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ORIGINAL ARTICLE

Screening and Characterization of Probiotic bacteria isolated from gut microflora of marine fishes collected from Parangipettai south east coast of India

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ABSTRACT
Aquaculture diseases cause serious threat to the entire culture pond thereby causing severe economic loss which could be treated by the use of probiotic bacteria. Phosphate in the original form does not favor consumption by the marine plants hence it has to be converted to solubilized form which is achieved by phosphate solubilizing bacteria. The present study was aimed at isolating probiotic bacteria and phosphate solubilizing bacteria from marine fish gut. A total of 25 probiotic bacteria were isolated from gut of 3 (*Epinephelus tauvina, Arius maculates* and *Gerres erythrourus*) different marine fishes. Among the 25 bacterial strains, 5 strains of *Lactobacillus* sp. (CASNG1, CASNG2, CASNG3, CASNG4 and CASNG5) were isolated and characterized for further work. Antibiotic tests for fish pathogen like *Aeromonas* sp.and human pathogens such as *Salmonella paratyphi, Staphylococcus aureus, Proteus mirabilis, Klebsiella pneumonia, Pseudomonas aeroginosa* were performed. The strains of CASNG2,CASNG5 showed good results against*Salmonella paratyphi, Staphylococcus aureus, Proteus mirabilis, Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Aeromonas* sp. Meanwhile phosphate solubilizing activity was also analyzed for all the isolated strains. CASNG5 exhibited good phosphate solubilizing activity. The *Lactobacillus* sp. serves as effective probiotics and as essential phosphate solubilizing bacteria.
Keywords: Probiotic Bacteria, Phosphate Solubilizing Bacteria, *Lactobacillus* sp., Antibiotic Activity

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INTRODUCTION

Aquaculture is the top most blooming aquatic food production sector and it is the billion dollar industry in the world. The leading problem in aquaculture sector is a viral disease that significantly causes the economic losses and limits the growth and expansion of aquaculture industries. Use of probiotics in aquaculture is the most essential disease management system in aquaculture practices. Probiotics are distinguished as living microbial food supplement which favourably impact the host by enhancing the gut microbial stability. Recent years, probiotics have been exhaustively premeditated as a medication for a range of infection [1]. Probiotics have wider applications in food, feed, dairy and fermentation industry, as non-pharmacological approaches for health management [2]. The use of probiotics in aquaculture is extremely increased due to the restriction of antibiotics usage. The microorganism such as *Bacillus* sp., *Lactobacillus* sp., *Enterococcus* sp., *Carnobacterium* sp. and the yeast *Saccharomyces cerevisiae* are the beneficial microorganisms, which are mostly utilized for the production of probiotics [3]. In India, the use of probiotics in aquaculture has been practiced for few decades. The present Indian probiotic market value is about INR 1.2 billion and the annual increasing speed is about 40%. Miserably, most of them are imported; the leading companies involved for the production utilize the bacteria of alien species. The alien probiotic bacteria facing the severe challenge since, they are screened from the community of diverse environmental conditions. However, the Indigenous bacterial strains are expected to become more active and productive since they have been screened from invariant environmental conditions.

Stimulation of immune system and anti-pathogenic activity and removal nutrients are the most significant criteria for the screening of probiotic species. Probiotic bacteria can synthesize a wide range of bio-toxins to suppress the growth of pathogens [4]. Phosphorus is an important nutriment involved in several biological processes such as photosynthesis, energy transfer and cell division [5]. Phosphorous is used in the form of fertilizers and is provided to the land as it enables the plants to improve the quality of fruits [6]. Phosphate solubilizing bacteria (PSB) is present in the soil and is capable of degrading phosphorous present in the soil and converts it into a less complex form which the plants are capable of absorbing. The most significant property of the phosphate solubilizing bacteria is the production of organic acids such as citric acid, glutamic acid, lactic acid, oxalic acid and succinic acid. Further, PSB is used as an effective biofertilizer as it is added into the soil to improve the soil fertility [7].

