



Review Article

Gastrointestinal Metagenomics of Fish: Methods, Research Advances and Applications

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Abstract

Fish microbiota function in fish nutrition and health, but research has been hindered by difficulties in culturing these microorganisms. New molecular techniques, like DDGE (Denaturation gradient gel electrophoresis) and FISH (Fluorescence in situ Hybridization), have produced limited detailed quantitative results. Next-generation sequencing based metagenomics and microarray offers more promise. These high throughput methods can obtain the overall genetic information within a microorganism community and there is no need to cultivate the individual microbes in advance. Metagenomics combines the sequencing of 16S rRNA genes and genomes of the sampled microbiota. We provide an overview of recent research advances in fish metagenomics and related applications. Many studies have already been done on individual fish species, but it is necessary to obtain a comprehensive profile of the microbiota of all fish species using metagenomics sequencing. © 2018 Friends Science Publishers

Keywords: Antimicrobial peptide; Fish; Gastrointestinal microbiota; Metagenomics; Next-generation sequencing; Probiotics

Introduction

Microorganisms are widespread within living organisms. However, most of the microorganisms cannot be cultured and are difficult to access and understand. Next-generation sequencing (NGS) and microarray techniques now make it easier to study the full range of microbial diversity. With the rapid development of metagenomic sequencing, we are now able to evaluate microbial communities, functional genes and their natural products.

Introduction to Metagenomics

The microbiota residing in an organism, especially in the gastro- and intestinal tract, are often viewed as equivalent to an integral organ. The gastrointestinal (GI) microbiota of fishes and vertebrates have been studied using various methods (culture independent or dependent). However, it is currently impossible to characterize over 70% of GI microbiota (Ringø and Olsen, 1999). This is mainly due to the impossibility of microbial culture using routine laboratory methods (Wu *et al.*, 2012). Usually, only the predominant microorganisms are specified and the majority

of the microbes remain unknown (Cardenas and Tiedje, 2008). Compared with the fish GI microbiome studies using a culture system, studies using metagenomics and 16S rRNA gene amplicon sequencing technologies are more likely to reflect overall community diversity (Hess *et al.*, 2011; Wrighton *et al.*, 2012). The phylum *Fusobacteria* occupy more than a half of the GI microbiota metagenomic data in captive carp, which could not be cultivated on synthetic media until recently (Sakata *et al.*, 1981; Van Kessel *et al.*, 2011).

Metagenomics is the study of genetic material recovered directly from environmental samples by molecular and bioinformatic techniques (Fig. 1, Thomas *et al.*, 2012). These approaches avoid the prolonged procedures of cloning in expression vectors (DeSantis *et al.*, 2006) by harvesting thousands of reads for one microbial sample in one run cycle. This allows the discovery of new microorganisms especially those unculturable on synthetic media (Fakruddin *et al.*, 2013). Thus we can now conduct in-depth analysis of microbial communities (Xing *et al.*, 2013). With the rapid development of NGS sequencing, a large amount of metagenomic data is now available to researchers (Wang and Jia, 2016).

Metagenomics combines sequencing of the 16S rRNA genes and genomes of sampled microbiota to generate taxonomic diversity and species structure within the examined microbiome (Chen and Pachter, 2005). Unlike genomics dealing with the genome sequencing of individual species, metagenomics studies the overall 16S rDNA and genomic sequences of microorganisms in samples.

The metagenomic approach by NGS sequencing aims to solve problems using integrated data analysis of multiple bacterial strains (Chen and Pachter, 2005). The main characteristic of metagenomics by NGS is that it typically does not require existing sequence information from the community of interest (Roh *et al.*, 2010), with which we can uncover new genes, pathways, taxa, and encoded functionality further. However, there are some challenges that require consideration, including the sampling choice, sequencing methods, assembly and coverage. Also, many microorganisms are still unknown and many host genomes are unavailable. Metatranscriptomics was developed to compensate for the shortcomings of metagenomics by NGS in detection of the expression status of microbial genes, microbial community activities and functions, as well as discovery of novel genes and regulatory elements (Wu *et al.*, 2015).

