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Review

Gnotobiotic models: Powerful tools for deeply understanding intestinal microbiota-host interactions in aquaculture

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ABSTRACT

Intestinal microbiota plays profound roles in host nutrition, physiology, and evolution. The development of DNA sequencing technologies has increased dramatically research on fish intestinal microbiota. However, most studies conducted so far have focused on the microbial structure and diversity. Studies targeting the exact function of commensal microbes in aquatic animals are still scarce, which limits the application of microbiota related knowledge in aquaculture. Gnotobiotic models (animals cultured in axenic conditions or with defined microbial lineages) are excellent tools for identifying the molecular interactions between intestinal microbiota and host, which drive studies of microbiota from correlation to causality in mammals. In recent decades, gnotobiotic fish models have been established and applied in aquaculture research. This review summarizes the colonization conditions in gnotobiotic zebrafish model and its application in understanding intestinal microbiota-host interactions in aquaculture. Furthermore, methods and research progress on other gnotobiotic models including freshwater and marine fishes, molluscs and crustaceans are also discussed. Application of gnotobiotic models in aquaculture has deepened our understanding of the relationship between the host and intestinal microbiota, which will facilitate the modulation of intestinal microbiota for production of healthy animals and sustainable development of aquaculture.

1. Introduction

Aquaculture, as the world's fastest growing food production sector, has contributed nearly half of the global food fish consumption for more than four decades (Tveteras et al., 2012). How to produce healthy and safe aquatic products has attracted more and more attentions. Animals are colonized at birth by complex and dynamic communities of microorganisms which take part in metabolism, development, immune regulation process, therefore, the function of symbiotic microbiota should not be ignored when we evaluate and manage the health of aquatic animals (Marques et al., 2006; O'Hara and Shanahan, 2006).

Disease outbreaks caused by intensive culture have produced serious economic losses (Ribas and Piferrer, 2014). Historically, antibiotics were used to prevent disease in commercial aquaculture facilities. However, recent concerns over the use of antibiotics have led to the development of natural immunostimulants, such as probiotics or prebiotics, which are supposed to regulate intestinal microbiota and enhance gut health, consequently improve the resistance against pathogens of aquatic animals (Haygood and Jha, 2018; Ran et al., 2015). Moreover, due to the high cost of fishmeal, the current aquaculture nutrition approaches have concentrated on replacing fishmeal with plant protein-based diets, which triggers intestinal inflammation, consequently generates negative influence on the development of healthy aquaculture (Booman et al., 2018; Ulloa et al., 2014). Multiple approaches have been taken to increase the utilization efficiency of plant protein and decrease the adverse effects, such as addition of essential amino acids (Jiang et al., 2015) or probiotics (Batista et al., 2016). However, due to the diversity of new protein ingredients and complex interactions among the genome, intestinal microbiota and diet, the effect of these strategies is still controversial and the understanding of mechanism underlying the whole digestion process is still limited (Ulloa et al., 2014). An essential part of that understanding should be provided by research involving the host-microbiota interactions (Rawls et al., 2004).

Characterization of the intestinal microbial communities has been explored in numerous gut microbial communities (Eckburg et al., 2005; Ley et al., 2008). Although aquatic animals represent the greatest taxonomic and ecological diverse group, the understanding of the

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Table 1
Studies based on gnotobiotic zebrafish.

Main idea	Method	Time	Bacteria	Concentration	Diet	Reference
To investigate the role of intestinal microbiota in the nucleotides-induced immune stimulatory effect.	Monoassociation Conventionalization	8dpf 3dpf	*Aeromonas veronii* Gut microbiota of zebrafish	10^7 CFU/mL 10^6 CFU/mL	Sterile diet	(Guo et al., 2019)
Colonization by commensals primes neutrophils and induces proinflammatory related genes.	Conventionalization	2dpf	Culture medium		Not mentioned	(Galindo-Villegas et al., 2012)
A key role for adhesion in protection by probiotic bacteria.	Monoassociation	4dpf	37 potential probiotic bacteria	2×10^7 CFU/mL	Sterile autoclaved fish food powder.	(Rendueles et al., 2012)
Relationship between the microbiota and innate immune response.	Monoassociation; Di-association; Tri-association	4dpf	*Aeromonas, Vibrio, and Shewanella*	10^6 CFU/mL	Not mentioned	(Rolig et al., 2015)
To investigate the functions of Mmp-9 in response to infection by L. *monocytogenes*.	Monoassociation (Immersion & microinjection)	5dpf 26hpf	*Listeria monocytogenes* or *Listeria innocua*	10^8 CFU/mL 100 CFU per fish	Not mentioned	(Shan et al., 2016)
Osmotic stress regulates the activation of immunity and host protection in newly hatched embryos.	Monoassociation (Microinjection)	1hpf	*Salmonella enterica* serovar *Typhimurium*	10 CFU/mL	Not mentioned	(Galindo-Villegas et al., 2016)
To reveal mechanisms involved in protection by two non-Saccharomyces yeasts.	Monoassociation	4dpf	*Debaryomyces hansenii* and *Yarrowia lypolitica 242*	10^4 to 10^7 CFU/mL	Not mentioned	(Caruffo et al., 2016)
Endogenous microbiota establish the normal homeostatic level of neutrophils in the intestine.	Monoassociation (Immersion & Microinjection)	5dpf 7dpf	*Aeromonas veronii, Pseudomonas fluorescens, Streptococcus* and *Staphylococcus* Filter-sterilized solutions of LPS	10^6 CFU/mL 3 to 250 μg/mL	Not mentioned	(Bates et al., 2007)
To examine the processes of microbial infections and host–pathogen interactions *in vivo*	Monoassociation microinjection	5dpf one-cell stage (brain) 26hpf(brain ventricle)	*Listeria monocytogenes* and *Listeria innocua*	10^{10} CFU/ml 11 CFU /embryo 11 to 1100 CFU per fish	Not mentioned	(Shan et al., 2015)
Effects of essential oils were mediated by the intestinal microbiota.	Conventionalization	3dpf	Intestinal bacteria of Nile tilapia	10^6 CFU/mL	Not mentioned	(Ran et al., 2016)
Anti-infective effect of EPSP from *Lactobacillus casei* BL23	Monoassociation	3dpf	17 potential probiotic bacterial strains	1×10^7 CFU/mL	Not mentioned	(Qin et al., 2017)
NF-κB activated by commensal microbiota.	Monoassociation Conventionalization	3dpf 3dpf	*Pseudomonas aeruginosa* A conventional zebrafish microbiota	10^4 CFU/mL 10^4 CFU/mL	Autoclaved ZM000 fish food	(Kanther et al., 2011)
Immune pathways elicited by intestinal microbiota.	Conventionalization Monoassociation	3dpf	Culturing media *Chryseobacterium* *Exiguobacterium*	4×10^3 CFU/mL 2×10^6 CFU/mL 12×10^6 CFU/mL	Not mentioned	(Koch et al., 2018)
Effects of the microbiota on behavior and stress.	Conventionalization	6dpf	Conventional fish water		Not mentioned	(Davis et al., 2016)
To investigated whether early life microbial disruption alters neurodevelopment and behavior in larval zebrafish.	Conventionalization Monoassociation	1,3,6,9dpf 1dpf	Conventional fish water *Aeromonas veronii* or *Vibrio cholera*	10^2 to 10^6 CFU/mL	Autoclaved ZM000 fish food	(Phelps et al., 2017)
To identify the role of microbiota in the intestinal cell differentiation.	Conventionallization Mono-association	5dpf	Culturing media *Aeromonas veronii, Pseudomonas fluorescens*	10^3 CFU/mL 10^6 CFU/mL	Filter-sterilized solutions	(Bates et al., 2006)
To identify the role of bacteria in epithelial cell proliferation	Monoassociation	4 or 6 dpf	*Aeromonas veronii*	10^5 to 10^6 CFU/mL	Not mentioned	(Cheesman et al., 2011)
To discover how *Vibrio cholerae* invade a host intestine.	Monoassociation	5dpf	*Vibrio Cholerae* *Aeromonas ZOR0001, Vibrio ZWU0020*	10^7 CFU/mL 10^6 CFU/mL	Not mentioned	(Logan et al., 2018)
Pathogenesis of *Aeromonas veronii*.	Monoassociation	4 or 5 dpf	*Aeromonas veronii, Aeromonas hydrophila*	10^7 to 10^8 CFU/mL	Not mentioned	(Ran et al., 2018)
Pathogenesis of *V. anguillarum*.	Monoassociation	5dpf	*Vibrio anguillarum*	10^8 CFU/mL	Not mentioned	(Oyarbide et al., 2015)
Factors contributing to bacterial adaptation in gut.	Monoassociation	4 or 5dpf	*Aeromonas veronii*	10^6 CFU/mL	No feeding	(Robinson et al., 2018)
To explore the effect of commensal yeasts on the structure and diversity of fish gut microbiota.	Monoassociation	2dpf	*Debaryomyces sp.*or *Pseudozyma sp.*	2×10^5 CFU/mL	Not mentioned	(Siriyappagouder et al., 2018)
Colonization ability of lactic acid bacteria.	Monoassociation	4dpf	*Lactobacillus plantarum* or *Lactobacillus fermentum*	10^7CFU/mL	Not mentioned	(Russo et al., 2015)
To track bacterial population sizes in different colonization regimes.	Monoassociation	5dpf	*Aeromonas veronii* or *Vibrio sp.*	10^5 CFU/mL	Not mentioned	(Stephens et al., 2015)
Bacterial cohesion predicts spatial distribution	Monoassociation	4dpf	Seven bacterial symbionts	10^6 CFU /mL	Germ-free *Tetrahymena thermophila*	(Schlomann et al., 2018)

(*continued on next page*)

Table 1 (*continued*)

Main idea	Method	Time	Bacteria	Concentration	Diet	Reference
The spatial and temporal dynamics of these microbial communities	Monoassociation	5dpf	*Aeromonas veronii*	10^6 CFU/ mL	Not mentioned	(Jemielita et al., 2014)
Establishment of gnotobiotic zebrafish model.	Conventionalization Monoassociation	3dpf	Culturing media *Aeromonas hydrophila* or *Pseudomonas aeruginosa*	10^4 CFU/mL 10^4 CFU/mL	Autoclaved ZM000 fish food	(Rawls et al., 2004)
The growth-promotion effect of nucleotides is related to microbiota	Conventionalization	3dpf	Intestinal bacteria from zebrafish	10^6 CFU/mL	Sterilized microparticulate diet	(Guo et al., 2017)
Intestinal microbiota promotes FA absorption.	Conventionalization Monoassociations	3dpf 3dpf	Zebrafish gut microbiota *Exiguobacterium sp.*, *Chryseobacterium* sp. or *Pseudomonas sp.*	10^4 CFU/mL	Irradiated artificial diet	(Semova et al., 2012)
Influence of microbiota on of Angptl4 transcription	Conventionalization	3 dpf	Zebrafish gut microbiota	10^4 CFU/mL	Autoclaved ZM000 fish food	(Camp et al., 2012)
The Hepatotoxicity of Palmitic Acid Involves the Intestinal Microbiota	Conventionalization	5–6,10 dpf	Zebrafish gut microbiota	10^6 CFU/mL	Sterilized Low-fat, high-fat diet or Palmitic Acid	(Ding et al., 2018)
The utility of gnotobiotic zebrafish to identify bacterial genes that affect these processes, and assessing the impact of these genes on host-microbial interactions.	Monoassociation	3dpf	*Pseudomonas aeruginosa*	10^4 CFU/mL	Autoclaved ZM000 fish food	(Rawls et al., 2007)
Microbiota-induced Serum amyloid A suppresses systemic neutrophil activation	Comparing germ-free zebrafish (GF) with conventionally rasied zebrafish (CV)				Autoclaved ZM000 fish food	(Murdoch et al., 2019)

composition and function of intestinal microbiota in fish has lagged behind that of terrestrial animals (Clements et al., 2014). The development of 16S rRNA gene-based approach with a next-generation sequencing approach has increased studies on the relationship between the intestinal microbiota and aquatic animals (Ahmed et al., 2019; Clements et al., 2014; Chen et al., 2019; Collins et al., 2012; Franzenburg et al., 2013;Lasa et al., 2019; Milligan-Myhre et al., 2011; Wong and Rawls, 2012). To date, intestinal microbial community has been investigated in a wide range of aquatic animals, including zebrafish (Stephens et al., 2015; Wong et al., 2015), Nile tilapia (Ran et al., 2015), rainbow trout (Ingerslev et al., 2014), grass carp (Wu et al., 2012), Atlantic cod (Star et al., 2013), Atlantic salmon (Navarrete et al., 2008), as well as shrimps (Rungrassamee et al., 2014; Zhang et al., 2014b), crabs (Zhang et al., 2016), *Euprymna scolopes* (Collins et al., 2012), Hydra (Franzenburg et al., 2013) and other Aiptasia (Ahmed et al., 2019). However, most of these studies have mainly focused on the microbial composition and diversity. Our understanding of the intestinal microbiota function in aquatic animals is still limited, which restricts the application of related knowledge in aquaculture (Romero and Navarrete, 2006).

