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## Recent advances in bivalve-microbiota interactions for disease prevention in aquaculture

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### ABSTRACT

In bivalves, no clear-cut functional role of microbiota has yet been identified, although many publications suggest that they could be involved in nutrition or immunity of their host. In the context of climate change, integrative approaches at the crossroads of disciplines have been developed to explore the environment-host-pathogen-microbiota system. Here, we attempt to synthesize work on (1) the current methodologies to analyse bivalve microbiota, (2) the comparison of microbiota between species, between host compartments and their surrounding habitat, (3) how the bivalve microbiota are governed by environmental factors and host genetics and (4) how host-associated microorganisms act as a buffer against pathogens and/or promote recovery, and could thereby play a role in the prevention of disease or mortalities.

## Introduction

Marine bivalves are ecological keystone species that fulfill essential roles in coastal ecosystems by providing habitat, mediating benthic-pelagic energy fluxes, and maintaining water quality. Their benthic filter-feeding lifestyle also makes them useful sentinels to detect pollution [1\*\*]. Bivalves are also economically highly important aquaculture species, with a world aquaculture production of molluscs for human consumption reaching 17.5 million tons in 2018 [2]. Aquaculture production and natural populations are strongly impacted by environmental fluctuations of biotic and abiotic factors. In recent years, infectious diseases have emerged as the major limiting factors for bivalve production, but very few tools are available to prevent and treat infections [3\*]. Techniques developed for the production of other animals cannot be directly applied to bivalve aquaculture, since the lack of antibody-mediated immune memory prevents individual vaccination approaches, and culture in the open sea prevents application of large-scale disease treatments.

In marine ecosystems, including open sea aquaculture, bivalves are exposed and colonized by a plethora of microorganisms [1\*\*] making host-microorganisms interactions a prime target for understanding bivalve health [3\*, 4–6]. Microorganisms can fulfill beneficial functions for the host and vice versa, and the holobiont concept captures these relationships by merging hosts and their associated microbiota into a single evolutionary unit of selection [7]. It suggests that microbiota composition provides benefits to the hosts for survival, but also homeostasis and development. For example, studies of resident microorganisms in the haemolymph of healthy bivalves suggest that the host-microbiome could limit settlement of pathogenic strains and play a role in preventing dysbiosis and aiding health recovery [8\*\*, 9].

Facilitated by the advances in high-throughput sequencing technologies, interactions between host immune responses and their associated microbiota are increasingly investigated within the context of disease [10]. Here, we review not only the methodologies used to characterize bivalve microbiota, but also the factors that structure microbiomes associated with bivalves under aquaculture conditions and highlight 1) their composition and host specificity, 2) interactions between environment and host-associated microbiomes, and 3) how these interactions may affect the ability of bivalves to resist pathogens. This review highlights critical gaps in our understanding of microbiome function, and identifies research priorities leading to innovative management practices of molluscan aquaculture in the future.

## Current methodologies

Typically, microbial communities associated with bivalves of aquaculture interest have been taxonomically characterized and analysed by massive amplicon sequencing of genes encoding small subunit ribosomal RNA gene regions (Figure 1) [1\*\*, 11\*, 12\*\*, 13\*]. While microbiota are often characterized by 16S sequencing of bacteria, the eukaryotic genetic diversity is rarely investigated [14, 15\*\*]. A more comprehensive approach than ribosomal amplicon sequencing is shotgun metagenomics, which offers the advantage to target the whole microbial gene pool within a sample with the possibility to run functional analysis as well as assembling genomes. Shotgun metagenome analysis applied to the study of Pacific oyster microbiome recently revealed that oyster hemolymph is a complex ecosystem containing diverse bacteria, protists and viruses [15\*\*]. However, the bioinformatic processing of metagenome data is not yet standardized and the application of shotgun metagenomics for the analysis of the bivalve microbiota is still challenging [15\*\*].

