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Original Article

Isolation, probiotic screening, molecular identification, and dietary evaluation of intestinal *Bacillus cereus* (LSPNCOF_P1) and its effects on growth performance, hematological, immunological, and carcass characteristics of *Cirrhinus mrigala* fingerlings

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Abstract
Probiotics have gained increasing attention as sustainable approaches for improving fish health and reducing reliance on antibiotics. This study aimed to isolate, characterize, and evaluate the probiotic potential of intestinal bacteria from *Cirrhinus mrigala*, with a focus on their effects on growth performance, hematological, immunological, and carcass characteristics. Fourteen bacterial isolates were obtained from the intestinal tract and screened for probiotic traits. Based on superior tolerance, isolate C8 (renamed LSPNCOF_P1) was selected and identified as *Bacillus cereus* through 16S rRNA gene sequencing. A feeding trial was conducted using three dietary inclusion levels (10^5, 10^8, and 10^{10} CFU mL^{-1}) along with a control group. Growth parameters showed no significant differences among treatments; however, fish fed the diet sprayed with *B. cereus* at 10^8 CFU mL^{-1} showed numerically better weight gain, specific growth rate, and feed conversion efficiency. Hematological parameters, including RBC, Hb, and Hct, were significantly enhanced ($p < 0.05$) in probiotic-fed groups, while WBC counts decreased, indicating reduced stress and improved physiological status. Immunological responses, including NBT, lysozyme, and MPO activities, were significantly elevated ($p < 0.05$), particularly in fish fed the diet sprayed with *B. cereus* at 10^8 CFU mL^{-1}, reflecting enhanced innate immunity. Carcass composition analysis revealed increased protein content and reduced lipid levels in probiotic-fed fish. Dietary administration of *B. cereus*, sprayed onto the feed at 10^8 CFU mL^{-1}, improved health status, immune response, and flesh quality of *C. mrigala* fingerlings. The findings highlight the potential of host-associated probiotics as sustainable alternatives to antibiotics in aquaculture systems.

Keywords: aquaculture; hematology; host-associated probiotic; Indian major carp; innate immunity

1 | INTRODUCTION

Aquaculture has emerged as one of the fastest-growing food production sectors globally, playing a crucial role in ensuring food security, nutritional supply, and livelihood generation (Ngasotter *et al*. 2020; Mohd and Mushtaq 2025). However, intensification of aquaculture practices has led to increased incidence of disease outbreaks, environmental stress, and reduced growth performance, thereby necessitating the development of sustainable and health-promoting strategies (Elgendy *et al*. 2024; Carlino-Costa and Belo 2025). Traditionally, antibiotics have been widely used to mitigate disease challenges; however, their excessive and indiscriminate use has raised serious concerns regarding antimicrobial resistance, environmental contamination, and food safety (Kawsar *et al*. 2026). Consequently, there is a growing emphasis on eco-friendly alternatives, among which probiotics have gained considerable attention in recent years.

Probiotics, defined as live microorganisms that confer health benefits to the host when administered in adequate amounts, have been widely explored in aquaculture for their ability to enhance growth performance, improve feed utilization, modulate gut microbiota, and strengthen immune responses (Madhulika *et al*. 2025; Mohammed *et al*. 2025). Among various probiotic candidates, *Bacillus* spp. has been extensively studied due to their spore-forming ability, high stability under harsh environmental conditions, and capacity to produce a wide range of extracellular enzymes and antimicrobial compounds (Vijayaram *et al*. 2024; Yu *et al*. 2025). These attributes enable them to survive feed processing, colonize the gastrointestinal tract, and exert beneficial effects on host physiology.

Bacillus cereus has emerged as a promising probiotic for aquaculture because of its ability to form highly resistant endospores, enabling survival during feed processing, storage, and passage through the gastrointestinal tract (Xie *et al*. 2023). Compared with many non-spore-forming probiotics, *B. cereus* exhibits superior stability under harsh environmental conditions, making it an attractive candidate for dietary supplementation in aquatic animals (Xie *et al*. 2023). Probiotic *B. cereus* produces extracellular digestive enzymes, including proteases, amylases, lipases, cellulases and xylanases, which improve nutrient digestion and feed utilization (NavinChandran *et al*. 2014; Xie *et al*. 2023). It also synthesizes antimicrobial compounds, competitively excludes pathogenic microorganisms, modulates the intestinal microbiota, and enhances intestinal barrier integrity, thereby promoting host health (Yang *et al*. 2020; Xie *et al*. 2023). Dietary supplementation with probiotic *B. cereus* has been shown to improve growth performance, antioxidant capacity, immune responses, intestinal health, and disease resistance in several cultured fish and shellfish species, highlighting its potential as a sustainable alternative to antibiotic growth promoters in aquaculture (Xue *et al*. 2020; Yang *et al*. 2020; Chattaraj *et al*. 2026).

Cirrhinus mrigala, an important Indian major carp, contributes significantly to freshwater aquaculture production in South Asia (Bais 2018). Owing to its high consumer demand, rapid growth potential, and adaptability to diverse culture systems, it is widely cultured across India and several other countries. However, like other cultured fish species, *C. mrigala* is susceptible to stress and disease under intensive farming conditions, which can adversely affect growth, health status, and overall productivity (Pulkkinen *et al*. 2010). Therefore, identifying effective probiotic strains that can enhance growth performance and health in this species is of considerable importance.

Therefore, the objectives of the present study were to (i) isolate and characterize potential probiotic bacteria from the intestinal tract of *C. mrigala*, (ii) screen the isolates for tolerance to gastrointestinal stress conditions and identify the selected isolate using 16S rRNA gene sequencing, and (iii) evaluate the effects of dietary supplementation with the selected probiotic isolate at different inclusion levels on growth performance, hematological and immunological responses, and carcass composition of *C. mrigala* fingerlings. We hypothesized that dietary supplementation with the selected host-associated bacterial isolate would improve growth performance and enhance physiological and immune responses of *C. mrigala* fingerlings compared with the control. The study will provide insights into the application of host-associated probiotics as a sustainable strategy to improve fish health and productivity, thereby contributing to the advancement of environmentally responsible aquaculture practices.

2 | METHODOLOGY

2.1 Sample collection and preparation

Healthy adult *C. mrigala* were randomly collected from earthen ponds at the LSPN College of Fisheries, Kawardha, Chhattisgarh and starved for 24 h prior to sampling. In the laboratory, fish were humanely euthanized using an overdose of clove oil (100–150 µL L^{-1}) and maintained in the solution until cessation of opercular movement, ensuring complete loss of consciousness and death, in accordance with CPCSEA guidelines. The fish were then thoroughly washed with sterile distilled water and aseptically dissected. The intestines were carefully excised, opened longitudinally, and cleared of excreta.

