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Diversity of microorganisms associated with freshwater fishes from Lake Surha, India

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Abstract

Studies were conducted to evaluate the microbial diversity of mucus from skin and gills collected from freshly catched carp species; *Cirrhinus mrigala, Labio rohita*, and *Cyprinus carpio* and tested for its antagonistic activities against certain pathogenic bacterial strains *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Bacillus cereus*, and *Aeromonas hydrophila*. The microbial population was found to be 2.3 x10^5 cfu/cm of skin mucus in *Cirrhinus mrigala* and was in same range with other two species. The antimicrobial activities of the purified isolates were measured in terms of zone of inhibition (ZOI) against pathogenic bacteria. The potential anatagonist (M0123) was also checked for its colonization potential on fish skin and gills by inoculating them in fish tank having fishes to record the changes in mucus secretion or its antibacterial effects and after inoculation an increase in mucus secretion was observed. The results indicate that the mucus from fish skin colonized with antagonistic strains shows strong antibacterial activity and may play an important role in protection of fishes against bacterial pathogens during fish farming.

Keywords: *Cirrhinus mrigala, Labeo rohita, Cyprinus carpio,* microbial diversity, mucus, antagonistic activity

Introduction

The increasing global population has caused enormous pressure on agriculture and allied production systems due to ever increasing demand for food. Aquaculture is the world's fastest growing food production sector, partly fulfilling the demand. Fish farming has a long history in India and dates back to Kautilya's Arthashastra (321–300 B.C.) and King Someswara's Manasoltara (1127 A.D.) The fisheries in small ponds in eastern India is in practice for centuries; however, significant advances were made in last centuries with assisted and controlled breeding of carp and raising he fingerlings in hatcheries (FAO, 2021). In recent past the Fish culture received notable attention as blue revolution in India. In the fresh water fisheries three Indian major carps, namely catla (*Catla catla*), rohu (*Labeo rohita*) and mrigal (*Cirrhinus mrigala*) contribute to 70-75% of production of the total fresh water fish production (FAO 2021). However, several diseases caused by pathogenic microorganisms like fungi, bacteria, viruses, and ectoparasites limits the production and quality of fish. Bacterial fish diseases are normally treated by antibiotics under controlled environment (Romero *et al.*, 2014), but antimicrobial resistance is a threat to the treatment worldwide. The augmentation or introduction of beneficial microorganisms is another strategy to manage the pathogenic population in the aquatic ecosystem in pond or other natural fish farming systems.

The microflora of fishes are largely unexplored or scant information exists on the biodiversity, and seasonality. Most of the studies conducted on microbial community and diversity of fish microbes focus on gut microbiome and are often conducted with the supplemented diets with probiotics/experimentally modified conditions in foavour of gut microbiome optimal for disease resistance and fish growth. Infectious diseases are among the most pressing concerns in aquaculture development.

The present study was aimed to evaluate the microbial diversity of skin mucus and gills collected from freshly catched carp species; *Cirrhinus mrigala, Labio rohita,* and *Cyprinus carpio* and tested for its antagonistic activities against certain pathogenic bacterial strains *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Bacillus cereus*, and *Aeromonas hydrophila*.

Materials and Methods

Site of study

The study was conducted on the fishes from Surha Taal Wildlife Sanctuary (25.8483° N, 84.1750° E), a natural rainfed lake, located north of Ballia town near village Rajpur in Ballia district Uttar Pradesh, India. It has an area of 1,528 ha. Surha Taal is surrounded by agricultural fields. This water body is host to several migratory and resident bird fauna, and fishing is very common from the lake by locals.

Fish collection and maintenance

Live specimen (300±25 g each fish) of carps species (*Cirrhinus mrigala, Labeo rohita, Cyprinus carpio*) were collected from three different site of the lake and transferred to the laboratory where they were held in Fish tanks (5 individuals $tank^{-1}$) with 1500 litre of fresh water. Exposure to chlorine was minimized by filling the tanks at least 4 days before the fish were introduced. The temperature was kept constant (28 ±2 ^{o}C). The fish were held in a 14 h: 10 h light: dark photoperiod. Mean values for additional parameters of water quality were: pH 7.6 ±0.2, Ca^{2+} 0.60 *mM* l^{-1} and Mg^{2+} 0.3 0.60 *mM* l^{-1}. Fish were fed with commercial fish food and were acclimatized for 7 days before the beginning of the experiments.

