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Microbial education for marine invertebrate disease prevention in aquaculture

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Short running title: An early life microbial exposure educates the immune system of invertebrate species of interest to aquaculture

Abstract:

The holobiont theory expands the notion of individual multicellular organisms as a community composed of a host and all its associated microorganisms. This concept has been extensively studied in the field of aquaculture, where increasing evidence has highlighted the importance of the host associated microorganisms in species fitness. Here, we focus our review on mollusc and crustacean species in which microbiota dysbiosis has recently been described in the context of various diseases, resulting in significant economic losses. Influencing the holobiont structure through the use of probiotics is a potential strategy that could improve the fitness or the robustness of cultivated species. We discuss here the possibility of developing microbiome targeted prophylactic approaches by promoting (1) methods to identify host microbial community that fosters good health status and (2) early life microbial education to favour long-term resistance to stress or disease. This review aims to inform the aquaculture industry about potential strategies in rearing practices to mitigate diseases and economic losses.

Keywords: mollusc, crustacean, holobiont, microbial education, probiotics

Introduction

Aquaculture production covers most of the increase in seafood needs since 1990 and has surpassed fisheries as a source of food for human consumption (Anderson et al. 2019). It has become the fastest growing food-producing sector in the world yielding 57.5 million tonnes of finfish, 17.7 million tonnes of molluscs and 11.2 million tonnes of crustaceans for the year 2020 (Food and Agriculture Organisation 2022). For the aquaculture industry, disease emergences pose a significant risk and can induce substantial economic losses. More concerning is the accumulation of evidence over the past decades that diseases affecting wild and cultivated animals have increased in frequency and severity in association with anthropogenic disturbances and global change (Harvell et al. 2002; Jones et al. 2008; Altizer et al. 2013; Burge et al. 2014; Kibenge 2019; Behringer and Duermit-Moreau 2021). As mentioned below, marine invertebrates constitute an important contribution to aquaculture production in the world. In these species, evidence for recurrent diseases inducing mass mortalities have accumulated, demonstrating the devastating impact of epizooties (Burge et al. 2014; Kibenge 2019). Diseases affecting farmed oysters, shrimp, abalone, and various fish cost billions of dollars each year to this industry (Lafferty et al. 2015). In some cases, these diseases

directly result from the emergence of viral, bacterial or parasitic pathogens (Soudant et al. 2013; Travers et al. 2015). For example, the White Spot Syndrome Virus (WSSV) is the most prevalent viral pathogen in shrimp leading to massive mortalities (up to 100% within 3-10 days) that have impacted productions for decades (Sánchez-Paz 2010). This highly virulent virus quickly infects gills, stomach, haemocytes, hematopoietic tissues and antennal gland (Pradeep et al. 2012). Another concerning infection known as acute hepatopancreatic necrosis disease (AHPDN) or Early Mortality Syndrome (EMS) has been reported in different shrimp species in Asia. It causes rapid and drastic mortalities causing losses of up to 90% of the livestock (Hong et al. 2016). This disease is caused by the bacterial agent *Vibrio parahaemolyticus* which damages the hepatopancreas. In molluscs, the abalone withering syndrome (WS) is another infectious disease caused by the bacteria *Xenohaliotis californiensis* responsible for moderate to mass mortality events depending on the species involved. Mortality rate of up to 99% have been observed in some species such as the black abalone *Haliotis cracherodii* in USA (Crosson and Friedman 2018). Several bacterial diseases caused by *Vibrio*, *Nocardia* and *Roseovarius* have also been reported in several bivalve species (Travers et al. 2015).

Other diseases that affect invertebrates of interest to aquaculture are multifactorial and polymicrobial: in this case, there is a destabilisation of the microbiota due to an initial harmful environmental pressure or to the presence of a pathogen. This dysbiosis eventually opens the door for the development or colonization by opportunistic pathogens or may even lead to a shift from commensalism to opportunism/pathogenicity for some members of the microbiota. In shrimp species, the White Feces Syndrome (WFS) is a gastrointestinal disorder affecting cultivated penaeid shrimp, leading to severe production losses worldwide. Shrimp with WFS usually show a reduced feed consumption and growth rate, hepatopancreatic discoloration, and loose shells. Diseased shrimp show significantly decreased microbial richness and diversity compared to healthy control (Huang et al. 2020). In molluscs, many epizooties affecting shellfish are polymicrobial. One of notable examples is the Pacific Oyster Mortality Syndrome (POMS) which emerged in 2008 and is still heavily impacting the Pacific oyster, *Crassostrea gigas*, production worldwide (Barbosa Solomieu et al. 2015; Petton et al. 2021). POMS is a multifactorial disease, influenced by biotic and abiotic factors (Petton et al. 2015, 2021). The central role of a herpes-like virus, OsHV-1- μ Var, in POMS has been demonstrated; viral infection triggers an immune-compromised state that leads to microbiota dysbiosis and subsequent bacteraemia caused by opportunistic bacteria, ultimately resulting in oyster death (de Lorgeril et al. 2018). Additional studies have shown that OsHV-1 infection leads to variations in the microbiota characterised by changes in the bacterial groups which are normally

the most abundant and an increase of rare OTU belonging to the *Vibrio* genus including known opportunistic pathogens such as *V. harveyi* (King et al. 2019a) and *Arcobacter* (Lasa et al. 2019). Other studies have shown that environmental factors such as exposure to pollutants or warming temperatures are other potential causes of dysbiosis in molluscs leading to increased mortality rate in farms. Temperature stress in *C. gigas* (rapid increase from 20°C to 25°C) alters in the bacterial composition of the microbiota towards dominance of opportunistic pathogens such as *Vibrionaceae* correlating with increased in mortality (Green et al. 2019). Heat stress also leads to decreased stability of the haemolymph microbiota in the Pacific oyster *C. gigas* (Lokmer and Wegner 2015) and induces lower diversity in the mussel *M. coruscus* (Li et al. 2019). These changes in the haemolymph microbiota contributes to increased mortality in these two bivalves in response to infections. Other studies performed in the clams *Chamelea gallina* (Milan et al. 2019) and *Tegillarca granosa* (Liu et al. 2022) have shown that chemical pollution leads to microbiota shift associated with the proliferation of opportunistic pathogens potentially contributing to the declining health of clams.

