The cryptic domain of gut microbiota in composite culture of Indian major carps

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According to official estimates, in the year 2016-17, India's total aquaculture production was nearly 6.5 million tons (~ 57% of the total fish production of 11.4 million tons), which is a remarkable ten-fold increase from 0.63 million tons in 1985. Over the same period, the contribution of poly-cultured Indian major carps has steadily grown from 0.38 million tonnes in 1985 to 3.53 million tonnes in 2015 (60.5 to 67.4% of total aquaculture production). This statistics is definite proof for the continuing predominance of carp polyculture systems in the Indian aquaculture scenario from its inception in the mid-1960s.

Fundamentally, the concept of growing compatible fish species in the same environment was to facilitate efficient use of all the ecological niches within the pond and augment the total fish production per unit area. Keeping this in mind, conventional composite fish culture in the Indian sub-continent included the three major carps that occupy unique feeding niches in the culture pond, namely *Catla catla* (catla), a surface feeder relying on zooplankton; *Labeo rohita* (rohu), a column feeder relying on periphyton and mostly plant matter; and *Cirrhinus mrigala* (mrigal), a bottom feeder relying on detritus. These agastric fish species belong to the same teleost family (Cyprinidae) and share several common features, but they also have certain distinguishable morphological and anatomical specifications that are suited to ingest and process their respective natural diet. For instance, the mouth position of catla is supra-terminal, rohu is terminal and mrigal is sub-terminal. Likewise, they differ in buccal cavity structure, relative length of their highly coiled intestine and gut microarchitecture. So logically, we hypothesise the presence of a host-specific microbiome which colonises the digestive tract with distinct functional relevance. But at present, scientific information on this biological aspect of Indian major carps is meagre and inadequate. Therefore, in this article, we make a strong case for in-depth investigations on the gut microbiota of polycultured major carps by presenting an overview of the present understanding about fish gut microbiome and observations of our preliminary study.

Gut microbiota and their functional significance

The digestive tract of all vertebrates including fish is known to harbour a complex microbial ecosystem with a large, diverse and dynamic collection of microorganisms. Over the course of life, these gut microbes become an integral component of the host animal with intimate host-microbe associations and key roles in the maintenance of normal gut function, physiology and health of the host. This includes their critical role in the digestion of complex nutrients like non-starch carbohydrates, intestinal nutrient acquisition, proliferation of enterocytes (intestinal cells), production of secondary metabolites (such as vitamins) and defence against pathogens (by stimulating the immune system and outcompeting opportunistic pathogens). Recently, gut microbes were also shown to influence food intake, energy homeostasis and weight gain through gastrointestinal chemosensing and nutrient-responsive signalling. Considering the fact that the collective genetic potential (metagenome) of the gut microbiota is several folds higher than the host genome (e.g., the human body contains 10 times more microbial cells than our body cells, with ~ 150 times more genes than our own genome), we are only beginning to understand their impact on host animal health and performance attributes. Lesser known aspects such as horizontal exchange of genes between co-residing symbionts and gene swapping from bacteria to eukaryotes might enable the host to gain characteristics which could help them to adapt to new environments. Thus, host-microbe and microbe-microbe beneficial associations (i.e., mutualistic symbioses) could underlie some of the major transitions in vertebrate evolution and ecology.

