0.56 a	12.76±0.55 a	13.46±0.73 a	14.16±0.72 a	32.93±0.96 a
LS2	17.51±0.91 a	12.20±0.73 ab	11.16±0.72 ab	13.73±0.78 a	33.16±0.73 a
BU	9.18±0.54 c	7.70±0.47 c	6.26±0.37 c	7.80±0.43 b	21.36±0.84 c
LS1+ BU	13.43±0.83 b	10.66±0.46 abc	9.63±0.66 b	14.40±0.34 a	25.90±0.95 b
LS2+ BU	14.46±0.75 ab	10.23±0.92 abc	10.06±0.58 b	13.76±0.64 a	26.73±0.63 b

Note: Total Ig: total immunoglobulin, ALP: alkaline phosphatase. Means in the same column with different letters differ significantly among groups ($P<0.05$) on the basis of Tukey's HSD test.

Two-way ANOVA (P-value)

	Lysozyme (U/mg pro)	Peroxidase (U/mg pro)	Protease (U/mg pro)	Ig (mg/ml)	ALP (U/mg pro)
LS	0.800	0.000	0.265	0.000	0.882
BU	0.950	0.000	0.461	0.000	0.048
LS × BU	0.632	0.937	0.247	0.954	0.506

Table 7. Effect of different treatments on hormonal antioxidant markers in *O. mykiss* in this study (mean±SE)

Treatment	SOD (U/ml)	CAT (U/ml)	MDA (μmol/l)
C1	44.33±2.08 b	17.5±2.5 b	10.03±0.85 a
LS1	60.50±1.80 a	23.83±2.02 a	5.93±0.90 b
LS2	61.17±1.04 a	19.53±1.50 b	7.4±0.96 b
BU	37.67±1.53 c	15.77±1.25 b	14.67±1.04 a
LS1+ BU	45.00±1 b	18.67±3.06 b	8.133±1.03 b
LS2+ BU	49.00±2 b	18.83±1.04 b	9.5±0.5 b

Note: SOD: superoxide dismutase, CAT: catalase, MDA: malondialdehyde. Means in the same column with different letters differ significantly among groups ($P<0.05$) on the basis of Tukey's HSD test.

Two-way ANOVA (P-value)

	SOD (U/ml)	CAT (U/ml)	MDA (μmol/l)
LS	0.001	0.000	0.0007
BU	0.000	0.001	0.021
LS × BU	0.053	0.064	0.177

Table 8. Results of liver enzymes and biochemical factors analysis with glucose and cortisol after exposure to different treatments in this study (mean±SE)

Parameters	C1	LS1	LS2	BU	LS1+BU	LS2+BU
AST (U/L)	163.33±7.02 b	126.17±6.53 c	129.67±4.73 c	193.67±6.03 a	155.33±5.03 b	161.33±6.03 b
ALP (U/L)	270.33±10.02 b	209.67±9.07 c	219±10.15 c	306.67±16.5 a	273±10.54 b	274.33±10.07 b
ALT (U/L)	47.67±3.51 b	30.17±2.75 c	33.17±2.02 c	57.5±2.29 a	49.17±3.25 b	47±2.00 b
LDH (U/L)	841.67±12.5 b	766.33±18.2 c	777.67±12.5 c	885.33±15.1 a	842±9.64 b	843±14 b
Glucose (mg/dL)	83.22±3.18 b	65.83±3.25 c	66.67±3.51 c	94.43±2.38 a	81.53±2.84 b	82.83±3.01 b
Cortisol (ng/ml)	172.83±3.01 b	147.67±2.52 c	148.5±4.09 c	186±6.00 a	151.5±3.12 c	153.5±3.12 c
TP (g/dL)	3.57±0.3 b	4.35±0.48 a	3.5±0.50 b	2.3±0.40 c	3.44±0.25 b	3.47±0.36 b
Albumin (g/dL)	2.17±0.25 a	2.6±0.56 a	1.7±0.17 b	1.4±0.20 c	1.83±0.32 b	2±0.17 b
Globulin (g/dL)	1.4±0.10 b	1.69±0.26 a	1.8±0.36 a	0.9±0.20 c	1.61±0.11 b	1.47±0.24 b

Note: AST: aspartate aminotransferase, ALP: alkaline phosphatase, ALT: alanine aminotransferase, LDH: lactate dehydrogenase, TP: total protein. Means in the same row with different letters differ significantly among groups ($P<0.05$) on the basis of Tukey's HSD test.

