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Progress in fish gastrointestinal microbiota research

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Abstract

The gastrointestinal (GI) microbiota of vertebrates plays critical roles in nutrition, development, immunity and resistance against invasive pathogens. In the past decade, research of the GI microbiota of mammals has drastically increased our knowledge on the microbiota and their relationship with health and disease. However, our understanding of fish intestinal microbiota is limited. This review provides an overview of research on fish gut microbiota, including microbial composition, formation, factors that affect the GI microbes and characteristics of fish intestinal microbiota compared with human and mice. Further, the updated research on gnotobiotic zebrafish is elaborated and the insights gained on functions of the fish intestinal microbiota are discussed. Understanding the intestinal microbiota of fish will guide the development of probiotics, prebiotics and hopefully probiotic effectors as novel additives to improve the health of fish.

Key words: fish, function, gastrointestinal microbiota, gnotobiotic zebrafish, structure.

Introduction

Vertebrates' gastrointestinal (GI) tract is a composite microbial ecosystem housing a complex and dynamic consortium of microorganisms, which play critical roles in the nutrition and health of the host (Brugman & Nieuwenhuis 2010; Cerf-Bensussan & Gaboriau-Routhiau 2010; Viney & Riley 2014). In animal GI micro-ecosystem, there are complex and relatively stable microbial–microbial and host–microbial relationships (Hooper & Gordon 2001; Ley *et al.* 2006; Pérez *et al.* 2010; Mazmanian & Lee 2014). Recent studies are revealing that many allergic, autoimmune and metabolic diseases in modern society are associated with changes in the gut microecosystem (Neish 2009; Nagalingam & Lynch 2011). Improved hygiene and the use of food chemical additives may induce dysbiosis of the gut microbiota, impair the activity of digestive enzymes, cause damage in gut tissue and barrier and increase infiltration of bacteria and luminal toxicants (Suez *et al.* 2014; Chassaing *et al.* 2015; Zhou *et al.* 2015). In the light of this, the relationship between changes in the gut micro-ecosystem and diseases has drawn more and more attention (Qin 2015). In the past decades, research into the mammalian GI microbiota provided much insight into the structure and functions of GI microbiota. To the author's knowledge, the earliest study of the microbial communities associated with fish intestine date back to the late 1910s (Reed & Spence 1929). Since this pioneer study, numerous effort has been dedicated to describing the microbial communities present in fish (e.g. Liston 1957; Trust & Sparrow 1974; Horsley 1977; Fishelson *et al.* 1985; Austin & Al-Zahrani 1988; Cahill 1990; Sakata 1990; Ringø *et al.* 1995, 2016; Austin 2006). In their review devoted to intestinal microbiota of salmonids, Ringø *et al.* (1995) put forward the statement that 'bacterial levels in fish are substantially lower than those reported for endothermic animals such as humans'. However, this statement can be question as it is based on the use of media to cultivate bacteria and such approaches are insufficient as the cultivable communities in the GI tract of several fish species can be as low as <0.1% based on recent studies using molecular methods revealing that the GI tracts of fish harbour an enormous variety of indigenous microorganisms (Nayak 2010; Star *et al.* 2013; Romero *et al.* 2014; Zhou *et al.* 2014; Ghanbari *et al.* 2015).

At present, GI microbiota study has been conducted in a wide range of fish species, including rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpio*), Atlantic cod (*Gadus morhua*), Atlantic salmon (*Salmo salar*), Arctic charr (*Salvelinus alpinus*), grass carp (*Ctenopharyngodon idellus*), zebrafish (*Danio rerio*; Table 1).

The GI microbiota of fish has become a frontier research field. Due to the complexity of the GI microbiota, the

Table 1 Studies of fish gastrointestinal microbial communities

Fish species	References
Rainbow trout (*Oncorhynchus mykiss*)	Spanggaard *et al.* (2000), Huber *et al.* (2004), Pond *et al.* (2006), Kim *et al.* (2007), Mansfield *et al.* (2010), Desai *et al.* (2012), Navarrete *et al.* (2012)
Common carp (*Cyprinus carpio*)	van Kessel *et al.* (2011), Kuhlwein *et al.* (2013), Li *et al.* (2013a,b), Ye *et al.* (2014)
Atlantic cod (*Gadus morhua*)	Lindsay and Gooday (1985), Ringø *et al.* (2006), Reid *et al.* (2009), Star *et al.* (2013)
Atlantic salmon (*Salmo salar*)	Hovda *et al.* (2007), Ringø *et al.* (2008), Skrodenyte-Arbaciauskiene *et al.* (2008), Navarrete *et al.* (2009), Cantas *et al.* (2011), Green *et al.* (2013), Zarkasi *et al.* (2014)
Grass carp (*Ctenopharyngodon idellus*)	Tsuchiya *et al.* (2008), Han *et al.* (2010), Zhang *et al.* (2013)
Zebrafish (*Danio rerio*)	Rawls *et al.* (2004, 2006, 2007), Bates *et al.* (2006), Pham *et al.* (2008), Roeselers *et al.* (2011), Cantas *et al.* (2012), Semova *et al.* (2012), Toh *et al.* (2013), Rieu *et al.* (2014), Russo *et al.* (2015)
Grouper (*Epinephelus coioides*)	Sun *et al.* (2009)
Stickleback (Gasterosteus aculeatus)	Bolnick *et al.* (2014a,b,c)
Siberian sturgeon (*Acipenser baerii*)	Geraylou *et al.* (2013)
Catfish (Silurus asotus)	Tsuchiya *et al.* (2008), Di Maiuta *et al.* (2013)
Guppy (*Peocilia reticulata*)	Sullam *et al.* (2015)
Reef fish (*Acanthurus nigricans*, *Chlorurusn sordidus*, *Lutjanusn bohar*)	Smriga *et al.* (2010)
Antarctic notothenioid (*Notothenia coriiceps*, *Chaenocephalus aceratus*)	Ward *et al.* (2009)
Sea trout (*Salmo trutta trutta*)	Skrodenyte-Arbaciauskiene *et al.* (2008)
River trout (*Salmo trutta fario*)	Skrodenyte-Arbaciauskiene *et al.* (2006)
Senegalese sole (*Solea senegalensis*)	Martin-Antonio *et al.* (2007)
African cichlid (*Astatotilapia burtoni*, *Ophthalmotilapia ventralis*)	Baldo *et al.* (2011)