An important limitation of metagenomic or eukaryotic metabarcoding studies is co-sequencing of host DNA that largely masks target DNA from host-associated microbial communities [16]. Ultra-deep sequencing or target sequencing implying removal of non-target DNA from samples before sequencing significantly overcomes this problem. In particular, hybridization-based capture with target-specific biotinylated probes is a versatile and cost effective technique for the sequencing of both

viruses and bacteria in complex samples [17]. Vezzulli et al. [18] demonstrates that by using such an approach, ultra-low abundance *Vibrio cholerae* genomes, including specific virulence factors, could be retrieved from raw river water approximately 2500 times more effectively than shotgun sequencing alone. By applying the same methodology, 884 phylogenetic and virulence markers of the potential microbial pathogenic community were targeted in oyster tissue allowing high taxonomic resolution analysis of the bivalve pathobiota [19\*]. For studying host-associated micro-eukaryotes, metabarcoding primers are available that reduce co-amplification of metazoan host sequences, enabling more thorough sequencing of micro-eukaryotic symbionts and parasites [14,20].

DNA sequencing technologies are rapidly evolving and are expected to contribute to new breakthroughs in the study of the bivalve microbiota. Latest technologies such as long-read targeted sequencing [21] or new ultra-deep sequencing platforms (e.g. NovaSeq 6000) are certainly paving the way to a more comprehensive analysis and understanding of the bivalve holobiont.

### **Host factors shaping bivalve microbiota**

The structure of bivalve-microbiota interactions is clearly stratified by host factors on different scales nested within each other: species [22\*\*], populations within species [15\*\*,23], and individuals/genotypes within populations [8\*\*,12\*\*,24\*\*]. Furthermore microbiomes change across developmental stages [25\*] and between organs [26,27\*].

Comparative studies have mostly been carried out on oysters and mussels [ex: 12\*\*,22\*\*,28\*,29] and to a lesser extent on clams [11\*,13\*]. Species collected from similar environments displayed a high degree of similarity and a high number of shared core OTUs [22\*\*,28\*], indicating that uptake from environmental strains by suspension feeding is a major determinant of microbiome composition. Yet, bivalve species clearly differ in microbiome composition and relative abundances of taxa, which may partly explain the better capacity of a particular species to defend against pathogens or to survive in specific environmental conditions [18\*\*,28\*]. Microbiome composition shows stable differences between individuals [8\*\*] and these host genotypic background signals can persist over months [23]. Indeed, genetics can partly explain microbiome structure in different oyster species (e.g. *Crassostrea gigas*, *Saccostrea glomerata*) [12\*\*,15\*\*,24\*\*].

Genotype associated patterns can however vary within individuals according to developmental stage or tissues [30]. Interestingly, species richness has been shown to decrease as larvae develop suggesting a shift towards a more specific microbiome [25\*]. This trend also continued between later stages (postlarvae and adults), with adults showing the lowest diversity [31]. In later developmental stages, tissue type becomes a strong determinant of bacterial community composition [23,32]. In particular, microbiota vary between internal organs such as gut and those that are in closer contact to the environment (e.g. hemolymph or gills). This pattern, consistent across individuals of different species [33], might suggest a role of microbiota in different physiological processes specific to the investigated organ.

On all levels, the effects of host-associated factors on compositional microbiome diversity are strongly influenced by environmental variation. Environmental disturbance for example can homogenize inter-individual differences [8\*\*] or deplete genetic signals [24\*\*]. In many cases, for example, on the population level, environmental and host genetic effects will be confounded, therefore, it is imperative to consider the interaction of host-specific and environmental effects when describing host-microbiota interactions.

### **Environmental factors modifying bivalve microbiota**

The influence of environmental factors on bivalve microbiota can be explained by the benthic lifestyle of bivalves and its heterogeneity at small spatial scales (ecological niches, sessile versus free-living life stages), and by the spatial and temporal variation of abiotic and biotic factors (season, transplantation, pollution, pathogens, water quality, and so on). Microbiota-environment interactions have repeatedly been studied by monitoring bivalves in the field, either over short or long periods. Specific diversity patterns suggest that bivalves select the microorganisms from the water column or sediment, depending on their microbiota compartment. This microbiota selection process is modified with the time of exposure for intertidal bivalves [13\*], but also with the seasons [11\*\*]. The influence of stochastic processes may also be a factor for microbiota diversity [1\*\*]. The transfer of bivalves from hatcheries to rearing sites plays an essential role in shaping the microbiome from juveniles to adults [29].