Two-way ANOVA (P-value)									
	AST (U/L)	ALP (U/L)	ALT (U/L)	LDH (U/L)	Glucose (mg/dL)	Cortisol (ng/ml)	TP (g/dl)	Albumin (g/dL)	Globulin (g/dL)
LS	0.000	0.001	0.000	0.001	0.000	0.000	0.001	0.000	0.000
BU	0.000	0.000	0.010	0.000	0.000	0.000	0.000	0.000	0.000
LS × BU	0.936	0.148	0.039	0.171	0.178	0.079	0.130	0.090	0.756

Antioxidant markers

The results of serum antioxidant markers in different treatments are illustrated in Table 7. SOD activity was markedly increased in LS1 (60.50 U/ml) and LS2 (61.17 U/ml) treatments ($P<0.05$); however, the minimum SOD content was found in the BU group (37.67 U/ml). Administration LS1 notably elevated CAT concentration (23.83 U/ml) compared to other treatments ($P<0.05$). Besides, feeding fish with LS significantly reduced MDA value compared to the control (10.03 $\mu\text{mol/L}$) and BU (14.67 $\mu\text{mol/L}$) treatments ($P<0.05$). No interaction was found between the effects of LS and BU on antioxidant status including SOD ($P = 0.053$), CAT ($P = 0.064$), and MDA ($P = 0.177$).

Liver enzymes factors

Liver enzymes and stress factors are shown in Table 8. Administration of LS1 and LS2 significantly reduced the activity of liver enzymes (ALT, AST, ALP, LDH) compared to other treatments ($P<0.05$). While, the maximum ALT (57.5 U/L), AST (193.67 U/L), ALP (306.67 U/L), and LDH (885.33 U/L) were recorded in BU treatment. According to Table 8, the concentration of stress factors declined in blood samples of LS1 (cortisol 147.67 ng/ml; glucose; 65.83 mg/dL) and LS2 (cortisol 148.5 ng/ml; glucose; 66.67 mg/dL) while blood samples of BU group (cortisol 186 ng/ml; glucose; 94.43 mg/dL) showed an increase in these parameters ($P<0.05$). The highest blood total protein (4.35 g/dL) and albumin (2.6 g/dL) was recorded in the LS1 group ($P<0.05$). Similarly, the maximum globulin level was recorded in LS1 (1.69 g/dL) and LS2 (1.8 g/dL) groups. These parameters decreased significantly in fish of the BU group (Table 8)

($P>0.05$). No interaction was found between the effects of LS and BU on AST ($P = 0.936$), ALP ($P = 0.148$), LDH ($P = 0.171$), glucose ($P = 0.178$), cortisol ($P = 0.079$), total protein ($P = 0.130$), albumin ($P = 0.090$), globulin ($P = 0.756$). However, there was an interaction between the effects of LS and BU on ALT activity ($P = 0.039$).

Discussion

Growth and nutritional performance are key parameters in determining the properties of a LAB supplement (De et al., 2014). According to the results, growth factors, including WG, FCR and SGR, were significantly increased in fish fed *L. sakei*. Similarly, several studies have reported the positive effects of feeding LAB bacteria such as *L. rhamnosus* (Hajirezaee and Khanjani, 2023) and *L. bulgaricus* (Mohammadian et al., 2020) in the rainbow trout. The enhancement of growth and nutritional indices in the groups supplemented with LAB may be as a result of an improvement in digestibility, absorption of nutrients, and protein retention (Ringø and Song, 2016; Yang et al., 2021). According to previous results, the amelioration of gut microbiota plays a significant role in improving protein retention and growth indices. Increasing the population of beneficial intestinal bacteria is one of the prerequisites for boosting the metabolic process (Yousefi et al., 2023 a). Several studies in aquaculture have shown that dietary LAB supplementation can help to support the dominance of LAB in the gut (Yang et al., 2021; Naiel et al., 2022).

