

Isolation and Characterization of Gut Micro Biota from Some Estuarine Fishes

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Abstract Gut flora has a continuous and dynamic effect on the host's gut and systemic immune systems. The gut microbiota of fish has been studied extensively using various techniques; little information is available on the correlation between gut microbiota and host activity. In the present investigation three species of estuarine fishes were taken with regard to their microbial population in the gut region and enzymatic analysis of those gut isolates. The total heterotrophic bacterial load ranged from 0.8×10^6 to 4.5×10^6 CFU/g of gut sample and it was found to be the maximum of 4.5×10^6 CFU/g in *Oreochromis mossambicus* gut followed by 3.1×10^6 CFU/g in *Oreochromis leucostictus* gut and minimum of 0.8×10^6 CFU/g in *Etroplus suratensis*. The isolates were identified by various biochemical tests. *Pseudomonas*, *Vibrio* and *Bacilli* are the common bacteria found in all three fishes. The bacterial isolates were subjected to quantitative estimation of enzymes viz., amylase, lipase and protease. Varied kinds of results were obtained in different fish species. However it was observed that 20% of the isolates produced only amylase. A collection of 10 bacterial isolates from three fishes were screened for antagonistic activity against human and fish bacterial pathogens. Upon testing all these strains, it became apparent that strains isolated from the fish *O. mossambicus*, exhibited antimicrobial activity against *Klebsiella pneumoniae* (17mm) and *Vibrio cholerae* (12mm). Thus the present preliminary study showed the variation in the gut micro-flora of different fish species and the microbes from marine environment may be useful in development of probiotics, drugs and also in the industrial enzyme production.

Keywords Estuarine fishes, Gut micro biota, THB, antimicrobial, Enzymes

1. Introduction

Fish receive bacteria in the digestive tract from the aquatic environment through water and food that are populated with bacteria. Being rich in nutrient, the environment of the digestive tract of fish confers favorable conditions for the microorganisms. The importance of intestinal bacteria in the nutrition and well-being of their hosts has been established for homeothermic species, such as birds and mammals. However, there is limited information for fish, the poikilothermic vertebrates. Gut micro flora plays an important role in the digestive process, growth and disease of the host. However some bacteria which possess the ability to tolerate the low pH in gastric juices resist the action of bile acids, lysozyme secreted in intestines, immune responses and adheres to the mucus or enteric wall surface could persist for a relatively long time and eventually make intestinal micro flora specific to each host animal[1]. It is clear that bacterial species presents in the gut can influence the health and robustness of the host.

The influence of the gut flora on the host is clearly of great

interest in aquaculture, particularly where poor productivity and/or stock losses are widespread. Within marine and other aquatic animals, the colonization of the digestive system by micro-organisms is influenced by a number of both host and non-host related factors. On the other hand, bacteria producing antibacterial substances were isolated from marine fish intestines[1,2] thereby suggesting that these bacteria may inhibit the establishment of invading bacteria in the fish intestine. However, it remains to be elucidated whether freshwater fish harbor the antibiotic-producers as well as marine fish. In the present study, an attempt has been made to investigate the variation of the gut bacterial species in the gastrointestinal (GI) tracts of three brackish water teleosts, namely the *Oreochromis leucostictus*, *Oreochromis mossambicus*, *Etroplus suratensis*. Further the intestinal isolates were evaluated for extracellular enzyme producing capacities and also examined for their antibacterial ability against fish and human pathogens.

2. Materials and Methods

2.1. Collection of Samples

Fishes *O. leucostictus*, *O. mossambicus*, *E. suratensis* were collected from the Vellar estuary (Lat.11°29' N and Long. 79°46' E), South east coast of India and transformed to ster-

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ile polythene bags using sterile forceps. All the samples were transformed immediately to the laboratory and subjected to various analyses.