2.2 Isolation, screening, and molecular identification of bacteria

2.2.1 Isolation of bacteria: Bacteria were isolated by following the methodology of Khan *et al*. (2021) with modifications. Intestinal epithelial swabs were collected aseptically using sterile cotton swabs and suspended in a fixed volume of 0.9% sterile normal saline (5 mL) to obtain an initial standardized dilution. The suspension was allowed to settle briefly to reduce particulate matter, and serial dilutions were prepared up to 10^{-6}. Aliquots of diluted samples were inoculated into nutrient broth and incubated at 30°C for 24 h for enrichment. Following incubation, 0.1 mL of the enriched culture was spread onto nutrient agar (HiMedia, Mumbai, India) plates and incubated at 37°C for 24 h. Morphologically distinct colonies were selected for further characterization. A total of 14 bacterial isolates were obtained and tested for Gram staining and tolerance test for presumptive probiotic characterization.

2.2.2 In vitro tolerance screening for probiotic potential: These tests were performed to assess acid, bile, and phenol tolerance of the isolates. Isolates exhibiting substantial growth under these stress conditions were considered tolerant, indicating their potential to survive gastrointestinal environments. Such physiological tolerance traits are key criteria for selecting robust probiotic strains. Detailed methodologies for the tolerance tests are provided in the Supplementary file (Notes S1).

2.2.3 Molecular identification by 16S rRNA sequencing: Molecular identification of the bacterial isolates was carried out through sequencing of the universal bacterial 16S rRNA gene. Briefly, genomic DNA was extracted using the pro-genome life science DNA extraction kit, and DNA quality was assessed by 1% agarose gel electrophoresis followed by visualization under gel documentation system (Vilber, Germany). DNA concentration was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). The 16S rRNA gene was amplified using universal primers 27F: 5′-AGAGTTTGATCMTGGCTCAG-3′ and 1492R: 5′-TACGGYTACCTTGTTACGACTT-3′ PCR amplification produced a single, distinct amplicon, which was confirmed by electrophoresis on a 1.2% agarose gel. The PCR product was subsequently purified to remove residual primers and impurities. Purified PCR amplicons were subjected to bidirectional sequencing using the BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI 3730xl Genetic Analyzer (Applied Biosystems, USA).

Sequence similarity searches were performed using the BLAST algorithm against the NCBI GenBank database. Multiple sequence alignment was carried out using ClustalW implemented in MEGA version 11, and phylogenetic analysis was performed using the Neighbor-Joining (NJ) method in MEGA 11, and the robustness of the tree was evaluated using 1,000 bootstrap replicates.

2.3 Preparation of probiotic-supplemented experimental diets

2.3.1 Incorporation of bacteria into commercial feed: The selected bacterial strain (*B. cereus*, LSPNCOF_P1) was aseptically incorporated into a commercial fish feed (manufactured by Maharaja Feeds, Dharshiva, Raipur, Chhattisgarh; 28.0% crude protein, 4.0% crude fat, 7.0% crude fibre, and 10.0% moisture). Briefly, the culture was grown in nutrient broth at 37°C for 24 h to obtain the required cell densities (10^5, 10^8, and 10^{10} CFU mL^{-1}). Cells were harvested by centrifugation at 4000 rpm for 10 min, washed with sterile distilled water, and resuspended in a minimal volume of sterile water. The concentrated bacterial suspension was uniformly sprayed onto pre-weighed feed pellets to achieve the target inclusion levels. The coated feed was air-dried under sterile conditions at room temperature for 1–2 h to facilitate bacterial adhesion and stability and subsequently stored in airtight containers at 4°C until use.

Following spraying and drying, the viable bacterial concentration in the treated feed was verified by total plate count to confirm the bacterial load delivered through the prepared diets. Feed samples were serially diluted in sterile saline and plated on nutrient agar, and the viable bacterial counts were determined after incubation at 37°C for 24–48 h.

2.3.2 Experimental design and feeding trial: The probiotic treatments were designated as BC-10^5, BC-10^8, and BC-10^{10}, corresponding to dietary inclusion levels of *B. cereus* at 10^5, 10^8, and 10^{10} CFU mL^{-1}, respectively, sprayed onto the commercial feed. The control group was fed the basal diet without probiotic supplementation and was designated as CTRL.

The study was conducted using a completely randomized design (CRD) comprising one control and three probiotic treatment groups, each in triplicate. Uniform glass aquaria measuring 0.6 × 0.3 × 0.45 m were used, and each tank was stocked with 10 fish. *C. mrigala* fingerlings with mean initial lengths of 7.90±0.21 cm (CTRL), 8.06±0.02 cm (BC-10^5), 8.17±0.16 cm (BC-10^8), and 7.82±0.33 cm (BC-10^{10}) were selected. The corresponding mean body weights were 4.82±0.10 g (CTRL), 4.66±0.09 g (BC-10^5), 4.92±0.48 g (BC-10^8), and 5.01±0.47 g (BC-10^{10}). All fish were acclimatized for five days prior to the initiation of the experiment. The feeding trial was conducted for a period of 90 days, during which adequate aeration was maintained, and the fish were fed twice daily at 4% of their body weight.

2.3.3 Blood collection and sample preparation: Prior to sampling, fish were anaesthetized with clove oil (50 µL L^{-1}; Merck, Germany). Blood samples were collected from randomly selected fish in each tank via the caudal vein. The blood was immediately divided into two aliquots: one

transferred into EDTA-coated vials for haematological analysis and the other into plain vials for serum collection.

2.4 Analytical measurements

2.4.1 Growth parameters: Growth performance parameters, including percentage weight gain, specific growth rate (SGR), feed conversion ratio (FCR), and survival rate were evaluated according to standard formulae described by Khademzade *et al*. (2020).

2.4.2 Haematological parameters: Haematological parameters were analysed to evaluate the physiological and health status of *C. mrigala* under different dietary probiotic treatments. Blood samples were collected from randomly selected fish in each treatment at the end of the experimental period. Key parameters assessed included total erythrocyte count (RBC), total leukocyte count (WBC), haemoglobin concentration (Hb), haematocrit (Hct), and erythrocyte indices such as mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC). Differential leukocyte counts (lymphocytes, monocytes, neutrophils, eosinophils, and basophils) were also determined. Detailed methodologies, calculation formulae, and analytical procedures for all haematological measurements are provided in the Supplementary file (Notes S1).

2.4.3 Immunological parameters: Serum and whole-blood samples were used to assess key non-specific immune indicators, including nitroblue tetrazolium (NBT) activity, lysozyme activity, myeloperoxidase (MPO) activity, and total immunoglobulin (Ig) levels. Detailed methodologies, assay conditions, and calculation procedures for all immunological analyses are provided in the Supplementary file (Note S1).