structure and function of the GI microbiota in fish have not been studied in depth, which limited the potential application of related knowledge in aquaculture (Romero & Navarrete 2006). Further research in this field will facilitate the selection of probiotics, prebiotics and chemical compounds with potentials to improve the gut homoeostasis and health of fish, which are promising alternatives of antibiotics that have been inhibited for use in food animals (Hoseinifar *et al.* 2015, 2016; Dawood & Koshio 2016). In this review, we will focus on structure and composition of fish microbiota, the influence of environmental factors on the composition of GI microbiota, as well as insights into the functions of fish intestinal microbiota gained from gnotobiotic zebrafish studies. The knowledge on fish intestinal microbiota will facilitate the development of effective strategies for manipulating GI microbial communities to promote fish health and productivity.

The GI microbiota structure

The GI microbiota composition

The distal GI tract in human houses up to 1000 distinct bacterial species and the number of bacterial cells can be up to 1×10^{14} microorganisms (Fujimura *et al.* 2010). The majority of bacterial species in a healthy human gut belong to Bacteroidetes (including *Bacteroides fragilis* and *Bacteroides thetaiotaomicron*) and Firmicutes (*Clostridia and Bacilli*) (Rajilic-Stojanovic *et al.* 2007). Proteobacteria, Actinobacteria, Fusobacteria, Cyanobacteria and Verrucomicrobia are less abundant phyla (Hsiao *et al.* 2008). In mice, up to 10^8 bacterial cells are contained in a gram of intestinal content. Similar with human, Bacteroidetes and Firmicutes are dominant phyla in the gut microbiota, with Deferribacteres, Tenericutes, Proteobacteria and Fusobacteriaas minor groups (Turnbaugh *et al.* 2008; Weldon *et al.* 2015).

In contrast to terrestrial vertebrates, aerobic, facultative anaerobic and obligate anaerobic bacteria are the principal colonizers in the GI tract of fish (Llewellyn *et al.* 2014). Studies have shown that the fish gut harbours an estimate of 10^7 to 10^{11} bacteria g^{-1} intestinal content (Nayak 2010). Aided by next-generation sequencing (NGS), studies have shown that the bacterial colonizers in fish GI tract include Proteobacteria, Fusobacteria, Firmicutes, Bacteroidetes, Actinobacteria, and Verrucomicrobia (Ringø *et al.* 2006; Desai *et al.* 2012; Li *et al.* 2013a,b; Carda-Diéguez *et al.* 2014; Ingerslev *et al.* 2014a,b). Among these, Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes and Fusobacterium are the dominant phyla. Notably, the composition of intestinal microbiota varies in different species, due to different environmental conditions and diet. For example, the dominant members of the microbiota in marine fish are facultative anaerobes, including *Vibrio*, *Pseudomonas*, *Acinetobacter*, *Corynebacterium*, *Alteromonas*, *Flavobacterium* and *Micrococcu*s (Onarheim *et al.* 1994; Blanch *et al.* 1997; Verner-Jeffreys *et al.* 2003). In contrast, the intestinal microbiota of freshwater fish species is dominated by *Aeromonas*, *Pseudomonas* and *Bacteroides* type A, with *Plesiomonas*,

Enterobacteriaceae, *Micrococcus*, *Acinetobacte*, *Clostridium*, *Bacteroides* type B and *Fusarium* as the less abundant groups (Austin 2006; Gómez & Balcázar 2008).

