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Intestinal microflora of farm raised Indian major carps, *Catla catla, Labeo rohita* and *Cirrhinus mrigala*

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Microflora associated with the intestine of farm raised Indian major carps such as *Catla catla* (Ham.), *Labeo rohita* (Ham.) and *Cirrhinus mrigala* (Ham.) were analyzed. The mean population of total intestinal microflora (TIM) in the intestine of *Catla catla* was 4.62×10^6 colony forming unit (CFU) per gram and that of *Labeo rohita* and *Cirrhinus mrigala* was 3.40×10^6 and 1.68×10^7 CFU per gram respectively. Morphological and physiological grouping of the isolates revealed dominance of Gram-negative rod shaped bacteria capable of elaborating various hydrolytic enzymes such as amylase, lipase and gelatinase. Ureolytic forms were relatively few. Characterisation of genera revealed the dominance of *Aeromonas* in the intestine of these fishes. Other genera encountered were *Vibrio, Bacillus, Moraxella, Micrococcus, Corynebacterium, Flavobacterium* and *Achromobacter*. Results of this experimental work might have valuable implications for the management practices in aquaculture ponds by using these established useful gut-flora.

Key Words : Indian major carps, intestine, microflora

INTRODUCTION

Fish always take a large amount of bacteria into their guts from water, sediments and or food (Sugita *et al.*, 1996). The indigenous microflora of fish in aquaculture has previously been studied for many purposes. This includes relationship between environment and fish microflora (Horsley, 1973), descriptions of microbial spoilage (Joseph *et al.*,1988), the monitoring of changes in fish farms (Allen *et al.*,1983), the nutritional role of the intestinal flora (Moriarty, 1990) and the antibiotic resistance profile of the indigenous flora (Spanggaard *et al.*,1993). During the last few decades, the intestinal microflora of reared fish has been studied with the purpose of finding disease preventive bacterial strains, which is popularly known as probiotics (Patra and Bandyopadhyay, 2003). The intestinal flora of fish has as a consequence received much attention by several authors (Sugita *et al.*, 1987 ; Onarhem and Raa, 1990 ; Ringo *et al.*, 1995), although the significance of the intestinal microflora with regard to disease protection still remains uncertain.

Many studies have shown that bacterial population in fish guts may be high (Trust *et al.*, 1979) and that fish may derive a substantial portion of their nutrition from bacteria. However, some bacteria, which possess the ability to tolerate the low pH in gastric juices, resist the action of bile acids and lysozyme secreted in the intestines, adhere to the

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mucous and entire wall surface and could persist for a relatively long time (Sugita *et al.,* 1980 ; Sugita *et al.,* 1988). Of these bacteria, some species seems to be indigenous and others are temporary. These facts strongly suggest that the ecology of these microorganisms in fish intestines should be determined in the interest of public health (Patra and Bandyopadhyay, 2002). Some non-pathogenic microflora has a disease preventing effect ; this protection is likely to be mediated by microorganisms that are dominant and present in high numbers (Sugita *et al.,* 1996). Studies of the composition and characteristics of the dominant microflora are, therefore, a crucial part in probiotic research (Spanggaard *et al.,*2000).

In addition to nutritional benefits, gut bacteria can facilitate ion transport across the host's gut wall (Haq *et al.,* 1986), enhance the host resistance to toxic effects of various chemicals (Dempsey and Kitting, 1987), and destroy opportunistic pathogens (Garriques and Arexalo, 1995). Those factors are related directly to the anatomy and physiology of the host, and include gut structure (Gunzl, 1991), gut passages time (Plante *et al.,* 1989). External environment conditions also affect gut microflora of fishes, including water salinity (Straub and Dixon, 1993), water temperature (Haq *et al.,* 1984), the presence of toxicants in the water (Atlas *et al.,* 1982) and food availability (Haq *et al.,* 1986).