Microarray analysis is a type of metadata analysis whose potential experimental outcome is defined in a specific range before analysis. The experimental results of microbial community from samples originate from the predefined probes fixed on array plates (Roh *et al.*, 2010). The main feature of metagenomics by microarray is that it requires existing sequence information and does not produce novel sequence data. This is because all sequence tags for the query are pre-designed (He *et al.*, 2010). DNA arrays, protein arrays and the classic quantitative PCR, are closely formatted methods.

NGS sequencing and microarray detection are two widely used representative metagenomic technologies. However, each category possesses advantages and deficiencies in operation. An attractive feature of NGS is that it is useful for novel gene discovery and bacteria exploitation (Hess *et al.*, 2011; Qin *et al.*, 2012; Wrighton *et al.*, 2012). However, contamination from the host and the environment is frequent in sample preparation, resulting in a low utilization rate of sequencing data and a waste of sequencing effort in NGS analysis.

In DNA microarray, microbial detection and microbiome community analyses are occasionally implemented (He *et al.*, 2011). rRNA genes are usually the targets of phylogenetic gene arrays and these are useful for distinguishing specific taxa and studying the phylogeny of microorganisms. The representative phylogenetic gene arrays are PhyloChip (Paliy *et al.*, 2009; Hazen *et al.*, 2010), which can detect many known microbial groups. The functional gene array, GeoChip, has recently been developed (Fig. 2). It targets functional gene categories for biogeochemical, ecological and environmental analyses

(He *et al.*, 2010; Lu *et al.*, 2012). It is also a potentially useful tool for fishery projects.

GeoChip studies have been applied without greatly varying the DNA amplification. Pure cultures, mixed cultures, and environmental samples are identified by hybridization signal intensities (Wu *et al.*, 2006). In addition, technical reproducibility of microarrays is less affected by inadequate random sampling than in NGS. One main defect of the microarray technologies is that it is not able to discover novel genes, taxa, and regulatory elements (Zhou *et al.*, 2015).

In this review, we mainly focus on metagenomic studies of the fish GI tract with NGS sequencing since this has been extensively reported. The GeoChip-based DNA array has also been employed in our labs for a Chinese program (Fish-M1K), which aims at establishing a comprehensive microbiota gene set from a metagenomic study on > 1,000 fish species.

Research Advances in Fish Metagenomics

Teleost fishes possess the largest taxonomic and ecological diversity among vertebrates but research on their GI microbiota and functional metagenomics has been delayed compared to studies on terrestrial vertebrates (Banerjee *et al.*, 2013). In contrast to the utilization of metagenomics on the GI microbiota in humans (Qin *et al.*, 2012) and other vertebrates (Kostic *et al.*, 2013), fish metagenomics studies have already been applied to microorganisms in gills (Kostic *et al.*, 2013), skin (Lokesh and Kiron, 2016) and mucosa (Gatesoupe *et al.*, 2016). However, the microorganisms in the digestive tract, which is also called the GI microbiota, still constitutes the majority of microbiota residing in fish and have a special influence on the health and growth of fish.

The intestinal morphologies of fishes show great variation (Clements and Raubenheimer, 2006). Some species have a delineated true stomach or hindgut chamber, while other species lack a delineated stomach or intestine. There are distinct microorganism populations with different metabolic functions in defined regions of fish alimentary tracts (Ye *et al.*, 2013). Among the different sections of the fish GI tract, the hindgut is often used in analyses of the fish gut microbiome. This is because it is more likely that there are targeted bacterial symbionts related to digestion and fewer environmental bacteria compared to the anterior region of the intestine (Zhou *et al.*, 1998).

