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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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Note S1

1. Tolerance test

1.1. pH tolerance test

To evaluate the potential probiotic properties of the bacterial isolates, acid tolerance assays were performed under *in vitro* conditions. For the pH tolerance test, bacterial cultures were inoculated into nutrient broth adjusted to pH 2.0, 3.0, and 4.0 using 1 N HCl or NaOH. The inoculated broths were incubated at 37 °C for 24 h, after which bacterial survival was assessed by measuring turbidity and/or viable cell counts (CFU/mL).

1.2. Phenol tolerance test

Phenol tolerance was evaluated by culturing the isolates in nutrient broth supplemented with 0.1%, 0.2%, and 0.4% (w/v) phenol. The inoculated cultures were incubated under identical conditions as above, and bacterial growth was assessed after 24 h by measuring optical density at 600 nm (OD_{600}) using a spectrophotometer.

1.3. Bile salt tolerance test

Bile tolerance assay was performed following the method of Nikoskelainen et al. (2001). Bacterial isolates standardized to an optical density (OD_{600}) of 0.25 were resuspended in phosphate-buffered saline (PBS; pH 7.4) supplemented with 2.5%, 5%, and 7.5% (w/v) bile salts (Hi-Media, India). The suspensions were incubated at 30 °C for 1.5 h, after which absorbance was measured at 600 nm using a spectrophotometer (Thermo Scientific, USA).

2. Haematological parameters

2.1. Total erythrocyte count (RBC)

The erythrocyte (RBC) count was determined following the method described by Schaperclaus (1991). Blood was aspirated into a dry erythrocyte pipette up to the 0.5 mark, after which Hayem's solution (Qualigens, India) was drawn to the 101 mark, resulting in a dilution of 1:200. The contents were thoroughly mixed by gently shaking the pipette for approximately 1 min. Subsequently, the diluted sample was loaded onto a counting chamber, and erythrocytes were enumerated under a trinocular microscope at 400× magnification. Cells were counted in five group squares, with each group square comprising 16 smaller squares. All erythrocytes within the selected group squares, including those touching the left and lower boundary lines, were included in the count. The final erythrocyte count was calculated using the following formula:

$$RBC\ (\frac{cells}{mm^3}) = \frac{Erythrocytes\ in\ 80\ small\ squares}{80 \times 1/400 \times 1/10 \times 1/200} \quad (1)$$

2.2. Total leucocyte count (WBC)

The total leukocyte count was determined following the method of Schaperclaus (1991). Blood was drawn into a WBC pipette up to the 0.5 mark, and WBC diluting fluid (Qualigens, Mumbai) was then added up to the 101 mark to obtain a dilution of 1:200. The contents of the pipette were gently mixed by rotating it for 2–3 min to ensure uniform dilution. A small drop of the diluted blood was then introduced into the counting chamber. After allowing the cells to settle for approximately 3 min, leukocytes were counted under a microscope in the four large corner squares of the chamber, each delineated by triple boundary lines (1 mm^3 area). The final leukocyte count (LC) was calculated using the following formula:

$$WBC\ (\frac{Cells}{mm^3}) = \frac{1\ no.of\ leucocytes\ in\ 4\ squares\ of\ 1\ mm^2}{24 \times 1/10 \times 1/200} \quad (2)$$

2.3. Hematocrit (HCT)

The hematocrit (HCT) value was determined following a modified method of Schaperclaus (1991). Blood was collected into a heparinized capillary tube up to the 100 mark (approximately 31 graduation units). Both ends of the tube were sealed with wax, and the tube was centrifuged at 3000 rpm for 3 min. After centrifugation, the capillary tube was placed on a hematocrit reader, and the packed cell volume was recorded. The hematocrit value was expressed as the percentage of the volume of blood cells relative to the total blood volume (volume %).

2.4. Total hemoglobin

Hemoglobin concentration was estimated using a Sahli's hemometer (Marinefield, Germany) following the method of Schaperclaus (1991). The graduated tube was initially filled with 0.1 N HCl up to the 2 mark, after which 20 µL of blood was added. The contents were mixed thoroughly to allow complete conversion of hemoglobin to acid hematin. Subsequently, distilled water was added dropwise to dilute the solution until its color matched the standard comparator. The hemoglobin concentration was then determined directly from the calibrated scale of the measuring tube.