Sampling for mucus

After acclimation for 7 days, the fishes were not fed for 24 h. Before collection of mucus from skin and gills the fishes were washed with 4% of potassium permanganate ($KMnO_4$) solution. The mucus was carefully taken by sterilized silicone spatula by scrapping the dorsal surface the body from head to tail. Separate samples were collected from the gills. One ml of sample was snap mixed with 9 ml of 1M phosphate buffered Saline (PBS) pH 7 and stored at -20 C to avoid contamination (Chong et al. 2005). The samples were collected in triplicate.

Microbial diversity assay

The samples were taken out and brought to room temperature. One ml aliquots of each sample was immediately mixed by pipetting into 9 ml sterilized distilled water, and a series of ten-fold dilutions (10^{-1} to 10^{-5}) of the suspension was prepared for each sample. An aliquot of 100 µl from $10^{-3,}$ $10^{-4,}$ 10^{-5} dilution of each sample was spread over solidified both selective and non-selective media like Nutrient Agar (NA), Nutrient Agar (10 times diluted), Halophilic agar (HA), and King's B Agar, Jensen's Agar and Yeast Extract Mannitol Agar medium respectively supplemented with 4% NaCl. Three replicate petri-dishes of each sample were incubated at 28 ± 2°C for 24 to 48 hrs. After the appropriate incubation time total bacterial counts were recorded on different growth medium. Once pure cultures of bacterial isolates were obtained, primary characterizations of the pure cultures of the all isolated bacterial strains were carried out through phenotypic morphological, biochemical and physiological characterization.

The morphological characteristics of all isolates were determined and compared to data of known organisms described in the Bergey's Manual of Systematic Bacteriology. Morphological features of colony *viz.* colony shape, size, edge, elevation, texture, opacity and pigmentation of bacterial isolates were recorded from the pure culture colonies. Gram staining, cell morphology and motility for all the bacterial isolates was carried out following the standard procedure.

Motility of bacteria was checked by hanging drop method. One drop of bacterial broth was put on a cover slip which was then inverted on concavity slide and motility was observed under compound microscope.

Test microorganisms

All microbial strains studied (five human pathogen including three Gram-negative bacterial strains *Escherichia coli*, *Pseudomonas aeruginosa*, and three Gram-positive bacterial strains *Staphylococcus aureus*, *Bacillus cereus Aeromonas hydrophila* were obtained from laboratory and grown at 30 °C in nutrient broth following Biosafety measures.

Antagonism assay

Dual plating method was followed to evaluate the antagonistic potential of isolated strains. The log phase culture of the pathogenic strains and bacteria isolated were inoculated as a line in a 90 mm diameter Petri dish 2.5cm apart from each other and incubated at 28±2 ^{o}C. Antagonist activity was observed after incubation up to 4 days. The activity of the strains was evaluated by measuring the growth inhibition against test organism and the percent growth inhibition was calculated by using the formula: (C-T/C) x100

Effect of Potential anatgonists on mucus antibacterial activity

The fish tanks having 5 healthy *C. mrigala* (300±25 g) fish were inoculated with the potential antagonistic bacteria (Isolate No M 0123) at a final concentration of water to 10^4 cfu/ml of water, rest of the culture conditions remained the same. After 7 days of inoculation, the fishes were sampled for mucus collection and measurement of its antibacterial property by well diffusion assay. The antibacterial effect of inoculated fish mucus and controlled fish mucus extracts of *C. mrigala* on the *P. aeuginosa* was assayed by Agar well diffusion method (Valgas et al. 2007). Petri plates containing 20 ml nutrient agar medium were inoculated with 10 µl culture of *P. aeruginosa* at 10^8 cfu ml^{-1} by spread plating. Then, wells with a diameter of 5 mm were punched aseptically with a sterile cork borer or a tip and fish skin mucus extracts were added. The plates were then incubated at 30 °C for 24 h. Evaluation of bactericidal effect was done by measuring the diameter of the zone of inhibition (ZOI) formed around the well (Jorgensen and Turnidge 2015).

