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Effect of multi-strain probiotics on the growth, hematological profile, blood biochemistry, antioxidant capacity, and physiological responses of *Clarias batrachus* fingerlings

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Abstract
This study was designed to evaluate the effects of multi-strain probiotic (MSP) on growth, whole-body composition, digestive and antioxidant enzymes, hematology, blood biochemistry, and physiological stress responses in *Clarias batrachus* fingerlings. Five experimental diets were prepared with supplementation of MSP (composed of *Bacillus subtilis*, *B. licheniformis,* and *Enterococcus faecalis*) powder at 0.5, 1.0, 1.5, and 2.0 g/kg. Healthy fingerlings ($N = 225$) were procured from a fish hatchery and acclimatized to laboratory conditions for 15 days. After acclimation, the fingerlings (10.13 ± 0.01 g; mean ± SD) were randomly stocked into five experimental groups (15 fish/aquarium) with three replicates and provided with experimental diets at a 5% body weight ration five times a day for 90 days. Fish fed with 2.0 g/kg MSP diets showed significantly higher growth performance in terms of weight gain % (493.98 ± 6.3%) and specific growth rate (1.97 ± 0.01%/day) and the lowest feed conversion ratio (1.72 ± 0.01) in comparison to the control (299.73 ± 5.17%, 1.53 ± 0.01%/day, and 2.28 ± 0.05, respectively). Similarly, higher activities ($p < 0.05$) of amylase (1.96 ± 0.02 U/mg protein), protease (10.51 ± 0.06 U/mg protein) and lipase (2.26 ± 0.02 U/mg protein) were also observed in the group fed 2.0 g/kg MSP than in the control group (1.20 ± 0.01, 8.61 ± 0.01, and 1.55 ± 0.02 U/mg protein, respectively). Dietary supplementation significantly ($p < 0.05$) enhanced the activities of superoxide dismutase (5.86 ± 0.04 U/mg prot), catalase (85.54 ± 0.58 U/mg prot), and glutathione peroxidase (105.69 ± 0.17 μU/mg prot) and reduced the malondialdehyde content (2.13 ± 0.06 mg/g prot) in fish fed 2.0 g/kg MSP-supplemented diets compared to the control group (4.54 ± 0.08 U/mg prot, 70.24 ± 0.53 U/mg prot, 89.61 ± 0.32 μU/mg prot, and 2.77 ± 0.02 mg/g prot, respectively). Dietary MSP did not affect the survival rate and proximate composition of fish. Hematological parameters such as white blood cells, red blood cells, hemoglobin, and hematocrit were also significantly increased in response to MSP supplementation in diets. Moreover, an increasing trend in blood biochemistry was observed in the activities of alanine phosphatase, while a decrease in alanine aminotransferase and aspartate aminotransferase was observed. It is concluded that MSP supplementation up to

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2.0 g/kg showed promising results on *C. batrachus* fingerlings. MSP supplementation significantly enhanced the growth performance, antioxidant and digestive enzyme activities, blood biochemistry, and hematological parameters of *C. batrachus* compared to the control. The physiological stress response did not show any significant alteration. Thus, MSP can be effectively recommended as an effective growth promotor for fish.

Keywords Multi-strain probiotics · Growth · *Clarias batrachus* · Digestive enzymes · Antioxidant status · Blood biochemistry

Introduction

The aquaculture industry has a key role in human nutrition and in meeting the future demand for aquatic food products and currently accounts for 50% of global fish consumption. The rapid increase in the human population has compelled the aquaculture industry to intensify its efforts to sustainably meet growing fish demand (Boyd et al. 2020). However, culturing a large number of fish in limited space leads to frequent collisions, competition for food, and degradation of water quality, which lead to oxidative stress in fish. Stress causes fish to exhibit depressed growth performance, a weakened immune system, and vulnerability to pathogens, which leads to frequent disease occurrence and causes huge economic losses in aquaculture (Martos-Sitcha et al. 2020). In the past, antibiotics were utilized to prevent bacterial diseases; however, due to serious health concerns such as antibiotic resistance and bioaccumulation, antibiotics have been banned in aquaculture (Manage 2018). Therefore, researchers have been actively seeking cost-effective, promisingly safe, and environmentally friendly alternatives to replace antibiotics in aquafeed. To address these concerns, dietary intervention with additives such as probiotics is considered the best approach (Mishra et al. 2015).

Probiotics, whether in viable or nonviable states, refer to nonpathogenic bacteria administered at a specific duration and optimal concentration that confer health benefits to their host. In fish farming, the incorporation of probiotics into feed offers a valuable nutritional approach and has become vital for various purposes (Nayak 2010). Probiotics help to maintain a healthy microbial balance by establishing beneficial microbiota in the gut. Probiotics help in disease prevention, reduce stress, support immunomodulation, and contribute to bioremediation efforts (El-Saadony et al. 2021; Tachibana et al. 2020)