It is believed that each fish species possesses “core essential GI microbiota” (Shade and Handelsman, 2012). Host-derived specific pressures play an important role in determining GI bacteria in fishes and thus fishes have a local microbiota mainly dependent on the host fish (Sakata *et al.*, 1981). Other studies have noted that fish GI communities are mostly occupied by indigenous organisms that are underrepresented in the environment (Cahill, 1990). Metagenomic analyses indicate that fish GI microbial communities are more similar to those of mammals,

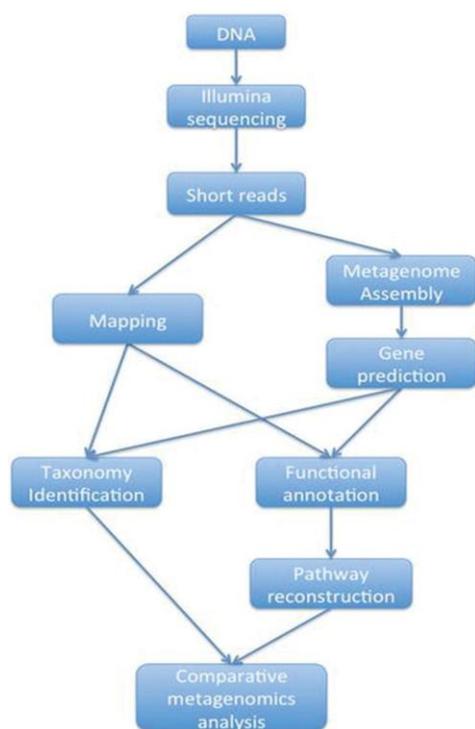


Fig. 1: Standard protocol for NGS-based metagenomics projects at BGI

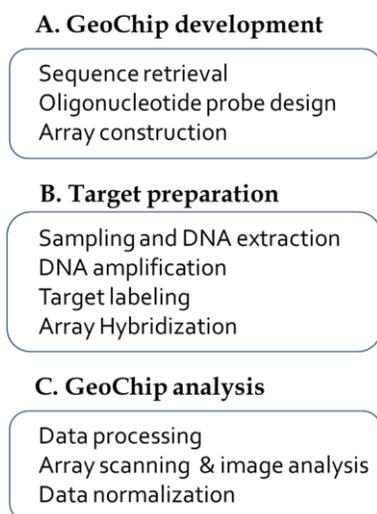


Fig. 2: Overview of the GeoChip-based DNA microarray (Zhou *et al.*, 2010)

particularly in the amount of Proteobacteria, Firmicutes and Bacteroidetes (Sullam *et al.*, 2012). Therefore, the GI microbiota composition of fish does not simply reflect the microorganisms in their habitat but develops from selective pressures within the gut such as species-specific behavior, immunity and metabolism (Ye *et al.*, 2013; Larsen *et al.*, 2014).

After long coevolution with the fish host, the fish gut microbiota assists in food digestion and provision of essential nutrients (Turnbaugh *et al.*, 2006; Ley *et al.*, 2008; Borsodi *et al.*, 2017). The fish GI tract harbors various bacteria, archaea, viruses and eukaryotic microorganisms, which are collectively called the GI microbiota. In fish, they are primary protective barriers and occur in the gut with both beneficial and opportunistic pathogenic microorganisms. In addition to defense against pathogens, the GI microbial community facilitates the proliferation of intestinal epithelium, digestion of complex carbohydrates, modulation of the immune system, regulation of the dietary energy intake, and even the generation of secondary metabolites such as vitamins (Sugita *et al.*, 1993; Flint *et al.*, 2008; Dawood *et al.*, 2016b). The cellulolytic bacteria in cyprinid fish intestines function in the digestion of cellulose (Wu *et al.*, 2015). The GI microbiota of fish harbor many opportunistic pathogens and the GI tract is open for pathogen invasion (Kim *et al.*, 2007; Roeselers *et al.*, 2011). For this reason, understanding the function and the composition of GI microbial communities is crucial for health management in aquaculture fish.

The internal and external factors affecting the composition of fish GI microbial communities include developmental stage, gut structure, diet composition, health condition, habitat, water temperature and salinity, rearing conditions and host genotypes (Nayak, 2010; Sullam *et al.*, 2012; Ni *et al.*, 2014). Among the external factors, host diet is a well-known factor determining microbiota composition (David *et al.*, 2014). Therefore, it is crucial to explain the relationship between host diet and the functional significance of fish intestinal communities by using the metagenomic approach.