2.4.4 Fish carcass analysis: At the end of the experimental period, representative fish samples from each treatment were collected and analysed for key nutritional components, including crude protein, crude lipid, ash, crude fibre, and moisture content. Detailed analytical procedures, instruments used, and calculation methods for proximate composition analysis are provided in the supplementary material (Notes S1).

2.5 Statistical analysis

All statistical analyses were conducted using SPSS software (Version 23.0). Each tank was considered an independent experimental unit, and the three replicate tanks in each treatment were used for statistical analysis (n = 3). Individual fish within the same tank were not considered independent replicates. Differences among treatment groups were analysed using one-way analysis of variance (ANOVA), with statistical significance set at $p < 0.05$. When significant differences were detected, Duncan's multiple range test (DMRT) was applied for post hoc comparison of means. Results are presented as mean ± standard error (SE).

3 | RESULTS AND DISCUSSION

3.1 Isolation, screening, and molecular identification of bacteria

A total of 14 bacterial colonies were isolated from the intestine of *C. mrigala* based on colony morphology and Gram-staining characteristics (Table S1). These isolates were designated as C2, C3, C5, C8, C9, C11, C12, C13, C14, C17, C20, C24, C25, and C27, and their morphological and Gram-stain features are summarized in Table S1. To screen for potential probiotic candidates, all isolates were further evaluated for acid (low pH), bile salt, and phenol tolerance (Table S2).

The pH tolerance test evaluated the ability of bacterial colonies to survive under acidic conditions (pH 2.0–4.0), a key criterion for probiotic suitability (Yumnam *et al*. 2025). Among all isolates, C8 and C24 exhibited the highest acid tolerance, showing strong growth (++) across all tested pH levels, indicating excellent survivability in highly acidic environments such as the gastrointestinal tract. This consistent tolerance highlights their strong potential as probiotic candidates (Khushboo *et al*. 2023). Colony C14 also demonstrated good acid resistance, particularly at pH 2 and 3, though a slight reduction in growth was observed at pH 4. Several colonies, including C5, C12, and C17, showed moderate but stable growth across the pH range. In contrast, other isolates displayed variable or poor acid tolerance, indicating limited suitability for probiotic applications.

The bile salt tolerance test evaluated the survivability of bacterial isolates under increasing bile salt concentrations (2.5, 5, and 7.5%). Among all colonies, C8, C9, and C24 showed strong and consistent growth (++) at all bile salt levels, indicating excellent resistance to bile stress and a high potential for intestinal survival. This robust tolerance underscores their suitability as promising probiotic candidates (Reale *et al*. 2015; Rachwal *et al*. 2024). A few other isolates exhibited moderate or variable tolerance, while several colonies showed poor or no growth under bile stress, suggesting limited adaptability.

The phenol tolerance test showed clear differences in chemical stress resistance among the isolates. C3, C8, C13, and C24 exhibited strong growth (++) at all tested phenol concentrations, indicating high tolerance and excellent adaptability to harsh gut-like conditions (Shehata *et al*. 2016). Their consistent performance highlights their suitability as potential probiotic candidates. In contrast, several isolates displayed moderate, variable, or no growth under phenol stress, suggesting limited resilience.

Based on superior tolerance to acidic pH, bile salts, and phenol test, isolates C8 and C24 were selected and designated as LSPNCOF_P1 and LSPNCOF_P2, respectively, for molecular identification. Partial 16S rRNA gene sequencing followed by BLAST analysis against the NCBI GenBank database identified both isolates as members of the *B. cereus* group. Where, LSPNCOF_P1 showed 100% sequence identity with *B. cereus* strain HYM84 (Accession No. KT982241.1) with 100% query coverage, whereas LSPNCOF_P2 showed 99.72% sequence identity with several members of the *B. cereus* group, including *B. cereus*, *B. thuringiensis*, *B. paramycoides*, and other closely related *Bacillus* spp., all with 100% query coverage (Figure S1). Phylogenetic analysis (Figure 1) demonstrated that LSPNCOF_P1 clustered closely with reference strains of *B. cereus*, whereas LSPNCOF_P2 clustered within the *B. cereus* group, consistent with the BLAST-based identification. The 16S rRNA gene sequences of LSPNCOF_P1 and LSPNCOF_P2 have been deposited in the NCBI GenBank database under accession numbers PZ724622 and PZ724623, respectively.

Both C8 (LSPNCOF_P1) and C24 (LSPNCOF_P2) were identified as *B. cereus* group isolates and showed similar performance in the acid, bile salt, and phenol tolerance assays (Table S2). As no clear difference was observed between the two isolates in the preliminary probiotic screening, C8 (LSPNCOF_P1) was selected for the subsequent feeding experiment as the representative isolate, while C24 (LSPNCOF_P2) was not taken forward for dietary evaluation.

3.2 Growth parameters

Growth performance of *C. mrigala* fed diets sprayed with *B. cereus* suspension is presented in Figure 2. All experimental groups exhibited comparable initial body weights (Table 1), ensuring uniformity at the start of the trial. At the end of the feeding period, no statistically significant differences ($p > 0.05$) were observed among treatments for the evaluated growth parameters, as indicated by the same superscript letters in the Figure 2 and Table 1. However, numerical variations suggested a positive trend in probiotic-supplemented groups, particularly at higher inclusion levels.

The highest weight gain (g) and percentage weight gain (%) were recorded in the BC-10^8 group, followed by BC-10^{10} and BC-10^5, while the control group exhibited comparatively lower values. Similarly, specific growth rate (SGR) and average daily weight gain (ADWG) followed the same trend, with BC-10^8 showing the best performance among all treatments. These improvements, although not statistically significant, indicate better growth efficiency in fish receiving probiotic-supplemented diets. Feed conversion ratio (FCR) showed an inverse trend, where lower values indicate better feed utilization (Fry *et al*. 2018). The lowest FCR was observed in the BC-10^8 group, suggesting improved feed efficiency compared to the control and other treatments. The control group recorded the highest FCR, reflecting comparatively less efficient feed utilization (Fry *et al*. 2018).

The absence of significant differences among treatments may be attributed to the relatively short experimental duration or adequate baseline nutrition provided by the control diet. Nevertheless, the numerical growth trends observed in the probiotic-fed groups, particularly at the 10^8 CFU mL^{-1} inclusion level, may indicate a potential beneficial effect; however, this observation requires further investigation because the differences in growth performance were not statistically significant.