In order to fight these diseases threatening aquaculture activities, a series of approaches have been developed. Strategies such as the use of probiotics, mutualist symbionts and bacteriophages have been proposed (Pérez-Sánchez et al. 2018). These approaches are based on the identification of beneficial microorganisms that directly benefit by exerting antagonistic effects against potential pathogens. These effects include the production of antimicrobial compounds, interference with quorum sensing or predatory actions (Touraki et al. 2012; Kiyimaci et al. 2018; Offret et al. 2018; Zhao et al. 2019; Goh et al. 2022). Other strategies are based on changes in aquaculture practices through the application of methods that aim to manage the microbial community within rearing tanks. For instance, the implementation of biofloc system improves water quality (Kumar et al. 2021; Rajeev et al. 2021; Khanjani et al. 2022) and has been shown to promote a better host immune response (Khanjani et al. 2023). Diverse technologies aim to create a K-selected microbial community that counteracts r-strategic opportunists. These microbial management strategies have resulted in improved performance and increased viability of larvae. (De Schryver et al. 2014; Bossier et al. 2016; Vadstein et al. 2018). Otherwise, in a broader context, several studies have evidenced the critical role of microbiota during the early developmental steps to educate and durably imprint the host innate immune system in mammals (Arrieta et al. 2014; Gensollen et al. 2016, Renz et al. 2017), in fish (Galindo-Villegas et al. 2012) in crustaceans (Ziaei-Nejad et al. 2006; Roy et al. 2020) and in molluscs (Yang et al. 2020; Fallet et al. 2022). Despite these evidence,

antagonistic approaches toward microbial communities, such as water sterilization and antibiotic treatment, have been favoured so far (Eljaddi et al. 2021; Cordier et al. 2021) .

In this review, we promote the potential application of natural microorganisms as a treatment during the early developmental stage (larval rearing) to shape and sustain lifelong innate immune competence in aquaculture. Firstly, we report recent studies which emphasize the critical role played by the microbiota in molluscs and crustaceans of economic interest for the aquaculture field. Then, we discuss the possibility of using the growing amount of data describing the host associated microbiota to predict health promoting bacteria. Finally, we argue that the application of these new generation probiotics for microbial education in early developmental steps (larval rearing) could be a novel strategy to induce long-term beneficial effects in aquaculture.

1. Host-microbiota interactions in health and disease of mollusc and crustacean species

a. Definition of the holobiont concept

Numerous studies have provided evidence that host associated microbial communities can influence multiple facets of animal physiology encompassing pathogen resistance, immunity, development, behaviour, and ecological functions such as stress tolerance (Sharon et al. 2010; Heijtz et al. 2011; Webster et al. 2011; McFall-Ngai et al. 2013; Kostic et al. 2013; Gilbert et al. 2015; Schmidt et al. 2015; Kohl and Yahn 2016; Webster and Reusch 2017; Gould et al. 2018). These impacts imply that microbiota can profoundly influence animal performance, fitness and adaptation capacities in changing environmental conditions (Rosenberg et al. 2009; Webster and Reusch 2017). These observations fall into the widely accepted holobiont concept which considers multicellular organisms as a complex community of species. Initially, this concept took into account only the mutualistic obligate associations between the host and its symbionts (Rohwer et al. 2002). however, with the advancements in sequencing methodologies and progress in microbiota research, the concept of holobiont was expanded to encompass the association between the host and all its associated microorganisms (bacteria, Archaea, viruses and yeasts) (Bordenstein and Theis 2015). Building on the holobiont concept, the hologenome theory of evolution considers the holobiont with its hologenome (all genomic entities in interaction) as the unit of selection during evolution (Zilber-Rosenberg and Rosenberg 2008; Theis et al. 2016). This theory implies that the host and the symbiont do not interact only for food or ecological niche, but also constitute the true unit of selection, thus co-evolving together

and shaping the genetic composition of each other. These concepts have enabled a re-evaluation of host-associated microorganisms beyond their association with diseases and have permitted the consideration of their role in host physiology (see Laukens et al. 2015). The influence of the microbiota on the host immunity is one of the best examples illustrating the contribution of associated microorganisms to host fitness. Interestingly, commensal microbiota has multiple ways to cooperate with the host immune system to prevent pathogen infection. This has been well described in model species, especially in mice where experiments on germ free animals is feasible. Commensal bacteria can boost the immune system either locally or systemically (e.g. in distant tissues) (Chung et al. 2012; Hooper et al. 2012; Abt and Artis 2013; Sommer and Bäckhed 2013). Additionally, commensals can also directly impact bacteria (either true pathogens or opportunistic ones) through interference with their entry and establishment via direct competition or production of antimicrobial compounds. The aquatic invertebrate model organism, the cnidarian *Hydra vulgaris* extensively addresses the beneficial effect of the microbiota. A substantial body of work clearly shows that mucosal innate immunity is shaped by host-microbiota interactions (Schröder and Bosch 2016).

b- Influence of the microbiota for health and disease outcomes in crustaceans and molluscs of aquaculture interests

Several studies focusing on host-associated microbiota have highlighted the beneficial effects of microorganisms for invertebrate species relevant to aquaculture. Beneficial properties of the microbiota have been observed in several context relevant for aquaculture such as enhanced growth, feed efficiency, increased survival in early life stages, settlement, shell quality, size, or organoleptic quality (Infante-Villamil et al. 2021; Rajeev et al. 2021; Paillard et al. 2022; Yu et al. 2022). We have focused our review work on host associated microorganisms which are beneficial in a context of health and infectious disease outcomes (Table 1). Several studies have indicated that higher microbiota diversity is associated with the host health status in both crustaceans and molluscs (Table 1, part 1). This may result from the capacity of a highly diversified microbial community to provide a greater number of beneficial functions. In different shrimp species, a greater diversity of metabolites with potential beneficial properties is produced by half of the core microbiota likely contributing to their immune homeostasis (Zhang and Sun 2022). Additionally, functional redundancy has been described in the more diverse microbial community of the yellow abalone compared to the blue abalone correlating with a lower prevalence of the Withering Syndrome disease (Cicala et al. 2018, 2022).

However, it's essential to note that while the mentioned studies are mostly correlative, only two of them have demonstrated a direct causality between healthy or diseased microbiota and health status. In penaeid shrimp, intestinal microbiota transfer (IMT) from WFS diseased shrimp to healthy ones led to the development of symptoms similar to those of diseased shrimp (Huang et al. 2020). In *C. gigas*, a microbiota transfer from healthy juvenile donor oysters to healthy recipient larval oysters for ten days, resulted in enhanced immune response at juvenile stages and a greater capacity to prevent OsHV-1 viral proliferation associated with reduced mortality to the POMS disease (Fallet et al. 2022).