We summarize recent research results on fish microbiome (Table 1) in which high throughput methods like 16S rDNA, metagenomics, and microarray were used. The metagenomics studies were carried out on fish species worldwide including freshwater and saltwater fishes like carp, rainbow trout, Siberian sturgeon, Atlantic salmon, catfish, Atlantic cod, reef fishes, and Antarctic notothenioids. Interestingly, the species attracting the most attention and publications are carps, including grass carp, silver carp, Prussian carp, and Jian carp. This is consistent with the large production and economic value of these cyprinid species in Asia (Tsuchiya *et al.*, 2008; Han *et al.*, 2010; Van Kessel *et al.*, 2011; Li *et al.*, 2013; Ye *et al.*, 2013; Ni *et al.*, 2014; Zhao *et al.*, 2014; Kashinskaya *et al.*, 2015; Li *et al.*, 2015; Wu *et al.*, 2015).

In recent studies on fish metagenomics, most microbiome samples were collected from the GI tract. The GI harbors most microorganisms in fishes and GI tract microbiomes are directly related to the metabolism and pathogens of the fish host. In some studies (Ringø *et al.*, 2006; Davis *et al.*, 2016), samples were obtained from larvae to determine the specific colonization processes that happen during the transition from the sterile larvae to the adult fish.

Table 1: Fish microbiome research by high throughput approaches

Species	Sample source	Method
Common carp (<i>Cyprinus carpio</i>); grass carp (<i>Ctenopharyngodon idellus</i>); transgenic common carp (<i>Cyprinus carpio</i>); grass carp (<i>Ctenopharyngodon et al.</i> , 2010)	GI tract	16S rDNA (Wu et al., 2015; Ye et al., 2013; Tsuchiya et al., 2008; Han et al., 2010; Van Kessel et al., 2011; Li et al., 2013, 2015; Ni et al., 2014; Zhao et al., 2014; Kashinskaya et al., 2015); Pyrosequencing; gene fingerprinting methods (such as DGGE); Metagenomics (Ni et al., 2014; Kashinskaya et al., 2015); microarray (Ni et al., 2014); Metatranscriptomics (Wu et al., 2015)
crucian carp (<i>Carassius cuvieri</i>); bighead carp (<i>Hypophthalmichthys nobilis</i>); juvenile Jian carp (<i>Cyprinus carpio</i> var. <i>Jian</i>); Prussian carp (<i>Carassius gibelio</i>); silver carp (<i>Hypophthalmichthys molitrix</i>)	Skin mucus (Lokesh and Kiron, 2016)	16S rRNA gene
Atlantic salmon (<i>Salmo salar</i>)	GI tract (Pond et al., 2006; Kim et al., 2007; Mansfield et al., 2010; Ingerslev et al., 2014; Gonçalves and Gallardo-Escárate, 2017)	16S rRNA pyrosequencing; DGGE
rainbow trout (<i>Oncorhynchus mykiss</i>)	GI tract (Roeselers et al., 2011; Cantas et al., 2012; Rawls et al., 2004) Larvae (Davis et al., 2004)	16S rDNA gene sequencing; microarray (Rawls et al., 2004)
zebrafish (<i>Danio rerio</i>)	Larvae (Ringø et al., 2006)	PCR-DGGE analysis
Atlantic cod (<i>Gadus morhua</i>)	GI tract	454 sequenced 16S rRNA library (Star et al., 2013)
Atlantic cod (<i>Gadus morhua</i>)	GI tract	454-pyrosequencing of the 16S rRNA gene (Geraylou et al., 2013)
Siberian sturgeon (<i>Acipenser baerii</i>)	GI tract	16S rDNA sequence (Tsuchiya et al., 2008)
catfish (<i>Panaque nigrolineatus</i>)	Faeces (Di Maiuta et al., 2013)	16S rRNA
catfish (<i>Panaque nigrolineatus</i>)	Feces and distal gut contents (Smriga et al., 2010)	Denaturing gradient gel electrophoresis profiles of 16S rRNA-
reef fish (<i>Acanthurus nigricans</i> , <i>Chlorurus sordidus</i> , <i>Lutjanus bohar</i>)	GI tract	16s cluster
Antarctic notothenioid		

The sample sources varied greatly, including wood eating catfish feces (Di Maiuta et al., 2013), three coral reef fish GI contents (Smriga et al., 2010), and salmon skin mucus during the transition from freshwater to saltwater (Lokesh and Kiron, 2016).

