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REVIEW

Fish disease prevention via microbial dysbiosis-associated biomarkers in aquaculture

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Abstract

Infectious diseases are a major burden in aquaculture, and represent a significant yield-limiting factor in production that is costly to manage as well as a potential vector for zoonoses. Over the past decades, a range of new diseases have emerged, alongside increased levels of antibiotic resistance, thus heightening the need for improved disease management methods supportive of the *One Health* concept. Simultaneously, recent advances in Next-Generation Sequencing have increasingly elucidated the role of the microbiome in regulating metabolism, immune function and resilience. Such work has included a plethora of studies on the potential for the management of pathogens through manipulation of the microbiome, as well as related studies of the mechanisms behind host resilience. There is now an increasing robust body of evidence recognizing the importance of a holistic framework in disease aetiology between the host, its environment and colonizing microorganisms, with perturbation increasingly associated with specific dysbiotic states and disease outcomes. Elucidating disease aetiology is a preliminary step towards the development of new prevention methods, with the main goal being early identification of dysbiosis-associated biomarkers prior to any physical signs of the disease. While acknowledging the challenges associated with using key microbial taxa as biomarkers, we review recent advances in the characterization of dysbiosis and associated microbiome signatures in the context of disease development, with an emphasis on early biomarkers for aquaculture disease prevention. Several promising strategies are suggested, including the use of functional genes or metabolic pathways that are conserved between microbial taxa as a potential proxy for homeostasis.

KEYWORDS

aquaculture, biomarkers, disease prevention, dysbiosis, fish, microbiome

1 | INTRODUCTION

For decades, disease control has been synonymous with the eradication of pathogens, a strategy that has hitherto proven to be an unsustainable and ineffective approach as it has increasingly resulted in

antibiotic resistance.^{1,2} In aquaculture especially, even after the complete eradication of pathogens, the risk of reinfection is high, since direct contact with the aqueous environment creates a unique opportunity for pathogen transfer and proliferation. As such, eradication measures must be repeated frequently, at considerable cost, and often

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result in damaging discharge of chemicals or antibiotics to the environment. As a result, effective integrated disease management strategies are urgently needed that are supportive of the *One Health* concept, to ensure human and animal welfare, as well as environmental sustainability.³

Advances in our understanding of microbial community ecology, host genetics, and disease-associated environmental and virulence factors have resulted in a paradigm shift in disease management strategies towards promoting system resilience rather than outright pathogen eradication.^{4,5} Indeed, as described by Snieszko,⁶ a disturbance of the normally balanced interactions between the environment, the host and associated microorganisms, may lead to pathogen invasion and subsequent disease outbreaks. The concept of system resilience thus highlights the fine line between commensal and pathogenic microorganisms, and sheds new light on the definition of opportunistic pathogens relative to the more conventionally accepted Koch's postulates that describe a causal relationship between an infectious agent and its associated disease.^{7,8}

Recent literature suggests a shift in focus towards describing pathogens as opportunists, able to proliferate when microbiome homeostasis is disturbed, a process known as 'dysbiosis'.⁴ Based on this concept, disturbance in the microbiome is associated with corresponding shifts in microbial community functionality, influencing physiological parameters within the host, including the immune system, and is directly correlated with disease resistance.⁹ Early detection of these disturbances, before reaching an irreversible diseased state, is of paramount importance for the prevention of disease. In light of the growing awareness of links between host immunity and the microbiome, there has been increased focus on elucidating hidden mechanisms involved in maintaining health^{10,11} and consequently the ability to manipulate the microbiome to reduce disease incidence, thanks to an increasing accessibility of Next-Generation sequencing (NGS).^{12–14} Such approaches (probiotics, prebiotics, postbiotics, synbiotics, etc.) are a major area of interest within the field of aquaculture and present very promising opportunities for a range of disease control strategies.^{13,15,16}

As well explored in many human medical studies, there is evidence for a correlation between dysbiosis in the host microbiota and infectious disease; however, questions remain regarding the potential for monitoring health and early detection of disease via the identification of dysbiosis-associated biomarkers.¹¹ Diagnostic biomarkers can be used as indicators of disease development prior to physical evidence of disease, and could play a key role in the prevention and optimization of prescribed treatments on aquaculture farms.¹⁷ In this review, we explore insights gained from recent advances in the characterization of dysbiosis in the context of fish disease development. Given the potential opportunities for disease prevention, we investigate the ways that such knowledge can potentially be used to diagnose disease in its early stages, and we contextualize strategies proposed in the literature based on their relative effectiveness. As such, we review mechanisms involved in the development of infectious diseases as they are currently understood, how they inform mitigation and prevention strategies, as well as future directions in microbiome research within the context of dysbiosis and disease management.

2 | WHAT IS A DYSBIOSIS?

In this review, we refer to the microbiome as a dynamic and interactive system consisting of a microbial community, as well as their activities and complex interactions within a given ecosystem characterized by host biotic and abiotic environments (holobiont inter-relationships).^{18,19} We distinguish this term from the definition of microbiota, which is generally considered the microbial assemblage (bacteria, archaea, viruses and fungi) inhabiting this habitat.^{12,20} A healthy microbiota community often demonstrates high taxonomic diversity, high microbial gene richness and stable core microbiota.²¹ So far, there is no consensus on the definition of the term 'dysbiosis' albeit it is most commonly described as an 'imbalance of the microbiota'.^{22,23} For instance, Petersen and Round²⁴ defined dysbiosis as '*any change in the composition of resident commensal communities relative to the community found in healthy individuals*'. However, as described by Levy et al.,²⁵ inter-individual variability of the microbiome between healthy organisms raises the problem of identifying a singular healthy microbiome as a reference point. The authors propose to define dysbiosis as '*a microbial community state that is not only statistically associated with a disease, but also functionally contributes to the etiology, diagnosis or treatment of the disease*'.²⁵ They highlight that this state is not only linked to abnormal taxonomic structure but also abnormal metagenomic function; hence, they propose the characterization of a disease-causing microbiome in line with Koch's postulates. Furthermore, it is often unclear whether dysbiosis is the cause or consequence of the disease.^{23,26} While the definition of dysbiosis may still be a matter of debate, the association between an unstable state of the microbiome and the disruption of 'healthy' pathways is clear. Petersen and Round²⁴ and Levy et al.²⁵ identified three types of dysbiosis that may occur concurrently: expansion of pathobionts, loss of microbial diversity and loss of beneficial microbes. In the following sections, we will explore the potential for early disease diagnostic signatures within these different types of dysbiosis.