Beyond the beneficial properties induced by a highly diversified microbial community, specific strains within the microbiota may also contribute to the host health through their direct antagonistic effect on pathogens (Table 1, part 2). For instance, the addition of Alterin-producing *Pseudoalteromonas hCg-42* correlates with lower mortality in *Vibrio* challenged *C. gigas* (Defer et al. 2013; Desriac et al. 2020 and Dantan et al., Not published). Injection of BALOs (*Bdellovibrio*-like organisms) in lobster juveniles results in lower *Vibrio* abundance compared to non-injected control group (Ooi et al. 2021). BALOs are a group of Gram-negative bacteria that prey on other Gram-negative bacteria and have been reported in the haemolymph microbiota of diverse shrimp species and the spiny lobster *Panulirus ornatus*. Moreover, in a broader context, extensive correlation analysis have revealed associations between the presence or abundance of specific bacteria and varying disease prevalence (King et al. 2019a; Clerissi et al. 2020; Infante-Villamil et al. 2021). Nevertheless, the precise role of these bacteria in contributing to the advantageous properties to their associated host has yet to be accurately delineated.

In summary, evidence suggests that the host-associated microbiota significantly contributes to the health of molluscs and crustaceans relevant to aquaculture. Disruption of a healthy and diversified microbiota can lead to disease states. Understanding the dynamic interplay of this interaction in these species is essential to implement disease mitigation strategies.

c. Innate immune effectors are key players which shape the microbiota of molluscs and crustaceans of interest to aquaculture.

In many species, host-microbiota associations appear relatively stable within an individual showing a strong correlation between host phylogeny and microbiota that co-evolved for beneficial outcomes, a concept known as phyllosymbiosis (Lim and Bordenstein 2020). This

concept implies that the host can selectively shape its microbiota, leading to convergence of host phylogeny and microbiota composition. The correlation between host genetic background and microbiota composition has been clearly observed in marine molluscs. In *C. gigas*, microbiota specific to oyster families has been described and perfectly segregates with the oyster's genetic background (King et al. 2019b; Clerissi et al. 2020; Dupont et al. 2020).

In a broader context, evidence from invertebrate models reveals that immune systems can control microbial communities and promote its long-term colonization through diverse strategies such as mechanical barriers provided by chitine or mucus layers, or mediated by conserved non-self recognition and antimicrobial mechanisms (Rosenstiel et al. 2009; Chu and Mazmanian 2013; Broderick 2016; Dishaw et al. 2016). Among all these mechanisms, antimicrobial peptides are major players identified as key regulators of a well-balanced interaction between commensal microbes and host tissues. Implication of these peptides for microbiota shaping has been well characterised in the two noteworthy invertebrate aquatic models, the squid *Euprymna scolopes* (Heath-Heckman et al. 2016) and the cnidaria *Hydra vulgaris* (Fraune and Bosch 2007; Augustin et al. 2017). In molluscs of aquaculture interest, several studies have shown an indirect link between microbiota and host immunity, especially in the context of abiotic stresses (Auguste et al. 2019, 2020; Yang et al. 2020; Li et al. 2022; Liu et al. 2022; Zhao et al. 2023; Dang et al. 2023) and disease-triggered dysbiosis (de Lorgeril et al. 2018; Milan et al. 2019). However, in these studies, whether the stress triggers a change in the microbiota resulting in an immune shift or conversely, whether the stress triggers an immune shift resulting in a change of microbiota structure remains to be determined. The few studies that have addressed the direct influence of the host innate immunity on microbiota composition in molluscs and crustaceans are listed in the Table 2. A substantial number of studies have been conducted in the kuruma shrimp *Marsupenaeus japonicus* and the king prawn *Litopenaeus vannamei* where RNAi invalidation has pointed to a causal link between innate immune effectors (reactive oxygen/nitrogen species -ROS and RNS- and antimicrobial peptides) and the balance of the bacterial microbiome (Table 2). In *L. vannamei*, similar studies involving inactivation of genes encoding for AMPs of the crustin family have shown that the type I and type II crustins were involved in regulating the host bacterial community (Lv et al. 2020b, a).

In molluscs, to our knowledge, studies have investigated the direct influence of the host innate immunity for microbiota composition in scallops and oysters. In the scallop *Argopecten purpuratus*, the big defensin ApBD1 and the bactericidal/permeability increasing protein ApLBP/BPI1 have been shown to regulate the proliferation of specific bacterial groups in the

haemolymph (Muñoz et al. 2019; González et al. 2020). In the same species, a g-type lysozyme is shown to participate in the microbial homeostasis (González et al. 2022). In *C. gigas*, big defensins have the potential to shape oyster microbiota. *Cg-BigDef1* induced a significant shift in oyster microbiota β -diversity 6h and 24h after its injection into oyster tissues (de San Nicolas et al. 2022).

In summary, a body of research in molluscs and crustaceans has revealed an interaction between host immunity and microbiota composition, particularly in response to abiotic stresses and dysbiosis triggered by diseases. Additionally, a direct causal link between innate immune effectors and the balance of the bacterial microbiome has been demonstrated in kuruma shrimp, king prawn, scallops, and oysters, where gene function invalidation serves as a method to establish causality between host innate immunity and microbiota composition.

d. Toward the use of next generation probiotics for an optimised microbial education

a- Probiotic usage: definition and limitation for shellfish aquaculture

In this article, we reviewed recent studies that emphasize the critical role played by the microbiota on health and disease resistance for economically important marine invertebrates. There is an increasing body of evidence that points toward the beneficial advantage conferred by the microbiota for crustaceans and molluscs in an aquaculture context and how a fine-tuned setting is necessary to ensure an optimal host-microbiota balance. Disruption of this balance by external stressors or pathogens induces a decrease in the fitness of the holobiont, eventually leading to death. In this sense, it is evident that we should now consider each organism as an holobiont where associated microorganisms not only provide a beneficial advantage in adverse conditions but are also essential players in maintaining host health. Experimentation with the microbial component of the holobiont has been active for decades using probiotics. According to the definition of the Food and Agriculture Organization (FAO), a probiotic is “live microorganisms, which when consumed in adequate amounts, confer a health benefit on the host” (Food and Agriculture Organisation of the United Nation 2006). Probiotics have been successfully applied for decades in human health, focusing on beneficial bacteria isolated from natural human microbiota. For instance, Lactic Acid Bacteria (LAB) have been extensively studied for their beneficial properties (as reviewed by Mousavi Khaneghah et al. 2020) and were recently used for potential application in depuration in *C. gigas* (Sorée et al. 2023). This has led to several commercial applications with proved beneficial effect in human health and in