Present knowledge of fish gut microbiota

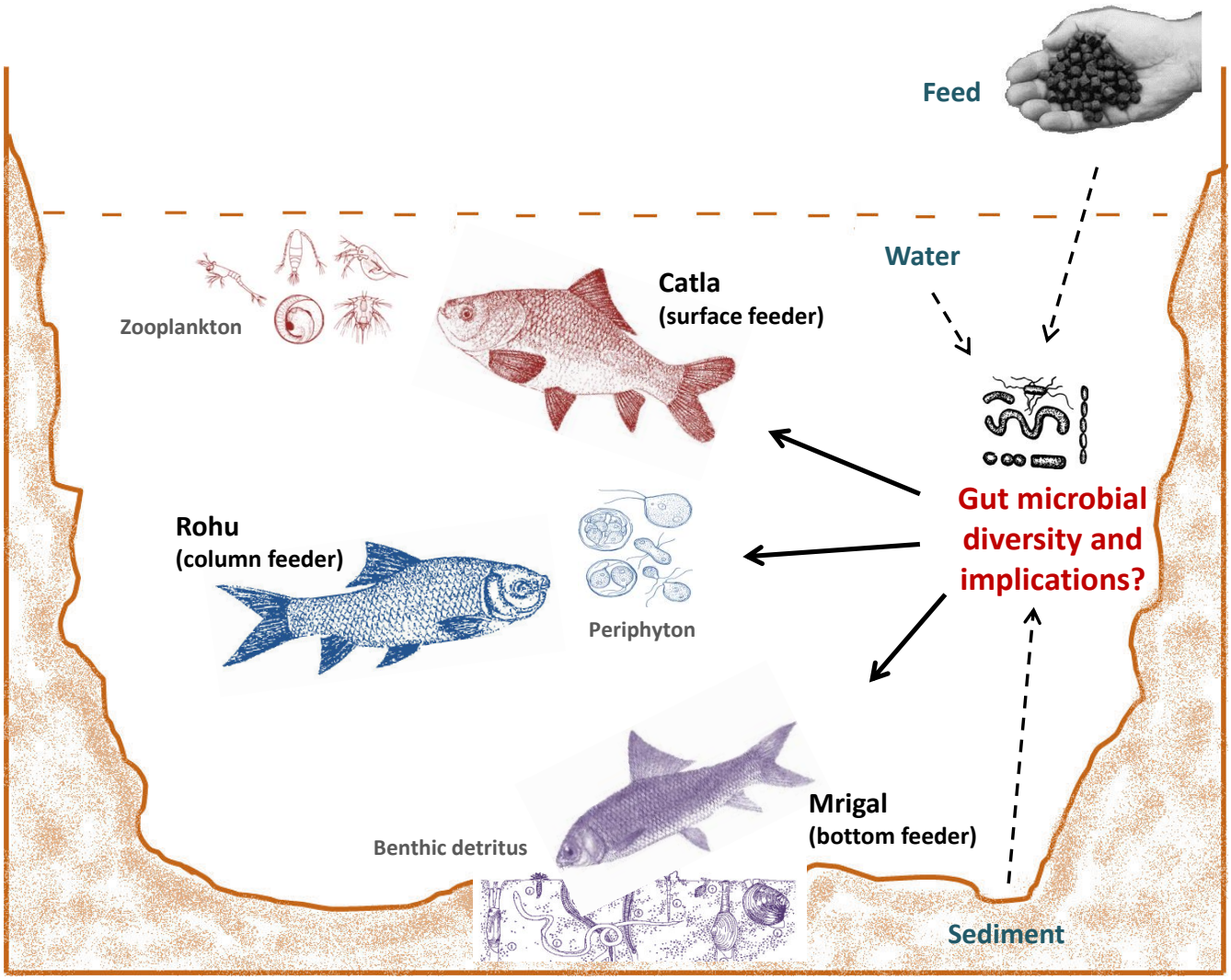
Fish are said to have a simple and less diverse microbial ecosystem as compared to the complex and dynamic one in terrestrial vertebrates. Numerous studies have shown that fish gut is colonised by specialised microbial communities that have not been detected in the environment. Distinct relationships have been found between host diet, trophic level, the anatomy of alimentary tract and gut microbiota composition. For instance, herbivorous and detritivorous fish species are known to harbour distinctive microbial populations due to host-specific selective pressures. Other exogenous and endogenous factors such as developmental stage, age, health status, phylogenetic position (e.g. species-specificity, genotype), domestication process, habitat or rearing environment (e.g. water temperature, salinity, culture system), geographical location, sampling time (i.e., season), starvation and probiotic-prebiotic-antibiotic treatment are also known to affect the composition of fish gut microbial communities. Further, there is evidence for the presence of discrete populations of microorganisms in defined regions of the digestive tract with different metabolic functions and despite high inter-individual variation, a core group of gut microbes has been discovered in some species like zebrafish. So in a nutshell, we know that the gut microbiome of fish is shaped by host genetics, gut physiology, nutritional and environmental factors. Function-wise, in a few herbivorous fishes, gut microbes have been associated with cellulolytic function or high levels of intestinal fermentation to convert indigestible plant matter to metabolically useful short chain fatty acids. Moreover, gut microbial symbionts were shown to be involved in fatty acid and protein uptake in the intestinal epithelium of fish. In terms of phenotype, gut microbiota was found to be associated with growth, anxiety-related behaviour and stress response. With all these findings, our understanding of the genomic, mechanistic and evolutionary aspects of fish gut microbiota is still limited and we are slowly catching up with the large volume of information in terrestrial vertebrates.