3 | MICROBIOTA COMMUNITY AS AN EARLY WARNING SIGNAL OF DISEASE

Different microbial communities can be observed in different parts of the fish body (e.g., skin, gills, gut), however, the gut microbiota has received more research attention due to its key role in metabolism, health and physiology.²⁷ Diverse interactions exist between microorganisms and their hosts which drive relationships that can be characterized as commensal, mutualistic or pathogenic.²⁸ Types of interactions, as well as inherent taxonomic diversity, and the functional expression profile of the microbiota therein all influence metabolic activity, and are dynamic throughout the host life cycle. Factors such as genetics,²⁹ diet,³⁰ antibiotics exposure,^{31,32} as well as environmental conditions,³³ especially domestication³⁴ are known to shape the gut microbiota of marine animals (Figure 1).

Several lines of evidence suggest that microbiota may also be shaped by infection status.^{35–38} Yellow croaker (*Larimichthys crocea*) were classified into two groups according to the variations of the gut microbial communities after infection with *Pseudomonas plecoglossicida*: the initial stage

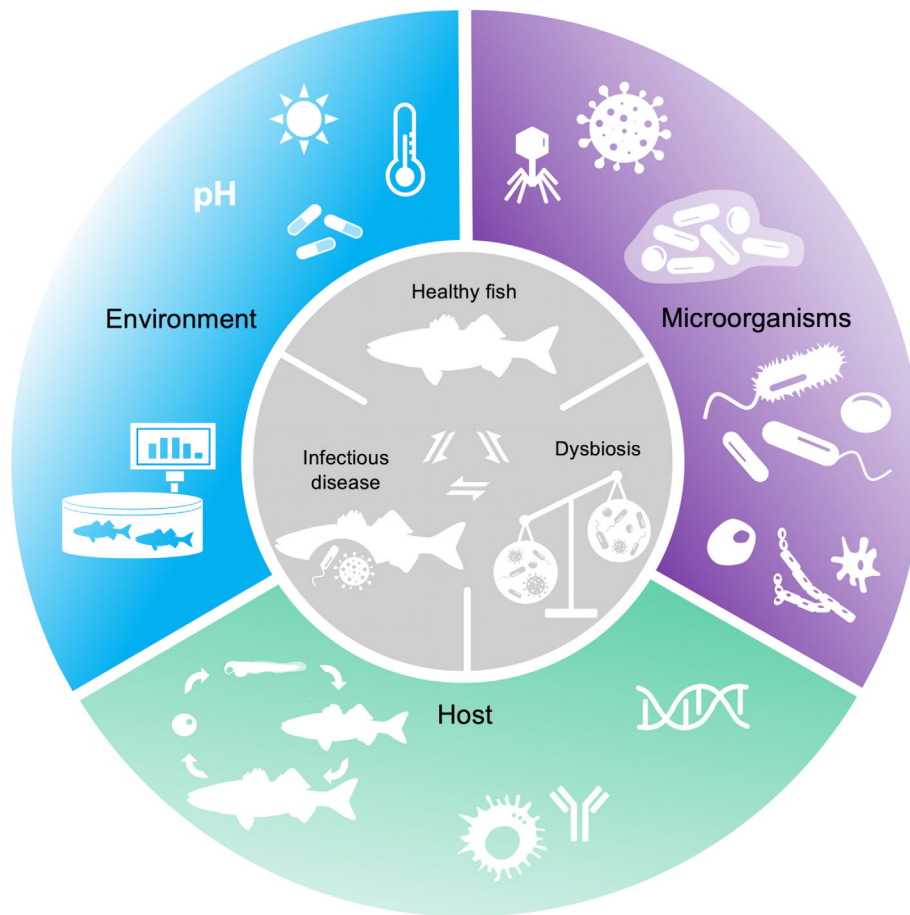


FIGURE 1 Holistic approach to characterize fish health and infectious disease development, as well as dysbiosis. Factors influencing fish health status: Environment (temperature, pH, antibiotics, water quality, fish density, feed, rearing conditions, etc.), microorganisms (phages, virus, bacteria, archaea, ‘micro-eukaryotes’, etc.), host (age, genetic, immune system, etc.)

of disease (24 h post infection) and disease progression (48, 72 and 96 h post infection), leading to an irreversibly disrupted state of the gut microbiota and ultimate mortality.³⁵ Similarly, during zebrafish (*Danio rerio*) infection with the parasite *Pseudocapillaria tomentosa*, variations in gut microbial communities were correlated with parasitic burden and disease severity.³⁶ Time of infection was likewise a significant factor driving microbial community structure during infection in Atlantic salmon (*Salmo salar*) by the salmonid alphavirus (SAV),³⁸ as well as the parasite *Lepeophtheirus salmonis*.³⁷ Moreover, the association between shifts in microbiota and subsequent infection has been observed across multiple studies, even when the microbiota sampled were distal to the primary site of infection.^{38–40} For example, an external bacterial skin infection of *Tenacibaculum* sp. was associated with dysbiosis within the gut microbiota of Atlantic salmon,⁴⁰ while similarly, gut enteritis was associated with changes in skin and gills microbiota of yellowtail kingfish (*Seriola lalandi*).³⁹ Such studies reinforce the need to track multiple factors over time using longitudinal studies, to better explain how microbial assemblages and host-microorganism relationships alter during disease progression. By studying the relationship between microbial dysbiosis and infection status, as well as internal and external factors shaping the microbiome (Figure 1), it may be possible to identify early biomarkers of dysbiosis

prior to physical signs of infection—allowing for faster and more targeted control measures.

Recent advances in bioinformatics have created novel applications for Next-Generation Sequencing (NGS) techniques to be utilized in a range of direct applications for disease prediction. Segata et al.⁴¹ developed an algorithm called linear discriminant analysis effect size (LEfSe) to identify metagenomic biomarkers (e.g., key species, genes or metabolic pathways) that can describe differences between communities. While this algorithm has been validated using human and mouse models, there is also interest in using this tool to investigate biomarkers in aquaculture.^{42–49}

4 | DEPRESSION OF BENEFICIAL BACTERIA AS BIOMARKERS OF DISEASE

4.1 | Gut microbiota variability

A wide range of recent studies have suggested that the microbial signature in gut microbiota can be an indicator of fish health,^{40,45,46,50} and comparisons of the bacterial gut composition between healthy

and diseased fish have demonstrated a loss of beneficial bacteria coinciding with overgrowth of opportunistic bacteria and subsequent disease progression (Table 1). For instance, Miyake et al.⁴⁵ suggested that the abundance of Fusobacteria and Firmicutes could be used as universal biomarkers in healthy fish. By comparing the taxonomic composition of the guts of healthy Asian seabass (*Lates calcarifer*) with individuals symptomatic of tenacibaculosis, they attempted to demonstrate that a relative decrease in Fusobacteria and Firmicutes, alongside an increase in Proteobacteria, can be correlated with a diseased state. Given that a high abundance of Fusobacteria and Firmicutes have been observed in other fish species, including Atlantic salmon⁵¹ and surgeonfishes (Acanthuridae),⁵² there is potential utility due to the universality of these biomarkers, although further research to establish this fingerprint method as a practical tool is clearly needed.⁴⁵