Indian major carps *Catla catla, Labeo rohita* and *Cirrhinus mrigala* are the most dominated and widely distributed freshwater fish species in the Indian subcontinent. Since the intestinal microflora play a significant role in the growth and nutrient utilization and also some potent organism responsible for disease resistance and immune responses, it is of importance to know the activity of the microflora in the intestine of Indian major carps of that environment. However, most studies are restricted to species belonging to temperate region or coldwater fishes such as salmon, rainbow trout and Arctic charr. Very little work has been done on the intestinal microflora of farm raised Indian major carps, such as *Catla catla, Labeo rohita* and *Cirrhinus mrigala.* Moreover, identification of the normal intestinal microflora of Indian major carps would be of great value in correct interpretation of physiology, nutritional requirements and disease resistance. Considering the above facts, the present investigation has been made to enumerate the generic composition, characterization of the microflora associated with the gastrointestinal tract of farm raised Indian major carps *Catla catla, Labeo rohita* and *Cirrhinus mrigala* in order to find out the role of intestinal microflora in the digestive process of these fishes.

MATERIALS AND METHODS

The experiment was conducted at Aquaculture Research Unit and Microbiology Research Unit of Vidyasagar University, Midnapore, India (Lat. 22°25′N and Long. 87°20′E). Samples were collected from an aqua farm (Mohanpur) located at the vicinity of Midnapore. The culture pond selected was a semi intensive freshwater aquaculture pond practicing mixed culture of *Catla catla, Labeo rohita* and *Cirrhinus mrigala.* The pond was fertilized with semidried cow dung and the fishes were fed with pelleted feed @ 5% of their body weight daily in two instalments which were prepared with an equal mixture of rice polish and mustard oil cake. Amount of pelleted feed was adjusted in month after sampling.

Indian major carps were causht by cast net. Fishes of medium size (435 ± 28 g) were taken for analysis assuming that they might have a well established pattern of intestinal microflora. Fishes were transferred to water collected from the pond and brought into the laboratory in live condition. Ten fishes of each sample were examined in this study. Upon reaching the laboratory, analysis of the intestinal microbial flora was done on samples consisting of excised and cut open with a pair of sterile scissors. Gut contents were removed by scrapping, and the intestines were washed three times with sterile saline solution to remove non-adherent microflora. The samples were then homogenized with 10 ml distilled water in Stomacher bags (Stomacher, Lab – Blender 400). Dilution series were prepared from the homogenates. To find out the total intestinal microfloral population (IMP), intestinal homogenate were first diluted serially (serial dilution) and then spread over nutrient agar (Hi-Media) plate by following the spread plate technique. After proper incubation period (room temperature at 28ºC - 32ºC for 24 h), the individual colonies were counted as colony forming units (CFU).

Morphologically different colonies were picked at random from the plates and restreaked to ensure purity. Smears were prepared on glass slides and stained adopting Gram's method for microscopic examination. Around fifty (50) colonies were selected for each fish, as it was sufficient to obtain a representative diversity of bacterial communities (Hatha *et al.,* 2000). The bacterial isolates were characterized on the basis of Gram stain, spore stain, motility, oxidase, catalase and O/F tests and grouped into various genera using Kaneko (1971) and Bergey's Mannual of Systematic Bacteriology (1986). The ability of the isolates to elaborate the synthesis of various hydrolytic enzymes such as amylase, gelatinase etc., was determined by substrate hydrolyzing assay. Urea splitting ability was determined by inoculating into Christiansen's urea agar medium. Isolates were maintained in nutrient agar slants and stored at 4ºC for further study.