Gut microbiome of carps is generally dominated by differing proportions of bacteria belonging to the phylum Proteobacteria, Firmicutes and Fusobacteria, with some species often having a higher abundance of Bacteroidetes and Cyanobacteria. Interestingly, a comparison between growth hormone transgenic and wild type fish indicated that the relative abundance of bacteria of the phylum Firmicutes over those of Bacteroidetes could be one of the factors that contribute to fast growth in growth hormone transgenic common carp. Further down the microbial taxa, the genus *Cetobacterium* and *Aeromonas* are commonly present in the gut microbiota of grass, common, bighead, Asian and crucian carps. Nevertheless, the same rearing environment does not result in similar intestinal microbiota compositions in polycultured carps. For instance, gut bacterial richness was higher in filter-feeding carp than in grazing carp from the same pond ecosystem. Hence, evolutionarily and functionally distinct symbionts could be critical elements in biological differences among carp species. On the other hand, dietary (e.g., nutrient levels and ingredient sources) and environmental factors (i.e., surrounding water and sediment) are also known to be strong determinants of carp gut microbial composition. Further, as in higher vertebrates, the intestinal microbiota of cyprinids have demonstrated significant roles in the digestion of plant material, fermentative metabolism, intestinal nutrient uptake and *de novo* vitamin production.

Technological advances in the investigative approach

Conventionally, culture-based methods and observations were employed to study the gut microbiota of fishes. Bacterial isolate identification based on biochemical and molecular characterisations were time consuming, had restricted discrimination power and also could not provide a complete insight on gut microbial composition and host-microbiota relationships. Further, as the detected species and the number of bacteria was dependent on culture conditions and culture media, our understanding of certain obligate and fastidious anaerobes was seriously limited. In fact, cultivable bacteria represented <10% of the total gut bacteria in fishes, as the majority could not be isolated and cultured under laboratory conditions. In order to overcome these disadvantages and underestimations, several culture-independent molecular techniques have been developed to characterise the microbial community that colonises fish gut by analysing pooled microbial DNA extractions. These methods were relatively

Figure 1: Schematic representation of the context of the study.



bias-free and allowed the identification and determination of the microbial diversity and phylogenetic affiliation of community members, without isolation. Techniques like fluorescence in situ hybridisation (FISH) involves the use of fluorescent-label probes that target specific regions of the ribosomal RNA (rRNA) and facilitates three dimensional observation of specific microbes using fluorescence or confocal microscopy. FISH has been used to track specific probiotics and their intricate spatial relationships with other microbes in the fish gut. For basic analysis of fish gut microbial communities, DNA fingerprinting methods such as restriction fragment length polymorphism (RFLP), denaturing gradient gel electrophoresis (DGGE) and ribosomal intergenic spacer analysis (RISA) have been extensively used. These techniques are based on targeted PCR amplification of variable regions within the ribosomal operons that are unique to bacterial species or strains. They are relatively inexpensive, fairly quick to perform and generally allow medium throughput analysis. The results obtained with these methods provide information on the complexity of the communities but not the specific operational taxonomic units (OTUs) that constitute each community (i.e., they are more qualitative than quantitative). Moving forward, over the past decade, high-throughput next generation DNA sequencing technologies of bacterial 16S rRNA genes have been instrumental in obtaining a comprehensive inventory of gut microbial diversity in several fishes, by identifying large numbers of gut microbial community members at phylogenetic level, irrespective of their biology. The cost-effectiveness and widespread application of this NGS technology and associated computing (bioinformatics) have triggered the generation of huge volumes of information on fish gut microbiome from myriad environments, with a level of detail which was hard to imagine in the past. Further, metagenomic approaches have provided a reference set of several million bacterial genes that allows targeted study of the activity and function of fish gut microbiota. Progressively, meta-transcriptomic discovery of plant biomass degrading capacity from grass carp intestinal microbiomes has also been reported. But surprisingly, to date, no notable culture-independent attempt has been made to elucidate the host-specific gut microbial diversity and dynamics of polycultured Indian major carps.

