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Application of Metagenomics to Chilean Aquaculture

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Abstract

Aquaculture is a rapidly expanding food production sector, facing the challenge of growth both increasing its efficiency and reducing its negative impact on the environment. Metagenomics is an emerging tool in aquaculture that helps to understand the complex host (fish-shellfish)-microbiota-pathogen-environment relationship underlying disease outbreaks, monitoring the dynamics of microbial diversity in farmed animals subject to different environmental conditions or perturbations. As Chile takes an important share of world aquaculture market, this chapter reviews the actual and potential applications of metagenomics to support a sustainable expansion and diversification of Chilean aquaculture. The focus is on (i) the role and function of the gut microbiota in the proper immunostimulation and disease control and (ii) the role of metagenomics in monitoring environmental microbial biodiversity and dynamics in relation to disease persistence and ecosystem stability. We conclude that despite the importance of the aquaculture sector in Chile, the application of metagenomics to deal with disease control and ecosystem preservation is still an emerging field of study. Understanding host (fish-shellfish)-microbiota-pathogen-environment diversity of interactions in a more holistic view, i.e., the holobiome approach, could be key to develop rational strategies to improve productivity by increasing resistance to diseases and reducing the use of antibiotics and their negative environmental impact.

Keywords: metagenomics, Chilean aquaculture, microbiota, disease, environment

1. Introduction

Aquaculture, the so-called blue biotechnology of the future [1], is the fastest growing food production sector and should continue expanding and diversifying to fulfill the need for good-quality protein of the ~8.5 billion people that will populate the earth by 2030. Such ascending trajectory of aquaculture will also help to achieve some of the United Nations (UN) sustainable developmental goals (SDGs) [2] that seek a better, less unequal, and more sustainable future for humanity by 2030, specifically in relation to food security, improved nutrition, and poverty alleviation in rural communities in particular. Therefore, aquaculture growth and management of aquatic genetic resources should be done in a sustainable manner, a goal that requires maximizing ecosystem goods and services in line with the Blue Growth Initiative of the Food and Agriculture Organization (FAO) [2]. Aquaculture has expanded at a rate of 5.8% annually (2001–2016), totalizing ~ 80 million of tons

in 2016, worth USD 231.6 billion (mainly finfish, crustacea, mollusk) [2]. Yet, the potential for expansion and diversification is immense considering the diversity of existing marine species [3]. For example, the total number of commercially farmed species increased in the last 10 years from 472 (2006) to 598 (2016) [2]. However, the sustainable aquaculture expansion is currently hampered by the impact of detrimental diseases causing high mortality and economic loss to the sector [4], together with the indiscriminate and inefficient use of antibiotics and chemicals to control them, which causes a negative ecosystem impact [5, 6]. To reevaluate this approach is the challenge ahead, and a good start is the ban imposed to the use of antibiotics by international organizations and consumers, which have stimulated the search for alternative microbial control strategies, like the use of probiotics [6]. Chile is a well-known and competitive salmon and trout producer. In fact, it's the world's second producer of salmon following Norway, and recently it has become a relevant mussel producer [2]. According to the FAO, in 2016 Chile ranked fourth among the world producers of marine and coastal finfish with 726.9 thousand tons (live weight) and the fourth marine mollusk producer (307.4 thousand tons [2]). The relevant aquaculture species are shown in **Figure 1**, with a total of 2162 centers distributed between the so-called Lake District (administrative region X); the northern part of Patagonia, where the salmon boom began; and the Magellan and Antarctic Region (XII). Region X is full of lakes that are intensively exploited as hatcheries for smolt production and also has protected coastal bays, fjords, and estuaries ideal for completing the marine phase of salmonid life cycle, not so far away from hatcheries. This region has 666 registered fish and 1171 mollusk centers. However, due to the ecosystem and disease consequences of the intensive salmon farming (high densities of fish per water volume) [7], the activity has moved to Region XI, with a total of 767 fish centers. In total (fish and mollusk), these centers harvested 1219.739 tons in 2016 [8].

One striking aspect of the farming of exotic salmonid species in Chile is the impressive expansion from the initial 80,000 tons harvested in the early 1990s to the 688,000 tons in 2004 and 900,000 tons in 2014 according to official statistics of the national service of fisheries, SERNAPESCA. In 2017 the production leveled at 791.103 tons with the following production figures by species (2017, tons): Atlantic salmon (*Salmo salar*) 582,350; coho salmon (*Oncorhynchus kisutch*), 134,235; and rainbow trout (*Oncorhynchus mykiss*) 74,518. Such successful story dates back to the last part of the twentieth century with the introduction of rainbow and brown trout

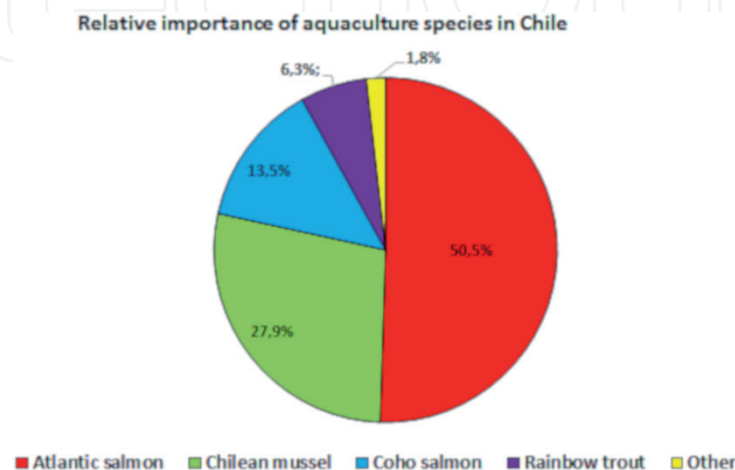


Figure 1. Main species produced by Chilean aquaculture. According to production figures (2017), farming of salmon and trout is by far the main aquaculture activity in Chile, followed by the Chilean mussel (*Mytilus chilensis*). Others correspond to seaweeds and the Chilean scallop.

and other salmonids, initially for recreational fisheries and later for aquaculture [9]. However, the industry almost collapsed in 2006–2007 by the outbreak of the deadly virus responsible of the infectious salmon anemia (ISA), a problem that some anticipated due to the unlimited expansion of the industry in an ecosystem with limited carrying capacity [10]. This has raised serious concerns on the environmental standards of an industry making intensive use of coastal, estuaries, and lakes that are shared by multiple users and due to the high rate of antibiotic consumption (0.53 Kg per ton harvested in 2016), one of the highest in the world [7]. The interaction between host (fish), microbiota, and environmental microorganisms could be a key factor to develop rational strategies to improve the productivity by increasing the resistance to infection and reducing the use of antibiotics and the environmental impact of aquaculture. However, in the production scale, the intensive use of water and the utilities of the sector does not match with the lack of scientific research available to deal with the problems caused.