The numerical growth trend observed in the present study may potentially be related to the reported ability of *B. cereus* to secrete extracellular digestive enzymes, including proteases, amylases, and lipases, which may enhance nutrient digestion and feed utilization (NavinChandran *et al*. 2014; Xie *et al*. 2023). In addition, previous studies have suggested that *B. cereus* may influence intestinal microbial balance and nutrient utilization, which could contribute to growth and feed efficiency (Xie *et al*. 2023). Similar improvements in growth performance following dietary supplementation with probiotic *B. cereus* have been reported in Pengze crucian carp (Yang *et al*. 2020), grass carp (Xue *et al*. 2020), tambaqui (Dias *et al*. 2018), and *Clarias magur* (Chattaraj *et al*. 2026). Previous studies also indicate that *Bacillus* spp. improve growth and feed efficiency in fish by enhancing digestive processes and nutrient bioavailability (Dighiesh *et al*. 2024; Rangel *et al*. 2024; Madhulika *et al*. 2025; Yu *et al*. 2025).

3.3 Hematological parameters

Hematological and immunological parameters reflect the overall health, nutritional condition, and physiological status of fish, and are influenced by factors such as age, size, species, diet, and culture environment (Taherpour *et al*. 2023; Madhulika *et al*. 2024). In the present study, the haematological parameters varied significantly ($p < 0.05$) among treatments, highlighting the influence of probiotics on physiological condition and stress response (Figure 3).

Red blood cell (RBC) count was highest in the probiotic-fed groups, particularly in BC-10^8 ($1.98\pm0.04 \times 10^6$ μL^{-1}) and BC-10^{10} ($1.95\pm0.03 \times 10^6$ μL^{-1}), while the lowest value was recorded in the control group ($1.39\pm0.01 \times 10^6$ μL^{-1}). Higher RBC levels indicate improved oxygen-carrying capacity of blood, which supports better metabolism, energy production, and overall fish performance (Madhulika *et al*. 2024). This improvement suggests that probiotics may enhance nutrient absorption and stimulate blood cell formation, as reported in earlier studies (Nayak 2010; Saravanan *et al*. 2021; Yu *et al*. 2025). Haemoglobin (Hb) levels followed a similar pattern. Fish in BC-10^8 group showed the highest Hb concentration

(9.16±0.14 g dL^{-1}), followed by BC-10^{10} (8.83±0.06 g dL^{-1}) and BC-10^{6} (8.60±0.11 g dL^{-1}), while the control group had the lowest value (6.49±0.24 g dL^{-1}).

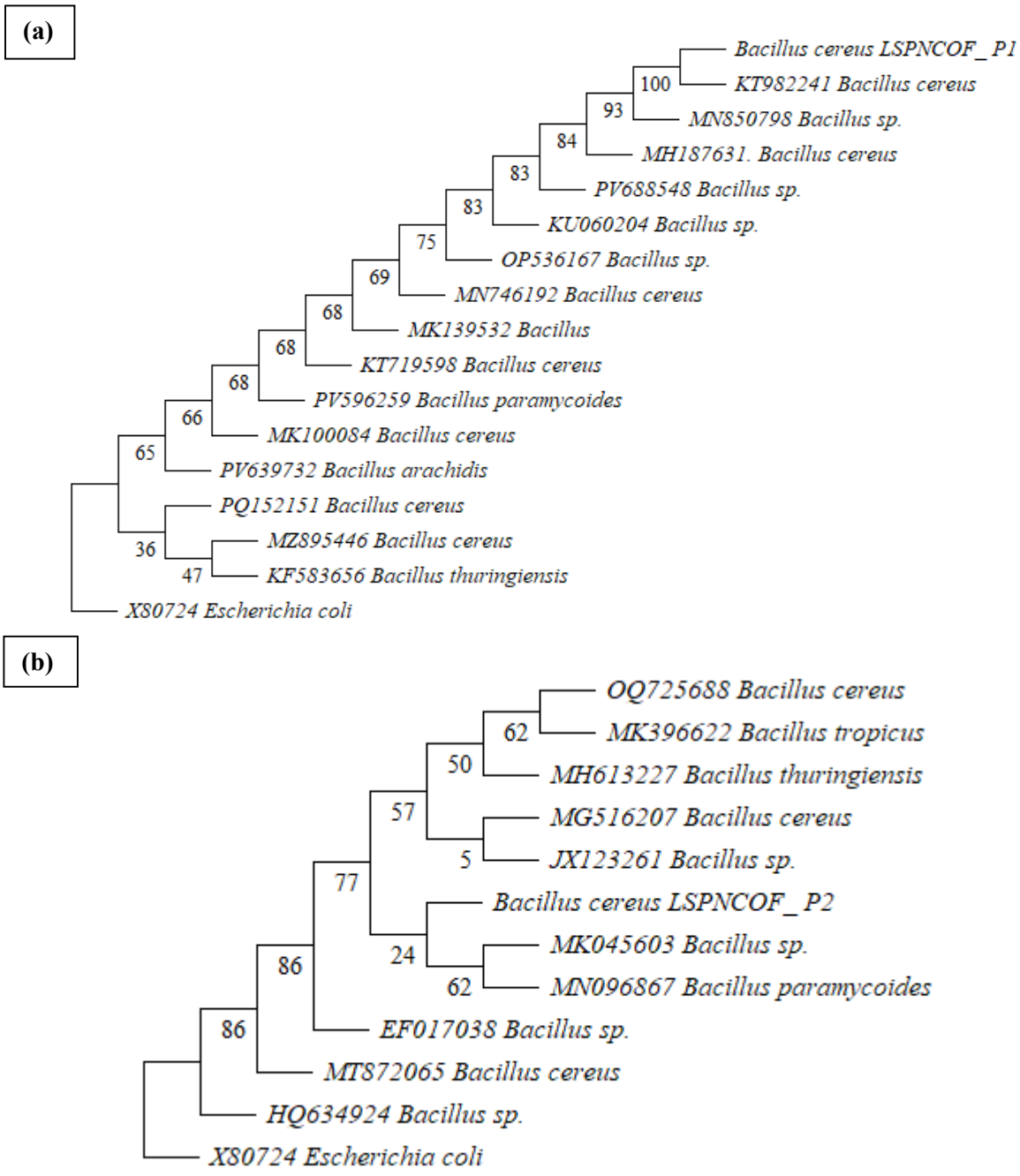

FIGURE 1 Neighbor-joining phylogenetic trees based on partial 16S rRNA gene sequences showing the phylogenetic relationships of probiotic isolates LSPNCOF_P1 (a) and LSPNCOF_P2 (b) with closely related *Bacillus* reference strains retrieved from the NCBI GenBank database.

TABLE 1 Initial and final body length and weight of *Cirrhinus mrigala* (mean ± SE, *n* = 3).

