Mucosal immunity and probiotics in fish

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A B S T R A C T

Teleost mucosal immunity has become the subject of unprecedented research studies in recent years because of its diversity and defining characteristics. Its immune repertoire is governed by the mucosa-associated lymphoid tissues (MALT) which are divided into gut-associated lymphoid tissues (GALT), skin-associated lymphoid tissues (SALT), and gill-associated lymphoid tissues (GIALT). The direct contact with its immediate environment makes the mucosal surfaces of fish susceptible to a wide variety of pathogens. The inherent immunocompetent cells and factors in the mucosal surfaces together with the commensal microbiota have pivotal role against pathogens. Immunomodulation is a popular prophylactic strategy in teleost and probiotics possess this beneficial feature. Most of the studies on the immunomodulatory properties of probiotics in fish mainly discussed their impacts on systemic immunity. In contrast, few of these studies discussed the immunomodulatory features of probiotics in mucosal surfaces and are concentrated on the influences in the gut. Significant attention should be devoted in understanding the relationship of mucosal immunity and probiotics as the present knowledge is limited and are mostly based on extrapolations of studies in humans and terrestrial vertebrates. In the course of the advancement of mucosal immunity and probiotics, new perspectives in probiotics research, e.g., probiogenomics have emerged. This review affirms the relevance of probiotics in the mucosal immunity of fish by revisiting and bridging the current knowledge on teleost mucosal immunity, mucosal microbiota and immunomodulation of mucosal surfaces by probiotics. Expanding the knowledge of immunomodulatory properties of probiotics especially on mucosal immunity is essential in advancing the use of probiotics as a sustainable and viable strategy for successful fish husbandry.

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1. Introduction

The aquatic environment harbors a wide array of biological, physical and chemical hazards. The constant exposure of fish to their environment typifies the importance of mucosal epithelia as a main organ of defense. The mucosal immune system of the fish is characterized by diverse and unique repertoire of innate and adaptive immune cells and molecules. They are orchestrated in the presence of antigenic factors such as bacteria or viruses to prompt specific and robust responses. In addition, the associated commensal microorganisms that are lining the mucosal surfaces serve as a biological reinforcement in protecting these surfaces against pathogens. An exceptional and interesting mechanism governs the maintenance of homeostasis between the immune-rich mucosal surfaces and their associated microbiota. Manipulation of the mucosal surfaces including their inherent and adherent factors have become key and emerging mode of disease control specifically in aquaculture where outbreak is a longstanding issue [1–5] Table 1.

Immunostimulants, vaccines and probiotics are believed to be ideal and effective disease control strategies that foster sustainability in aquaculture. The popularity of these alternatives was brought forth when call for reduction on the use of antibiotics and for the development of an eco-friendly industry arose. Antibiotics have been the conventional and popular bacterial control agents in aquaculture for almost three decades until evidences were presented on their risks to the consumers and environment [6,7]. The use of probiotics is regarded as a very promising strategy and their wide acceptance for use in aquaculture is evidently shown in the number of research studies published over the last ten years [8–11]. The ability of probiotics in modulating the immunity of the host has revolutionized the application of probiotics on a wider scale. The immunomodulatory features of probiotics presents two interesting scientific domains: *i*) the properties of probiotics reveal

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Table 1
Immunological influences of probiotics on the mucosa-associated lymphatic tissues (MALT) of the fish.