2.2. Isolation of the Gut and Their Homogenization

Fishes were anaesthetized in an ice bath for 5–10 min and each individual surface sterilized by immersion for 30 seconds in 70% ethanol. The gut was aseptically dissected from the animal's musculature. Gut was weighed and placed into a 10 ml sterile double strength phosphate-buffered saline (PBS) solution (disodium phosphate, 2-3% (w/v); sodium phosphate, 0-6% (w/v) and sodium chloride, 1-2% (w/v)). Gut was homogenized in tissue homogenizer.

2.3. Isolation and Identification of Bacteria

One ml aliquot of the gut homogenate was aseptically spread with 9ml sterile double strength PBS onto Nutrient agar. All plates were incubated at 37°C for 24-48hrs. Colonies developed on the plate were counted and expressed as cfu/g. The isolates were purified on the agar slants. The isolated bacterial strains were identified up to species level[3] and were stored in nutrient agar slants for further study. As an initial step in the identification of isolates, the following properties or activities were recorded: Gram stain, motility, oxidase activity, catalase activity, oxidation/ fermentation, glucose acid, glucose gas, pigment production and citrate utilization. A series of secondary tests was used, when required, to complete the genus level identification of isolates. These tests included growth on 0% sodium chloride media, production of amylase, production of protease, lipase activity and gelatinase activity.

2.4. Antagonistic Activity of Gut Isolates

A spectrum of antibacterial activity was studied using test agent range of 10 different strains of human bacterial pathogen and 5 fish bacterial pathogenic gram positive and gram negative bacteria (*Vibrio parahaemolyticus*, *Proteus mirabilis*, *Staphylococcus aureus*, *Vibrio cholera*, *Klebsiella pneumonia*, *Klebsiella oxytoca*, *Staphylococcus pyogens*, *Escherichia coli*, *Salmonella typhi*, *Salmonella paratyphi*, *Pseudomonas fluroscences*, *Aeromonas hydrophila*, *Vibrio alginolyticus*, *Enterobacter sp.*, *Proteus sp.*). In vitro antibacterial assay was carried out by well diffusion technique[4]. The inhibition zone was measured and expressed in terms of diameter using caliper or a scale and recorded.

3. Results

3.1. Total heterotrophic Bacteria (THB)

The numbers of cultivable bacterial cells present in fish gut were estimated after isolation and growth on nutrient agar (NA) plates incubated at room temperature at 37°C. The total heterotrophic bacterial load ranged from 1.8×10^6 to 4.8×10^6 CFU/g of gut sample and it was found to be the maximum of 4.5×10^6 CFU/g in *O.mossambicus* fish gut, 3.1×10^6 CFU/g in *O.leucostictus* fish gut and minimum of 0.8×10^6 CFU/g in *E.suratenis* (Fig 1).

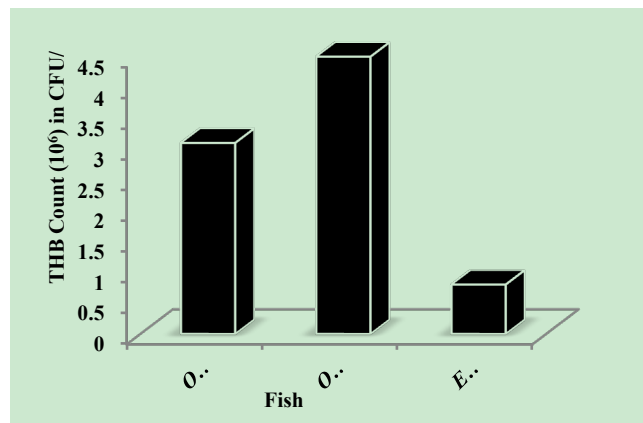


Figure 1. THB load in various fish species

A total of 188 bacterial strains were isolated from the three estuarine fish and classified in to nine taxonomic groups. *Acinetobacter* (2), *Bacillus* (22), *Enterobacteriaceae* (43), *Vibrio* (21), *Alcaligenes* (12), *Photobacterium* (9), *Pseudomonas* (33), *Aeromonas* (32) and *Flavobacterium* (14) (Table 1).

