

Fish Gut Microbiome: Current Approaches and Future Perspectives

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Abstract In recent years, investigations of microbial flora associated with fish gut have deepened our knowledge of the complex interactions occurring between microbes and host fish. The gut microbiome not only reinforces the digestive and immune systems in fish but is itself shaped by several host-associated factors. Unfortunately, in the past, majority of studies have focused upon the structure of fish gut microbiome providing little knowledge of effects of these factors distinctively and the immense functional potential of the gut microbiome. In this review, we have highlighted the recently gained insights into the diversity and functions of the fish gut microbiome. We have also delved on the current approaches that are being employed to study the fish gut microbiome with an aim to collate all the knowledge gained and make accurate conclusions for their application based perspectives. The literature reviewed indicated that the future research should shift towards functional microbiomics to improve the maximum sustainable yield in aquaculture.

Keywords Fish · Microbiome · Gut · Metagenomics · High-throughput sequencing

Introduction

Due to their vast ecological adaptations, microorganisms present enormous diversity, thereby fascinating the microbiologists to explore their residence in and on the animal bodies. After first insights into diversity of microbial communities were given by the “Human Microbiome Project”, it followed that most of the bacterial symbionts and commensals populate the gastro-intestinal (GI) tracts and their interactions among themselves and with the host finds great significance to human biology [1]. Since then, intensive research has contributed to an enhanced understanding of gut microbiome for its potential complexity and functional contributions only to develop as an attractive area of research among other vertebrates. Representing more than half of the vertebrates with vast ecological diversities and distinctive structural features within their intestinal tracts [2, 3], fish has justifiably emerged to be a significant class for the examination of the confederation of microorganisms with their hosts. In the past, the traditional culture-dependent methods [4–7] and the use of Denaturing Gradient Gel Electrophoresis (DGGE) and Temporal Temperature Gradient Gel Electrophoresis (TTGE) techniques [8, 9] revealed very low fraction of these significant microorganisms. While these approaches only aimed to reveal the “variety” constituted in the microbiome, some early studies also correlated the taxonomic abundance of fish gut flora with specific host functions [10, 11]. Over the years, the culture based assessments have led to identification of several probiotic and pathogenic bacterial strains and still continue being employed in studies of fish gut microbiome [12–14]. In recent years, however, the culture-independent methods have replaced these traditional techniques as the direct mining of community DNA hold promise to unveil the low abundance and rare taxa [15–19].

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While the next generation sequencing (NGS) platforms have vested ease in deciphering the whole community structure, they also compel researchers to devise ways to mine the complete functional potential of fish gut microbiome. Further, to test the efficiency and sustainability of the beneficial flora is an emerging need. In 2016, the Food and Agriculture Organisation (FAO) of the United Nations reported that the aquaculture yield worldwide has been increasing. However, at the same time, about 31.4% of the commercial wild fish stocks were reported to be fished at biologically unsustainable levels in 2013. As the demand of fish for human consumption continues to increase, the aquaculture research must focus on the application of latest technologies to improve the maximum sustainable yield (MSY). The gut microbiome is known to play crucial roles in development of fish immune system and aid in optimal nutrient absorption [20]. Despite this, the use of NGS platforms in studies aimed at exploiting the beneficial flora to improve overall fish health lags far behind. Too often, these studies only focus on their species specificity and effect on fish health while neglecting the change in the overall gut microbiome structure. The microbiome is influenced by a myriad of factors and it is difficult to ascertain the individual effects of each of these factors. Tarnecki et al, recently summarized the different factors affecting fish gut microbiome while also highlighting the potential sources of bias in the results from sample processing [21]. Apparently, the choice of the study design also greatly impacts the results. It is therefore important to contemplate at the study design prior to noting the important inferences. In this review, we aim to summarize the different study designs currently being employed in order to delineate the influence of the different selective pressures affecting fish gut microbiome. The important findings from recent metagenomics studies are noted. The review also discusses the future perspectives of fish gut microbiome research and sheds light on the need to focus on applied microbiomics.

Composition of Fish Gut Microbiome

The colonization of fish gut starts early in the larval stage and is continuously driven towards achievement of a complex assemblage of gut associated microbes [22]. Approximately 10^8 bacterial cells belonging to over 500 different species are reported to populate the fish GI tract, which are dominated by aerobes or facultative anaerobes although strict anaerobes have also been detected [7, 23]. While considering a metagenomic sample, the diversity is defined in terms of number of OTUs. OTUs are number of clustered similar sequences (> 97%) that define a taxonomic unit on the basis of divergence. OTU analyses are

done to reveal the alpha diversity (within-sample diversity) and beta diversity (diversity among different samples) while deciphering the composition of gut microbiome. Most studies corroborate to the domination of bacterial sequences in NGS sequencing data from fish gut sources with negligible representatives of archaeal and eukaryal origins [17, 19, 24].

To date, the analyses of sequencing data have revealed a peculiarly low phylogenetic diversity with Proteobacteria, Firmicutes and Bacteroidetes representing up to 90% of the fish intestinal microbiota across different species and Fusobacteria, Actinobacteria, and Verrucomicrobia among the represented phyla [20, 24–31]. This is not very surprising with our knowledge of the challenges imposed by the gut environment onto the microorganisms to whittle down the diversity in the niche. The diversity generally increases as the diet of the fish changes from carnivorous to omnivorous to herbivorous [19]. The composition also differs due to different environmental conditions. *Acinetobacter*, *Aeromonas*, *Flavobacterium*, *Lactococcus*, and *Pseudomonas*, obligate anaerobes *Bacteroides*, *Clostridium*, and *Fusobacterium*, and members of family Enterobacteriaceae dominate the gut of freshwater species [32]. The guts of marine fish are dominated by *Aeromonas*, *Alcaligenes*, *Alteromonas*, *Carnobacterium*, *Flavobacterium*, *Micrococcus*, *Moraxella*, *Pseudomonas* and *Vibrio* [32]. A summary of the major bacterial phyla composing the gut microbiome as revealed by studies in different species can be referred in review by Llewellyn et al, [33] and much recent studies in Table 1. Abundance of similar bacterial phyla irrespective of the taxonomic position or geographical location of the fish indicates a role of microbiome in important host functions such as nutrient absorption, digestion and generation of immune response. Further, the structural similarity of fish gut microbiome with that of the mammals indicates towards the vertebrate core gut microbiotas.