MALT	Key findings	Probiotics used	Origin of probiotics	Fish species under study (Age[a]; administration strategy[b])	References
Gut-associated lymphatic tissues (GALT)	Increased T-cells and acidophilic granulocytes; Lowered transcription of pro-inflammatory cytokines	*Lactobacillus delbrueckii* ssp. *delbrueckii* (AS13B)	host gut	*Dicentrarchus labrax* (LV, *nm*; LF)	[3]
	Lowered lactate dehydrogenase activity and caspase-3 during *V. anguillarum* infection	*Pseudomonas* sp. *(GP21)* and *Psychrobacter* sp *(GP12)*	host microbiota	*Gadus morhua* (JV, 300−400 g; IV)	[29]
	Increased expression of chemokines but no change with the interleukins	*Pseudomonas* sp. *(GP21)* and *Psychrobacter* sp *(GP12)*	host microbiota	*G. morhua* (JV, 300−400 g; IV)	[27]
	Increased villi height; Increased population of intraepithelial lymphocytes and acidophilic granulocytes	*Lactobacillus rhamnosus* GG (ATCC 53103)	human intestine	*Oreochromis niloticus* (JV, 30−50 g; FF)	[5]
	No pronounced effect on gut integrity and leukocyte level	*Pediococcus acidilactici*	commercial[c]	*O. niloticus* (JV, ∼175 g; FF)	[89]
	Elevated intraepithelial leukocytes; Influenced goblet cell population; Upregulated *tnfα* expression	*P. acidilactici*	commercial[c]	*O. niloticus* (JV, ∼9 g; FF)	[90]
	Modulated expression of *il1β*, *tgfβ* and *tnfα*	*Bacillus subtilis* C-3102	commercial[d]	*O.niloticus* × *Oreochromis aureus* hybrid (JV, ∼1 g; FF)	[97]
	Increased level of leukocytes infiltration, number of goblet cells and villi height	*Bacillus cereus* var. *toyoi*	soil isolate	*Oncorhynchus mykiss* (JV; FF)	[91]
	Increased lysozyme activity of the mucus	*B.s subtilis*	host digestive tract	*O. mykiss* (JV, ∼30 g; FF)	[96]
	Increased phagocytic activity of the mucosal leukocytes	*Lactococcus lactis* subsp. *lactis* CLFP 100, *Leuconostoc mesenteroides* CLFP 196, and *Lactobacillus sakei* CLFP 202	intestine of healthy salmonids	*O. mykiss* (JV, ∼50 g; FF)	[93]
	Influenced expression of *il8* during feeding and during infection	*Lactobacillus plantarum*	host origin	*O. mykiss* (JV, ∼26 g; FF)	[22]
	Unchanged pro-inflammatory cytokine expression	*Carnobacterium maltaromaticum* B26 and *C. divergens* B33	host intestine	*O. mykiss* (JV, ∼300 g; IV)	[76]
	Increased microvilli length	*P. acidilactici*	commercial[c]	*O. mykiss* (JV, ∼100 g; FF)	[92]
	Increased mucosal fold length and infiltration of epithelial leukocytes	*P. acidilactici (administered with short chain fructooligosaccharides)*	commercial[c]	*Salmo salar* (JV, ∼250 g; FF)	[103]
	Alleviated epithelial cell damage caused by the pathogens	*Carnobacterium divergens*	Arctic charr gut	*S. salar* (JV, ∼73 g; IV)	[94]
	Pronounced abundance leukocyte-like cells in the intestinal epithelium; Prevented the damaging effect of *Aeromonas salmonicida*	*L. delbrueckii* subsp. *lactis*	culture collection strain	*S. salar* (JV, ∼140 g; IV)	[95]
	Increased population of Ig$^+$ and acidophilic granulocytes	*Lactobacillus fructivorans* (AS17B) *L. plantarum*	host gut human feces	*Sparrus aurata* (LV, *nm*; LF)	[4]
	Influenced the expression of *il8*, *casp1*, *actb*, *ocln*, *cox2* and *tf*	*B. subtilis* (administered with inulin and microalgae)	culture collection strain	*S. aurata* (JV, ∼50 g; FF)	[99]
Skin-associated lymphatic tissues (SALT)	Increased myeloperoxidase activity, lysozyme activity and total protein content of the mucus	*Bacillus amyloliquefaciens* FPTB16	fermented fish product	*Catla catla* (JV, 20−30 g; FF)	[107]
	Mitigated *V. anguillarum*-induced apoptosis; Modulated the expression of immune-related genes	*Pseudomonas* sp. (GP21)	host microbiota	*G. morhua* (JV, 300−400 g; IV)	[106]
	Increased protein content of mucus	*Lactobacillus casei*	commercial[e]	*Poecilopsis gracilis* (LV, ∼47 mg; LF)	[105]
Gill-associated lymphatic tissues (GIALT)	Influenced *defb* expression in the gills	*Pseudomonas* sp. (GP21)	host microbiota	*G. morhua* (JV, ∼150 g; RW)	[57]
	Maintained gill structure and promoted regenerative gill filaments during pesticide exposure	*B. subtilis*, *L. lactis* and *Saccharomyces cerevisiae*	culture collection strain	*Labeo rohita* (JV, ∼7.5 g; FF)	[108]

nm = not mentioned.
additional note: the weight shown in the table is the initial weight of the fish or the weight of the fish where mucosal cells were isolated for *in vitro* studies.
[a] Age of fish: **LV** = larvae; **JV** = juvenile.
[b] Administration strategy: **RW** = rearing water; **FF** = formulated feed; **LF** = live feed; **IV** = *in vitro*.
[c] Added as Bactocell®.
[d] Added as Calsporin®.
[e] Added as Yakult®.

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how intricate and specific the recognition mechanism of the host (*i.e.*, discrimination of pathogenic and non-pathogenic factors) towards the introduced microorganism (*i.e.* probiotics), the commensals and most importantly, the pathogens; and *ii*) the immune system of fish is a biological pool of immune cells and molecules of remarkable functions and specificity.

In this review paper, the current knowledge on immunomodulation in fish by probiotics is revisited. A number of review papers discussed the immunomodulatory properties of probiotics [2,8,9,11–14], however no significant attention was given to their impact on mucosal immunity. Mucosal immunology of more evolved vertebrates such as fish has been strongly explored in the last decades [13] and the effects of stimuli such as nutrition and commensal microflora are the sub-topics that are widely discussed. On the other hand, the actions of probiotics on mucosal tissues are least explored. Brief discussions on the current understanding of teleost mucosal immune system and mucosal microbiota are provided in this paper to build a strong link on the discussion of mucosal immunity and probiotics in fish. The application of probiotics has been gaining significant interest in fish and the synthesis that this review provides will further this initiative and allow platforms for future research endeavors in this area particularly on the capability of probiotics to modulate the mucosal immunity of the host.

2. Application of probiotics in aquaculture and their relevance to host immunity

Probiotics are traditionally defined as "a live microbial feed supplement which beneficially affects the host animals by improving microbial balance" [15]. This terrestrial-based definition of probiotics has been modified through the years particularly on its applicability in aquaculture. The diversification of the definition of probiotics to meet the biological issues in aquaculture was discussed in a recent review paper [2] and presented a simplified definition based on the proposal of Merrifield and colleagues [10]. The proposed definition of probiotics explicitly states, "live or dead, or even a component of the bacteria that act under different modes of action in conferring beneficial effects to the host or to its environment". The present review paper adheres on this simplified definition as this addresses the concerns on *i*) administration strategies; *ii*) multifaceted benefits; and *iii*) bacterial viability.

