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Comparative study of seasonal variation in bacterial flora concomitant with farm raised fingerlings of *Cyprinus carpio* at tarai region of Uttarakhand

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Abstract

The bacterial infection is an important economic and limiting factor in intensive fish production. The present study focuses on investigation of the bacterial population associated with farmed common carp fingerlings, its environment and limnological quality of pond, during winter and summer season. It was found that the bacterial count in the pond sediment (6.40 cfu x 10^4) was about 10 times higher in comparison of pond water (6.93 cfu x 10^3). Further, the intestinal bacterial count was about 100 times higher (6.67 cfu x 10^5) during winter and 1000 times higher (2.33 cfu x 10^6) during summer season in comparison to the surfacial skin of fish during winter and summer (3.39 and 8.87 cfu x 10^3), respectively. The isolated bacteria were both Gram negative and Gram positive, mostly aerobic rods. Furthermore, the temperature showed a significant relation with the bacterial counts of pond water. In the summer season, higher bacterial counts (8.72 cfu x 10^3) were recorded as compared to winter (5.13 cfu x 10^3). The dominant bacteria isolated from the sample of pond water, pond sediment and fish were identified as *Aeromonas hydrophila*, *Pseudomonas fluorescens*, *Pseudomonas* sp., *Flavobacter* sp., *Bacillus* sp., *Micrococcus* sp., *Corynebacterium* sp. Moreover, the bacterial density was dependent on C:N values, and the optimum range of C: N ratio was found between 16-23, for the carp culture ponds. Among the isolated bacterial flora, the presence of strains which were well known for their probiotic properties suggested an autochthonous source for use in aquaculture. Further, analysis of various physico-chemical parameters of pond water revealed that they were within the suitable range for the freshwater fish culture throughout farming phase.

Key words

Bacterial flora, Cyprinus carpio fingerlings, C:N values, Seasonal variation

Introduction

India has witnessed an overwhelming growth of aquaculture sector during the last two decades and ranks second in aquaculture production. Carp culture plays an indispensable role with a percentage contribution of 93.6 to freshwater aquaculture production of India (FAO, 2005). While this growth is much appreciated in terms of food security, the health risk associated with the aquaculture produce is another important concern.

Microbial quality of farmed fish is largely determined by the quality of water in which they cultivated (Black, 2009). The water quality influences the bacterial load in fish ponds (Roy *et al.,* 2011). Similarly, feed and feeding to cultured fishes have been reported to have a considerable impact on the load of bacteria (Fafioye, 2011**).** Water quality is an important indicator in appraising the eutrophic situation, primary productivity and fish yield potential. The relationship between bacteria and water environment has received attention of researchers, but the studies undertaken are limited (Surendraraj *et al.,* 2009).

Microbiological investigations are of importance both from view point of maintaining a proper environment as well as optimum utilization of available and added nutrients for fish

production. They also help in understanding the delicate balance between the host and environment relationship. Bacteria are extremely diverse with respect to aquatic habitat. Within pond environment bacteria inhabit the water phase, the bottom sediment and of course live upon plants, animals and detritus. Aquatic animals such as fish are in direct contact with the microflora which is already present in their environment.

With intensification of culture practices, the numbers of outbreak of bacterial diseases in cultured fish have also increased. The use of probiotic bacteria, isolated from naturally-occurring bacterial communities, is gaining in popularity in the aquaculture industry as the preferred, environmentally-friendly management alternative to the use of antibiotics and other antimicrobials for disease prevention. Beneficial bacteria, in the best cases, can be used to substitute the use of antibiotics, as preventive agents of disease (Nikoskelainen *et al.*, 2001) and as growth promoters (Byun *et al*., 1997). Various studies have been undertaken to analyze the bacterial flora associated with gut of culturable fishes as probiotic usage of bacteria is an integral aspect of environment friendly and sustainable aquaculture (Gomez-Gil *et al*., 2000).

Common carp (*Cyprinus carpio*) is one of the most cultured fish in the world. In 2008, the world and the European production were 2987433 and 144747 tons, respectively (FAO, 2011). It is well established cultured species with a well known production cycle. It is consumed as a traditional food in Central Europe. Common carp is an omnivorous species eating plankton and benthos (worms, insects, molluscs) as well as detritus in under natural conditions (Adamek *et al.,* 2004a).