Functional Potential of Fish Gut Microbiome

Till date, majority of studies on fish gut microbiome are restricted to diversity analysis and most of our knowledge comes from its correlation with the necessary host functions (Table 1). In this regard, computational tools have been developed that may provide important predictions of the functional capabilities of the community, once the taxonomic composition is deciphered using marker gene based approach e.g. PICRUSt [34] and Tax4Fun [35]. The most widely studied functional attributes of microbiota include digestion and immunity. In grass carp, the ability to digest plant matter is long been associated with the higher abundance of cellulolytic bacteria in the gut of herbivorous

Table 1 List of some recent studies concerning fish gut microbiome. In all cases, the targeted region used for amplicon sequencing is the hypervariable region(s) of bacterial 16S rRNA gene sequence which is denoted by its standard code(s). AU and AL, here, stand for autochthonous and allochthonous microbiomes respectively

Host	NGS platform	Amplicon/shotgun Sequencing	Microbiome	Study design	Problem addressed	Dominant phyla	Important inferences	References
<i>Silurus meridionalis</i>	Illumina HiSeq	Amplicon: V4–V5	AU and AL	Gut microbiome of lab-reared fish over developmental stages	Effect of host age and—associated factors on gut microbiome	Tenericutes, Fusobacteria, Proteobacteria, and Bacteroidetes	Microbial diversity increases with host age; is significantly Abundance of Tenericutes decrease while Fusobacteria, Proteobacteria and increase with host age	[107]
<i>Silurus meridionalis</i>	Illumina HiSeq	Amplicon: V4–V5	AL	Gut microbiome of stomach and intestine at different time intervals after feeding	Spatial and temporal microbial dynamics within gut	Fusobacteria, Firmicutes, Proteobacteria, and Bacteroidetes	Distinguishable communities between stomach and intestine; higher diversity in stomach. Firmicutes increase and Fusobacteria decrease after feeding	[108]
<i>Danio rerio</i>	Illumina Miseq	Amplicon: V4	Not defined	Microbiome of formulated diet fed group compared with that of control	Effect of gluten formulated diet (GFD) on gut microbiome	Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes dominated control samples. Planctomycetes, Fusobacteria, and Verrucomicrobia dominated GFD samples	Legionellales, Rhizobiaceae, and <i>Rhodobacter</i> abundant in GFD fed group. Cobalamin synthesizing <i>Bacteroides</i> and <i>Lactobacillus</i> present in control group and absent in experimental group	[109]
<i>Siganus fuscescens</i>	Illumina Miseq	Amplicon: V4	AU and AL	Microbiome of different regions in GI tract	Differences at the level of individuals, gut locations and sample types	Proteobacteria, Cyanobacteria and Firmicutes	Midgut communities highly diverse for both adherent and non-adherent microbiome; Greater diversity of adherent microbiome accounting for its active selection by host	[110]
<i>Gambusia affinis</i>	Illumina HiSeq	Amplicon: V4	AU and AL	Effect of broad range antibiotic rifampicin on gut microbiome	Microbiome composition and its response towards antibiotic stress	Proteobacteria, Planctomycetaceae; <i>Myroides</i> genus of Flavobacteria dominated during treatment	Antibiotic treatment lowers the diversity and unstably alters the composition to become antibiotic resistant during treatment while the microbiome takes long to recover	[111]
<i>Oncorhynchus mykiss</i>	Illumina Miseq	Amplicon: V6–V8	AU and AL	Juvenile fish fed with plant- and animal-derived dietary proteins	Role of dietary nutrients source in microbiome composition	Proteobacteria, Firmicutes, Bacteroidetes, Fusobacteria and Actinobacteria	Gut microbial diversity decreases as the source of nutrient derivation in diet is restricted	[72]