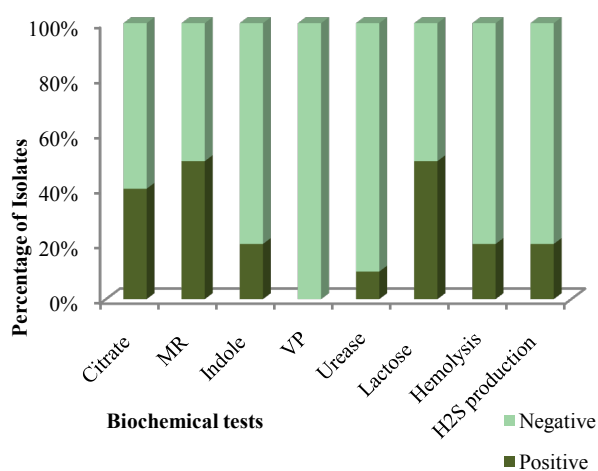
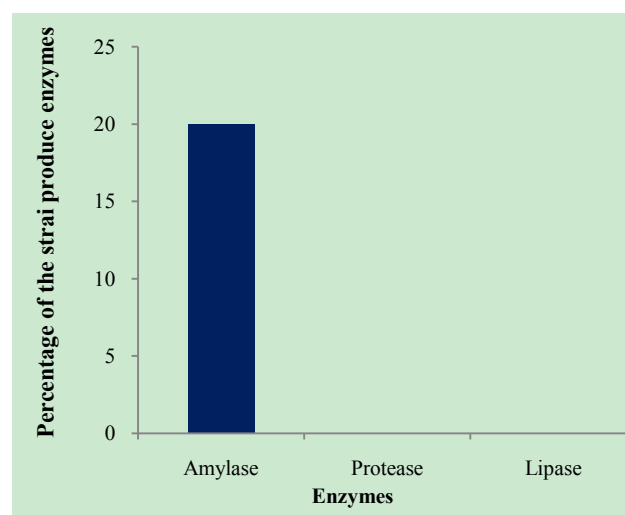
All fish isolates were tested for their biochemical characters and their production of enzymes such as amylase, protease and lipase (Table 2). Biochemical test shows 90 % of the isolates were gram negative bacilli and 6% were gram negative cocci and remaining 4% were gram positive bacilli. None of the isolate was gram positive cocci. About 40 % of the strains utilize citrate and 20% of strains showed positive in indole, Haemolysis and H₂S production test. Methylred test and Lactose fermentation showed positive on 50 % of the isolates. No isolates were show positive results on VP test (Fig 2). About 20% of the isolates were produces the amylase enzymes (Fig 3). There was no production of protease and lipase enzymes by the isolates.

Table 1. Number of isolates in different fishes

Bacterial Isolates	Number of strains isolated from Various fishes		
	<i>O.mossambicus</i>	<i>O.leucostictus</i>	<i>E.suratenis</i>
<i>Acinetobacter</i>	2	0	0
<i>Bacillus</i>	8	8	6
<i>Enterobacteriaceae</i>	22	13	8
<i>Vibrio</i>	10	7	4
<i>Alcaligenes</i>	7	3	2
<i>Photobacterium</i>	6	2	1
<i>Pseudomonas</i>	17	11	5
<i>Aeromonas</i>	18	8	6
<i>Flavobacterium</i>	7	4	3
Total	97	56	35