This chapter focuses on Chilean aquaculture to evaluate how metagenomics, a recently developed genomic subdiscipline, is actually contributing, or could potentially contribute, to develop more efficient aquaculture practices in relation to disease control and the environmental burden such practices have brought about. Metagenomics has made possible and cheaper the analysis of the complex genomes of microbial communities to unravel their diversity, dynamics, and functioning in different environments. The application of this tool to the aquaculture microcosm in particular has been reviewed by Martinez-Porchas and Vargas-Albores [11]. One of the benefits of metagenomics is to provide access to unculturable species, the vast majority of disease-related microbes in aquaculture whose diversity and function were unknown so far. We first provide an overview of Chilean aquaculture, the microbiological threats faced by the salmon farming industry, mainly the symbiotic and antagonist interrelationship between microbes and farmed animals. The focus is placed on how the gut microbiota of farmed and native species contribute to their fitness and overall performance in production-related traits like disease or stress resistance. Finally, we seek to evaluate the application of metagenomics to monitoring environmental biodiversity and microbial dynamics in a scenario of climate oscillations and other ecosystem perturbations such as the development of harmful algal blooms.

2. Microbiological threats facing Chilean aquaculture

As stated earlier, Chilean aquaculture is a successful industry. In fact, Chile is the first world producer of rainbow trout and coho salmon, is the second world producer of Atlantic salmon, and, recently, has become the world fourth producer of the Chilean mussel (*Mytilus chilensis*) [2, 8]. This level of production is achieved by the natural condition of the water present in the south of Chile and by the intensive farming strategy used. In fact, using less water surface, Chile competes to Norway in salmon production [12]. As consequence, Chilean aquaculture has faced several sanitary problems, related with outbreaks of viruses such as infectious pancreatic necrosis virus (IPNV) [13] and infectious salmon anemia virus (ISAV) [14]; bacterial pathogens such as *Piscirickettsia salmonis* [15], *Flavobacterium psychrophilum* [16], and *Renibacterium salmoninarum* [17]; and parasites such as *Caligus rogercresseyi* [15]. Diseases caused by viruses are one of the great challenges of Chilean salmon farming industry; the first virus being identified in Chile was IPNV, which is highly persistent and causes severe mortality. However, the introduction of individuals with QTL related to the resistance to the disease and the administration of vaccines has partially reduced mortalities in Atlantic salmon [15, 18, 19]. Due to its prevalence and because IPNV has been found in healthy fishes, it is now considered endemic in

the Chilean Coast [13]. The ISAV, an orthomyxovirus related with influenza, caused several outbreaks between 2007 and 2008 forcing to close about 90% of the Atlantic salmon hatcheries located in Region X [14, 20]. To say the least, the industry almost collapsed. New outbreaks of ISAV have been detected in the last years; however, its pathogenicity is not comparable to the 2007–2008 outbreaks [15]. Recently, piscine reovirus (PRV) has been found in some Atlantic salmon with the so-called heart and skeletal muscle inflammation (HSMI) and also rainbow trout [21], although this virus has not been related to outbreaks with important losses [22].

Regarding bacterial pathogens, *P. salmonis* accounts for about 15% of production loss of post-smolt Atlantic salmon [15], while *F. psychrophilum* produces mortalities between 20 and 80% in rainbow trout and Atlantic salmon during the freshwater phase [23]. *R. salmoninarum* is the etiological agent of the bacterial kidney disease (BKD) and is responsible of the systemic and chronic infection in rainbow trout, Atlantic salmon, and coho salmon, with about 40% of prevalence in the latter species [24]. Several types of vaccines have been produced to control viral- and bacterial-caused diseases that, however, have proved inefficient as disease outbreaks continue to appear. This is the reason why antibiotics are still the main therapeutic strategy against bacterial pathogens. More than 186 tons were used only in the first semester of 2018 [25, 26].

Regarding farmed mussels (*Mytilus chilensis*) in Chile, there are no serious disease outbreaks reported so far, as it has occurred in major producer centers in Spain where congeneric species are intensively cultivated (*M. galloprovincialis* and *M. edulis*). Most of the registered mussel centers [8] located in the De Los Lagos Region (Lake District), southern Chile, cultivate mussels from ropes hanging from long lines. Spats are obtained either by the existing natural beds or by natural larva settlement. A recent review on bacteria associated to mollusk farming concentrated in the Chilean scallop (*Argopecten purpuratus*), a species cultivated in the north that showed potential aquaculture relevance prior to mussels [27]. This study also reports bacteria with probiotic effect on the pathogens listed. More recently, Lohrmann et al. [28] provided baseline information on the symbionts and other conditions of cultivated mussels from the Lake District, such as parasites (protozoan *Marteilia* sp., coccidian, and gregarines), intestinal copepods, castrating trematodes (*Proctoeces* sp.), intracellular bacteria in gills and digestive glands, ciliates on gills, microsporidian and metazoan parasites, and other conditions.

Currently, the factors that preclude bacterial and viral outbreaks remain unknown. With no doubt, the intensive culture strategy used by the industry accounts for disease prevalence and economic losses. But still, the cost–benefit ratio is extremely positive for the industry. Very likely, several unknown factors affect the host-pathogen-environment relationship. While the physicochemical factors enhancing disease outbreaks are relatively easy to determine, either by direct or satellite monitoring, the analysis of the microbiological factor is still biased to cultivable bacteria (see below), which means that more than 99% of the all micro-organism present in the environment are left out. Metagenomics provides a more holistic view of the microbial ecosystem and their interactions, and so it is expected to shed light on this complex problem for the industry, for the environment, and also to humans that eat fish with antibiotic traces.