agro-industries. The use of probiotic strains has further expanded to animal production, and is considered a promising eco-responsible and prophylactic alternative to antibiotics (Getachew 2016). The extensive use of antibiotics has been identified as a major cause for emergence of antibiotic resistant pathogens in shrimp aquaculture (Thornber et al. 2020). In this field, the application of probiotics has been used for decades as an environmentally-friendly alternative strategy to fight pathogens (Akhter et al. 2015; Goh et al. 2022). The use of probiotics has also been successfully tested on oysters (Aguilar-Macías et al. 2010; Kesarcodi-Watson et al. 2012b; García-Bernal et al. 2019; Modak and Gomez-Chiarri 2020). The mode of action of probiotics and their beneficial effects on disease resistance and species performance in aquaculture have been extensively reviewed (Akhter et al. 2015; Yeh et al. 2020; Goh et al. 2022; Sumon et al. 2022). Several limitations regarding the application of probiotics, which is not always beneficial, have been reported. One major limitation is that probiotic strains do not persist in the endogenous microbiota (Table 3). The addition of non-autochthonous strains (bacteria isolated from other host species) is a possible explanation considering the tight association between the host and its microbiota, as explained in the first part of this review. Regular addition of probiotics supplemented with prebiotics (compounds in food that induce the growth or activity of the probiotics) has been a strategy applied so far to maintain the long-term beneficial properties of the added strains. However, such an approach increases the economic cost of the probiotic solution (Pérez-Sánchez et al. 2018). Another major limitation in the shellfish farms is the impossibility of using probiotics in open sea culture. In this sense, the use of probiotics in molluscs industry is limited to hatcheries during larval rearing or necessitate land-based facility to apply the treatment on spat or adult stages. Except for the study performed by Fallet et al. (2022), the literature on probiotics added during larval stages do not report long-term beneficial effect for molluscs and crustacean health (Table 3). Several reasons might explain this potential caveat: (i) The long-term beneficial properties have not been addressed in the published study mentioned in table 3 and the authors have overlooked the potential of their probiotics to confer long-term benefits to their host (ii) The author have added a single probiotic strain and considering the importance of the microbial diversity for host fitness, the use of multi-strain probiotics (MSP) should lead to more beneficial effects. MSP consist of a mix of two or more strains, that have proven to offer various beneficial effects for their host. The improved performance of MSP exposure compared to single strain has been intensively described in fish (Puvanasundram et al. 2021) (Padeniya et al. 2022) and is starting to be described in crustaceans and molluscs (Kesarcodi-Watson et al. 2012a; Grandiosa et al. 2020). For example, the use of a mix of four different probiotics has more efficiently improved the

growth and immune status of *L. vannamei* in comparison with single-strain probiotics (Wang et al. 2019). The addition of MSP during the larval stage conferred a protective effect in Spiny Lobster (*P. ornatus*) larvae against *Vibrio owensii* infections (Goulden et al. 2012).

In summary, probiotics have proven to be promising tools for mitigating disease risks in mollusk and crustacean aquaculture. However, there are challenges that still need to be addressed, such as the limited persistence in the endogenous microbiota, the impossibility of applying them in open sea culture, and the associated economic costs. The use of multi-strain probiotics isolated from the endogenous microbiota is a promising way to enhance host performance. Additionally, exploring the long-term effects on immune status after probiotics addition during larval rearing is another avenue that deserves further exploration.

b-Next generation probiotics: emerging tools for a better prediction of health promoting bacteria

The recent advances on host-microbiota interactions have brought new insights, that should broaden the use of probiotics in the most updated strategies. A substantial number of large scale analyses applied to diverse crustacean (Holt et al. 2021) or mollusc species (Yeh et al. 2020) has led to multiple descriptions of bacteria associated with different tissues, at several life stages, related to different health statuses, in different environments. These datasets constitute tremendously valuable resources for predicting of stage and species-specific beneficial bacteria. Correlation studies that identify association between health status and microbiota composition should lead to deciphering consortia of beneficial microorganisms. This approach has been applied to microbial communities associated with health and disease in shrimp in order to identify stage specific disease or bioindicators of health (Zheng et al. 2017). Based on a Linear Discriminant Analysis (LDA) Effect Size (*LEfSe*) (Segata et al. 2011), Zheng et al. identified that the genus *Meridianimaribacter* was enriched in healthy shrimp and with good water quality. In agreement with other culture-dependent study (Zheng et al. 2016), this genus was identified as a beneficial bacterium for shrimp larvae and was suggested as a probiotic candidate for shrimp larval rearing. Similarly, based on the the *LEfSe* method applied to the bacterial community associated with POMS-sensitive and resistant oysters, we identified stage specific bacterial taxa that are overrepresented in more resistant oysters during experimental infection (Fallet et al. 2022). In another study, we performed a differential analysis of taxa abundance between conditions in microbiome and identified three bacterial families (*Colwelliaceae*, *Cyanobacteria* (Subsection III, family I), and *Rhodobacteraceae*) significantly associated with

oysters resistant to POMS disease (Clerissi et al. 2020). Overall, we suggest that predictive analysis based on correlation studies in combination with literature information, should be an upstream step to identify putative beneficial bacteria that can be then isolated by culture-based approaches (Figure 1). We anticipate an improved benefit of the probiotics designed in such a way considering the concept of the holobiont and the long co-evolutionary history between the host and the bacteria (Box 1). However, this is only a predictive strategy, and the beneficial effect of these probiotics requires validation in an experimental framework, as we describe in Figure 1.

c- Microbial education during early stages: An opportunity to sustain long-term beneficial effects.

Here, we also explore another possibility, which involves educating the host immune system by applying beneficial microorganisms throughout the course of immune system development. This “microbial education” plan is not just another probiotic strategy but is a real concept rooted in robust recent scientific studies that span several fields, as summarised in the box 1: (1) the microbial community management in aquaculture (2) the holobiont theory (3) The biological embedding concept (4) the possibility to prime the innate immune system of invertebrate species to induce long-term immune protection. The “microbial education” outline is to add host specific microorganisms during the larval development. This strategy aims to influence the immune system toward better defence capacities. The possibility to influence the developmental trajectory of individuals through environmental manipulation during early development has been previously discussed by others (Gavery and Roberts 2017; Eirin-Lopez and Putnam 2021). This strategy can be used to produce a desired phenotype and applied to critical issues in aquaculture with some benefits but also, some barriers as summarised in Table 4. The possibility to educate the host immune system through a microbial education has been recently demonstrated in oysters (Fallet et al. (2022)). In this study, we showed that it was possible to increase *C. gigas* immune competence until the juvenile stage, four months after a 10 days natural microbial exposure during the larval stages. This study clearly highlighted that an interaction with a diversified and autochthonous microbiota during the early developmental stages has lifelong consequences, even transgenerational impact, and could be instrumental in fighting against POMS disease. Such an approach remains to be investigated for other pathogens commonly described from the larval to adult stages in *C. gigas* (Dégremont et al. 2021), and other oyster species (Dégremont et al. 2015). This early life microbial education is

somewhat in contradiction with some hatchery current practices, which seek to limit the introduction of both non-pathogenic and pathogenic bacteria, viruses, and eukaryotes as well as to eliminate unwanted plankton and zooplankton in seawater, from the conditioning of the broodstock to the larval and spat stages (Bourne et al. 1989; Helm et al. 2004; Eljaddi et al. 2021; Cordier et al. 2021). Additionally, the depletion of bacteria in the rearing environment and in farmed animals is often accentuated by the addition of antibiotics. This process was tested in response to the risk of mortality in livestock (Le Pennec et al. 1973; Bourne et al. 1989; Nicolas et al. 1992) and was quickly associated with the risk of selecting antibiotic-resistant bacteria (Martin and Mengus 1977; Bourne et al. 1989). Today, however, this use remains a common practice, mainly at the larval stage of various species of marine invertebrates, as well as their broodstock.

