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Probiotics

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ABSTRACT

Gut probiotic improves growth and health of *B. gonionotus* fishes

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Euthanasia methods

1 Pure clove oil was first dissolved in ethyl alcohol in 1:9 ratio (clove oil: ethyl alcohol)

2 This solution then diluted in water in order to obtain concentrations of 0.05 mL (50 mg), and 0.20 mL (200 mg) of clove oil per 500 mL of water

3 For hematological study, experimental fish were anesthetized by using 0.05 mL clove oil per 500 mL of water

4 For histological, reproductive and intestinal microflora study, fish were euthanized by using 0.20 mL of clove oil per 500 mL of water, and death was confirmed by the destruction of the brain

Collection of probiotic samples from the gut of B. gonionotus

5 The abdomens of fish were cut aseptically by sterile scissors and the gut was taken out with care to avoid any distortion of gut and contamination with blood.

6 The gut was cut into small pieces and rinsed with 0.9% (w/v) saline solution and placed in a conical flask containing 10 ml distilled water.

7 The sample was stirred with a stirrer to make a homogenous solution.

Isolation of probiotic bacteria from the gut of B. gonionotus

8 One gram of sample was diluted in 10 ml sterilized water and inoculated on De Man, Rogosa, and Sharpe agar (MRS) media (Himedia, India)

9 The agar plates were incubated at 28^{O}C for 24 h in an incubator and the colony characteristics were observed carefully to choose desired colonies

10 The isolates were routinely sub-cultured on NA (Nutrient agar) plates and incubated at 28^{O}C and stored in a freezer at -20^{O}C supplemented with 10% glycerol.

Molecular identification of probiotic strains

11 Genomic DNA of the selected isolates was extracted by using a commercial GenJET genomic DNA purification kit (Thermo Fisher Scientific, USA) #K0721

12 DNA was amplified by using universal primer 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGATACCTTGTTACGACTT-3').

13 The PCR amplification condition was done by an initial denaturation at 94 °C for 5 min; 35 cycles of a denaturation at 94 °C for 1 min, an annealing at 57 °C for 40 sec and an extension at 72 °C for 1 min and a final extension step at 72 °C for 10 min.

14 Then the PCR amplicons were purified by using a commercial kit (Thermo Fisher Scientific, USA)

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15 *16S rRNA* gene sequencing was done by Sanger Sequencing

Preparation of probiotic strains

16 each strain was cultured in 1 litre nutrient broth in an orbital shaker and incubated at 28°C for 24 h

17 Then the broth media was centrifuged at 8000 × g for 5 min. The pelleted probiotic bacterial strains were collected and washed twice in sterile water

18 The pellets were then suspended in sterile distilled water and were added to the dough

19 A spread plate technique was used to assess the viability of cells according to the cell concentrations measured at OD600.

Acidic pH tolerance test and preparation of simulated gastrointestinal juice of host

20 The five gut probiotic bacteria were grown in MRS broth in an orbital shaker and incubated at 28°C for 24 h

21 The cultures were centrifuged at 8000 × g for 5 min at 4^0C

22 The pellets were washed and suspended in sterile phosphate-buffered saline solution (PBS).

23 Each probiotic strain was diluted 10^{-2} in sterile PBS at pH 1.0, 2.0, 3.0, 4.0 and 5.0.

24 Gastrointestinal juices were prepared fresh by dissolving pepsin (Thermo Fisher, USA) from *B. gonionotus* stomach mucosa (3 g/L) in sterilized saline solution (5g/L)

25 The pH of the gastrointestinal preparation was adjusted to 2.0 with 12 M HCl

Exposure of gut probiotics to simulated gastrointestinal juice and total viable counts

26 A 1-mL aliquot of each culture was centrifuged at 5000 × g for 10 min at 4^0C and washed three times in sterile PBS

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27 The tolerance of five probiotic bacteria to simulated gastrointestinal juices was determined by mixing 0.2 mL of each washed cell suspension with 1 mL of gastric juice.

Histological analyses of intestine and liver of the probiotic treated silver barb

28 The experimental fish were humanely killed by using clove oil (0.20 mL per 500 mL of water),

29 Death was confirmed by the destruction of the brain

30 The whole liver and part of the intestine from each fish were dissected carefully, cut to separate each other, and stored in bouins solution for 24 h

31 These samples were dehydrated in ascending grades of alcohol and cleared in xylene

32 The fixed tissues were embedded in histoparaffin (Paraplast plus; Sigma-Aldrich) and sections (7 µm) were cut using a microtome (CUT-5602, Germany)

33 Then the sections of intestinal villi and liver were selected and stained with Delafield's hematoxylin-eosin for observation under a light microscope (DM 100; Leica, Wetzlar, Germany).

34 Ten slides were prepared from the intestine of each fish through histological method.

35 Then the slides were observed under a trinocular microscope.

36 Images were captured using a digital camera (DFC 290, Leica) and the villi length of the intestine was measured using AmScope software (Version 3.7; Carl Zeiss Primo Star, Germany)

Measurement of Hematological Parameters

37 A total of 27 fish from each treatment were anesthetized with the clove oil (0.05 mL per 500 mL of water) for hematological analysis

38 Blood was collected from fish using a 3 cc syringe containing 10% blood anti-coagulant (EDTA) inserted into the caudal peduncle region to drag out blood.

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39 The blood was transferred to a test tube coated with EDTA, and stored at -30 ^{O}C until use.

40 Red blood cells (RBCs) and white blood cells (WBCs) were counted using an improved Neubauer hemocytometer (MarienFeld Company Germany) under the light microscope (DM 100; Leica, Wetzlar, Germany)

41 To measure hemoglobin, fresh blood was collected from fish from each treatment and was poured in the edge of a strip of hemoglobin meter before the coagulation of blood.

42 The glucose level of blood was measured through a glucose meter from the sample.

43 To measure packed cell volume (PCV) (%), blood was taken in a capillary tube at the marked level and sealed with gum.

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