Most previous microbiome studies on fish involved a high throughput metagenomic approach, especially 16S rDNA sequencing (Table 1). Many of these studies were combined with culture independent techniques, such as DDGE (Denaturation gradient gel electrophoresis) and FISH (Fluorescence in situ Hybridization). Only a few whole metagenomic studies have been reported. One of these was used to decipher the biosynthesis and metabolism pathways of carbohydrates, amino acids and lipids in ryegrass fed grass carp (Ni et al., 2014). In this research, the microarray method was also applied to detect the changing mode of metabolic products. Another study dealt with the role of the GI microbiome on metabolizing cellulose using metatranscriptomics (Wu et al., 2015). A comparison study was done on Prussian carp using both metagenomics and 16S sequencing. This study indicated that metagenomics is more accurate in taxonomic assignment than 16S analysis only (Kashinskaya et al., 2015). Microarray, including GeoChip and PhyloChip, are additional high throughput approaches but they have not been used in previous studies of fish metagenomics. However, their merits would be useful in future fish microbiome studies. Microarray will complement the 16S and NGS-based metagenomic approaches with its beneficial aspects for microbiome research.

Factors Shaping GI Microbiota and Core Microbiome

The gut microbiome of fish varies considerably from the

microbiome of other vertebrates and the microflora in water and soil (Sullam et al., 2012). Both trophic level and salinity have great impact on its composition (Wong and Rawls, 2012; Li et al., 2015). In addition, animal feed, which is the direct source of animal gut microorganisms, plays a vital role in the genesis of GI microbiota, but it has variable effects on different animals (Geraylou et al., 2013; Wong et al., 2013; Baldo et al., 2015). The bacteria identified in gut microbiome can reflect the food preference of the host (Ye et al., 2013). Prey items of the three-spined stickleback carry microbiota that can influence the host gut microbiome, but the host genotype has a relatively greater impact (Smith et al., 2015). In two freshwater fish species, the relationship of gut microbiome diversity and dietary diversity was inversely correlated (Bolnick et al., 2014); even in Trinidadian guppies, the effect of diet on GI microbiota was so small to be ignored (Sullam et al., 2015a).

Other important factors determining fish GI microbiota include physiological condition of the fish and environmental components. In silver carp, components of the gut microbiome were geographically and temporally dependent (Ye et al., 2013). Dramatic changes of the fish GI microbiota can occur when habitats change including shifts from natural to man-made environments (Cantas et al., 2012; Clements et al., 2014; Sullam et al., 2015b).

Although the composition and structure of fish GI microbiomes are now better understood, the factors shaping these GI microbiomes require further study. Many factors have substantial influence on the composition of GI microbiota in vertebrates, including host genetics, environment and nutrition, but the host genetic background and the house keeping physiological function of GI microbiota play decisive roles in selecting for the essential

(core) microbial taxa. As core GI microbiota are present in all GI communities with individuals of one species, it has been an interesting goal in this realm (Turnbaugh *et al.*, 2007). In research on zebrafish, a core microbiome was determined by comparing laboratory raised and wild fish. The data indicated that this species of fish harbors a core GI microbiome as do other vertebrates (Roeselers *et al.*, 2011). This finding was supported by a reciprocal microbiota transplant experiment between zebrafish and mice (Rawls *et al.*, 2006).