The gut microflora is an essential constituent of the entire living being. A great verity of endogenous and exogenous factor determining the number and species composition of microbe population and affecting physiological and biochemical features of the microorganisms themselves influence it. Many different bacteria get into an organism from the environment. Hence, the present study has been planned to screen the probiotic bacteria from gut flora of selected marine fish and to evaluate the potential anti-pathogenic and phosphate solubilizing activity.

MATERIAL AND METHODS

Collection of marine fish

Live marine fish was collected from Annankovil landing center, Parangipettai, Tamil Nadu. The collected fishes were identified as *Epinephelus tauvina, Arius maculatus* and *Gerres erythrourus* by using theFAO species identification guide and were selected for the present study.

Isolation and identification of bacteria

The ventral surfaces of the fishes were sterilized using 70% ethanol and aseptically dissected to remove intestines. The intestines were opened by a longitudinal incision and thoroughly flushed with sterilized chilled phosphate buffer solution (PBS) to remove feed materials, dirt and other impurities. Then the intestines were homogenized with normal saline solution (0.87% NaCl) in sterile mortar and pestle. The homogenized intestines were centrifuged at 5000 rpm for 5 minutes. The supernatants were serially diluted up to 10^{-6} dilutions. From 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilutions, 100 µl of samples were plated by spread plate method on prepared MRS agar media incubate at 48 hr.

Physical and biochemical characterization

The *Lactobacillus* species were identified based on physical (Gram staining) and biochemical (indole ,catalase, methyl red , voges proskauer's ,citrate utilisation , triple sugar iron, skim milk agar) tests outlined in Bergey's Mannual of Systematic Bacteriology [8].

Antibiotic susceptibility test

Bacterial antibiotic resistance was determined on MHA (Muller Hinton Agar) using agar well diffusion method against five different types of human pathogens (*Pseudomonas aeruginosa, Salmonella paratyphi, Klebsiella pneumoniae, Staphylococcus aureus, Proteus mirabilis)* and fish pathogen *Aeromonas* sp*.* The Antimicrobial agents, Amoxicillin and Ampicillin were used as control [9]

Estimation of phosphate solubilizing bacteria

Quantitative Analysis The quantitative estimation or abilities of the isolated phosphate solubilizing bacterium to solubilize TCP on Pikovskaya's agar media was determined in terms of solubilization index (SI). Phosphate solubilization index was calculated by measuring the colony diameter and the halo zone diameter and the colony diameter, using the following formula [10].

Qualitative Analysis

The qualitative analysis of phosphate solubilization potential of selected PSB isolate was measured in vitro by determining available soluble phosphate in the Pikovskaya's broth supplemented with 0.5% TCP. The flasks were incubated at 37ºC for 5 days on rotary shaker at 180 rpm and centrifuged at 10,000 rpm for 10 min. Absorbance was noted at 410nm. Phosphomolybdate method was used for determination of available soluble phosphate in culture supernatant [11].

RESULTS

The live fish were collected and the gut was dissected to obtain the probiotic bacteria. The fishes were selected based on its availability, the species such as *Gerres erythrourus* and *Arius maculatus* are commonly available along the shoreline but *Epinephelus tauvina* is available in the deeper part of the ocean(Fig.1). About 25 live specimens were collected from each species for obtaining the probiotic bacteria from the gut. The gut microflora of all the three fish species were enumerated and best five potential probiotic strains of *Lactobacillus* sp. were screened namely CASNG1 from *Epinephelus*

tauvina,CASNG2- *Gerres erythrourus*, CASNG3-*Arius maculatus*, CASNG-4 *Gerres erythrourus* and CASNG5 from *Epinephelus tauvina*. All the five strains were isolated and cultured separately (fig. 2).

Biochemical characterization of the *lactobacillus* species

The *Lactobacillus* sp. was characterized by physiological and biochemical tests. By gram staining method it was observed that the isolated strains were rod shaped gram positive bacteria. Test results of biochemical tests were presented in Table.1.