Microbiome-wide associations are moving beyond correlations and beginning to address causation in mammals (Surana and Kasper, 2017), which not only implies the function of specific microbes in improvement of host metabolic health, but also affords microbiome-targeted dietary interventions (Zhao et al., 2018). Similar to mammals, the identification of intestinal microbiota function will constitute a new frontier in the field of aquaculture nutrition and health, although it is still in its infancy (Haygood and Jha, 2018; Wang et al., 2018).

Gnotobiotic models, which refer to animals cultured in axenic conditions or with defined microbial lineages, have been widely used to identify the function of intestinal microbiota in a variety of animals, including mice, rats, guinea pigs, goats and chickens (Pham et al., 2008). Gnotobiotic models have been applied in aquaculture animals research since 1940's (Gee and Sarles, 1941) and multiple gnotobiotic aquatic animal models have been established (Marques et al., 2006). The development of the sterile and colonization methods has made gnotobiotically grown aquatic animal models as popular for studying intestinal microbiota function. In fact, the application of gnotobiotic models has facilitated our understanding that intestinal microbiota is involved in nutrients harvesting (Semova et al., 2012), intestinal epithelial cell proliferation (Rawls et al., 2004), neurobehavioral development (Phelps et al., 2017) and immune regulation in fish (Kanther et al., 2011). However, further studies are needed to improve our understanding on gnotobiotic models establishment in other aquatic animals, including the appropriate method and time to colonize the germ-free models and the application of gnotobiotic models in aquaculture by utilizing the development of “omics” technologies (Martin and Krol, 2017). This review further provides methods and research progress on gnotobiotic models in other aquatic animals and furnishes challenges of gnotobiotic models in aquaculture perspective.

2. Gnotobiotic zebrafish model

Zebrafish (*Danio rerio*) is a freshwater teleost of the family *Cyprinidae* and is a model organism in many research fields due to their large brood size, high fecundity, external fertilization, rapid external development, optical transparency and a wealth of genomic information (Leulier et al., 2017; Pham et al., 2008). Zebrafish have been applied in the research related to reproduction, development, toxicology, nutrition and genetics (Grunwald and Eisen, 2002). It has been recommended by Ribas and Piferrer (2014) that a good fish model for aquaculture should: 1) has the basic biological characteristics shared by the most important cultured species, 2) exhibits similar physiological response to cultured fish, 3) has a short life span and easy to breed and 4) has many resources to deepen the research in most area. Zebrafish fulfil to a large extent on most of the above conditions than any other

species.

As might be expected, due to its appropriate qualities, Rawls et al. (2004) first established germ-free zebrafish model and published the protocol for generating and colonizing gnotobiotic zebrafish (Pham et al., 2008). This model opened new era on gnotobiotic science in aquatic animals and facilitated the application of zebrafish as a model organism in microbiota related researches (Pham et al., 2008; Melancon et al., 2017). Until now, gnotobiotic zebrafish models have been applied in the research related to immune regulation (Koch et al., 2018), growth condition evaluation (Guo et al., 2017), pathogenesis identification (Oyarbide et al., 2015), nutrients absorption (Semova et al., 2012), as well as behavior development (Phelps et al., 2017). Gnotobiotic zebrafish is the most mature model for revealing the function of intestinal microbiota in aquaculture, and the method to produce the axenic zebrafish has been fully stated (Pham et al., 2008; Melancon et al., 2017). However, the colonization conditions for gnotobiotic zebrafish models vary among studies, which may cause confusion to the new researchers in this field. Therefore, we summarized the colonization conditions, including the colonization mode, inoculation time point and microbial load (Table 1). A critical overview of the findings obtained by using gnotobiotic zebrafish is also provided for fully understanding the application of this model.

3. Colonization of germ-free zebrafish

3.1. Modes of colonization

One of the methods used for colonization is harvesting intestinal microbiota contents from other fish and adding them into the culture media of germ-free zebrafish, which is referred as conventionalization (Pham et al., 2008; Guo et al., 2019). This inoculum contains microbiota together with feces or intestinal contents, representing the microbial community normally encountered by zebrafish (Pham et al., 2008; Guo et al., 2019). Colonizing germ-free zebrafish by inoculating microorganism in culture media is a convenient method, but it is not easy to accurately monitor or control the route or timing of microbe delivery in immersion method. Direct microinjection has also been used for microbe colonization. For microinjection, larvae are anaesthetized with Tricaine, and then a standard capillary needle is employed to inoculate about 10 to 100 nL of bacterial culture either through the anal pore or directly into the developing intestinal tract through the body wall (Toh et al., 2013). As susceptible and microscopically visible infection models, bacteria are injected *via* yolk sac, brain ventricle and blood island for constructing the infection models in zebrafish (Shan et al., 2015; Shan et al., 2016). Direct injection through the abdominal wall may cause tissue damage, and therefore, microgavage was conducted to introduce microbes into the intestine. Microgavage utilized embryo microinjection and stereomicroscopy equipment and gavage can be performed at a rate of 7–10 fish per minute (Cocchiaro and Rawls, 2013). Microinjection and microgavage are both labor intensive, but result in a targeted delivery of microbes without escapees of microbes into the medium (Toh et al., 2013). Furthermore, microgavage permit the loading of higher concentration of microbes which may be lethal to fish when exposed by immersion (Cocchiaro and Rawls, 2013).

The use of conventionalization as a mode of colonization only allows the evaluation of the effect of total microbial community. For studies which are interested in detecting the function(s) of a specific microorganism, mono-association should be used. Unlike conventionalization, mono-association means a single microbial species is used to colonize fish. In order to detect the function of a combination of microbes, Di-association or Tri-association may also be used for colonization (Rolig et al., 2015).

3.2. Time of colonization

Three days post fertilization (dpf) and 5 dpf are always selected as appropriate colonization time because zebrafish normally hatch from their protective chorions at 3 to 4 dpf, while begin feeding by 5 dpf (Pham et al., 2008). For example, in order to determine whether the intestinal microbiota mediated the nucleotides-induced immune or growth-promoting effect, germ-free zebrafish were colonized at 3 dpf with the intestinal content of zebrafish (Guo et al., 2017; Guo et al., 2019). Jemielita et al. (2014) colonized germ-free larval zebrafish at 5 dpf with enhanced green fluorescent protein (EGFP)-expressing *Aeromonas veronii* to explore the dynamics of early microbial colonization of the zebrafish gut. Notably, zebrafish larvae at 1 dpf have also been used for colonization when the effect of early life microbial disruption is evaluated (Phelps et al., 2017). Zebrafish larvae younger than 1 dpf were also selected when microinjection was employed for colonization (Shan et al., 2015; Galindo-Villegas et al., 2016; Shan et al., 2016). These studies indicate that although 3 dpf and 5 dpf of germ-free zebrafish larvae are more often used, time for colonization also depends on the research target and colonization modes.

3.3. Microbes concentration

Considering that exposing fish to high microbe titers could cause fish mortality, while low titers may not elicit enough responses, microbial concentration must be considered before the experiment (Pham et al., 2008). The initial idea to determine the concentration of microbes is based on the knowledge of the microbial concentration in recirculating media or related research (Pham et al., 2008). We summarized the published studies and found that 10^3 to 10^6 CFU/mL microbes were the normally used concentrations for colonization in nutrition or metabolism related researches (Guo et al., 2017; Cheesman et al., 2011; Rawls et al., 2004). However, concentrations from 10^7 to 10^8 CFU/mL or even higher titer of microbes have also been used for some pathogenicity or immune regulation related studies (Oyarbide et al., 2015; Shan et al., 2015; Ran et al., 2018). Our laboratory performed a study to evaluate the efficiency of different concentrations of *Escherichia coli* and *Bacillus subtilis* on germ-free zebrafish at 3 dpf and 5 dpf, and found that the final concentrations of 10^4 CFU/mL guarantee high survival rate of zebrafish larvae and colonization efficiency. Higher bacterial inoculation densities (more than 10^6 CFU/mL *E. coli* DH5α or *B. subtilis* WB800N) reduced sharply the dissolved oxygen level in media of mono-associated zebrafish, which may be lethal to zebrafish (Tan et al., 2019). Despite this, the effect of microbial titer may vary depending on microbial species, microbial genotype, media component and culture conditions (Pham et al., 2008). In general, these studies show that, the inoculation concentrations are variable depending on the research targets, microbial species and colonization conditions Tan et al., 2019.

4. Applications of gnotobiotic zebrafish models

Since intestinal microbiota composition varies depending on environmental factors, gnotobiotic models allow an increased control of variables, enhanced reproducibility of results and more accurate experimental designs by separating an animal into the host and microbiota (Marques et al., 2006). Gnotobiotic models facilitate analysis of a range of parameters. For example, gnotobiotic zebrafish models have been used to study the influence of microbial colonization or microbial products on host biological processes such as intestinal epithelial maturation, lipid absorption and immune homeostasis (Semova et al., 2012; Kanther et al., 2011; Koch et al., 2018). Gnotobiotic zebrafish models have also been used to study factors related to bacterial colonization (Stephens et al., 2015), resistance against pathogens (Rendueles et al., 2012) as well as behavior development (Phelps et al., 2017).

4.1. Intestinal epithelial maturation

Animal-associated microbial communities are involved in the development of the host, but are often overlooked by developmental biologists and physiologists as critical developmental determinants (Bates et al., 2006). Gnotobiotic zebrafish have been used to describe several intestinal epithelial maturation function in animals. Bates et al. (2006) found that the gut of 8 dpf germ-free larval exhibited immature patterns just like that of 5 dpf conventional animals, while colonization of 5 dpf germ-free larvae with microbe from conventional siblings was sufficient to restore alkaline phosphatase activity, indicating that intestinal microbiota play essential roles in gut development. Moreover, Rawls et al. (2006) found differences in the extent to which individual bacteria induced intestinal cell proliferation in mono-associations, suggesting that intestinal microbiota have different proliferative capacities. Cheesman et al. (2011) demonstrated that intestinal epithelial cell proliferation was regulated by both Wnt signaling and intestinal microbiota. Furthermore, intestinal microbiota enhanced the stability of β-catenin in intestinal epithelial cells and promoted cell proliferation in the developing vertebrate intestine (Cheesman et al., 2011).