3. Microbiota of cultivated species in Chile

3.1 Microbiota

Multicellular eukaryotes (plants and animals) have traditionally been classified as highly complex organisms independent of the community of commensal

microorganisms that colonize them [29, 30]. This community of microorganisms is known as microbiota and represents between 50 and 90% of an individual's cells in pluricellular forms of life [31]. Our initial failure to appreciate its importance is derived from biases arising from analyzing only cultivable microorganisms, which represent less than 1% of the diversity in a determined environment [32]. The development of non-culturing methods for microbial identification like PCR amplification of rRNA genes (16S or 18S) [33] and subsequent DGGE/TGGE analysis [34] and the development of massive sequencing techniques [35] allowed to broaden our knowledge, making possible to assess the complex community of microorganisms colonizing animals and plants such as bacteria, archaea, yeasts, and fungi. In humans, the gut microbiota is now considered as a complex endocrine organ that has coevolved with us through time, including cultural evolution [36–38], secreting several molecules that modulate human physiology [39]. The intimate and indissoluble relationship between microbiota and its host led to redefining the term organism and the emergence of the concept holobiont, which is used to define a community composed of host and hosted microbiota [40].

A healthy host has a stable microbiota which is altered when the metabolism or behavior of the host changes. In turn, changes in the microbiota composition caused by imbalances in the microorganisms that compose it (dysbiosis) can also produce metabolic changes in the host [41]. To date, there is abundant evidence showing that microbiota from mammalian participates directly in four processes: (a) protection against pathogens [42], (b) behavior [43], (c) energy balance [44], and (d) stimulation and maturation of the immune system [45, 46].

Much less is known about the characteristics and role of microorganisms that normally colonize the Atlantic salmon or rainbow trout and other teleost fishes [47]. Despite this, there is evidence showing a functional similarity between the roles of commensal microorganisms of salmonids and mammals [48]. In both cases, a complex community is established at the mucosal level that changes according to diet [49–56], temperature [57], season [57, 58], geographical location [59], culture condition [60–63], genetic [64], and stage of growth [58, 59, 65–67]. Microbiota composition also varies depending on the mucosal surface and epithelial location [68, 69]. High-resolution maps using next-generation sequencing (NGS) have identified around 950 operational taxonomic units (OTUs) in Atlantic salmon, with a slightly higher number in the skin [65, 68]. In the gastrointestinal tract (GT), these OTUs belong mainly to the phyla *Proteobacteria*, *Firmicutes*, and *Tenericutes* [55], while in the skin the main phylum is *Proteobacteria* [65]. As expected, the exposure to antibiotics such as oxytetracycline produces profound changes in culturable [70] and non-cultivable microbiota (Tello unpublished).

3.2 Function of the microbiota in salmonids

Although the function of microbiota in the physiology of salmonids has not been deeply studied, some evidence in Atlantic salmon and rainbow trout and other teleost fishes shows that it may play a similar role as described in mammals [48]:

- a. *Antagonist against pathogens*: In salmonids and other fishes, the mucosa covers the entire organism; thus, it is estimated that microbiota colonizing it forms a main defense line against pathogens, by mechanisms that include competition for space or nutrients [71], interruption of communication signals, and production of inhibitory and antimicrobial substances, such as reported in mammalian [72]. However, few studies have focused on understanding the role of microbiota in salmonids. Some of them, performed in rainbow trout, show that bacteria from microbiota are able to protect against bacterial and fungal

pathogens specially in egg or larval stages when the immune system is not fully developed [69, 73, 74]. On the other hand, some probiotic bacteria that have direct antagonism against salmonid pathogens confer protection in challenge assays, for example, *Clostridium butyricum* protects rainbow trout against infection with *Vibrio* sp. [75].

- b. *Behavior*: Conditions that elicit stress in Atlantic salmon, rainbow trout, and zebra fish cause significant changes in the microbiota [76, 77]. This is probably due to increased mucosal secretion. Although it is not yet known if the commensal microbiota of rainbow trout or Atlantic salmon can modulate behavior, the administration of *Lactobacillus rhamnosus* IMC 501 significantly alters social and explorative behavior in zebra fish [78]. Moreover, in *Solea senegalensis*, *Shewanella putrefaciens* Pdp11 prevents mortality caused by increased population density [79], and in other fishes the administration of bacterial probiotics reduces levels of cortisol, a molecule with immunosuppressive activity that is secreted under stress conditions [80]. It remains to be determined if this protection is due to reduced sensitivity to the stress caused by high population density, increased resistance to opportunistic pathogens through competition or immunostimulation, or both.
- c. *Energy balance*: In Atlantic salmon or rainbow trout, there is some evidence showing the microbiota is implicated in harvesting energy. The administration of probiotics for 10–12 weeks increases the rate of conversion in rainbow trout [81]. In zebra fish, the microbiota stimulates genes related to nutritional metabolism [82], increasing fat uptake and storage in adipose tissue [83], and also stimulates maturation of the gastrointestinal tract by increasing the production of digestive enzymes [84].
- d. *Stimulation and maturation of the immune system*: In mammals and zebra fish, the role of microbiota in the development of a proper immune response, especially in the maturation of innate immune response, is well known. In the gnotobiotic zebra fish model, the treatment with nonabsorbable antibiotics to deplete normal microbiota improves epithelial proliferation [85]; the innate immune response [82]; the expression of immunoglobulin M gene [86], neutrophils, macrophages, and phagocytosis [87–89]; and its composition also determined by B- and T-cell receptors [90]. In other fishes, the administration of probiotics helps to improve phagocytosis and the alternative complement pathway [91].

In Atlantic salmon or rainbow trout, it is unknown if normal microbiota modifies the immune response, but some indirect evidence suggests:

1. Gut inflammation occurs as a consequence of changes in diet that alter the microbiota [92, 93]. It remains to be determined if inflammation is caused by the change in microbiota or not.
2. Probiotics improve the immune function and innate immune response [75, 94, 95].
3. Most of the elements that affect the host-microbiota relationship in mammals and zebra fish are also observed in salmonids (see below).

As mentioned earlier, evidence shows that microbiota should play several roles in the salmonid physiology. However, functional association between metagenomic

characterization and physiological parameters is an amazing challenge poorly explored. Likewise, the identification of the microorganism or consortium unique to fishes that could be administrated as probiotics to improve the performance of salmonid aquaculture is, therefore, a challenge ahead.

3.3 Effect of antibiotics

In agricultural systems, the use of chemical products, especially antibiotics, has become widespread as a form of disease prevention and treatment [96]. While, in cattle breeding, antibiotics are preferably used as growth promoters [97], in aquaculture these properties have not been convincingly demonstrated. Historically, due to its intensive practice, the Chilean salmon industry is identified as one that uses more antibiotics per ton of harvested product [7]. In 2016, the amount of antibiotic used in the Chilean salmon industry reached 382.5 tons. The administration of antibiotics in aquaculture farms is mainly done through food, the remaining of which accumulates in the environment together with excretions [98]. Such accumulation of antibiotics in the marine sediments can persist for months, acting as a selection pressure that favors the establishment of resistant microorganisms that alter the endemic microbiota and the natural biogeochemical processes [99]. In Chile, this phenomenon is of great importance, since most of the salmon production is concentrated in the south of Patagonia, an area of high biological diversity.