Considering the above facts, the present investigation was planned to observe the bacterial biomass associated with the fish, *Cyprinus carpio* (fingerlings) and its environment in two major seasons, so as to get an insight into health of the fish and condition of their surrounding environment. Qualitative investigation would also help in fish probiotic research since it is a general consensus that probiotics from autochthonous source have a greater chance of competing with resident microbes and of becoming predominant within a short period of intake, which can significantly improve disease resistance, growth and survival of fish.

Materials and Methods

Experimental site and plan : Present study was conducted at instructional fish farm of the College of Fisheries, Pantnagar (29°N latitude, 79.3°E longitude and an altitude of 243.8 msl) in two rectangular earthen ponds (A and B) of size, 8.73×1.6×0.85 m, where composite fish culture is carried out under semi-intensive culture technique. The source of water supply was an artesian tube well which was regularly used to maintain the water level (1.2 m). The study was carried out in two trials of 60 days each, conducted in two major seasons i.e. winter (December to January) and summer (April to May). Natural food alone was not sufficient to achieve the expected fish production hence, supplementary feed (approximately 30% protein) consisting of groundnut oil cake, soybean oil cake, rice bran, and fishmeal were fed to the fishes during the culture period @ 5% of body weight.

Sampling and data collection : Simultaneously with the bacteriological analysis, the chemical parameters of soil viz. pH, organic carbon, nitrogen and phosphorous (fortnightly) and physico-chemical parameters of water (weekly) such as do, free CO_2 alkalinity were analyzed till the end of the experiment. Soil/mud samples were collected from bottom of both ponds A and B, with Ekman dredge. Water samples were collected weekly in glass stoppered sampling bottles and analyzed in laboratory following the standard method of APHA (2005). Water temperature was recorded with the help of digital thermometer (Cooper-Atkins, USA) having range of 0-50 °C, with mark up to 0.1 °C. The pH of water samples was measured with a pH meter (Sartorius, Germany).

Bacteriological analysis : The bacteria from water and soil samples were isolated following the serial dilution plating method and the cell number of bacteria was determined and expressed in cfu ml^{-1} on nutrient agar plates with the help of Quebec colony counter. The plates having more than 30 and less than 300 colonies were used to calculate bacterial population numbers and were expressed in expressed as cfu. The plate counts were carried out in triplicates and the average of the three respective readings were taken.

Bacteria that were morphologically different and well isolated on plates were transferred to slants and incubated at 30±2°C for 24-48 hr. All the isolates were rechecked for purity by streaking them on a second fresh nutrient agar plate and then transferred on slants and maintained at 4°C temperature. Further, the bacterial isolates were studied and identified following Bergey's Manual of Determinative Bacteriology (Breed *et al.,* 1957) for their colony morphology (size, shape, chromogenesis, edge and elevation of colony), motility (by hanging drop method) and cell morphology (simple, Gram's and spore staining). However, biochemical and physiological tests are considered as essential tools for identification of bacterial genera and species. Various isolates were tested for 10-12 biochemical and physiological tests namely catalase test, acid and gas production from carbohydrates, starch hydrolysis, gelatin hydrolysis, cecarboxylation of amino acids, indole production, MRVP test, urea hydrolysis and H_2S production following microbes in action (Harry and Paul, 1962). The whole process was done in aseptic conditions to avoid contamination during isolation. Further, the experimental data were subjected to statistical analysis (Gomez and Gomez, 1984). Level of significance used for F and T test were P=0.05 from the table given by Fisher. The critical difference of means has been worked out.

Results and Discussion

The results of total plate counts indicated that the viable count of pond water samples was found in low range (4.43±0.15×10^3 to 5.50±0.09×10^3 cfu ml^{-1}) during winter phase in comparison to summer phase (7.43±0.03×10^3 to 9.66±0.09×10^3 cfu ml^{-1}) (Table 1). Further, in both the ponds sediment, the bacterial counts were in the range of 3.46 ± 0.15×10^4 to 4.46±0.15×10^4 cfu g^{-1} during the winter phase and 8.3±0.26×10^4-9.43±0.24×10^4 cfu g^{-1} during the summer phase (Table 2).

Moreover, total viable counts in the fish skin ranged between 3.2±0.12×10^3 to 3.73±0.03×10^3 cfu cm^{-2} during winter season and 8.2±0.09×10^3 to 9.53±0.11×10^3 cfu cm^{-2} during summer phase (Table 3). The bacterial counts in the intestine of fish were found in the range of 6.53±0.18×10^5 to 7.76±0.2×10^5 cfu g^{-1} during the winter phase and 1.85±0.38×10^6 to 2.21±0.01×10^6 cfu g^{-1} during summer phase (Table 4), respectively.