In a nutshell, these studies demonstrated that intestinal microbiota were involved in the regulation of intestinal epithelium development. Considering the adverse effects caused by plant-based protein on fish intestinal inflammation, gnotobiotic zebrafish allow the evaluation of a large numbers of feed ingredients *in vivo* at a cost-effective way. These models also afford the possibility to identify the molecular mechanisms, which will provide insights into possible solutions for the existing problems resulting from nutrition interventions in aquaculture industry.

4.2. Feed additives identification

To fully understand the function of new additives on fish growth or physiology, a shift in approach is needed to determine the molecular mechanism. The evaluation of a large amount of feed additives in aquaculture has caused high-cost and long-term assays. The use of gnotobiotic zebrafish is more suitable for high throughput experiment in aquaculture (Ulloa et al., 2014). For example, although some feed additives, including thymol and carvacrol cause beneficial effects on fish, their possible mechanisms may involve a combination of both the host tissue stimulation and intestinal microbiota mediated effect (Ahmadifar et al., 2011; Ezzat Abd EI-Hack et al., 2016). Knowledge on the mechanisms may guide the efficient use of these feed additives. In order to identify the exact mechanism, the effect of the host can be detected in germ-free animals, while the function of microbiota can be evaluated by colonizing germ-free zebrafish with intestinal microbiota (Ran et al., 2016). Using a similar approach, Guo et al. (2017) found dietary nucleotides promoted growth of fish by decreasing the standard metabolic rate of zebrafish, which also included the contribution from the nucleotide-altered microbiota. Ding et al. (2018) showed that palmitic acid (PA) incorporated into high fat diet induced liver damage and endoplasmic reticulum stress, which was related to the PA-altered microbiota. The underlying mechanisms on feed additives will facilitate both screening and evaluating the safety of novel feed ingredients. In addition, many microbes are able to generate new products by degrading substrates in the diet, and the secondary metabolites from microbiota can serve as hormones or signaling molecules to influence the host physiology (Ikeda-Ohtsubo et al., 2018). Therefore, the proof of the mechanisms based on gnotobiotic *in vivo* model is more accurate than *in vitro* ones and gnotobiotic fish is regarded as the potential powerful model for *in vivo* research.

4.3. Nutrients absorption

The genome of microbes within the host encode physiological traits that are not represented in host genome. As the majority of these commensals are present in digestive tract, they contribute a lot to the harvest of dietary nutrients (Rawls et al., 2006). The metabolites of intestinal microbiota during the digestion have often been measured *in vitro*, which may not reflect the interaction between host and intestinal microbiota and their influence on nutrients digestion. With the development of gnotobiotic models, nutrients absorption and digestion can be measured *in vivo* (Semova et al., 2012).

The contributions of intestinal microbiota to degradation of complex dietary carbohydrates have been studied extensively, because the dietary carbohydrates are not easily degraded by the host (Zhang et al., 2014a). The impact of the microbiota on dietary protein or lipid metabolism has received less attention. The development of germ-free models, may allow for discovery of the function of microbiota in nutrients metabolism (Semova et al., 2012). For example; germ-free zebrafish larvae failed to take up protein macromolecules in the distal intestine, while addition of intestinal microbiota at a later development stage reversed all of the germ-free phenotypes (Bates et al., 2006). Similar to protein absorption, the colonization of intestinal microbiota stimulated fatty acids uptake and caused lipid droplet accumulation in the intestinal epithelium and liver of zebrafish, which may be caused partly by the microbial suppression of circulating inhibitor of lipoprotein lipase (Angiopoietin- 4, Angptl4/Fiaf) in intestinal epithelial (Semova et al., 2012; Galindo-Villegas et al., 2012). Moreover, mono-associated zebrafish revealed that different bacteria induced lipid accumulation *via* distinct mechanism (Semova et al., 2012). Compared to other gnotobiotic models, gnotobiotic zebrafish larvae are transparent and a large amount of transgenic zebrafish lines also facilitate the identification of the role of microbiota in the intestinal epithelium or extra intestinal tissues (Semova et al., 2012). To address the possible molecular mechanisms, genes related to lipid metabolism have been detected in germfree zebrafish and conventional fish (Sheng et al., 2018). As might be expected, the expression of some genes were influenced by intestinal bacteria in fish, suggesting the possible molecular mechanism regulated by intestinal microbiota during energy harvest (Sheng et al., 2018). Previous studies have suggested that species- or strain-level variation should be considered when we evaluate the influence of microbes on the health or growth performance of animals (Ikeda-Ohtsubo et al., 2018). The development of gnotobiotic models will further broaden our understanding of intestinal microbiota during nutrients absorption.

4.4. Immune homeostasis and resistance against pathogens

Intestinal microbiota plays an important role in regulating immune system in fish. An important concept in host-microbiota interaction is the presence of conserved innate sensors including Toll-like receptors (TLRs) (Kanther et al., 2011). Colonization of germ-free transgenic zebrafish with a commensal microbiota caused dynamic temporal and spatial patterns of NF-κB transcriptional activation and led to up-regulation of its target genes (Kanther et al., 2011). In addition, colonization by commensals increased neutrophils and enhanced several genes encoding pro-inflammatory and antiviral mediators, increasing the resistance of larvae to viral infection (Galindo-Villegas et al., 2012). A study by Bates et al. (2007) demonstrated that brush border enzyme, intestinal alkaline phosphatase (Iap), which dephosphorylates lipopolysaccharide (LPS), was induced by the intestinal microbiota in zebrafish. Moreover, the normal homeostatic level of neutrophils in the intestine was influenced by intestinal microbiota through a process involving myeloid differentiation primary response gene 88 (Myd88) and tumor necrosis factor (TNF) receptor in zebrafish (Rendueles et al., 2012). Consistent with this work, Koch et al. (2018) found that colonization of germ-free zebrafish larvae with the intestinal microbiota altered the host immune state and the microbiota-induced changes in the expression of genes were dependent on the innate immune adaptor gene Myd88 (Koch et al., 2018). Furthermore, *per capita* immunostimularoty effect of individual bacteria within a community also

varied widely (Rolig et al., 2015), suggesting that the functional identification of a specific bacteria should be detected *in vivo* by using a powerful gnotobiotic zebrafish model.

The identification of novel probiotics for aquatic animals is increasing with the demanding for environment-friendly aquaculture. However, studies on protective effects of a wide range of potentially probiotics are time-consuming and low-throughput (Rendueles et al., 2012). These weaknesses can be solved by using gnotobiotic zebrafish models, which have easy husbandry condition and short generation time. Gnotobiotic zebrafish models will be helpful for high-throughput screening of the function of probiotics *in vivo*. For example, thirty-seven potential probiotic bacterial strains were used to pre-colonized zebrafish larvae to detect their protective function against *Edwardsiella ictaluri* and three of them were found to robustly protect zebrafish larvae (Rendueles et al., 2012). Furthermore, Caruffo et al. (2016) found 13 different yeast strains isolated from the intestinal microbiota of healthy fish could protect zebrafish against a *Vibrio anguillarum* challenge and two probiotic candidates, *Debaryomyces hansenii* and *Yarrowia lypolitica* were used to colonize germ-free zebrafish larvae to determine whether the protection of probiotics against pathogens was mediated by the intestinal microbiota. These studies showed that gnotobiotic models afforded a high-throughput *in vivo* approach to oro-intestinal infection so as to identify the molecular basis underlying pathogen exclusion by probiotics (Rendueles et al., 2012). The question whether the identified probiotics are beneficial to fish cannot be determined only by 16S rRNA gene-based or *in vitro*-based analyses, the influence of probiotics on host *in vivo* should be investigated systematically.

4.5. Pathogenicity of pathogens in aquaculture

Most pathogens in aquaculture are opportunistic and cause outbreaks mainly through water and foodborne routes. Skin and gastrointestinal tract as the first line against pathogens, have received more attention for pathogenesis analysis. However, the interaction between host and pathogen is extremely complicated due to the diversity of the symbiotic microbiota, which hampers the interpretation of the results. Application of gnotobiotic fish models helps to overcome these difficulties (Oyarbide et al., 2015; Logan et al., 2018; Shan et al., 2016; Shan et al., 2015; Ran et al., 2018). For example, *Vibrio anguillarum* (VAN), which provokes one of the highest mortality rates in fish, entered through the larvae mouth at 5 dpf and after three hours post infection (hpi), *V. anguillarum* was detected mainly in the first part of the digestive tract and after 24 hpi throughout the digestive tract (Oyarbide et al., 2015). The combination of gnotobiotic zebrafish and three-dimensional live imaging techniques were successfully used to identify the mechanism used by *Vibrio cholera* to invade the intestine, suggesting that zebrafish is a powerful model for direct observation and experimental control of microbiome interactions (Logan et al., 2018). Moreover, Ran et al. (2018) found *Aeromonas veronii* isolates were more virulent than *Aeromonas hydrophila* isolates when infecting germ-free zebrafish larvae. Furthermore, authors identified aerolysin as the key virulence factor in their study. Using gnotobiotic zebrafish, it was found that immune effector serum amyloid A (Saa) could suppress expression of pro-inflammatory markers and bactericidal activity and the effects of Saa on neutrophil depended on microbiota colonization (Murdoch et al., 2019). Study based on germ-free zebrafish embryos model indicated that genes encoding antimicrobial effectors in skin are constitutively expressed during development, independently of commensal microbes and osmotic stress, suggesting that the influence of microbe on different tissues varies (Galindo-Villegas et al., 2016).

Compared with the nongnotobiotic zebrafish, gnotobiotic models had more homogenous response to bacterial infection and higher reproducibility of results, suggesting that gnotobiotic fish has already become a powerful platform for mechanistic studies of host-pathogen interactions (Oyarbide et al., 2015; Murdoch et al., 2019). In addition, the high-throughput of gnotobiotic zebrafish empowered the screening the pathogenicity of a large amount of pathogens.

4.6. Microbial colonization ability

The intestine is the “home” to gastrointestinal microbial community, which is essential in host development and health. However, little is known about the spatial and temporal dynamics of intestinal microbiota not only in fish but also in mammals. Gnotobiotic models provide the possibility for tracing the colonization characteristics of microbes, but in germ-free mice, interrogation of intestinal contents can be done only by dissection, which yields single-time-point data. Gnotobiotic zebrafish models offer a system in which monitoring of the spatial and temporal dynamics of intestinal microbiota in a living organism can be performed (Rawls et al., 2007). Using a gnotobiotic zebrafish-*Pseudomonas aeruginosa* model, Rawls et al. (2007) quantify bacterial colonization and behavior in the zebrafish intestine for the first time. Fluorescently labeled strains of *Aeromonas* species were used to colonize germ-free zebrafish larvae to measure the growth kinetic of a bacterial species *in vivo* (Jemielita et al., 2014).

Except for the growth kinetic of specific bacteria, the interspecies interactions of intestinal microbiota are more important in aquaculture. For example, the ability of probiotics to colonize the zebrafish intestinal tract is essential for the evaluation of potential beneficial effects (McFall-Ngai, 2006). Germ-free zebrafish larvae at 4 dpf were colonized with 10^7 CFU/mL lactic acid bacteria including two stains from *Lactobacillus plantarum* and one strain from *Lactobacillus fermentum* (Russo et al., 2015). The authors showed that, the occurrence of all strains were detected in the intestinal tract of the larvae after 6 hpi and different bacteria had differential adhesion capabilities (Russo et al., 2015). Furthermore, transient early-life exposure to yeast induced remarkable alterations in the microbial assemblage in fish (Siriyappagouder et al., 2018). These studies suggest the important role of gnotobiotic zebrafish models in evaluation and development of novel probiotics.