Establishment of GI microbiota

It is well recognized that human intestinal microbiota is seeded before birth and maternal microbiota forms the first microbial inoculum (Mackie *et al.* 1999). Following birth, the infant intestine is rapidly colonized by an array of microbes. The intestinal microbiota of newborn is characterized by low diversity and a relative dominance of the phyla Proteobacteria and Actinobacteria. With time, the microbiota becomes more diverse, in parallel with and the emergence and dominance of Firmicutes and Bacteroidetes. By the end of the first year of life, infants possess an individually distinct microbial profile, gradually forming the characteristic microbiota of an adult (Palmer *et al.* 2007). By 2–5 years of age, the microbiota fully resembles that of an adult in composition and diversity (Rodríguez *et al.* 2015). Similarly, during the birth process and rapidly thereafter, the gut of a newborn mouse is immediately colonized by microbes from the mother and surrounding environment, which mainly are facultative anaerobes. Following the uptake of diets, strict anaerobes such as Bacteroides, *Clostridium* begin to colonize, forming the characteristic microbiota of an adult mouse (Singer & Nash 2000).

In fish, there are several possible sources for the intestinal microbiota, and it is generally believed that the processes of bacterial colonization in early developing fish larvae are complex and depend upon the microbiota of: (i) eggs, (ii) the larval rearing water and (iii) the live feed. In the early development stage, fertilized eggs are released into the water. Both cultivation and ELISA studies have revealed that the gut microbiota of larvae rapidly established after hatching, and based on cultivation, the colonization of the larval intestine seems to follow a two-step pattern (e.g. Strøm & Ringø 1993; Bergh *et al.* 1994; Ringø *et al.* 1996; Ringø & Vadstein 1998), with a stable indigenous microbiota forming at the metamorphosis and post-larval stage (Eddy & Jones 2002). Fish larval uptake feeds from water through the gill and mouth prior to the complete development of GI tract. Romero and Navarrete (2006) showed that the stable microorganisms are established after first feeding stages, and its major components are acquired from the environment at hatching.

It has long been known that the surface of fish eggs is a habitat for bacterial colonization (e.g. Oppenheimer 1955; Bell *et al.* 1971; Yoshimizu *et al.* 1980; Hansen & Olafsen 1989). The first steps of interactions and colonization of progeny occur as soon as the eggs are laid, and according to Yoshimizu *et al.* (1980), the number of culturable bacteria colonizing salmonid eggs ranges between 10^3 and 10^6 bacteria g^{-1}. The major bacteria of healthy eggs are as follows: *Cytophaga*, *Flavobacterium* and *Pseudomonas* (Bell *et al.* 1971; Yoshimizu *et al.* 1980). Early studies have demonstrated that GI bacteria of non-fed marine fish larvae originate from the resident egg epiflora at the time of hatching (Olafsen 1984; Hansen & Olafsen 1989). As the larval gut is sterile at the time of hatching, it is rapidly colonized by microbiota present in the environment, as well as those originally present on the chorion (Hansen & Olafsen 1989). Moreover, the studies of Fernandez *et al.* (1996) have demonstrated that the dominant *Pseudomonas* species of the bacterial flora of yolk-sack larvae of milkfish, *Chanoschanos* (Forsskal), were similar to those of the rearing water. Additionally, several studies have revealed that, once feeding begins, the intestinal microflora was derived from the live feed ingested rather than the bacteria present in water (Muroga *et al.* 1987; Tanasomwang & Muroga 1988; Munro *et al.* 1993, 1994; Bergh *et al.* 1994; Bergh 1995; Griez *et al.* 1997).

Factors affecting GI microbiota of fish

In fish, it is well known that GI microbiota is affected by a range of factors, including host factors (e.g. genetics, gender, weight, age, immunity and intestinal motility) (Li *et al.* 2012, 2014a,b; Navarrete *et al.* 2012; Bolnick *et al.* 2014a,c; Li *et al.* 2015, 2016; Stephens *et al.* 2016), environmental factors (e.g. water, diet and medicine/antibiotics) (Sullam *et al.* 2012; Ringø *et al.* 2016; Dehler *et al.* 2017), microbial factors (e.g. adhesion capacity, enzymes and metabolic capacity) (Prakash *et al.* 2011) and displayed individual variations and day-to-day fluctuations (Sugita *et al.* 1987a, b; Sugita *et al.* 1990; Ringø *et al.* 1995; Ringø & Birkbeck 1999). In addition, a recent study revealed that the intestinal microbial communities of wild largemouth bronze gudgeon (*Coreius guichenoti*) were significant different between male and female fish (Li *et al.* 2016). Stephens *et al.* (2016) demonstrated stage-specific signatures in the zebrafish and extensive inter-individual variation. Furthermore, we elaborated the influence of fish microbiota by water and diet, which have been mostly studied as the environmental factors affecting the fish microbiota.

The influence of water

Earlier studies have indicated that microbes in water may affect the fish GI microbiota (Tanasomwang & Muroga 1988; Wang *et al.* 1993). This finding was later confirmed by Navarrete *et al.* (2009) reporting that *Pseudomonas* spp. in the gut of juvenile Atlantic salmon was derived from water influent. Wu *et al.* (2012b) also revealed that the intestinal microbiota composition of grass carp (*Ct. idellus*) resembles that in the culture water and sediment.

