Isolation, Identification and Biochemical Characterization of Probiotic Bacteria from the Gut region of *Channa marulius* and *Mugil cephalus*

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Abstract:
The culturable microorganisms was isolated from the intestinal content of fingerlings of *Channa marulius* and *Mugil cephalus.as well as from adult Channa marulius* length 34.4 to 44 cm and weight 275 g 305 g) *Mugil cephalus* (length 17.5 to 20 cm, weight 120 g to 425 g) In the present study, 10 bacterial strains were isolated from the gut of *Mugil cephalus* were screened for antibacterial and antagonistic activity against two major fish pathogens namely,. **PATHO-1-*Aeromonas Spp*, and PATHO-2 *Vibrio Spp*** Out of 10 isolates from *channa marulius* , 07 isolates showed zone of inhibition greater than 10 mm against the fish pathogens. Better results were obtained from *channa marulius* as compare to *Mugil cephalus.* Cross streak method revealed that the five strains in *Mugil cephalus* (CM-2, CM-4, and CM-10) show zone of inhibitions. The strain CM-3 exhibited inhibition zone greater than 10 mm against all the test pathogens, Followed by the strains CM-9, CM-10, and CM-5 which produce inhibition zone greater than 5 mm against the test pathogens. By virtue of its greater inhibition zone in the cross streak method and positive antibacterial activity, CM-3 was selected for further experiments.
Key Words: Probiotic**,** *channa marulius, Mugil cephalus,* gut region.

1. Introduction

Sanz and Palma (22) were recorded that, the gut micro organisms by means of the epithelium and mucosal immune system place network of immunological and non-immunological defenses, providing defense in opposition to pathogens, tolerance to commensal bacteria and harmless antigens.

The intestinal micro organisms investigations of fish have been motivated by the possible use of the biologically significant bacteria as supposed Probiotics **(9).** Attempts carried out on the intestinal micro organisms in fishes have shows that bacterial inhabitants influence the establishment of pathogenic microorganisms in the intestinal tract **(21, 23, 24,).** Further the focus had been given on the stability of the intestinal flora in intestine .as the pathogens enters in intestine the natural resistance of fish to infections produced by bacterial pathogens in the digestive tract **(21**). Channa marulius and Mullets (*Mugil cephalus*) have worldwide distribution and inhabit tropical and temperate waters and. They has good economical importance. Snakehead recent capture was said to be improving more hence taking spotlight amongst the aquaculture scientists. the production yields have increased from 16 tons in 1998-2000 to 42 tons in 2010-12 FAO, 2014.similarly Global aquaculture production was approximately 121365 tons while capture production was 23905 tons for grey mullet of the world in the year of 2002 (**6**).

The use of commercial probiotics made in market used to feed fish is relatively fruitless since most of the commercial preparations are obtained or prepared by using probiotic source from the strains from non-fish sources. These micro organisams unable to survive or viable at high cell density in the gut environment of fish during the active growth phase of the fish (**15**). For this reason, there is elegant logic in isolating assumed probiotics from the host in which the probiotic is intended for use. Such strains should perform better because they have already adhered to the gut wall of the fish. consequently, they are well-adapted to compete with pathogens for nutrients. Presumably, strains that develop dominant colonies in the fish intestine are good candidates for preventing the adhesion of pathogens to the gut wall.

Keeping these views in the mind the present chapter is designed to assess prospective probiotic bacteria from the gut region of the *Channa marulius* and *Mugil cephalus* further the focus on to check antibacterial activity in opposition to fish pathogenic micro organisms there was attempts carry with regard to record antagonist activity, pathogenicity test, morphological and biochemical identification of prospective probiotic bacteria isolated from both the fish species.