Table 1 continued

Host	NGS platform	Amplicon/ shotgun Sequencing	Microbiome	Study design	Problem addressed	Dominant phyla	Important inferences	References
<i>Carassius auratus</i>	Illumina Miseq	Amplicon: V4–V5	AU and AL	'Red-operculum' disease affected individuals compared with healthy individuals	Differences in healthy and diseased gut microbiome	Fusobacteria, Proteobacteria and Bacteroidetes	Distinct differences in microbiome composition in two groups; potential of some species as disease-specific bacterial signatures	[112]
<i>Ctenopharyngodon idellus</i> , <i>Megalobrama amblycephala</i> , <i>Carassius auratus</i> , <i>Hypophthalmichthys molitrix</i> and <i>H. nobilis</i> ,	Pyrosequencing	Amplicon: V4	AL	Species with three different feeding habits raised under identical husbandry conditions	Relationship among host evolutionary distance, gut microbiota and metabolic profiles	Fusobacteria, Proteobacteria, Bacteroidetes and Firmicutes	Gut microbiome composition as well as metabolite profiles are significantly altered by host species and feeding behaviour	[71]
<i>Danio rerio</i>	Illumina Hiseq	Amplicon: V4	Not defined	Adaptive immune compromised individuals compared with wild-type individuals	Ecological filtering of microbiome composition by adaptive immunity of the host	Proteobacteria, Fusobacteria and Actinobacteria	Functional adaptive immunity affects the neutral assembly processes; Adaptive immunity specifies the microbiome composition in each host which otherwise seems to be much similar; Co-housing individuals of different genotypes increases the microbiome diversity	[113]
<i>Salmo salar</i> L.	Illumina Miseq	Amplicon: V3 and V4	AL	Lab reared and commercial freshwater fish farm	Effect of habitat on microbiome composition	Firmicutes, Proteobacteria, Tenericutes	Presence of core gut microbial flora regardless of the habitat type indicating operational host selective forces	[61]
<i>Salmo salar</i>	Ion Torrent	Amplicon: V1 and V2	AU and AL	Reference and experimental groups fed with alternative protein sources	Effect of alternative diets on microbiome structure and function	Firmicutes, Proteobacteria, Fusobacteria, Bacteroidetes, Actinobacteria	Significantly different adherent and non-adherent communities; Non-adherent microbiome much diverse and diet-dependent than adherent microbiome	[114]
<i>Oreochromis niloticus</i>	Illumina MiSeq	Amplicon: V4–V5	AL	Cadmium(Cd)- exposed, Cd- exposed and probiotic fed, only probiotic fed and control groups	Toxic effects of pollutants on microbiome and application of probiotics for treatment	Fusobacteria, Proteobacteria, Bacteroidetes and Firmicutes	Probiotic supplementation improved the gut health status in Cd-exposed fish to prevent death	[115]

Table 1 continued

Host	NGS platform	Amplicon/ shotgun Sequencing	Microbiome	Study design	Problem addressed	Dominant phyla	Important inferences	References
<i>Carassius auratus gibelio</i>	Illumina Miseq	Amplicon: V4	AL	Development of microbiota in a bacteria-free fish gut ecosystem over a year	Factors governing colonization of germ- free gut	Proteobacteria, Fusobacteria and Firmicutes	Gut microbial diversity increases as the fish develop and is less affected by surrounding environment than by host diet and development	[62]
<i>Oncorhynchus mykiss</i>	Illumina MiSeq	Amplicon: V4	AL	Fish from different sources i.e. reared in farm and in aquarium	Complexity of microbiome composition in differently sourced individuals of a species	Tenericutes, Firmicutes, Proteobacteria, Spirochaetae Bacteroidetes	Similar gut microbiota regardless of source; shaped by host factors; Differences in composition highlight the habitat specific taxa	[116]
Antarctic Fish spp.: <i>Trematomus bernacchii</i> , <i>Chionodraco hamatus</i> , <i>Gymnodraco acuticeps</i> and <i>Pagothenia borchgrevinkii</i>	Illumina Miseq	Amplicon: V4–V5	AL	Analysis of gut microbiota	Complexity of microbiome composition in different species	Proteobacteria, Actinobacteria, Firmicutes, Thermi, Bacteroidetes and Tenericutes	Gut microbial communities in different species are not exactly same but also not different altogether	[117]
<i>Ictalurus punctatus</i>	Illumina MiSeq	Amplicon: V4	AU and AL	Microbiome at different life stages with different diet formulations to study gut microbiota across developmental ontogeny	Influence of age as well as the dynamic dietary and environmental factors on gut microbiome	Bacteroidetes, Firmicutes, Fusobacteria, and Proteobacteria	Age has a significant influence on the intestinal microbiota; Water microbiota strongly influence gut microbiota at early life stages	[118]
<i>Oreochromis niloticus</i>	Illumina HiSeq	Amplicon: V4	AU and AL	Gut microbiome of cultured fish in axenic, probiotic-supplemented and later active life phases	Effect of short-term probiotic administration on gut microbiome	Firmicutes, Actinobacteria and Proteobacteria (control groups)	Probiotic administration for a short period significantly affects the gut microbiota composition at later stages of life	[119]

Table 1 continued

Host	NGS platform	Amplicon/ shotgun Sequencing	Microbiome	Study design	Problem addressed	Dominant phyla	Important inferences	References
<i>Dicentrarchus labrax</i>	Pyrosequencing	Amplicon: V3–V4	AU	Gut microbiome of two different nutritionally stressed groups and effect on host's growth or resistance to hypoxia	Correlation between gut microbiota composition, dietary stress and host's health	Proteobacteria, Bacteroidetes, Actinobacteria and Firmicutes	Gut microbiome is dependent upon the host life history or genetic background; Different nutritional stresses affect host microbiome and health differently	[120]
<i>Ctenopharyngodon idellus</i> , <i>Siniperca chuatsi</i> , <i>Silurus meridionalis</i> , <i>Carnis megalobrama</i> , <i>Carassius auratus</i>	Illumina Miseq	Amplicon: V4	AL	Assembly of gut microbiota in larval and adult fish	Effect of ecological processes on gut microbiota assembly	Proteobacteria, Firmicutes and Bacteroidetes	Gut environment and other host development processes shape the microbiome	[44]
<i>Cyprinus carpio</i> and <i>Canna micropeltes</i>	Illumina HiSeq	Amplicon: V4	AU and AL	Survey of intestinal bacteria at key developmental time points	Microbiome composition across developmental stages	Proteobacteria, Firmicutes, Fusobacteria, Actinobacteria and Bacteroidetes	Environmental factors more strongly influence microbiome at early life stages	[121]
<i>Salmo salar</i>	Illumina HiSeq	Amplicon: V6	AU and AL	Influence of alternative diet on microbiome, recirculating waters and biofilters	Effect of alternative feeds on microbiome in fish cultured in recirculating water systems	Proteobacteria and others	Gut microbiome differs by diet treatment but communities in biofilters remain stable independent of diet; Gut communities less diverse than those of water and biofilters	[122]