The global trend in salmon production has been to reduce the use of chemical products to comply with biosecurity and animal welfare policies. In Chile, however, the use of antibiotics has continued to increase, accumulating an average of 343.4 tons of antibiotics per year (period 2005–2016) [100], 95% of which is used in marine fish farms. During this period, a high rate of infection by the intracellular bacterium *Piscirickettsia salmonis*, which causes the salmonid rickettsial syndrome (SRS), was observed, for which there is no effective vaccine or antibiotic treatment [101].

The main antibiotics used in Chile correspond to florfenicol and oxytetracycline. According to the national service of fishing and aquaculture from Chile (SERNAPESCA) in 2016, florfenicol and oxytetracycline represented 82.5 and 16.8%, respectively, of all the antibiotics used in Chilean aquaculture [100]. Both are broad-spectrum antibiotics used to combat infections of aquatic pathogens such as *Aeromonas salmonicida*, *Aeromonas hydrophila*, and *Yersinia ruckeri*, among others [102]. The predominance of florfenicol in recent years is mainly due to the fact that this antibiotic is the main agent against *P. salmonis*.

Pathogens cause immense economic losses that also have social impact. The crisis of the ISA virus in 2007 represented US\$ 600 million and 16,000 jobs lost. The infection by *P. salmonis* caused 70% of the mortality of Atlantic salmon and rainbow trout in recent years, amounting to US\$ 450 million per year, including vaccination, antibiotics, and other measures to mitigate the disease [103].

Although preventive measures have been implemented with the mandatory use of vaccines, the results have not been as promising as in mammals. The reason behind would be the less developed immunological memory in salmonids than mammals [104]. A corollary of this is the continued massive use of antibiotics. As mentioned, a critical problem is the propagation in the environment of microorganisms with resistance genes. Antibiotic residues have been reported in the muscles of fish and can be transferred directly to humans if the fish is not cooked properly [105]. Studies using massive sequencing to detect antibiotic resistance genes from fish sediment identified that more than 90% had mobile genetic elements of high homology to human pathogens [106]. This confirms the high rate of genetic exchange, or horizontal transmission, of antibiotic resistance between microbes and fishes that in the end affects human health.

Some studies identify the presence of antibiotic resistance genes in isolates from areas where salmonids are cultivated [107]; however, in-depth studies on the impact of antibiotics on the composition of microbial communities in farmed animals are still lacking. This field is open for metagenomics, especially for environmental DNA monitoring, in order to evaluate the impacts on the native microbial communities and their dynamics in places where salmon cages are located. It is particularly important to establish how environmental microbial communities recover after antibiotic treatment or after cages have moved to another place to allow recovery of the site, according to the actual practice.

In relation to the effect of antibiotics on the salmonid microbiota, studies conducted with culturable bacteria indicate that oxytetracycline decreases bacterial diversity, facilitating the proliferation of opportunistic pathogenic bacteria [70]. Studies conducted in our laboratory using broad-spectrum antibiotics such as bacitracin/neomycin showed the bacterial load in the intestine is reduced 10 times, in particular, the population of *Proteobacteria*, which favors the increase of the phylum *Firmicutes*. At the level of genera, the predominance of *Pseudomonas* and *Aeromonas* is replaced by *Lactococcus*. The interesting data is that the microbial composition is not recovered after 15 days of antibiotic treatment, which suggests that changes in the microbiota could be irreversible, at least within a short window of time (Tello, unpublished data). At the functional level, we were able to see that antibiotics increase the diversity of genes related to general metabolic pathways (amino acid biosynthesis, secondary metabolites, enzyme synthesis, etc.) and antibiotic metabolisms.

In farming systems, the impact of antibiotics on the fish's normal microbiota and its effects on the long term is unknown. It remains to be determined if the dysbiosis induced by the antibiotics generates a favorable scenario for other pathogens or if it affects the immune response. Preliminary work from our laboratory indicates that the administration of bacitracin and neomycin induces the expression of the inflammatory immune response judging by the increase in the expression of interleukin IL-1b and the reduced amount of leukocytes in the immunological organs. Similar effects are observed when administering florfenicol, a broad-spectrum antibiotic widely used in salmon farming (Figure 2).

3.4 Effect of heavy metals

It is a well-known fact that the presence of metals in different environments generates a toxic effect on both the biota and microbiota. Metagenomic analyses show

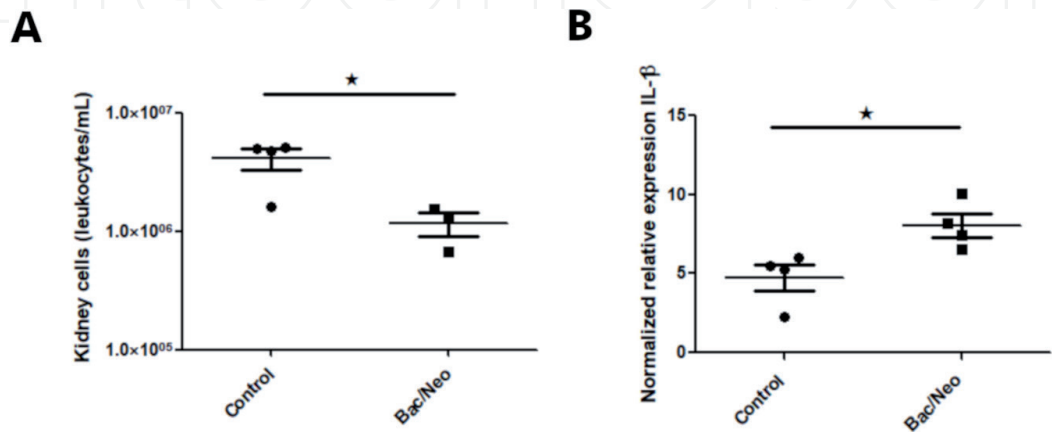


Figure 2. Effects of bacitracin/neomycin on the immune system of Atlantic salmon. The figure shows the effects of bacitracin/neomycin administered by 14 days on the amount of leukocytes (A) and expression of IL-1b (B). Both measures were determined on the head kidney.

these effects are persistent in the gut microbiota and in the environment, principally in water bodies where the toxicity of these metals modulates the microbial community composition [108, 109]. Previous studies have demonstrated that environmental pollutants affect the gut microbiota even at low concentration [109]. The metagenomic analyses of the gut microbiota from *Cyprinus carpio* have made evident the prevalence (or selection) of bacterial genera containing genes associated to metal resistance as well as genes involved in heavy metal biotransformation pathways that tend to attenuate their toxicity in fishes exposed to heavy metals [110, 111].