However, there are many teleost species and the ecology within the fish GI community can be more complex (Shade and Handelsman, 2012). It is more difficult to find a common core microbiome in fishes than in mammals (Turnbaugh and Gordon, 2009; Tap *et al.*, 2009). In a study on teleost GI microbiota (Roeselers *et al.*, 2011), comparisons of GI microbiome between wild and domestic zebrafish populations revealed significant differences. A total of 21 shared operational taxonomic units (OTUs) are considered to be the core community present in all fish. In invasive carp species (Eichmiller *et al.*, 2016), the core GI microbiome shared by laboratory-reared and wild individuals comprised up to 40% OTU abundance, while there were merely five shared OTUs, thus interpreting their critical role. Some researches suggested that the genetic background of the fish host plays a decisive role in determining GI microbial communities (Roeselers *et al.*, 2011). Other studies revealed that the living environment is the primary driver for construction of GI microbiota population structure (Eichmiller *et al.*, 2016), and some authors indicate that the main influence on GI microbiome of species is nutrition or trophic level (Liu *et al.*, 2016). In fish-associated microbiomes, there are more similarities among freshwater fishes, regardless of their phylogenetic relationship to fish in marine environments (Sullam *et al.*, 2012). *Aeromonas* spp. was predominant in the GI microbiota of freshwater fishes, while *Vibrio* spp. was the largest group in the GI microbiota of marine fish species. The evolutionary origin of the GI microbiota was not likely relevant to their roles in the gut (Sullam *et al.*, 2012).

If a core microbiome does exist among fish species across distinct environments and distant phylogenetic relationship (Roeselers *et al.*, 2011; Hennersdorf *et al.*, 2016), the factors shaping the GI microbiota needs further investigation. Studies should include evolutionary forces dictated by host genetics, gut physiology and bacterial symbionts. If this hypothesis is true, it would be easier to manipulate the GI bacterial communities, and this would be helpful to promote fish health and yields in aquaculture operations. Members of the phyla *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Verrucomicrobia* and *Fusobacteria* have been shown to occupy the fish GI microbiome (Sullam *et al.*, 2012; Llewellyn *et al.*, 2014). *Proteobacteria*, *Fusobacteria* and *Firmicutes* had the greatest abundance in the GI microbiome of most fish species (Hennersdorf *et al.*, 2016; Eichmiller *et al.*, 2016).

Applications of Metagenomics: Antimicrobial Peptides (AMPs) and Probiotics

The fish GI microbiota has probably coevolved with the host for millions of years. The microbiota competes with the host for common resources, but it is an integral component of the host. The GI tract also harbors opportunistic pathogens within the gut microbiota and so it is a potential pathway for pathogen invasion (Wu *et al.*, 2010; Roeselers *et al.*, 2011).

The autochthonous bacteria among the gut microbiota are more likely to confer health benefits, like robust temporal stability, than allochthonous or non-fish bacteria (Fjellheim *et al.*, 2010; Sun *et al.*, 2010, 2015). Therefore, the beneficial autochthonous GI microbiota or their bacteriocin products warrant further investigation in farmed fish because of the potential significance of GI microbiota for disease control in aquaculture (Ringø *et al.*, 1995; Ray *et al.*, 2012; Llewellyn *et al.*, 2014; Ringø and Song, 2016). Diverse probiotic strains of bacteria within the genera *Lactobacillus*, *Bacillus*, *Citrobacter*, *Enterobacter*, *Streptococcus* (Ghosh *et al.*, 2010; Ray *et al.*, 2010; Khan and Ghosh, 2012; Ray *et al.*, 2012) and yeasts (*Pichia kudriavzevii*, *Candida tropicalis*, *C. parapsilosis* and *C. rugosa*) (Mandal and Ghosh, 2013; Banerjee and Ghosh, 2014; Das and Ghosh, 2014) have been identified or characterized from the GI tracts of Indian major carp, exotic carp species and other teleosts. *Lactobacillus* comprises over 50 species of lactic acid bacteria, which is reported to stimulate the host immune system and fight against intestinal pathogens with their antimicrobial activity (Kemgang *et al.*, 2014). *Pediococcus acidilactici* can balance microecology by acid production (Zacharof and Lovitt, 2012). *Bacillus subtilis* is widely accepted in agriculture and food products as a form of microecologies (Field *et al.*, 2015).