Parameters	Treatments			
	Control	BC-10^{5}	BC-10^{8}	BC-10^{10}
Initial body length (cm)	7.9±0.21^{a}	8.06±0.02^{a}	8.17±0.16^{a}	7.82±0.33^{a}
Final body length (cm)	10.0±0.13^{a}	10.39±0.02^{a}	11.56±0.37^{a}	10.76±0.20^{a}
Initial body weight (g)	4.82±0.10^{a}	4.66±0.09^{a}	4.92±0.48^{a}	5.01±0.47^{a}
Final body weight (g)	8.86±0.67^{a}	9.04±0.10^{a}	9.91±0.33^{a}	9.56±0.15^{a}

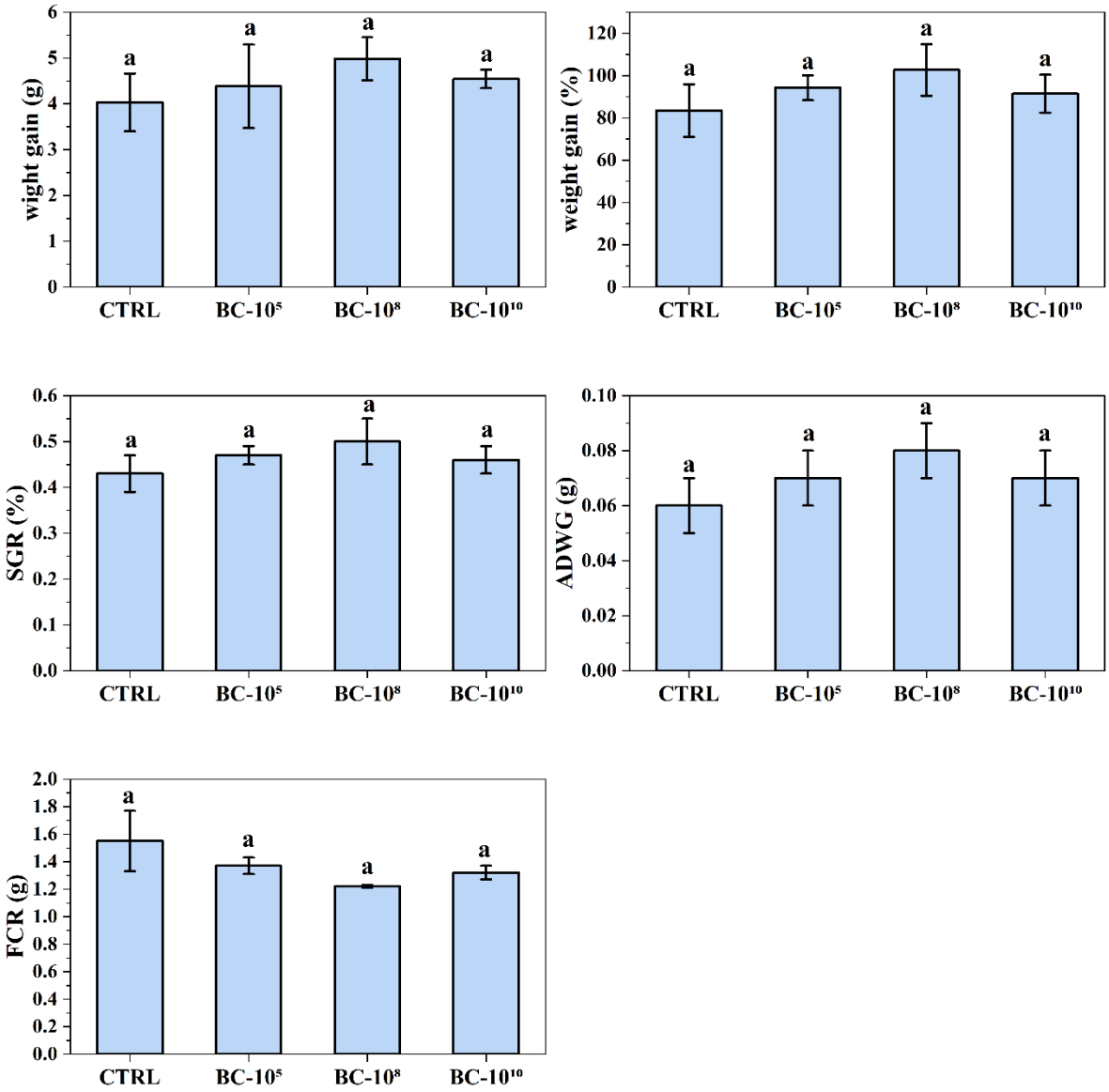

FIGURE 2 Growth parameters of *Cirrhinus mrigala* in control and *Bacillus cereus* fed groups. Data are represented as mean ± SE (n = 3). Different letters indicate significant difference (p<0.05). Bars sharing the same letter are not significantly different.

Higher Hb levels improve oxygen transport efficiency and support faster growth and better health, indicating that probiotic-fed fish were in a stronger physiological condition (Taherpour *et al*. 2023). In contrast, white blood cell (WBC) counts were highest in the control group (30.38±0.06 ×10^3 µL^{-1}) and significantly lower in probiotic treatments, with the lowest count observed in BC-10^8 (24.95±0.23 ×10^3 µL^{-1}). Elevated WBC levels in the control group may reflect higher stress or immune challenge, whereas lower and balanced WBC counts in probiotic-fed fish suggest reduced pathogen pressure and better health status (Dügenci *et al*. 2003). Haematocrit (Hct) values also improved with probiotic supplementation. The highest Hct was recorded in BC-10^8 (36.54±0.11%), compared to the lowest value in the control group (29.63±0.88%). Higher Hct indicates improved blood volume and red cell concentration, which is associated with better oxygen supply and overall vitality (Merrifield *et al*. 2010; Madhulika *et al*. 2024). Mean corpuscular indices (MCV, MCH, and MCHC) showed overall improvement in probiotic-fed groups, indicating healthier red blood cell structure and haemoglobin content. Differential leukocyte counts further supported immune enhancement, as monocytes, neutrophils, and eosinophils were generally

higher in BC-108 and BC-10^{10}. The increase in hematological parameters in the probiotic-supplemented groups suggests improved innate immune responses (Yu *et al*. 2025).

Probiotic supplemented groups, particularly BC-10^{8} and BC-10^{10}, showed significantly higher levels of haematological parameters. Similar results have been seen in studies with *Bacillus* spp. as probiotics in *Oreochromis niloticus* (Yu *et al*. 2025), *Oncorhynchus mykiss* (Taherpour *et al*. 2023), *Labeo rohita* (Saravanan *et al*. 2021).