Materials and Methods

1. Sample collection and fish acclimatization

Channa marulius fingerlings were collected from Godavari River Nanded District (MS) (19.1383° N, 77.3210° E). The fingerlings of *Mugil cephalus* were collected from Mandovi and Zuari estuaries (latitude 15° 25' to 15° 31' N and longitude 73° 45' to 73° 59'E on the west coast of state Goa. India. The healthy fingerlings were isolated from infected ones and used for the identification of probiotic bacteria and assessment of growth associated physiological and biochemical changes in these fishes due to the incorporation of different doses of probiotics supplements. In the present investigation, *Channa marulius* and *Mugil cephalus* fingerlings fingerlings were Selected randomly here we were select infected and healthy fishes during the first week of August 2012 using a cast net. All the infected and healthy fishes were then examined at the laboratory (RSML, Latur 18.4088° N, 76.5604° E) for detail study of disease-causing agents and probiotic bacteria.

2. Isolation of culturable intestinal micro organisams

The culturable micro organisams was isolated from the intestinal content of fingerlings of *Channa marulius* and *Mugil cephalus.as well as from adult Channa marulius* length 34.4 to 44 cm and weight 275 g 305 g) *Mugil cephalus* (length 17.5 to 20 cm, weight 120 g to 425 g)

The acclimatized live fish were brought to the laboratory alive further these all fishes were sacrificed. The belly region was sterilized by using 70% ethanol and the gut region was aseptically removed from the fish abdominal cavity. The gut region, washed with the help of sterilized chilled normal saline solution (NSS) to remove feed materials, and gut impurities. The moisture blotted by using filter paper. The total weight of the gut were recorded, after weight record it was macerated with a sterile glass rod and homogenized in sterile NSS (1:10 w/v) by the use of a vortex mixer. Samples which were thoroughly macerated, homogenized gut were serially diluted in normal saline solution (NSS) after that aseptically plated by the spread plate technique on nutrient agar to determine the total plate count. The inoculated agar plates were incubated at 22–24°C for 24 to 48 h and the total bacteria were counted. The healthy fingerlings of both the species were selected and sacrificed in the laboratory; the bacterial samples were isolated by using sterile swabs on *Lactobacilli* MRS agar, MRS broth, TCBS agar, and SAA. The agar plates and broth were incubated at 37°C for 24-48 hours after that the bacterial colonies had growth on plates were examined for further characterization and identification.

3. Disc diffusion method for the study of Antibacterial activity:

The Agar well diffusion method **(13)** was used to record antibacterial activity the isolated bacteria's from both the fishes. Pathogenic bacterias like *Aeromonas spp* and Vibrio *spp* isolated from infected *Channa marulius* and *Mugil cephalus* from the study areas.

4.Isolation and identification of bacterial samples:

The healthy fingerlings of both the species were selected and sacrificed in the laboratory; the bacterial samples were isolated by using sterile swabs on *Lactobacilli* MRS agar, MRS broth, TCBS agar, and SAA. After that the agar plates and broth were incubated at 37°C for the period 24-48 hours after that the bacterial colonies had growth on plates were examined for further characterization and identification.

The morphological characteristics of bacterial isolates were recorded. The bacterial isolates were also analyzed for various biochemical tests like oxidase tests, catalase test, motility test, indole producing test, and carbohydrates fermentation (arabinose, fructose, galactose, lactose, mannitol, salicin, sucrose and trehalose). These characteristics are considered for the species level identification of bacterial isolates.

By using sterile swabs the specimen which had sacrificed the bacterial sample collected from gut region particularly intestinal region for probiotic bacteria sampling and for disease-causing agents oral region, skin, gills, fins were examined. Further inoculated the swab on *Lactobacilli* MRS agar, MRS broth, TCBS agar, and SAA.

The agar plates and broth were incubated at 37°C for 24-48 hours after that the bacterial colonies had growth on plates were examined for further characterization and identification. The various identification parameters such as colony morphology, colour, size and margin were recorded correctly. The bacterial colonies were also studied by using Gram staining and motility test along with catalase test, oxidase tests, motility test, indole producing test, and carbohydrates fermentation (arabinose, fructose, galactose, lactose, mannitol, salicin, sucrose, and trehalose) test. Lastly, all cultures were analyzed by using biochemical analysis.