Bacteria are the most predominant members of the probiotic strains of gut microbiota. They use different mechanisms to modulate the microbial community and improve the microbial balance and maintain the robust temporal stability (Mohapatra *et al.*, 2013). One such mechanism is the production of bacteriocins to antagonize closely related competing bacteria *in vivo*. Bacteriocins are AMPs ribosomally synthesized both in Gram-negative and Gram-positive bacteria (Nes *et al.*, 2007). There are two kinds of bacteriocins with different antimicrobial spectra. One has relatively narrow spectra, including most bacteriocins targeting species only related to their producers. Another type, with a wider spectrum, includes the lactococcal bacteriocin nisin, which has inhibitory activity on pathogenic or problematic species of *Staphylococcus* and *Listeria* (Jack *et al.*, 1995; Nes and Johnsborg, 2004; Arqués *et al.*, 2015). Bacteriocins have no effects on their producers with protection mechanisms or on other beneficial cells and eukaryotic cells, while they are active against pathogenic bacterial strains (Cotter *et al.*, 2013). Hence, they are promising components of antibiotics for the prevention and control of diseases in aquaculture.

Since bacteriocins and bacteriocin-like peptides are ribosomally translated by gene regulation, they are good candidates for metagenome data mining (Donia *et al.*, 2014; Walsh *et al.*, 2015). The sequencing data facilitated by NGS has enabled high throughput *in silico* mining of putative antimicrobial substance genes and probiotics that modulate the gut microbiota (Erejuwa *et al.*, 2014; Walsh *et al.*, 2014), or can help moderate the increasing risk to host health caused by antibiotic resistance. Therefore, research on the abundance and categories of bacteriocins encoded in the fish GI microbiome could estimate beneficial or harmful microorganism in the GI community and identify the key organisms that help maintain the integral composition and union. Using NGS, we can study probiotic strains by homology-based searches with the antimicrobial peptide sequences obtained from the metagenome data.

In the commercial fishery industry, unfavorable conditions can decrease production and increase the rate of infectious diseases (Bondad-Reantaso *et al.*, 2005). However, the resistance that has developed from the overuse of antibiotics (Romero *et al.*, 2012; Lyapparaj *et al.*, 2013) requires discovery of antibiotic alternatives, such as phage therapy, immunostimulants, plant extracts, pro-, pre- and symbiotic concepts, and bacteriocins (Balcázar *et al.*, 2006; Ringø *et al.*, 2014; Newaj-Fyzul and Austin, 2014; Ringø and Song, 2016). AMPs or bacteriocins isolated from the GI tracts of Japanese coastal fish (Sugita *et al.*, 1998) and Indian major carp (*Labeorohita*) (Giri *et al.*, 2011), which were produced by bacilli, are possible alternative antibiotics to control intestinal pathogens in fish (Ghanbari *et al.*, 2013). The mortality of fish challenged with a virulent strain of *Aeromonas salmonicida* was reduced by oral delivery of *Lactobacillus rhamnosus* (Nikoskelainen *et al.*, 2001). Because the healthy fish gut is resilient and dynamic, the microbiota composition may not be affected by bacteriocinogenic microbes, but at lower taxonomic levels, significant changes were observed.

In this post genomic era, we need greater detection of probiotics and AMPs in the reservoir of GI microbiota in fish species of economic value, and we need to integrate bioinformatics data from different levels (gene, metagenome, peptide, strain, microbiota, literature mining). We recently identified 5 AMPs in the metagenome of the grass carp GI tract (Dong *et al.*, 2017) and found lactococin 972, pediocin, aureocin-like bacteriocins and subtilosin A. To confirm strains producing them, 16S rDNA analysis was used by bacteriocinogenic microbiota. We demonstrated that many commensal microorganisms in grass carp, especially three species of *Lactococcus* (*L. raffinolactis*, *L. lactis*, *L. garvieae*), and many members of *Streptococcus*, *Bacillus*, *Lactobacillus* and *Enterococcus*, were potential probiotics for grass carp, as they were closely related to those characterized probiotic strains already used as microecologics (Mansfield *et al.*, 2010).