Aquaculture researchers have successfully evaluated the use of single-strain probiotics (SSPs) as valuable supplements (Kwoji et al. 2021). However, the effect of probiotics on the host immune response depends on various factors, such as their source, type, strain, and species, which means that supplementing SSPs to a specific host may not necessarily yield a beneficial impact on the host immune system. Therefore, combining diverse species and genera of probiotics as multi-strain probiotics (MSPs) can be used in harmony and synergistically strengthen the host's immune system (Ouwehand et al. 2018). Currently, in the fish farming industry, there has been a growing trend in the utilization of a large range of bacterial species, such as gram-positive spore-forming bacteria belonging to the genus *Bacillus* and lactic acid bacteria (LAB) (Abdel-Tawwab et al. 2020; Ringø et al. 2018). Among all of the species, Bacillus species have gained much importance, especially *Bacillus subtilis,* which has shown efficiency in various fish species. It has been used to enhance the growth, nutritional profile, immunity, and overall health status of fish. It exhibits the ability to prevent pathogen colonization and

promote a balanced gut microbial environment (Kuebutornye et al. 2019; Telli et al. 2014; Zaineldin et al. 2018). Likewise, *Bacillus licheniformis* has emerged as a highly promising probiotic known for its endospore production, which enhances the immunity of fish and promotes the production of enzymes and the synthesis of antimicrobial compounds (Darafsh et al. 2019; Dawood et al. 2018; Gobi et al. 2018). Furthermore, LAB such as *Enterococcus faecium* can be effectively used in aquaculture (Ringo et al. 2020; Ringø et al. 2018). *E. faecium,* commonly found in the gastrointestinal tract, is a potential probiotic because it acts as a growth promotor, performs immunomodulatory activities, and produces bacteriocins to counteract the risk of infection (Costa Sousa et al. 2019; Tarkhani et al. 2020).

Clarias batrachus is an economically important freshwater air-breathing fish that belongs to the family *Clariidae*. Although it is considered an invasive species, its adaptability has led to its introduction in many countries beyond its native range for aquaculture purposes (Paul et al. 2015). In Asia, it is famous for its good nutritional content, taste, and consistent availability throughout the season and has gained immense popularity with high market demand among consumers (Gupta and Verma 2020; Sinha et al. 2014). Furthermore, it has a high growth rate, an efficient feed conversion ratio, and the capacity to survive in oxygen-depleted water conditions, which make it a desirable culture species. Therefore, this experiment was planned to assess the effect of MSP on growth performance, blood biochemistry, and digestive and antioxidant enzyme activities in *C. batrachus.*

Materials and methods

Preparation of probiotic-based diets

A commercial MSP powder (Compro Napro^{TM} Aqua Probiotics (PR-24), China) was used, and each 1.0 kg of this product contained *Bacillus subtilis* (5.0×10^9 CFU/gram), *Enterococcus faecalis* (5.0×10^8 CFU/gram), and *B. licheniformis* (5.0×10^9 CFU/gram). Five experimental diets with 40% CP were formulated by incorporating different levels of MSP powder: 0.0, 0.5 g/kg, 1.0 g/kg, 1.5 g/kg, and 2.0 g/kg, labelled T_0 (control diet), T_1, T_2, T_3, and T_4, respectively. Ingredients were taken from the local market in Lahore and analyzed for proximate composition following AOAC (2016). All the ingredients (Table 1) were weighed and ground to a particulate size of 0.05 mm and mixed in an electrical mixer (km 280, Kenwood) with the gradual addition of oil. Then, MSP powder was added according to the formulation described in Table 1. The ingredients were combined and mixed thoroughly by adding 15% distilled water to make a stiff dough. The dough was then passed through a meat mincer (Ag 3060, Anex) for pellet formation. Pellets were air dried with 10% moisture, and the experimental diets were packed in well-sealed plastic-labelled zipper bags in a refrigerator at 4 °C and then used throughout the research period. The samples of formulated feed were also analyzed for proximate analysis according to the method followed by AOAC (2016). The moisture content was estimated by using a hot-air oven (Wise Ven). Crude protein (CP) was analyzed using the micro-Kjeldahl method with an N percent of 6.25 by using the Kjeltec autoanalyzer (KjeltecTM 8100), crude fat (CF) was assessed through the use of a Soxhlet apparatus (Behro Test 901745) with diethyl ether extraction (40–60 °C), and the ash contents were determined by using a muffle furnace (Vulcan D-550) at 660 °C for 5 h.

Table 1 Feed formulation and proximate composition of the experimental diets used in the present study

Ingredients (g/kg)	Diets with MSP levels				
	0.0 g/kg	0.5 g/kg	1.0 g/kg	1.5 g/kg	2.0 g/kg
Soybean meal	250	250	250	250	250
Fish meal	250	250	250	250	250
Corn gluten	230	230	230	230	230
Wheat flour	100	99.5	99.0	98.5	98.0
Sunflower meal	080	080	080	080	080
Fish oil	060	060	060	060	060
Choline chloride	005	005	005	005	005
[1]Vitamin premix	010	010	010	010	010
[2]Mineral mixture	010	010	010	010	010
Vitamin C	005	005	005	005	005
MSP	0.00	0.50	1.00	1.50	2.00
Nutrient composition (%)					
Dry matter	90.57±0.58	90.88±0.62	90.60±0.57	90.87±0.44	90.79±0.47
Crude protein	40.18±0.21	40.28±0.15	40.28±0.19	40.30±0.25	40.26±0.22
Crude fat	8.11±0.15	8.14±0.17	8.11±0.14	8.15±0.16	8.13±0.17
Ash	7.26±0.59	7.23±0.41	7.40±0.36	7.18±0.65	6.73±0.54
Metabolizable energy (kcal/kg)	3490±67	3490±54	3490±59	3490±63	3490±62