5. Antagonistic activity:

Two sets of the experiment, *Aeromonas sp*., *Vibrio sp*., were conducted for 15 days. For each bacterium, 12 conical flasks (100 ml) having a culture medium (50 ml) containing a pure strain of bacterial fish pathogens, to which 2 ml of probiotic bacterial culture was added to the flask. One flask was containing only pathogenic bacteria it do not contain Probiotics micro organisams.it serve as control**.** The entire flasks were incubated at 37ºC for 15 days. Starting from the first day, the number and growth of organism were monitored using standard dilution technique i.e. 10^{-4} to 10^{-5} in a sterilized test tube and finally colony forming units (CFU/ml) was enumerated by pour plate method, similar methods were followed for both pathogens in every 5 days intervals.

6. Pathogenicity test

The pathogenicity of the most effective probiotic strains was determined by challenging *Channa marulius and Mugil cephalus* in an immersion assay and observing their health status. The experiment was conducted in glass aquaria (60×30×30 cm) with aeration facilities in the laboratory where the temperature was kept as 25±1ºC and a lighting schedule of LD 12:12..After an initial 10-day acclimation period, *Channa marulius* (mean body weight: *11.75 ± 0.75)* and *Mugil cephalus* fingerlings (mean body weight: 6.4 ± 0.5 g) were randomly distributed among the aquaria, with 10 fish per aquarium.

The *Lactobacilli* species were inoculated in MRS broth and incubated for a week on rotary shaker where the temperature was keep in between 28± 2ºC. After that culture were centrifuged and the supernatant fluid sterilized by syringe filter to collect the bacteriocin. The Muller Hinton agar plates were seed with 24hours bacterial pathogen of *Aeromonas sp* and *Vibrio sp* the culture fluid was loaded in the wells made on the agar surface. The plates were incubated at temperature 37ºC for the period of 24 – 48 hours.

7. Preliminary identification of the isolates

1. Gram's staining

Bacterial smear was taken on a clean and dryed glass slides. The Culture made by using distilled water. Smear was dried and heat fixed. Primary stain crystal violet applied on the smear further it stand for one minute, excess of the stain washed off. In next step application of the Gram"s iodine solution on smear .it was kept for one minute. next after one minute stand, Gram"s iodine washed off and slide was discolored by using 70% alcohol for fifteen seconds. At last saffranin stain (counter stain) was added, it kept for one minute further the excessive stain washed off. The prepared slide air dryed for the study. Finally observed under oil immersion microscope.

2. Endospore staining

The endospores were isolated by Schaeffer–Fulton stain technique. Bacterial strains placed on a slide heat well to fix it. Slide allowed to steam in water bath covering porous paper over it. Malachite green applied to the slide, which help to penetrate the walls of the endospores. After five minute, slide was removed from the steam, and the paper towel was removed.it allows for cooling then slide was rinsed thoroughly by using water up to thirty second. Slide was stained by diluted safranin for two minute; it stains other micro organic bodies red or pink. Slide then rinsed again, blotted dry with the help of bibulous paper. Allowed it for drying.

Prepared slides were observer under light microscope. Endospores observed were green in color. Bacterial bodies look red in color.

3. Motility test: Motility test was done by preparing a dilute suspension of fresh bacterial culture on a clean glass slides further it had covered by using cover slip. Prepared slide were observed under microscope having camera attached to the computer moniter (MIPS-OLYMPUS)

4. Morphological /Biochemical identification of the potential probiotic strain

The morphological characteristics of bacterial isolates were recorded. The bacterial isolates were also analyzed for various biochemical tests *viz.* catalase test, oxidase tests, motility test, indole producing test, and carbohydrates fermentation (arabinose, fructose, galactose, lactose, mannitol, salicin, sucrose

and trehalose). These characteristics are considered for the species level identification of bacterial isolates.

Results

From the 10 gut samples collected, 70 *Lactobacillus* isolates were isolated. The maximum *Lactobacillus* isolates were observed in *Channa marulius* isolates as compare with the *Mugil cephalus* which indicates that the probiotic (*Lactobacillus)* bacterial distribution may be varies according to the generic variation of fishes.