RESULTS AND DISCUSSION

Total microfloral population (TMP) in the intestines of farm raised *Catla catla, Labeo rohita and Cirrhinus mrigala* is presented in Table 1. The mean population of TMP in *Catla catla* and *Labeo rohita* was 4.62×10^6 and 3.40×10^6 CFU g $^{-1}$ respectively. The TMP load in the intestine of *Cirrhinus mrigala* was 1 log higher than IMP population of the intestine of *Catla catla* and *Labeo rohita.* TMP population of such magnitude was reported earlier by Trust and Sparrow (1974) in the intestine of Salmonids, Trust *et al.* (1979) in the intestinal tract of *Carassius auratus* and *Ctenopharyngodon idella,* Sugita *et al.* (1988) in *Carassius auratus,* Hossain *et al.* (1999) in *Labeo rohita* and Hatha *et al.* (2000) in *Catla catla.* However, the IMP load was less than those reported in the intestine of salmonids (Trust *et al.,* 1979) as well as that of Arctic charr in the natural environment (Ringo and Strom, 1994). The TMP load in the intestine of *Hypopthalmicthys molitrix* was 2 log higher than the present study. The variations in the microfloral counts between individual fish had been observed previously (Trust and Sparrow, 1974; Yoshimizu and Kimura, 1976). The present studies showed up to 2-log unit difference, which was even lesser than observed by Yoshimizu and Kimura (1976) who found 3 – 4 log units difference when examining the microflora of healthy salmonids. This might be due to the different environmental conditions in the aquaculture farms as the fishes acquire their intestinal microflora from the ambient environment (Patra and Bandyopadhyay, 2003).

Table 1 : Mean and range of population of total intestinal microflora (TIM) in the intestine of *Catla catla, Labeo rohita* and *Cirrhinus mrigala*

Sample source Intestine of	Mean population (CFU g^{-1})	Range of IMP
Catla catla	4.62×10^6	2.89×10^5–4.52×10^7
Labeo rohita	3.40×10^6	1.47×10^5–4.40×10^7
Cirrhinus mrigala	1.68×10^7	1.20×10^6–4.02×10^7

Morphological and physiological grouping of microflora found in the intestine of *Catla catla, Labeo rohita* and *Cirrhinus mrigala* is presented in Table 2. There was predominance of Gram-negative rods in the intestine of these fishes. The occurrence of Gram-negative rods was around 75% in the intestine of *Catla catla, Labeo rohita* but around 90% in the case of *Cirrhinus mrigala.* Similar data were found in the intestines of various fishes such as Arctic charr (Ringo, 1993; Ringo and Strom, 1994). *Labeo rohita* (Hossain *et al.,* 1999), rainbow trout (Spanggaard *et al.,* 2000) and grass carp (Hatha *et al.,* 2000).

Ability of the microfloral isolates to elaborate various hydrolytic enzymes indicated that majority of them were capable of utilizing various substrates such as starch, gelatin and lipid (Tween 80). Urea splitting forms were relatively less (Table 2). The beneficial effects of some intestinal microflora, which is popularly known as probiotics in disease resistance, were well documented (Gatesoupe, 1999; Patra and Bandyopadhyay, 2003). Some investigators had also suggested that micro-organisms exert beneficial effects on the digestive process of fish (Riquelme *et al.,* 1996; Gibson *et al.,* 1998; Gram *et al.,* 1999). Flora of the digestive

Table 2 : Per cent incidence of various morphological and physiological groups in the intestine of *Catla catla, Labeo rohita* and *Cirrhinus mrigala*

Morpholotical and physiological characteristics	Per cent incidence of various morphological and physiological groups in the intestine of *Catla catla*	*Labeo rohito*	*Cirrhinus mrigala*
Gram positive	32	28	10
Gram negative	68	72	90
Rods	86	84	96
Cocci	14	16	04
Motile	72	78	76
Oxidase positive	80	86	78
Calalase producers	68	64	84
Glucose fermenters	86	92	92
Amylolytic forms	62	69	86
Gelatenolytic forms	80	87	98
Lipolytic forms	70	66	66
Ureolytic forms	40	54	40

tract could act on lipolysis by way of contributing to triglyceride breakdown. Lipolytic activity of bacterial isolates from gastrointestinal tract in grass carp, *Ctenopharyngodon idella* had been previously reported by Trust *et al.,* (1979). Flora of the digestive tract could act on lypolysis in different ways by contributing to triglyceride break down through bacterial action and by changing pancreatic lipase secretion or inactivating it with bacterial protease. The intestinal bacteria with antibacterial activity might inhibit the growth of invading bacteria in intestines of freshwater fish (Sugita *et al.,* 1996).