The Chilean aquaculture industry uses heavy metals such as copper due to its antimicrobial properties in, for example, antifouling paints that avoid the formation of bacterial biofilms which also are considered potential pathogen reservoirs [112, 113]. Another application of copper is for coating cages and recirculation systems (RAS) used in land-based salmon farming sites. On a less positive side, it has been observed that small variations in the concentration of copper in incoming freshwater from underground wells that feed a hatchery modify the fish behavior and reduce food intake, such as in cascade effect that ends up affecting the production. Such behavior impairment by copper seems to affect the nervous system of the fish [114]. Other studies in *Cyprinus carpio* show that low copper concentrations also affect the microbiota diversity and lipid metabolism [110].

Owing to the importance of recirculation systems in aquaculture facilities, it is imperative to understand how environmental pollutants affect the dynamic of the gut microbiota and health of reared fishes as well as the microbiota of the biofilter [115]. The metagenomic approach combined with metabolomic studies may help to understand the complex changes that occur at different levels in a hatchery exposed to environmental pollutants.

3.5 Potential use of microbiota of wild and farmed fishes in Chile

3.5.1 Metagenomics in the study of microbiota of wild and farmed fishes in Chile

Despite the importance of the gut microbiota in the fitness of wild and farmed animals, as seen for salmonids in Section 4, few studies have characterized the microbiota composition of farmed and wild fish and shellfish that are commonly consumed in Chile [107, 116–121]. Most of the best characterized microbiota corresponds to foreign species such as Atlantic salmon and rainbow trout, in agreement with their economic and social importance. With few exceptions, most of the microbiota reported in these species corresponds to studies done in Europe, Canada, the USA, and New Zealand. Even for rainbow trout, a species introduced in Chilean rivers more than 100 years ago [9], there are no studies characterizing and comparing the microbiota of feral rainbow trout living in Chilean rivers with the microbiota present in wild rainbow trout from regions where the species is autochthonous [122].

Since rainbow trout is a species considered “naturalized” in Chile, the obvious question would be if naturalization or adaptation to local conditions is associated with a particular microbiota composition. This is, perhaps, another dimension to consider in the study of the adaptation of rainbow trout in Chile. Given the wild north–south distribution of the species in Chile, it would be also necessary to understand if microbiota composition varies with latitude. The same sort of questions are pertinent to understand the microbiota composition of farmed trout and naturalized ones.

A similar situation happens with farmed Atlantic salmons which escaped from Chilean hatcheries. It is unknown if they show a microbiota composition similar or different to those present in the wild Atlantic salmon from Canada

or Norwegian rivers or closer to the Atlantic salmon farmed in Chile. In the case of coho salmon, another heavily cultivated species in Chile, studies on the microbiota have been done using 16S rRNA PCR coupled to DGGE [123], which gives a sort of qualitative approach to the microbiota diversity. This study shows that stable microbiota is established after first feeding and comes mainly from bacteria located in eggs and water. A more accurate analysis of the genomes of the microbiota would be important to understand the function that a particular group of bacteria could play in the species adaptation to the aquaculture or wild environment.

In short, the study of microbiota of farmed salmonids, as well as of other species, and their naturalized or wild relatives should provide evidence on how the host-microbiota-environment relationships evolve [64, 124]. The microbiota from wild fishes (marine or freshwater) living in the Chilean territory could give us some clues to understand how these fishes have adapted to local conditions. Such studies should help to optimize its nutrition and protection against pathogens in the artificial environment under the environmental conditions present in Chile.

The intestinal microbiota of *Seriola lalandi* and *Paralichthys adspersus* (fine flounder) has been sequenced. Both species distributed in the southern Pacific Ocean have, respectively, actual or potential aquaculture interest in Chile. In both cases, bacteria belonging to the phylum *Proteobacteria* were the most abundant group in wild specimens, while under aquaculture conditions, members of the phylum *Firmicutes* predominated. Under aquaculture conditions, the fine flounder shows a reduction of *Actinobacteria*, a group known to produce antimicrobial compounds [117, 119]. Further, metagenomic characterization of the microbiota through the complete sequencing of all the microbial genomes is necessary to properly predict the function of the microorganisms that colonize wild or farmed fishes and so to have a better approach to the evolution of the host-microbiota relationship in *Seriola lalandi* and *Paralichthys adspersus*.

3.5.2 Potential uses of the metagenomics for identification of new probiotics

The term microbiota refers to a complex and dynamic ecosystem of microorganisms that colonize the exposed surfaces and epithelia of an organism [125]. The role of these microorganisms has been studied mainly in the gastrointestinal tract (GT), contributing greatly to the general welfare of the host, participating in the absorption of nutrients, functioning as a protective barrier against potential pathogens, and regulating the expression of genes involved in epithelial proliferation in addition to having a role in the stimulation of the immune system and the prevention of diseases [126]. It is for these reasons that the microbiota is a good source of probiotic potentials.

Probiotics are defined as live microbiological food supplements with beneficial effects for the animal host [127], which confer protection (antagonism) against pathogens, helping in the development of the immune system and providing nutritional benefits [128]. In the aquaculture sector, probiotics began to be used at the end of the 1980s, as a prophylactic method against pathogens, mainly due to their ability to stimulate the innate immune response, which is characterized by having a nonspecific mode of action against various microorganisms [129].

The microorganisms used as probiotics in the aquaculture can have different origins, microorganisms previously used in mammals (allobiotic, probiotic) or commensal microorganisms that colonize the GT and the mucous membranes of the fish (autochthonous probiotic). In the case of aquatic animals, probiotics of autochthonous origin have adaptive advantages against foreign microorganisms, since they are adapted to factors such as water temperature and salinity [91], a situation that

allows them to compete adequately with the resident organisms of the GT [130], thus ensuring adequate colonization and persistence of the microorganism.

