# Time to integrate biotechnological approaches into fish gut microbiome research

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# Time to integrate biotechnological approaches into fish gut microbiome research

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Like for other vertebrates, the fish microbiome is critical to the health of its host and has complex and dynamic interactions with the surrounding environment. Thus, the study of the fish microbiome can benefit from the new prospects gained by innovative biotechnological applications in human and other animals, that include manipulation of the associated microbial communities (to improve the health, productivity, and sustainability of fish production), *in vitro* gut simulators, synthetic microbial communities, and others. Here, we summarize the current state of knowledge on such biotechnological approaches to better understand and engineer the fish microbiome, as well as to advance our knowledge on host–microbes interactions. A particular focus is given to the most recent strategies for fish microbiome manipulation to improve fish health, food safety and environmental sustainability.

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## Introduction

Fish have unique and relatively stable interactions with the vast variety of microorganisms that surround them, inhabit their skin, gills, and gastrointestinal tract [1•]. While fish microbiome research, particularly in their gut, dates back to pioneer studies in the early half of the 20th century [2] and to later descriptive papers [3], knowledge on fish-associated microbes has grown significantly in the last two decades, due to the advent of nucleic acids-based techniques to describe aquatic prokaryotes [4], coupled to the exponential growth of aquaculture. Most knowledge on fish microbiome has, indeed, been gained from aquaculture, an industry representing one of the fastest growing and highly traded food sectors globally [5•], with volumes predicted to double the current production by 2050.

Teleost microbiome research lags well behind that in humans and mouse models [6•,7•] and current knowledge of fish microbiome is still far from being complete. Yet, there is considerable and growing interest in understanding more on this exciting topic, from both the standpoint of basic research and biotechnological applications. Several studies have accumulated in the last years, most of which carried out from an observational perspective and focused on gaining an understanding of microbes associated to the gut [6•,7•], where the vast majority of microbial biomass is located (typically $10^7$ to $10^{11}$ bacterial cells per gram of intestinal content). Moreover, gut microbes have an important impact on the host health through their involvement in biological processes such as nutrient processing, detoxification, gut motility modulation, immune function, development, and mucosal tolerance [6•,8]. A recent review highlighted that gut bacterial communities have been assessed in over 145 species of teleosts from 111 genera [8]. Yet, a very high frequency of studies is dealing with aquaculture fish [9,7•], due to the increasing importance of this industry as source of animal protein in the global food supply and the continuous growth of the seafood farming. In addition, the aquaculture setting serves as a large-scale controlled experimental environment where fish can grow in their full life cycle under various conditions that can be manipulated providing, thus, a unique research opportunity that cannot be met wild fish investigations. Other studies in both aquaculture and wild fish have focused on microbial topographies, that is, diverse niches throughout the animal body other than the gut, such as skin [10–12], that represent habitats for specific host-associated microbes and where the role of microbes remains today mostly underexplored.

Despite the increasing research effort on the fish microbiome over the years, the notion that studying the fish microbiome is extremely challenging still lingers This holds particularly true when studies are performed directly in nature, given that aquatic environments and

**Glossary**
**Gnotobiotic fish:** Microbe-free fish which have derived from sterile eggs and have grown under sterile conditions.
**Gut-on-a-chip:** A microfluidics-based technological device which allows engineering manipulations of an artificial gut system.
**Gut simulatorA:** n artificial *in vitro* dynamic construction that mimics the various stages of food processing along the gastrointestinal tract.
**Organoids:** A 3D *in vitro* produced tissue that mimics its corresponding *in vivo* organ.
**Fecal Material Transplant (FMT):** Any practice which involves the transfer of fecal material from one individual to another one, usually for treating a dysbiotic condition.
**Phage Therapy:** The use of bacteriophages for treating a specific bacterial pathogen.