The influence of interaction between the host and microbiota on the microbial colonization characteristics was also evaluated on gnotobiotic zebrafish. Schlomann et al. (2018) used fluorescently labeled strains of seven bacterial symbionts to mono-associate with germ-free zebrafish. These authors found that, strains showed large difference in both cohesion and spatial distribution, which may be driven by peristaltic fluid flow, tubular anatomy, bacterial growth and aggregation kinetics (Schlomann et al., 2018). All above results indicate that gnotobiotic zebrafish can be used not only to measure the growth kinetic of specific bacteria, but also to reveal the influence of bacteria-bacteria or bacteria-host interactions on the colonization characteristics of intestinal microbiota.

4.7. Behavior

The microbiome helps integrate the gut-brain coordination (Fung et al., 2017). It has been found that antibiotic treatment or early-life microbiota composition could modulate anxiety behavior and symptoms of stress in fish (Almeida et al., 2019; Phelps et al., 2017). However, the influence of the microbiota on behavior and stress responses is poorly understood. Zebrafish is a premier model for exploring cellular and molecular mechanisms underlying a variety of behaviors (Davis et al., 2016; Melancon et al., 2017). Axenic zebrafish exhibited hyperactivity compared with the conventional ones and colonization of *Lactobacillus plantarum* attenuated anxiety-related behavior, indicating disruption of intestinal microbiota in early life influenced the neurodevelopment and behavior in larval zebrafish (Phelps et al., 2017). The high-throughput assays in zebrafish will be helpful to uncover genetic or environmental factors that influence fish behavior. Furthermore, gnotobiotic zebrafish will enable unprecedented resolution of mechanism by which commensal microbiota affect the nervous system or behavior (Melancon et al., 2017). With increased attention about the

Table 2
Gnotobiotic models for other species in aquaculture.

Common name	Species	Object	Treatment	Diet	Reference
Freshwater fishes					
Zebrafish	*Danio rerio*	Eggs	0.1% PVP-I + 0.003% bleach solution +antibiotics	Ref to Table 1	Pham et al. (2008)
Trout	*Salmo irideus*& *Salmo fario*	Eggs	0.185% Acriflavine or 0.015% Sulfo-merthiolate	Not mentioned	Gee and Sarles (1941)
	Salmo trutta fario	Eggs	0.1–0.2 ppm ozone/ 50 mg/L bronopol	Not mentioned	Forneris et al. (2003) Aller-Gancedo and Fregeneda-Grandes (2007)
	Oncorhynchus mykiss	Eggs	250 ppm iodine/ 2000 mg/L iodine/ 30,000 mg/L sodium chloride	Not mentioned	Marking et al. (1994) Wagner et al. (2010)
Platyfish	*Platyaoecilus maculatus*	Adults	tincture of iodine	Agar particles/ Commercial fish food/ Nematodes/ Algae	Baker et al. (1942)
Coho salmon	*Oncorhynchus kisutch*	Adults	1% PVP-I	Not mentioned	Trust (1974)
		Eggs	0.4% PVP-I	Not mentioned	Lesel and Lesel (1976)
	Oncorhynchus mykiss	Eggs	25, 50, 100, 200 ppm glutaraldehyde/0.0002%–0.02% tannic acid	Not mentioned	Cline and Post (1972) Wagner et al. (2012b)
Northern pike	*Esox lucius*	Eggs	0–6000 μl/L hydrogen peroxide	Not mentioned	Rach et al. (1998)
Nile tilapia	*H. macrocephala* *Oreochromis niloticus*	Eggs	0.04% formaldehydeaquarium 2000 mg/L active hydrogen peroxide, 10 mg/L (Amp + Rfp + Tmp + Gen) + 0.5 mg/L Amph B + 25 mg/L FB 28, 75 mg/L active chlorite solution	Yolk *Artemia nauplii*	Shaw (1957) Situmorang et al. (2014)
Lake sturgeon	*Acipenser fulvescens* & *Polyodon spathula*	Eggs	0–6000 μl/L hydrogen peroxide	Not mentioned	Rach et al. (1998)
Carp	*Lepidomeda copei*	Eggs	5000 mg/L hydrogen peroxide/ 40 mg/L, 60 mg/L copper sulphate/ 0–6000 μl/L;Hydrogen peroxide/	Not mentioned	Wagner et al. (2012a)
	Catostomus commersoni *Cyprinus carpio*		0–6000 μl/L hydrogen peroxide/ 0–200 mg/L iodine/ 0–400 mg/L formaldehyde		Rach et al. (1998) Khodabandeh and Abtahi, 2006
Marine fishes					
Sheepshead minnow	*Cyprinodon variegatus*	Eggs	950 units/mL Pen G + 950 μg/mL Stre +5 μg/mL Fun +50 μg/mL Gen	Dry flake food or *Artemia*	Battalora et al. (1985)
Turbot	*Scophthalmus maximus*	Eggs	Antibiotic solution (not mentioned in detail)/ 400–800 mg/L glutaraldehyde	Rotifers (*Brachionus plicatilis*) Rotifers (*Brachionus plicatilis*)	Munro et al. (1995) Salvesen et al. (1997)
Red drum	*Sciaenops ocellatus*	Eggs	3% Hydrogen peroxide	Rotifers	Douillet and Holt (1994)
Atlantic wolffish	*Anarhichas lupus*	Eggs	600 mg/L glutaraldehyde	Not mentioned	Pavlov and Moksness (1993)
Atlantic halibut	*Hippoglossus hippoglossus*	Eggs	400 ppm glutaric dialdehyde 400–800 mg/L glutaraldehyde 25% Glutaraldehyde, 10 mg/L (OA + Kan + Ery) + 150 mg/L Pen G + 75 mg/L Stre	*Artemia* and zooplankton Not mentioned Axenic and gnotobiotic *Artemia*	Harboe et al. (1994) Salvesen et al. (1997) Verner-Jeffreys et al. (2003)
Atlantic salmon	*Salmo salar*	Eggs	50 mg + 100 mg iodine	Not mentioned	Cipriano et al. (2001)
Gilthead sea bream	*Sparus aurata*	Eggs	0.3 mg min/L ozone/0.5 mg/L ozone	Not mentioned	Ben-Atia et al. (2007) Can et al. (2012)
			300 ppm iodine	Not mentioned	Can et al. (2010)
			300 ppm hydrogen peroxide	Not mentioned	Can et al. (2010)
Sea bass	*Dicentrarchus labrax*	Eggs	20 mL/L 0.5% active iodine, 100 mg active glutaraldehyde l-10	The larvae were not fed during the experiment	Dierckens et al. (2009), Li et al. (2014)
		Eggs, larvae	10 mg/L Amp + 10 mg/L Rif, 10 mg/L Rif	*Artemia*	Rekecki et al. (2009)
Atlantic cod	*Gadus morhua*	Eggs	0.2 to 10 mg/L ozone 3% hydrogen peroxide 3.5 mL/L peracetic acid 10 to 150 mg/L iodine 10 mg/L Rfp + 10 mg/L Amp	Not mentioned	Grotmol et al. (2003) Peck et al., 2004 Brown et al. (2005) Overton et al. (2010) Rekecki et al. (2012)
			400 ppm glutaraldehyde/100 to 800 mg/L glutaraldehyde	Gnotobiotic rotifers/Newly hatched *Acartia tonsa*	Forberg et al. (2011) Overton et al. (2010)
California halibut	*Paralichthys californicus*	Eggs	50 mg/L iodine	Not mentioned	Stuart et al. (2010)
Threespine stickleback	*Gasterosteus aculeatus*	Eggs	100 μg/mL Amp + 5 μg/mL Kan + 250 ng/mL Amph B, 0.2% PVP-I, 0.003% bleach	Not mentioned	Milligan-Myhre et al. (2016)

(*continued on next page*)

Table 2 (*continued*)

Common name	Species	Object	Treatment	Diet	Reference
Molluscs					
Hard clam	*Mercenaria mercenaria*	Eggs	100 mg/L Pen G + 100 mg/L Stre S + 50 mg/L Chl	Bacteria-free alga (*Isochrysis galbana*)	Guillard (1959)
European oyster	*Ostrea edulis*	Larvae	670 μg/mL Pen +50 μg/mL Stre +5 μg/mL Chl	Algae	Milliar and Scott (1967)
Pacific oyster	*Crassostrea gigas*	Eggs	0.5% hypochlorite solution	Artificial diets/ alga (*Chaetoceros calcitrans*)	Langdon (1983)
		Adults	1% NaOCl	Axenic algae (*Isochrysis galbana*)	Douillet and Langdon (1993)
Perlemoen abalone	*Haliotis midae*	Juveniles	600 μg/mL Amp + 250 μg/mL Chl + 250 μg/mL Cef	*Ecklonia maxima/ Gracilaria verrucosa*	Erasmus et al. (1997)
Pacific oyster, Portuguese oyster	*Crassostrea gigas, Crassostrea angulata*	Adults	PVP-I, 0.1 mg/L OA, 0.1 mg/L Kan, 0.1 mg/L Ery, 0.1 mg/L Stre S, 30 μg/L Flu and 100 units/L Pen	Not mentioned	Arzul et al. (2001)
Crustaceans					
Artemia	*Artemia salina*	Cysts	1 ppt Merthiolate +10% 2 mL/L aerosol OT/20% NaOCl (2% chlorine)	Not mentioned	Provasoli and D'Agostino (1969) Rahat and Diementman (1982)
	T. japonicus	Adults/larvae	5000 Dhstre +5000 units Pen G + 150 units Pol B S/250 μg/mL Tet + 50 μg/mL Chl + 250 μg/mL Neo	Not mentioned	Jung and Hagiwara (2001)
	Artemia franciscana	Cysts	30 mg/L Chl + 40 mg/L Tmp + 8 mg/L Sfm	Not mentioned	Soto-Rodriguez et al. (2003)
			10% NaOCl +0.25 mol/L NaOH +0.1 mol/L HCl	Not mentioned	Verner-Jeffreys et al. (2003)
Daphnia	*Daphnia magna*	Adults	10 μg/mL Neo + 1500 μg/mL Stre +6000 μg/mL Pen +400 μg/mL Tet	Not mentioned	D'Agostino and Provasoli (1970)
		Adults	5 IU/mL Tet	Sterile *Chlorella*	Avtsyn et al. (1982)
		Eggs	1 mg/L Amp + 50 μg/mL Kan + 50 μg/mL Tet	Autoclaved algae	Sison-Mangus et al., 2014
		Adults	0.25–2 mg/L Tmp	*P. subcapitata*	Gorokhova et al. (2015)
		Eggs	0.01% peracetic acid	Axenic algae	Callens et al. (2016) Callens et al. (2018) Macke et al. (2017)

Amp, ampicillin; Amph B, amphotericin-B; Cef, cefotaxime; Chl, chloramphenicol; Ery, erythromycin; FB 28, fluorescent brightener 28; Flu, flumequine; Fun, fugizone; Gen, Gentamycin; Kan, kanamycin; Neo, neomycin; OA, oxolinic acid; Pen, penicillin; Pen G, penicillin G; Pol B S, polymyxin B sulphate; ppm, parts per billion; ppt, parts per trillion; PVP-I, polyvinylpyrrolidone iodine; Rfp, rifampin; Sfm, sulfamethoxaxole; Stre, streptomycin; Stre S, streptomycin sulphate; Tet, tetracycline; Tmp, trimethoprim.

intestinal microbiota and neurodevelopment, the gnotobiotic models will show their power in behavior related research.

5. Gnotobiotic models from other aquatic animals and their application

5.1. History of germ-free fish and disinfection/sterilization methods

Apart from zebrafish, gnotobiotic models from other species in aquaculture were reported during the last century (Gee and Sarles, 1941). Since then, series of axenic researches have been conducted in many aquatic animal species (Table 2). Gee and Sarles (1941) developed a new and safe method for the disinfection/sterilization of living trout (*Salmo irideus*, and *S. fario*) eggs, which provided an idea for obtaining germ-free larvae. In fact, the original intention of their research was to restrict a highly infectious disease caused by *Bacterium salmonicida* and eggs were supposed to be the carriers of pathogens. Germ-free model of platyfish (*Platyaoecilus maculatus*), which is viviparous fish, was established in 1942 (Baker et al., 1942). Researchers extracted young fish through the ruptured walls of the ovary and reared them by using heat-killed algae or autoclaved housefly larvae. The life span of germ-free platyfish could reach more than four months (Baker et al., 1942). Although contamination occurred in some circumstance, the germ-free models were successfully established for fish produced either by egg or foetus.