In salmonids, another taxon proposed as a biomarker is the *Mycoplasma* spp., grouped under the phylum Firmicutes. A recent study by Bozzi et al.⁴⁰ compared the distal gut microbiota composition of healthy Atlantic salmon with that of individuals who had an ulcerative skin infection most likely caused by *Tenacibaculum dicentrarchi* and suggested that this biomarker may be effective even if the gut is not the primary infection site. A negative correlation was observed between the relative abundance of *Mycoplasma* spp. and *Aliivibrio* spp., with a decrease of *Mycoplasma* spp. in diseased fish. In a similar study, a high relative abundance of *Mycoplasma* spp. in the midgut was linked to a *Flavobacterium psychrophilum*-resistant line of rainbow trout (*Oncorhynchus mykiss*).⁵³ Other studies have proposed a mutualistic relationship between *Mycoplasma* spp. and their salmonid hosts,^{54–56} while Gaulke et al.³⁶ even discovered a positive correlation between the relative abundance of *Mycoplasma* spp. and the parasite burden in the gut of zebrafish infected with *Pseudocapillaria tomentosa*. In such cases, it is difficult to draw conclusions, as some researchers have identified *Mycoplasma* spp. as part of the core intestinal microbiota of salmonids,⁵⁷ while others indicate sporadic incidence of these species in Atlantic salmon, even when sampled from the same farm.⁵⁸ Furthermore, several authors have concluded that the healthy Atlantic salmon gut is not characterized by a high abundance of *Mycoplasma* spp., suggesting that the presence and abundance of *Mycoplasma* spp. could be host-, environment- or infection-dependent.^{2,59} Despite the wide divergence of conclusions in this field, many researchers continue to attempt to characterize a stable versus diseased gut microbiota, and to identify specific biomarkers. For instance, *Cetobacterium* spp. is another taxon often associated with healthy gut microbiota. In crucian carp (*Carassius auratus*), specific gut bacterial changes were associated with the development of 'Red-Operculum' disease.⁴³ *Cetobacterium* spp. were found in relatively high concentrations in healthy individuals, whereas high concentrations of *Vibrio* spp., *Aeromonas* spp. and *Shewanella* spp. were found in diseased individuals. In a similar study, a significant reduction in *Cetobacterium* spp. was observed in the intestinal tract of diseased yunlong grouper (*Epinephelus moara* ♀ × *E. lanceolatus* ♂).⁴⁴ Interestingly, *Cetobacterium* spp. have been implicated in vitamin B12 production and its beneficial effects on host metabolism,⁶⁰ however, few researchers have looked specifically at this type of metabolic role,

while many continue to investigate its presence or absence in the gut of healthy and diseased fish. Similar to the salmon case above, a wide range of discrepancies exist between studies, wherein results are often contradictory even in the same species or with the same pathogens. For instance, no significant difference was identified in relative abundance of *Cetobacterium* spp., and *Mycoplasma* spp. between tapeworm infected and non-infected common carp (*Cyprinus carpio*)⁶¹ whereas high levels of *Cetobacterium* spp. were detected in the gut microbiota of grass carp (*Ctenopharyngodon idellus*) infected by a reovirus (GCRV),⁶² as well as in the intestine of zebrafish infected by non-O1/O139 *Vibrio cholerae* strains.⁴² The latter study concluded that infection by different strains of *V. cholerae*, specifically El Tor (O1) strains, was related to a lower abundance of *Cetobacterium* spp., suggesting a variable impact within the intestine. However, considering the conflicting evidence, the presence or abundance of *Cetobacterium* spp. is potentially infection- or strain-infection-dependent. Identifying a taxonomic biomarker is thus rendered more difficult given a wide range of pathogens, including different serotypes and strains, and an even more diverse array of potentially species-specific host responses. The only conclusion that can be drawn from such widely divergent findings relates to difficulties in the identification of a single biomarker, and even if such biomarkers could be identified, the limitation of such work if markers are strain and species-specific, and likely also subject to variations in environmental conditions.

4.2 | Skin and gill surface microbiota variability

Even though the gut microbiota had been studied extensively for its impact on fish metabolism,⁶³ other research has also focused on mucosal surfaces such as the skin and gills that are similarly colonized by different commensal microorganisms, and can provide an important barrier against infection (Table 1).^{37–39,47,48,64–67} In several studies, a decrease in specific bacteria has been identified as commensurate with various skin or gill infections.^{47,66} For instance, the relative abundance of *Shewanella* spp., *Acinetobacter* spp. and *Pseudomonas* spp. decreased on the skin of rainbow trout infected with parasitic Ich (*Ichthyophthirius multifiliis*).⁴⁷ In contrast, a decrease in *Shewanella* spp. was associated with an increase in *Alteromonas* spp. in the ulcerated skin of gilthead seabream (*Sparus aurata*).⁶⁶ Two further studies also suggested that *Rubritalea* spp. on mucosal surfaces in European seabass (*Dicentrarchus labrax*) could be used as a biomarker in healthy fish.^{64,67} Indeed, a lower abundance on the skin of diseased fish was associated with microbiota changes in European seabass infected with *Vibrio harveyi*.⁶⁴ Likewise, a decrease of *Rubritalea* spp., as well as *Polaribacter* 4, *Pseudomonas* spp. and *Stenotrophomonas* spp., was recorded in the gills of European seabass, putatively infected with *Photobacterium damsela*.⁶⁷ Other studies have similarly identified *Rubritalea* spp. as part of the core microbiota in the mucosal surfaces of European seabass and seabream.^{68,69} The beneficial effects of *Rubritalea* spp. are most likely associated with the production of carotenoids and squalene production (i.e. antioxidants and precursors for important vitamins or metabolites).^{70,71} However, to our

TABLE 1 Summary of the literature characterizing the changes in fish gut, gills and skin microbiota in a context of infectious disease