[1]Mineral mixture contained the following per kilogram: magnesium 200,000 mg, selenium 100 mg, cobalt 2000 mg, manganese 23,750 mg, iodine 2750 mg, zinc 75,000 mg, copper 5000 mg

[2]Vitamin premix contained the following per kilogram: vitamin D3 480,000 IU, 60,000 mg inositol, 2400 mg vitamin E, 10 mg vitamin B12, vitamin A 10,000 mg, 4,000,000 IU, 2400 mg vitamin K3, 4000 mg vitamin B1, 4000 vitamin B6, 1200 mg folic acid, 40,000 mg vitamin C, 100 mg D-biotin, 4000 mg niacin, Cal. D. Pantothenate

Fish acclimatization

Two hundred twenty-five *C. batrachus* fingerlings (10.13 ± 0.01 g; mean + SD) were procured from the local fish farm of the Sindhwan fish hatchery, Head Balloki, Department of Fisheries, Government of Punjab, and transferred to the Fish Seed Rearing Unit, University of Veterinary and Animal Sciences, Ravi Campus, Pattoki , Pakistan. Fingerlings were placed in a $KMnO_4$ bath (5 g/L) for 1–2 min and stocked into a cemented flow through circular tanks with 0.3 L/mint water exchange for acclimatization. Fish were fed a control diet (T_0) during a 15-day acclimatization period.

Experimental design, fish rearing, and husbandry

At the beginning of the feeding trial, fingerlings were weighed and randomly distributed into glass aquaria (315 liter capacity) with dimensions 89 × 58 × 61 (cm) (length × width × depth) and a stocking density of 15 fish per aquarium. The fish were hand fed with test diets in triplicate at 5% of their body weight, and feed was given to fingerlings five times (every 4 h) a day for 90 days. On a daily basis, any uneaten feed was siphoned daily to calculate feed intake (g) and then feed conversion ratio. Physicochemical parameters such

as dissolved oxygen, temperature, and pH (7.1 ± 0.2 mg/L, 28.5 ± 0.3 °C, and 7.1 ± 0.1, respectively) were monitored on a daily basis.

Growth indices

After the termination of the 90-day feeding trial, the fish weight was measured, and the number of fish in each replicate was also recorded. Growth parameters, such as weight gain percent (WG%), feed intake (FI), feed conversion ratio (FCR), specific growth rate (SGR), and survival rate (SR), were determined using the following formulae:

$$WG\% = \frac{final\ body\ weight - initial\ body\ weight}{initial\ body\ weight} \times 100$$

$$FI(g/fish) = feed\ given\ (g) - unconsumed\ feed(g)$$

$$\mathrm{FCR} = \frac{\mathrm{FI\ (g)}}{\mathrm{WG\ (g)}}$$

$$SGR\ (\%/day) = \frac{\ln(average\ final\ weight) - \ln(average\ final\ weight)}{Number\ of\ days} \times 100$$

$$SR\ (\%) = \frac{Number\ of\ fish\ at\ the\ end\ to\ trial}{Number\ of\ fish\ at\ the\ initiation\ of\ trial} \times 100$$

Whole-body composition

The four fish were arbitrarily selected for whole-body proximate analysis in terms of moisture content, crude protein, crude fat, and ash content following the protocol AOAC (2016) described above in the feed proximate analysis.

Digestive enzyme activities

Intestinal digestive enzyme analysis, such as amylase activity, was evaluated using starch solution as a substrate at 2% (w/v) (Bernfeld 1955). Lipase activity was measured using the spectrophotometric technique with the substrate p-nitro phenyl palmitate (pNPP) (Mahadik et al. 2002). The activity of protease was determined using the García-Carreño (1992) casein digesting technique. At 37 °C, the enzymatic unit hydrolyzes casein and produces a color identical to 1 mol/mint tyrosine (pH = 7.5).

Antioxidant enzyme activities

Each 2-g liver sample was mixed with 6 ml phosphate buffer (pH 7.4), homogenized, filtered using Whatman filter paper no. 1, and centrifuged at 10,000 × g for 15 min. The supernatant was separated, and all enzyme isolation steps were carried out at 4 °C. The activity of CAT was estimated by using the method of Maehly and Chance (1954). Briefly,

the reduction in H_2O_2 concentration at a wavelength of 240 nm was measured using an Analytik Jena Specord 200 Plus UV/VIS spectrophotometer. SOD activity was measured as described by Giannopolitis and Ries (1977). The activity of SOD was determined by measuring its ability to inhibit the photoreduction of nitroblue tetrazole (NBT). The activity of GPx was determined by calculating its capacity to decrease the H_2O_2 concentration at a wavelength of 470 nm following the method of Civello et al. (1995).

Thiobarbituric acid reactive substance contents in the liver and muscles were determined by following Gatta et al. (2000). Briefly, samples were homogenized in a solution of KCl and Tris-maleate, followed by the addition of ascorbic acid and incubation at room temperature. Thiobarbituric acid (TBA) and HCl were added to the samples, which were boiled for 25 min and then cooled and centrifuged after the addition of trichloroacetic acid. Finally, TBA values, expressed as l g malondialdehyde equivalents/mg tissue, were determined photometrically at 530 nm.