The search for sustainable disease control strategies has been the fundamental driving force why the application of probiotics in fish has generated unprecedented research studies. The use of probiotics is considered a worthwhile and sustainable alternative to the traditional dependence of the aquaculture industry to synthetic antimicrobials, which were shown to have harmful effects to the environment through their residuals. Unlike the other two disease control alternatives (*i.e.*, vaccines and immunostimulants), probiotics are not limited to disease control as they have several other favorable benefits [8,9,11,14]. Probiotics act on different modes of actions such as inhibition of pathogenic bacteria through adhesion interference or/and production of antagonistic metabolites, growth improvement, nutritional contribution, improvement of water quality, enhancement of immune responses and many others [10,11,14]. These multifaceted dimensions of the actions of probiotics in fish affirm their significance in key areas of successful fish husbandry including nutrition, environmental control and immunity.

The results that have been documented through the years elevated probiotics from mere growth enhancers or biological control agents to being immunomodulatory agents. Consequently, the effects of probiotics in the host resulted in a research niche in fish immunology. Probiotics can influence both the systemic and local immunity of the host whether they are administered *i*) orally or through the rearing water, or *ii*) as live or as dead cells. Earlier studies on the immunomodulatory features of probiotics in humans and other animal models [16] laid the solid foundation in exploring the same mechanisms and possibilities in teleosts. One of the pioneering review papers discussing the use of probiotics in aquaculture appeared during the early 2000s [14]. The influence of probiotics on host immunity was not widely explored then, thus only a small section of the review discussed this topic. Nevertheless, it provided a springboard in furthering this research area as manifested by over a hundred papers published since then. The innate immune system of the fish is the main target in profiling the immunomodulatory properties of a candidate probiotics. For example, the respiratory burst activity of the red blood cells, serum-mediated killing against *Escherichia coli* and serum immunoglobulin were enhanced in rainbow trout, *Oncorhynchus mykiss* following dietary administration of *Lactobacillus rhamnosus* [17]. A different strain of the same species of *L. rhamnosus* was administered in diets of rainbow trout and significant enhancement of serum lysozyme, complement activities and head kidney leukocyte phagocytic activity were observed [18]. The influences of probiotic on various humoral and cellular defenses as well as in peroxidase and anti-protease activities of the fish were discussed comprehensively in an earlier review by Nayak [12].

Cytokines are one of the key regulators in orchestrating the immune response in fish [19] and probiotics are also shown to be actively involved in triggering potent responses from this group of chemical messengers. Probiotic application could modulate the expression of pro-inflammatory cytokines such as interleukin-1(*il1*), *il6*, *il12*, tumor necrosis factor α (*tnfα*) and gamma interferon (*ifnγ*) and also the anti-inflammatory cytokines such as *il10* and transforming growth factor β (*tgfβ*) in fish [12,20–22].

There are close to 22,000 different fish species, and most of them have their "immune peculiarities" [23]. Case in point is Atlantic cod, *Gadus morhua*. The lack of antibody production during vaccination is due to the deficiency of the major histocompatibility class II (MHC II) in the immune system of this fish species [24,25]. In addition, immunological differences were observed between the different regions of the gut [26–29] and skin [27,30], thus, influencing the responses of cod to different stimuli. This example is an indication that it is necessary to understand the immune system of a particular fish before extrapolating the immunomodulatory features of a given probiotics as their actions might not only be dependent on the inherent features of the bacteria but also on the complexity of the immune system of the host as well. This makes immunity and probiotics in fish an area worthy of considerable research.

3. Mucosal immune system of fish

The build-up of knowledge on the importance of mucosal immunology in mammals prompted the exploration of its teleostean counterpart. The fact that fish have direct interaction with the immediate environment makes the study of teleost mucosal immunity of particular interest. The mucosal surfaces of the fish include the epithelia and associated tissues of the gills, gut and skin and the reproductive tract [31]. The gut, skin and gills of fish share many distinguishable characteristics with the type I mucosal surfaces of mammals despite some functional and structural differences [32]. These differences and similarities are fully detailed in the review papers of Gomez et al. [20,33].

The mucosal immune system has a key role in the defense mechanism against pathogens [34] and thus considered as a very active immunological site [35]. The vertebrate immune system is defined by lymphoid organs and is categorized as either primary or

secondary according to their ontogeny and functional characteristics. One of the secondary organs is the MALT or the mucosa-associated lymphoid tissue [36]. This lymphoid-associated tissue is further sub-divided into three distinct associations mainly based on morphological distinctions: *i*) gut-associated lymphoid tissue (GALT), *ii*) skin-associated lymphoid tissue (SALT), and *iii*) gill-associated lymphoid tissue (GIALT) [35]. These mucosal surfaces are covered with a protective overlay of immune-enriched mucus layer, which serves as the first line of defense against pathogens [37,38]. The fish mucus is enriched with a multitude of immune-related factors such as lectins, mucins, antimicrobial peptides, toxins and immunoglobulins.