In water environment, water temperature and salinity are two main factors that affect fish GI microbiota. Hagi *et al.* (2004) reported that the intestinal lactic acid bacteria (LAB) composition varied with seasons in four fish species, that is silver carp (*Hypophthalmichthys molitrix*), common carp (*Cy. carpio*), channel catfish (*Ictalurus punctatus*) and deep bodied crucian carp (*Carassius cuvieri*). It was revealed that abundance of predominant LAB depended on the water temperature, irrespective of fish species. Seasonal variations in the intestinal microbiota have also been revealed in farmed Atlantic salmon (Hovda *et al.* 2012). Zarkasi *et al.* (2014) revealed that the intestinal composition of LAB within Atlantic salmon also varied with seasons. Al-Harbi and Uddin (2004) analysed the total viable counts (TVC) of bacteria in the intestine of hybrid tilapia (*Oreochromis niloticus* × *Oreochromis aureus*) cultured in earthen ponds in Saudi Arabia, and the results showed that the TVC of bacteria varied in different seasons (autumn, summer and winter). Recently, Neuman *et al.* (2016) showed that number of bacteria generally increases with water temperature, when not considering the influence of diet.

Several previous studies have revealed that the gut microbiota of freshwater and seawater fish are different (Yoshimizu *et al.* 1976a,b; Sakata *et al.* 1980; Sakata 1990; Ringø & Strøm 1994). Specification and discussion of the results are presented in the review of Ringø *et al.* (1995). More recently, Sullam *et al.* (2012) also reported that variation in fish gut bacteria composition was correlated with water salinity. The intestinal microbiota of fish from estuarine habitats appears to be more similar to that of freshwater fish, while the intestinal microbiota of fish from mixed salinity habitats more resembles intestinal microbiota of saltwater fish. However, as they used different fish from freshwater or marine water, it is difficult to evaluate the exact role of salinity in shaping the intestinal microbiota. Recently, Zhang *et al.* (2016) further investigated the gut microbiota composition of Nile tilapia reared under different salinity. The results showed that the abundance of *Devosia*, *Pseudomonas* and *Cetobacterium* increased in high salinity environment.

The influence of diet and feeding habit

As early as 1953, it was reported that fasting has influence on fish intestinal bacteria (Margolis 1953). Currently, a number of studies have demonstrated that diet could strongly influence the fish GI microbiota (Campbell & Buswell 1983; Sugita *et al.* 1987a,b; Onarheim *et al.* 1994; Ringø & Birkbeck 1999; Ringø *et al.* 2006, 2016; Uchii *et al.* 2006; Martin-Antonio *et al.* 2007; Muegge *et al.* 2011; Sullam *et al.* 2012; Xia *et al.* 2014; Ye *et al.* 2014). The GI tract of fish is colonized at an early stage and guided in new and different directions depended on diet type (Brunvold *et al.* 2007; Reid *et al.* 2009). Ingerslev *et al.* (2014a,b) examined the gut microbiota change in rainbow trout (*On. mykiss*) during the onset of first feeding, and the authors revealed that microbial abundance and diversity increased after first feeding. Furthermore, Firmicutes dominated the gut of fish fed plant source oils while Proteobacteria was the dominant phyla in fish oil fed fish, which is consistent with previous reports (Desai *et al.* 2012).

Moreover, feeding habit is also an important factor influencing GI microbial diversity, and an increasing trend in diversity was observed following the order of carnivores, omnivores and herbivores (Ward *et al.* 2009; Larsen *et al.* 2014; Li *et al.* 2014a,b; Miyake *et al.* 2015). The study of He *et al.* (2013) revealed that herbivorous grass carp (*Ct. idellus*) possessed more bacterial species than the exclusively omnivorous gibel carp and black bream and carnivorous black carp under the same rearing environment. Furthermore, feeding habit also influences the structure and composition of GI microbiota. Recently, research has reported that cellulose-degrading bacteria *Clostridium*, *Citrobacter* and *Leptotrichia* were dominant in the herbivores, while *Cetobacterium* and protease-producing bacteria *Halomonas* were dominant in the carnivores (Liu *et al.* 2016a).

The regional difference of GI microbiota

The microbial density varies in different regions of the GI tract of fish depending on the physico-chemical conditions (Zhou *et al.* 2007). Generally, a progressive increase in bacterial levels from the stomach to the posterior intestine was observed in fish (Trust & Sparrow 1974; MacDonald *et al.* 1986; Cahill 1990; Molinari *et al.* 2003). Navarrete *et al.* (2009) analysed the bacterial composition of stomach, pyloric caeca, and intestine from ten juvenile (30 g) Atlantic salmon, and the average total bacterial density was 1×10^7, 8×10^6 and 5×10^7 CFU g^{-1}, respectively. Ye *et al.* (2014) investigated the microbiota composition in the foregut and hindgut of gizzard shad (*Dorosoma cepedianum*) and Asian silver carp (*H. molitrix*). The results showed that gizzard shad hindgut samples exhibited the highest alpha-diversity indices followed by Asian silver carp foregut ($n = 15$), gizzard shad foregut ($n = 9$) and Asian silver carp hindgut ($n = 24$). Tao *et al.* (2013) investigated the microbial communities of eight parts of brown croaker (*Miichthys miiuy*) digestive tract and revealed that the intestine harbours the highest number of bacterial cells, followed by midgut (27.4%), foregut (25.2%), hindgut (22.9%), stomach (21.4%), pylorus (15.6%), proventriculus (2.2%) and oropharyngeal cavity (3%). However, there are also some early studies reporting contrary results, Austin and Al-Zahrani (1988) revealed a progressive decline in numbers of culturable aerobic bacteria along the rainbow

trout digestive tract from oesophagus to lower intestine, while Ringø and Strøm (1994) reported that the number of culturable autochthonous bacteria of posterior and distal intestine of Arctic charr (*S. alpinus* L.) was constant.