A glimpse of what is known and what we have observed in Indian major carps

In Indian major carps (catla, rohu and mrigal), hitherto, culture-based identification of few aerobic and facultative anaerobic bacteria has been carried out, either based on biochemical properties or 16S rRNA gene sequence analysis of the enumerated colonies. The microbes that were isolated and genetically identified from Indian major carps belong to the genus *Citrobacter sp., Enterobacter sp.* and *Bacillus sp.* of the phyla Proteobacteria and Firmicutes. Moreover, the hindgut of Indian major carps was found to be highly colonised than the foregut. Some of the isolated microbes were demonstrated to be a distinct source of exogenous digestive enzymes for the host, apparently assisting digestive processes. Apart from this, there is no proper information on 1) the presence of host-specific microbial populations; 2) the source or origin of the gut microflora; 3) the resident (autochthonous) and transient (allochthonous) microbial groups; and 4) the existence of symbiotic host-microbe associations and functional contributions. Therefore, in the following study, we employed both bacterial enumeration and culture-independent DNA fingerprinting (PCR-DGGE) approach to examine the presence of host-specific gut microbiota in IMCs related to their occupancy of distinct ecological niches (figure 1). Also, we looked at the source/origin (from water, sediment and diet) and colonising ability (resident-transient forms) of bacteria in the digestive tract of the IMCs.

Table 1: Details of the sampled fish.

Species	No. of fish	Average body weight (g)	Average body length (cm)	Relative gut length (cm)
Catla	5	76	19.8	5.3
Rohu	4	95	20.8	8.4
Mrigal	5	68	21.8	13.6

Fish used for the study

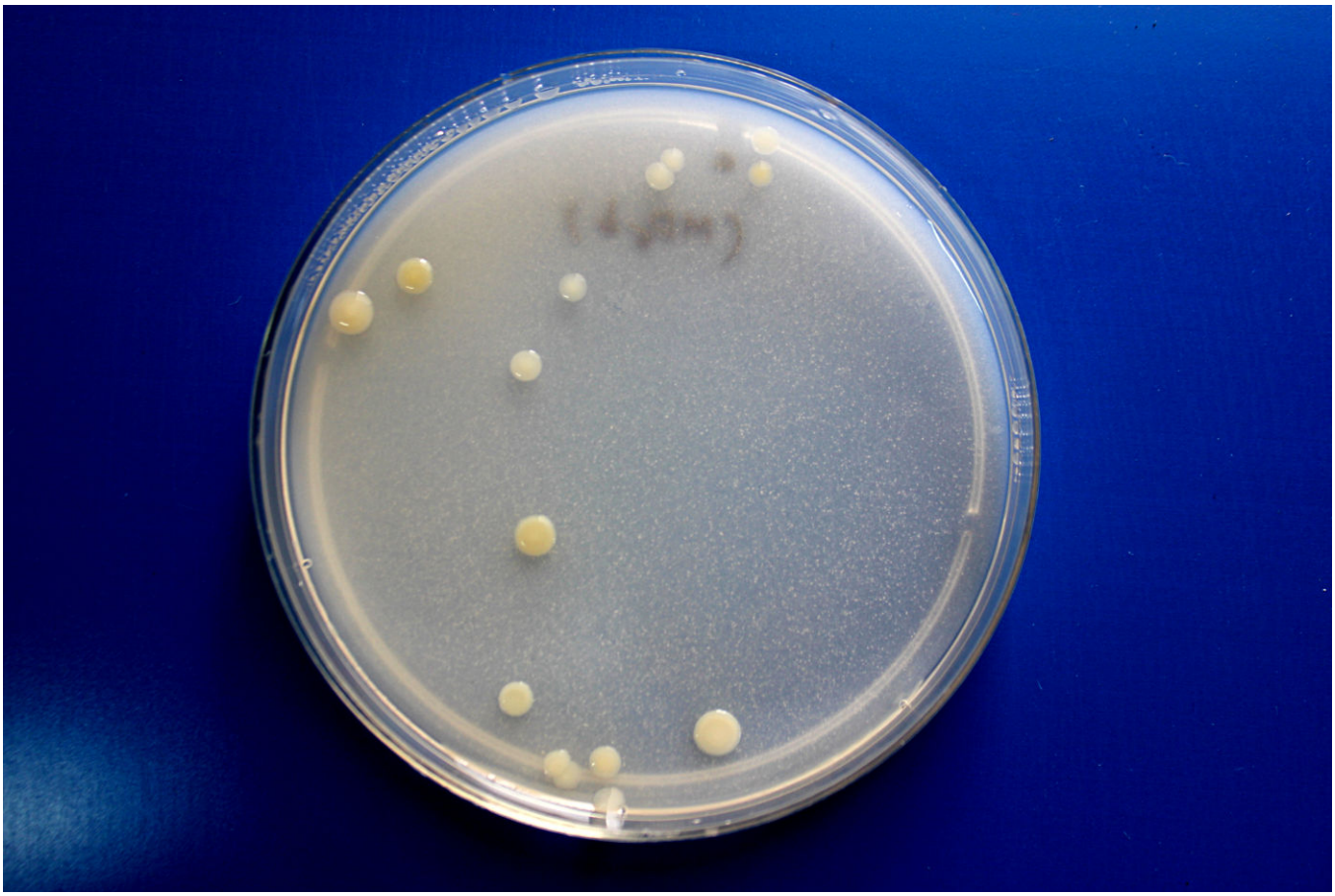
We performed the present investigation on juveniles of the three Indian major carp species, namely catla, rohu and mrigal, which were raised in an earthen carp poly-culture pond of 0.2 ha area and 1 m water depth (Regional Research Station of Central Institute of Freshwater Aquaculture, Bengaluru, India). The pond was annually fertilised with cattle manure and the fish were daily provided a supplementary feed mash made up of rice bran, groundnut oil cake, fish meal and vitamin-mineral mixture. During sampling in the post-summer month of June, the temperature of the rearing water varied between 25 to 30°C. Basic information of the experimental fishes is given in table 1. We observed clear differences in relative gut length between the three species in the following order, mrigal > rohu > catla, reflecting their distinct feeding habits.