Results and Discussion

Fish collection and maintenance

Live specimen (300±25 g each fish) of carps species *Cirrhinus mrigala, Labeo rohita, Cyprinus carpio*) were collected from three different site of the lake and brought in the laboratory where they were reared Fish tanks (5 individuals per tank) with 1500 litre of water. The level of chlorine in water was minimized by filling the tanks at least 4 days before the introduction of fishes. The temperature was kept constant (28 ±2 ^{o}C). The fish were held in a 14 h: 10 h light: dark photoperiod. The water quality was maintained to pH 7.6 ±0.2, Ca $^{2+}$ and Mg $^{2+}$ to 0.60 *mM* l^{-1} and 0.3 0.60 *mM* l^{-1}, respectively. Fish were fed on commercial fish food and before the beginning of the experiments were acclimatized for 7 days. .

Sampling for mucus

The mucus secreted by different species (*C. mrigala*, *L. rohita*, and *C. carpio*) exhibited variation in appearance and amount, *C. mrigala* and *L. rohita* secreted colourless to whitish mucus with froth whereas, *C. carpio* mucus was pale yellowish having and odour (Table 1). The quantity of mucus is highest in *C. arrpio* and lowest in *C. mrigala.* It was also noted that mucus was more viscous and secretion was more during breeding season followed by summer and winters. The tissues secreting mucus (mucosa) like gills, olefactory system and skin are in direct contact with the surrounding aqauatic environment and are the point of contact of the microbes with the host. Therefore, the mucus layer over tissues might be the first defense to external environmental influences. Biochemeically, it consists of the glycoprotein of high molecular weight (mucin) in addition to other lipids, proteins and ion. The mucus contains immune components like complement proteins, lectins, NARPs, immunoglobulins (Uribe et al. 2011; Brinchmann 2016).

Table 1 The mucus scrapped from surface in three attempts a day

Fishes	Mucus (ml/fish/day))		
	Summer (May)	Breeding period (July)	Winters (December)
C. mrigala	3	3.9	2.4
L. rohita	3.6	4.2	3
C. carpio	3.9	4.5	3.6

Microbial diversity assay

A total of 48 different isolates were isolated from the mucus. Differential staining (Grams stain) of the isolates from mucus revealed that the Gram's positive bacterial isolates were dominant over Gram's-negative bacteria which produced semi translucent smooth circular colonies on growth medium at 28ºC after 48 h of incubation. The details of the colonial characteristics of bacterial isolates are given in Table 2.

The isolate produced small to large and semi translucent to opaque, smooth circular to irregular margins colonies and had convex, lobate, undulate and entire colonies, while flat, hilly, raised or drop like colonies were also found. The colonies showed wide variation in their colour like White, cream, pale yellow, yellow, yellowish green, orange, brown and pink in colours. Wide variations were observed among the isolates in their colony morphology and showed resemblance to different bacterial genera like *Enterobacteria, Kocuria, Microbacterium, Bacillus, Pseudomonas*, and *Serratia* etc. (Table 2).