With the construction of germ-free models, different disinfection/sterilization methods and chemicals have been used. Shaw (1957) disinfected *Tilapia macrocephala* eggs by using formaldehyde solution to produce germ-free fry. Two different methods were employed for producing bacteria-free salmonid species. The first method is to collect bacteria-free eggs and sperm and then to fertilize aseptically *in vitro* (Trust, 1974), while the second is to disinfect fertilized eggs by using polyvinylpyrrolidone iodine (PVP-I) (Lesel and Lesel, 1976). Moreover, Douillet and Holt (1994) disinfected eggs by using hydrogen peroxide at 3% concentration and no adverse long-term effects on either survival or growth of Red drum larvae were observed. Ozone dissolved in seawater was also evaluated as an egg disinfectant method on spawning sea bream (Ben-Atia et al., 2007). Situmorang et al. (2014) used a double disinfection procedure including hydrogen peroxide and sodium hypochlorite solution to fish eggs, and then immersed eggs in a cocktail of antibiotics and antifungal agents. Nowadays, the common method for obtaining germ-free larvae is to collect fertilized eggs, disinfect and incubate them in a combination of disinfectants and antibiotics.

5.2. Application of gnotobiotic freshwater fishes

Except for zebrafish, the majority of research related to gnotobiotic freshwater fishes focused on testing the resistance against pathogens or identifying the function of probiotics. Situmorang et al. (2014) inoculated four candidate pathogenic bacteria strains onto Nile tilapia larvae to construct a consistent infection model for evaluating the protection role of probiotics. Their results indicated that, the developed gnotobiotic Nile tilapia model can be used to extend the understanding of the mechanisms involved in host-microbe interaction and to evaluate new methods of disease control.

Compared with terrestrial animals, assembly of fish gut microbiota is also dependent on the aquatic environmental factors. Therefore, the exposure of fish to environmental free-living microbiota and its effect on early colonization in the gut is important for understanding host-microbiota interaction in fish (Giatsis et al., 2016). In their study, Giatsis et al. (2016) reared germ-free tilapia larvae under conventional conditions or exposed them to a single probiotic strain. Results indicated that although the added probiotic was only transiently detected, gut microbiota of the exposed fish still differed from that of the control group (Giatsis et al., 2016), suggesting that the early probiotics treatment contributed to the assembly of intestinal microbiota. All these studies suggest that gnotobiotic Nile tilapia larva is a powerful tool for investigating the effects and modes-of-action of probiotics under controlled conditions (Situmorang et al., 2014). Similar to the gnotobiotic zebrafish, gnotobiotic Nile tilapia is also a high-throughput tool, and more suitable for aquaculture nutrition related research.

5.3. Application of gnotobiotic marine fishes

The establishment of gnotobiotic marine fishes was later developed than that of freshwater fishes, but their models are more widely applied than the latter. Similar to the freshwater species, the original of axenic larvae was also from the disinfected eggs due to frequency and high mortality of fry caused by infectious disease in the mariculture industry (Ben-Atia et al., 2007). The axenic larvae of marine fishes have been established for sheepshead Minnow (*Cyprinodon variegatus*) (Battalora et al., 1985), Red drum (*Sciaenops ocellatus* Linnaeus) (Douillet and Holt, 1994), turbot (*Scophthalmus maximus*) (Munro et al., 1995) and halibut (*Hippoglossus hippoglossus L.*) (Harboe et al., 1994; Salvesen et al., 1997). Bacteria-free larvae from halibut were used to test the toxicity of 20 bacterial isolates from hatcheries and the results showed that most of the bacteria isolated from halibut hatcheries are not harmful to yolk-sac larvae (Verner-Jeffreys et al., 2003). The high-throughput features of gnotobiotic models, offer opportunities for use of germ-free marine fish models to detect the influence of multiple pathogens or factors.

Gnotobiotic marine fishes have been used to identify the pathogenesis of a specific pathogen on a specific fish. For example, the location and damage caused by *Vibrio anguillarum* as the causative agent for the newly hatched sea bass have been studied extensively. A standardized challenge test with the pathogen *V. anguillarum* has been constructed (Dierckens et al., 2009). The presence and location of this pathogen in gnotobiotic sea bass (*Dicentrachus labrax*) at different time intervals after the infection have been defined (Rekecki et al., 2012). Furthermore, studies on gnotobiotic sea bass also indicated that indole signaling and the alterative sigma factor RpoS have a significant impact on the virulence of *V. anguillarum* (Li et al., 2014). Apart from *V. anguillarum*, other intestinal microbiota has also been studied in sea bass. For example, gnotobiotic sea bass larvae were fed with axenic *Artemia* sp. for one week and one of the three opportunistic pathogens, *Aeromonas hydrophila*, two *Listonella anguillatum* were inoculated *via* water and encapsulated in *Artemia* sp. (Dierckens et al., 2009). Using this approach, the influence of native microbial communities was excluded, suggesting that, gnotobiotic fishes are more suitable for understanding the effects and modes-of-action of other bacteria (Dierckens et al., 2009).

Except for the pathogenesis analysis, gnotobiotic marine fishes have also been used for nutrition or development related research. A study on bacteria-free sea-bass (*Dicentrarchus labrax L.*) larvae reported higher growth and more developed digestive tracts compared to conventionally raised larvae (Rekecki et al., 2009), which was inconsistent to the findings in mammals and zebrafish. These results necessitated increased research attention to elucidate the role played by host-microbiota in marine fish. Under this circumstance, Forberg et al. (2011) generated bacteria-free Atlantic cod (*Gadus morhua L.*) larvae without continued addition of antibiotics and developed an experimental system allowing addition of live feed to the larvae. Furthermore, the effects of eight combinations of either live or dead bacteria were inoculated to germ-free cod larvae and 17 different gnotobiotic conditions were investigated. The results indicated that the host response to the probiotic effect of a strain is not strictly dependent on bacterial activity, using dead probiotic bacteria also have advantages under some circumstance (Forberg et al., 2012).

Gem-free model of threespine stickleback (*Gasterosteus aculeatus*), which has complex traits during the repeated evolution from ancestral oceanic to derived freshwater, has been established (Milligan-Myhre et al., 2016). The innate immune response of these fish to resident gut

microbiota has been detected and the results indicated that oceanic fish mount a strong immune response to residential microbiota, whereas freshwater fish frequently do not. The gnotobiotic stickleback model has the potential to be used for research on immune response in future (Milligan-Myhre et al., 2016).

Compared with gnotobiotic zebrafish, gnotobiotic models from other fishes have more specific features, for example, stickleback has natural genetic variation among population, which allows the determination of immune response of varied microbiota across families within the same population (Milligan-Myhre et al., 2016). Furthermore, some fish, including stickleback or Nile tilapia have a wide salinity tolerance, which facilitate the assessment of the relationship among host, microbiota and environmental factors. Sea bass has serious infection caused by *Vibrio anguillarum,* and thus it is a suitable model for identifying the pathogenesis or screening the protective probiotics (Rekecki et al., 2012). However, compared to large amount of aquatic animals, the diversity of germ-free fish models is still limited and much more effort should be directed towards establishment of germ-free models.

6. Challenges facing fish gnotobiotic models

Gnotobiotic models offer the possibility to understand the mechanisms by which intestinal microbiota regulate the host, but compared with the mammals, gnotobiotic fish models are still far from mature. Zebrafish may be reared for up to 30 days in a sterile environment (Rawls et al., 2006), but only the larvae (< two weeks) have been used in most studies, partly due to the challenges on long-term germ-free husbandry methods (Melancon et al., 2017). Supplying adequate nutrition to GF zebrafish is the greatest challenge. Although GF culture of *Tetrahymena thermophile* and *Artemia* have been used by several groups (Milligan-Myhre et al., 2011; Rendueles et al., 2012), the nutritional content of these live food is poorly defined or variable across facilities (Melancon et al., 2017). Sterilized artificial diets were also used by some studies (Rawls et al., 2004; Rawls et al., 2007; Semova et al., 2012; Camp et al., 2012; Guo et al., 2019; Phelps et al., 2017; Rendueles et al., 2012; Murdoch et al., 2019), but we still noticed that most studies did not mention the diet components or did no feed germ-free larvae during the experiment (Table 1). Zebrafish larvae hinder the discovery of various responses to microbes from different organs or the interactions between intestinal microbiota and adult fish because of small size and limited life-span of germ-free ones. Due to the limited knowledge of zebrafish stage-specific nutritional requirements, the diet that can sustain gnotobiotic zebrafish into adult stages is absent, which is a major impediment to the application of germ-free zebrafish. Great efforts should be afforded to define zebrafish nutritional requirements and develop a standardized artificial diet (Melancon et al., 2017).

Furthermore, gnotobiotic models availability are limited to a few species, which are far from enough considering the diverse trophic levels of fish. The application of gnotobiotic models in aquaculture is still limited, which hampers the functional identification of commensal microbiota in different aquatic animals.

7. Gnotobiotic models of molluscs

Similar to the gnotobiotic fishes, the bacteria-free hard clam model was constructed with the original thought for identifying pathogens for larval mortality (Guillard, 1959). Two species of bacteria-free oysters larvae were established in 1967 (Milliar and Scott, 1967) and 1983 (Langdon, 1983). Accordingly, oysters models were applied to detect the effects of marine bacteria or virus on the culture of axenic Oyster *Crassostrea gigas* larvae (Arzul et al., 2001; Douillet and Langdon, 1993). In 1997, Erasmus et al. produced gnotobiotic abalone and indicated that the bacteria in the digestive system of *Haliotis midae* assisted the digestion efficiency of alginate, laminarin, agarose, carrageenan and cellulose, which was the first research to address the function of bacteria in the nutrition of molluscs (Erasmus et al., 1997).

8. Gnotobiotic models of crustaceans

Daphnia magna straus was used to study relations between water fleas and *vibrios* and the results indicated that water fleas act as biological scavengers for preventing and controlling of intestinal infection even without symbiotic bacteria (Avtsyn et al., 1982). Germ-free *Daphnia* has been widely used for studying microbiota-host interaction. Gorokhova et al. found that the decrease of microbiota in the daphnia gut induced by antibiotics caused compromised nutrition and ultimately, growth, suggesting the important function of intestinal microbiota (Gorokhova et al., 2015). Macke et al. pointed that both environmentally and host genotype-induced variations in gut microbiota structure mediate Daphnia tolerance to toxic cyanobacteria (Macke et al., 2017). Considering that *Daphnia magna* is a model species used to study stress responses, physiological adaptations, and genome-environment interactions, germ-free *Daphnia magna* has more widely application potential in identifying the function of intestinal microbiota.

Artemia salina was able to be grown aseptically to adults in a non-living medium (Provasoli and Shiraishi, 1959). Similar to axenic Daphnia model, nutritional requirements and development studies were performed on gnotobiotic *Artemia* (Provasoli and D'Agostino, 1969; Defoirdt et al., 2006). Soto-Rodriguez *et al* used germ-free *Artemia* as a model for virulence evaluation (2003) and Defoirdt *et al* showed 5 short-chain fatty acids protect gnotobiotic *Artemia franciscana* from *Vibrio campbellii* infection (2006), suggesting *Artemia* served as a good model for controlling *Vibrio* infection. In fact, searching for natural compounds which could protect farm (aquatic) animals from bacterial diseases has great potential for disease control and gnotobiotic *Artemia* model system afforded more information compared with *in vitro* models (Baruah et al., 2015; Baruah et al., 2017).