Species	Disease/symptoms	Pathogen	Sample	Change in specific bacterial relative abundance in infected fish		Change in α -diversity in infected fish	Functional prediction analysis	Reference
				↓ Decrease	↑ Increase			
Gut microbiota								
Asian sea bass (<i>Lates calcarifer</i>)	Tenacibaculosis	<i>Tenacibaculum singaporense</i>	Gut	Fusobacteria, Firmicutes ^a	Proteobacteria <i>Vibrio</i>	↓	-	45
Atlantic salmon (<i>Salmo salar</i>)	Tenacibaculosis	<i>Tenacibaculum dicentrarchi</i>	Distal gut	Mycoplasma ^a	<i>Aliivibrio</i>	-	-	40
Atlantic salmon (<i>Salmo salar</i>)	Various symptoms	-	Intestinal content	<i>Sphingomonas</i> <i>Streptomyces</i> <i>Pedobacter</i> <i>Janthinobacterium</i> <i>Burkholderia</i> <i>Balneimonas</i>	<i>Aliivibrio</i> <i>Vibrio</i> Aeromonadaceae	↓	-	2
Brown trout (<i>Salmo trutta</i>)	Salmonid myxozoan parasite infection	<i>Tetracapsuloides bryosalmonae</i>	Intestinal content	<i>Yersinia</i>	-	↑ richness	-	81
Common carp (<i>Cyprinus carpio</i>)	Tapeworm infection	<i>Atractolytocestus tenuicollis</i> <i>Khawia japonensis</i>	Hindgut	No significant correlation	No significant correlation	No significant difference	-	61
Crucian carp (<i>Carassius auratus</i>)	'Red-Operculum' disease	-	Intestine	Aeromonadaceae <i>Cetobacterium</i> <i>Clostridium</i>	<i>Vibrio</i> ^a Aeromonas <i>Shewanella</i>	↓ diversity and stability	Cf Table 2	43
Gibel carp (<i>Carassius gibelio</i>)	CyHV-2 infection	Cyprinid herpesvirus 2 (CyHV-2)	Intestine (Hindgut)	<i>Planctothrix</i> <i>Synechococcus</i>	<i>Plesiomonas</i> ^a <i>Bacteroides</i> <i>Shewanella</i>	↓ diversity and richness	-	75
Grass carp (<i>Ctenopharyngodon idellus</i>)	Enteritis	-	Intestinal content	<i>Spironema</i> <i>Parabacteroides</i> <i>Chitinilyticum</i> <i>Cetobacterium</i> <i>Erwinia</i> <i>Turicibacter</i> <i>Citrobacter</i>	<i>Dechloromonas</i> <i>Methylocaldium</i> <i>Planctomyces</i> <i>Rhodobacter</i> <i>Caulobacter</i> <i>Pseudomonas</i> <i>Flavobacterium</i>	↓ diversity and richness	Cf Table 2	49
Grass carp (<i>Ctenopharyngodon idellus</i>)	Grass carp reovirus (GCRV) infection	Grass carp reovirus (GCRV)	Intestine	<i>Acinetobacter</i> <i>Stenotrophomonas</i> <i>Aquabacterium</i>	<i>Cetobacterium</i>	↑	-	62

(Continues)

TABLE 1 (Continued)

Species	Change in specific bacterial relative abundance in infected fish				Reference
	Disease/symptoms	Pathogen	Sample	Change in α -diversity in infected fish	
Indian major carp, rohu (<i>Labeo rohita</i>)	Ectoparasite infection	<i>Argulus siamensis</i>	Intestine	↓ Decrease	46
				↑ Increase	
Largemouth bronze gudgeon (<i>Coreius guichenoti</i>)	Furunculosis	<i>Aeromonas salmonicida</i> subspecies <i>salmonicida</i>	Intestine	↓ Decrease	77
				↑ Increase	
Orange-spotted grouper (<i>Epinephelus coioides</i>)	NNV infection GIV infection Vibriosis	Nervous necrosis virus (NNV) Groupers iridovirus (GIV) <i>Vibrio harveyi</i>	Intestine	↓ diversity	50
				↑ richness	
Rainbow trout (<i>Oncorhynchus mykiss</i>)	External (skin lesions, necrosis of fins/gills) and internal sign of disease	-	Intestine	↓ diversity and richness	59
				No significant difference	
Yellow croaker (<i>Larimichthys crocea</i>)	Bacterial infection	<i>Pseudomonas plecoglossicida</i>	Intestine	↑ diversity	35
				No significant difference	
Yunlong grouper (<i>Epinephelus moara</i> ♀ × <i>E. lanceolatus</i> ♂)	Visual observation of disease	-	Intestinal content	72 hours post infection (hpi)	44
				No difference at 24, 48, 96 hpi	

TABLE 1 (Continued)

Species	Disease/symptoms	Pathogen	Sample	Change in specific bacterial relative abundance in infected fish		Change in α -diversity in infected fish	Functional prediction analysis	Reference	
				↓ Decrease	↑ Increase				
Zebrafish (<i>Danio rerio</i>)	Vibriosis- <i>Vibrio cholerae</i> (different strains)	V. cholerae O1 El Tor Strain 254-93	Intestine	Fluviicola Aeromonas Cloacibacterium		-	-	42	
		Strain AM-19226			Plesiomonas Vibrio Novosphingobium	↓ diversity			
		Strain V52			Plesiomonas Vibrio Cetobacterium	↑ richness			
		V. cholerae non-O1/O139							
		Strain E7946			Plesiomonas Vibrio Enterobacteriaceae Fictibacillus	↑ richness			
Zebrafish (<i>Danio rerio</i>)	Intestinal parasitic infection (Helminth)	<i>Pseudocapillaria tomentosa</i> Strain N16961	Digesta	Plesiomonas		↑	-	36	
Mucosal surface: Skin and gills									
Atlantic salmon (<i>Salmo salar</i>)	Winter-ulcer disease	-	Distal intestine	-	Alivibrio Alcaligenes	↑ diversity	-	65	
		-	Skin	No significant difference (high relative abundance of <i>Tenacibaculum</i> and <i>Arcobacter</i>)		↓ diversity			
Atlantic salmon (<i>Salmo salar</i>)	Salmon lice infection (parasitic copepod)	<i>Lepeophtheirus salmonis</i>	Skin	-	Vibrio Flavobacterium Tenacibaculum Pseudomonas	↑ diversity ↓ richness		37	
Atlantic salmon (<i>Salmo salar</i>)	Viral infection	Salmonid alphavirus (SAV) subtype 3	Skin	Proteobacteria- Oceanospirillaceae such as <i>Oleispira</i> Enterobacteriaceae Pseudoalteromonas	Flavobacteriaceae such as <i>Tenacibaculum</i> Streptococcaceae Vibrionaceae	No significant difference	-	38	
European Seabass (<i>Dicentrarchus labrax</i>)	Skin lesions	<i>Vibrio harveyi</i>	Skin	Rubritalea^a <i>Acinetobacter</i>	Vibrio	No significant difference	-	64	

(Continues)

TABLE 1 (Continued)