The initial microbiome (bacteria, protist and virus) is deeply modified by transplantation (strongly for transient microbiome, and less for resident microbiota such as those from digestive gland), according to the environmental conditions of the new rearing sites [12\*\*,15\*\*,27\*,34\*].

Altogether, results indicate that bivalve microbiota is at least partly originated from the marine microbiota where these organisms are living. Water pollution could also impact bivalve microbiota. Titanium dioxide nanoparticles (nTiO<sub>2</sub>) — very widely used in a diverse range of products — and PS-NH<sub>2</sub> nanoplastic have been shown to affect the *Mytilus galloprovincialis* microbiota [35,36]. Similarly, oysters exposed to atrazine, a prevalent herbicide in agricultural practice, result in a significant loss of key mutualistic microbial species and a subsequent colonization of pathogenic bacteria [37]. Exposure to estrogenic chemicals such as 17 $\beta$ -estradiol, Bisphenol A and Bisphenol F is also leading to changes in the microbiota composition of first larval stages of *M. galloprovincialis* [38].

Water temperature seems to be a major driver and strongly influences the composition of oyster microbiota both in the haemolymph [8\*\*,33] and other tissues [19\*], and only a small fraction of the microbial species persist as a 'core' community throughout the different seasons.

Accordingly, climate change was observed to alter the microbiome of the Sydney rock oyster *S. glomerata* [39]. Warmer temperatures are also favoring the proliferation of potential pathogenic bacteria (e.g. *Vibrio*) within bivalve microbiota, thus increasing susceptibility to microbial diseases [40,41].

### **Microbiota and bivalve health**

Microbiota are hypothesized to influence disease susceptibility [1\*\*,7]. However, in bivalves few studies have explicitly described the role of these host-microbial interactions in buffering the host against fluctuating conditions (enantiostasis). In particular, investigations into the roles of microbiota in host health and disease have so far mostly focused on oysters (*Crassostrea* and *Saccostrea* species) from a solely bacterial perspective. However, it is clear that the bivalve symbiome also includes viruses and microeukaryotes, the identities and roles of which are only just starting to be investigated [15\*\*].

Oyster bacterial microbiota are dynamic and were shown to be modified in response to multiple stressors, including pathogens [8\*\*,15\*\*,24\*\*,27\*,34\*,42–45]. Recently, [43] demonstrated that Pacific Oyster Mortality Syndrome affecting *C. gigas* oyster throughout the world is associated with a disruption of the host microbiota. This study showed that infection by an Ostreid herpesvirus compromises the oyster antibacterial defence and is followed by a microbiota shift leading to bacteremia and oyster death [43]. Field analysis performed on *C. gigas* also revealed that oysters experiencing mortality outbreaks at different life stages displayed signs of dysbiosis [19\*,46]. Nevertheless, it is often unclear whether an altered microbiota is a cause or result of the impact of invading pathogen(s). Examples are known of both situations, and it is important to consider both

options in each case. Experimental investigations and temporal sampling are required to better understand causal relationships [47].

Recent studies highlight the potential involvement of oyster microbiota in disease resistance. They showed that the microbiomes of disease-susceptible oyster families were significantly different from the microbiomes of disease-resistant families [48,49\*]. The differences observed suggested that both the structure and the composition of the microbiota might predict oyster mortalities [49\*]. Interestingly, the microbiota of oysters originating from the same hatchery picked up genotype-specific signals of their different rearing environment in the field and this was correlated with different outcomes to Ostreid herpesvirus infection [34\*]. Bacterial taxa explaining genotype-specific differences offer the opportunity to identify putative microbial determinants of disease resistance. Accordingly, a row of key bacterial taxa were identified, which may provide a protective or detrimental role in disease outbreaks of oysters (Table 1) [45,48,49\*].