2.5. Mean corpuscular volume (MCV)

Mean corpuscular volume (MCV) was estimated to determine the average volume of individual erythrocytes and is expressed in femtoliters (fL or 10^{-15} L). The MCV was calculated using the standard formula described by Lewis et al. (2001).

$$MCV = \frac{1\ Haematocrit\ or\ PCV\ (\%) \times 10}{RBC\ count\ (EC\ in\ million/mm^3)} \quad (3)$$

2.6. Mean corpuscular hemoglobin (MCH)

Mean corpuscular hemoglobin (MCH), also referred to as mean cell hemoglobin, represents the average mass of hemoglobin present per RBC in a given blood sample. It indicates the absolute amount of hemoglobin contained within an individual erythrocyte and is expressed in picograms (pg) per cell. The MCH value was calculated using Hb concentration and RBC count, following the standard equation described by Lewis et al. (2001).

$$MCH = \frac{Haemoglobin\ (g/dL) \times 10}{RBC\ count\ (EC\ in\ million/mm^3)} \quad (4)$$

2.7. Mean corpuscular hemoglobin concentration (MCHC)

Mean corpuscular hemoglobin concentration (MCHC) represents the average concentration of hemoglobin within packed RBCs and is expressed as grams of hemoglobin per 100 mL of packed cells. The MCHC was calculated using the standard formula described by Lewis et al. (2001).

$$MCHC = \frac{1\ Hemoglobin\ (g/dL) \times 10}{2\ Hematocrit\ (or)\ PCV\ (\%)} \quad (5)$$

2.8. Total lymphocyte count

The total leukocyte count was initially determined using a hemocytometer with an appropriate diluting fluid, such as Turk's solution, which lyses erythrocytes and stains leukocyte nuclei to facilitate clear visualization. A known volume of diluted blood was then introduced into a Neubauer counting chamber, and leukocytes were enumerated under a microscope at low magnification. Following this, a differential leukocyte count (DLC) was performed by preparing a well-spread peripheral blood smear and staining it with a Romanowsky-type stain (Giemsa, Wright, or Leishman stain). A minimum of 100 leukocytes were examined under oil immersion, and the proportion of lymphocytes was recorded. The absolute lymphocyte count was subsequently calculated by multiplying the total leukocyte count by the percentage of lymphocytes obtained from the differential count.

2.9. Monocyte count

Fresh venous blood was collected in an anticoagulant (EDTA), and the total leukocyte count was initially determined using a hemocytometer following dilution with a leukocyte diluting fluid such as Turk's solution, which lyses erythrocytes and stains leukocyte nuclei for improved visualization. A peripheral blood smear was subsequently prepared, air-dried, and stained using a Romanowsky-type stain (Wright, Giemsa, or Leishman). Under oil immersion, a minimum of 100 leukocytes were examined, and the percentage of monocytes was recorded. The absolute monocyte count (AMC) was then calculated by multiplying the total leukocyte count by the percentage of monocytes obtained from the differential leukocyte count.

2.10. Eosinophil count

A peripheral blood smear was prepared, air-dried, and stained using a Romanowsky-type stain (Giemsa, Wright, or Leishman). Under oil immersion, at least 100 leukocytes were examined, and the percentage of eosinophils was recorded.

The absolute eosinophil count (AEC) was calculated by multiplying the total leukocyte count by the percentage of eosinophils obtained from the differential leukocyte count. For instance, with a total leukocyte count of 9,000 cells/µL and 4% eosinophils, the AEC would be 360 cells/µL. Additionally, earlier direct counting methods employed specialized diluting fluids (such as Pilot's or Dunger's fluid containing phloxine or eosin) to selectively stain eosinophils for enumeration using a hemocytometer.

2.11. Neutrophil count

A thin peripheral blood smear was prepared, air-dried, and stained using a Romanowsky-type stain (Giemsa, Wright, or Leishman). The stained smear was examined under oil immersion, where at least 100 leukocytes were differentiated into neutrophils, lymphocytes, monocytes, eosinophils, and basophils. The percentage of neutrophils was then recorded.

2.12. Basophil count

A peripheral blood smear was prepared, air-dried, and stained using a Romanowsky-type stain (Wright, Giemsa, or Leishman). The smear was examined under oil immersion, where leukocytes were differentiated into their respective subtypes, and the percentage of basophils was recorded. As basophils are relatively rare (typically <1% of circulating leukocytes), counting a larger number of cells (200–300 leukocytes) is recommended to improve accuracy. The absolute basophil count (ABC) was calculated by multiplying the total leukocyte count by the percentage of basophils using the following formula:

3. Immunological parameters

3.1. Nitroblue tetrazolium (NBT) assay

A volume of 0.1 mL of heparinized blood was dispensed into a microtiter plate well, followed by the addition of an equal volume of 0.2% Nitroblue tetrazolium (NBT) solution (Sigma, St. Louis, MO, USA). The mixture was incubated at room temperature for 30 min. Subsequently, 0.05 mL of the NBT–blood cell suspension was transferred to a glass tube containing 1.0 mL of N,N-dimethylformamide. The mixture was centrifuged at 3000 × g for 5 min, and the absorbance of the supernatant was measured at 540 nm using a spectrophotometer (Anderson and Siwicki 1995).