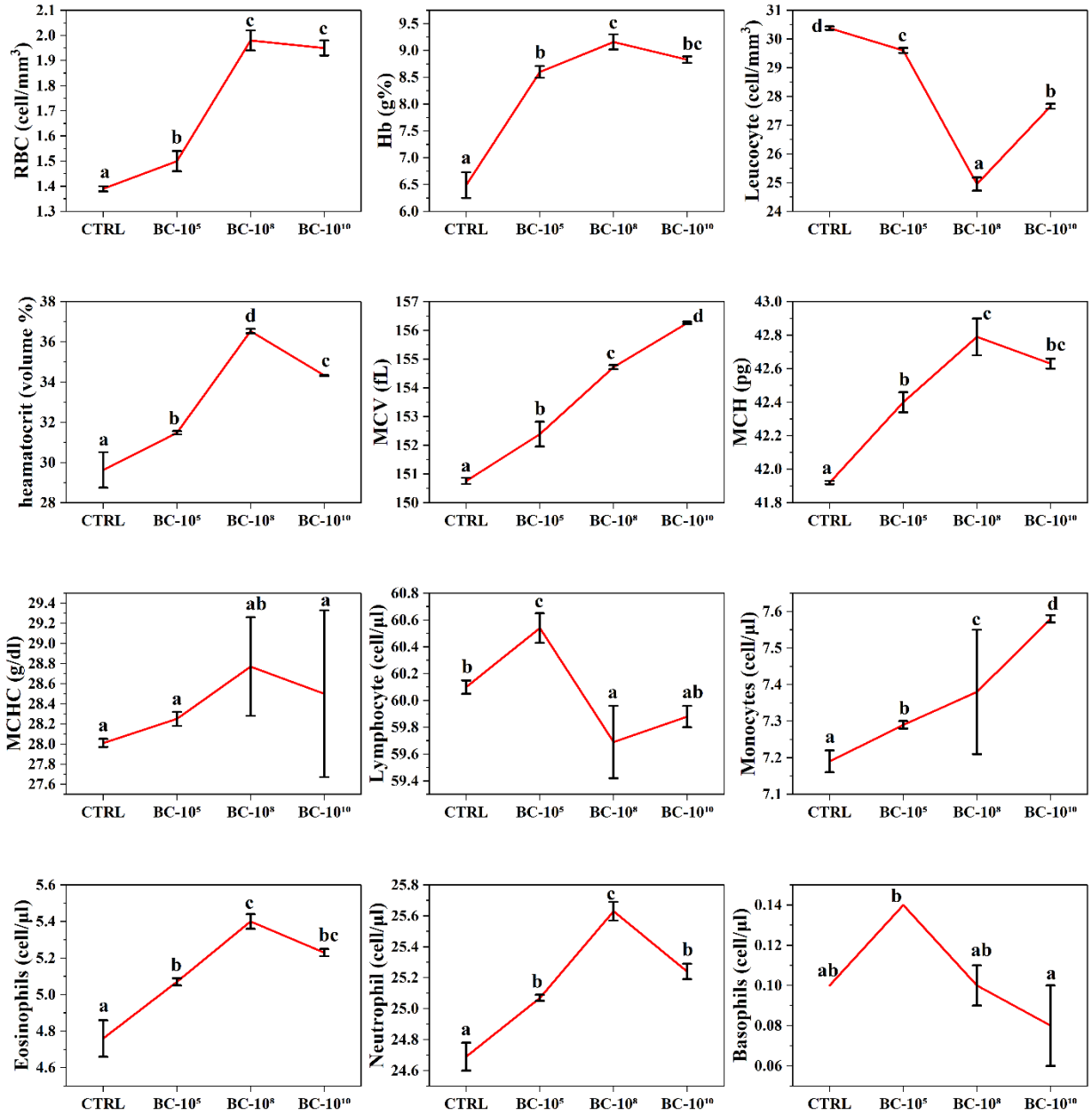

FIGURE 3 Hematological parameters of *Cirrhinus mrigala* in control and *Bacillus cereus* fed groups. Data are represented as mean ± SE (n = 3). Different letters indicate significant difference ($p < 0.05$).

3.4 Immunological parameters

Studies have suggested that probiotic supplementation enhances the immune response in fish (Nayak 2010; Madhulika *et al*. 2025). The assessment of immune parameters revealed significant differences ($p < 0.05$) among the experimental groups (Figure 4). Nitro blue tetrazolium (NBT) activity indicates the respiratory burst and phagocytic ability of immune cells and is an innate

immune response in fish (Bhatnagar and Mann 2025). NBT levels were lowest in the control group (0.42±0.17) and highest in BC-10^8 group (0.72±0.01), followed by BC-10^{10} (0.65 ± 0.18). The elevated NBT activity in probiotic-fed groups suggests enhanced oxidative killing capacity of neutrophils and macrophages, allowing fish to mount a stronger defence against invading pathogens. Similar probiotic-induced stimulation of respiratory burst activity has been reported in fish (Shadrack *et al*. 2023; Bhatnagar and Mann 2025).

Lysozyme activity was also significantly different (p < 0.05) in treatment groups. The highest activity was observed in BC-10^8 (9.43±0.75), followed by BC-10^{10} (8.55±0.08) and BC-10^5 (6.69±0.14), whereas the control group showed the lowest value (3.41±0.26). Lysozyme activity is an important antimicrobial enzyme responsible for degrading bacterial cell walls and is a measure of non-specific immune defence response (Mokhtar *et al*. 2023; Shawky *et al*. 2023; Shadrack *et al*. 2023; Elbahnaswy *et al*. 2024). Enhancement in lysozyme levels suggest that probiotic supplementation may stimulate the production of natural antibacterial enzymes, thereby strengthening the primary defence mechanisms of fish against infections (Mokhtar *et al*. 2023). Comparable findings have been reported in earlier studies demonstrating the role of probiotics in enhancing lysozyme-mediated immunity in fish (Jinendiran *et al*. 2019; Jinendiran *et al*. 2021; Shadrack *et al*. 2023; Elbahnaswy *et al*. 2024).

Myeloperoxidase (MPO) activity reflects neutrophil activation and microbial killing through reactive oxygen species (Jinendiran *et al*. 2021). MPO activity was significantly (p < 0.05) highest in BC-10^8 (0.75±0.02) and BC-10^{10} (0.65±0.03), while comparatively lower values were observed in the control and BC-10^5 groups. These results suggest that probiotic supplementation enhances cellular immune responses, thereby improving the capacity of fish to eliminate pathogens, consistent with findings reported by (Jinendiran *et al*. 2021; Jinendiran *et al*. 2019).