Similar with density, the microbial composition varied significantly among different GI tract regions. In tilapia (*Or. niloticus*), Molinari *et al.* (2003) revealed that *Plesiomonas shigelloides* was present in all GI regions, but the abundance was higher in the posterior gut (76%) compared to anterior gut (4.8%) and stomach (0.6%). *Aeromonas hydrophila*, *Escherichia coli* and *Flavimonas oryzihabitans* were present only in the stomach, while *Citrobacter freundii* and *Burkholderia cepacia* were detected only in the posterior gut. Studies on the autochthonous microbiota in the GI tract of adult yellow grouper (*Epinephelus awoara*) revealed that *Empedobacter* sp. PH7-1 and *Acinetobacter* sp. N15 were unique for the stomach section, while uncultured bacterium clone F6-37 and γ–*Proteobacterium* and *Acinetobacter radioresistens* Philippines-11 were only observed in intestine sections (Zhou *et al.* 2009).

Due to the differentiation of GI tract structures and functions following development, we speculate that the bacterial communities of GI tract also differentiate concurrently with differentiation digestive tract. Li *et al.* (2013a,b) analysed the GI microbiota alteration of southern catfish (*Silurus soldatovi meridonalis* Chen) during the differentiation procedures of GI tract and showed clear differentiations of GI microbiota, structures following the GI differentiation. Meanwhile, temporal discrepancy was observed for the microbiota differentiation in stomach and intestine.

The fish GI microbiota functions

Gnotobiotic zebrafish gut microbiota transplants models

In recent years, gnotobiotic models have been emerging as an excellent tool for host–microbe interaction studies (Falk *et al.* 1998; Cebra 1999; Marques *et al.* 2006; Dierckens *et al.* 2009). In 2004, Rawls *et al.* first developed protocols to establish the gnotobiotic zebrafish (*D. rerio*) model system (Rawls *et al.* 2004). Among the germ-free animal models, gnotobiotic zebrafish model system offers many advantages, such as external fertilization, high-fecundity (rapid development after hatching), small size, the organization of the gut similar to mammals, early optical transparency, as well as a wealth of genetic and genomic resources (Howe *et al.* 2013; Phillips & Westerfield 2014). These advantages made the gnotobiotic zebrafish an effective tool to exploit the functions of GI microbiota and host–microbial relationships (Table 2).

Larval zebrafish hatches from its chorion at ~3 days post fertilization (dpf), and the intestine is colonized by microbes within 3–4 dpf (Bates *et al.* 2006). At ~ five dpf, larval zebrafish begins food ingestion and metamorphosis starts at ~14 dpf. Zebrafish may be reared for up to 30 days in a sterile environment (Rawls *et al.* 2006). The rearing protocol depends on the length of the experiment, number of fish involved and equipment available. For experiments with fewer than 300 fish and lasting less than 2 weeks, fish may be reared in sterile flasks. For larger experiments (up to 1200 fish) and longer time commitments (up to 30 dpf), fish should be reared in a gnotobiotic isolator. If experiments are carried out prior to eight dpf or earlier, zebrafish need not to be fed. However, zebrafish reared for more than eight dpf will require a food source because the yolk will be depleted by then (Milligan-Myhre *et al.* 2011). Zebrafish can be fed with sterilized dry powder diets, germ-free paramecia and brine shrimp (Pham *et al.* 2008).

Role of GI microbiota in fish: gnotobiotic approaches

Gnotobiotic zebrafish model system provides opportunities for exploring the molecular foundations of host-microbial interactions, including the host–microbial metabolism and the synergy evolution of the immune system. Researches of Rawls *et al.* (2004) demonstrated that microbiota in fish can regulate the expression of 212 genes, in which some were related to stimulation of epithelial proliferation, promotion of nutrient metabolism and innate immune response. In accordance, the absence of GI microbiota in fish may lead to impaired physiological functions, such as intestinal epithelial cell dysfunction, compromised nutrient absorption, metabolism and weaker immune responses (Table 3).

Role of GI microbiota in epithelial renewal

Studies in gnotobiotic zebrafish clearly demonstrated a role for the microbiota in stimulating rates of intestinal cell proliferation during normal development (Rawls *et al.* 2004, 2006). Cheesman *et al.* (2011) reported that cell proliferation in the developing zebrafish intestine is stimulated both by the presence of the resident microbiota and by the activation of Wnt signalling, which induce cytoplasmic accumulation of β-catenin. It is suggested that the resident intestinal microbiota enhances the stability of β-catenin in intestinal epithelial cells and promotes cell proliferation in the developing vertebrate intestine.