Globally, research efforts remain to be performed for microbial education to be applied during larval rearing. The microbial management plan aligns with our proposal, aiming to increase the performance and viability of larvae by applying ecological theories and optimising larvae-microbiota interactions (De Schryver and Vadstein 2014; De Schryver et al. 2014). Diverse methods (MMS: Microbially Matured Water System, BFT: Bio Floc Technology, RAS: Recirculating Aquaculture System) allow for the stable presence of naturally K-selected microbial community into the rearing tanks. It clearly has a long-term beneficial impact and favours the production of high quality juveniles (Bossier et al. 2016; Vadstein et al. 2018). The immunomodulatory properties and health benefits of such practices have been demonstrated (Kumar et al. 2021). However, using these methods, the added microflora is not controlled and not specific to the raised species and it is hazardous since it may contain pathogenic microorganisms. In perspective of this review, we propose a simple conceptual model to summarize the possible strategy that could be applied to perform the most appropriate early-stage microbial education (Figure 1). Full-scale trials will be necessary and should consider several parameters that could impact hatching and larval growth/survival. The parameters should include: the definition of the multi-strain bacterial mix to be applied, the way the bacterial mix should be administered, the most appropriate window to be targeted, the duration of the exposure, and the aquaculture rearing conditions. In Fallet et al. (2022), the microorganisms to which recipient oyster larvae were exposed came from healthy *C. gigas* spat sampled in their natural environment and used as microbiota donors. Those donor oysters were held upstream of the larval tanks but downstream the filter systems. This strategy allowed transferring the whole oyster associated microorganisms' community to recipient larvae and accordingly, the immunomodulatory potential of the overall microbial diversity (including

viral, prokaryotic, eukaryotic and uncultivable microorganisms) was exploited. Such a strategy could be applied to hatcheries. However, a major limitation of such a practice should be considered: the microorganism community is not controlled and remains hazardous. A real health risk remains since donor oysters of microbiota may also carry opportunistic or latent pathogens while appearing to be healthy. In this sense, a controlled environment rich in microorganisms used during the larval rearing is recommended. This can be achieved using donor oysters of microbiota which were always kept in biosecured facilities (Figure 1). In this way, the oysters were shown to be devoid of the three main pathogens of *C. gigas* from larvae to juveniles (Azéma et al. 2017; Dégremont et al. 2021). Alternatively, a controlled culture-based bacterial formulation, a multi-strain bacterial mix can be added during the larval rearing (Figure 1). For this purpose, it will be necessary to first build a collection of host-associated bacteria and screen several bacterial cocktails for their ability to induce long term immunomodulatory effects. Early development is the preferential stage to be targeted to induce long lasting effects, as previously reported (Galindo-Villegas et al. 2012; Arrieta et al. 2014; Gensollen et al. 2016; Fallet et al. 2022). Targeting this stage also offers the possibility to treat several millions of larvae at the same time.

In summary, we discuss here an alternative approach to mitigate infectious disease in aquaculture. We propose to educate the host immune system by introducing host beneficial microorganisms during the maturation of the innate immune system. This concept of “microbial education” goes beyond conventional probiotics strategies and is grounded in recent scientific studies that intersect various fields, such as the management of microbial communities in aquaculture, the holobiont theory, the biological embedding concept, and the ability to prime the invertebrate innate immune system. We further suggest a potential strategy for early-stage microbial education, with an emphasis on controlled environments and health considerations when transferring microorganisms.

Conclusions:

Diseases of farmed shellfish induce significant economic losses. To address these recurring threats, the exploration of alternative and environmentally friendly solutions has been ongoing for a long time. In this review, we delve into how host-associated microorganisms are not only greatly contribute to improving the fitness of their host but also part of a much more complex evolutionary unit called the holobiont. We discuss the possibility of implementing advanced

techniques and the concept of the holobiont field as potential new strategies to mitigate diseases in farmed animals. We suggest (i) using the increasingly expansive data describing microbiota in diverse species as a tool to predict the most natural beneficial bacteria for their associated host and (ii) applying an exposure of these predicted beneficial microorganisms during the larval rearing step in the hatchery to benefit from biological embedding. If properly applied, this microbial education is anticipated to induce lifelong protection and could be a natural and sustainable strategy for safeguarding aquaculture efforts from infectious disease.

List of abbreviations:

AHPDN: Acute Hepatopancreatic Necrosis Disease

BALOs: Bdellovibrio and like organisms

BLIS: Bacteriocin-Like Inhibitory Substance

Cg-BigDef1: *C. gigas* Big Defensin 1

EMS: Early Mortality Syndrome

LAB: Lactic Acid Bacteria

LC_MS: liquid chromatography-coupled mass spectrometry

LEfSe: Linear discriminant analysis (LDA) Effect Size

MSP: multi-strain probiotics

OTU: Operational Taxonomic Unit

POMS: Pacific Oyster Mortality Syndrome

RNS: Reactive Nitrogen Species

ROS: Reactive Oxygen Species

TNF: Tail Fan Necrosis

WFS: White Feces Syndrome

WS: Withering Syndrome

WSSV: White Spot Syndrome Virus

Competing interests

The authors declare that they have no competing interests.