Species	Disease/symptoms	Pathogen	Sample	Change in specific bacterial relative abundance in infected fish		Change in α -diversity in infected fish	Functional prediction analysis	Reference
				↓ Decrease	↑ Increase			
European seabass (<i>Dicentrarchus labrax</i>)	Natural disease outbreak	<i>Photobacterium damsela</i> ssp. <i>piscicida</i> and <i>Vibrio harveyi</i>	Skin	-	Vibrionaceae <i>Vibrio</i> Flavobacteriaceae <i>Aureispira</i>	↑ diversity	Cf Table 2	48
European seabass (<i>Dicentrarchus labrax</i>)	Infectious disease outbreak	<i>Photobacterium damsela</i> identified as the most probable causative agent	Skin	No significant difference of relative abundance of <i>Photobacterium</i>		↓ diversity	-	67
			Gills	<i>Rubritalea</i> <i>Polaribacter</i> 4 <i>Pseudomonas</i> <i>Stenotrophomonas</i> NS3a marine group	<i>Pseudomonas</i> <i>Stenotrophomonas</i> <i>Photobacterium</i>	No significant difference		
Gilthead seabream (<i>Sparus aurata</i>)	Skin ulcers	Ulcers were performed in the fish skin with an electric toothbrush for 30 s	Skin	<i>Staphylococcus Lactobacillus</i> <i>Alteromonas</i> <i>Thalassabius</i> <i>Winogradskyella</i>	<i>Streptococcus Gramulicatella</i> <i>Flavobacterium</i> <i>Chryseobacterium</i> <i>Tenacibaculum</i>	↓ richness	-	66
Rainbow trout (<i>Oncorhynchus mykiss</i>)	Parasitic Ich infection	<i>Ichthyophthirius multifiliis</i>	Skin	<i>Acinetobacter</i> <i>Shewanella</i> <i>Pseudomonas</i>	<i>Flavobacterium</i> <i>Bdellovibrio</i>	↑ diversity	-	47
Yellowtail Kingfish (<i>Seriola lalandi</i>)	Enteritis	-	Skin	Proteobacteria: Bacteroidetes (P/B)^a ratio at early stages <i>Corynebacterium</i> <i>Glaciecola</i> <i>Ferrovum</i>	<i>Litoricola</i> <i>Candidatus Aquiluna</i> <i>Simplicispira</i>	↑ diversity and richness at early stages	-	39
			Gills	P/B^b ratio at early stages <i>Psychrobacter</i> <i>Glaciecola</i> <i>Polynucleobacter</i> <i>Roseovarius</i> <i>Ascidilaceihabitans</i>	<i>Microcella</i> <i>Pelagibacter</i> <i>Loktanella</i> <i>Marivita</i> <i>Planktomarina</i> <i>Pseudospirillum</i>	No significant difference		

The biomarkers identified by the authors.

knowledge, *Rubritalea* spp. have been identified as a potential biomarker only in European seabass and seabream, thus their universality as a marker is potentially limited.

In a similar study of Atlantic salmon (*S. salar*), a significant decrease of *Oleispira* spp. in the skin of salmonid alphavirus (SAV)-infected fish was observed.³⁸ To date, *Oleispira* spp. is thought to have a role in the smoltification process, although its beneficial effect at this life stage has not been fully elucidated. A study of Atlantic salmon found the prevalence of *Oleispira* spp. to be dependent on ploidy.⁷² These findings further point to the distinct problem of identifying universal biomarkers as the microbiome differs across individuals and as a function of environmental factors. Developing universal methods for fish species requires considerable work in mapping indigenous microbiota before generic biomarkers may be suggested.

To that end, Legrand et al.³⁹ propose to use the ratio of the relative abundance of Proteobacteria and Bacteroidetes (P/B ratio) as an indicator of fish disease. By investigating the impact of enteritis on the skin and gills of yellowtail kingfish, they demonstrate a decrease in P/B ratio at early stages of the disease, particularly in the skin. Interestingly, Proteobacteria and Bacteroidetes are found in most mucosal fish surfaces and could potentially be interesting for monitoring disease more broadly.^{47,66,67,73} Such an approach has already been proposed in humans and mammals, such as the Firmicutes/Bacteroidetes (F/B) ratio that is used as a biomarker of intestinal dysbiosis.⁷⁴ As with other proposed biomarkers, further studies are needed to confirm the observed trends across a diversity of species and environmental conditions.

5 | OPPORTUNISTIC BACTERIA AS BIOMARKERS OF DISEASE

5.1 | Gut microbiota variability

A number of authors have considered the presence of opportunistic bacteria as an early-warning signature for diseased individuals. In such studies, a comparison of the microbiota in diseased and healthy fish highlights how an increase in potential pathogens, mostly belonging to Proteobacteria, occurs in the gut microbiota of diseased fish.⁴⁵ For instance, She et al.⁷⁵ proposed the use of *Plesiomonas* spp. as a bacterial biomarker for Cyprinid herpesvirus 2 (CyHV-2) infection in gibel carp (*Carassius gibelio*). Similarly, a high relative abundance of *Plesiomonas* spp. and *Lawsonia* spp., was observed in common carp infected by the parasite *Khawia japonensis*⁶¹ although elsewhere a significant decrease of *Plesiomonas* spp. was correlated with parasite burden and an increase in *Pseudomonas* spp. in the gut of zebrafish.³⁶ The same trend between *Plesiomonas* spp. and *Pseudomonas* spp. was also highlighted in zebrafish after antibiotic exposure, suggesting that this phenomenon could be a biomarker of disturbed microbiota instead of an infection-specific biomarker.⁷⁶ Similarly, a significant increase in *Pseudomonas* spp. was recorded in diseased yunlong grouper (*E. moara* ♀ × *E. lanceolatus* ♂)⁴⁴ as well as grass carp.⁴⁹ Together, these studies emphasize the difficulties in discriminating between bacterial

biomarkers specific to an infection, and bacterial biomarkers specific to an unbalanced microbiota.

Many researchers have identified associations between potential pathogens within the gut microbiota of diseased fish. *Vibrio* spp., *Aeromonas* spp., and *Shewanella* spp. were characterized as a bacterial signature for 'Red-Operculum' disease in crucian carp.⁴³ In a similar prior study an increase in *Aeromonas* spp. was observed in bronze gudgeon (*Coreius guichenoti*) suffering from furunculosis,⁷⁷ while a relatively high abundance of *Vibrio* spp. found in diseased Asian sea bass (*Lates calcarifer*) was correlated with symptoms of tenacibaculosis.⁴⁵ Interestingly, these taxa were not detectable in the surrounding environment, suggesting that there are opportunistic intestinal bacteria commonly present in the microbiota that become pathogenic only under specific conditions.⁴³ Other opportunistic genera, such as *Allivibrio* spp. have also been associated with disease in Atlantic salmon.^{2,40} Similarly, increases in *Flavobacterium* spp. associated with other opportunistic taxa have been reported.^{49,75} Overall, these studies reveal a range of associations between potential pathogenic genera and specific microbial infections, as summarized in Table 1. These suggest that the gut microbiota may be a reservoir for opportunistic pathogens. However, to date, the causality remains unclear between the abundance of opportunistic bacteria and infection and further research is needed into the interactions between beneficial/opportunistic microbiota and disease states for the development of more precise preventive control measures.