Table 2. Biochemical characteristics of the Bacterial isolates

Characteristic feature	Isolate I	Isolate II	Isolate III	Isolate IV	Isolate V
Gram Stain	-	-	-	+	-
Morphology	R	C/B	R	R	R
Motility	+	-	+	+	+
Indole	+	-	+	-	-
VP	-	-	-	-	+
Citrate	+	-	-	+	+
Methyl red test	+	-	+	+	-
H ₂ S Production	-	-	-	-	-
Oxidase	+	-	-	-	+
Urease	-	-	-	-	-
Catalase	+	+	-	+	-
Starch utilization	-	-	-	+	-
Acid from glucose	-	-	-	-	-
Gas from glucose	-	-	-	-	+
Hemolysis	β	-	-	β	-
Identified as	<i>Aeromonas</i>	<i>Acinetobacter</i>	<i>Alcaligenes</i>	<i>Bacillus</i>	<i>Enterobacter</i>
Characteristic feature	Isolate VI	Isolate VII	Isolate VIII	Isolate IX	
Gram Stain	-	-	-	-	
Morphology	R	R	R	R	
Motility	-	+	+	+	
Indole	-	+	+	-	
VP	-	+	+	+	
Citrate	-	-	+	-	
Methyl red test	-	+	-	-	
H ₂ S Production	-	-	-	+	
Oxidase	+	-	+	-	
Urease	+	+	-	+	
Catalase	+	+	+	+	
Starch utilization	-	-	-	+	
Acid from glucose	+	-	-	+	
Gas from glucose	-	+	+	+	
Hemolysis	-	β	β	β	
Identified as	<i>Flavobacterium</i>	<i>Photobacterium</i>	<i>Pseudomonas</i>	<i>Vibrio</i>	

**Figure 2.** Percentage of isolates showing biochemical activities**Figure 3.** Enzyme Production by the Isolates

3.2. Antagonistic Activity of Gut Isolates

The bacterial strains isolated from the gut of three fish species were assayed for their antimicrobial properties. Antimicrobial abilities were detected by well diffusion technique. A total of nine bacterial isolates were tested for their antagonistic ability against highly virulent 10 human bacterial and 5 fish bacterial pathogens. Maximum zone of inhibition was recorded against *K.pneumoniae* (17mm) and minimum against *V.cholerae* (12mm). No activity was recorded against the fish pathogens (Table 3).

Table 3. Antagonistic activity of Bacterial isolates from various fishes against human and fish bacterial pathogens

Pathogens	Isolates of <i>O.leucostictus</i> (mm)	Isolates of <i>O.mossambicus</i> (mm)	Isolates of <i>E. suratenis</i> (mm)	Control (mm)
Human Pathogens				
<i>S.aureus</i>	—	—	—	24
<i>P.mirabilis</i>	—	—	—	24
<i>V.parahaemolyticus</i>	—	—	—	24
<i>V.cholerae</i>	—	12	—	24
<i>K.pneumoniae</i>	—	17	—	25
<i>K.oxytoca</i>	—	—	—	22
<i>S.pyogens</i>	—	—	—	21
<i>E.coli</i>	—	—	—	22
<i>S.typhi</i>	—	—	—	18
<i>S.paratyphi</i>	—	—	—	17
Fish Pathogens				
<i>A. hydrophila</i>	-	-	-	23
<i>P.fluorescences</i>	-	-	-	21
<i>V. alginolyticus</i>	-	-	-	22
<i>Enterobacter sp</i>	-	-	-	19
<i>Proteus sp</i>	-	-	-	19

4. Discussion

In contrast to terrestrial animals, fishes have closer contact with the environmental micro-biota due to their aqueous habitat. Surrounding bacteria are continually ingested with food or water. For this reason, transient microorganisms probably have more constant and important interaction with fish gastrointestinal ecosystems compared to terrestrial animals. In general, genus *Acinetobacter*, *Vibrio* and *Pseudomonas* were the most common bacteria in the gut of marine fishes[5, 6, 7], and *Vibrio* was considered as one of the predominant bacteria[7, 8, 9, 10]. The experiment measured total bacterial numbers in the gastro intestinal region of three estuarine fishes. The percentage of isolated species and the percentage of isolate from any particular species varied significantly among gastro intestinal tract region. *Aeromonas hydrophila*, *Aeromonas veronii* were the most frequently isolated Gram negative bacilli[11]. In the present study, the micro-biota in the gut of three estuarine fishes was studied. The results showed that *Acinetobacter*, *Vibrio* and *Pseudomonas* were also isolated from the gut of fishes.