The gut-associated lymphoid tissue (GALT) of fish lacks specialized structures such as the Peyer's patches in mammals, however the mucosal immune molecules such as lymphocytes, plasma cells, granulocytes and macrophages lines the epithelium or distributed in the lamina propia [13,39,40] and these potentiate this mucosal surface as an active immune organ. The GALT component of mucosal immunity could be traced back to the evolution of jawed vertebrates and this had played a crucial event in shaping the diversity of the molecules present in it [41].

The teleost skin is a metabolically active tissue [42] and possesses uniqueness and histological diversity [43]. In addition, fish integument is a multifunctional organ, and its components may serve important roles not just in protection but also for communication, sensory perception, locomotion, respiration and ion regulation [44]. The constant exposure of the skin to the external environment makes it the most susceptible mucosal immune organ to different kinds of pathogens (*e.g.* bacteria, virus and parasites) and stressors (*e.g.*, chemical and physical). The skin-associated lymphoid tissue (SALT) is typified by localized antigen recognition in the skin, homing in of specific types of T cells to sites and the presence of different types of immune cells/regulatory molecules [45]. The cutaneous/epidermal layer is made up of significant number of immunocompetent cells such as the epithelial cells, mucus cells, club cells, goblet cells and several other cell types that make the complexity of the cutaneous immune defense [27,46–48]. Recently, it was reported that teleost skin elicits gut-like immune responses as shown by the prevailing role in skin mucosal immunity of IgT, a teleost immunoglobulin specialized in gut immunity [49].

After the skin, the gill is the mucosal organ that has close interaction with the external environment. This feature increases the susceptibility of this organ to infection because it serves as a portal of entry for numerous pathogens. For instance, one of the portals of entry of two common fish pathogens *Vibrio anguillarum* and *Aeromonas salmonicida* is through the gills [50,51]. This enables the gill-associated lymphoid tissue (GIALT) to develop into an immunologically active tissue made up of potent immune factors and immunoreactive cells. The cellular morphology of teleost GIALT is composed of lymphocytes, macrophages, eosinophilic granulocytes, neutrophils and antibody-secreting cells (ASC) [52–55]. In addition, it possesses numerous immune-related molecules such as antimicrobial peptides [56,57], acute surface reactants [58] and cytokines [56,59] just like the other two mucosal immune systems. The gills of modern bony fishes is made up of four paired arches, each containing two rows of posteriolaterally oriented filaments with lamellas covered by respiratory epithelium [60].

4. Commensal microbiota of the mucosal surfaces

Aside from the immune-related molecules and factors characterizing the mucosal immune system of fish, these mucosal surfaces harbor a large population of commensal microbiota, which serves as a biological bulwark against invading pathogens. Hence, the commensal microbiota is considered beneficial to the host when homeostasis is maintained. The importance of these microorganisms in development, homeostasis and protection particularly in the gut gave them the distinction of being the "extra organ" of the host [61]. It is also important to mention that the normal microbiota of these mucosal surfaces contains "bad" commensal microorganisms and the close contact of these surfaces to the immediate environment plays an important part in shaping the overall microbiological make-up of the mucosal surfaces. With this, they could also be considered a liability when "bad" microorganisms overpopulate and out-compete the beneficial commensal population and eventually disarray the microbial homeostasis. Biological and physico-chemical factors are key modulators of the commensal microbiota of the fish, therefore they must be gearing up for the promotion of beneficial bacteria rather than the pathogenic bacterial population. As in the gut, the coexistence of these commensal bacteria in a dynamic equilibrium is maintained through continued and active signaling [62] and this is might also be present in the skin and gills [48,63,64]. Conversely, the inherent recognition repertoire of the mucosal surfaces is an important regulatory mechanism that maintains mucosal homeostasis and this is discussed in a separate section of this review paper.

Studies on the gut microbiota of the fish was traditionally dependent on culture-based techniques but the rise in popularity of culture-independent methods such as denaturing gradient gel electrophoresis (DGGE) provided more valuable insights. For instance, it was initially believed that there was a difference between the microbial community profile of freshwater and saltwater fish [65–67] but recent data through more discriminating techniques are proving this otherwise [64]. The gut microbiota of fish is influenced by genetic, nutritional, microbiological and environmental factors [64,68].

The skin of the fish harbors commensal bacteria with interesting diversity [65,69]. Generally, the bacterial population of the skin is around 10^2 to 10^4 cm^{-2} [70]. However, by being the principal organ with close contact to the environment makes the cutaneous microflora a reflection of the microbial population of the surrounding environment thus wide variety exists between species [65,71].

Little is known on the microflora of fish gills [64,72]. Gill tissue has been found to harbor high bacterial populations of up to 10^6 bacteria g^{-1} of gill tissue [73]. The diversity of the commensal population also reflects the immediate environment and the diversity could easily be manipulated given the nature on how the organ interacts with the aquatic environment [71,73,74]. In salmon, *Salmo salar* the gills harbor antagonistic bacteria against pathogens. These antagonistic bacteria were mostly *Carnobacterium piscicola*-like [63], thus oftentimes the gills are considered a rich source of potential probiotics [75].

In recent years, the advances in probiotics research in aquaculture have been directed towards the utilization of commensal bacteria as probiotics. At present, there are several candidate probiotics in aquaculture that are of host origin specifically isolated from the mucosal surfaces [1,2,10,76,77] and their number is rapidly increasing. Though there is no universal acceptance that probiotics should be of host origin, the impressive amount of data that were gathered in the last years provided solid evidences of their significance in fish probiotics research.