9. Other models for host-microbiota interactions

Antibiotics have been used to deplete the intestinal microbiota and antibiotic-treated animals are widely used to explore the function of commensal microbiota in mammals (Brandl et al., 2008; Morgun et al., 2015; Ruiz et al., 2017). However, the application of antibiotic-treated models in aquaculture is not common. Recently, lipid accumulation and the expression of related genes were detected among germ-free zebrafish, antibiotics-treated zebrafish and conventional ones (Sheng et al., 2018). The results indicated that both models (germ-free & antibiotics treated) provided valuable information for detecting the relationship between gut microbiota and lipid metabolism (Sheng et al., 2018). Furthermore, Phelps et al. (2017) found that impairment of host colonization using antibiotics also caused hyperactivity in conventionally colonized larvae, which was similar to axenic zebrafish, suggesting that disruption of host-associated microbial communities may exert influence on neurobehavioral development. However, antibiotic-induced effects can be explained by either depletion of microbiota, direct effects of antibiotics on the host or the effect of remaining antibiotic-resistant bacteria (Morgun et al., 2015). Therefore, care must be taken when antibiotics-treated animals are used to identify the function of commensal microbiota (Sheng et al., 2018).

10. Conclusion

The goal of aquaculture is to produce the highest quality of food for consumers by using the lowest production cost and causing less environment pollution. Modulation of the intestinal microbiota is a possible way to promote the growth and health of the aquatic animals by the use of probiotics, prebiotics and other feed ingredients (Haygood and Jha, 2018). Revealing the interactions between the intestinal microbiota and the host (aquatic animal) is essential for the development of aquaculture. A better understanding of interactions of host-

microbiota will facilitate the development of effective solutions for disease control or a healthy aquaculture growth.

The gnotobiotic models have showed successful uses as (i) identifying the function of intestinal microbiota in the metabolism, immune regulation, intestinal health, development and behavior of fish; (ii) evaluating the protective function of probiotics, prebiotics or other additives and identifying the underlying mechanism;(iii) revealing the interactions among the host, microbiota and environment factors (diet component, salinity, etc); and (iv) screening major factors that shapes the microbial assembly, which is useful for microbial modulation. Future works on intestinal microbiota are expected from correlation to causality, including investigating the function of specific microbial strains or the metabolites produced by intestinal microbiota. More gnotobiotic aquatic animals are required and endeavors should be performed to overcome the shortcomings of the present models. The gnotobiotic models provide useful information on host-microbiota interactions and facilitate the modulation of intestinal microbiota in aquaculture.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Ahmadifar, E., Falahatkar, B., Akrami, R., 2011. Effects of dietary thymol-carvacrol on growth performance, hematological parameters and tissue composition of juvenile rainbow trout, Oncorhynchus mykiss. J. Appl. Ichthyol. 27, 1057–1060.

Ahmed, H.I., Herrera, M., Liew, Y.J., et al., 2019. Long-term temperature stress in the coral model Aiptasia supports the “Anna Karenina Principle” for bacterial microbiomes. Front. Microbiol. 10, 975. https://doi.org/10.3389/fmicb.2019.00975.

Aller-Gancedo, Fregeneda-Grandes, 2007. Comparative efficacy of Pyceze (bronopol) in controlling mortality of brown trout Salmo trutta eggs. Aquac. Res. 38, 618–624.

Almeida, A.R., Tacão, M., Machado, A.L., et al., 2019. Long-term effects of oxytetracycline exposure in zebrafish: a multi-level perspective. Chemosphere. 222, 333–344.

Arzul, I., Renault, T., Lipart, C., 2001. Experimental herpes-like viral infections in marine bivalves: demonstration of interspecies transmission. Dis. Aquat. Org. 46, 1–6.

Avtsyn, Shakhlamov, Trager, Petrova, 1982. Water fleas as biological antagonists of NAG vibrios. B. Exp. Biol. Med. 93, 48–50.

Baker, J.A., Ferguson, M.S., TenBroeck, C., 1942. Growth of platyfish (platyaoecilus maculatus) free from bacteria and other microijrganism. Exp. Biol. Med. 51, 116–119.

Baruah, K., Duy Phong, H.P., Norouzitallab, P., et al., 2015. The gnotobiotic brine shrimp (Artemia franciscana) model system reveals that the phenolic compound pyrogallol protects against infection through its prooxidant activity. Free Radic. Biol. Med. 89, 593–601.

Baruah, K., Norouzitallab, P., Duy Phong, H.P., et al., 2017. Enhanced resistance againstVibrio harveyiinfection by carvacrol and its association with the induction of heat shock protein 72 in gnotobioticArtemia franciscana. Cell Stress Chaperones 22, 377–387.

Bates, J.M., Mittge, E., Kuhlman, J., et al., 2006. Distinct signals from the microbiota promote different aspects of zebrafish gut differentiation. Dev. Biol. 297, 374–386.

Bates, J.M., Akerlund, J., Mittge, E., et al., 2007. Intestinal alkaline phosphatase detoxifies lipopolysaccharide and prevents inflammation in zebrafish in response to the gut microbiota. Cell Host Microbe 2, 371–382.

Batista, S., Medina, A., Pires, M.A., et al., 2016. Innate immune response, intestinal morphology and microbiota changes in Senegalese sole fed plant protein diets with probiotics or autolysed yeast. Appl. Microbiol. Biotechnol. 100, 7223–7238.

Battalora, M.S.J., Ellender, R.D., Martin, B.J., 1985. Gnotobiotic maintenance of sheepshead minnow larvae. Prog. Fish-Cult. 47, 122–125.

Ben-Atia, I., Lutzky, S., Barr, Y., et al., 2007. Improved performance of gilthead sea bream, Sparus aurata, larvae after ozone disinfection of the eggs. Aquac. Res. 38, 166–173.

Booman, M., Forster, I., Vederas, J.C., et al., 2018. Soybean meal-induced enteritis in Atlantic salmon (Salmo salar) and Chinook salmon (Oncorhynchus tshawytscha) but not in pink salmon (O-gorbuscha). Aquaculture. 483, 238–243.

Brandl, K., Plitas, G., Mihu, C.N., et al., 2008. Vancomycin-resistant enterococci exploit antibiotic-induced innate immune deficits. Nature. 455, 804–807.

Brown, Kling, Skonberg, 2005. Demonstration of Sustainable Cod Farming from Egg to Grow-Out in Maine.

Callens, Macke, Muylaert, Bossier, Lievens, Waud, Decaestecker, 2016. Food availability affects the strength of mutualistic host-microbiota interactions in *Daphnia magna*. ISME J. 10, 911–920.

Callens, Watanabe, Kato, Miura, Decaestecker, 2018. Microbiota inoculum composition affects holobiont assembly and host growth in daphnia. Microbiome. 6, 56.

Camp, J.G., Jazwa, A.L., Trent, C.M., et al., 2012. Intronic cis-regulatory modules mediate tissue-specific and microbial control of angptl4/fiaf transcription. PLoS Genet. 8, e1002585.

Can, Saka, Firat, 2010. Disinfection of gilthead sea bream (*Sparus aurata*), red porgy (*Pagrus pagrus*), and common dentex (*Dentex dentex*) eggs from sparidae with different disinfectants. Kafkas. Univ. Vet. Fak. 16, 299–306.

Can, Karacalar, Saka, Firat, 2012. Ozone disinfection of eggs from gilthead seabream Sparus aurata, sea bass Dicentrarchus labrax, red porgy, and common dentex Dentex dentex. J. Aquat. Anim. Health 24, 129–133.

Caruffo, M., Navarrete, N.C., Salgado, O.A., et al., 2016. Protective yeasts control V. anguillarum pathogenicity and modulate the innate immune response of challenged zebrafish (Danio rerio) larvae. Front. Cell. Infect. Microbiol. 6, 127.

Cheesman, S.E., Neal, J.T., Mittge, E., et al., 2011. Epithelial cell proliferation in the developing zebrafish intestine is regulated by the Wnt pathway and microbial signaling via Myd88. Proc. Natl. Acad. Sci. U. S. A. 108, 4570–4577.

Chen, H., Li, C., Liu, T., et al., 2019. A metagenomic study of intestinal microbial diversity in relation to feeding habits of surface and cave-dwelling sinocyclocheilus species. Microb. Ecol. https://doi.org/10.1007/s00248-019-01409-4.

Cipriano, Novak, Flint, Cutting, 2001. Reappraisal of the federal fish health recommendation for disinfecting eggs of Atlantic Salmon in Iodophor. J. Aquat. Anim. Health 13, 320–327.

Clements, K.D., Angert, E.R., Montgomery, W.L., et al., 2014. Intestinal microbiota in fishes: what’s known and what’s not. Mol. Ecol. 23, 1891–1898.

Cline, Post, 1972. Therapy for trout eggs infected with Saprolegnia. Progress. Fish-Cult. 34, 148–151.

Cocchiaro, J.L., Rawls, J.F., 2013. Microgavage of zebrafish larvae. J. Vis. Exp. 20, e4434. https://doi.org/10.3791/4434.

Collins, A.J., LaNarre, B.A., Won, B.S., et al., 2012. Diversity and partitioning of bacterial populations within the accessory nidamental gland of the squid Euprymna scolopes. Appl. Envrion. Microbiol. 78, 4200–4208. https://doi.org/10.1128/AEM.07437-11.

D'Agostino, Provasoli, 1970. Dixenic culture of *Daphnia magna*, straus. Biol. Bull. 139, 485–494.

Davis, D.J., Bryda, E.C., Gillespie, C.H., et al., 2016. Microbial modulation of behavior and stress responses in zebrafish larvae. Behav. Brain Res. 311, 219–227.

Defoirdt, Halet, Sorgeloos, Bossier, Verstraete, 2006. Short-chain fatty acids protect gnotobiotic *Artemia franciscana* from pathogenic Vibrio campbellii. Aquaculture 261, 804–808.

Dierckens, K., Rekecki, A., Laureau, S., et al., 2009. Development of a bacterial challenge test for gnotobiotic sea bass (Dicentrarchus labrax) larvae. Environ. Microbiol. 11, 526–533. https://doi.org/10.1111/j.1462-2920.2008.01794.x.

Ding, Q., Zhang, Z., Ran, C., et al., 2018. The hepatotoxicity of Palmitic acid in zebrafish involves the intestinal microbiota. J. Nutr. 148, 1217–1228. https://doi.org/10.1093/jn/nxy084.

Douillet, P., Holt, G.J., 1994. Surface disinfection of red drum (Sciaenops ocellatus Linnaeus) eggs leading to bacteria-free larvae. J. Exp. Mar. Biol. Ecol. 179, 253–266. https://doi.org/10.1016/0022-0981(94)90118-X.

Douillet, P., Langdon, C.J., 1993. Effects of marine bacteriaon the cultureof axenic oyster crassostrea gigas (thunberg) larvae. Biol. Bull. 184, 36–51. https://doi.org/10.2307/1542378.

Eckburg, P.B., Bik, E.M., Bernstein, C.N., et al., 2005. Diversity of the human intestinal microbial flora. Science. 308, 1635–1638.

Erasmus, J.H., Cook, P.A., Coyne, V.E., 1997. The role of bacteria in the digestion of seaweed by the abalone Haliotis midae. Aquaculture. 155, 377–386.

Ezzat Abd EI-Hack, M., Alagawany, M., Ragab Farag, M., Tiwari, R., et al., 2016. Beneficial impacts of thymol essential oil on health and production of animals, fish and poultry: a review. J. Essent. Oil Res. 28, 365–382.