5.2 | Skin and gill surface microbiota variability

A number of recent studies have examined the abundance of opportunistic taxa in the fish mucosal surface microbiota (e.g., skin and gills) during pathogen infection. For instance, after parasitic Ich (*I. multifiliis*) infection, an increase in the relative abundance of Flavobacteriaceae, specifically *Flavobacterium* spp., have been found on rainbow trout skin.⁴⁷ Another parasitic infection caused by *Lepeophtheirus salmonis* was associated with an increase in *Vibrio* spp., *Flavobacterium* spp., *Tenacibaculum* spp. and *Pseudomonas* spp. on the skin of Atlantic salmon.³⁷ Interestingly, in Atlantic salmon, viral infection caused by SAV has been associated with an increase in Streptococcaceae, Vibrionaceae, Flavobacteriaceae and more particularly *Tenacibaculum* spp.³⁸ An increase in *Flavobacterium* spp. and *Tenacibaculum* spp., as well as *Chryseobacterium* spp., *Streptococcus* spp. and *Granulicatella* spp., were also observed in ulcerated skin of gilthead seabream.⁶⁶ Similar results were recorded during bacterial infection in European seabass in which diseased individuals exhibited an increase in Vibrionaceae and Flavobacteriaceae.⁴⁸

The observation that different pathogen infections (parasitic, viral, bacterial) can be associated with an increase in the relative abundance of opportunistic microbial taxa presents a challenge for the use of relative abundance as biomarkers. It cannot be ruled out that opportunists causing secondary bacterial infections are responsible for surface lesions, ulcers, or that the associated secondary pathology is independent of the factors causing primary infection. Pathogen co-infection, as well as synergistic and antagonistic mechanisms, are still poorly

investigated in an aquaculture context, with the situation further complicated by the fact that the appearance of opportunistic species depends additionally on the autochthonous constellation of symbionts in the microbiota.⁷⁸ Universally, however, the appearance of co-infections is common,^{59,72} and perhaps inevitable once homeostasis has been significantly perturbed and disease is established.

6 | DIVERSITY AND STABILITY AS BIOMARKERS OF DISEASE

A number of studies have postulated a convergence between diversity of the gut microbiota and fish health based on the concept that the microbiota reflects metabolic activity and interactions with the host immune system. In studies using high-throughput sequencing, different indices can evaluate the diversity-stability relationship in an ecosystem, such as the alpha-diversity index commonly used to evaluate species richness and evenness within a sample.^{79,80}

A decrease of alpha-diversity within the gut microbiota of infected fish relative to healthy fish has been observed in a multitude of studies,^{2,43,45,59,62,75,77} albeit many other studies also report the opposite trend^{36,46,49,81} and further studies even suggest that there is no difference between healthy and diseased fish.⁴⁴ For instance, low diversity has been recorded in healthy Atlantic salmon, suggesting that diversity may be not a good proxy for health.⁴⁰ Furthermore, the impact of pathogens on this diversity may also be strain-dependent, since different strains of *Vibrio cholerae* in zebrafish were related to distinct differences in bacterial diversity and richness.⁴² A further unresolved challenge is the ability to discriminate between observed discrepancies attributed to environmental, host and virulence factors, and those originating from the study approaches themselves such as the number of samples analysed, and consequently the statistical power of the results.

Literature on diversity changes in surface microbiota (gills and skin) of healthy and diseased fish shows a clear lack of consensus. For instance, an increase in diversity was associated with winter-ulcer disease on the skin of Atlantic salmon,⁶⁵ as well as during parasitic Ich (*I. multifiliis*) infection in rainbow trout.⁴⁷ In European seabass, increasing diversity was observed during infection with *Photobacterium damsela* ssp. *piscicida* and *Vibrio harveyi*⁴⁸ while decreasing diversity was observed during what was most likely *Photobacterium damsela* infection.⁶⁷ Cámara-Ruiz et al.⁶⁴ reported no significant differences in diversity between healthy European seabass and those infected with *V. harveyi*. Contradictory results obtained within the same species and with similar pathogens suggest that diversity may be driven by yet unknown genetic and environmental factors. Diversity and richness may also vary over the course of an infection. Indeed, in investigating differences in the microbiota between early and late enteritis in yellow-tail kingfish, Legrand et al.³⁹ observed significantly lower diversity and richness during early stages of disease progression compared to later stages. We can thus speculate that disease drives different immunological changes impacting the host microbiota, and as such may limit the applicability of microbial diversity indices as a biomarker.

7 | FUNCTIONAL ANALYSIS OF CHANGES IN THE MICROBIOTA

In prior sections, this review has highlighted difficulties in the identification of universal taxonomic biomarkers of dysbiosis given that complex internal and external factors (Figure 1), along with confounding factors during disease progression, drive taxonomic composition within the microbiota. Nonetheless, knowledge from the field of ecosystem ecology suggests that although there is divergence in the microbial taxonomic community, these communities may encode a similar function, a phenomenon referred to as functional redundancy.^{82,83} For instance, Burke et al.⁸³ identified core functional genes within bacterial communities associated with the green macroalga *Ulva australis*, despite divergent taxonomic composition across samples. The observed functional redundancy across marine bacterial taxa suggests that these genes are not monophyletic and can be represented in different taxa. Thus, rather than taxonomic variations, it may be worth identifying and studying functional alterations in the microbiota of infected fish. By identifying consistent changes in the functional profile of the microbiota with greater discriminatory capacity during disease progression, it may be possible to propose functional biomarkers such as specific genes or metabolites.

Thanks to recent advances in bioinformatics, the functional potential of microbial communities can be studied with functional predictive tools such as PICRUSt,⁸⁴ revealing putative metagenome functionality using 16S rRNA data within a reference database.⁸⁵ Alongside alterations in the functional profile under different feeding or rearing/living conditions,^{86–90} functional alterations have been observed in the microbiota of fish after infection,^{43,44,46,49,59} while no significant differences were observed in the functional profile of the skin microbiota⁴⁸ (Table 2).

In crucian carp (*C. auratus*) affected with 'Red-Operculum' disease, bacterial functionality profiles were used to discriminate between diseased and healthy fish, as the relative abundance of pathways associated with bacterial motility and chemotaxis, membrane transport (bacterial secretion system), as well as signalling molecules and interactions (bacterial toxins) were more abundant in diseased fish.⁴³ These pathways are commonly associated with the virulence of opportunistic bacteria and pathogens, playing a key role in colonization and competition.^{91–93} This is all the more interesting given that the plasticity of the bacterial genome and virulence associated genes are often due to mobile genetic elements acquired via horizontal gene transfer, suggesting that these elements—rather than specific species—could be suitable biomarkers for infection.