3.2. Myeloperoxidase (MPO) activity

A 10 µL serum sample was diluted with 90 µL of phosphate-buffered saline (PBS) in a 96-well microplate. To this, 35 µL of 20 mM 3,3',5,5'-tetramethylbenzidine hydrochloride (TMB) (HiMedia) and 5 mM H_2O_2 were added. After incubation for 2 min, the reaction was terminated by adding 35 µL of 4 M sulfuric acid. The plate was centrifuged for 10 min, and the supernatant was collected. The optical density (OD) was then recorded at 450 nm (Quade and Roth 1997).

3.3. Serum lysozyme activity

A 50 µL sample was mixed with 150 µL of *Micrococcus lysodeikticus* suspension (adjusted to an OD of 0.6–0.7). The decrease in absorbance was monitored at 450 nm for 5 min at 1-minute intervals (Ellis 1990).

3.4. Total immunoglobulin

Total immunoglobulin content was estimated following the modified method of Anderson and Siwicki (1995). Briefly, 0.1 mL of plasma was mixed with 0.1 mL of 12% polyethylene glycol prepared in deionized water and incubated at room temperature for 2 hours. The mixture was then centrifuged at 7000 rpm for 10 minutes to obtain a clear supernatant. The protein content in the supernatant was determined using a total protein estimation kit. Total immunoglobulin was calculated as:

$$Total\ immunoglobulin\ =\ protein\ in\ blood\ serum\ -\ protein\ content\ in\ the\ supernatant \quad (6)$$

4. Fish carcass analysis

4.1. Crude protein

The crude protein content of the samples was determined by estimating total nitrogen (N) using the Kjeldahl digestion and distillation method. The nitrogen percentage obtained was converted to crude protein by multiplying with a conversion factor of 6.25 (casein factor: 5.75).

$$Crude\ protein\ (\%) = N(\%) \times 6.25 \quad (7)$$

4.2. Ash content

A clean, dry crucible was first weighed (W_1), and approximately 2 g of the sample (W) was added. The sample was then incinerated in a muffle furnace at 600 °C for 24 h. After cooling in a desiccator, the crucible containing the ash was reweighed (W_2).

$$Ash = \frac{W_2 - W_1}{W} \times\ 100 \quad (8)$$

4.3. Crude fat

Crude fat content was determined as ether extract using a Soxhlet apparatus (SOCS PLUS, SCN 6, Pelican). The extracted fat was weighed, and the percentage was calculated as follows.

$$Crude\ fat\ (\%) = \frac{weight\ of\ extracted\ fat\ (g)}{weight\ of\ sample\ (g)} \times\ 100 \quad (9)$$

4.4. Crude fibre

Crude fibre content was estimated using FIBRA-PLUS and FES-4 (Pelican). The fibre content was calculated based on the weight difference before and after digestion.

$$Crude\ fiber\ (\%) = \frac{W_1 - W_2}{W} \times\ 100 \quad (10)$$

Where, W_1 = weight of residue after acid and alkali digestion (before ashing), W_2 = weight of ash after incineration of that residue, and W = sample weight.