Forberg, T., Arukwe, A., Vadstein, O., 2011. A protocol and cultivation system for gnotobiotic Atlantic cod larvae (Gadus morhua L.) as a tool to study host microbe interactions. Aquaculture. 315, 222–227.

Forberg, T., Vestrum, R.I., Arukwe, A., et al., 2012. Bacterial composition and activity determines host gene-expression responses in gnotobiotic Atlantic cod (Gadus morhua) larvae. Vet. Microbiol. 157, 420–427.

Forneris, Bellardi, Palmegiano, Saroglia, Sicuro, Gasco, Zoccarato, 2003. The use of ozone in trout hatchery to reduce saprolegniasis incidence. Aquaculture 221, 157–166.

Franzenburg, S., Walter, J., Kunzel, S., et al., 2013. Distinct antimicrobial peptide expression determines host species-specific bacterial associations. Proc. Natl. Acad. Sci. U. S. A. 24, E3730–E3738.

Fung, T.C., Olson, C.A., Hsiao, E.Y., 2017. Interactions between the microbiota, immune and nervous systems in health and disease. Nat. Neurosci. 20, 145–155. https://doi.org/10.1038/nn.4476.

Galindo-Villegas, J., Garcia-Moreno, D., de Oliveira, S., et al., 2012. Regulation of immunity and disease resistance by commensal microbes and chromatin modifications during zebrafish development. Proc. Natl. Acad. Sci. U. S. A. 109, E2605–E2614.

Galindo-Villegas, J., Montalban-Arques, A., Liarte, S., et al., 2016. TRPV4-mediated detection of hyposmotic stress by skin keratinocytes activates developmental immunity. J. Immunol. 196, 738–749.

Gee, L.L., Sarles, W.B., 1941. The disinfection of trout eggs contaminated with bacterium salmonicida. J. Bacteriol. 44, 111–126.

Giatsis, C., Sipkema, D., Ramiro-Garcia, J., et al., 2016. Probiotic legacy effects on gut microbial assembly in tilapia larvae. Sci. Rep. 6, 33965. https://doi.org/10.1038/srep33965.
Gorokhova, Rivetti, Furuhagen, Edlund, Ek, Breitholtz, 2015. Bacteria-mediated effects of antibiotics on daphnia nutrition. Environ. Sci. Technol. 49, 5779–5787.
Grotmol, Dahl-Paulsen, Totland, 2003. Hatchability of eggs from Atlantic cod, turbot and Atlantic halibut after disinfection with ozonated seawater. Aquaculture 221, 245–254.
Grunwald, D.J., Eisen, J.S., 2002. Headwaters of the zebrafish — emergence of a new model vertebrate. Nat. Rev. Genet. 3, 717–724. https://doi.org/10.1038/nrg892.
Guillard, R.R.L., 1959. Further evidence of the destruction of bivalve larvae by bacteria. Biol. Bull. 117, 258–266.
Guo, X., Ran, C., Zhang, Z., et al., 2017. The growth-promoting effect of dietary nucleotides in fish is associated with an intestinal microbiota-mediated reduction in energy expenditure. J.Nutr. 147, 781–788. https://doi.org/10.3945/jn.116.245506.
Guo, X., Li, J., Ran, C., et al., 2019. Dietary nucleotides can directly stimulate the immunity of zebrafish independent of the intestinal microbiota. Fish. Shellfish Immunol. 86, 1064–1071. https://doi.org/10.1016/j.fsi.2018.12.058.
Harboe, T., Huse, I., Øie, G., 1994. Effects of egg disinfection on yolk sac and first feeding stages of halibut (*Hippoglossus hippoglossus* L.) larvae. Aquaculture 119, 157–165. https://doi.org/10.1016/0044-8486(94)90172-4.
Haygood, A.M., Jha, R., 2018. Strategies to modulate the intestinal microbiota of tilapia (Oreochromis sp.) in aquaculture: a review. Rev. Aquac. 10, 320–333.
Ikeda-Ohtsubo, W., Brugman, S., Warden, C.H., et al., 2018. How can we define "optimal microbiota?": a comparative review of structure and functions of microbiota of animals, fish, and plants in agriculture. Front. Nutr. 5, 90. https://doi.org/10.3389/fnut.2018.00090.
Ingerslev, H.C., Strube, M.L., Jorgensen, L.V., et al., 2014. Diet type dictates the gut microbiota and the immune response against Yersinia ruckeri in rainbow trout (Oncorhynchus mykiss). Fish. Shellfish Immunol. 40, 624–633. https://doi.org/10.1016/j.fsi.2014.08.021.
Jemielita, M., Taormina, M.J., Burns, A.R., et al., 2014. Spatial and temporal features of the growth of a bacterial species colonizing the zebrafish gut. MBio. 5https://doi.org/10.1128/mBio.01751-14. (e01751–14).
Jiang, W.D., Hu, K., Zhang, J.X., et al., 2015. Soyabean glycinin depresses intestinal growth and function in juvenile Jian carp (Cyprinus carpio var Jian): protective effects of glutamine. Br. J. Nutr. 114, 1569–1583. https://doi.org/10.1017/S0007114515003219.
Jung, Hagiwara, 2001. The effect of bacteria on interspecific relationships between the euryhaline rotifer Brachionus rotundiformis and the harpacticoid copepod Tigriopus japonicus. Hydrobiologia 446 (447), 123–127.
Kanther, M., Sun, X., Muhlbauer, M., et al., 2011. Microbial colonization induces dynamic temporal and spatial patterns of NF-kappaB activation in the zebrafish digestive tract. Gastroenterology. 141, 197–207.
Khodabandeh, S., Abtahi, B., 2006. Effects of sodium chloride, formalin and iodine on the hatching success of common carp, *Cyprinus carpio*, eggs. J. Appl. Ichthyol.
Koch, B.E.V., Yang, S., Lamers, G., et al., 2018. Intestinal microbiome adjusts the innate immune setpoint during colonization through negative regulation of MyD88. Nat. Commun. 9, 4099. https://doi.org/10.1038/s41467-018-06658-4.
Langdon, C.J., 1983. Growth studies with bacteria-free oyster (crassostrea gigas) larvae fed on semi-defined artificial diets. Biol. Bull. 164, 227–235.
Lasa, A., di Cesare, A., Tassistro, G., et al., 2019. Dynamics of the Pacific oyster pathobiota during mortality episodes in Europe assessed by 16S rRNA gene profiling and a new target enrichment next-generation sequencing strategy. Environ. Microbiol. https://doi.org/10.1111/1462-2920.14750.
Lesel, R., Lesel, M., 1976. Obtention d'alevins non vésiculés axéniques de salmonides. Ann. Hydrobiol. 7, 21–25.
Leulier, F., MacNeil, L.T., Lee, W.J., et al., 2017. Integrative physiology: at the crossroads of nutrition, microbiota, animal physiology, and human health. Cell Metab. 25, 522–534. https://doi.org/10.1016/j.cmet.2017.02.001.
Ley, R.E., Hamady, M., Lozupone, C., et al., 2008. Evolution of mammals and their gut microbes. Science 320, 1647–1651.
Li, X., Yang, Q., Dierckens, K., et al., 2014. RpoS and indole signaling control the virulence of vibrio anguillarum towards Gnotobiotic Sea bass (Dicentrarchus labrax) larvae. PLoS ONE 9, e111801. https://doi.org/10.1371/journal.pone.0111801.
Logan, S.L., Thomas, J., Yan, J., et al., 2018. TheVibrio choleraetype VI secretion system can modulate host intestinal mechanics to displace gut bacterial symbionts. Proc. Natl. Acad. Sci. U. S. A. 115, E3779–E3787. https://doi.org/10.1073/pnas.1720133115.
Macke, Callens, Meester, De, Decaestecker, 2017. Host-genotype dependent gut microbiota drives zooplankton tolerance to toxic cyanobacteria. Nat. Commun. 8, 1608.
Marking, Rach, Schreier, 1994. American fisheries society evaluation of antifungal agents for fish culture. Progress. Fish-Cult. 56, 225–231.
Marques, A., Ollevier, F., Verstraete, W., et al., 2006. Gnotobiotically grown aquatic animals: opportunities to investigate host-microbe interactions. J. Appl. Microbiol. 100, 903–918.
Martin, S.A.M., Krol, E., 2017. Nutrigenomics and immune function in fish: new insights from omics technologies. Dev. Comp. Immunol. 75, 86–98. https://doi.org/10.1016/j.dci.2017.02.024.
McFall-Ngai, M., 2006. Love the one you're with: vertebrate guts shape their microbiota. Cell. 127, 247–249.
Melancon, E., Gomez De La Torre Canny, S., Sichel, S., et al., 2017. Best practices for germ-free derivation and gnotobiotic zebrafish husbandry. Methods Cell Biol. 138, 61–100.
Milliar, R.H., Scott, J.M., 1967. Bacteria-free culture of oyster larvae. Nature 216, 1139–1140.
Milligan-Myhre, K., Charette, J.R., Phennicie, R.T., et al., 2011. Study of host-microbe interactions in zebrafish. Methods Cell Biol. 105, 87–116.
Milligan-Myhre, K., Small, C.M., Mittge, E.K., et al., 2016. Innate immune responses to gut microbiota differ between oceanic and freshwater threespine stickleback populations. Dis. Model. Mech. 9, 187–198.
Morgun, A., Dzutsev, A., Dong, X., et al., 2015. Uncovering effects of antibiotics on the host and microbiota using transkingdom gene networks. Gut. 64, 1732–1743. https://doi.org/10.1136/gutjnl-2014-308820.
Munro, P.D., Barbour, A., Birkbeck, T.H., 1995. Comparison of the growth and survival of larval turbot in the absence of culturable bacteria with those in the presence of vibrio anguillarum, vibrio alginolyticus, or a Marine Aeromonas sp. Appl. Environ. Microbiol. 61, 4425–4428.
Murdoch, C.C., Espenschied, S.T., Matty, M.A., et al., 2019. Intestinal serum amyloid a suppresses systemic neutrophil activation and bactericidal activity in response to microbiota colonization. PLoS Pathog. 15, e1007381. https://doi.org/10.1371/journal.ppat.1007381.
Navarrete, P., Mardones, P., Opazo, R., et al., 2008. Oxytetracycline treatment reduces bacterial diversity of intestinal microbiota of Atlantic salmon. J. Aquat. Anim. Health 20, 177–183.
O'Hara, A.M., Shanahan, F., 2006. The gut flora as a forgotten organ. EMBO Rep. 7, 688–693.
Overton, Bruun, Dalsgaard, 2010. Chemical surface disinfection of eggs of Baltic cod, *Gadus morhua* L. J. Fish Dis. 33, 707–716.
Oyarbide, U., Iturria, I., Rainieri, S., et al., 2015. Use of gnotobiotic zebrafish to study vibrio anguillarum pathogenicity. Zebrafish. 12, 71–80. https://doi.org/10.1089/zeb.2014.0972.
Pavlov, Moksness, 1993. Bacterial destruction of common wolffish during incubation. Aquac. Int. 1, 178–186.
Peck, Buckley, O'Bryan, Davies, Lapolla, 2004. Efficacy of egg surface disinfectants in captive spawning Atlantic cod *Gadus morhua* L. and haddock *Melanogrammus aeglefinus* L. Aquac. Res. 35, 992–996.
Pham, L.N., Kanther, M., Semova, I., et al., 2008. Methods for generating and colonizing gnotobiotic zebrafish. Nat. Protoc. 3, 1862–1875. https://doi.org/10.1038/nprot.2008.186.
Phelps, D., Brinkman, N.E., Keely, S.P., et al., 2017. Microbial colonization is required for normal neurobehavioral development in zebrafish. Sci. Rep. 7, 11244. https://doi.org/10.1038/s41598-017-10517-5.
Provasoli, D'Agostino, 1969. Development of artificial media for *Artemia Salina*. Biol. Bull. 136, 434–453.
Provasoli, Shiraishi, 1959. Axenic cultivation of the brine shrimp *Artemia salina*. Biol. Bull. 117, 347–355.
Qin, C., Zhang, Z., Wang, Y., et al., 2017. EPSP of L. casei BL23 protected against the infection caused by Aeromonas veronii via enhancement of immune response in zebrafish. Front. Microbiol. 8, 2406. https://doi.org/10.3389/fmicb.2017.02406.
Rach, Gaikowski, Howe, Schreier, 1998. Evaluation of the toxicity and efficacy of hydrogen peroxide treatments on eggs of warm- and coolwater fishes. Aquaculture 165, 11–25.
Rahat, Diementman, 1982. Cultivation of bacteria-free *Hydra viridis*: missing budding factor in nonsymbiotic hydra. Science 216, 67–68.
Ran, C., Huang, L., Liu, Z., et al., 2015. A comparison of the beneficial effects of live and heat-inactivated Baker's yeast on Nile tilapia: suggestions on the role and function of the secretory metabolites released from the yeast. PLoS ONE 10, e0145448. https://doi.org/10.1371/journal.pone.0145448.
Ran, C., Hu, J., Liu, W., et al., 2016. Thymol and carvacrol affect hybrid tilapia through the combination of direct stimulation and an intestinal microbiota-mediated effect: insights from a germ-free zebrafish model. J. Nutr. 146, 1132–1140. https://doi.org/10.3945/jn.115.229377.
Ran, C., Qin, C., Xie, M., et al., 2018. Aeromonas veronii and aerolysin are important for the pathogenesis of motile aeromonad septicemia in cyprinid fish. Environ. Microbiol. 20, 3442–3456. https://doi.org/10.1111/1462-2920.14390.
Rawls, J.F., Samuel, B.S., Gordon, J.I., 2004. Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. Proc. Natl. Acad. Sci. U. S. A. 101, 4596–4601.
Rawls, J.F., Mahowald, M.A., Ley, R.E., et al., 2006. Reciprocal gut microbiota transplants from zebrafish and mice to germ-free recipients reveal host habitat selection. Cell. 127, 423–433.
Rawls, J.F., Mahowald, M.A., Goodman, A.L., et al., 2007. In vivo imaging and genetic analysis link bacterial motility and symbiosis in the zebrafish gut. Proc. Natl. Acad. Sci. U. S. A. 104, 7622–7628.
Rekecki, A., Dierckens, K., Laureau, S., et al., 2009. Effect of germ-free rearing environment on gut development of larval sea bass (Dicentrarchus labrax L.). Aquaculture. 293, 8–15.
Rekecki, A., Gunasekara, R.A.Y.S.A., Dierckens, K., et al., 2012. Bacterial host interaction of GFP-labelled vibrio anguillarum HI-610 with gnotobiotic sea bass, Dicentrarchus labrax (L.), larvae. J. Fish Dis. 35, 265–273.
Rendueles, O., Ferrieres, L., Fretaud, M., et al., 2012. A new zebrafish model of oro-intestinal pathogen colonization reveals a key role for adhesion in protection by probiotic bacteria. PLoS Pathog. 8, e1002815. https://doi.org/10.1371/journal.ppat.1002815.
Ribas, L., Piferrer, F., 2014. The zebrafish (Danio rerio) as a model organism, with emphasis on applications for finfish aquaculture research. Rev. Aquac. 6, 209–240.
Robinson, C.D., Klein, H.S., Murphy, K.D., et al., 2018. Experimental bacterial adaptation to the zebrafish gut reveals a primary role for immigration. PLoS Biol. 16, e2006893. https://doi.org/10.1371/journal.pbio.2006893.
Rolig, A.S., Parthasarathy, R., Burns, A.R., et al., 2015. Individual members of the microbiota disproportionately modulate host innate immune responses. Cell Host