Table 1 continued

Host	NGS platform	Amplicon/ shotgun Sequencing	Microbiome	Study design	Problem addressed	Dominant phyla	Important inferences	References
<i>Megalobrama amblycephala</i> , <i>Ctenopharyngodon idellus</i> , <i>Siniperca chuatsi</i> , <i>Culter alburnus</i> , <i>Cyprinus carpio</i> , <i>Carassius auratus</i> , <i>Hypophthalmichthys molitrix</i> , <i>H. nobilis</i>	Illumina MiSeq	Amplicon: V4	AL	Influence of trophic level on the gut microbiome	Differences in gut microbiota in herbivorous, carnivorous, omnivorous and filter feeding species	Proteobacteria, Firmicutes, Fusobacteria, Acidobacteria, Bacteroidetes, Actinobacteria, Verrucomicrobia and Cyanobacteria	Trophic level strongly influence the microbiome composition of fish from same habitats; Evidence of a large core gut microbiota in multiple species	[19]
<i>Salmo salar</i> L.	Pyrosequencing	Amplicon: V3 to V6	AL	Effect of diet on the gut microbiome	Diet as a factor controlling microbiome composition	Bacteroidetes, Firmicutes and Proteobacteria	Diet, time of sampling and host specific factors influence the microbes	[123]
<i>Oreochromis niloticus</i>	Pyrosequencing	Amplicon: V1–V2	AU and AL	Gut microbiome development in larvae	Impact of rearing environment on microbiota assembly in early life stages	Proteobacteria and Actinobacteria	Water microbial communities strongly shape those in the gut; Correlation between water and gut microbial community dynamics	[63]
<i>Salmo salar</i>	Illumina MiSeq	Amplicon: V4	AL	Gut microbiomes of freshwater and marine specimens	Effect of geographical distance on microbiome	Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria	Geographical distance has less impact on gut microbiome; Diversity and identity of microbial communities is more strongly determined by life-cycle stage	[124]
<i>Pimephales promelas</i>	Illumina MiSeq	Amplicon: V3–V4	AU and AL	Gut microbiome of triclosan exposed fish	Effect of antimicrobial compounds on gut microbiome	Proteobacteria, Bacteroidetes and Fusobacteria	Microbiome is significantly altered even at low level of environmental changes but has strong resilience power	[125]

species [20, 36, 37]. Li et al, showed that gut bacterial community of grass carp is dominated by cellulolytic *Aeromonas*, followed by *Enterobacter*, *Enterococcus*, *Citrobacter*, *Bacillus*, *Raoultella*, *Klebsiella*, *Hydrothalea*, *Pseudomonas*, *Brevibacillus* and that an increase in intake of plant-fibre increases the diversity of cellulolytic bacteria [36]. Cellulose degrading bacteria *Clostridium*, *Aeromonas*, *Cellulomonas* and *Bacteroides* along with other nitrogen fixing species are reported to provide assimilable carbon in the wood eating fish *Panaque nigrolineatus* [38, 39]. Clostridia also dominate the gut microbial flora in different marine herbivorous fish species [40]. In contrast to the cellulolytic function of microbiome in herbivorous species, lipase and protease producing bacteria and trypsin activity are observed to be much higher in carnivorous species [19], which further confirms the role of microbiota in host digestion. In Atlantic salmon fed a plant-based diet, Lactic acid producing bacteria (LAB) are shown to be present in higher abundance as compared to those fed with fishmeal-based diets suggesting their potential role in digestion [41]. Besides digestion, changes in microbiome composition resulting from environmental stress results in challenged immunity in the host. The gut microbiota produce important short-chain fatty acids (SCFAs) while breakdown of complex sugars which are absorbed in the intestine by simple diffusion or specific receptors and confer resistance against pathogenic invaders [42, 43]. Therefore, the functional repertoire of gut microbiota appears to be synergic with the host needs.

Fate of Colonizing Members and the Factors Affecting Fish Gut Microbiome

The gut colonization may either be driven by (1) stochastic or neutral assembly that derives from random dispersal of microorganisms or chance events that land the microbes into the intestine that are solely responsible for the final shape of intestinal community; or (2) deterministic or non-neutral model assumes that the assembly is determined by the host selective pressures, active dispersal by the host and host-microbe and microbe–microbe interactions. Studies on zebrafish, *Danio rerio*, herbivorous *Ctenopharyngodon idellus*, carnivorous Chinese perch, *Siniperca chuatsi*, and catfish *Silurus meridionalis* over developmental time suggest that the gut colonization among larvae is governed by seeding from surrounding environments which then transits to be progressively determined by the non-neutral processes as the host matures to become adult [44, 45]. Therefore, suggesting stochastic towards deterministic colonization of GI tract.