the microbial assemblages in those environments are highly dynamic and diverse, making it difficult to monitor microorganisms outside the fish body. Research has also highlighted that individual variability in gut fish microbiome may be conspicuous within the same species, fish population or even under the same dietary conditions [13,14•]. Moreover, different microbial compositions may also exist among the different types of fish alimentary canals and along the different gut parts, which can be differently affected by manipulation [1•,15]. All these aspects make it extremely difficult to generalize the results obtained from one fish species to all the others. The coupling of animal welfare issues [16] and the increasing restrictions in the use of experimental animals [17], along with the climate change impacts on marine biota [18••], leads to an increasing interest in *in vitro* and *ex vivo* approaches, emerging as particularly helpful alternatives to gain knowledge on the fish gut microbiome and their relation to nutrition, growth and health. Among these approaches, *ex-vivo* in combination with *in vivo* trials will increase our understanding of the role of the fish gut microbiome in digestion and gut health. *In vitro* and *ex vivo* testing allows expansion or generation of research hypotheses rather than overly testing similar quests, while microbial growth and microbial interactions of inferred potential beneficial microbes can be tested more accurately. Therefore, the integration of ecological information that is obtained through population level microbiome studies with physiological information obtained through *in vitro* and *in vivo* experiments [19•], may help designing better microbiome modulation strategies, as it is being done for humans [20] with synthetic and minimal microbiomes.

In this opinion, we summarize the current knowledge and advances on the biotechnological approaches to explore the fish gut microbiome, by focusing mostly on the available knowledge, but also on future research trajectories of experimental, manipulative and engineering approaches in the fish gut ecosystem. Within this framework, we place attention on experimental rather than observational studies (for which excellent reviews have been recently made available, some cited above), and discuss understanding and future perspectives of studies that have focused on fish microbiome manipulation and engineering for disease control and fish production optimization.

## Use of gnotobiotic models to understand host–microbe interactions

Gnotobiotic fish models have emerged over the past 20 years as an excellent tool to study host–microbe interactions, with the zebrafish (*Danio rerio*) being the first fish species that protocols were established [21] (Table 1). Most research so far has been performed on zebrafish, where protocols to rear sterile zebrafish for up to 30 days exist [21], thus, providing excellent opportunities to explore host–microbe interactions in a more mechanistic approach. Using gnotobiotic zebrafish models, important aspects of host–microbe interactions were verified so far, such as the positive impact of microbial colonization on enterocyte renewal, along with the effect on nutrient metabolism and development of the innate immune system [21,22••]. Moreover, gnotobiotic models were used to understand host habitat selection of the gut microbiome, after reciprocal gut microbial transplants between gnotobiotic zebrafish and mice [22••], highlighting the importance of such tools.

Protocols that attempted to establish gnotobiotic fish or larvae were reported already back in 1960s (as reviewed by Zhang *et al.* [23]), with several successful examples in different aquaculture species such as the European seabass (*Dicentrarchus labrax*) [24], Atlantic cod (*Gadus morhua*) [25], Atlantic halibut (*Hippoglossus hippoglossus*) [26], Nile tilapia (*Oreochromis niloticus*) [27] and rainbow trout (*Oncorynchus mykiss*) [28]. Although most of these protocols are established at the yolk-sac larvae stage (before external feeding), important findings have been reported so far regarding to gut microbiome importance for disease resistance [29,28]. More specifically, the use of monoassociated or defined communities consisting of several microbes to understand the impact of specific microbial population in the gut will enable us to disentangle fish–microbe interactions and better understand the role of certain communities such as probiotic strains. However, due to the health status of these animals and the limitation regarding the breeding and maintenance of gnotobiotic models, such a tool can only be limited to the early life stages of the animals. Therefore, such studies can be combined with *in vitro* approaches in order to be able to target-specific questions with relation to defined microbial communities.

## Tools to study *in vitro* and *ex vivo* microbial interactions

Gnotobiotic models are an important tool to study host–microbe interactions, however, before selecting the communities to study in gnotobiotic models, it is important to understand the nature of microbial interactions in the gut.

**Table 1**

**Overview of biotechnological approaches to explore fish microbiome, including details on the fish species tested, the scientific aims and the most important outcomes from research in the field**