Instead of genes associated with virulence, another hypothesis makes use of genes associated with host defence mechanisms. In Indian major carp (*Labeo rohita*), pathways related to a chitin binding protein, osmotically inducible protein OsmC, osmoprotectant transport system proteins, endoglucanase and endo 1, 4, beta xylanase were associated with *Argulus siamensis* parasitic infection.⁴⁶ Importantly, chitin is a component of the exoskeleton of the parasite, and the presence of a chitin binding pathway could be associated with the abundance of chitinase-producing bacteria in the gut. Similarly, the abundance of osmotically inducible protein OsmC and the

TABLE 2 Summary of the literature relating the effects of disease on functional prediction analysis based on 16S rRNA sequences of fish gut, gills and skin microbiota

Species	Disease/symptoms	Pathogen	Sample	Functional prediction analysis—change in relative abundance pathway in diseased fish—PICRUS _T based on 16S rRNA sequences	References
Gut microbiota					
Crucian carp (<i>Carassius auratus</i>)	'Red-Operculum' disease	-	Intestine	<ul style="list-style-type: none"> ↑ cell motility: bacterial chemotaxis and motility proteins ↑ membrane transport: bacterial secretion system ↑ signalling molecules and interaction: bacterial toxins 	43
Indian major carp, rohu (<i>Labeo rohita</i>)	Ectoparasite infection	<i>Argulus siamensis</i>	Intestine	<ul style="list-style-type: none"> ↑ endocrine system, energy metabolism, nucleotide metabolism, translation and transport and catabolism (KEGG level 2) ↑ chitin binding protein, osmotically inducible protein OsmC, osmoprotectant transport system proteins, endoglucanase and endo 1, 4, beta xylanase (KEGG level 3) ↓ signal transduction, cell growth and death, neurodegenerative diseases, circulatory system and cardiovascular diseases were significantly (KEGG level 2) ↓ methyl accepting chemotaxis protein and purine binding chemotaxis protein CheW (KEGG level 3) 	46
Grass carp (<i>Ctenopharyngodon idellus</i>)	Enteritis	-	Intestinal content	<ul style="list-style-type: none"> ↑ amino acid metabolism: valine, leucine and isoleucine degradation, phenylalanine metabolism ↑ xenobiotics biodegradation and metabolism: aminobenzoate degradation ↑ carbohydrate metabolism: glyoxylate and dicarboxylate metabolism ↓ pentose phosphate pathway 	49
Rainbow trout (<i>Oncorhynchus mykiss</i>)	External (skin lesions, necrosis of fins/gills) and internal sign of disease	-	Intestine	At level 1 and 2, no significant difference between healthy and unhealthy fish. At level 3, there were few significant differences.	59
Yunlong grouper (<i>Epinephelus moara</i> ♀ × <i>E. lanceolatus</i> ♂)	Visual observation of disease	-	Intestinal content	<ul style="list-style-type: none"> ↑ metabolism: terpenoids and polyketides, biosynthesis of other secondary metabolites ↑ 'human diseases': metabolic diseases; ↑ transport and catabolism ↑ carbohydrate metabolism 	44
Mucosal surface: Skin and gills					
European seabass (<i>Dicentrarchus labrax</i>)	Natural disease outbreak	<i>Photobacterium damselae</i> ssp. <i>piscicida</i> and <i>Vibrio harveyi</i>	Skin	No significant increased pathways in the infected state Amino acid degradation and fatty acid and lipid biosynthesis in healthy fish	48

osmoprotectant transport system pathway could be associated with the host defence through the abundance of bacteria associated with osmoregulation. At present, however, there is insufficient research to determine how pathways related to host defence mechanisms following pathogen colonization could be used as indicators of infection, despite this being a promising area of research that is being actively pursued in other fields such as human medicine.

Infection has also been correlated with an increase in amino acid and carbohydrate metabolism associated with transfer in energy metabolism.^{44,49} Ma et al.⁴⁴ identified a correlation between the increase in carbohydrate metabolism in the intestine of infected yunlong grouper (*E. moara* ♀ × *E. lanceolatus* ♂), and an increase in immune system energy requirements. They suggested that energy transfer within the host resulted in a reduction in energy available for growth. Similarly, by recording an expansion of pathways related to amino acid metabolism, xenobiotics biodegradation and metabolism, as well as carbohydrate metabolism along with cell growth and death, infectious disease and immune system, Tran et al.⁴⁹ suggested that infection may be associated with an interference in energy metabolism and an inflammation of gut microbiota.

The aforementioned findings were obtained using functional predictive tools based on 16S rRNA data. As described by Langille et al.⁸⁴ functional prediction analysis suffers from some basic limitations. For instance, databases may lack taxonomic coverage, or results may not reflect the actual expression of genes or the presence of associated proteins and metabolites within the microbiota. Further research is required to determine if this strategy agrees with a procedure whereby microbiota functionality would be invested with the combined perspectives of metagenomics, metatranscriptomics and metabolomics, research areas that could provide more insight into the implication of genes in disease development.

8 | TOWARDS PREVENTIVE METHODS

8.1 | Non-invasive methods to detect dysbiosis

Although in prior sections, we have discussed the potential to develop biomarkers for dysbiosis in different fish microbiota, in practice the implementation of such method requires that the biomarkers should be easily monitored and identified via non-invasive methods. Nonetheless, the gut microbiota is largely inaccessible through non-invasive means, since direct extraction of fish gut content may lead to mortality, especially in small specimens. Potentially, the sampling of faeces could be used as a non-invasive method to gain insight into the gut microbiota, a practice that is currently being investigated in humans.^{94,95} However, some questions remain as to whether samples from faeces are a suitable proxy for the fish gut microbiota. To that end, Anslan et al.⁹⁶ evaluated the taxonomic differences between several samples: faeces and gut content for the characterization of the gut microbiota of two fish *Gymnocypris* cf. *namensis* and *Triplophysa* spp. While their results show substantial differences between samples, the authors also state that faeces may provide useful information about gut content, but also caution about assimilating results from

respective gut and faecal samples. Moreover, contamination of faecal samples with pond water may introduce biases.

Monitoring the surrounding microbial environment, such as the water, would be a convenient strategy if it worked. Similarities between planktonic microbiota and gut microbiota have been observed, suggesting that finding a biomarker for dysbiosis may yet be possible.^{97–100} However, variations due to feeding patterns and throughout the culture period exist, as the microbiota is shaped by both external and internal factors. For instance, the correlation between fish gut microbiota and surrounding pond water was found to be between 1% and 73% depending on the culture period, thereby indicating considerable variability.⁹⁹ The extent to which the fish microbiota can be shaped by the surrounding planktonic microbiota remains wholly unclear, as well as the delay between a change in a stressor and the resulting taxonomic shift.