In this study, the administration of *L. sakei* increased the proliferation of LAB and TBC in LS1 and LS2 group

at the end of the experiment, which may indicate the ability of *L. sakei* in regulating the intestinal microbial flora during this period. Increasing the gut LAB count could modulate some vital tasks involved in metabolic activities such as digestive enzymes (Yousefi et al., 2023 a). Gut-beneficial bacteria can generate several extracellular or intracellular enzymes to break down diet ingredients into absorbable components (Assan et al., 2022). According to the results, the level of digestive markers including protease, amylase, and lipase was higher than LS1 and LS2 treatments. Similar results were also obtained in fish fed diets containing LAB such as *L. helveticus* (Yousefi et al., 2023 a) and *L. sakei* (Salih et al., 2023) in common carp, *L. lactis* subsp. *lactis* (Yeganeh et al., 2021).

Furthermore, as expected, fish exposed to butachlor in the BU group showed a decline in all growth parameters, digestive enzymes, and LAB count. Interestingly, no significant differences between the aforementioned parameters in LS1+BU and LS2+BU groups were found compared to the control. The ability of LAB to alleviate the adverse effects of toxic compounds on growth indices of rainbow trout has also been documented in other studies. For instance, Madreseh et al. (2019) stated that dietary supplementation of rainbow trout with *L. fermentum* can markedly mitigate the deleterious effects of the heavy metals (i.e. cadmium, lead, nickel, and zinc) on growth. It seems that *L. sakei* mitigates the negative effect of butachlor on the growth indices of rainbow trout through improving gut LAB proliferation, digestive enzyme activities as well as immune responses (Lugert et al., 2016). In support of these results, *L. sakei* had positive effects on the growth markers and digestive enzymes and immune capacity in common carp exposed to acetamiprid (Salih et al. 2023).

Fish skin mucus is an essential component of the protective system and is considered as the first line of defense, containing a collection of potent defense molecules (Caipang et al., 2011; Rajan et al., 2013; Lazado et al., 2015). Results of this study indicated that pellets containing of *L. sakei* significantly enhanced some immunity parameters in the mucus of the fish. Lysozyme is a protein enzyme that is involved in the death of microbes by destroying the cell wall of bacteria (Sun et al., 2010). It was observed that dietary *L. sakei* markedly enhanced the amount of mucus LYZ in LS1 and LS2 groups. However, fish exposed to butachlor showed significant depletion in skin mucus lysozyme, reflecting the adverse effect of butachlor on serum immunity of the fish. Jalil et al. (2022) reported that *Cyprinus carpio* exposed to the herbicide pretilachlor showed a significant decrease in lysozyme level after 21 days. Contrarily, no significant changes were observed between LS1+BU and LS2+BU compared to control group in serum lysozyme level. This suggests that dietary *L. sakei* caused an improvement in the immune status of the rainbow trout and helped to control the destructive impact of butachlor, which was consistent with the effects of this strain on common carp exposed to acetamiprid (Salih et al., 2023).

The ACH50 and total complement and immunoglobulin are effective proteins of the humoral immune system and are considered as indicators of immune status in fish (Wang et al. 2014; Sharifian et al. 2015). Increases in ACH50 and Ig levels as a result of the oral administration of probiotic in fish have been described by several researches (Ramesh and Souissi, 2018; Lee et al., 2020; Yousefi et al., 2023 a). In this study, significant elevations in total Ig and ACH50 levels were observed in LS1 group and LS1 and LS2 treatments. Although total Ig and ACH50 showed a significant decrease in BU group, no changes were observed in LS1+BU and LS2+BU in comparison with control, suggesting a protective function for *L. sakei* against adverse effects of butachlor. Yousefi et al. (2023 a) reported a remarkable increase in Ig and ACH50 activities in common carp after feeding with *L. helveticus* diet. The main proteins of the complement system including C3 and C4 have a central activity in the setting up of the complement system (Boshra et al., 2006). In this study, concentrations of C3 and C4 in serum were raised in the probiotic treatments over 30 days feeding period. These results were consistent with the results of Hajirezaee and Khanjani (2023), where *L. rhamnosus* mitigated the effects of ammonium in the exposed rainbow trout (RB and MPO are the main products of the phagocytosis process burst). In this study, the elevated levels of RB and MPO in supplemented fish confirmed the prompting effects of *L. sakei* on non-specific immune responses. In spite of reduction in BU group, immune parameters in LS1+BU and LS2+BU were similar to the control group, indicating the effective effects of *L. sakei*. Similarly, *L. rhamnosus* protected the RB and MPO levels in rainbow trout exposed to environmental ammonium (Hajirezaee and Khanjani, 2023).