Among the 70 *Lactobacillus* isolates, cultural characteristically distinct 10 isolates were from *Channa marulius*, and 10 isolates from *Mugil cephalus* selected for further growth study. These 20(*Mugil cephalus* + *Channa marulius*) *Lactobacillus* isolates morphologically characterized, were gram-positive, non-motile, the shape of the isolates were varied rod, stout rod, bacilli, short bacilli.total isolated colony are grouped as 10 sample from *Channa marulius* which named as CM-1 to CM -10 by considering first letter of genus and species name . And 10 samples from *Mugil cephalus* which also named according to first letter from genus and species name as MC-1 to MC-10

1. Antibacterial activity by disc diffusion method

Out of 70 Lactobacillus we choose 20 bacterial strains. The antibacterial activity of the 20 bacterial strains isolated from the intestinal region of *Channa marulius* isolates as compare with the *Mugil cephalus show* different zone of inhibition against two fish pathogens *Aeromonas spp* and *Vibrio spp* .(Table 1).

Among all the isolates, **CM-3** and **CM-7** showed maximum zone of inhibition (21.4 ± 0.5 and 19.9 ± 1.5 mm) against *Aeromonas spp,* followed by CM-2, CM-4 which showed minimum zone of inhibition (5.3±0.5 mm , 6.2. ± 0.5 mm).

In *Vibrio spp,* maximum zone of inhibition was observed in **CM-3** and (19.9 ± 1.5 and minimum zone of inhibition was observed in CM-2, CM-4 which showed minimum zone of inhibition (7.2 ± 0.57 mm, 6.20. ± 0.5 mm). Which show extensive of inhibitory activity screen for antagonistic assay

2. Antagonistic activity

Cross streak method revealed that the five strains in *Mugil cephalus* (CM-2, CM-4, and CM-10) show zone of inhibitions (Table-1). The strain CM-3 exhibited inhibition zone greater than 10 mm against all the test pathogens, Followed by the strains CM-9, CM-10, and CM-5 which produce inhibition zone greater than 5 mm against the test pathogens. By virtue of its greater inhibition zone in the cross streak method (Table-3) and positive antibacterial activity, CM-3 was selected for further experiments.

3. Determination of pathogenicity for CM-3

The addition of CM-3 cells to the rearing water of *Channa marulius* and *Mugil cephalus,* there was no any observation of pathological symptoms, behavioral alternations, abnormalities, or mortality. The fishes were seemed to be healthy .it showed that the isolated CM-3 stain was not pathogenic in nature. Previous mortality recorded in *Mugil cephalus* was due to acclimatization in laboratory conditions.

4. Preliminary and biochemical identification of CM-3

The strain CM-3 was gram positive, aerobic, endospores forming and motile, while spores are ellipsoidal and central in position CM-3 was positive for the Vogues-Proskaeur reaction and negative for the methyl red test. The able strain produced catalase, indole and capable of utilizing citrate and reduce nitrate to nitrite. It is negative for oxidase. It cannot utilize hydrogen sulphide. The sugar fermentation test confirms the capability of CM-3 to utilize glucose, maltose, sucrose, mannitol, and fructose as the carbohydrate source for their growth (Table 4)

Table 1: study of antibacterial activity of the isolates from gut region of *Mugil cephalus* against fish pathogens

Sr.No	**Isolated organism**	**PATHO-1** Zone of inhibition (in mm)	**PATHO-2** Zone of inhibition (in mm)
1.	MC-1	10.9 ± 1.3	9.3 ± 1.11
2.	MC-2	10.3± 1.11	10.3 ± 1.14
3.	MC-3	5.3±1.1	5.44±1.3
4.	MC-4	5.2±0.5	5.33±0.6
5.	MC-5	16.3 ±1.4	16.6±1.3
6.	MC-6	12.2 ±0.5	12.2 ±0.5
7.	MC-7	14.3±0.51	14.2 ±0.41
8.	MC-8	5.6 ±0.47	8.22 ±0.47
9.	MC-9	10.9 ± 1.55	11.2 ±1.16
10.	MC-10	10.2± 0.51	10.6± 1.42