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1 **Tables and figures:**

2 **Table 1: Studies which support that host-associated microbiota influences the health of molluscs and crustaceans of aquaculture interest**

3 **Part 1: Studies which highlight how the microbiota diversity influences health and disease outcomes.**

Species	Disease	Microbiota change	Effect on the Host	Reference
<i>L. vannamei</i>	WSSV	Increased microbial diversity induced by dietary supplementation with brown seaweeds composition	Reduction of the mortality rates after a WSSV challenge in the diet-fed group compared to the control shrimp	(Schleder et al. 2020)
<i>M. japonicus</i> , <i>L. vannamei</i> , <i>Macrobrachium rosenbergii</i> , <i>Procambarus clarkii</i>	WSSV	Production of metabolites with antiviral potential by half of the core microbiota in response to viral challenge	Likely contributes to a better immune homeostasis and potential host resistance	(Zhang and Sun 2022)
<i>Homarus gammarus</i>	Infection with <i>Homarus gammarus nudivirus</i> (HgNV)	Increased species richness and diversity observed in sea-based container culture compared to land-based culture	Lower viral prevalence in sea-based container culture compared to land-based culture	(Holt et al. 2020)

<i>Penaeid</i> shrimp	WFS	Decreased in microbial richness and diversity during the WFS disease	Intestinal microbiota transplants from WFS diseased to healthy shrimp lead to symptoms similar to those of the diseased ones	(Huang et al. 2020)
<i>Haliotis fulgens</i> <i>Haliotis corrugata</i>	WS	Higher microbial diversity in the yellow abalone (<i>Haliotis corrugata</i>) compared to blue abalone (<i>Haliotis fulgens</i>)	Increased susceptibility to the WS in the blue abalone correlates with structural and functional alterations in their microbiota, in contrast to yellow abalone which maintains a stable microbiota under WS stress	(Cicala et al. 2018, 2022)
<i>C. gigas</i>	POMS	Higher microbial diversity in larvae after a microbiota transfer compared to control group	Lower mortality in the microbiota transferred group compared to control	(Fallet et al. 2022)

4

5 **Part 2: Studies which highlight the beneficial contribution of a specific bacterial species for the health of its autochthonous host by**
6 **antagonistic effects.**

Species	Disease	Microbiota change	Effect on the Host	Reference
<i>C. gigas</i>	Vibriosis	Addition of <i>Pseudoalteromonas</i> (hCg-42 and hCg-6) (producer of antimicrobial molecules (Alterins)) to <i>C. gigas</i>	<i>In vitro</i> antimicrobial effect against vibrio and decrease of the mortality rates after a vibrio challenge in <i>C. gigas</i> incubated with <i>hCg-42</i>	(Defer et al. 2013; Desriac et al. 2020; Not

				published results)
<i>P. ornatus</i>	Vibriosis	Addition of <i>BALOs</i> (<i>Bdellovibrio</i> and like Organisms) to <i>P. ornatus</i>	Abundance decreases of vibrio in haemolymph of host injected with <i>Halobacteriovorax</i> sp. (BALOs)	(Ooi et al. 2021)
<i>Litopenaeus vannamei</i>	Vibriosis	Addition of <i>Bacillus</i> sp. YC5-2 initially isolated from the guts of healthy wild adult shrimp	<i>In vitro</i> antibacterial effect against vibrio and decreased mortality after a vibrio challenge	(Luis-Villaseñor et al. 2011)
<i>C. gigas</i>	<i>V. coralliilyticus</i> infection	Addition of <i>Pseudoalteromonas</i> (D16 and DM14), and Epibacterium (B11) initially isolated from <i>C. gigas</i> oysters	Improving survival of oysters during <i>V. coralliilyticus</i> infection	(Madison et al. 2022)

7

8 **Legend of Table 1:** Studies that underscore the microbiota's impact on health and disease outcomes in invertebrates relevant to aquaculture. The
9 part 1 comprises studies demonstrating how a shift in the microbiota diversity (reported in column 3) impacts the host health status (reported in
10 column 4). The part 2 comprises studies demonstrating how the introduction of antimicrobial-producing strains into the host microbiota (indicated
11 in column 3) counteracts pathogens and contributes to the health benefits of the host (indicated in column 4).

12

13 **Table 2: Studies which support direct consequences of the innate immune system for the host associated microbiota structure.**

Organisms	Innate immune effectors	Influence on the microbiota structure	Reference
<i>M. japonicus</i>	MjHeCL (protein that combines a C-type lectin domain, and an antimicrobial peptide)	The down-regulation of MjHeCL expression by RNAi leads to the proliferation of the haemolymph microbiota, ultimately resulting in shrimp death	(Wang et al. 2014)
<i>M. japonicus</i>	The catalase enzyme by regulating the Reactive Oxygen Species (ROS) level	High level of ROS in shrimp intestine (obtained after inactivation of MjCAT which encodes for the catalase) correlates with a reduction in bacterial load of the shrimp intestinal lumen	(Yang et al. 2015)
<i>M. japonicus</i>	Nitric Oxide (possibly through its positive regulation of AMPs production)	After RNA interference of the Nitric Oxide Synthase (NOS) or treatment with an inhibitor of NOS, NO production decreased, and the gut bacterial load increased significantly in shrimp	(Hong et al. 2021)
<i>L. vannamei</i>	Antimicrobial peptides of the Crustin family	Inactivation of the gene encoding for the antimicrobial peptide LvCrustin II-1 leads to change in the microbiota composition of the shrimp gills	(Lv et al. 2020a)
<i>L. vannamei</i>	Antimicrobial peptides of the Crustin family	Inactivation of the gene encoding for the antimicrobial peptide LvCrustin I-1 leads to change in the microbiota composition of shrimp intestines	(Lv et al. 2020b)

<i>A. purpuratus</i>	Antimicrobial peptide from the big defensin family (ApBD1)	Inactivation of the gene encoding for ApBD1 leads to changes in bacterial composition of the hemolymph microbiota	(González et al. 2020)
<i>A. purpuratus</i>	The bactericidal/permeability increasing protein ApLBP/BPI1	Inactivation of the gene encoding for ApLBP/BPI1 leads to changes in bacterial composition of the hemolymph microbiota	(González et al. 2020)
<i>A. purpuratus</i>	g-type lysozyme ApGlys	Inactivation of the gene encoding for ApGlys leads to higher diversity in hemolymph bacterial community and imbalance in certain bacterial groups	(González et al. 2022)
<i>C. gigas</i>	Antimicrobial peptide from the big defensin family (<i>Cg</i> -BigDEf1)	Injection of synthetic <i>Cg</i> -BigDEf1 into the oyster tissues induced a significant shift in oyster microbiota β -diversity	(de San Nicolas et al. 2022)

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16 **Legend of the Table 2:** Studies indicating that certain host innate immune effectors (specified in column 2) directly influence microbiota
17 composition. The repercussions of the inactivation (or addition) of these effectors on host microbiota structure are detailed in column 3.