In the last three decades, several microorganisms with probiotic activity have been identified and characterized for the aquaculture sector [131]. These microorganisms include both Gram-positive and Gram-negative bacteria, bacteriophages, microalgae, and yeasts [132, 133]. Among the most used probiotic species in the production of salmonids are the genera *Lactobacillus*, *Bifidobacterium*, *Aeromonas*, *Plesiomonas*, *Bacteroides*, *Fusobacterium*, *Carnobacterium*, *Eubacterium*, *Bacillus*, *Enterococcus*, *Bacteroides*, *Clostridium*, *Agrobacterium*, *Pseudomonas*, *Brevibacterium*, *Microbacterium*, *Staphylococcus*, *Streptomyces*, *Micrococcus*, *Psychrobacter*, *Pediococcus*, *Saccharomyces*, *Debaryomyces*, *Alteromonas*, *Tetraselmis*, *Roseobacter*, *Weissella*, and *Aspergillus* [75].

The use of these and other microorganisms with probiotic activity has generated a reduction in the levels of antimicrobial compounds, particularly antibiotics, used in the salmon industry. In addition, there has been an improvement in the appetite and/or growth of farmed salmonids [134]. Many of these microorganisms have antagonistic activity in vivo in salmonids against pathogens such as *A. salmonicida*, *V. anguillarum*, *V. ordalii*, and *F. psychrophilum*.

Marine bacteria also have the potential to be used as probiotics. These bacteria have the ability to store the biodegradable polymer polyhydroxybutyrate (PHB), which exhibits the ability to neutralize pathogens in *Artemia*, fish, or shrimps. Such probiotic effect seems associated with the breakdown of PHB into monomers (short-chain fatty acids (SCFA)) in the gut of the target species; this breakdown changes pH and improves bacterial richness [135] or enhances immunological defense and provides energy to cells [6, 135–139]. Baruah et al. demonstrated that a commercial PHB source enhanced the survival of *Artemia* challenged with pathogen by triggering the expression of the heat shock protein, Hsp 70, which is associated with protective innate immune responses [135]. Another positive PHB effect has been reported in the European sea bass (*Dicentrarchus labrax*) in an experiment in which the diet of juveniles was supplemented with 2 and 5% PHB (w/w). Juveniles showed better growth performance correlated to a high bacterial richness in the gut [135]. Similar results were observed in the Siberian sturgeon (*Acipenser baerii*) fingerling, also with a diet supplemented with increasing amounts of PHB [137], as well as in shrimps [136, 139].

There are currently commercialized probiotics for use in aquaculture, such as Mycolactor Dry Probiotic®, which corresponds to a mixture of *Saccharomyces cerevisiae*, *Enterococcus faecium*, *Lactobacillus acidophilus*, *L. casei*, *L. plantarum*, and *L. brevis*; INVE Sanolife® MIC that includes a mixture of *Bacillus* strains (Biogen®), *Bacillus licheniformis* and *Bacillus subtilis*; and BACTOCELL® (*Pediococcus acidilactici*), the first probiotic approved by the European Union for use in aquaculture, as an additive in the feeding of salmonids [140].

In the case of mollusks, there is a history of a bacterial strain isolated from the gonads of Chilean scallops (*Argopecten purpuratus*) and characterized as *Alteromonas haloplanktis* which shows inhibitory activity in vitro against the known pathogens *V. ordalii*, *V. parahaemolyticus*, *V. anguillarum*, *V. alginolyticus*, and *A. hydrophila* [141]. The combination of *A. haloplanktis* and *Vibrio* strain 11 showed in vitro inhibition against *V. anguillarum*-protected scallop larvae in in vivo assays [142]. A recent example in the European blue mussel (*Mytilus edulis*) showed high poly- β -hydroxybutyrate levels regulating the immune response of mussels challenged with *Vibrio coralliilyticus* [143].

Other studies test the protective capacity of *Aeromonas media* A199 in vitro against other 89 strains of *Aeromonas* and *Vibrio*, in addition to preventing the

death of oyster larvae (*Crassostrea gigas*) when challenged in vivo with *Vibrio tubiashii*. However, *A. media* A199 was not detected in the host after 4 days of the administration of the probiotic treatment, indicating that it would be necessary to administer the probiotic at regular intervals of time if a prolonged protective effect is required [144].

The functional relationship between the immune system of teleost and mammals and innate and cellular response present in shellfish makes plausible that microbiota plays these roles in all cultured species. Characterization of the microbiota by a metagenomic approach has helped to identify microorganisms or consortia that can be used to improve the absorption of nutrients, have an antagonistic effect against bacterial pathogens, or can stimulate the innate and cellular response [145]. Metagenomics based on sequencing the 16 s rRNA associated to a host biological property or condition such as resistance to pathogens could help to identify bacteria or consortium with antimicrobial properties and look for ways to culture this bacteria to isolate potential probiotics. This analysis can also be complemented with a prediction of the metabolic pathway using the software PICRUSt [146]. It may also help to predict a particular condition of the fish if associated to a particular group of microorganisms with different metabolic properties, such as the production of vitamins, use of different carbon sources, or production of metabolites with immunomodulator properties such as SCFA or PUFA [118]. This analysis could be improved using metagenomics based on the complete sequence of the whole microbial DNA. This analysis, associated to ORF prediction, metabolic reconstruction, and prediction of secondary metabolites and antimicrobial peptides using antiSMASH [147], could help to improve the metabolic characterization of the microbiota associated to a particular condition and help to guide the identification of cultivable microorganisms that can be used as probiotics. Currently this approach began to be applied in the identification of potential probiotics to the aquaculture, for example, from eggs of Rainbow trout resistant to the infection with the fungus *Saprolegna*, was isolated a cultivable bacterium belonging to the genus *Actinomyces* that produce antifungal compounds and confer protection against this pathogen [73].

4. Importance of microbiota-host-environment interactions for the development of a sustainable aquaculture

4.1 Microbiota and immune system interaction in salmonids

Most of the current information about the host-microbiota communication comes from studies in mammalian models. These studies show that the interplay between the microbiota and the immune system is a sort of chemical cross talk [148] which involves from microbiota to host (a) the specific stimulation of host receptors by molecules from microbial organisms (bacteria, fungus, viruses, and archaea), (b) bioactive molecules secreted by the microbiota metabolism, and (c) stimuli of epigenetic mechanisms to control expression of immune genes and fate of immune cells. In salmonids the interplay between immune system and microbiota has not been studied yet. However, the immune system of salmonids shows the same elements that allow the host-microbiota communication in mammals.