Occurrence of various bacterial genera in the gastrointestinal tract of *Catla catla, Labeo rohita* and *Cirrhinus mrigala* is presented in Table 3. *Aeromonas* was the dominant genus in the intestines of all these fishes. *Corynebacterium* was the second dominant genus in *Catla catla* while *Vibrio* in *Labeo rohita* and *Cirrhinus mrigala*. Presence of *Pseudomonas* was only recorded in *Labeo rohita.* Smith and Davey (1993) reported that the intestinal flora *Pseudomonas fluorescens* might be reduced by *Aeromonas* sp. It had been observed that the increasing trend of *Vibrio* from *Catla, Labeo* and *Cirrhinus* while the reverse was found in case of *Bacillus*. Other bacteria present were *Moraxella, Micrococcus, Flavobacterium* and *Achromobacter* while 2% of the bacteria were unidentified in *Labeo*. In the earlier report, *Vibrio* and *Aeromonas*

Table 3 : Per cent occurrence of different bacteria in the intestine of *Catla catla, Labeo rohita* and *Cirrhinus mrigala*

Sample source Intestine of	Name of the organisms	Percentage of occurrence
Catla catla	*Aeromonas* sp.	58
	Corynebacterium sp.	12
	Micrococcus sp.	8
	Moraxella sp.	8
	Vibrio sp.	8
	Bacillus sp.	6
Labeo rohita	*Aeromonas* sp.	62
	Vibrio sp.	12
	Flavobacterium sp.	8
	Pseudomonas sp.	6
	Lactobacillus sp.	6
	Bacillus sp.	4
	Unidentified	2
Cirrhinus mrigala	*Aeromonas* sp.	68
	Vibrio sp.	16
	Flavobacterium sp.	10
	Achromobacter sp.	2
	Bacillus sp.	2
	Micrococcus sp.	2

were the dominant genera in the intestine of gray mullet (*Mugil cephalus*) (Sakate *et al.,* 1988); *Aeromonas* was in grass carp (Hatha *et al.,* 2000) and Coryneform in *Labeo rohita* (Hossain *et al.,*1999). The composition of intestinal bacteria may vary from fish to fish (Hossain *et al.,* 1999), and environment to environment (Sugita *et al.,* 1987). MacMillan and Santucci (1990) found that the composition of the intestinal microflora of farm raised channel catfish varied with season. Sugita *et al.,* (1987), detected day-to-day high fluctuation in the microflora of goldfish. It had been argued that the fish intestine did not have a stable microflora (Yoshimizu *et al.* 1980), although the gastrointestinal tract provided an ecosystem distinctly different from the surrounding water. Different types of bacteria were found in different species which might be due to ontogenetic changes exhibited by the host (Yasuda and Kitao, 1980) or gut passage time might have been relatively slow and gut might have provided a more stable environment for the proliferation of aerobic bacteria (Sugita *et al.,* 1987). Occurrence of intestinal microflora also varied due to age, diets, emotional stress, mucin of host fish and anthropogenic activity on water bodies (Paul, 2002). Austin and Al-Zalrani (1988) distinguished between the flora of the gut content and associated gut wall flora in rainbow trout and noted that scanning electron microscopy showed only spare colonization of the wall.

In the present study *Bacillus* was one of the genera in all the fishes. In the earlier report it was observed that *Bacillus* provided both cellular and humoral immune defence. which was found in shrimp gut (Rengpipat *et al.,* 1998). Moreover, there were many reports of isolation of *Bacillus* strains from the intestine of fish (Strom and Olafsen, 1990, Sugita *et al.,* 1988). Queiriz and Boyd (1998) confirmed that a commercial inoculum of *Bacillus* spp. used as probiotics for terrestrial livestock had telluric organs and they were not autochthonous in the gastrointestinal tract. but they might be active during intestinal transit (Gournier-Chateau *et al.,* 1994).

Assuming that the associated intestinal microflora has a disease preventing effect, this protection is likely to be mediated by microorganisms that are present in higher numbers. Studies of the composition and characteristics of the dominant microflora are, therefore, a crucial part in probiotic research. The results would provide information about the 'normal' gut flora, which may act as inhibitory to disease pathogenesis. This would eventually help in designing therapeutic trials as probiotic supplement.

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