5. Discrimination between good and bad bacteria in the mucosal surfaces

It is generally assumed that probiotic action is solely dependent on the inherent properties of the bacteria, but in reality it is a collective product of the probiotic bacteria, the host and the cross

talks in between. First and foremost, the probiotic bacteria must be recognized by the host to be non-pathogenic because all the downstream processes will not take place unless the bacteria pass this stage. This recognition also works in order that the good bacteria of the commensal microbiota dominate the mucosal surfaces. In consequence, the precise recognition of the host to these bacteria mounts proper and appropriate responses. The vertebrate innate immune system recognizes pathogenic and non-pathogenic microorganisms through a defined germline encoded pathogen pattern recognition receptors (PRRs) that identify peculiar structures of the microorganisms called microbe-associated molecular patterns (MAMPs) [33,78,79]. Some of the well-studied MAMPS include lipopolysaccharides (LPS), peptidoglycan, flagellin, and microbial nucleic acids [61,80]. There are several PRRs that have already been identified in teleost and the four main types to date include *i*) toll-like receptors; *ii*) NOD-like receptors (NLR); *iii*) C-type lectin receptors (CLRs); and *iv*) peptidoglycan recognition proteins (PGRPs) [81]. The continuous interactions between pathogenic and non-pathogenic bacteria require the coordination of multiple PRR signaling pathways that will command whether microbial colonization will result in symbiotic coexistence, asymptomatic infection or virulent disease [82]. Specifically, the binding of PAMPs to these recognition repertoires triggers intracellular signaling cascade thereby prompting the release of specific cytokines and transmit signals to neighboring cells whether to exert anti-viral, pro- or anti-inflammatory effects [83]. The ligands for PRRs are not exclusive to pathogens and are abundantly produced by the resident microbiota during normal colonization [84]. Further, the activation status and immune regulatory function of the mucosal surfaces are dependent on the kind of bacteria and the immune-derived stimuli they receive [85]. In a collective sense, *i*) the communication between bacteria, *ii*) the specificity of the information transmitted to the host and *iii*) the signals that are transferred from one cell/molecule to another cell/molecule in the orchestrated response network should be in ascertained fine tuning to initiate and mount rapid, selective, specific and potent immune responses (Fig. 1). The utilization of host-derived bacteria as probiotics does not simply stand on the fact that microorganisms perform well at their optimum levels in their natural habitats [2,14]. Moreover, the fact that they are already recognized by the host as non-pathogenic make their application more desirable.

6. Influences of probiotics on the mucosal immunity of teleosts

6.1. Gut-associated lymphoid tissues (GALT)

The intestine is the primary target organ during oral administration of probiotics. Therefore studies on gut immunity in relation to oral delivery of probiotics warranted high consideration [3] and this is manifested on the bulk of research studies in this mucosal tissue.

The use of lactic acid bacteria (LAB) as probiotics in fish has been one of the well-accepted and common practices. To date, numerous candidate bacteria have been identified and characterized, yet LAB remains the most popular choice. This group of beneficial bacteria could manipulate the histo-architectural structure of the gut for efficient nutrient utilization and effective immunoreactivity as initially shown in humans and higher vertebrates [86–88]. *Lactobacillus rhamnosus* GG, LAB of human origin, is popularly used in aquaculture was incorporated to the diets of tilapia, *Oreochromis niloticus* at a rate of 10^{10} CFU/g in feed and administered for 30 days [5]. The supplementation of LAB resulted in the promotion of intestinal structure and mucosal immunity as shown by the increased villous height in the proximal and mid intestine of the probiotic-fed

group. It was further demonstrated that the population of intra-epithelial lymphocytes and acidophilic granulocytes increased significantly in the probiotic-fed group leading to a conclusion that probiotic supplementation could modulate the population of the intestinal immune cells. The results of this study corroborated with the earlier studies on the capability of probiotics in modulating the lymphoid cells and granulocytes of teleost gut. In a study by Picchietti et al. on the probiotic influences to GALT of larval gilthead seabream, *Sparus aurata* they found that co-administration of *Lactobaccillus fructivorans* (host origin) and *Lactobacillus plantarum* (human origin) influenced the population of Ig$^+$ cells and acidophilic granulocytes in the gut. Among the population of acidophilic granulocytes, the G7$^+$ cells were accounted for the majority of intraperitoneal population that were significantly influenced by probiotic treatments. Further, it was shown that the extent of cellular influences depended on the age of the larva, duration of the treatment and the type of live vectors [4]. The use of live vectors was also employed by using rotifers and *Artemia* in delivering *Lactobacillus delbrueckii* ssp. *delbrueckii* (AS13B) to the developing sea bass, *Dicentrarchus labrax* [3]. This strategy had no detrimental effect on gut integrity and significantly increased the population of T-cells and acidophilic granulocytes in the intestinal mucosa. A known probiotic, *Pediococcus acidilactici* was supplemented into the diets and administered to on-growing tilapia for a period of 32 days [89]. The gut integrity and leukocyte levels remained unchanged and the effect was more pronounced in the systemic immunity as shown by the increase in blood leukocyte levels and serum lysozyme activity. The same probiotic strain was used in another study in tilapia focusing on the localized and peripheral immunomodulatory features of the bacteria [90]. Microscopic evaluation showed that *P. acidilactici* -fed tilapia had elevated intestinal intraepithelial leukocyte and a less pronounced increase in the population of goblet cells. The influence on gut morphology was also shown in the intestinal mucosa of rainbow trout fingerling fed with *Bacillus cereus* var. *toyoi* wherein the probiotic-supplemented diet increased the level of leukocyte infiltration in the lamina propria of the intestinal mucosa, the number of goblet cells and villi height [91]. Influence on gut villi morphology was also shown in rainbow trout fed with Bactocell®, a commercial probiotics composed of *P. acidilactici* [92].