Though the host GI tract provides for shelter of microbes, certain adaptations are demonstrated to be

exigent for this possession. Ley et al. [46], intricately reviewed the demands imposed on microbial flora by the GI tract viz. in order to firmly attach to the mucosal epithelium of gut wall, microorganisms must possess cell surface molecules for adhesion; the efficient utilization of all the nutrients calls for the production of an enzyme arsenal; the microbes must also be armed with genetic tools for adapting to the ecological plasticity offered by the GI tract and for immunity against bacteriophages. The fittest microorganisms that are able to meet the ecological demands grow and survive in the GI tract and are able to appropriate most niche space to become permanent dwellers, also referred to as “autochthonous”. While others, known as “allochthonous” are visitors of the gut that derive from the surrounding environment [22]. Thus, microbes associate with the host in diverse ways which may be simply fortuitous at first and may become obligatory later. The adherence of the bacteria onto the epithelial cells is considered an essential factor in determining their effector functions within host. A link between the biofilm forming ability of bacteria and successful colonization and functions of gut flora is suggested [47, 48]. The ability to form biofilms helps bacteria to survive in hostile environment offered by the GI tract and is seen in cases of both pathogenic and probiotic bacteria [49, 50]. To unravel the allochthonous communities, fecal samples are generally examined while mucosal epithelium scraped after rinsing are used for analyzing the adherent residents. So far, studies on characterization and comparison of the two community types suggest presumably different composition and that both serve different purposes within the GI tract [51]. High species richness in the digesta (allochthonous) is observed as compared to the mucosa (autochthonous) [52]. This can be understood from the knowledge of the exasperating conditions within the GI tract outlined above. Further, there are evidences that the microbial composition also differs in different regions along the GI tract, the foregut communities being significantly different from hindgut communities [53]. However, the degree by which these two population types differ is not completely understood and needs further clarification.

Interplay of a variety of factors determines the constitution of the fish gut microbiome. Quality of surrounding water and microbial communities directly influence the gut microbiome of fish [24, 54]. Strong evidences of host genetics, developmental stage, immune status and other host specific pressures on the gut microbiome also persist [33, 45, 53, 55, 56]. In addition, the diet also shapes the gut microbiome which displays differential composition with difference in dietary intake [38, 57–60]. Hence, the factors influencing gut microbiome may be broadly grouped into three classes, which are (1) ecology and environmental conditions, (2) host specific and (3) trophic level and/or

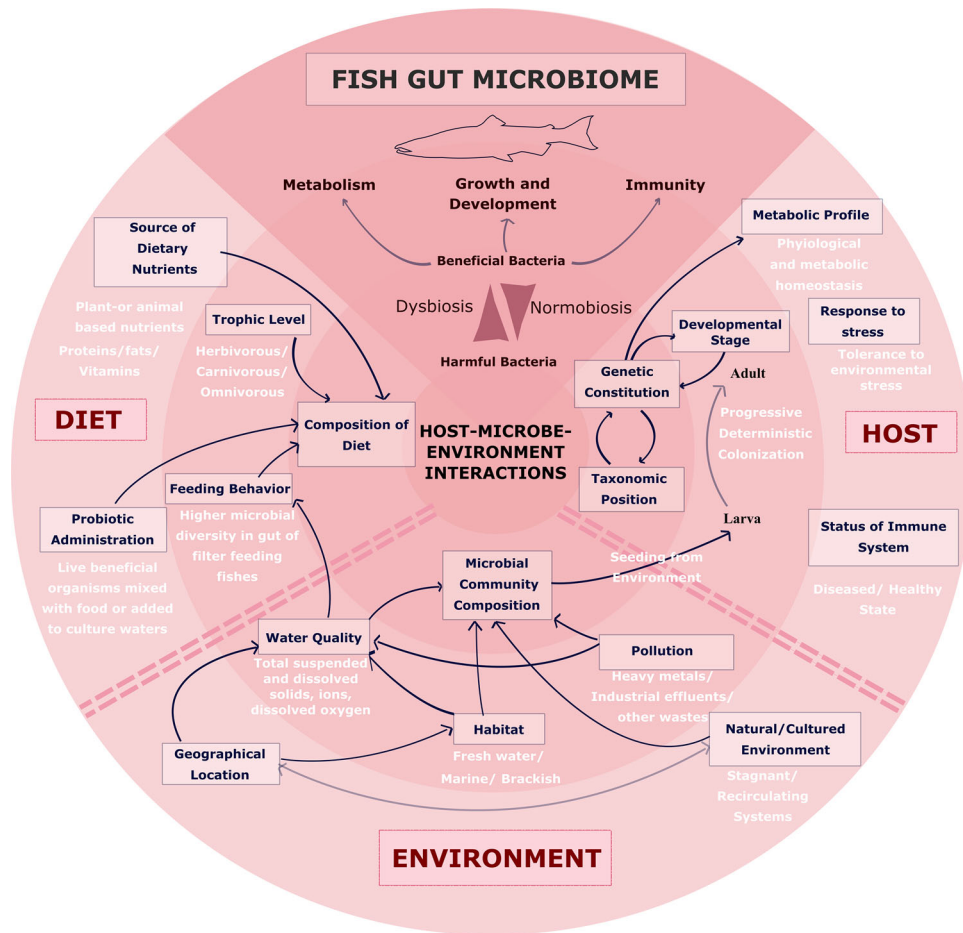


Fig. 1 Factors influencing the diversity and function of the gut microbiome of fish. The factors may be broadly categorized into environmental, diet-associated or host—associated. All these intrinsic

and extrinsic factors are responsible for either healthy state (normobiosis) or altered microbiota (dysbiosis) both of which affect the growth and development of the fish host

feeding behavior as illustrated in Fig. 1. Each of these factors are discussed in detail in the following section.

Study Designs Currently Employed to Study the Factors Affecting Fish Gut Microbiome

While some recent attempts highlight both the environmental and host associated factors to be significantly contributing to the microbial composition in gut [61], others suggest a stronger influence of the host selective pressures [62]. The exact degree with which each of these factors influence the gut microbiome is not known. Clearly, it is difficult to distinguish the host specific and environmental effects on fish gut microbiota. The difference in feeding behavior of different species adds to the problem of investigating the role of each of these factors. Several different approaches are currently employed to study the degree of their influence on fish

gut microbiome. Most approaches rely on the fact that is strongly determined by the environment, the gut microbiome must vary among members of a species, both spatially and temporally, in correlation with the surrounding waters and vice versa if strongly shaped by host selective pressures. The different approaches that are currently employed to study the different determining factors are outlined below.