Blood analysis

Five live fish were randomly selected from each aquarium, and their blood was collected from the caudal vein by using 3-ml syringes and stored in EDTA vacutainers. Hematological parameters such as red blood cells (RBCs), white blood cells (WBCs), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), hemoglobin (HB), mean corpuscular hemoglobin (MCH), and platelets (PLT) were determined by an autohematological analyzer (Celltac α, MEK-6550 Ltd., Japan) in the general laboratory, Department of Fisheries and Aquaculture, UVAS Ravi Campus, Pattoki.

Aside from hematological testing, blood samples were also collected in Eppendorf tubes without anticoagulant and centrifuged at 3000 rpm for 15 min within 30 min of collection. The resulting samples were then stored at − 80 °C for analysis. Serum was used for aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alanine phosphatase (ALP) by using the kit method (BioScien) followed by Hossain et al. (2016). Serum was also used for stress parameters such as cortisol and glucose assays for the determination of stress by an automatic biochemical analyzer (Hitachi 7600-110 Ltd., Japan).

Statistical analysis

Data presented in the description and tables are the means and standard deviation of the three replicates. Data obtained in this study were subjected to one-way ANOVA in SAS (version 9.1). Significant parameters ($p < 0.05$) were compared using Duncan's multiple range (DMR) test (Duncan 1955). Linear regression analysis (best fitter model based on R^2) was performed on WG% data.

Results

Growth and feed utilization

Dietary supplementation with MSP significantly ($p < 0.05$) enhanced the growth performance and feed utilization of *C. batrachus* compared to the control group (Table 2). Significantly higher ($p < 0.05$) weight gain % was observed in fish fed with 0.5 (340.04

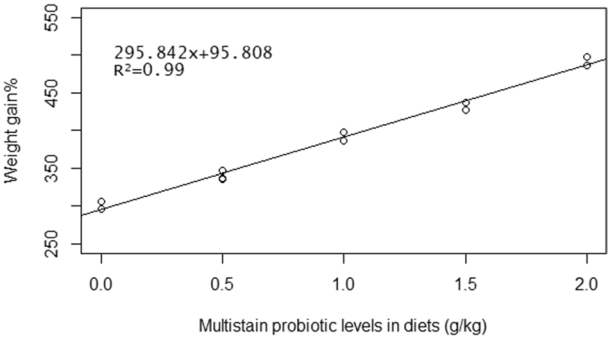

Fig. 1 Linear regression analysis on the WG% data in response to multi-strain probiotics

Table 2 Growth performance and feed utilization of *C. batrachus* fed diets supplemented with MSP

Parameters	Diets with MSP levels					*p* value
	0.0 g/kg	0.5 g/kg	1.0 g/kg	1.5 g/kg	2.0 g/kg	
IBW (g)	10.13 ± 0.01	10.12 ± 0.00	10.13 ± 0.01	10.13 ± 0.01	10.13 ± 0.01	0.588ns
FBW (g)	40.52 ± 0.57^{a}	44.57 ± 0.57^{b}	50.05 ± 0.58^{c}	53.74 ± 0.57^{d}	60.21 ± 0.58^{e}	< 0.001
WG (%)	299.73 ± 5.17^{a}	340.04 ± 5.68^{b}	393.92 ± 6.42^{c}	430.56 ± 5.69^{d}	493.98 ± 6.39^{e}	< 0.001
SGR (% day^{-1})	1.53 ± 0.01^{a}	1.64 ± 0.01^{b}	1.77 ± 0.01^{c}	1.85 ± 0.01^{d}	1.97 ± 0.01^{e}	< 0.001
FI (g fish^{-1})	69.35 ± 0.57^{a}	73.88 ± 0.58^{b}	81.78 ± 0.58^{c}	85.51 ± 0.51^{d}	86.30 ± 0.56^{d}	< 0.001
FCR	2.28 ± 0.05^{a}	2.14 ± 0.04^{b}	2.04 ± 0.02^{c}	1.96 ± 0.01^{d}	1.72 ± 0.01^{e}	< 0.001
SR (%)	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	ns

Values are shown as the mean ± SD, $n = 3$. Values in the same row with various superscripts are different significantly ($p < 0.05$). Initial body weight = IBW; final body weight = FBW; weight gain percentage = WG %; feed intake =FI; feed conversation ratio = FCR; specific growth rate = SGR; survival rate = SR are acronyms

± 5.68), 1.0 (393.92 ± 6.42), 1.5 (430.56 ± 5.69), and 2.0 (493.98 ± 6.39) g/kg MSP-supplemented diets compared with the control group (299.73 ± 5.17). The highest weight gain was observed in fish fed 2.0 g/kg MSP based on linear regression analysis (295.842x + 95.808; $R^2 = 0.99$) (Fig. 1). Similarly, significantly ($p < 0.05$) higher SGR (%/day) was observed in fish fed with 0.5 (1.64 ± 0.01), 1.0 (1.77 ± 0.01), 1.5 (1.85 ± 0.01), and 2.0 (1.97 ± 0.01) g/kg MSP-supplemented diets compared with the control group (1.53 ± 0.01). Dietary supplementation significantly reduced ($p < 0.0.5$) the FCR linearly in fish fed with 0.5–2.0 g/kg MSP-supplemented diets in comparison to the control group. The lowest FCR was observed in the 2.0 g/kg MSP treatment (1.72 ± 0.01), and the highest FCR was observed in the control group (2.28 ± 0.05). Dietary supplementation of MSP showed significantly higher ($p < 0.05$) feed intake (g/fish) of 0.5 (73.88 ± 0.58), 1.0 (81.78 ± 0.58), 1.5 (85.51 ± 0.51), and 2.0 (86.30 ± 0.56) g/kg compared with the control group (69.35 ± 0.57) after 90 days of the feeding trial. However, the highest feed intake was observed in the 1.5 and 2.0 g/kg MSP-supplemented groups, which showed nonsignificant ($p > 0.05$) differences.