| Biotechnological approach | Fish species tested | Scientific aim(s) | Main knowledge acquired | Reference |
|---|---|---|---|---|
| GNOTOBIOTIC FISH | Zebrafish (*Danio rerio*) - post fertilization to late juvenile stages | To investigate the molecular mechanisms underlying biological processes such as nutrient processing and absorption, development of the mucosal immune system, angiogenesis, and epithelial renewal, linked to gut microbiota | (i) more than 200 genes regulated by the microbiota (stimulation of epithelial proliferation, promotion of nutrient metabolism, and innate immune responses); (ii) colonization of germ-free zebrafish with individual members of its microbiota revealed the bacterial species specificity of selected host responses | Rawls et al., 2004 |
| | Atlantic cod (*Gadus morhua*) - larvae | (i) To set up a protocol to generate bacteria-free Atlantic cod larvae; (ii) to set up an experimental system that allows addition of live feed to the larvae without compromising the gnotobiotic state | First protocol to generate bacteria-free cod larvae, and first protocol for marine larvae to be independent on continued addition of antibiotics | Forberg et al., 2011 |
| | Seabass (*Dicentrarchus labrax*) - larvae | To test *Listonella anguillarum* and *Aeromonas hydrophila* strains on gnotobiotic seabass to assess their impact on fish mortality | Only a few of the tested strains were connected to fish mortality | Dierckens et al., 2009 |
| | Atlantic halibut (*Hippoglossus hippoglossus*) - larvae | (i) Assessing the toxicity of 20 bacterial isolates from halibut hatcheries towards halibut yolk-sac larvae; (ii) assessing the activity of bacterial growth inhibiting strains in protecting halibut yolk-sac larvae against invasion by *V. anguillarum*; (iii) investigating how particular bacteria influence their start-feed response | (i) most of the bacteria (except a *V. anguillarum* strain) routinely isolated from halibut hatcheries are not harmful to yolk-sac larvae and (ii) no protective effect against *V. anguillarum*; (iii) bacterial contamination of the live food does not appear to influence initiation of the feeding response. | Verner-Jeffreys et al., 2003 |
| | Nile tilapia (*Oreochromis niloticus*) - larvae | To develop a gnotobiotic Nile tilapia larvae model system to investigate the impact of microbes under controlled conditions | The developed model can be used as a tool to extend understanding of the mechanisms involved in host–microbe interactions and to evaluate new methods of disease control | Situmorang et al., 2014 |
| | Rainbow trout (*Oncorhynchus mykiss*) - larvae | To explore host-microbiota-pathogen interactions in a germ-free and gnotobiotic model of rainbow trout. | (i) germ-free larvae were extremely sensitive to infection by *Flavobacterium* columnare, a common freshwater fish pathogen; (ii) recolonization with 11 species from trout microbiota conferred resistance to *F. columnare* infection; (iii) single strain colonization of germ-free trout highlighted that this protection was determined by a *Flavobacterium* strain | Perez-Pascual et al., 2021 |
| *IN VITRO* AND *EX-VIVO* MANIPULATION | Rainbow trout (*O. mykiss*) | To setup a new method for the establishment and long-term maintenance of *ex vivo* cultures from intestinal regions of rainbow trout | (i) metabolic characterisation of cells; (ii) basic morphology of growing cells characterised by histology, immunofluorescence, TEM and TEER; (iii) significant regional differences in intestinal enzymatic activities after exposure to model inducers were found | Langan et al., 2018 |
| | Rainbow trout (*O. mykiss*) | To realize the first fish-gut-on-chip model, based on the reconstruction of the intestinal barrier of rainbow trout in an artificial microenvironment | (i) a controllable innovative microfluidic platform to study critical barrier functions in the presence of relevant physiological cues was realized; (ii) physiological, realistic fluid flow and shear stress was sufficient to promote stable intestinal epithelial tightening; (iii) the device may facilitate studies of, e.g., xenobiotic uptake or immunological defense mechanisms | Drieschner et al., 2019 |
| | Atlantic salmon (*Salmo salar*) | To develop an in vitro gut model (SalmoSim) to simulate gut compartments and associated microbial communities | (i) the response of the in vitro system to two different diets were comparable to a parallel in vivo trial in real salmon | Kazlauskaite et al., 2021 |
| | Medaka (*Oryzias latipes*) and zebrafish (*D. rerio*) | To derive organoids from rapidly developing teleosts | (i) primary embryonic stem cells from zebrafish and medaka efficiently self-organized into anterior neural structures, particularly retina; (ii) within days, cell aggregates executed key steps of eye development; (iii) the number of aggregated cells and genetic factors impacted the morphological changes reflecting the in vivo situation; (iv) the system was highly reproducible | Zilova et al., 2021 |
| FECAL MATERIAL TRANSPLANT | Zebrafish (*D. rerio*) | To test how factors specific to host gut habitat shape microbial community structure by performing reciprocal transplantations of microbiotas into germ-free zebrafish and mouse recipients | (i) communities assembled in predictable ways; (ii)the transplanted community resembled its community of origin in terms of the lineages present, but with relative abundance resembling those of normal gut microbial community composition of the recipient host; (iii) differences in community structure between zebrafish and mice arise in part from distinct selective pressures imposed within the gut habitat of each host | Rawls et al. 2006 |
| | Zebrafish (*D. rerio*) | To evaluate if zebrafish larvae can be colonized by human gut microorganisms | (i) some members of human gut microbiota were transferred to zebrafish larvae; (ii) the sporulating bacteria *Bacillus clausii* and Clostridioides difficile were the most persistent microorganisms | Valenzuela et al., 2018 |
| | African turquoise killifish (*Nothobranchius furzeri*) | To apply FMT from young to old fish to understand the role of the gut microbiota during host aging | (i) the gut microbiota played a key role in modulating vertebrate life span; (ii) recolonizing the gut of middle-age individuals with bacteria from young donors resulted in life span extension and delayed behavioral decline by contrasting the decrease in microbial diversity associated with host aging and maintaining a young-like gut bacterial community | Smith et al., 2017 |