The total plate counts revealed that the bacterial density was 10 times higher in the pond sediment in comparison to pond water and 100 times higher in the fish intestine than on the surface of skin. The results also showed a direct and significant relation of the temperature with the bacterial counts. Higher counts were recorded during summer phase as compared to winter in all the isolated bacterial biomass. Further, the findings are in confirmation with the earlier reports of Jun *et al.*, (2000) and Al-Harbi and Uddin (2008).

The bacterial flora obtained from pond water sediment and fish skin and intestine consisted mainly of Gram negative rods. Almost similar bacterial composition was found in soil and

Table 1 : Bacterial count (cfu × 10^3) in earthen pond water of College of Fisheries, Pantnagar

Days	Winter season		Summer season	
	Pond A	Pond B	Pond A	Pond B
0	5.13 ± 0.0.12	5.2 ± 0.1	8.4 ± 0.34	9.26 ± 0.09
15	5.20 ± 0.20	5.0 ± 0.21	8.56 ± 0.11	9.33 ± 0.09
30	4.93 ± 0.35	4.96 ± 0.15	8.26 ± 0.09	8.96 ± 0.09
45	5.06 ± 0.24	5.26 ± 0.12	8.16 ± 0.36	8.86 ± 0.06
60	5.1 ± 0.43	5.5 ± 0.09	8.56 ± 0.20	9.66 ± 0.09
Average	5.08 ± 0.44[a]	5.19 ± 0.10[a]	8.83 ± 0.07[b]	8.61 ± 0.14[b]

Values in the same row having same superscripts do not differ significantly ($P > 0.05$). Values are mean of 3 samples ±S.D.

Table 2 : Bacterial count (cfu × 10^4) in earthen pond sediment of College of Fisheries, Pantnagar

Days	Winter season		Summer season	
	Pond A	Pond B	Pond A	Pond B
0	3.83 ± 0.11	3.96 ± 0.12	8.3 ± 0.26	8.53 ± 0.06
15	3.53 ± 0.12	4.1 ± 0.12	8.6 ± 0.06	9.2 ± 0.1
30	3.46 ± 0.15	3.8 ± 0.27	9.25 ± 0.15	9.25 ± 0.09
45	3.76 ± 0.18	4.26 ± 0.03	8.6 ± 0.07	8.5 ± 2.89
60	4.06 ± 0.15	4.46 ± 0.15	9.3 ± 0.18	9.43 ± 0.24
Average	3.72 ± 0.10[a]	4.11 ± 0.11[a]	8.81 ± 0.19[b]	8.98 ± 0.19[b]

Values in the same row having same superscripts do not differ significantly ($P > 0.05$). Values are mean of 3 samples ±S.D.

Table 3 : Bacterial count (cfu × 10^3) ± S.D. in fish skin cultured in earthen pond of College of Fisheries, Pantnagar

Days	Winter season		Summer season	
	Pond A	Pond B	Pond A	Pond B
0	3.23 ± 0.09	3.32 ± 0.06	8.2 ± 0.09	8.49 ± 0.09
15	3.43 ± 0.18	3.46 ± 0.15	8.33 ± 0.21	9.23 ± 0.09
30	3.2 ± 0.12	3.26 ± 0.12	8.0 ± 0.15	8.96 ± 0.06
45	3.3 ± 0.21	3.56 ± 0.0.12	9.16 ± 0.20	8.63 ± 0.06
60	3.46 ± 0.12	3.73 ± 0.03	9.53 ± 0.11	9.3 ± 0.19
Average	3.32 ± 0.05[a]	3.46 ± 0.08[a]	8.63 ± 0.29[b]	8.92 ± 0.15[b]

Values in the same row having same superscripts do not differ significantly ($P > 0.05$). Values are mean of 3 samples ±S.D.

water, however, intestinal bacteria showed more diversification in contrast to bacteria present on the skin. The bacteria isolated during both the seasons from water, sediment and fish samples were identified as *Aeromonas hydrophila, Pseudomonas fluorescens, Pseudomonas sp., Flavobacter sp., Bacillus sp., Micrococcus sp.* and *Corynebacterium sp.* respectively. Among the 7 species of bacteria *Pseudomonas* and *Aeromonas* formed the dominant group (>60 %) of the total bacterial population. Thus, this study also reveals that the pond environment consisted of diverse flora while, fish inhabits a limited range of isolates.