Microbe 18, 613–620.
Romero, J., Navarrete, P., 2006. 16S rDNA-based analysis of dominant bacterial populations associated with early life stages of coho salmon (Oncorhynchus kisutch). Microb. Ecol. 51, 422–430.
Ruiz, V.E., Battaglia, T., Kurtz, Z.D., et al., 2017. A single early-in-life macrolide course has lasting effects on murine microbial network topology and immunity. Nat. Commun. 8, 518. https://doi.org/10.1038/s41467-017-00531-6.
Rungrassamee, W., Klanchui, A., Maibunkaew, S., et al., 2014. Characterization of intestinal bacteria in wild and domesticated adult black tiger shrimp (Penaeus monodon). PLoS ONE 9, e91853. https://doi.org/10.1371/journal.pone.0091853.
Russo, P., Iturria, I., Mohedano, M.L., et al., 2015. Zebrafish gut colonization by mCherry-labelled lactic acid bacteria. Appl. Microbiol. Biotechnol. 99, 3479–3490.
Salvesen, I., Øie, G., Vadstein, O., 1997. Surface disinfection of Atlantic halibut and turbot eggs with glutaraldehyde: evaluation of concentrations and contact times. Aquac. Int. 5, 249–258.
Schlomann, B.H., Wiles, T.J., Wall, E.S., et al., 2018. Bacterial cohesion predicts spatial distribution in the larval zebrafish intestine. Biophys. J. 115, 2271–2277.
Semova, I., Carten, J.D., Stombaugh, J., et al., 2012. Microbiota regulate intestinal absorption and metabolism of fatty acids in the zebrafish. Cell Host Microbe 12, 277–288.
Shan, Y., Fang, C., Cheng, C., et al., 2015. Immersion infection of germ-free zebrafish with listeria monocytogenes induces transient expression of innate immune response genes. Front. Microbiol. 6, 373.
Shan, Y., Zhang, Y., Zhuo, X., et al., 2016. Matrix metalloproteinase-9 plays a role in protecting zebrafish from lethal infection with listeria monocytogenes by enhancing macrophage migration. Fish. Shellfish Immunol. 54, 179–187.
Shaw, E., 1957. Potentially simple technique for rearing “Grem-free” fish. Science. 125, 987–988.
Sheng, Y., Ren, H., Limbu, S.M., et al., 2018. The presence or absence of intestinal microbiota affects lipid deposition and related genes expression in zebrafish (Danio rerio). Front. Microbiol. 9, 1124.
Siriyappagouder, P., Galindo-Villegas, J., Lokesh, J., et al., 2018. Exposure to yeast shapes the intestinal bacterial community assembly in zebrafish larvae. Front. Microbiol. 9, 1868. https://doi.org/10.3389/fmicb.2018.01868.
Sison-Mangus P., M., Mushegian A., A., Ebert, D., 2014. Water fleas require microbiota for survival, growth and reproduction. The ISME Journal.
Situmorang, M.L., Dierckens, K., Mlingi, F.T., et al., 2014. Development of a bacterial challenge test for gnotobiotic Nile tilapia Oreochromis niloticus larvae. Dis. Aquat. Org. 109, 23–33.
Soto-Rodriguez, Roque, Lizarraga-Partida, Guerra-Flores, Gomez-Gil, 2003. Virulence of luminous vibrios to *Artemia franciscana* nauplii. Dis. Aquat. Org. 53, 231–240.
Star, B., Haverkamp, T.H., Jentoft, S., et al., 2013. Next generation sequencing shows high variation of the intestinal microbial species composition in Atlantic cod caught at a single location. BMC Microbiol. 13, 248. https://doi.org/10.1186/1471-2180-13-248.
Stephens, W.Z., Wiles, T.J., Martinez, E.S., et al., 2015. Identification of population bottlenecks and colonization factors during assembly of bacterial communities within the zebrafish intestine. Mbio. 6https://doi.org/10.1128/mBio.01163-15. (e01163–01115).
Stuart, Keller, Drawbridge, 2010. Efficacy of formalin and povidone-iodine disinfection techniques on the eggs of three marine finfish species. Aquac. Res. 41, e838–e843.
Surana, N.K., Kasper, D.L., 2017. Moving beyond microbiome-wide associations to causal microbe identification. Nature. 552, 244–247. https://doi.org/10.1038/nature25019.
Tan, F., Limbu Mchele, S., Qian, Y., Qiao, F., Du, Z.-Y., Zhang, M., 2019. The responses of germ-Free zebrafish (Danio rerio) to varying bacterial concentrations, colonization time points, and exposure duration. Frontiers in Microbiology.
Toh, M.C., Goodyear, M., Daigneault, M., et al., 2013. Colonizing the embryonic zebrafish gut with anaerobic bacteria derived from the human gastrointestinal tract. Zebrafish. 10, 194–198. https://doi.org/10.1089/zeb.2012.0814.
Trust, T.J., 1974. Sterility of salmonid roe and practicality of hatching gnotobiotic salmonid fish. Appl. Microbiol. 28, 340–341.
Tveteras, S., Asche, F., Bellemare, M.F., et al., 2012. Fish is food - the FAO’s fish price index. PLoS ONE 7, e36731. https://doi.org/10.1371/journal.pone.0036731.
Ulloa, P.E., Medrano, J.F., Feijoo, C.G., 2014. Zebrafish as animal model for aquaculture nutrition research. Front. Genet. 5, 313. https://doi.org/10.3389/fgene.2014.00313.
Verner-Jeffreys, D.W., Shields, R.J., Birkbeck, T.H., 2003. Bacterial influences on Atlantic halibut Hippoglossus hippoglossus yolk-sac larval survival and start-feed response. Dis. Aquat. Org. 56, 105–113.
Wagner, Oplinger, Arndt, Forest, Bartley, 2010. The safety and effectiveness of various hydrogen peroxide and iodine treatment regimens for rainbow trout egg disinfection. N. Am. J. Aquac. 72, 34–42.
Wagner, Bartley, Oplinger, 2012a. Egg disinfection to improve conservation aquaculture of leatherside chub. N. Am. J. Aquac. 74, 199–207.
Wagner, Oplinger, Bartley, 2012b. Effect of single or double exposures to hydrogen peroxide or iodine on salmonid egg survival and bacterial growth. N. Am. J. Aquac. 74, 84–91.
Wang, A.R., Ran, C., Ringo, E., et al., 2018. Progress in fish gastrointestinal microbiota research. Rev. Aquac. 10, 626–640.
Wong, S., Rawls, J.F., 2012. Intestinal microbiota composition in fishes is influenced by host ecology and environment. Mol. Ecol. 21, 3100–3102.
Wong, S., Stephens, W.Z., Burns, A.R., et al., 2015. Ontogenetic differences in dietary fat influence microbiota assembly in the zebrafish gut. Mbio. 6, e00687.
Wu, S., Wang, G., Angert, E.R., et al., 2012. Composition, diversity, and origin of the bacterial community in grass carp intestine. PLoS ONE 7, e30440.
Zhang, M., Chekan, J.R., Dodd, D., et al., 2014a. Xylan utilization in human gut commensal bacteria is orchestrated by unique modular organization of polysaccharide-degrading enzymes. Proc. Natl. Acad. Sci. U. S. A. 111, E3708–E3717.
Zhang, M.L., Sun, Y.H., Chen, L.Q., et al., 2014b. Characterization of the intestinal microbiota in Pacific white shrimp, Litopenaeus vannamei, fed diets with different lipid sources. Aquaculture. 434, 449–455.
Zhang, M.L., Sun, Y.H., Chen, L.Q., et al., 2016. Symbiotic bacteria in gills and guts of Chinese mitten crab (Eriocheir sinensis) differ from the free-living bacteria in water. PLoS ONE 11, e0148135.
Zhao, L., Zhang, F., Ding, X., et al., 2018. Gut bacteria selectively promoted by dietary fibers alleviate type 2 diabetes. Science. 359, 1151–1156.