In addition to innate immune responses, total immunoglobulin (Ig) levels were also significantly different (p < 0.05) across the treatments. The highest Ig concentration was recorded in BC-10^8 (0.56±0.01), followed by BC-10^5 (0.46±0.01), control (0.43±0.02) and BC-10^{10} (0.42±0.01) exhibited comparatively lower values. Immunoglobulins are glycoproteins produced by white blood cells that help prevent disease by recognizing and binding to specific antigens (Nayak 2010; Jinendiran *et al*. 2021; Mokhtar *et al*. 2023). Other studies have also found alteration in total immunoglobulin levels in fish fed with probiotic supplemented diets (Jinendiran *et al*. 2021; Das *et al*. 2013). These results suggest that probiotics may also enhance antibody production, thereby contributing to improved adaptive immune defence in fish.

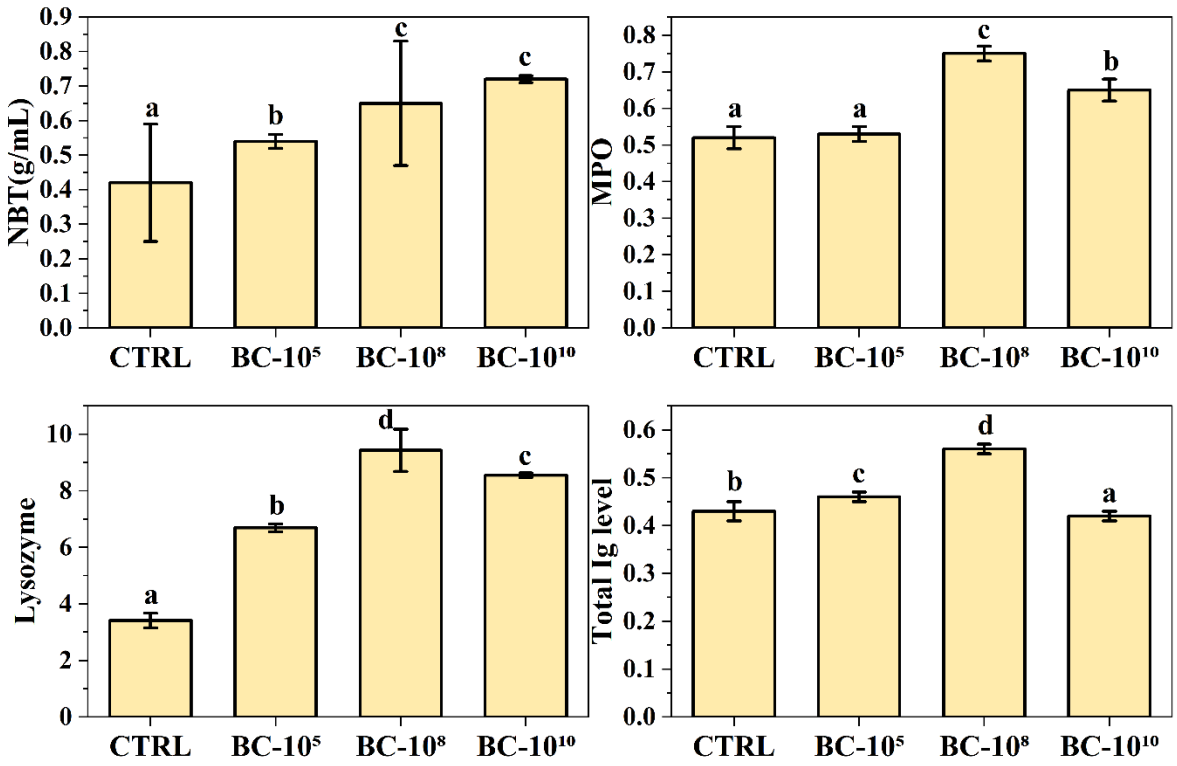

FIGURE 4 Immunological parameters of *Cirrhinus mrigala* in control and *Bacillus cereus* fed groups. Data are represented as mean ± SE (n = 3). Different letters indicate significant difference (p < 0.05).

The enhanced innate immune responses observed in this study are consistent with the immunomodulatory properties of probiotic *B. cereus*. Cell wall components and bioactive metabolites produced by *B. cereus* stimulate phagocytic cells and enhance respiratory burst activity and antimicrobial enzyme production (Yang *et al*. 2015; Zhao *et al*. 2016). Furthermore, *B. cereus* has been reported to strengthen intestinal barrier function by upregulating tight-junction proteins while suppressing inflammatory responses through modulation of antioxidant pathways (Xue *et al*. 2020; Yang *et al*. 2020). These mechanisms collectively contribute to enhanced disease resistance and overall fish health (Chattaraj *et al*. 2026).

3.5 Fish carcass analysis

The fish carcass analysis showed significant difference ($p < 0.05$) among treatments (Figure 5). Protein content was significantly higher in probiotic-fed groups, with the highest value in BC-10^8 (18.37±0.26%), followed by BC-10^{10} (16.61±0.24%) and BC-10^5 (15.34±0.48%), while the control group showed the lowest value (14.06±1.09%). Fat content showed the opposite trend, with the lowest level in BC-10^8 (4.16±0.04%) and the highest in the control group (5.45±0.02%). Fibre content was significantly higher in BC-10^8 (7.87±0.01%), and lowest in control group (6.71±0.11%). Ash content slightly declined in probiotic treatments, with the lowest value in BC-10^8 (1.99±0.01%) compared to the control (2.39±0.03%); moisture content was also higher in probiotic-fed fish, particularly in BC-10^8 (75.9±0.07%). Hussain *et al*. (2021) have reported similar results in *C. carpio* fed with probiotics. Other studies also report significant changes in fish carcass analysis when fed with probiotics supplemented feed (Islam *et al*. 2021; Shawky *et al*. 2023; Ye *et al*. 2023).