Table 2: Colony Morphology of isolates

Strains Codes	Size	Shape	Margin	Elevation	Opacity	Colour
M0107	Large	Irregular	Lobate	Raised	Opaque	Light cream
M0108	Large	Circular	Undulate	Flat	Opaque	White
M0109	Medium	Circular	Undulate	Umbonate	Opaque	White
M0110	Medium	Circular	Undulate	Raised	Opaque	Cream
M0111	Large	Circular	Entire	Raised	Opaque	Light cream
M0112	Medium	Irregular	Lobate	Raised	Opaque	Creamy white
M0113	Medium	Irregular	Lobate	Raised	Opaque	White
M0114	Medium	Circular	Entire	Raised	Opaque	White
M0115	Medium	Circular	Undulate	Raised	Opaque	Light cream
M0116	Medium	Circular	Entire	Raised	Semi translucent	Light cream
M0117	Medium	Circular	Entire	Convex	Opaque	Cream
M0118	Large	Irregular	Lobate	Raised	Opaque	White
M0119	Large	Circular	Lobate	Raised	Opaque	White
M0120	Large	Irregular	Lobate	Raised	Opaque	White
M0121	Large	Irregular	Lobate	Raised	Opaque	Cream
M0122	Large	Circular	Undulate	Flat	Opaque	White
M0123	Medium	Circular	Undulate	Raised	Opaque	Creamy white
M0124	Medium	Irregular round	Lobate	Raised	Opaque	White
M0125	Medium	Circular	Lobate	Raised	Opaque	Cream
M0126	Large	Circular	Undulate	Flat	Opaque	Light orange
M0127	Large	Circular	Undulate	Flat	Opaque	cream
M0128	Large	Irregular	Lobate	Raised	Opaque	Light orange
M0129	Large	Irregular	Lobate	Raised	Opaque	White
M0130	Large	Irregular	Lobate	Raised	Opaque	White
M0131	Medium	Circular	Undulate	Flat	Opaque	White
M0132	Medium	Circular	Entire	Raised	Semi translucent	Creamy white
M0133	Large	Irregular	Lobate	Flat	Opaque	White
M0134	Medium	Circular	Entire	Flat	Semi translucent	White
M0135	Large	Circular	Undulate	Flat	Opaque	Cream
M0136	Medium	Circular	Entire	Raised	Opaque	Creamy white
M0137	Medium	Circular	Undulate	Raised	Opaque	Creamy white
M0138	Large	Irregular	Entire	Raised	Opaque	Cream
M0139	Medium	Circular	Lobate	Umbonate	Opaque	Cream

Earlier studies of Merrifield and Rodiles (2015) exhibited that the mucus from skin and gills are habitat for aerobic microbes than that of anaerobes. However, it is difficult to compare in absolute numbers. Normally the skin contains 100-1000 bacteria per centimeter, whereas, the gills contains bacterial population till up to 10^6 cfu per gram of the tissues (Austin 2006; Merrifield and Rodiles 2015). Further other studies have shown seasonal effect on the population e.g the microbial diversity was in a Portuguese marine aquaculture system was higher in warmer season (Pereira et al. 2011). Turner et al. 2009 reported variations and correlation in Vibrio populations with the season and plankton composition. Potential mechanisms underlying seasonal effects on fish microbes are many including temperature and dissolved oxygen, it can be indirectly correlated with the variations in mucus secreted by fish in different seasons.

Antagonism assay

Dual plating method was followed to evaluate the antagonistic potential of isolated strains. The log phase culture of the pathogenic strains and bacteria isolated were inoculated as a line in a 90 mm diameter Petri dish 2.5 cm apart from each other and incubated at 28±2°C. Antagonist activity was observed after incubation at 28 ± 2°C up to 4 days. The activity of the strains was evaluated by measuring the growth inhibition against test organism and the percent growth inhibition was calculated by using the formula: (C-T/C) x100. Isolate M0123 exhibited maximum inhibition against all the test pathogens followed by isolate M0129, M0138, M0125 and M0134.

Table 3: Antagonism assay

Strains Codes	***Inhibition**				
	Escherichia coli,	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Bacillus cereus*	*Aeromonas hydrophila*
M0107	+	+	-	-	-
M0108	-	-	-	-	-
M0109	+	+	+	-	-
M0110	++	++	+	-	+
M0111	-	+	-	-	-
M0112	+	+	+	-	-
M0113	++	+	+	+	+
M0114	+	+	+	-	-
M0115	-	+	-	-	-
M0116	++	++	+	-	+
M0117	++	+	+	+	+
M0118	-	-	-	-	-
M0119	-	-	-	-	-
M0120	-	-	-	-	-
M0121	+	+	-	-	-
M0122	+	+	+	-	-
M0123	+++	+++	++	+	++
M0124	-	+	-	-	-
M0125	++	+	+	+	+
M0126	+	+	+	-	-
M0127	-	-	-	-	-
M0128	++	++	+	-	+
M0129	+++	++	++	+	+
M0130	-	+	-	-	-