Table 3 Whole-body proximate analysis of *C. batrachus* fed diets supplemented with MSP

Parameters (%)	Diets with MSP levels					*p* value
	0.0 g/kg	0.5 g/kg	1.0 g/kg	1.5 g/kg	2.0 g/kg	
Moisture	74.91 ± 0.57	74.80 ± 0.54	74.60 ± 0.56	74.54 ± 0.52	74.41 ± 0.57	0.813
Crude protein	16.75 ± 0.44	16.95 ± 0.51	17.37 ± 0.44	17.40 ± 0.45	17.64 ± 0.59	0.245
Crude fat	5.11 ± 0.35	5.24 ± 0.37	5.31 ± 0.44	5.11 ± 0.26	4.98 ± 0.16	0.766
Ash	3.93 ± 0.57	3.89 ± 0.37	3.92 ± 0.56	3.88 ± 0.55	3.69 ± 0.56	0.978

Values are shown as the mean ± SD, $n = 3$. Values in the same row without superscripts are nonsignificant to each other ($p > 0.05$)

Whole-body proximate analysis

Dietary supplementation of MSP in the diet did not influence ($p > 0.05$) the whole-body composition, as presented in Table 3. Values of proximate composition were observed as moisture (74.41–74.91%), crude protein (16.75–17.64%), crude fat (4.98–5.31%), and ash contents (3.69–3.93%).

Intestinal digestive enzyme activity

Dietary incorporation of MSP in the diets significantly improved ($p < 0.05$) the digestive enzyme activities (U/mg protein)) in the intestine of *C. batrachus* (Table 4). Amylase activities were significantly enhanced ($p < 0.05$) with increasing levels of MSP such as 0.5 g/kg (1.35 ± 0.03), 1.0 g/kg (1.55 ± 0.03), 1.5 g/kg (1.78 ± 0.03), and 2.0 g/kg (1.96 ± 0.02) compared with the control group (1.20 ± 0.01). A similar trend ($p < 0.05$) was observed in protease and lipase activities. The highest protease activities ($p < 0.05$) were observed in the 2.0 g/kg treatment group (10.51 ± 0.06), and the lowest activity was observed in the control group (8.61 ± 0.01). Similarly, fish fed the 2.0 g/kg MSP-supplemented diet showed the highest ($p < 0.05$) lipase activities (2.26 ± 0.02), and the lowest activities were observed in the control group (1.55 ± 0.02).

Table 4 Intestinal digestive enzyme activity of *C. batrachus* fed diets supplemented with MSP

Parameters	Diets with MSP levels					*p* value
	0.0 g/kg	0.5 g/kg	1.0 g/kg	1.5 g/kg	2.0 g/kg	
Amylase (U/mg protein)	1.20 ± 0.01^{a}	1.35 ± 0.03^{b}	1.55 ± 0.03^{c}	1.78 ± 0.03^{d}	1.96 ± 0.02^{e}	< 0.001
Protease (U/mg protein)	8.61 ± 0.01^{a}	8.92 ± 0.03^{b}	9.19 ± 0.03^{c}	9.59 ± 0.03^{d}	10.51 ± 0.06^{e}	< 0.001
Lipase (U/mg protein)	1.55 ± 0.02^{a}	1.85 ± 0.03^{b}	1.95 ± 0.03^{c}	2.16 ± 0.02^{d}	2.26 ± 0.02^{e}	< 0.001

Values are shown as the mean ± SD, $n = 3$. Values in the same row with various superscripts are different significantly ($p < 0.05$)

Antioxidant enzyme activity

Dietary supplementation of MSP in diets of *C. batrachus* demonstrated a significant enhancement ($p < 0.05$) in liver SOD, CAT, and GPH-X activities (Table 5). Significantly higher ($p < 0.05$) activities of CAT (U/mg prot) were observed in fish fed with 0.5 g/kg (73.86 ± 0.56), 1.0 g/kg (79.0 ± 0.59), 1.5 g/kg (83.09 ± 0.57), and 2.0 g/kg (85.54 ± 0.58) compared with the control group (70.24 ± 0.53). Similarly, significantly higher ($p < 0.05$) activities of SOD (U/mg prot) were also observed in fish fed with 0.5 g/kg (4.98 ± 0.09), 1.0 g/kg (5.21 ± 0.08), 1.5 g/kg (5.64 ± 0.09), and 2.0 g/kg (5.86 ± 0.04) MSP-supplemented diets compared with the control group (4.54 ± 0.08). Dietary supplementation of MSP at 2.0 g/kg showed the highest ($p < 0.05$) activities (μU/mg prot) of GPH-x (85.54 ± 0.58), and the lowest activities were observed in the control group (89.61 ± 0.32). Supplementation with MSP significantly reduced ($p < 0.05$) the MDA concentrations (mg/g prot) with increasing levels of MSP such as 0.5 g/kg (2.56 ± 0.03), 1.0 g/kg (2.43 ± 0.02), 1.5 g/kg (2.28 ± 0.03), and 2.0 g/kg (2.13 ± 0.06) in comparison to the control group (2.77 ± 0.02).