(Note: PATHO-1: - *Aeromonas spp*, PATHO-2:- *Vibrio spp*)

Table 2: study of antibacterial activity of the isolates from gut region of *channa marulius* against fish pathogens

Sr.No	**Isolated organism**	**PATHO-1** Zone of inhibition (in mm)	**PATHO-2** Zone of inhibition (in mm)
1.	CM-1	10.6 ± 0.6	11.5 ± 1.2
2.	CM-2	5.3± 0.5	7.2 ± 0.57
3.	CM-3	21.4±0.5	19.9±1.5
4.	CM-4	6.2±0.5	6.20±0.5
5.	CM-5	9.1 ±1.70	10.1±1.0
6.	CM-6	12.3±1.50	12.1±1.3
7.	CM-7	14.1±1.16	11±0.5
8.	CM-8	12±1	13.3±1.15
9.	CM-9	8.2±1.21	10.1±1.5
10 .	CM-10	7.6 ± 0.51	12.2±1.1

(Note: PATHO-1:-*Aeromonas spp*, PATHO-2:- *Vibrio spp*)

Table 3: Inhibition patterns produced by the isolates in Cross streak method

S.No	Isolated organism	PATHO-1 Zone of inhibition (in mm)	PATHO-2 Zone of inhibition (in mm)
1.	MC-1	+++	+
2.	MC-2	+++	+
3.	MC-3	+	+
4.	MC-4	+	+
5.	MC-5	+++	+++
6.	MC-6	+++	+++
7.	MC-7	+++	+++
8.	MC-8	+	+
9.	MC-9	+++	+++
10.	MC-10	+++	+++
11.	CM-1	+++	+++
12	CM-2	+	+
13.	CM-3	+++	+++
14.	CM-4	+	+
15.	CM-5	++	++-
16.	CM-6	+++	+++
17.	CM-7	+++	+++-
18	CM-8	+++	+++
19	CM-9	++	+++
20	CM-10	++	+++

Note: +++ 10mm; 5-10mm

Table 4: Biochemical identification of CM-3 isolate

S.No.	Characteristic features	Observation
1	Colony shape	Round
2	Colony margin	Entire
3	Colony texture	Moist
4	Colony color	White, shiny
5	Gram staining	+
6	Shape of cell	Rod shape
7	Endospores formation	+
8	Cell size	1.5~3.0 µm

9	Catalase	+
10	Oxidase	-
11	Methyl red test	-
12	Voges-proskauer test	+
13	Indole	+
14	Triple sugar ion agar	No H_2Sproduction
15	Citrate utilization	+
16	Urea	-
17	Sugar utilization test	
	Glucose	+ve
	Sucrose	+ve
	Fructose	+ve
	Lactose	-ve
	Mannitol	+ve .
	Mannitol	+ve

(Note: + Positive ,– Negative)

The beneficial effects of probiotic use in fish aquaculture are growth performance improvement (10), The fish intestine is a favorable ecological niche for microorganisms, the colony is more than in the surrounding water (3). In the present investigation, isolation of potent probiotic bacteria from the intestinal tract of *Mugil cephalus* indicate diverse occurrence of bacterial strains. Earlier studies suggest that, microorganisms form an important dietary component for deposit feeding animals. Near about 15–30% of organic basis in the stomach of mugil cephalus has been contribute by microorganisms. Such occurrence of the diversified population of bacteria also indicates their possible role in the breakdown of plant matter as the gut content of mullets also harbour sizable quantity of plant matter (7). Similar studies on the intestinal content of mullets were carried out by Moriarity (14). He reported that the muramic acid in the gut of *Mugil cephalus* was high; indicating ingestion of bacterial strains associated with sediments thus forming an important component of detritus based food chain. He also reported that the microbial community in the intestinal tract comprises of both gram positive and gram negative bacteria. Occurrence of diseases in fish culture systems poses a great threat to fish farmers, in terms of heavy loss.