Role of GI microbiota in nutrition

In fish, several reviews have hinted that the GI microbiota plays a crucial role in nutrition (e.g. Ray *et al.* 2012; Clements *et al.* 2014). Ray *et al.* (2012) provide an overview information of the enzyme-producing microbiota from fish GI tract, and extensive range of enzymes (e.g. amylase, cellulase, lipase, proteases, chitinase and phytase) produced by GI bacteria might have a significant role in digestion. On

Table 2 Studies of gnotobiotic zebrafish gut microbiota transplants

Bacterial association of gnotobiotic zebrafish	Processing period (dpf)	Biomarkers	Zebrafish husbandry	References
Aeromonas hydrophila (ATCC 35654) and *Pseudomnas aeruginosa* (strain PA01) were grown overnight under aerobic conditions in tryptic soy broth (TSB) at 30°C and in nutrient broth at 37°C, respectively. Add to beaker containing 3-dpf gnotobiotic zebrafish at final concentrations of 10^4 CFU mL^{-1} of sterile GZM	3–6	S-phase cells, and 212 genes related to epithelial proliferation, nutrient metabolism, Xenobiotic metabolism and innate immune responses	Temperature: 28.5°C; equipment: sterile isolator; density: At 14 dpf, about 0.4 individual mL^{-1} of static water, at 28 dpf, about 0.03 individual mL^{-1}; light cycle: 14 h; feeding: At 3dpf, zebrafish were fed with rotifers (Aquatic Biosystems); at 14 dpf, fed with brine shrimp (Aquafauna Bio-Marine); at 28dpf, advanced to a diet of brine shrimp, TetraMin flakes (Tetra), and Hikari micropellets (Hikari)	Rawls *et al.* (2004)
Caecal contents were pooled from adult CONV-R Swiss-Webster female mice under aerobic conditions, diluted 1:1200 in PBS and added directly (1:100 dilution) to GZM containing 3 dpf gnotobiotic zebrafish (final density: 10^2 CFU mL^{-1} aerobic culture; 10^3 CFU mL^{-1} anaerobic culture	3–6	Lipid metabolism biomarkers: fasting-induced adipose factor (*fiaf*), carnitine palmitoyltransferase 1a (*cpt1a*), trifunctional enzyme hydroxyacyl CoA dehydrogenase/3-ketoacyl CoA thiolase/enoyl CoA hydratase α (*hadha*); innate immune response biomarkers: serum amyloid a (*saa*), myeloperoxidase (*mpo*), complement component factor b (*bf*); gut epithelial cell renewal biomarker: hymidylate kinase (*dtymk*), minichromosome maintenance genes (*mcm2*, *mcm3*, *mcm5*, *mcm6*), origin-recognition complex subunit 4 (*orc4l*), proliferating cell nuclear antigen (*pcna*)	Temperature: 28.5°C; equipment: sterile isolator; density: At 14 dpf, about 0.4 individual mL^{-1} of static water, at 28 dpf, about 0.03 individual mL^{-1}; light cycle: 14 h; feeding: At 3dpf, zebrafish were fed with rotifers (Aquatic Biosystems); at 14dpf, fed with brine shrimp (Aquafauna Bio-Marine); at 28dpf, advanced to a diet of brine shrimp, TetraMin flakes (Tetra) and Hikarimicropellets (Hikari)	Rawls *et al.* (2006)
An unfractionated gut microbiota harvested from CONV-R adult C32 zebrafish; *P. aeruginosa* PA01 containing pMF230; *Escherichia coli* MG1655 containing pRZT3; wild- type *P. aeruginosa* PAK or the isogenic △*fliC* strain carrying pSMC21; isogenic wild-type or mutant *P. aeruginosa* PAK strains without plasmids; all of them were grown overnight at 37°C in Luria–Bertani broth before inoculation. Microbes were introduced at a density of 10^4 CFU mL^{-1} GZM	3–6	*Saa*, *mpo*, *fiaf*, *cpt1a*	Temperature: 28.5°C; equipment: air incubator; density: 1.3 individuals mL^{-1} GZM; Light cycle: 14 h in sterile vented tissue culture flasks; Feeding: at 3 dpf, fish were fed daily with a sterilized solution containing 0.1 mg of ZM000 fish food (ZM Ltd., Winchester, United Kingdom) per millilitre of GZM, and 90% water change was performed before each daily feeding	Rawls *et al.* (2007)

Table 2 (continued)