Microbial Composition and Quality of Surrounding Waters

Due to being constantly exposed to alterations in water quality resulting from various anthropogenic and natural causes, the microbial communities in GI tract of fish should change repeatedly. Thus, a comparison of the water quality and host gut microbiome is expected to improve the understanding of environmental influence. Shared Operational Taxonomic Units (OTUs) between water and gut

microflora have suggested the possible role of surrounding water in steering of gut microbiota [63]. Salinity of water also largely determines the microbial composition in fish gut as revealed from analysis of a large number of species with different ecological needs for survival [24]. Studies on wild and lab-reared invasive carp species also establish environment as a key factor in shaping the gut microbiome [64]. On the contrary, studies also suggest otherwise that the abundant microbial taxa in surrounding waters are not found in the gut of habitant fish and vice versa suggesting a much stronger influence of host associated factors than environment [65].

Species that are known to survive in different stressed habitats are considered apt to assess if a change in quality of surrounding waters other than microbial composition also affect the host gut microbiota. For instance, the endogenous microbiota in Amazonian tambaqui, *Colossoma macropomum* that is tolerant to significant variation in pH levels has been shown to be significantly altered at experimental low pH levels; however, it also displays a strong resilience once pH levels are restored [66].

Variations in Different Cohabiting Species

The second approach employs the use of different species inhabiting same water systems. Many studies have revealed a larger influence of host selective pressures and trophic level in constraining the gut microbiota than the environment as different cohabiting species are observed to comprise different microflora in gut. For instance, cohabiting species of silver carp, grass carp, bighead carp, and blunt snout bream revealed distinct gut microbiome composition [67] providing for the influence of host specific factors on the gut micro-organismal communities. Similar results were obtained in paddle fish, *Polyodon spathala* and bighead carp, *Aristichthys nobilis* with similar feeding behavior when fed same food and reared in same pond [68]. Thus host associated factors outplay the influence of environment.

Variations Within a Species at Different Geographical Locations

Third approach employs studying the microbiome of a single species found in different geographical locations to study if it is defined by a core set of microbial communities that stably reside in the GI tract due to host-specific selective pressures or if the environment shapes the communities. In this regard, zebrafish, *Danio rerio*, has been extensively studied to establish the strong influence of host associated factors that shape the core gut microbiome of the species [25]. Although differences occur, however, the

peculiarly low diversity presented by the gut microbiome of different fish species of ecologically and geographically different origins with respect to the bacterial phyla further warrants the argument of host selective pressures on the gut microbiome [19, 23, 26, 27, 69, 70].

Variations at Different Trophic Levels and Feeding Behavior

The microbial diversity in GI tract increases as the diet changes from carnivorous to omnivorous to herbivorous [53]. Cellulose-decomposing bacteria such as *Anoxybacillus*, *Leuconostoc*, *Clostridium*, *Actinomyces*, and *Citrobacter* populate the gut of herbivorous species such as grass carp, *Ctenopharyngodon idellus* [20]. On the other hand, carnivorous species are found to harbor lipase and protease-producing bacteria such as *Halomonas* [19]. Feeding behavior is also shown to affect the gut microbiome composition in a habitat in closely related but different species. Filter feeding fishes display higher diversity as they filter large volumes of water and cover large areas by swimming rapidly [71].

Administration of Different Dietary Components

The effect of dietary intake on gut flora is not only restricted to the nutritional composition but also the source of nutrients. In general, plant-derived dietary proteins have been linked to significantly reduced diversity of microbial flora [28] with an increase in relative abundance of Lactobacillales, Bacillales and Pseudomonadales [72]. While animal-derived proteins nurture Bacteroidales, Clostridiales, Vibrionales, Fusobacteriales and Alteromonadales in the gut [72]. A study by Mansfield et al., revealed that fish fed with synthetic casein based diet have larger diversity as compared to those fed with fish meal or soyabean meal based diet [73]. Effects of a large number of other nutritional components have been reviewed in detail [74].

Patterns of Gut Colonization in Gnotobiotic Models

The use of gnotobiotic fish models incomparably fit to serve the purpose of delineating the processes that fabricate the gut microbiome structure. Analysis of patterns of colonization in germ-free zebrafish GI tract revealed that the host responses may be attributed to specific bacterial members of the community [23]. Transplantation experiments of gut microbial flora between gnotobiotic zebrafish and mouse and between gnotobiotic mouse and zebrafish hint towards reconstruction of the microbiome after transplantation to resemble that of conventionally raised fish thereby concealing the microbial communities which were transplanted in their gut [55].

Thus, it can be inferred that the microbial composition of water influences the fish gut microbiome to some extent. In addition, diet and genetic variations among individual hosts also shape the gut microbiome. To elucidate the exact degree of influence by each factor would be an interesting area of study. Even though the microbiome composition of two individuals might differ at the species level, there occur significant level of similarity among the microbial genes that are shared which suggest for a molecular link between the microbial genes and host functions [75]. Thus, there is an emerging need for multifaceted analyses to clearly demarcate the host-, environment- and feeding behavior derived manifestations of the gut microbiome. It may be viewed as environment, host specific factors and diet, act in concert to constrain the acquisition of gut microbiota.

Profiling the Fish Gut Microbiome

An illustration of the steps followed to analyze the gut microbiome of a fish species by direct analysis of the community DNA is shown in Fig. 2. The choice of amplicon or shotgun sequencing depends on the goal of the study. To date, the studies of fish gut microbiome have remained limited to deciphering the composition. However, shotgun assessments of the gut microbial genetic repertoire are needed to provide crucial insights into their functional potential and can be further aided by the genomic sequencing and analysis of the cultured isolates as has been investigated in other niches [76–85].