The diversity and roles of microeukaryotic symbionts are both almost certainly much greater than currently appreciated. It is increasingly recognized that bivalves are host to a diversity of protistan parasites that have an indeterminate effect on the host. Some of these can occur at relatively high prevalence without a clearly discernible effect on the populations (e.g. *Minchinia mytili* in *Mytilus edulis*; [50], and another currently undescribed *Minchinia* species in *Cerastoderma edule*; [51]). The effects of these parasites may become more apparent under conditions of environmental or other physiological stress on their hosts. Further research is now required to elucidate the dynamic reciprocity between the whole microbiota and host in the context of pathogenesis, and host susceptibility and resistance to disease.

## Conclusion

Over the last few years many studies have investigated the bacterial microbiome of a wide range of bivalves, in both healthy and diseased states (Figure 1). These have shown that the structure and the composition of microbiota is influenced by the ecology and geography of the sampling site, season, host life stage, and health status among other factors (Figure 2). Studies of the microbiomes of farmed bivalves to date have focused on oysters with or without associated mortality events. It will thus be important to complement these with new studies underway on other bivalves affected by chronic diseases including microparasites (e.g. Perkinsosis and Brown R Disease in Manila clams, Juvenile Oyster Disease and Perkinsosis in *C. virginica*). Integrating the dynamics of the whole symbiota — bacteria, viruses, and eukaryotes — and its interaction with the host and surrounding environment, should be the ultimate aim to achieve a comprehensive understanding of bivalve physiological states, including health [47].

Research on microbiome modulator drugs (prebiotics, probiotics, synbiotics) for the health of cultured bivalves should be developed as its value has been demonstrated for other invertebrates such as shrimp [52]. So far, our understanding of bivalve microbiota has focused on its structural aspects, but the large diversity and variability of these microbiota have precluded the identification of probiotic taxa providing protection against disease. Alternative approaches using *Bacillus pumilus* RI06-95 as a probiotic in oyster hatcheries has shown some evidence of decrease of pathogenic microorganisms [45]. Several studies on bivalves — and also on other invertebrates and vertebrates — suggest that the gut compartment harbors a more stable microbiota, including symbionts, and could be a good lead for the identification of a potential microbiota profile. Manipulation of the gut microbiome could also improve immunity and health of bivalves as has been done for shrimps [52]. Probiotics interact with the immune system and prevent pathogen colonization in multiple ways [53]. They can directly compete with pathogens for the same niche (competition for nutrients or production of antimicrobial molecules) and also stimulate the host immune system. These contributions to bivalve defenses are still poorly understood and could be developed as one of the additional strategies to combat disease

in bivalve aquaculture. Phage therapy using specific or cocktails of bacteriophages, could be also used to limit pathogen colonization [3\*].

Complementary approaches (including various 'omics, microscopy techniques, (epi)genetics, and immunology) integrating microbiome studies with host and environmental factors will allow us to better understand the dynamics of microbiota across a diversity of environments and hosts and should help us to identify, for example through network interaction studies, taxa predictive of survival as well as mortality events [54,55]. The more comprehensive understanding that is resulting from these integrated approaches will enable us to better manage bivalve aquaculture, and mitigate problems. Pathobiotic systems can be reversed within certain boundaries, and host health status is a result not just of individual pathogens but a greater diversity of influences, of which we have increasing understanding and ability to manipulate. Collaboration between producers, farm managers, and scientists from diverse disciplines (e.g. microbial ecology, pathology, environmental science, modeling, chemistry, genetics, immunology, and so on) will be key to this success.