Table 2: Cultivable aerobic bacterial count (CFU/g or ml*).

Sample	TPC	Proteolytic	Lipolytic	Amylolytic	Cellulolytic
Water*	7×10^2	7×10^2	5.5×10^2	6×10^2	6×10^2
Sediment	0.9×10^5	1.2×10^5	0.6×10^5	0.7×10^5	0.7×10^5
Feed mash	2.5×10^6	-	-	-	-
Catla digesta	2.8×10^7	2.6×10^7	-	2.1×10^7	2.4×10^7
Rohu digesta	2×10^6	1.5×10^6	0.8×10^6	1.1×10^6	0.3×10^6
Mrigal digesta	2.8×10^6	2.3×10^6	2.4×10^6	5.5×10^6	2.6×10^6
Catla intestine	3×10^6	2.9×10^6	2.5×10^6	2.6×10^6	2.2×10^6
Rohu intestine	4.6×10^5	4.5×10^5	-	6.8×10^5	5.2×10^5
Mrigal intestine	5.6×10^5	6.2×10^5	5.6×10^5	6.8×10^5	5.1×10^5

Figure 2: Collection of fish, intestinal tract and digesta samples.

Figure 3: Methodology for enumeration of aerobic cultivable bacteria.



Sampling procedure

The experimental fishes were randomly collected from the culture pond using drag net, euthanised with cold-shock and transferred to the laboratory. Soon after, they were dissected under aseptic conditions and the entire digestive tract was carefully removed and uncoiled. The intestinal contents were gently squeezed out and collected. Then the empty intestine was washed thoroughly in physiological saline (0.9% NaCl) and pooled together. At the time of field sampling, water and surface sediment samples were also collected from five spatially distinct locations in the same fish pond and pooled

together. Likewise, a small portion of the supplementary fish feed mash was taken for bacterial count and composition analysis. After allocating a small portion of all the samples for serial dilution and bacterial enumeration, the remaining samples were stored at -80°C until DNA extraction.

Culture-dependent approach: Methodology and observations

For enumerating the aerobic heterotrophic bacteria present in the collected samples, first we prepared well homogenised and diluted fractions of each sample (intestine and digesta of the test carp species; water, sediment and feed). From three appropriate serial dilutions (1:10), we took 0.1 ml of sample and aseptically pour plated in sterilised agar plates, in duplicates. We used tryptone soy agar media for total plate count (TPC); and starch, cellulose, peptone gelatin and crude coconut oil agar media to enumerate bacteria that produces amylase, cellulase, protease and lipase, respectively. The inoculated plates were then incubated at 26°C for 24 hours. Subsequently, the plates were examined for the development of bacterial colonies and well separated colonies were counted, multiplied by the correct dilution factor and expressed as colony forming units per g or ml of the sample.