The gut produces and receives an arsenal of enzyme secretions while performing functions such as grinding the food mechanically and chemically and extracting and absorbing nutrients, which creates a major challenge in extraction of quality community DNA from the microbial populations. For instance, bile salts and complex polysaccharides present in gut inhibit the downstream processes of PCR amplification [86]. Therefore, a DNA extraction method should be employed after envisaging (1) the correct representation of genomes from entire community with no over or under-representation of individual microbial populations, (2) its efficiency on the sample source (foregut, midgut or hindgut) and type (luminal contents only or gut wall with contents), (3) the design of study which involves the type of sequencing i.e., whole genome or targeted sequencing and (4) the quality data required for downstream analysis. The choice of DNA extraction method may strongly influence the analysis of the community composition. There are convincing evidences that a method for extracting DNA does not conform to changes in host species or sample type due to variability in the ingested food and microbial load in the host GI tract [87–90]. As the

ingested food may vary among individual hosts and may comprise of semi-digested particulate matter and bones, the availability of the starting mass of fecal or gut content samples for DNA extraction needs to be taken into consideration while comparing the extraction protocols to avoid false interpretations. Perceivable differences are reported to occur in the purity and concentrations of the extracted DNA employing different methods [88]. Further, the purity of extracted DNA does not guarantee a successful amplification and sequencing [88]. Sample preparations pose as a source of variability in testing the extraction efficiency of a method. Processing the entire GI tract along with the luminal content may result in isolation of a large amount of eukaryotic DNA from fish, therefore, diluting the bacterial DNA for sequencing and analysis. The structural composition of microbiota also affects precise extraction of DNA as the gram-positive bacteria are more difficult to lyse than gram-negative bacteria due to presence of a thick peptidoglycan layer in their cell walls. This spurs a necessity to ensure complete lysis of all bacterial cells in the community by the chosen extraction method.

It is critical to use an appropriate method of extraction of microbial community DNA which lays the foundation for accurate characterization of the gut microbiota both structurally and functionally. Essentially, the sample source and type need to be emphasized upon before relying upon the results from DNA extraction methods comparison studies as not all the results are comparable owing to the sample variability.

Host-Microbe Interactions in Fish Gut

The gut-associated microbes might have potential beneficial or harmful effects on the host. When the beneficial micro-organisms constitute majority of the microbiome, a state of “normobiosis” prevails. Any perturbations in its normal composition result in “dysbiosis” in which state the harmful microbes predominate giving rise to a diseased condition. While on one hand host intrinsic factors and environment shape the gut microbiome, the microbiota has equal influence on the host biology. Thus, a three way interaction involving host- microbe-environment operates to maintain homeostasis in the fish gut.

Colonization of the gut with specific microorganisms to gain insights into the effects of microbiota on host cellular responses in mice revealed that they modulate the expression of several cellular genes that endows host with important functions including metabolism, nutrient absorption, immune response generation, and intestinal maturation [1]. Similar findings have been obtained using zebrafish models [23, 91, 92] and in other fish species