Hematological parameters

The hematological parameters of *C. batrachus* in response to MSP in the diets are shown in Table 6. Values of WBC, RBC, HB, and HCT showed a significant linear increase ($p < 0.05$) with increasing levels of MSP in diets compared to the control group, and the highest values were observed in 2.0 g/kg MSP-supplemented diets. Moreover, MCH showed a significant decrease ($p < 0.05$) in response to increasing MSP supplementation in diets compared to the control group. Dietary supplementation with MSP did not influence ($p > 0.05$) the MCV, MCHC, or PLT values.

Serum biochemistry

Dietary MSP supplementation significantly ($p < 0.05$) increased the activities (U/L) of ALP and reduced the activities of AST and ALT in *C. batrachus* (Table 7). The activities

Table 5 Antioxidant enzyme activity and MDA concentrations of *C. batrachus* fed diets supplemented with MSP

Parameters	Diets with MSP levels					*p* value
	(0.0 g/kg)	(0.5 g/kg)	(1.5 g/kg)	(1.5 g/kg)	(2.0 g/kg)	
SOD U/mg prot	4.54 ± 0.08^{a}	4.98 ± 0.09^{b}	5.21 ± 0.08^{c}	5.64 ± 0.09^{d}	5.86 ± 0.04^{e}	< 0.001
CAT U/mg prot	70.24 ± 0.53^{a}	73.86 ± 0.56^{b}	79.0 ± 0.59^{c}	83.09 ± 0.57^{d}	85.54 ± 0.58^{e}	< 0.001
GPH-x μU/mg prot	89.61 ± 0.32^{a}	91.96 ± 0.36^{b}	98.36 ± 0.12^{c}	102.35 ± 0.13^{d}	105.69 ± 0.17^{e}	< 0.001
MDA mg/g prot	2.77 ± 0.02^{a}	2.56 ± 0.03^{b}	2.43 ± 0.02^{c}	2.28 ± 0.03^{d}	2.13 ± 0.06^{e}	< 0.001

Values are shown as the mean ± SD, $n = 3$. Values in the same row with various superscripts are different significantly ($p < 0.05$). Superoxide dismutase = SOD; catalase = CAT; glutathione peroxidase = GPH-x; and malondialdehyde = MDA are acronyms

Table 6 Hematological parameters of *C. batrachus* fed diets supplemented with MSP

Parameters	Diets with MSP levels					*p* value
	0.0 g/kg	0.5 g/kg	1.0 g/kg	1.5 g/kg	2.0 g/kg	
WBC (10^3 μL^{-1})	30.31 ± 0.01^{a}	30.48 ± 0.09^{b}	30.67 ± 0.05^{c}	30.89 ± 0.03^{d}	31.05 ± 0.04^{e}	< 0.001
RBC (10^6 μL^{-1})	2.08 ± 0.07^{a}	2.20 ± 0.02^{b}	2.37 ± 0.08^{c}	2.46 ± 0.04^{cd}	2.57 ± 0.06^{d}	< 0.001
HB (g/dL)	8.47 ± 0.04^{a}	8.63 ± 0.05^{b}	8.86 ± 0.05^{c}	9.13 ± 0.05^{d}	9.33 ± 0.05^{e}	< 0.001
HCT (%)	24.72 ± 0.58^{a}	25.83 ± 0.56^{b}	26.58 ± 0.57^{bc}	27.00 ± 0.60^{c}	28.12 ± 0.56^{d}	< 0.001
MCV (fl)	118.60 ± 5.20^{b}	117.28 ± 3.78^{ab}	112.09 ± 4.17^{ab}	109.47 ± 1.03^{a}	109.85 ± 5.35^{a}	0.075
MCH (pg)	40.65 ± 1.52^{c}	39.18 ± 0.54^{bc}	37.39 ± 1.44^{ab}	37.03 ± 0.50^{ab}	36.74 ± 0.64^{a}	0.008
MCHC (g dL^{-1})	34.29 ± 0.62	33.42 ± 0.6	33.36 ± 0.9	33.83 ± 0.67	33.47 ± 1.04	0.588
PLT (10^5 μL^{-1})	1.62 ± 0.18	1.57 ± 0.00	1.68 ± 0.02	1.73 ± 0.07	1.77 ± 0.18	0.307

Values are shown as the mean ± SD, $n = 3$. Values in the same row with various superscripts are different significantly ($p < 0.05$). White blood cell = WBC; red blood cell = RBC; hemoglobin = Hb; hematocrit = Hct; mean cellular volume = MCV; mean cellular hemoglobin = MCH, mean cellular hemoglobin concentration = MCHC, and PLT = platelets are acronyms

Table 7 Serum biochemistry of *C. batrachus* fed diets supplemented with MSP

Parameters	Diets with MSP levels					*p* value
	0.0 g/kg	0.5 g/kg	1.0 g/kg	1.5 g/kg	2.0 g/kg	
ALP (U/L)	80.79 ± 0.42^{a}	82.82 ± 0.31^{b}	83.91 ± 0.16^{c}	86.90 ± 0.15^{d}	90.30 ± 0.45^{e}	< 0.001
AST (U/L)	13.95 ± 0.23^{a}	13.11 ± 0.23^{b}	12.10 ± 0.24^{c}	11.17 ± 0.24^{d}	10.68 ± 0.29^{e}	< 0.001
ALT (U/L)	36.23 ± 0.38^{a}	31.70 ± 0.35^{b}	27.94 ± 0.37^{c}	26.03 ± 0.33^{d}	24.05 ± 0.38^{e}	< 0.001