#### **Conflict of interest statement**

Nothing declared

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

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Vezulli et al., carried out a comparative analysis of haemolymph and digestive gland microbiota in *C. gigas* and *M. galloprovincialis* co-cultivated at the same aquaculture site using 16SrDNA pyrosequencing. It was found that *Vibrio*, including potential pathogenic species, accounted for a larger fraction of the microbiota in *C. gigas* compared to *M. galloprovincialis*. In addition, microbiome analyses performed on bivalve specimens subjected to commercial depuration highlighted the ineffectiveness of such practice in removing *Vibrio* species from bivalve tissues.

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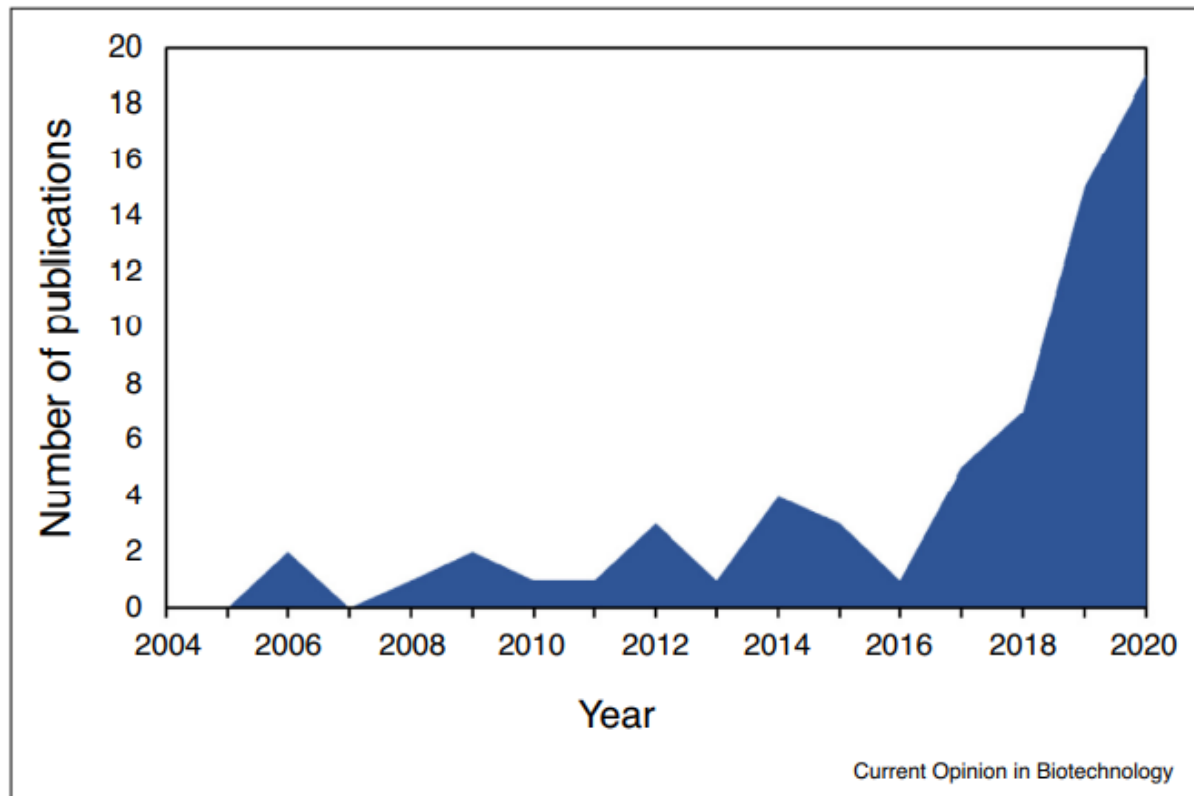
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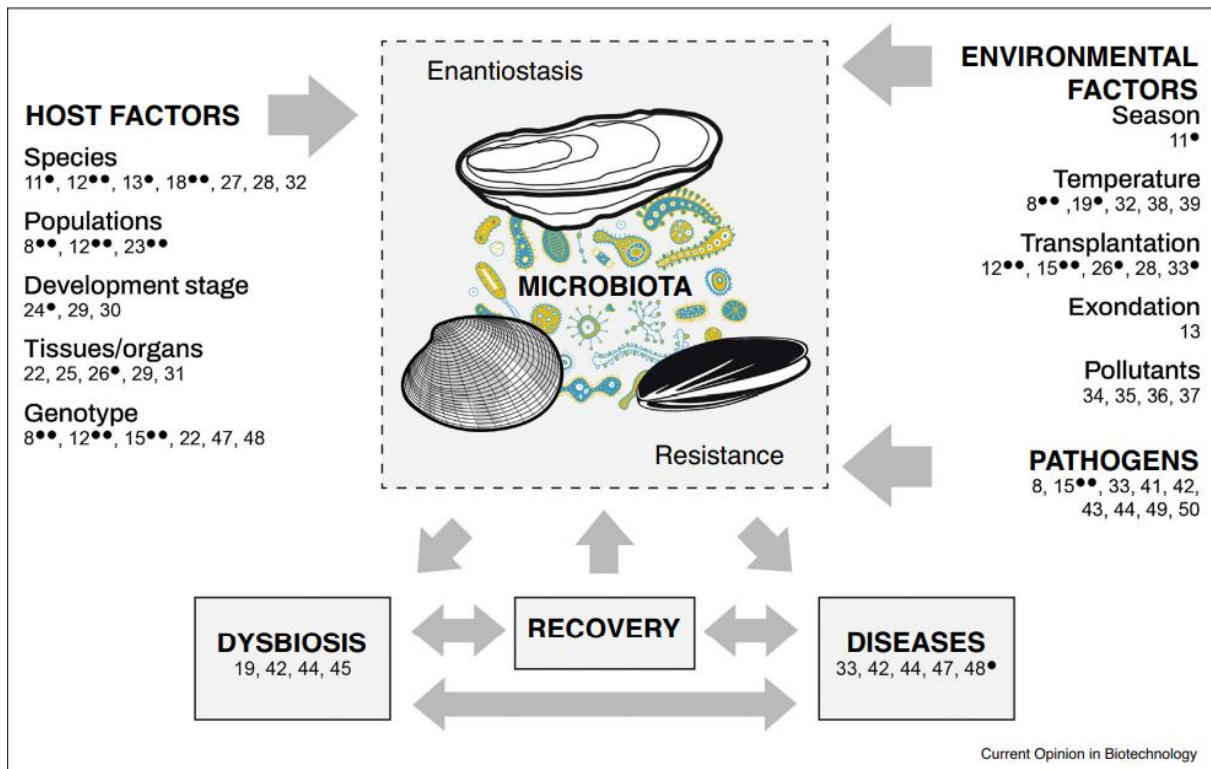
**Figure 1**

Articles published since 2006 reporting the microbial community in bivalve molluscs. Source Scopus May 2021, Query: 16S AND bivalve AND (microbiota OR microbiome OR community OR metabarcoding), output has been checked, removing non-relevant articles.



**Figure 2**

Host and environmental factors acting on bivalve microbiota. Some of these factors, alone or in combination, have been shown to lead to dysbiosis, diseases and recovery.



**Table 1**

Bacterial microbiota taxa associated with a better resistance of *Crassostrea gigas* oyster during disease outbreaks or experimental challenge.

Bacteria taxa (family or genus level)	life stage	Disease	Mortality outbreak	References
Cyanobacteria (Subsection III, family I)	juveniles	Ostreid herpesvirus Disease	Field outbreak	[48*]
Rhodobacteraceae	juveniles	Ostreid herpesvirus Disease	Field outbreak	[48*]
Colwelliaceae	juveniles	Ostreid herpesvirus Disease	Field outbreak	[48*]
Oceanospirillales	larvae	Vibriosis	Experimental challenge	[44]
Winogradskyella	juveniles	Ostreid herpesvirus Disease	Field outbreak	[47]
Bradyrhizobiaceae	juveniles	Ostreid herpesvirus Disease	Field outbreak	[47]