As mucosal surfaces such as skin and gills are the first line of fish defence against the external environment, changes within these mucosal microbiotas could also provide an interesting non-invasive method to gain insight into fish health. Many studies have investigated the transcriptional and metabolic pathway comprising fish mucosal secretions as potential focal points for fish health and welfare monitoring. Metabolomic profiling of mucus has been shown to be highly correlated with fish plasma.^{101,102} Mucus sampling may thus work as an alternative to blood, which is considered a good indicator of fish physiological health due to its circulation through all organs.¹⁰³ Nonetheless, the extent to which such compounds are present in the mucus, and how these may also shape the microbiota—and consequently influence homeostasis—is still unclear.

8.2 | Limits and future prospects

8.2.1 | Characterization of a healthy and diseased microbiome

The fish microbiota is composed of commensal, mutualistic and opportunist microorganisms. Most studies reviewed here compare taxonomic profiles between the microbiota of controls (presumed healthy) and diseased individuals over variable temporal and spatial distributions. The concept of dysbiosis stems from these types of study, in which a disturbance of the microbiome is associated with diseased individuals. Community stability and resilience, concepts borrowed from the field of ecology, have been considered the hallmark aspects of a healthy microbiome and its associative role in homeostasis. Nonetheless, the contribution of these studies remains limited in answering whether dysbiosis is the cause or consequence of disease progression. Yet the great interest in this field, and its enormous potential, make it clear that unravelling the mechanisms behind dysbiosis remain of paramount importance to better understand the development of disease within a ‘pathobiome’ concept^{104,105} (Figure 1), and ultimately to inform methods for preventive disease control.

Moving away from a patho-centric approach, the characterization of a healthy or a diseased microbiota on a merely taxonomic level is too simplistic. Interestingly, a recent study has proposed to apply the Anna Karenina principle to the animal microbiome in the sense that ‘*all healthy microbiomes are similar; each dysbiotic microbiome is dysbiotic in its own way*’ (derived from Tolstoy’s *Anna Karenina*).¹⁰⁶ According to this principle, dysbiosis is a stochastic, rather than deterministic phenomenon that always leads to a decline in health.¹⁰⁶ Inter- and intra-variability across fish species and populations makes it difficult to establish a healthy microbiome reference point, from which deviations could be considered ‘dysbiosis’. Instead, characterizing a healthy microbiome relative to functional genes and metabolic pathways that are conserved and expressed through different taxa could be a better alternative,⁸³ despite the paucity of studies today that actually characterize these functional genes or metabolic pathways relative to healthy and unhealthy microbiota. In the future, a holo-omics approach combining metabolomics and metatranscriptomics data may assist in identifying functional states associated with dysbiosis and ultimately, disease.¹⁰⁷ Such approaches could lead to the development of new aquaculture functional feed additives such as metabiotics, or bacteria-free metabolic probiotics that have a direct role in the maintenance of homeostasis.¹⁰⁸

8.2.2 | Importance of monitoring different kingdoms

Overall, the studies reviewed here have focused on prokaryotes and specifically bacteria, but the role of archaea in the microbiome, referred to as the archeome, may also play a more important role in metabolic processes than currently understood.¹⁰⁹ Elucidating this dynamic in the context of fish disease could open new doors into the potential of archaea as biomarkers and probiotics in aquaculture.¹¹⁰ Micro-eukaryotes such as protists, yeasts, or fungi have also been described as important microbiome constituents with a clear capacity to interact with the host immune system.^{111,112} A recent study by Xiong et al.¹¹³ demonstrated that eukaryotic taxa could be a relevant discriminatory indicator for shrimp diseases, but equivalent studies in fish are still lacking.

Virus and especially phages are vectors for genetic mobile elements via horizontal gene transfer, singling them out as important drivers for microbial evolution. The virus equivalent of the microbiome is referred to as the virome, and has been implicated as a strong shaper of the fish microbiota though is comparatively ill-studied. Interestingly, Dinsdale et al.¹¹⁴ observed a number of metabolic pathways encoded by the virome—mostly composed of phages—within the metagenome of different communities. Phages have been implicated in the transfer of antibiotic resistance genes when fish are exposed to antibiotics, suggesting a potentially significant role in disease and antibiotic resistance.¹¹⁵ Unravelling the functional composition of the virome is thus essential to understand interactions within microbial communities, as well as individual impact on the fish host.

8.2.3 | Challenges in sampling and methodology

The concept of dysbiosis within aquaculture is relatively new and as such, still lacks a rigorous reference methodology for the study and evaluation of fish microbiota. Results may be biased due to methodological differences in sampling, preservation, sequencing methods, reference databases, or subsequent bioinformatic analysis. It is therefore of paramount importance to develop standardized guidelines to ensure the reliability of different assays and facilitate comparison between studies, as we have recently described elsewhere.¹¹⁶ Much of the current research in this field has instead focused on human medical applications, though the field of aquaculture can clearly benefit from similar efforts to develop more specific tools and databases to address coverage and reference shortcomings.

9 | CONCLUSION

The microbiome has long been considered a black box in aquaculture. With recent advances in Next-Generation Sequencing (NGS), we have the potential to unravel hidden mechanisms that are involved in maintaining health or that conversely, lead pathogen proliferation to disease. Health is a dynamic state, and microbiota composition and stability are driven by external and internal factors (Figure 1). Based on these dynamics, we understand that disturbance may result in perturbations of the microbiota resulting in dysbiotic states, and that early detection of these harmful changes is of paramount importance for the prevention of disease. Future research in this field will directly improve our ability to implement disease control measures (e.g., probiotics, prebiotics, synbiotics, phage therapy and metabiotics), as well as early-warning systems to detect disease before physical signs emerge and the damage becomes irreversible.

In this review, we have described recent insights into the potential use of key microbial taxa as biomarkers for dysbiosis and the challenges with these markers owing to high variability observed within the microbiota among fish species. One of the most promising directions is in the use of functional genes or metabolic pathways conserved between taxa as a more robust proxy for homeostasis. Further research is needed to investigate the link between microbial community structure and its metabolomic function, as well as the mutualistic relationship between fish and their microbiota. In line with the *One Health* concept,³ these advancements can render future aquaculture disease management more accurate, preventative and accountable.

AUTHOR CONTRIBUTIONS

Julia Mougin: Conceptualization; investigation; writing – original draft.
Alyssa Joyce: Supervision; writing – review and editing.

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DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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