- a. Specific stimulation of host receptors by molecules from microbial organisms.** The immune system is able to recognize the pattern of some structural molecules from microorganisms either commensal or pathogens.

Characterized originally in pathogens, these pathogen-associated molecular patterns (PAMP), which are specific for each type of microorganism, are recognized by a family of receptors located in the immune cells denominated as pattern recognition receptor (PRR), in particular a subtype of them, namely, Toll-like receptors (TLR). This interaction appears to be key in stimulating NF- κ B, interferon-response factors, and the inflammasome, which, depending on the cell type, context, and microorganism involved, responds through the production of different inflammatory cytokines (e.g., interferon, IL-1 β , IL-22), which can have systemic or local effects [149–153]. On the other side, commensal microbiota controls the immune response through the exposure or secretion of molecules that stimulate anti-inflammatory response. Polysaccharide A of *Bacteroides fragilis* can induce an anti-inflammatory response by increasing IL-10 production and the population of regulatory T lymphocytes and by decreasing the inflammatory response mediated by Th-17 [154, 155].

There is a greater diversity of TLR in salmonids than in mammals (20 versus 10) [156, 157], which suggests that in Atlantic salmon or rainbow trout, the cellular immune response mediated by TLR stimulation should be more complex than in mammals. This might be because fish live in an aqueous environment where they are exposed to a greater quantity and diversity of microorganisms that interact with a proportionally greater surface of mucosa. In addition, salmonids have a poorly developed immune response at the level of antibodies, strongly suggesting that in these organisms the cellular immune response and microbiota are the main barriers against pathogens. As in mammals, TLR expression is also stimulated by microbial infections [158]; however, how the pattern of TLR gene expression changes as consequences of variation in microbiota composition is unknown.

b. Bioactive molecules secreted by the salmonid microbiota. In mammals microbiota secretes several bioactive molecules able to modify the cell metabolism and immune response [159]; among them the molecules with the most significant impact are short-chain fatty acids (SCFA, formate, acetate, n-propionate, n-butyrate, and n-valerate). SCFA are generated by the anaerobic fermentative metabolism of bacteria that are part of the intestinal microbiota and, because they are hydrophobic, are absorbed by epithelial cells and rapidly disseminate throughout the organism causing effects in different organs [160]. The microbial SCFA best characterized is butyrate; this molecule induces the production of regulatory T lymphocytes beyond the thymus [161], stimulates microglia maturation and function and PMN lymphocyte activity [162, 163], and decreases the production of proinflammatory cytokines in macrophages (INF- γ , IL-1 β , TNF α) [164]. Butyrate also decreases the proliferation and increases apoptosis of T CD4 lymphocytes [165, 166], increases the production of anti-inflammatory cytokines in dendritic cells (IL-10, IL-23), and decreases exposure of MHCII [167, 168]. Although in general the effect of butyrate and other SCFA is to promote anti-inflammatory responses, the exact role depends on the type of cell and SCFA. The generic effects of butyrate can be explained by its capacity to stimulate the free fatty acid receptors (GPR41, GPR43, and GPR109a), which in turn stimulate a cascade of phosphorylation by Gai to activate at ERK1/ERK2 MAP kinase. In mammals, these receptors are expressed in immune cells, specially GPR43, which is highly expressed in macrophages/microglia, neutrophils, and monocytes [162, 164]. Bioinformatic assays performed in our laboratory indicated that Atlantic salmon genome encodes for 13 proteins which are homologous to the butyrate receptors present

in mammals. This expansion suggests an important role of this molecule in Atlantic salmon physiology [Tello et al. unpublished]; however, the pattern of expression of these genes is currently unknown in both the salmonid gut and its immune organs. Also it is unknown if butyrate is able to induce ERK1/ERK2 phosphorylation in salmonids.

Besides its interaction with its receptors, n-butyrate is also a strong inhibitor of histone deacetylase (HDAC), inducing chromatin remodeling and changes at an epigenetic level [169, 170]. HDAC is a highly conserved protein among different species. Human and Atlantic salmon HDAC shares a 97% of sequence identity, thus making highly plausible that HDAC from salmonids can also be inhibited by butyrate.

It is unknown if the salmonid microbiota produces butyrate or other bioactive molecules able to modulate the immune response; evidence from other fish suggest that butyrate is also a bioactive molecule in teleost. Butyrate is found in the intestinal tract of herbivorous and carnivorous fish [171, 172]. In *Sparus aurata* it increases intestinal microvilli and nutrient absorption [173] and in *Cyprinus carpio* increases the expression of shock protein-70 (HSP70), proinflammatory cytokines (IL-1 β and TNF- α), and anti-inflammatory cytokines (transforming growth factor- β) [174]. The mechanism by which butyrate can induce these changes is currently unknown.

Preliminary experiments performed in our laboratory show that butyrate modifies the antiviral response in SHK-1 cells, in a mechanism that is independent of the expression of the putative butyrate receptors [Tello unpublished], suggesting that Atlantic salmon cells could be sensible to this microbial metabolite.

c. Stimuli epigenetic mechanisms to control gene expression and fate of immune cells. In mammals microbiota also controls the immune system through several epigenetic mechanisms: DNA methylation [175], histone modification [169, 170, 176, 177], and control of gene expression by noncoding RNA [178]. In most of cases, this control is achieved by metabolic products of the microbiota, such as butyrate or vitamin precursor [179]. In simple terms, DNA methylation of cytosine impedes transcription factor binding and favors the recruitment of methylated binding domain proteins, which in turn prevents the binding of transcription factors by inactivating the chromatin configuration around genes. Through changes in DNA methylation pattern, microbiota may control the proliferation of Treg [180] and the function of NK cells [181].

Changes in the histone modification pattern produced by inhibiting HDAC with SCFA stimulate changes in the chromatin structure increasing the expression of *foxP3*, which promotes the differentiation of T CD4⁺ lymphocytes in Treg lymphocytes, favoring the anti-inflammatory response [161]. In intestinal macrophages, SCFA reduces the production of proinflammatory mediators (cytokines) via HDAC inhibition [182]. Deleting histone deacetylase 3 in intestinal epithelial cells alters normal microbiota, changes the expression patterns of antimicrobial peptides, and increases inflammatory processes, suggesting that epigenetic control of the host by microbiota is a fundamental element in homeostasis maintenance [183]. Although the epigenetic regulation in Atlantic salmon or rainbow trout has been poorly studied, at genetic level, both species show a more complex DNA methylation and histone acetylation/deacetylation systems than mammals, with several gene duplications [184], suggesting that this mechanism could also be implied in the host-microbiota communication.