M0131	++	++	+	-	+
M0132	+	+	+	-	-
M0133	+	+	+	-	-
M0134	++	++	+	-	+
M0135	+	+	+	-	-
M0136	++	++	+	-	+
M0137	+	+	+	-	-
M0138	++	+	+	+	+
M0139	-	-	-	-	-

* + = up to 25% inhibition, ++ = up to 50%, +++ = up to 75%, - = less than 25% or no inhibition

Effect of Potential antagonist on mucus antibacterial activity

The mucus collected from the fishes from inoculated tank exhibited more than 75% inhibition towards the growth of *P. aeruginosa*. Earlier studies confirm that the mucus secreted from fish skin, and gills create an inhibitory interface between fish and pathogen and act as mechanical barrier (Reverter et al. 2018). The study presented is in agreement to the findings that the skin mucus also acts as the reservoir of antimicrobial components which acts in different ways and is gifted with innate antibacterial ability as reported by Nagashima et al. (2001, 2003). In conclusion, the study shows that beneficial microorganism in aquatic system contributes towards the health of fishes.

References

Austin B. (206) The bacterial microflora of fish, revised. *Sci World J*. **6**:931–45.

Brinchmann MF. (2016).. Immune relevant molecules identified in the skin mucus of fish using -omics technologies. *Mol Biosyst* 2 **12**:2056–63.

Chong K, Ying TS, Foou J, Jin LT, Chong A (2005) Characterization of proteins in epidermal mucus of Discus fish (*Symphosodon* spp.) during parental phase. Aquaculture 249:469–476. https://doi.org/10.1016/j.aquaculture.2005.02.045

FAO fisheries division (2021): National aquaculture sector overview: India. http://www.fao.org/fishery/countrysector/naso_india/en

Jorgensen J, Turnidge J (2015) Susceptibility Test Methods: Dilution and Disk Diffusion Methods. In: Jorgensen J, Pfaller M, Carroll K, Funke G, Landry M, Richter S, Warnock D (eds) Manual of clinical microbiology, 11th edn. ASM Press, Washington, DC, pp 1253–1273

Merrifield DL, Rodiles A. (2015) The fish microbiome and its interactions with mucosal tissues. In: Peatman BHB (ed.) *Mucosal Health in Aquaculture*. San Diego: Academic Press, 273– 95.

Nagashima Y, Sendo A, Shimakura K, Kobayashi T, Kimura T, Fujii T (2001) Antibacterial factors in skin mucus of rabbit fishes. J Fish Biol 58:1761–1765. https://doi.org/10.1111/j.1095-8649.2001.tb02331

Nagashima Y, Kikuchi N, Shimakura K, Shiomi K (2003) Purification and characterization of an antibacterial protein in the skin secretion of rockfish *Sebastes schlegelii*. Comp Biochem Physiol C Toxicol Pharmacol 136:63–71. https://doi.org/10.1016/ S1532-0456

Pereira C, Salvador S, Arrojado C *et al.* (2011)Evaluating seasonal dy- namics of bacterial communities in marine fish aquaculture: a preliminary study before applying phage therapy. *J Environ Monitor*; **13**:1053–8

Reverter M, Tapissier-Bontemps N, Lecchini D, Banaigs B, Sasal P (2018) Biological and ecological roles of external fish mucus: a review. Fishes 3(4):41. https://doi.org/10.3390/fishes/3040041

Romero J, Ringø E, Merrifield DL. (2014) The gut microbiota of fish. In: *Aquaculture Nutrition*. John Wiley & Sons, Ltd, New Yersey, USA, 75–100.

Turner JW, Good B, Cole D *et al.* (2009) Plankton composition and environmental factors contribute to *Vibrio* seasonality. *ISME J* **3**:1082–92.

Uribe C, Folch H, Enriquez R *et al.* (2011) Innate and adaptive immunity in teleost fish: a review. *Vet Med* **56**:486–503.

Valgas C, De-Souza SM, Smania EFA, Jr Smania (2007) Screening methods to determine antibacterial activity of natural products. Braz J Microbiol 38(2):369–380. https://doi.org/10.1590/S1517-83822007000200034.

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