Aside from the mentioned influences of probiotics on gut integrity and architecture, this group of beneficial bacteria has the capacity to modulate the physiological activities of gut mucosal cells. For example, the mucosal leukocytes of rainbow trout fed with LAB mixture composed of *Lactococcus lactis* subsp. *lactis*, *Lactobacillus sakei*, and *Leuconostoc mesenteroides* was shown to have a better phagocytic activity than the control group [93]. This physiological influence in gut muscosal cells was also observed in studies using non-LAB probiotics. Host-derived probiotics in cod (GP21 and GP12) did not only demonstrate adhesion interference towards *V. anguillarum* in the intestinal epithelial cells (IEPC) but they could also lower lactate dehydrogenase activity and caspase-3 activity of the *V. anguillarum*-infected cells [29]. The protective capacity of probiotics against the damaging effect in the gut epithelia of pathogens was also demonstrated in rainbow trout wherein the probiotic, *Carnobacterium divergens* alleviated epithelial cell damage, cell debris in the lumen, and disorganization of the microvilli [94] and in Atlantic salmon fed with LAB were protected against the damaging effects of *A. salmonicida* [95]. A *Bacillus* sp (*Bacillus subtilis*) could stimulate immune parameters in rainbow trout by increasing gut mucus lysozyme activity following feeding [96].

Cytokines are crucial chemical messengers that transmit signals between cells and they are key molecules in the immunomodulatory cascade in the fish gut following exposure
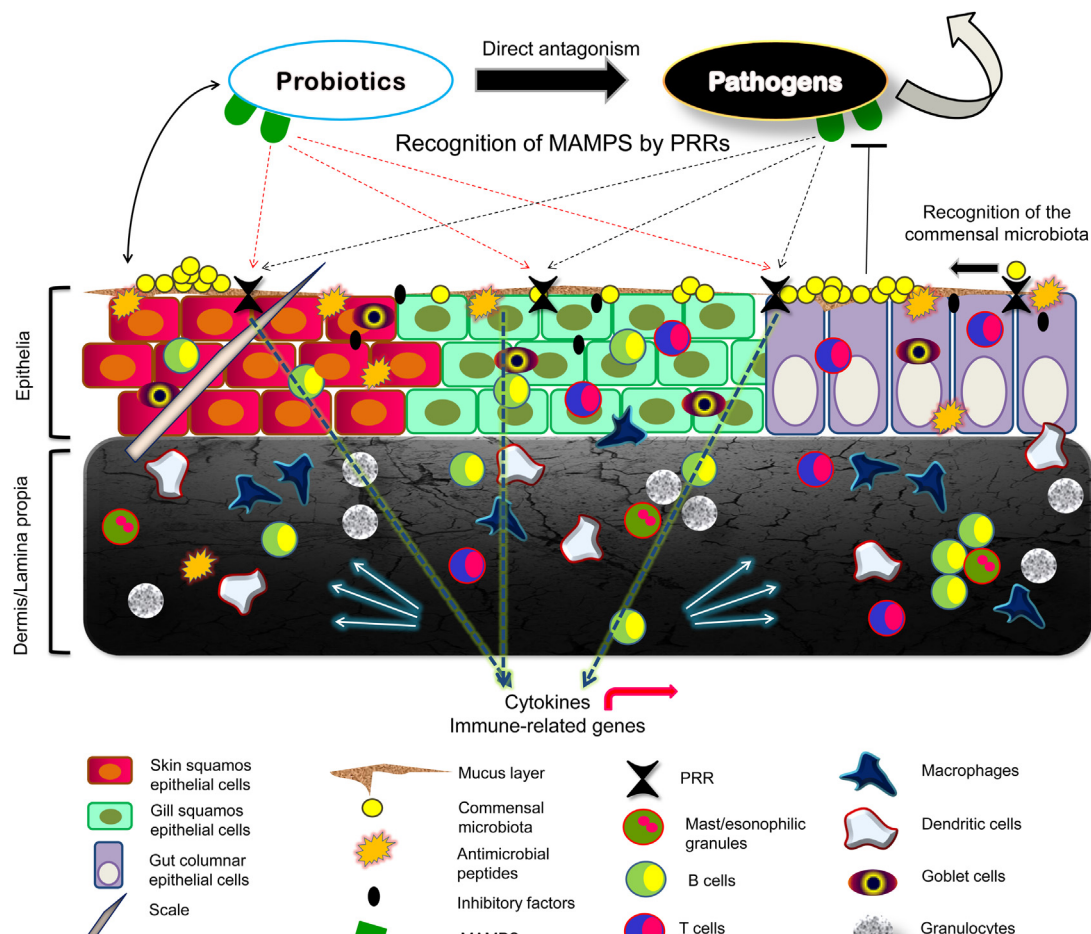
Fig. 1. Host-microbe interactions in teleost mucosal immune system. The teleost lymphoid-associated mucosal tissue is divided into three distinct associations mainly based on morphological distinctions: *i*) gut-associated lymphoid tissue (GALT), *ii*) skin-associated lymphoid tissue (SALT), and *iii*) gill-associated lymphoid tissue (GIALT). The mucosal surfaces of fish may share differences with their predominant morphology, but the associated immune cells and factors share a plethora of similarities. There are two identifying features lining the fish mucosa: *i*) The mucus layer envelops the majority of the epithelia. The fish mucus is enriched with diverse immune-related factors such as lectins, mucins, antimicrobial peptides, toxins and immunoglobulins. *ii*) The commensal bacteria serve as biological sentinels and could interfere with pathogen adhesion through different mechanisms. These barriers serve as the first line of defense against pathogens. Evolution shaped the functionality and specificity in responding to hazards of the present pool of immune cells and molecules (B cells, T cells, macrophages, dendritic cells, goblet cells and granulocytes). Probiotics are beneficial bacteria capable of not only inhibiting pathogens but also in modulating the immune system of the host. Immunomodulation through probiotics could be regarded as a communal effort of the introduced microorganism, the host and the commensals. The host can recognize whether the introduced microorganism is pathogenic or not through pathogen pattern recognition receptors (PRRs). These recognition repertoires identify the microbe-associated molecular patterns (MAMPs) that are present in both pathogenic and non-pathogenic microorganism. Some MAMPS include lipo-polysaccharides (LPS), peptidoglycan, flagellin, and microbial nucleic acids. The binding of PAMPs to PRRs trigger intracellular signaling cascade thereby prompting the release of specific cytokines and transmit signals to neighboring cells whether to exert anti-viral, pro- or anti-inflammatory effects. The same mechanism of recognition governs the homeostasis of the commensal microbiota in the mucosa. In addition, probiotics could also manipulate the richness and diversity of the commensal microbiota. (The illustration is a product of the composite information from Perez et al. (2010), Gomez et al. (2013), Rombout et al. (2010), and Nayak (2010)).