Values are shown as the mean ± SD, $n = 3$. Values in the same row with various superscripts are different significantly ($p < 0.05$). Alkaline phosphatase = ALP, aspartate aminotransferase = AST, and alanine aminotransferase = ALT are acronyms

of ALP were significantly ($p < 0.05$) increased in fish fed with 0.5 g/kg (82.82 ± 0.31), 1.0 g/kg (83.91 ± 0.16), 1.5 g/kg (86.90 ± 0.15), and 2.0 g/kg (90.30 ± 0.45) compared with the control group (80.79 ± 0.42). Significant ($p < 0.05$) increases in AST activities were observed in fish fed 0.5 g/kg (13.11 ± 0.23), 1.0 g/kg (12.10 ± 0.24), 1.5 g/kg (11.17 ± 0.24), and 2 g/kg (10.68 ± 0.29) MSP-supplemented diets compared with the control group (13.95 ± 0.23). Similarly, a significantly ($p < 0.05$) decreasing trend was also observed in activities, and the lowest activities were observed in the 2.0 g/kg (24.05±0.38) MSP group, while the highest activities were observed in the control group (36.23 ± 0.38).

Stress parameters

The effect of dietary MSP supplementation on the stress biomarkers is given in Table 8. Dietary supplementation with MSP decreased ($p > 0.05$) the glucose level with increasing levels of MSP, and the lowest glucose level was observed in the 2.0 g/kg (47.85 ± 0.84 g/dL) MSP group, while the highest glucose level was observed in the control group (47.26 ± 0.94 g/dL). Cortisol levels were observed to decrease ($p > 0.05$) with increasing supplementation of MSP, and the lowest level was observed in the 2.0 g/kg (8.33 ± 0.18 ng/mL^{-1}) treatment group, while the highest level was observed in the control group (8.58 ±

Table 8 Effect of dietary MSP on the stress biomarkers of *C. batrachus* fingerlings

Parameters	Diets with MSP levels					*p* value
	0.0 g/kg	0.5 g/kg	1.0 g/kg	1.5 g/kg	2.0 g/kg	
Cortisol (ng/mL^{-1})	8.58 ± 0.10	8.53 ± 0.12	8.48 ± 0.11	8.42 ± 0.18	8.33 ± 0.18	0.355
Glucose (g/dL^{-1})	47.85 ± 0.84	47.56 ± 0.51	47.41 ± 0.87	47.36 ± 0.70	47.26 ± 0.94	0.902

Values are shown as the mean ± SD, $n = 3$. Values in the same row with various superscripts are different significantly ($p < 0.05$)

0.10 ng/mL^{-1}). Both glucose and cortisol showed nonsignificant responses ($p > 0.05$) with MSP supplementation.

Discussion

Single-strain probiotics are usually used in aquaculture; however, with advancements in aquaculture, multi-strain probiotics (MSPs) may provide additional benefits due to the synergistic effects of different probiotic strains (Mohammadi et al. 2021; Puvanasundram et al. 2021). In the current study, the supplementation of MSP in the diet significantly ($p < 0.05$) improved the growth performance in terms of FW, WG%, SGR, and FCR with increasing levels up to 2.0 g/kg. Consistent with our results, MSP has been reported to enhance growth performance, such as MSP consisting of *B. subtilis*, *L. plantarum*, and *E. faecium* in *Pangasianodon hypophthalmus* (Abdel-Latif et al. 2023); *B. subtilis and B. licheniformis* in *O. niloticus* (Tachibana et al. 2020); *B. subtilis, B. licheniformis,* and *E. faecium* in *Oncorhynchus mykiss* (Merrifield et al. 2010); *Lactobacillus plantarum* and *Pediococcus pentosaceus* in *O. niloticus* (Muhammad et al. 2022); and *Lactobacillus* and *Bifidobacterium* in *Clarias gariepinus* (Ayoola et al. 2013). Probiotics have been reported to enhance digestive enzyme activities in the fish gut, which leads to improved nutrient absorption and growth (El-Haroun et al. 2006). Therefore, the enhancement of growth might be due to improvements in growth factors, absorption, and assimilation in fish (Balami et al. 2022).

In the present study, a significant ($p < 0.05$) improvement in digestive enzyme activities (amylase, lipase, and protease) was observed in *C. batrachus* fed MSP-supplemented diets. The findings of this study are consistent with previous investigations in *L. rohita* (Mukherjee et al. 2019; Saravanan et al. 2021; Ullah et al. 2020), *P. hypophthalmus* (Abdel-Latif et al. 2023; Akter et al. 2019), *O. mossambicus* (Yaqub et al. 2021), *O. niloticus* (Liu et al. 2021), and O. *mykiss* (Adel et al. 2017). Probiotics help to boost the activation and secretion of digestive enzymes (Vazirzadeh et al. 2020). Moreover, bacteria have the ability to release protease enzymes to breakdown peptide bonds within proteins, facilitating the breakdown of proteins into component monomers and free amino acids, which can help the animal's nutritional condition (Macfarlane and Cummings 1991). The majority of probiotics may secrete lipase enzymes, which stimulate the digestion of lipids, resulting in increased growth and immunity in fish (Sharma et al. 2010).