Noncoding RNAs (miRNA, lncRNA, and snRNA) are a group of RNAs highly expressed in cells with several regulatory functions. Among them, microRNAs (miRNAs) are implicated in the cross talk between mammalian microbiota and its host immune system [178, 185, 186], while lncRNA are involved in the cross talk with gut epithelial cells [187]. Commensal microbiota is able to regulate the expression of several miRNAs that target genes involved in the inflammatory process, generating a tolerance state in the gut [188–190]. Among them, two important miRNAs that regulate the inflammation process are miRNA146 and miRNA155. miRNA146 expression is induced by low doses of LPS and acts as an anti-inflammatory regulator by targeting TNF receptor-associated factor 6 (TRAF6) and IL-1R-associated kinase 1 (IRAK1), which are involved in the NF- κ B pathway. miRNA146 allows the establishment of postnatal intestinal microbiota in the newborn gut, preventing inappropriate inflammation. miRNA155 is induced by high concentrations of LPS and plays an opposite role stimulating the inflammatory process by targeting the negative regulator of the NF- κ B pathway. Recent works using NGS (RNAseq) from different organs of Atlantic salmon identified 180 distinct mature miRNAs belonging to 106 families of miRNAs [191]. These miRNAs were deposited in the miRBase database (<http://www.mirbase.org>). This database currently contains 371 miRNAs from Atlantic salmon. Among the miRNAs identified in Atlantic salmon, orthologous of miRNA146, miRNA155, and other 15 of 27 miRNAs that participate in the microbiota-host communication in mammals were found. Currently it is unknown if the expression of these miRNAs changes according to the composition of Atlantic salmon microbiota.

The study of the mechanisms underlying the stimulation of immune system by microorganism that conform the microbiota is an open field for metagenomic studies searching for association between microorganism, consortia, or microbial metabolites and the proper immunological function. This approach could help to understand or help to predict more accurately the impact of environmental factors triggering outbreaks and to design either new prophylactic or therapeutic strategic based on microorganism or microbial metabolites. In a more holistic approach, this also could help to understand if changes in the environmental microbiota are sensed by the fish or other species which should help to properly assess their impact on the aquaculture ecosystem.

4.2 Prediction systems: an ecosystem approach

FAO's ecosystem approach to aquaculture [192] is a “strategy for the integration of the activity within the wider ecosystem such that it promotes sustainable development, equity, and resilience of interlinked social-ecological systems.” The so-called aquaculture ecosystem of southern Chile is shared by multiple users, notably by the salmon and mussel industry and a significant part of the national fishermen task force that is concentrated in Region X (De Los Lagos Region or Lake District). The latter depends on seafood collection and commercialization for their subsistence, and so any serious ecosystem perturbation ends up in conflicting situations affecting all users, including tourism, also an important player depending on the marine ecosystem. One of the most serious harmful algal blooms (HABs) of *Pseudochattonella verruculosa* occurred in the austral summer of 2016 (February–April) killing nearly 12% of the Chilean salmon production (106,000 tons), causing severe mortality of other fish and shellfish in the coastal waters and interior sea of western Patagonia [193]. This event exemplifies the inherent complexity of ecosystem perturbations and its socioecological consequences. But not only users like aquaculture producers should be blamed by such perturbations since climatic change seems to have created

the oceanographic conditions that amplified this HAB event. Indeed, the event was associated to El Niño and the climatic and oceanographic conditions associated to it [193]. In spite of the ongoing monitoring protocols carried out by different institutions, it has been difficult to understand what factors influence the diversity and abundance of harmful microalgae population, which is understandable due to the dynamic and complexity of the marine ecosystem, so a HAB event cannot be understood from the analysis of few variables. Metagenomic studies offer new insights into the complexity of the marine ecosystem and HAB events by allowing a deeper view to the microbial diversity that cannot be approached by the traditional microscopic analysis often used for microalgae identification. Additionally, different sorts of interactions can be discovered at all levels, particularly between microbes and microalgae [194–198]. It is now known that some bacterial populations could promote the growth of specific harmful microalgae in species, while some bacteria related to disease in fish or mollusks could also promote blooms. But, also virus controls the abundance and activity of microbial populations and microalgae in nature. In short, the ecosystem associated to HABs is complex and needs a more holistic or integrated approach. The term holobiome has been suggested to address this complexity: holo = entity; biome = biological community. An ongoing project funded by the Japanese government in Chile integrates different Japanese and Chilean universities, the Instituto de Fomento Pesquero (IFOP), and private and governmental bodies, under the holobiome concept with the goal to shed light on the mechanisms involved on algal bloom formation and, at the same time, predict HAB blooms (www.mach-satreps.org/en/).

5. Conclusions

1. Chilean aquaculture is the focus of this review considering its local and world relevance, particularly the farming of exotic salmonids, one of the highly regarded productive clusters.
2. Given the intensive farming strategy, the cost-effectiveness of the salmon industry is affected by three critical situations: the outbreak of mortal viruses, the occurrence and persistence of bacterial diseases, and the scale of antibiotic usage, one the highest in the world, with serious environmental consequences.
3. Understanding the interaction between host (fish), microbiota, and environmental microorganisms could be a key factor to develop rational strategies to improve the productivity by increasing the resistance to infection, reducing the use of antibiotics and their negative environmental impact.
4. The application of metagenomics to understand host (fish)-microbiota-environment interaction in salmon farming is limited, a reality opposed to the economic gains of the industry. Nevertheless, it has contributed to evaluate (i) the skin (the first barrier to pathogens or parasites) and gut microbiota of farmed salmonid subject to different nutritional and growth conditions, (ii) the function of the microbiota as antagonist against pathogens, and (iii) the potential function in the nutrition and environmental adaption.
5. Metagenomics has contributed to the identification of probiotic bacteria in both salmonids and marine fish species with significant benefits to the industry.

6. Metagenomics is essential to assess and predict critical environmental perturbations affecting fish and mollusk species like harmful algal blooms (HABs). Addressing such a problem requires to have a holistic view of the ecosystem components and their interactions, including the microbial diversity.
7. The sustainable expansion and diversification of Chilean aquaculture require to incorporate metagenomics and other omics tools to successfully deal with current and new diseases. This is required for aquaculture to be a really efficient and environment-friendly industry.

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Conflict of interest

The authors declared no conflict of interest.

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