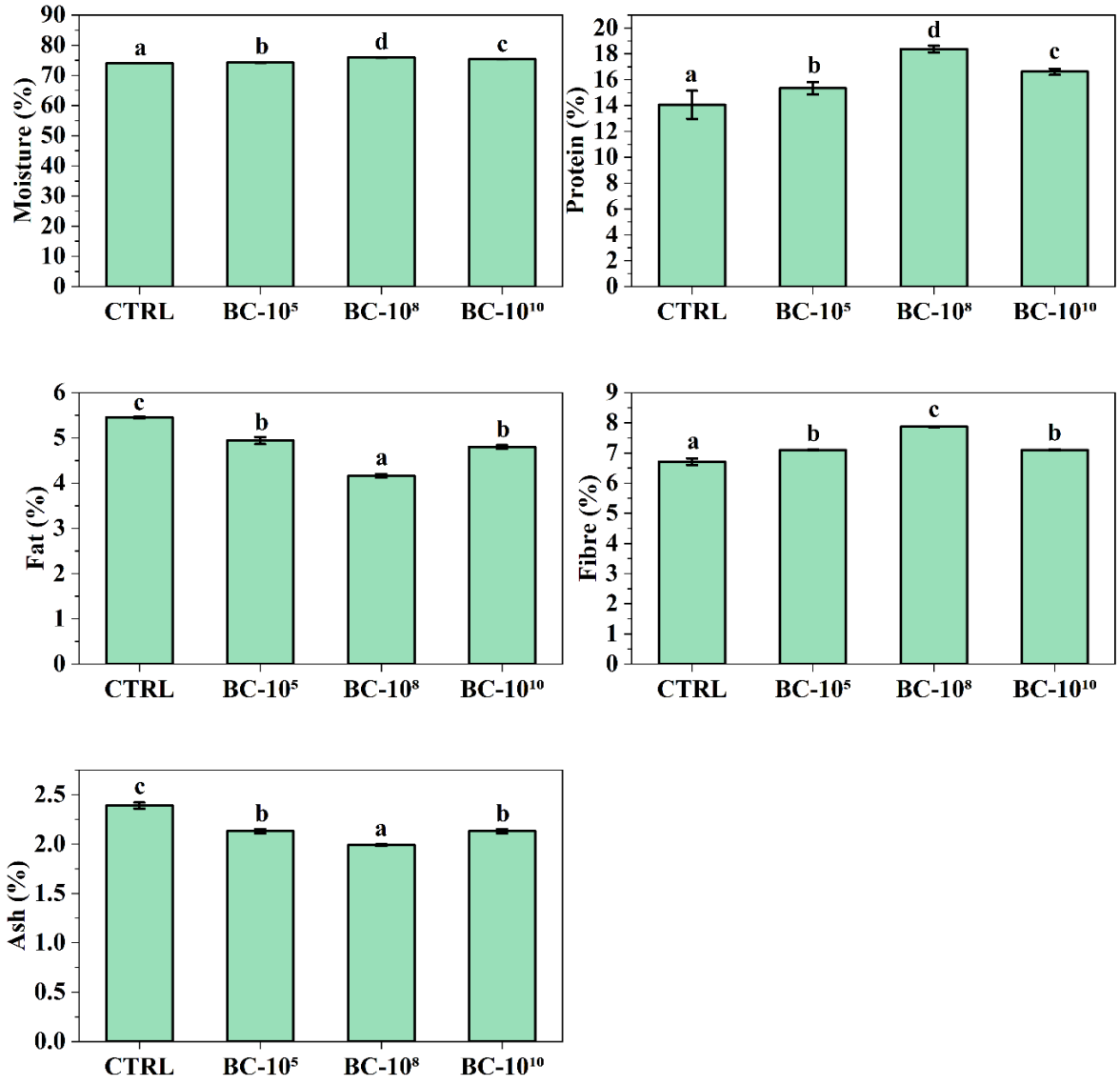

FIGURE 5 Fish carcass analysis of *Cirrhinus mrigala* in control and *Bacillus cereus* fed groups. Data are represented as mean ± SE ($n = 3$). Different letters indicate significant difference ($p < 0.05$).

3.6 Safety considerations and study limitations

Although LSPNCOF_P1 exhibited favourable acid, bile salt, and phenol tolerance and produced beneficial responses in *C. mrigala*, these characteristics alone do not establish the safety of the strain as a probiotic. The *B. cereus* group comprises strains with considerable genomic and phenotypic diversity, including strains capable of producing virulence-associated toxins (Baldwin 2020; Chang *et al*. 2026). In the present study, dedicated safety characterization, including screening for toxin- and virulence-associated determinants, was not performed. Therefore, LSPNCOF_P1 should be considered a potential probiotic candidate rather than a confirmed safe probiotic strain. Further investigations involving comprehensive strain-level safety assessment, including virulence/toxin gene profiling and other appropriate pathogenicity-related tests, are required before recommending this isolate for practical aquaculture use.

4 | CONCLUSIONS

The present study demonstrated the successful isolation, characterization, and application of a host-associated probiotic, *B. cereus* (LSPNCOF_P1), from the intestine of *C. mrigala*. The isolate exhibited strong tolerance to gastrointestinal stress conditions, confirming its suitability as a potential probiotic candidate for aquaculture applications. Dietary supplementation of *B. cereus* at different inclusion levels revealed notable improvements in physiological and health-related parameters of the experimental fish. Although growth performance parameters did not show statistically significant differences among treatments, numerical differences in weight gain, specific growth rate, and feed conversion ratio were observed, with the most favourable numerical values recorded at the 10^8 CFU mL^{-1} inclusion level. More importantly, significant enhancements in hematological parameters, including RBC, Hb, and Hct, along with reduced WBC counts, indicated improved physiological condition and reduced stress in probiotic-fed fish. The marked elevation in innate immune responses, such as NBT, lysozyme, and MPO activities, further confirmed the immunostimulatory potential of the probiotic. Additionally, improvements in carcass composition, characterized by higher protein and lower lipid content, highlight the beneficial role of *B. cereus* in enhancing flesh quality. The study highlights the potential of host-derived probiotics as candidates for sustainable and effective alternatives to antibiotics, contributing to improved fish health, productivity, and environmentally responsible aquaculture practices.

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ETHICAL APPROVAL

The use of animals conforms to the existing laws in India. The care and treatment of fish used in this study were in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Fisheries, Animal Husbandry and Dairying, Department of Animal Husbandry and Dairying, Government of India, on the care and use of animals in scientific research.

CONFLICT OF INTEREST

The author declares no conflict of interest.

AUTHORS' CONTRIBUTION

Jaya Khutiyare: Investigation, Methodology, Software, Writing-Original draft; Pabitra Barik: Conceptualization, Formal Analysis, Resources, Supervision, Validation, Writing-review and editing; T. Madhulika: Visualization, Validation, Writing-review and editing. Soibam Ngasotter: Visualization, Validation, Writing-review and editing; Deepika Kurre: Investigation, Methodology, Writing-original draft; Md. Idrish Raja Khan: Visualization, Validation, Writing-review and editing; Mutum Deepti: Visualization, Writing-review and editing; Kamalesh Panda: Supervision, Validation; Jitender Kumar Jakhar: Supervision, Validation; Dushyant Kumar Damle: Supervision, Validation; and Kishore Kumar Krishnani: Supervision, Validation.

DATA AVAILABILITY STATEMENT

The 16S rRNA gene sequences generated in this study have been deposited in the NCBI GenBank database under accession numbers PZ724622 and PZ724623. All other data supporting the findings of this study are included in this article and are available from the corresponding author upon reasonable request.

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