18 **Table 3: Current limitations of probiotic application in mollusc and crustacean aquaculture**

The probiotics do not persist in the endogenous microbiota	
Exposure of the European abalone <i>Haliotis tuberculata</i> to the <i>Pseudalteromonas</i> hCg-6 exogenous strain leads to a transient establishment of this probiotic strain in the haemolymph rather than the establishment of a long-term interaction	(Offret et al. 2018)
Exposure of <i>C. gigas</i> larvae to bacteriocin-like inhibitory substance (BLIS) - producing <i>Aeromonas</i> , showed that the probiotic strain concentration decreased right after it was added to the oyster and was not detectable 72h after its addition	(Gibson et al. 1998)
The probiotics display short-term beneficial effects	
In <i>L. vannamei</i> , larval exposure to <i>Bacillus subtilis</i> E20 or to <i>Bacillus sp.</i> YC5-2 is beneficial to the shrimp during larval stages but subsequent beneficial effects on further stages have not been tested.	(Liu et al. 2010; Luis-Villaseñor et al. 2011)
Immunomodulation is observed in <i>L. stillostris</i> after a larval exposure to <i>Pseudoalteromonas</i> probiotic candidates but it has not been tested on later stages.	(Pham et al. 2014)
Addition of <i>Bacillus pumilus</i> RI06–95 and <i>Phaeobacter inhibens</i> S4 during larval development of the eastern oyster <i>C. virginica</i> for either 6h or 24h prior to pathogen challenge leads to an effective immune response and protection against <i>V. coralliilyticus</i> and <i>Vibrio tubiashii</i> . The beneficial effect was observed	(Karim et al. 2013; Modak and Gomez-Chiarri 2020)

immediately after exposure, but no significant protection was observed when the larvae were challenged 48 and 96h after removal of the probiotic.	
The exposure of <i>C. corteziensis</i> larvae to a mix of two bacteria <i>Pseudomonas aeruginosa</i> , strain YC58 and <i>Burkholderia cepacia</i> , strain Y021, leads to a better survival during the larval rearing stages.	(Campa-Córdova et al. 2011)
A 20-hours larval exposure to <i>Pseudoalteromonas sp.</i> D41 or <i>Phaeobacter gallaeciensis</i> , has conferred a significant protection to <i>C. gigas</i> larvae against <i>V. coralliilyticus</i>	(Kesarcodi-Watson et al. 2012b).
An immunomodulation was also observed on the Yesso scallop <i>Patinopecten yessoensis</i> fed with a diet of microalgae supplemented with the strain <i>Pseudoalteromonas sp.</i> F15 during larval stages	(Ma et al. 2019).
In the sea cucumber <i>Apostichopus japonicus</i> , an exposure to bacterial strains <i>Pseudoalteromonas elyakovii</i> HS1, <i>Shewanella japonica</i> HS7, or <i>Vibrio tasmaniensis</i> HS10 leads to an enhanced cellular and humoral immune response and to an improved survival and growth rate	(Chi et al. 2014)

20 **Box 1: Scientific rationale behind the microbial education**

Description of the concept	
<p>(1)</p> <p>Microbial community management in aquaculture</p>	<p>The microbial management in aquaculture consist in controlling the water microbiota in rearing system according to ecological selection principles. Methods which favour K-selection in the rearing environment have been shown to select against r-strategic microbes and promote healthy microbe-larvae interactions (De Schryver and Vadstein 2014; De Schryver et al. 2014; Bossier et al. 2016; Vadstein et al. 2018).</p>
<p>(2)</p> <p>Holobiont concept</p>	<p>Host associated microbial communities can influence multiple facets of animal physiology including development, behaviour, ecological functions such as stress tolerance as well as pathogen resistance and immunity. (Sharon et al. 2010; Heijtz et al. 2011; Webster et al. 2011; McFall-Ngai et al. 2013; Kostic et al. 2013; Gilbert et al. 2015; Schmidt et al. 2015; Kohl and Yahn 2016; Webster and Reusch 2017; Gould et al. 2018). The beneficial effect of the microbiota for host immune homeostasis is clearly one of the best illustration of the holobiont theory (Chung et al. 2012; Hooper et al. 2012; Abt and Artis 2013; Sommer and Bäckhed 2013).</p>
<p>(3)</p> <p>Biological embedding</p>	<p>The biological embedding is the process by which the life history of an individual during ontogeny impacts its development and modifies its phenotype (Aristizabal et al. 2020). The environment has a long-lasting influence on the functions of biological systems and this environmental imprint has major consequences for the behaviour and health of individuals. The early stages of development are sensitive windows for recording this environmental information (Dolinoy et al. 2011; Fellous et al. 2022). Microbial colonization during early life is an example of biological embedding and many studies have emphasized its critical role in educating and durably imprinting the immune system (Arrieta et al. 2014; Gensollen et al. 2016).</p>

(4)

Immune priming

The immunological memory as described in the adaptive immune system of vertebrates does not exist *per se* in invertebrates and the fight against pathogens relies on innate immune mechanisms. It has been recently shown that this innate immunity can be trained to retain the memory of a primary exposure to better respond to infections encountered later (Netea et al. 2020). In molluscs and crustaceans, recent studies have shown that it is possible to train the innate immune system (Zhang et al. 2014; Norouzitallab et al. 2016; Lafont et al. 2017, 2020). The possibility of using the trained immunity as a potential strategy to fight disease in shrimp aquaculture has been recently reviewed by Roy et al. (2020).

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23 **Table 4: Benefits and barriers for practicability in aquaculture**

Benefits

The microbial education is a probiotic strategy that must be applied during the larval stages in hatcheries/nurseries. This plan is feasible for shellfish as it represents small volume of animals to be treated using bath, even those which will be transferred in open sea at later stages.

The use of donors of microorganisms is the most promising strategy, it is easy to implement, and it is not costly.

The probiotic exposure can be performed on numerous animals (several hundreds of millions of larvae) at the same time by bath or on their diet.

The microbial education aims at providing individuals with an increased robustness against infectious diseases. It is a strategy to better face the epizooties without using antibiotics.

The exposure benefice during larval stages is expected to be lifelong but also multigenerational.

Barriers

The larval stage is identified as the most sensitive rearing phase, and inappropriate probiotic exposure during this stage may be more detrimental than beneficial.

The microbial education approach is empirical and require time for optimisation. The optimal setup for one species might not be applicable to another species. Similarly, the response of the host to the exposure of probiotics could depend on their broodstock, used to produce the shellfish.

The exposure to multi-strain probiotic could lead to increase resistance toward particular pathogens and may not protect against other diseases.

The use of donors of microorganisms is the most promising strategy, but the transferred microbiota is not controlled and there is a risk for pathogen transmission.