Antioxidant enzymes (SOD, GPx, CAT) are involved in the defense mechanism of fish and play an important role in protecting fish from oxidative stress. Moreover, MDA serves as an indicator of lipid peroxidation in fish species (Hoseinifar et al. 2020). The increased SOD, CAT, and GPx activities and decreased MDA content in our study are consistent

with previous research studies reported in different fish species fed probiotic-supplemented diets (Giannenas et al. 2015; Gobi et al. 2018; Kuebutornye et al. 2020; Wang et al. 2017). Probiotics are capable of producing metabolites such as glutathione, butyrate, folate, and exopolysaccharides, which are known for their antioxidant properties (Hoseinifar et al. 2020). Furthermore, probiotics also have the ability to produce or stimulate the release of GSH from the intestines, which has a favorable effect on the production of antioxidants in the liver of fish species (Mishra et al. 2015).

MSP supplementation showed nonsignificant ($p > 0.05$) results on the whole-body proximate composition of *C. batrachus*. These findings are aligned with previous studies on probiotic supplementation in *O. mykiss* (Ramos et al. 2015), *P. hypophthalmus* (Boonanuntanasarn et al. 2019), *O. niloticus* (El-Haroun et al. 2006), and *Paralichthys olivaceus* juveniles (Niu et al. 2019). In contrast, Mukherjee et al. (2019) reported a significant increase in the crude protein and crude lipid content of *L. rohita* fed a diet containing *a Bacillus* strain. The variation in the results could be attributed to differences in probiotic composition, fish species, experimental design, feed formulations, and rearing conditions.

The hematological parameters of fish are known to fluctuate in response to changes in physiological, nutritional, and environmental conditions and can serve as valuable indicators of the overall health of fish (Fazio 2019). The current results revealed a significant ($p < 0.05$) improvement in the hematological profile of *C. batrachus.* Similar to our study, previous investigations on MSP supplementation in fish diets have also reported enhanced levels of Hb, RBCs, MCH, and WBCs (Dahiya et al. 2012; Tabassum et al. 2021; Yaqub et al. 2021). Furthermore, previous research has also demonstrated that the use of MSP consisting of *L. sporogenes*, *L. acidophilus*, *B. subtilis*, *B. licheniformis*, *and S. cerevisiae in C. mrigal* (Sharma et al. 2010), as well as *L. acidophilus* and *B. subtilis* in *O. niloticus* (Aly et al. 2008), can lead to an increase in WBCs, which is also consistent with the findings of the present study. Furthermore, improvements in MCV, MCH, and MCHC suggest that fish fed the MSP diet were healthier, similar to the study reported by Gabriel et al. (2004). It can be inferred that MSP may play a role in the increase in HB and RBC content through the promotion of iron absorption in the gut. This could be attributed to the release of organic acid, which enhances the availability of iron, thus facilitating the production of more RBCs and HB (Dahiya et al. 2012). Furthermore, the observed increase in WBC levels may indicate improved innate immunity (Rajikkannu et al. 2015).

In serum biochemistry, AST, ALP, and ALT are key enzymes that serve as important biomarkers for evaluating liver and kidney functions. The reduced ALT and AST levels in our study are in line with previous studies, which demonstrated a significant reduction in AST and ALT levels in different fish species fed a combination of probiotics, i.e., *B. subtilis*, *B. licheniformis*, and *E. faecalis* (Liu et al. 2021; Wang et al. 2017). Dietary supplementation enhanced ALP activities in the current study, which is consistent with previous studies on probiotics (Gobi et al. 2018; Panigrahi et al. 2004; Sangma and Kamilya 2015; Sheikhzadeh et al. 2012). ALP activity is commonly associated with the increased production of enzymes by macrophages.

The blood glucose level is thought to be a sensitive indicator of stress (Abdel Rahman et al. 2020). Cortisol is classified as a chronic stress hormone, and its level has been used to measure stress in aquatic animals (Sadoul and Geffroy 2019). In the current study, no significant differences ($p > 0.05$) in the levels of glucose and cortisol were observed in fish fed MSP, which indicates the absence of any stress condition in fish during the rearing period. Our results are in line with the study of Tachibana et al. (2020).

Conclusion

In conclusion, MSP supplementation up to 2.0 g/kg significantly enhanced the growth performance and feed utilization of *C. batrachus*. Furthermore, MSPs also enhanced hematology, serum biochemistry, and digestive enzyme activities in fish fed MSP-supplemented diets, which indicates better health conditions. Moreover, antioxidant enzyme activities were also enhanced with the MSP-supplemented diet, indicating better stress coping capabilities. Therefore, it is recommended to supplement the diet of *C. batrachus* with 2.0 g/kg MSP to enhance growth performance and health.

Author contribution Ayesha Tanveer: Investigation, data curation, writing—original draft. Noor Khan: Supervision, methodology. Mahroze Fatima: Conceptualization, supervision. Wazir Ali: Formal analysis, writing—review and editing. Sadia Nazir: writing—review and editing. Sheeza Bano: writing—review and editing. Muhammad Asghar: writing—review and editing.

Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethical approval This study was performed after approval from the Ethical Review Committee of the University of Veterinary and Animal Sciences, Lahore.

Competing interests The authors declare no competing interests.

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