to probiotics. Earlier studies on the immunomodulatory properties of probiotics mainly focused on their influence on systemic cytokine-mediated responses [21] and it was only recently that their significance has been explored in understanding the crosstalk between probiotics and gut immunity in teleost. *Lactobacillus plantarum* significantly upregulated *il8* transcription in the intestine of rainbow trout and stimulated the expression of several cytokines in the head kidney. In addition, the expression of *il8* in the probiotic-fed group after *Lactococcus garvieae* infection was stimulated suggesting that this immune molecule was important in the probiotic-mediated immune response to infection in the intestine [22]. Inclusion of *Bacillus subtilis* C-3102 in the diets of hybrid tilapia (*O. niloticus* × *Oreochromis aureus*) induced upregulation of intestinal cytokines such as *il1β*, *tgfβ*, and *tnfα* [97]. This increase in the mRNA level of pro-inflammatory cytokine was also

observed in tilapia fed with *P. acidilactici* with the upregulation of *tnfα* [90]. However, some studies also showed that exposure of the fish to probiotics could lower or have negligible effects on the expression of pro-inflammatory cytokines. Probiotic administration of *Lactobacillus delbrueckii* in gilthead seabream resulted in lower transcription of pro-inflammatory cytokine genes such as *il1β*, *il10*, *cox2* and *tgfβ* in the intestine [3]. The decrease in the expression of these cytokines is likely an indication that inclusion of probiotics in the diets did not promote intestinal inflammation. In another study, host-derived potential probiotics (GP21 and GP12) in viable and heat-inactivated form did not elicit significant changes in the expression of interleukins (*il1β*, *il8*, *il10* and *il22*) in the intestinal epithelial cells (IEPC) of Atlantic cod. However, the heat-activated form significantly downregulated the transcription of CC chemokines and such response was attributed to the chemical compounds

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that might have been released during heat-inactivation [27]. The unresponsiveness of cytokines to probiotics in the gut cells was also shown in rainbow trout. The expression of $il1\beta$, $il8$, $tnf\alpha$ and $tgf\beta$a remained unchanged when the gut cells were exposed with *Carnobacterium maltaromaticum* B26 and *C. divergens* B33 at a multiplicity of infection of 25 for 6 and 12 h, respectively [76].