Bacterial association of gnotobiotic zebrafish	Processing period (dpf)	Biomarkers	Zebrafish husbandry	References
Aeromonas veronii biovarsobria and *Pseudomonas fluorescens* were cultured from homogenized zebrafish larvae, injected into flasks of gnotobiotic 5dpf larvae to a final concentration of 10^6 CFU mL^{-1}	5–8	Intestinal alkaline phosphatase, glycoconjugate (Galα1, 3Gal)	Equipment: tissue culture flasks; feeding: without feeding during experimental period	Bates *et al.* (2006)
Lactobacillus strains were grown at 30°C in MRS medium for 24 or 48 h, pelleted and washed once in water and then diluted at a final concentration of 2.10^7 CFU mL^{-1}	4–9	TNF-α, IL-1, IL-22, IL-10	Temperature: 28°C; equipment: fish were kept in vented cap culture flasks or 24-well microtiter plates in autoclaved mineral water (Volvic); feeding: at 3–4 dpf, fish were fed every 2 days with axenic *Tetrahymena thermophila*; after 10 dpf, larvae were fed with *Artemia salina*	Rendueles *et al.* (2012)
Lactobacillus paracasei and *Eubacterium limosum* were grown in fastidious anaerobe agar media supplemented with 5% defibrinated sheep blood (Hemostat Laboratories, Dixon, CA, USA), suspended in sterile, pre-reduced to attain an approximate density of 10^9 CFU mL^{-1}. Static immersion: the community was added to zebrafish larvae in multiwell plates at a final density of 10^6 CFU mL^{-1}; Microinjection: a standard capillary needle was loaded with the defined community at a density of 10^9 CFU mL^{-1}, and each fish was inoculated with 10–100 nL of culture	5–12		Temperature: 28.5°C; light cycle:14 h; larvae could survive to a maximum of 13 dpf without food	Toh *et al.* (2013)
Lactobacillus casei ATCC334 were grown for 24 h in MRS medium at 30°C, then pelleted and washed once in sterile water, diluted at a final concentration of 10^7 CFU mL^{-1}	5–6	TNF-α, IL-1β	Temperature: 28°C; light cycle: 14 h; feeding: fish were fed twice per day with A. salina nauplii and dried flake food (Europrix)	Rieu *et al.* (2014)
Lactobacillus plantarum Lp90, *L. plantarum* B2 and *Lactobacillus fermentum* PBCC11.5 were fluorescently tagged by transfer of the pRCR12 plasmid, grown overnight on MRS broth containing chloramphenicol at 10 μg mL^{-1} and 0.05% cysteine at 37°C, washed three times in PBS and then diluted to 10^7 CFU mL^{-1}	4–7	mCherry protein	Temperature: 27°C; equipment: 60-L tanks with aerated freshwater; feeding: pellet-formulated diet (Gemma Micro 300, Skretting); light cycle: 12 h	Russo *et al.* (2015)

Table 3 Host transcriptional responses to the microbiota

Host transcriptional responses	References
Decreasing expression of genes involved in epithelial proliferation, that is thymidylate kinase (*dtymk*), minichromosome maintenance genes (*mcm2*, *mcm3*, *mcm5*, *mcm6*), origin-recognition complex subunit 4 (*orc4l*), proliferating cell nuclear antigen (*pcna*), ribonucleotide reductase subunit M2 (*rrm2*)	Rawls *et al.* (2004, 2006), Cheesman *et al.* (2011)
Regulation expression of genes involved in cholesterol metabolism and trafficking, that is fasting-induced adipose factor (*fiaf*), carnitine palmitoyl transferases (*cpt1a*, *cpt2a*), trifunctional enzyme hydroxyacyl CoA dehydrogenase/3-ketoacyl CoA thiolase/enoyl CoA hydratase a (*hadha*) (up-regulated); farnesyl-diphosphate synthetase (*fdps*), apolipoprotein B (*apob*), cholesterol 7α-hydrolase (down-regulated)	Rawls *et al.* (2004, 2006), Bates *et al.* (2006)
Decreasing expression of genes involved in innate immune responses, that is serum amyloid a1 (*saa1*), C-reactive protein (*crp*), complement component 3 (*C3*), angiogenin 4 (*ang4*), suppressor of cytokine signalling 3 (*socs3*), myeloperoxidase (*mpo*), complement component factor b (*bf*), glutathione peroxidase	Rawls *et al.* (2004, 2006, 2007), Kanther *et al.* (2011)
Reducing the expression of intestinal immune cells and factors, that is intestinal neutrophils, IL-1β	Bates *et al.* (2007), Galindo-Villegas *et al.* (2012)
Down-regulate intestinal alkaline phosphatase activity, up-regulate Galα 1, 3 Gal expression and decrease the number of goblet cells and enteroendocrine cells	Bates *et al.* (2006, 2007)

the other hand, GI microbiota may stimulate nutrient material uptake, especially in cholesterol metabolism and trafficking (Rawls *et al.* 2004). Bates *et al.* (2006) reported that germ-free zebrafish larvae failed in uptake of protein macromolecules, with a lower level of farnesyl-diphosphate synthetase and apolipoprotein B (apoB) compared with conventional larvae. However, all these traits were reversed by addition of microbiota later in development. Moreover, recent studies have revealed that the community of microorganisms in the intestine regulates fat storage. Semova *et al.* (2012) revealed that colonization with microbiota stimulates fatty acid (FA) uptake in the intestinal epithelium, resulting in accumulation of lipid droplet (LD) in enterocytes and increased accumulation of dietary FA in extraintestinal tissues. To determine how microbes control fat storage, Camp *et al.* (2012) took advantage of the zebrafish model to define the expression of a circulating inhibitor of lipoprotein lipase called angiopoietin-like 4 (Angptl4/Fiaf). The results showed that zebrafish *angptl4* gene is specifically suppressed in the intestinal epithelium upon colonization with a microbiota. This study provides a new mechanism insight into how intestinal microbes influence fat storage.