Physiological parameters and host selective pressure along with nutritional effects may affect those communities and their dynamics. Understanding microbial interactions and their dynamics *in vitro* may offer a better understanding of the prevalence of certain microbial communities, that is, the core communities [19•], trophic interactions in the fish gut [20] or even assist in the development of next generation probiotics [30]. Therefore, the development of *in-vitro* gut simulations can provide an important tool for mechanistic microbiome research, by closely mimicking the *in vivo* situation, trying to reproduce the physiological parameters of the gut environment that influence the microbial communities. Moreover, using such simulators in an *in vitro* setting, the dynamics of the microbial communities can be studied over time, separated from the host physiological impact. There are several gut simulators developed for the human gastrointestinal tract [31–33], while recently, gut simulators for poultry were also reported [34]. In fish, although several *in vitro* gut simulators that imitate digestion were developed over the years for several fish species [35], only recently an *in vitro* gut microbiome simulator, SalmoSim, was developed by Kazlauskaite *et al.* [36••] (Table 1). SalmoSim simulates the stomach, the pyloric caeca, and the midgut regions of the gastrointestinal tract of farmed Atlantic salmon. Such a tool that can maintain stable microbial communities *in vitro* can be used to study fundamental ecological processes that underpin microbiome dynamics and assembly for multiple fish species. In combination with synthetic communities, or the minimal microbiome concept, a mechanistic understanding of the microbial networks and their role in ecosystem functioning is possible, as is already happening in humans and mice.

To understand complex interactions between the hosts and their associated microbiomes, tools to measure the direct interactions between the gut microbial communities and the host cell responses are important. To achieve this, methods that can sustain these complex microbial communities in direct contact with mucosal intestinal cells *in vitro* enable the investigation of host–microbiome interactions. Several existing *in vitro* models, such as Transwell inserts, have been used to study host–microbe interactions in human studies, but with limitations in the duration, since within a few hours bacterial growth negatively affects cell growth [37]. The development of gut-on-a chip or intestinal organoids can serve as important tools towards that direction. Gut-on-a-chip concept was developed for human gut models around 10 years ago, which uses microfluidic technology, by reconstructing the intestinal barrier using intestinal cell lines in an artificial controlled microenvironment [38]. Only recently the full complexity of the gut microbiota was able to be co-cultured in intestinal organoids including aerobic and anaerobic communities, by imitating the physiological conditions and low oxygen level in the human intestine, successfully maintained for up to five days within the chip [39]. Intestinal organoids can be also an *in vitro* tool to study host–microbe interactions and are currently developed in several farming animal studies besides human cultures [40–42]. The limitation of this model is that the period of co-culturing with microbes within the organoids, as it has been reported for the time being, is usually short (around 1 day) and moreover, they do not sustain low oxygen levels, which is important to grow anaerobic bacteria [43]. More recently a successful development of a gut-on-a-chip was reported in fish using cell lines from rainbow trout [44••], while attempts to develop organoids for fish have been also reported [40,45]. Although currently none of these tools involve microbial communities, these tools could be of great value for future microbiome research.

To further understand the impact of microbiome modulation, current microbiome engineering methods can be used to introduce a specific perturbation to cause intentional shifts in human studies [46]. Such perturbations can be either biotic (microbial transplants, probiotics, phages) or abiotic (dietary changes, antibiotics/xenobiotics use). Moreover, a combination of gnotobiotic fish models with defined communities, selected from *in vitro* simulations, with or without applied perturbations, can enhance our understanding in host–microbe interactions, and assist in defining the role of those communities for the host.