Antibiotic test

The screened probiotic strains of *Lactobacillus* sp. was subjected to antimicrobial tests against five human pathogens namely *Pseudomonas aeruginosa*, *Salmonella paratyphi*, *Klebsiella pneumoniae*, *Staphylococcus aureus, Proteus mirabilis* and one fish pathogen(*Aeromonas* sp). All the five of the recognized probiotic strains showed the peak inhibition against most of the pathogens. The inhibition results were measured and given in mm. In this experiment, the formed inhibition zones were ranged between 0 and 4 mm. at 10µg concentration no inhibition activity was observed for all the strains except CASNG2. The overall observation showed that the strain CASNG2exhibited good inhibition activityagainst at each tested pathogens at all the concentrations. The highest peak (4mm) was observed against them at 75µg concentration.Besides,CASNG5 also showed high inhibition against all the tested pathogens at all the concentrations excluding 10µg concentration.

Estimation of phosphate solubilizing ability

Qualitative analysis:

All the five probiotic strains were assessed for the Phosphate solubilising ability using Pikovskaya's (PKV) agar plates. The isolate CASNG5 was found to be potent phosphate solubilizer showing clear halo zone around its colony (11mm) fig. 3. The SI of the screened strain CASNG5 was also estimated and the detectedphosphate SI was 3.66 (Table .3). The formation halo zone around the bacterial colony could be owing to the synthesis of organic acids or because of the production of polysaccharides or due to the phosphatase enzyme action PSB.

Quantitative Analysis:

The efficiency of phosphate solubilizing activity of isolated PSB strain CASNG5 in Pikovskaya's broth confirmed that the strain can efficiently solubilizeinorganic phosphateafter 120h of incubation(fig.4).