Based on the results of the bacterial enumeration (table 2), firstly, we observe that the bacterial count in the intestine and digesta of all the three Indian major carps was several orders higher than in the water and sediment. This reaffirms the fact that the digestive tract of fish is a nutrient rich abode for microbes than the culture pond habitat. Secondly, the surface filter feeder catla was found to have a higher abundance of gut microbes (both in intestine and digesta) as compared to rohu and mrigal. This corresponds to a previous observation in polycultured Chinese carps, where gut microbial diversity of filter feeding bighead carp was found to be higher than that of grass carp and crucian carp with grazing habits. Between rohu and mrigal, the latter had a relatively higher bacterial count in the digesta samples possibly linked to their detritivorous feeding habit. Overall, this result serves as an indication for host-specific and feeding habit specific selective pressures on the gut microbiota of IMCs, just as we had hypothesised. Thirdly, in all the carps, the bacterial count of intestine samples was less than the digesta samples, implying the possible existence of resident and transient bacterial groups with differences in colonising ability. Fourthly, in all the samples, the order of bacterial cell count was similar between the plates with different nutrient substrates, i.e., amino acids, fatty acids, starch and cellulose. This suggests that the enumerated bacterial strains could use different nutrients for their survival and growth, and perhaps benefit the host by contributing exogenous digestive enzymes.

For microbial DNA extraction, we followed a simple conventional protocol which included mechanical, chemical and enzymatic lysis of the microbial cells. Briefly, 400 µl sample of centrifuged and concentrated organic matter from pond water, sediment and feed; and manually crushed and homogenised digesta and intestine were added to small (2 ml) screw cap tubes containing both 0.1 and 0.5 mm sterile zirconia/silica beads. To this suspension, two volumes of lysis buffer (2% cetyl trimethyl ammonium bromide, CTAB) was added and the mixture was bead beaten twice for 2 minutes in a mini bead beater for mechanical cell disruption. The sample-buffer mixture was then incubated at 70°C for 30 minutes, with occasional mixing for chemical lysis. After that, lysozyme (10 mg/ml) was added and the mixture was incubated at 42°C for an hour for enzymatic cell lysis. After mechanical (beat beating), chemical (CTAB) and enzymatic (lysozyme) disruption of microbial cells to improve extraction yield and the quality of the community DNA, proteinase K (2 mg/ml) and RNase (1 mg/ml) was added to the mixture and incubated at 37°C for 45 minutes, for degrading protein and RNA contaminants. Thereafter, we followed the conventional phenol-chloroform method and the extracted DNA was precipitated with ice-cold ethanol. For each sample, DNA was extracted in four technical replicates, pooled together and purified by gel elution. The freshness of the sample and extraction method was found to be highly critical to obtain good quality microbial DNA. Subsequently, we amplified the bacterial 16S ribosomal RNA gene and V3-V4 conserved regions from each sample using ~100 ng DNA template, universal bacterial primers and Taq polymerase enzymes in a touch-down PCR reaction.

Finally, we employed DNA fingerprinting method (denaturing gradient gel electrophoresis of the amplified V3 region of the 16S rRNA bacterial gene) to generate preliminary information on the bacterial diversity of Indian major carp intestine-digesta samples, pond water, sediment and feed. For this, a denaturing gradient acrylamide gel was prepared by mixing a low (30%) and high (60%) gradient solution made up of urea and formamide. From each sample, 45 µl of the amplified 16S rRNA V3 region template was loaded in the gel and run for nearly 14 hours at 60 V/cm and 60oC to maintain the denaturing conditions. After that the gel was washed, stained using silver staining protocol and bacterial diversity was visualised.