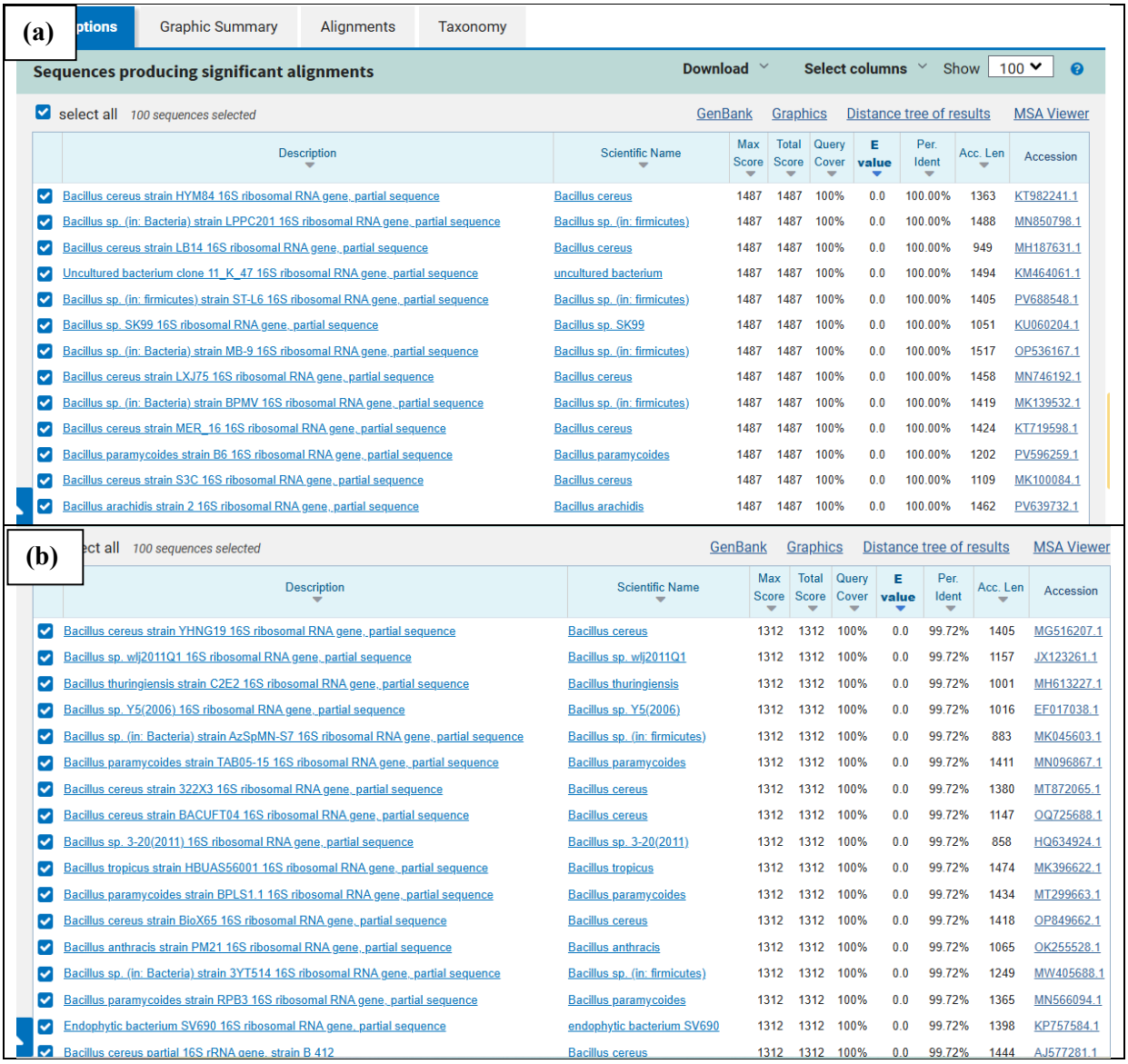

FIGURE S1 BLAST analysis of the partial 16S rRNA gene sequences of probiotic isolates LSPNCOF_P1 (a) and LSPNCOF_P2 (b) against the NCBI GenBank database showing the closest sequence matches, percentage sequence identity, query coverage, and accession numbers of the top BLAST hits.

Table S1. Morphological and Gram-stain characteristics of bacterial isolates.

Code	Shape	Colour	Texture	Gram staining
C2	Circular	White, shiny	Moist	Gram positive
C3	Irregular round	Dull creamy	Dried	Gram positive
C5	Spherical or ovoid	Creamy white	Moist	Gram negative
C8 (renamed LSPNCOF_P1)	Circular	Whitish yellow, shiny	Moist	Gram positive
C9	Spherical or ovoid	Dull, creamy	Dried	Gram negative
C11	Irregular round	Creamy white	Moist	Gram positive
C12	Circular	Dull creamy	Moist	Gram positive
C13	Spherical or ovoid	White shiny	Dried	Gram negative
C14	Circular	Shiny and creamy white	Dried	Gram negative
C17	Spherical or ovoid	Slightly yellow, shiny	Dried	Gram negative
C20	Irregular round	Creamy white	Dried	Gram negative
C24 (renamed LSPNCOF_P2)	Circular	White creamy and slightly yellow	Moist	Gram positive
C25	Circular	Slightly yellow, shiny	Moist	Gram negative
C27	Spherical or ovoid	Dull creamy	Dried	Gram positive

TABLE S2 Tolerance of bacterial isolates to acidic pH, bile salts, and phenol during probiotic screening.

Colonies	pH			Bile salt conc. (%)			Phenol conc. (%)		
	2.0	3.0	4.0	2.5	5	7.5	0.1	0.2	0.4
C2	+	+	++	-	-	-	+	+	+
C3	-	+	++	+	+	-	++	++	++
C5	+	+	+	+	+	+	-	-	+
C8 (renamed LSPNCOF_P1)	++	++	++	++	++	++	++	++	++
C9	-	-	-	++	++	++	-	-	-
C11	+	+	-	++	++	+	-	-	+
C12	+	+	+	-	-	-	-	-	-
C13	-	++	+	-	-	+	++	++	++
C14	++	++	+	+	+	+	+	+	+
C17	+	+	+	++	++	-	+	-	-
C20	-	-	-	-	-	-	+	++	+
C24 (renamed LSPNCOF_P2)	++	++	++	++	++	++	++	++	++
C25	-	+	+	++	+	+	+	+	+
C27	+	-	-	-	++	++	+	+	+

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