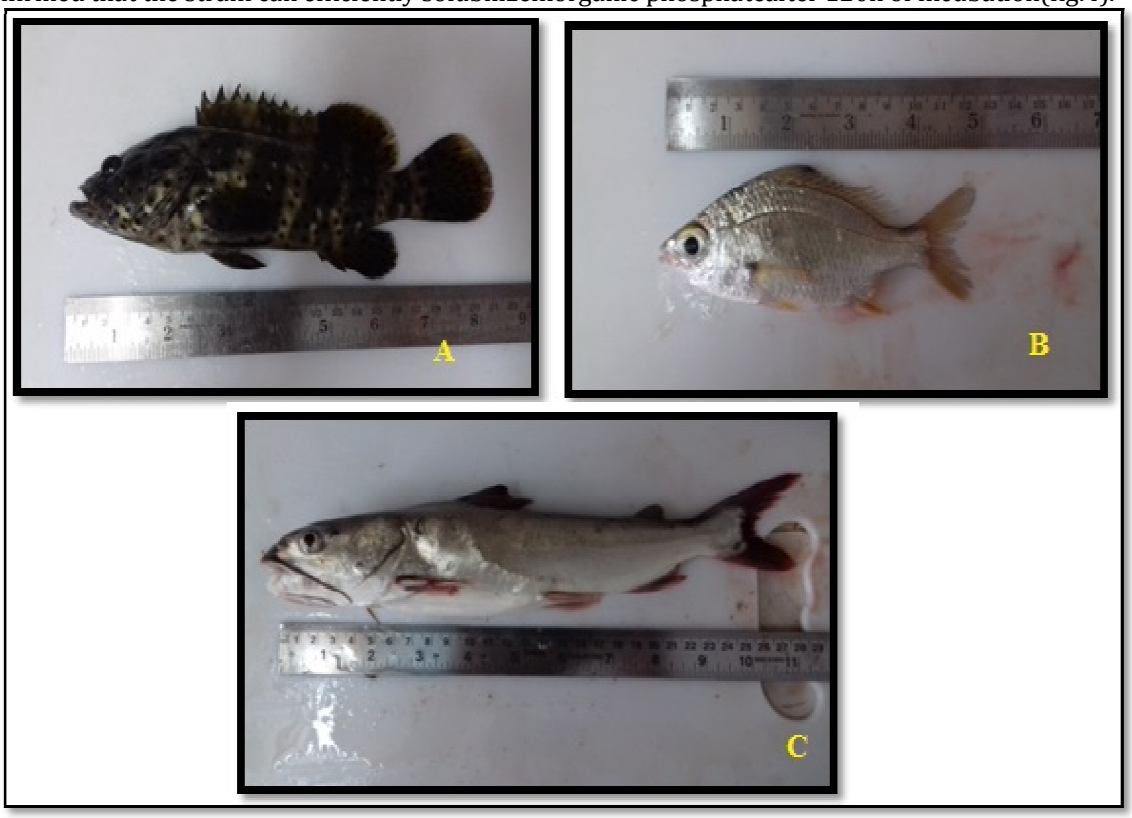

Fig.1. Fish samples A. *Epinephelus tauvina* B. *Gerreserythrourus,* C. *Arius maculates*

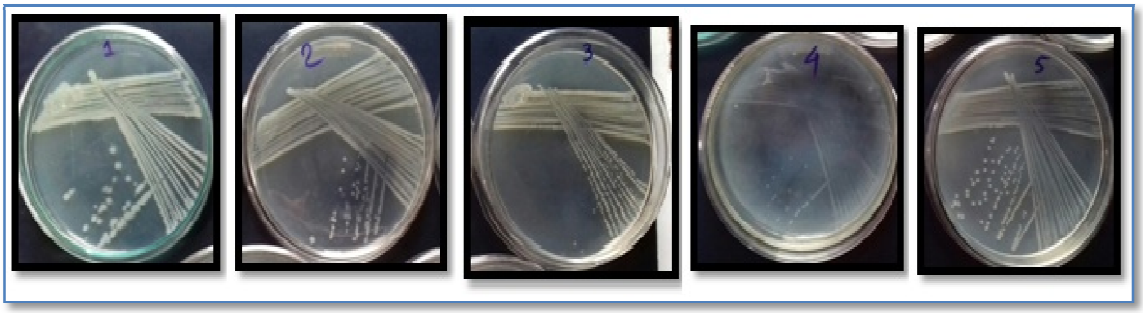

Fig.2. Selected *lactobacillus* sp. from fish sample

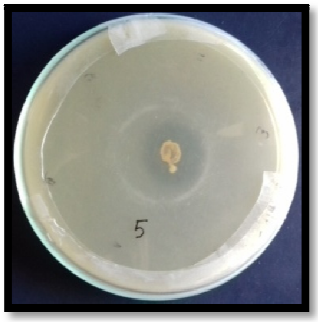

Fig.3. Qualitative analysis of Phosphate solubilizing bacteria from CASNG5

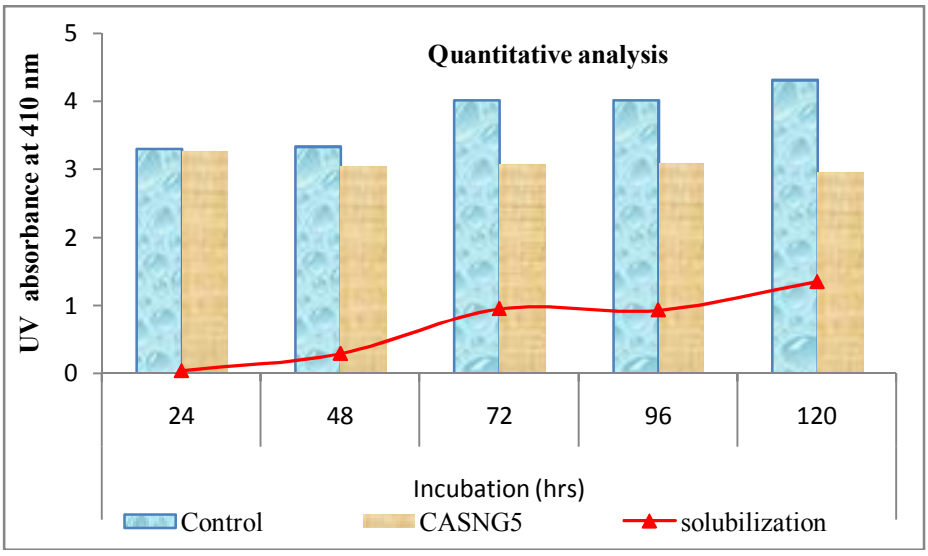

Fig.4. Quantitative analysis of phosphate solubilizing bacteria from CASNG5

Table:1. Physical and biochemical characterization of the L*actobacillus* sp.