Though phylogenetic identification of bacterial diversity was not possible, we were able to derive certain salient observations from the DGGE study. As observed in the bacterial count, bacterial diversity in the fish gut was higher than in the environment (see the number of bands). While some of the gut microbes (bands 3 and 4) were sourced from the environment (pond water, sediment and feed), others were apparently unique to the fish intestine and digesta (bands 2, 5, 6 and 7). Based on the band pattern differences between the digesta and intestine samples, we can say that not all bacteria (band 4 and 7) are able to colonise the intestine of Indian major carps. But at the same time, there are groups which are efficient (band 6) in colonising the intestine. This is a clear indication for the presence of resident and transient forms of gut bacteria in major carps, with a possible core group. In the intestine samples, the difference in band intensities (band 1, 2 and 3) and banding pattern suggests the possible host-specificity of gut microbes in Indian major carps. Overall, our preliminary culture-independent observation provides few notional answers for the questions concerning the gut microbiota of polycultured Indian major carps and there are similarities between our culture-dependent and independent observations. But, still we need to carry out high-throughput NGS investigations on major carp gut microbiome to characterise the phylogenetic diversity and host-specific dynamics.

Prospects and applications

Continuous advancement in culture-independent high-throughput technologies are opening the doors to a microbial world which is far beyond our expectations and we are just starting to understand the great diversity of gut microbiome and the way they shape animal biology. In Indian major carp polyculture systems, first, we need to generate a comprehensive understanding of gut bacterial diversity and community

structure, with which we can assemble a clear picture of host-microbe and microbe-microbe interactions. Based on this foundation, we can then probe the factors that influence the gut microbiome phylogenetics and their metagenomic functional roles in fish nutrition, metabolism, growth and health. Ultimately, using this understanding of the intricate host-microbe symbioses and core microbiome functions in healthy conditions, we can monitor husbandry conditions in farms and manipulate gut microbes to decrease disease susceptibility and increase feed efficiency/productivity. Also, we can relate the carp gut microbes to many other production aspects such as changes in nutritional requirements, environmental adaptation, microbial spoilage and antibiotic resistance. In the long run, we need to look beyond bacterial communities and explore yeast, virus, archaea and protozoan populations that are functionally associated with fish gut microbiome.

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Suggested readings

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Figure 5: PCR-DGGE gel showing bacterial diversity of the samples.

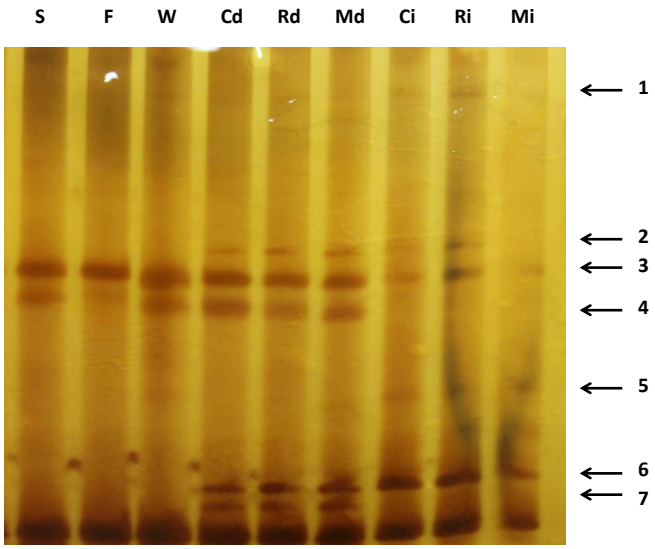
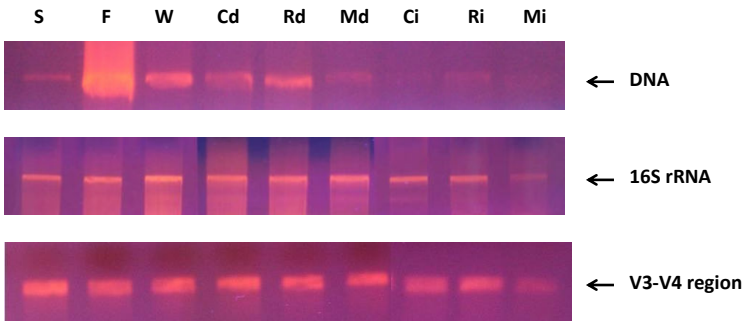


Figure 4: Extracted genomic DNA and amplified bacterial 16S rRNA gene and V3-V4 region.



S, sediment; **F**, feed; **W**, water; **Cd**, catla digesta; **Rd**, rohu digesta; **Md**, mrigal digesta; **Ci**, catla intestine; **Ri**, rohu intestine; **Mi**, mrigal intestine