NGS accelerates the discovery and development of novel AMPs and potential probiotics in the GI. Traditionally,

purified bacteriocins are usually acquired in a case by case fashion (Zendo *et al.*, 2008). Now the developed genome mining bioinformatics tools, including the Bacteriocin Operon and gene block Associator (BOA; Walsh *et al.*, 2017), Hidden Markov Model (HMM) (De Jong *et al.*, 2010) and BAGEL2 (Scheffler *et al.*, 2013), are employed to discover functional AMPs or bacteriocin genes (Li *et al.*, 2016). The high throughput and trans-omic approach would be beneficial to determine the bacteriocins and bacteriocinogenic microbes that exist in fish GI. These are also the subjects of metagenomics studies. In order to assess the antimicrobial potency of a bacterial strain, the characterization of the bacteriocin-coding gene is indispensable. Bacteriocinogenic microbiota are a valuable resource for exploring potential probiotics (Więckowicz *et al.*, 2011).

Probiotics, containing living beneficial bacteria, have been widely used as animal feed additives. They can adhere to the intestinal epithelial cells, stimulate gut mucosal immune tissues and induce cellular or humoral immune responses (Guarner *et al.*, 2010; Neu, 2014). Fish intestinal microbiota have maintained a balance with the mucosa immunity. This protects fish health and inhibits colonization by foreign pathogenic strains. However, when external adverse factors like deterioration of the water quality or a decreased oxygen level happen, the balance will be upset and enable pathogens to rapidly grow, translocate and infect other fish organs. Hence, addition of probiotic microbiota to restrain proliferation of pathogens and adjust the immune functions of fish intestines is important to maintain healthy conditions. This should provide economic returns to the fish aquaculture industry and the side effects of using chemical products, including antibiotics and chemotherapeutics, could be minimized (Cerezuela *et al.*, 2013). Probiotic supplements consisting of multi-species components could be more efficient than those containing only one species or strain (Dawood *et al.*, 2016a).

Many commercial probiotic products do not provide effective or stable results. This may be due to the inappropriate selection or application of the probiotics. Many probiotic strains are isolated from the environment or endotherm rather than from a fish digestive tract. They are thus incapable of colonizing in the fish digestive tract or only capable of colonizing for a short time. The profile and function of microbiota in the fish digestive tract have not been thoroughly studied, and the composition and distribution of beneficial and pathogenic microbiota are not well known. Hence, the successful application of probiotics in fish farming has been hindered. For example, the probiotics in use may share no identical colonization sites with the targeted pathogens, and they are therefore ineffective.

The fish gastrointestinal microbiota is an important resource for screening potential probiotics for cultured fish species. We must determine the composition, distribution and function of microbiota in fish digestive tract. The

metagenomic approach is the best way to achieve this goal. We initiated the China Fish-M1K program to sequence colonizing metagenomes from 1,000 fish species. It will be completed within 5 years with help from the Chinese Academy of Fishery Sciences using a combination of NGS, GeoChip and 16S rDNA methods. Like our previous Fish-T1K Project (Sun *et al.*, 2016) and China Aquatic 10-100-1,000 Genomics Program (Liu *et al.*, 2017), the Fish-M1K Program will involve international collaboration from both academic and commercial organizations.

Concluding Remarks

Fish metagenomics is a rapidly developing field that has been advanced by high throughput sequencing technology. Community profiles of the GI microbiota in many fish have been documented, but understanding of their functions in digestion and immunity, as well as the causation of intraspecific composition variations and interspecific differences have not been clarified.

Many studies have been performed on individual fish species to characterize the profiles of GI-related microbiota. However, describing the microbiota of the more than 25,000 teleost fish species living in many different environments will require substantially more research than what has already been performed. Prior to designing strategies to manipulate the microbiota, a more in-depth understanding of how this system is organized, how it is influenced and how it affects fish health will be required. These aspects are not clearly understood in fish. It is necessary to set up a unitary and comprehensive project to normalize the fish metagenomics searches, including sampling, shipment, sample storage, analytic pipelines and data networks. This will be useful for comparison of metadata across species and geographic locations, and building an international platform for fish metagenomics research.

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