Characteristics	CASNG1	CASNG2	CASNG3	CASNG4	CASNG5
Physical Characterization					
Gram staining	Gram+ve rod	Gram+ve rod	Gram+ve rod	Gram+ve rod	Gram+ve rod
Biochemical characterization					
Methyl red test	+	+	-	+	+
VP test	-	-	-	-	-
Indole test	-	-	-	-	-
Citrate utilization test	-	+	-	-	+
TSI test	+	+	-	+	+
Skim milk test	+	-	-	+	-
Catalase test	-	-	-	-	-

Table: 2. Antibacterial activity against human pathogen and fish pathogen

pathogens	Control	Ampicillin	Amoxicillin	10µg	25µg	50µg	75µg
	***Lactobacillus* sp. CAS NG 1(mm)**						
Salmonella paratyphi	0	2	3	0	1	2	2
Staphylococcus aureus	0	2	2	0	1	1	2
Klebsiella pneumoniae	0	2	2	0	2	2	2
Proteus mirabilis	0	2	2	0	2	2	2
Pseudomonas aeruginosa	0	2	2	0	2	1	2
***Aeromonas*sp.**	0	3	3	0	2	2	3
	***Lactobacillus* sp. CAS NG 2(mm)**						
Salmonella paratyphi	0	2	2	1	3	3	4
Staphylococcus aureus	0	2	2	2	3	4	4
Klebsiella pneumoniae	0	2	3	2	3	4	4
Proteus mirabilis	0	2	2	2	3	4	4
Pseudomonas aeruginosa	0	2	3	2	3	4	4
***Aeromonas*sp.**	0	3	3	3	4	4	4
	***Lactobacillus* sp. CAS NG 3(mm)**						
Salmonella paratyphi	0	2	2	0	1	1	2
Staphylococcus aureus	0	2	2	0	1	2	2
Klebsiella pneumoniae	0	2	2	0	1	2	2
Proteus mirabilis	0	2	2	0	1	2	2
Pseudomonas aeruginosa	0	2	2	0	1	2	2
***Aeromonas*sp.**	0	3	3	0	1	2	2
	***Lactobacillus* sp. CAS NG 4(mm)**						
Salmonella paratyphi	0	2	2	0	1	2	2
Staphylococcus aureus	0	2	2	0	1	1	2
Klebsiella pneumoniae	0	2	2	0	1	1	2
Proteus mirabilis	0	2	2	0	1	2	2
Pseudomonas aeruginosa	0	2	2	0	2	2	2
***Aeromonas*sp.**	0	2	2	0	1	1	2
	***Lactobacillus* sp. CAS NG 5(mm)**						
Salmonella paratyphi	0	2	2	0	1	2	2
Staphylococcus aureus	0	2	2	0	1	2	3
Klebsiella pneumoniae	0	2	2	0	2	3	4
Proteus mirabilis	0	2	2	0	1	2	3
Pseudomonas aeruginosa	0	2	2	0	2	3	4
***Aeromonas*sp.**	0	2	2	0	2	3	4

Table.3.Qualitative estimation of phosphate solubilization efficiency of *Lactobacillus* sp.

PSB isolate	Colony diameter (mm)	Halo zone diameter (mm)	Solubilization index (SI)
CASNG5	3.00±0.577	8.00±0.577	3.66

DISCUSSION

Several researchers have performed experiments to obtain probiotic bacteria from the gastrointestinal tract of most of the freshwater and marine fishes (12,13, 14, 15). However, still there is a heavy demand in probiotic bacterial production and also there have been limited studies on *Gerres erythrourus, Arius maculatue* and *Epinephelus tauvina*. Hence for the present study, these fishes were selected for obtaining the gut content analysis and the five different *Lactobacillus* strains have been isolated and screened.