Plasmid-mediated resistance to antimicrobials has been identified in a number of bacterial fish pathogens including *A. salmonicida, A. hydrophila, V. anguillarum, V. vulnificus, V. alginolyticus, V. parahaemolyticus, V. harveyi, Pseudomonas fluorescens, Pasteurella piscida* and *Edwardsiella tarda* (2). In clinical terms, there is plenty proof about antibiotic-resistant strains of fish pathogens have developed in which antibiotics have been used to control fish disease.
In the present study, 10 bacterial strains were isolated from the gut of *Mugil cephalus* were screened for antibacterial and antagonistic activity against two major fish pathogens namely,. **PATHO-1-*Aeromonas Spp*,and PATHO-2 *Vibrio Spp*** Out of 10 isolates from *channa marulius* , 07 isolates showed zone of inhibition greater than 10 mm against the fish pathogens.better results were obtained from *channa marulius* hence we had been focused on the *channa marulius* as compare to *Mugil cephalus*. A similar frequency of inhibitory bacteria was observed for the isolates from halibut larva (**5**) and in shrimp (19, 20). It has been reported that the inhibition was caused by the release of chemical substances with bactericidal or bacteriostatic effects.

In the cross streak method, the strain CM-3 exhibited a wide spectrum of inhibitory activity against the test pathogens. Inhibitory zone produced by CM-3 was similar to those reported by (1) against *Altermonas* sp. and by **Rengpipat *et al.*** (18) for *Bacillus* strain BY-9 against the pathogenic *V. harveyi.* In another study, **Galindo** (8) reported that, *L. plantarum* produce wide range of inhibition zone against *A. hydrophila* and *L. lactis* exhibited inhibition against *A. hydrophila, E. tarda* and *S. aureus* respectively. It has also been found that, the

interruption of pathogen growth was caused by production of one or more of the following factors: antibiotics, antimicrobial peptides, bacteriocins, siderophores, lysozymes, proteases, hydrogen peroxide and organic acids (26). Nakamura *et al.*, (16) reported that, the reduction of pathogen growth and cell densities in antagonistic assay were because of the extracellular bacteriolytic product produced by the bacterium. The *in vitro* production of compounds that inhibit known pathogens was often used in the selection of putative probiotic strains (20). In the present *in vitro* study, CM-3 inhibited all the five pathogenic strains and hence was selected to check the probiotic efficacy of the strain.

Determination of pathogenicity of CM-3 revealed that, there is no evidence of harmful effect on *Mugil cephalus* including mortality, pathology or abnormality. Similar studies on immersion assay were carried out in rainbow trout for 14 days with commercial fish feed supplemented with putative probiotics by Newaj-Fyzul *at.al* (17) to determine the pathogenicity of the probiotic strain. **Austin *et al.*** (4) presented that, isolated probiotic strains were non-pathogenic. The non-pathogenic nature of the strain CM-3 highly ensures the safety of the strain for use as a probiotic.

Biochemical identification and molecular characterization of CM-3 strain using 16S rRNA gene sequencing followed by homology search *via.* BLAST database shared the maximum identity with the *B. subtilis* and hence characterized as *B. subtilis. Bacillus* is distinguished from other endospores forming bacteria by being a strict or facultative aerobe, rod shaped and catalase positive organism. The bacilli are not autochthonous to the gastrointestinal tract have been isolated from carps (12), shrimp culture ponds (25), and shrimp larvae rearing medium (19). Most *Bacilli* produce antibiotics such as difficidin, oxydifficidin, bacitracin, polymyxin, subtilin, mycobacillin, gramcidin, or bacillomycin B and are antagonistic to pathogenic bacteria in both *in vivo* and *in vitro* environment (11, 27). Hence, this strain could be a potential candidate as probiotic organism *in vivo*, thereby enhancing the immunity of the fish.

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