Humoral antioxidant markers including SOD and CAT are the major factors to support fish against oxidative stress (Gobi et al., 2016). SOD has a central function in changing O_2^- to H_2O (Li et al., 2015; Wang et al., 2017), and CAT is involved in catalyzing H_2O_2 into H_2O and O_2 (Wang et al., 2017). Probiotics help to enhance antioxidant capability by increasing CAT and SOD levels in both skin mucus and intestine (Weifen et al., 2012; Wang et al., 2017; Hindu et al., 2018). On the other hand, MDA reveals the toxicity volume as a result of the activity of ROS and increases when fish are exposed to harsh environmental conditions (Couto et al., 2013). According to the results, dietary addition of *L. sakei* could boost the antioxidant capacity of the fish and also reduces the stress conditions after exposure to butachlor. In support of our results, dietary *L. sakei* significantly boosted antioxidant capacity in the common carp exposed to acetamiprid (Salih et al., 2023). Overall, the improvement of the antioxidant capacity of SL treatments may be associated with increases in the cellular enzyme activity and scavenging ROS by various metabolites such as folate and exopolysaccharides (Yousefi et al., 2023 b).

In this research, *L. sakei* significantly raised the amount of albumin, globulin and total proteins in se-

rum of the fish. Ahmadifar et al. (2019) also observed significant increases in serum total protein and albumin levels, in common carp supplemented with LAB (*L. fermentum*). These indices can reflect health status of fish especially for liver conditions. Hematology and immunology improvements in fish after application of probiotics have been documented (Kumar et al., 2006; Bandyopadhyay et al., 2009; Sayed Hassani et al., 2020). The AST, ALT, and ALP are notable enzymes that their high levels in serum could indicate liver damage (Tahir et al., 2021). Furthermore, AST or ALT fluctuation reveals potential stress responses in many fishes (Kumar et al., 2019). According to the results, AST, ALT, and ALP levels decreased after *L. sakei* supplementation, demonstrating potential health benefits of *L. sakei* in the fish, as reported by Lee et al. (2020). As expected, fish exposed to butachlor showed high level of stress, as AST, ALT, and ALP levels were high in the exposed fish; LDH is one of main agents in glycolysis process (Yang et al., 2019). Lower activity of LDH in LS1 and LS2 group may indicate the prompting function of *L. sakei* on metabolism of carbohydrates. The positive impacts of *L. sakei* supplementation were magnified in LS1+BU and LS2+BU group as the levels of measured liver enzymes were maintained and did not show significant difference compared to control group. This phenomenon was also observed for serum cortisol and glucose levels which indicates the beneficial effects of *L. sakei* supplementation in reducing the chronic stress in the fish. Several data in former works have also demonstrated that administration of probiotics significantly reduces cortisol and glucose levels in fish (Telli et al., 2014; Sadat Hoseini Madani et al., 2018).

Conclusion

Overall our findings revealed that *L. sakei* significantly improved the growth markers, the level of digestive enzymes, LAB population, immunological and antioxidant activities, and ameliorated the butachlor stress. However, it is suggested to design more researches to study the mode of action of the probiotic with butachlor toxicity and related mechanisms.

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Conflicts of interest/competing interests

There is no conflict of interest to declare.

Ethics approval

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

Consent for publication

All authors give consent for publication.

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