Role of GI microbiota on immunity

The gut immune system, also named gut-associated lymphoid tissues (GALT), not only protects GI tract from infectious agents but also regulates immune system in the GI tract. The GI microbes play a critical role in the development and maturation of GALT, which in turn mediate a variety of host immune functions.

Bates *et al.* (2006, 2007) reported that gut microbiota induces the expression of intestinal alkaline phosphatase (IAP) in zebrafish, which functions to dephosphorylate LPS associated with gut bacteria, thereby modulating intestinal inflammation in response to the resident microbiota. Kanther *et al.* (2011) reported that colonization of germ-free transgenic zebrafish with a commensal microbiota induced dynamic temporal and spatial patterns of NF-κB transcriptional activation. Galindo-Villegas *et al.* (2012) showed that colonization by commensals in newly hatched zebrafish primes neutrophils and induces several genes encoding proinflammatory and antiviral mediators, increasing the resistance of larvae to viral infection.

Methods used to assess the bacterial communities

It is generally accepted that the GI microbiota of fish plays an important role in nutrition and immunity. In-depth knowledge of the structure and relationships between GI microbiota and their host fish can provide insight into both the function and dysfunction of the host organism. For this purpose, a comprehensive and detailed view of fish GI microbiota, including both taxonomic composition and genetic potential, is a prerequisite. In the past few decades, most of the studies on the intestinal microbiota of fish were carried out by conventional culture-dependent methods (Cahill 1990; Ringø & Gatesoupe 1998). However, the fish GI microbiota has been reported to be of low cultivability; it only represent <0.1% of the total microbial community in the GI tract of some fish species (Romero & Navarrete 2006; Navarrete *et al.* 2009; Zhou *et al.* 2014; Ghanbari *et al.* 2015). Recently, with the development of DNA sequencing technologies and bioinformatic analysis, a wide range of molecular ecology methods based on the 16S and 23S rRNA genes have become more commonly used. These

culture-independent molecular-based techniques have substantially improved our knowledge of the structure and diversity of bacterial communities within the gut of fish (Austin 2006; Kim *et al.* 2007; Namba *et al.* 2007; Wu *et al.* 2010, 2012a,b; Lan & Love 2012; Larsen *et al.* 2013; Zarkasi *et al.* 2014; Zhou *et al.* 2014; Parma *et al.* 2016; Ringø *et al.* 2016). Zhou *et al.* (2014) reviewed the methodological approaches which have been used in evaluations of fish gut microbiota. The main methodologies utilized have depended on the aim of the studies: (i) clone libraries have been used to identify the microbiota composition; (ii) finger printing methods such as denaturing gradient gel electrophoresis (DGGE) and temporal temperature gradient electrophoresis (TTGE) have been used to analyse microbial community structure and diversity; (iii) quantitative real-time PCR (qPCR) and fluorescent *in situ* hybridization (FISH) have been used to determine the abundance of particular taxa or total microbial levels; and (iv) FISH and immunohistochemistry have been used to assess bacterial–host interactions at the mucosal brush border.

Recently, rapid and low-cost approaches for NGS technologies have been introduced to study the composition and genetic potential of densely populated microbial communities such as gut microbiota (Foster *et al.* 2012). Ghanbari *et al.* (2015) have highlighted the potential of NGS platforms for the analysis of fish gut microbial ecology. The promising results produced by rapid, low-cost and reliable NGS techniques will continue to improve our knowledge of the bacterial community profiles of fish GI microbiota.

Conclusion

Aided by NGS technologies, research on fish intestinal microbiota has drastically increased in the past few years. Although more insights have been gained, many questions remained to be elucidated in this field. We discussed differential composition and density of bacteria in different regions of the fish GI tract. Factors responsible for such differences, either biotic factors such as nutrition or immunity or abiotic ones (pH, O_2), deserve further investigation. The fish microbiota is characterized by large diversity among individuals. The impact of such diversity on the overall function of the microbiota, such as the digestion or effect of diets and additives, is an interesting area for fish microbiota research.

Until now, most of the studies on fish intestinal microbiota were descriptive and only concerned the composition of the microbial community. The functional studies in gnotobiotic zebrafish model have mainly focused on the functions of the whole microbiota. Further works are warranted to investigate the functions of subpopulations in the microbiota and ultimately the functions to the species level. This will facilitate the development of novel probiotics for fish use and guide more rational design of prebiotics targeting the beneficial subgroups in the intestinal microbiota. Moreover, administration of the probiotic effector ingredients might be an alternative way to obtain the health benefits, especially considering the possible risks associated with probiotic administration suspension in fish (Liu *et al.* 2016b). Anticipatedly, further elucidation of the fish intestinal microbiota and host–microbiota interactions would lead to the development of more refined and efficacious microbiota-intervention strategies to improve the health and performance of fish.

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Conflict of interest

The authors have declared no conflict of interest.

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