## Manipulating the fish microbiome for disease control

Gut manipulation for health promotion of humans and animals, includes indirect (diet, prebiotic, probiotic and synbiotic dietary inclusion, antibiotics and antimicrobials) and direct (fecal material transplant, FMT) practices. FMT has been applied in fish model organisms for understanding animal–microbe interactions [47•] (Table 1). Reciprocal fecal transplant involving fish and other animal species, has been applied for experimental reasons showing gut microbiota assembly mechanisms [22••], colonization of human beneficial microbes in zebrafish [48,49]. A fish targeted FMT study has shown that the older African turquoise killifish (*Nothobranchius furzeri*) individuals benefited after transplant with fecal material from younger individuals which actually extend life longevity [50]. These studies, along with the available knowledge on the farmed fish species microbiomes [7•] set the perspectives for FMT application in real-world scale for the aquaculture sector.

The major positive perspectives of FMT in aquaculture include:

a) to directly evaluate the impact of an almost indigenous microbiome specific for the reared fish species, from lab to real-world scale

b) to use fecal material from other species which are known to be beneficial for the specific species
c) to supplement the fecal material with external probiotic or beneficial strains, selected by their requirements and how they perform in the specific fish species
d) can be used only when in need, in contrary with the constant dietary supply of beneficial microbes
e) it is a practice that is aligned with some of the principles of organic aquaculture [51].

As every biological intervention, FMT, is not free of limitations and especially for farmed fish is expected to be slightly more challenging [52••] than for humans and other domesticated animals due several reasons:

a) as fish microbiomes seem to be host-specific, that is, each fish species selects for its own microbiome [22••], the need for species-specific microbiome manipulations is imperative,
b) the high heterogeneity in the strategies being currently used [53], dictates for streamlined and standardized protocols to avoid diverging primary data that require deep knowledge of the microbiomes of healthy and diseased fish in specific life stages/phases of rearing,
c) any potential impacts on the aquatic environment of the farmed fish after FMT interventions (e.g. risk assessment of uncontrolled release of the transferred microorganisms) should be assessed and minimised as possible,
d) issues of animal ethics regarding FMT should also need to be recognised and clarified specifically for fish.

It is noteworthy that alternative, indirect strategies to modulate microbiome for disease control are being currently explored and acting on the environmental (e.g. water, sediment) microbiome. Exposure of fish to environmental free-living microbes, such as probiotic strains added to the water, has been performed for some farmed fish species, including tilapia (*Oreochromis niloticus*) and salmonids [54,55•], indicating an enhanced promotion of immune responses, growth rates and protection against infection [56,55•]. Phage therapy, which has been indicated to be very effective in liquid conditions, is also a promising sustainable solution to control pathogenic aquaculture bacteria [57•] although limitations in the sustainability of this approach is still questionable [58]; tests using phages have been recently applied in aquaculture by immersion on farmed rainbow trout, Atlantic cod and turbot (*Scophthalmus maximus*) [59,60••,61]. In addition, although a limited number of immersion vaccines are today commercially available, fish vaccines are considered a promising tool to indirectly modulate microbiome by acting on fish pathogens in the surrounding environment [62].

## Conclusions

Fish microbiome research is mature enough to move from its 'descriptive era' to more experimental, manipulative and engineering approaches. Because of the variety in fish life cycle and the fish interaction with the highly dynamic aquatic environment, we advocate for a demanding integration of these ever-needed descriptive studies with those based on *in vitro* gut simulators, synthetic microbial communities, and *in vitro* and *in vivo* systems, increasing our predictive potential for improving production and eliminating risks in fish production. Further manipulative approaches, including targeted approaches indirectly modulating the fish microbiome such as phage therapy and vaccination, are prompted to be explored to enable a complete understanding of compositional and functional alterations of the microbiome and their effects on the health and safety of fish. Potential benefits from this approach enhance our understanding and managing practices of public health importance related to novel feed design and test, antimicrobial resistance and transfer, management of pathogens, parasites and pests, and environmental footprint of aquaculture. Advancing knowledge specifically in aquaculture species microbiome will open new doors for the design and implementation of more sustainable, productive, and healthier aquaculture systems, and will facilitate increasing production of aquaculture species under more efficient food production and lower environmental footprints scenarios.

## Conflict of interest statement

Nothing declared.

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Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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