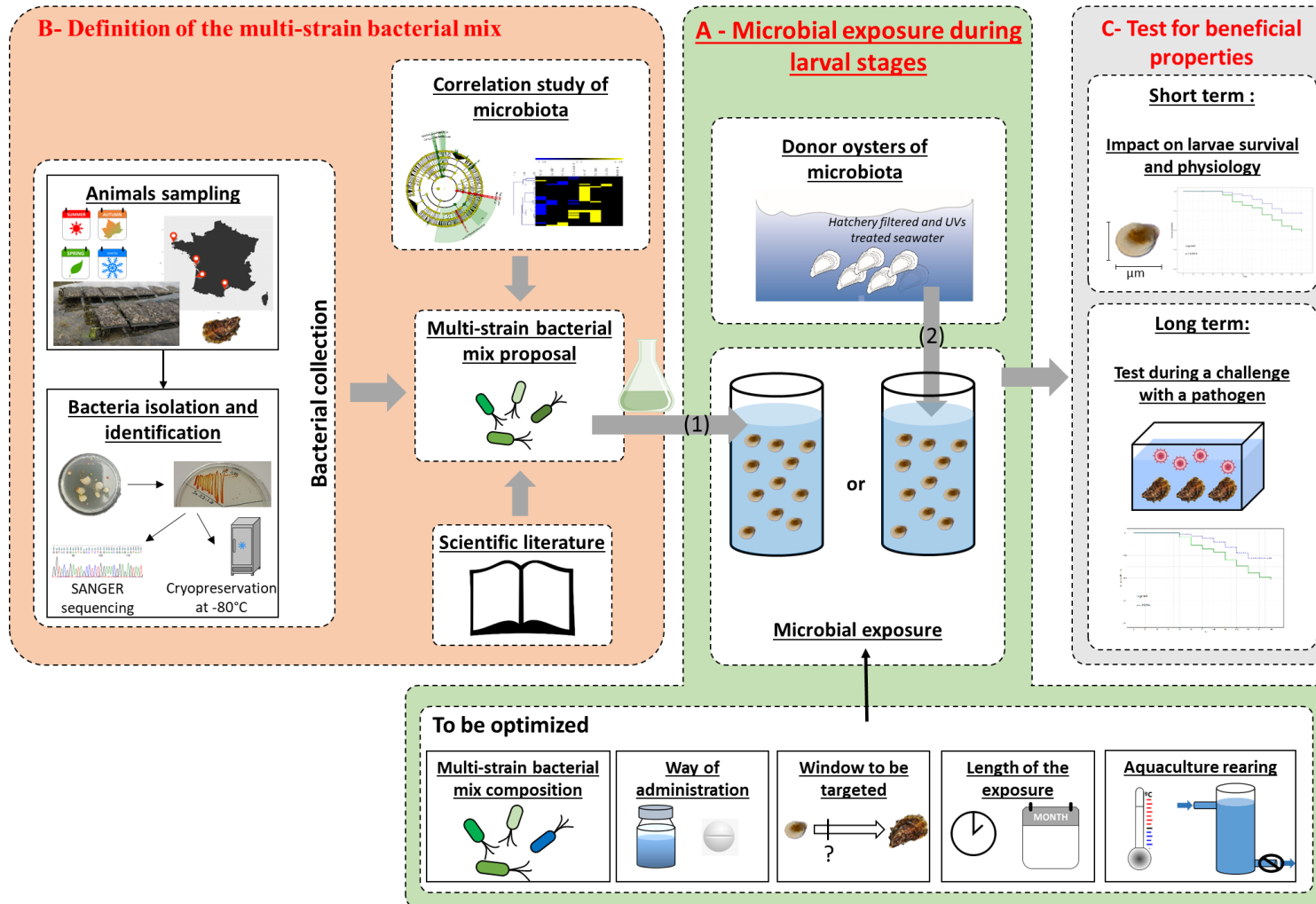
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27 **Figure 1: Full scale experimental design to identify beneficial bacteria for microbial education.**

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29

30 **Figure legend**

31

32 **Figure 1: Full scale experimental design to identify beneficial bacteria for microbial**
33 **education.**

34 For a long-term beneficial effect, it is recommended to perform the microbial education during
35 larval stages (Part A, green coloured). The microorganisms to be added to the seawater during
36 larval rearing can be introduced either (1) by pathogen-free donor oysters that were always kept
37 in controlled facilities using UV-treated seawater, strict biosecurity zoning and management
38 procedures or (2) by adding cultured based multi-strain bacterial mix that has been carefully
39 selected. The method of administration of the mix and its composition has to be optimised to
40 maximize its absorption by the larvae (Dipping or in freeze-dried form, delayed or simultaneous
41 to the feeding, biofloc form). The exposure window (from embryogenesis to larval stages) and
42 the duration of the exposure to the bacterial cocktails must be adjusted. Rearing conditions are
43 other parameters that should be tested (Temperature, continuous flow or batch rearing system).
44 The definition of the multi-strain bacterial mix (Part B, orange coloured) is a necessary
45 upstream step to better anticipate beneficial properties. First, a library of cultivable bacteria
46 must be created. These bacteria will be preferentially isolated from the host to be targeted.
47 Pathogen-resistant animals (if probiotics aims at improving resistance toward a particular
48 infectious disease) must be collected from several geographical sites and at different seasons in
49 order to maximize the bacterial diversity. The bacteria thus obtained will then be cultivated,
50 purified, and cryopreserved. Several physicochemical parameters (culture media, temperature)
51 for bacterial culture may be tested to increase the potential biodiversity in the bacterial library.
52 Identification of each cultivated strain from the collection will be performed by SANGER
53 sequencing of the 16S rRNA coding gene. In parallel, *in silico* predictive analysis must be
54 performed to predict which bacteria are generally associated to resistant phenotype in the host
55 (if probiotics aims at improving resistance toward a particular infectious disease). This
56 correlative study will necessitate that several (meta)barcoding analyses have been previously
57 generated on microbiota sampled from resistant and sensitive animals toward a specified
58 disease. These correlative analyses, coupled with an exhaustive study of the scientific literature,
59 should make it possible to predict bacteria from the collection that could be beneficial probiotic
60 candidates. Then, beneficial effects of microorganism exposure will have to be tested (Part C,
61 grey coloured). Short term effect will be tested during the larval stages. Particular attention
62 should be paid to the effect of the multi-strain bacterial mix on the survival and physiology of
63 the larvae, to test whether the exposure is deleterious, beneficial, or neutral for larval

64 development and growth properties. Sampling for molecular analysis (*ie* transcriptomic,
65 barcoding, metabolic, epigenomic analysis,) may be worth it to decipher molecular basis of the
66 microbial effect. Finally, long-term beneficial effects will be tested on subsequent life cycle
67 stages: juveniles and adults will be challenged by pathogens.

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