The screened *Lactobacillus* strains were subjected to biochemical characterization such as Methyl red, VP, Indole, Citrate utilization, TSI, Skim milk and Catalase test. These tests showed positive results in Methyl red test (CASNG1, CASNG2, CASNG4, CASNG5), Citrate utilization test (CASNG2, CASNG5), TSI test

(CASNG1, CASNG2, CASNG4, CASNG5), Skim milk test (CASNG1, CASNG4). Whereas, catalase tests, VP test and Indole tests showed no positive results in any of the culture strains. (16) performed biochemical assays on *Bacillus cereus* and showed good results as positive activity was noted in only in few tests. However, in this study, biochemical assays exhibited better results as positive results were exhibited in about 5 of the 7 tests and among 4 out of the 5 *Lactobacillus* sp strains.

The earlier investigation made by (17, 18,19, 20), screened the probiotic bacteria based on the antagonistic activity against the pathogens. They considered an anti-pathogenic activity as the prime factor for the screening of probiotic isolates. (18) stated that the probiotic exhibiting a wide antibacterial activity against various pathogens was considered as potential probionts. Therefore in this study also the antagonistic activity was considered as the key factor for the selection of probiotic strains. Thus, the isolates with wider antibacterial activity against most of the target pathogens were chosen and sorted out as sturdy probionts. Out of 25 isolates, only 5 exhibited strong antibacterial activity. The strains CASNG2 and CASNG5 isolated from *Gerres erythrourus* and *Epinephelus tauvina* respectively showed the excellent antagonistic activity against human and fish pathogens.

Previously, (21) isolated bacteriocin producing *Lactobacillus*. They screened two strains namely, *Lactobacillus lactis* and *Lactobacillus plantarum* from fish muscle and both the strains exhibited good bacteriocin activity. (19) studied the antimicrobial activity of Lactic Acid Bacteria (LAB) isolated from the intestine of *Oreochromis niloticus*. (20) collected *Perca* sp. and *Tuna* sp. for retrieving *Lactobacillus* species from their gut and intestine. The obtained cultures were tested for antibacterial activity against some pathogens such as *Klebsiella* and *Bacillus* species and satisfactory results were obtained.

The results illustrated satisfactory results against *Pseudomonas*, *Vibrio* and *Mycobacterium* strains. (22) obtained LAB from the gut of estuarine fish namely *Mugil Cephalus*. These bacteria exhibited antibacterial activity against certain fish pathogens such as *Vibio harveyi*, *Vibrio parahaemoloyticus*, *Aeromonas hydrophila* and *Pseudomonas aeruginosa*. The results indicated significant activity against all gram negative organisms.

Further, the potential strains of *Lactobacillus* sp. were tested for its phosphate solubilizing potential. Several studies have been conducted on the phosphate solubilizing potential most of the environmentally screened bacteria. However, there has been limited or no work regarding the phosphate solubilizing potential of probiotic bacteria. In the present study, all the five probiotic bacteria (*Lactobactillus* strains) were assessed for phosphate solubilizing activity and the results showed significant activity was noticed in CASNG5. This study suggests that the phosphate solubilizing potential of the probiotic bacteria is another dimension which requires further comprehensive investigations.

From the literature obtained, it can be noticed that, the results of the present study are in line with other recent studies. However, better results are observed in comparison with certain other studies. Hence, it could be stated that from the present study, probiotic bacteria could be isolated from marine fishes and used against certain human and fish pathogens. The probiotic *Lactobacillus* sp.possesses effective antimicrobial activities and this potential could be used as a replacement for certain products in the pharma industry.

CONCLUSION

The indigenous bacteria screened from the gut of marine fishes showed a potential antagonistic activity against pathogens as well as phosphate solubilising ability. Hence, the screened strains can be used as probiotic bacteria in aquaculture. However, derailed investigation is very much needed to assess the efficiency and feasibility of the production.

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