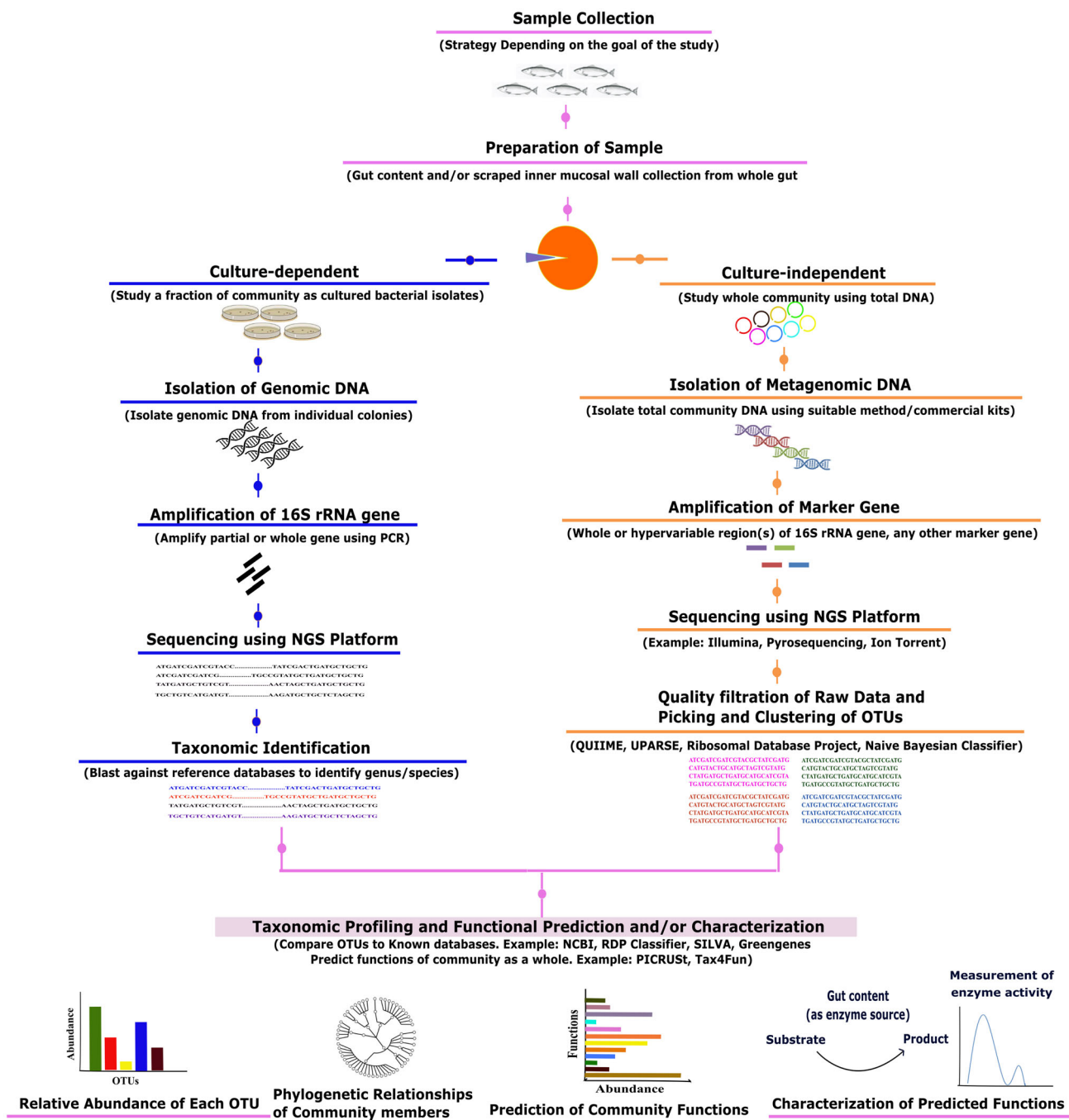


Fig. 2 Schematic representation of the workflow for analyzing the fish gut microbiome. The currently employed procedures for fish gut microbiome studies include the traditional culture-dependent analysis as well as culture-independent analysis of the total DNA obtained directly from gut contents and mucosal wall. The culture-dependent techniques widely use sequencing of 16S rRNA gene to identify bacteria. For defining the uncultured microbiota, amplification and sequencing of whole or partial [hypervariable region(s)] of 16S rRNA gene is widely employed. Highly similar sequences are then grouped

into Operational Taxonomic Units (OTUs) and compared against databases. The widely used databases and tools include NCBI [126], QIIME [127], UPARSE [128], Silva [129], Green Genes [130], RDP Classifier [131] and Naïve Bayesian Classifier [132]. The community is thus profiled based on the relative abundance of each OTU and their phylogenetic relationships. Using advanced 'omic' tools, functions of the community can also be predicted such as PICRUSt [34] or Tax4Fun [35]. Important microbial functions may then be characterized through wet lab experiments

where microorganisms demonstrated to regulate metabolism [29]. Studies on transgenic fast-growing common carp, *Cyprinus carpio* L. suggest an important role of gut

flora on growth of fish and that the Firmicutes confer fast growth of fish over Bacteroidetes [29]. The microbial members are known to aid in digestion of cellulose in

herbivorous fishes [20, 22, 60]. They are also known to influence innate immune responses in fish [32] and educate the host-immune system for better protection against pathogenic invaders. These findings provide strong perspectives of the interactions between resident microorganisms and their host fish.

Conclusion and Future Perspectives: Towards Applied Research

The gut microbiota is influenced by a myriad of factors but the appeal of each of these factors on the behavior and physiology of fish remain poorly understood. For drawing meaningful conclusions, one of the crucial challenges is to establish a correlation of microbiome structure and function with health status, age, genetic background, geographical location and other individual differences of the host. Furthermore, creating a comparative picture based on studies employing different methods of DNA extraction from different sample types within a species needs careful analyses. As the marker gene based approach limits the analysis to predictions of microbiome structure, there has emerged a need for complete shotgun sequencing efforts to fully explore the metabolic potential of the gut microflora and uncover functional variation with diet or host associated factors.

Fish are affected by several pathogenic bacteria including *Aeromonas*, *Edwardsiella*, *Pseudomonas*, *Flavobacterium*, *Vibrio* and *Streptococcus* and *Yersinia* causing diseases in different tropical freshwater fishes [93, 94]. These are often treated with antibiotics. However, their overuse has encouraged antibiotic resistance which makes the fight against pathogens even more difficult [93]. Pathogenicity of these bacteria such as *Aeromonas hydrophila*, *A. salmonicida*, *Vibrio anguillarum*, *V. harveyi*, *Yersinia ruckeri* and *Tenacibaculum maritimum* is mediated by quorum sensing systems that are important for biofilm formation [95]. The use of quorum quenching enzymes has also been successful in reducing pathogenicity of these pathogens [96–98]. Bacteria derived from fish microbiome have also been shown to produce natural products that inhibit formation of biofilms and therefore promote detachment of gram-negative pathogens [50]. However, more efforts focusing on their application in fish GI tracts are needed. From the recent studies on isolation of *Bdellovibrios* that naturally feed on gram-negative bacteria from experimental diseased fish models, we envisage the use of these microbial predators as natural cure of diseases [99–101]. *Bdellovibrios* are also found to be prevalent in intestines of human and other animals and have been reported from diverse habitats [76, 102–104]. The emerging concept of “forward microbiomics” that involves

manipulating the gut flora to promote fish health finds important applications in aquaculture. Several *Lactobacillus* species have been identified as probiotics in fish and other animals including humans [105, 106]. However, their potential needs to be fully explored. Collating inferences from different studies would help in identifying microbial biomarkers and would augment the application of probiotics. It is becoming increasingly clear that the microbiome affects its host in more than one ways and its study is thought to bring a plenitude of understanding of its functional potential in the host and expand current notions of the fish gut microbiome.

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Author Contributions RL and RKN conceived the idea. CT wrote the manuscript and SN helped shape the manuscript. RL and RKN critically reviewed the manuscript.

Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

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