Recently, *Psychrobacter* has been successfully isolated from the gut of marine fish, such as Arctic charr (*Salvelinus alpinus*)[12] and Atlantic cod (*Gadus morhua*)[13], but its role in the gut of fish is unclear. *Pseudomonas sp.* was isolated and dominated in the gut of all the three fishes used for present study. Therefore, the number and composition of gut micro-biota were discussed below. The total heterotrophic bacterial load ranged from 1.8×10^6 to 4.8×10^6 CFU/g of gut sample and it was found to be the maximum in *O. mossambicus* fish gut, and minimum in *E.suratenis*. Several factors, such as bacterial host specificity, food type and water resource[14] may explain these differences. Bacteria in the surrounding environment and feeding habit may have influence on the composition of the gastrointestinal microbiota in fish.

O.mossambicus typically feed on the detritus which are

rich in organic matter compared to the other fishes. *O. leucostictus* grazing on aquatic plants which are the rich in the starch content and *E.suratenis* naturally feeds on the tiny green plants (algae) present in the mid water rather than the bottom mud. It does not freely feed on large plant leaves. The feeding habitat is one of the factors may responsible for the presence of gut biota in these fishes. Both marine and freshwater fish have been shown to have a specific indigenous gut micro-biota[8, 15] and it may change with fish age, nutritional status, and environmental conditions[8]. The most common genera such as *Vibrio*, *Pseudomonas* and *Acinetobacter* were also isolated from the gut of *E.suratenis*. Interestingly, besides Gram positive *Lactobacillus*, *Bacillus* and several Gram negative bacteria normally in the gut of fish were also isolated and identified, such as *Pseudomonas*, *Enterobacter* and *Flavobacterium*. This normal indigenous flora can act competitively, excluding pathogens, inhibiting colonization and, consequently, preventing infection.

Many workers have isolated bacteria which produce antibiotics from marine sources, including seawater, sediment; seaweed and fish gut[2,16,1,17]. In present study the isolates of estuarine fishes shows a broad range of activity against the pathogens. Maximum zone of inhibition was recorded against *K.pneumoniae* and minimum against *V.cholerae*. These results suggest that intestinal bacteria with antibacterial abilities may inhibit the growth of invading bacteria in intestines of freshwater fish, as well as marine fish, to some extent. The antibacterial effect of bacteria is generally due to any of the following factors, either singly or in combination: production of antibiotics, bacteriocins, siderophores, lysozymes or proteases, and alteration of pH values by the organic acids produced.[18] reported that *Pseudomonas fluorescences* reduced diseases caused by *A. salmonicida*. [19] also observed a similar phenomenon that *Vibrio alginolyticus*, used as a probiotic strain, reduced diseases caused by *A. salmonicida*, *V. anguillarum* and *V. or-*

dalii. *Bacillus* has been successfully isolated from the gut of several marine fish and applied as probiotics[20,21]. In the present study, *Bacillus* were the most dominant bacteria in the gut of these fishes and demonstrated antagonistic effect on pathogenic *Vibrio* (Table 3). Previous studies have showed that many *Bacillus* strains isolated from marine fish could inhibit potential pathogens[20,22] and one strain (*Bacillus* no. 48) has been successfully used as probiotics to exclude pathogenic *Vibrio* from common snook (*Centropomus undecimalis*)[20]. This work strongly suggests that effective control of microflora in fish intestines is possible using antibiotic-producing bacteria. A similar approach may be possible in freshwater fish using intestinal bacteria with an inhibitory effect against pathogenic bacteria[23]. Further studies along these lines should be undertaken in the near future in order to find an effective way of preventing outbreaks of infectious diseases mediated by cultured fish. Thus the present findings showed the variation in the micro-flora of different fish species and the microbes from marine environment might contribute to the incorporation of these bacteria in commercial aquaculture as supplement in formulated fish feed or in form of bacteria biofilm to achieve colonization in the fish gut at a higher degree and may be useful in development of probiotics, drugs as well as in the industrial enzyme production. However, further research involving potent bacterial strains should be conducted for evaluating their efficacy under actual farm conditions.

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