The microflora of fish (skin and intestine), are a reflection of pond environment (water and sediment) Austin (2006). Al-Harbi and Uddin (2008) also reported dominance (76% of the populations) of Gram negative rods with more diversified intestinal bacterial population and *A. hydrophila* alone as one fourth (25%) of the total bacterial populations. Denev *et al.* (2009) and Wu Shangong *et al.* (2012) stated that the genera present in the gut generally seem to be those from the environment or diet which can survive and multiply in the intestinal tract of the fish. Among the reported bacteria, *Bacillus* is well known for its probiotic properties and hence may be selected as a probiotic candidate for aqua-feeds.

Water and soil characteristics determine the productivity of an aquatic ecosystem. Although each factor plays its individual role but it's the synergistic effect of various parameters, which determine the composition and productivity of flora and fauna

Table 4 : Bacterial count (cfu × 10^5-10^6 in winters and summers respectively) ± S.D. in fish intestine cultured in earthen pond of College of Fisheries, Pantnagar

Days	Winter season		Summer season	
	Pond A	Pond B	Pond A	Pond B
0	6.53 ± 0.22	7.76 ± 0.15	1.87 ± 0.37	2.19 ± 0.07
15	6.76 ± 0.19	7.63 ± 0.15	1.89 ± 0.48	2.21 ± 0.01
30	6.53 ± 0.18	7.6 ± 0.23	1.85 ± 0.38	2.19 ± 0.12
45	6.66 ± 0.15	7.66 ± 0.33	1.86 ± 0.37	2.16 ± 0.08
60	6.9 ± 0.12	7.76 ± 0.20	1.90 ± 0.39	2.22 ± 0.01
Average	6.67 ± 0.07[a]	6.68 ± 0.03[a]	2.47 ± 0.10[b]	2.19± 0.01[b]

Values in the same row having same superscripts do not differ significantly ($P > 0.05$). Values are mean of 3 samples ±S.D.

Table 5 : Chemical characteristics of earthen pond sediment of College of Fisheries, Pantnagar

Parameter	Winter phase		Summer phase	
	Pond A	Pond B	Pond A	Pond B
pH	6.6-7.1	6.7-7.2	6.9-7.4	6.9-7.4
Organic carbon (%)	1.12-1.37	1.17-1.41	0.93-1.12	0.98-1.15
Nitrogen (mg $100g^{-1}$)	35.2-38.8	37.8-41.7	49.8-55.7	51.1-54.4
Phosphorus (mg $100g^{-1}$)	4.8-6.9	4.7-6.5	5.0-7.2	5.0-6.9
C : N ratio values	28.8-38.9	28.0-37.3	17.7-22.5	18.0-22.5

Values are mean of 3 samples

Table 6 : Physico-chemical characteristics of earthen pond water of College of Fisheries, Pantnagar

Parameter	Winter phase		Summer phase	
	Pond A	Pond B	Pond A	Pond B
Temperature (ºC)	18.2-19.4	18.3-19.4	26.2-29.1	26.4-28.7
Transparency (cm)	20-24.5	20.5-24.8	16.2-18.7	17.0-19.5
pH	6.9-7.2	7.0-7.3	7.1-7.4	7.1-7.5
Dissolved O_2 (mgl^{-1})	4.6-6.2	4.9-6.4	5.8-6.8	5.9-7.2
Free CO_2 (mgl^{-1})	4.2-6.2	4.0-6.0	0.0-4.2	0.0-4.0
Total alkalinity (mgl^{-1})	122.0-142.0	121.0-140.0	130.0-146.0	128.0-142.0
Nitrate- nitrogen (mgl^{-1})	0.09-0.14	0.10-0.15	0.18-0.25	0.19-0.26

Values are mean of 3 samples

(Banerjee and Chattopadhyay, 2001). In the present study, the values of chemical characteristics of pond sediment (Table 5) were within the conducive range for fresh water fish culture (Ayyappan, 2010 and Pillay, 2005). Further, analysis of physico chemical parameters of pond water (Table 6) also depicted favourable environment for fast growth of fish throughout the farming phase and are in agreement with Sachidanandamurthy and Yajurvedi (2006).

The results, thus can be used as guidelines for isolating and screening potential probiotic candidates for aquaculture applications, and provide the basis for developing functional foods for use in finfish and shellfish hatcheries that incorporate a naturally occurring, probiotic bacteria.

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