To increase the functionality of fish feeds, probiotics are being incorporated to the diets together with prebiotics in recent years. Prebiotics could be used conjunctionally with probiotics (syn-biotics) to elicit synergistic and more favorable actions [1,10]. These compounds mainly consist oligosaccharides that have been proven to promote bacterial growth within the gastrointestinal tract of higher vertebrates [98], thus their value-added potential with regards to probiotic addition was desirable. In most cases, they were studied in isolation and only a handful discussed the effects particularly in mucosal surfaces when given at the same time. Inulin, two microalgae (*Tetraselmis chuii* and *Phaeodactylum tri-cornutum*) and *Bacillus subtilis* (solely or combined with inulin or microalgae) were given through diets to gilthead seabream [99]. It was demonstrated that combinations were able to modulate the expression of immune-related genes in the intestine particularly $il8$, $casp1$, $actb$, $ocln$, $cox2$ and tf. Microlagae had been previously shown to influence the expression of several immune-related genes such as $ef1\alpha$, igm_h, $tcr\beta$, $mhci\alpha$, $mhcii\alpha$, $csf1r$ and $defb$ in the gut of gilthead seabream [100]. In another study, the same researchers were able to show that inulin and *Bacillus subtilis* could influence not just the microbial community structure but at the same time the intestinal morphology, though most of the changes observed were not favorable [101]. These observations concurred with the preceding study on the probiotics and microalgae in gilthead seabream specifically on the apparent demonstration of edema and inflammation following synbiotic feeding [102]. Simultaneous administration of *P. acidilactici* and short chain fructooligo-saccharides (scFOS) in salmon showed that the mucosal fold length and the infiltration of epithelial leucocytes were significantly higher in the anterior and posterior intestine, respectively, in the synbiotic fed group. Consequentially, modulated expression of $il1\beta$, $tnf\alpha$, $il8$, $tlr3$ and mx-1 in the intestine was also demonstrated in synbiotic fed group [103].

6.2. Skin-associated lymphatic tissues (SALT)

The skin is a tissue of interest in the study of mucosal immunity in relation to the administration of probiotics because this organ is directly in contact with the environment and it is one of the primary and largest mucosal defense systems of the fish. Despite of these key premises, studies on the effect of probiotics on the fish skin are sparse. The physical appearance of the fish skin may appear to be totally different from mammals, but similarities exist particularly in development, architecture and protective functions [104]. Thus, the earlier observations in human skin could be used as a basis in exploring the mechanisms involved in the mucosal immune responses in the fish skin.

Lactobacillus casei, a popular probiotic bacteria commercially available as Yakult®, was tested in a popular baitfish Porthole livebearer, *Poecilopsis gracilis* [105]. The strain was delivered either as bacterial cells (probiotic fermented milk eliminated by centrifugation) or as the initial form of the product (fermented milk included), and *Artemia nauplii* was used as live vector. Though most of the parameters remained unchanged following probiotic feeding, the protein content of the skin mucus in the group fed with bacterial cells showed significant elevation. It would have been more interesting if the authors were able to show what specific components of the proteinaceous part had increased significantly.

The immunomodulatory features of probiotics in the fish skin were also demonstrated by two Atlantic cod host-derived probiotics, GP21 (*Pseudomonas* sp.) and GP12 (*Psychrobacter* sp.). Interaction of these probionts with *V. anguillarum* elicited remarkable immune responses in the epidermal (EP) cells of the host. In particular, the probiotics were able to mitigate pathogen-induced cellular apoptosis and influenced myeloperoxidase production of the EP cells. In addition, probiotics–pathogen interactions modulated the expression of immune-related genes especially those involved in bacterial defenses and inflammation [106]. In an *in vivo* study, GP21 was directly added to the rearing water of juvenile Atlantic cod. Even though most of the antimicrobial protein genes remained unaltered in the skin, a significant upregulation of $defb$ was observed [57]. In a probiotic feeding experiment in catla (*Catla catla*), *Bacillus amyloliquefaciens* FPTB16 was added to the diets at three different inclusion levels [107]. The systemic and cutaneous immune responses manifested differential modulation and the inclusion level of the bacteria influenced the extent of the effect. Myeloperoxidase activity, lysozyme activity and total protein content of the mucus were significantly stimulated by probiotic inclusion. Despite not showing specific mucosal immune markers, the authors speculated that the increase in total protein level could be attributed to mucosal proteins such as agglutinins, lectins, lysozyme and immunoglobulins, which are key defense molecules and the increase in the levels of these proteins was believed to be a contributing factor to the resistance observed during pathogen challenge.

6.3. Gill-associated lymphatic tissues (GIALT)

Among the three mucosal surfaces mentioned in this paper, the influences of probiotics in fish gills are the least explored. One of the main reasons why investigation on the immunological influence of probiotics in fish gills is scarce could be attributed to the fact that dietary supplementation (*i.e.* through formulated or live feeds as vector) is the most popular mode of administration of probiotics especially during the larval to juvenile stages. Upon dietary administration, the exposure of gills to probiotic bacteria becomes minimal compared with direct inclusion of the probiotics to rearing water. The advantage of dietary inclusion is that the fish receive almost same amount of bacteria and the probionts are in a relatively stable vector. On the other hand, direct addition of the probiotics to the rearing water increases the likelihood of the gills to be exposed to the probionts especially if the administered bacteria have high affinity to gill mucosa. Therefore, the effects of probiotics to the gills and how the bacteria trigger the mucosal immune response also deserve considerable attention.

Probiotics could mitigate the gill-related necrotic effects of the pesticide fenvalerate in *Labeo rohita* [108]. Administration of multispecies probiotic mixture (*Bacillus subtilis*, *Lactococcus lactis*, *Saccharomyces cerevisiae*) in the diets kept the histo-architectural structure of the gills intact and promoted regeneration of the gill filaments during fenvalerate exposure. As a mucosal immune-related tissue, antimicrobial peptides are one of the key defense molecules in the gills of the fish and probiotics could modulate the expression of these molecules. For instance, the defensin (*defb*) gene in the gills of Atlantic cod was significantly downregulated after bath exposure to probiotics (GP21) [57]. Korkea-aho and colleagues showed that pathogens and probiotics adhere on the same mucosal surface particularly on the gills [109]. It would have been interesting if the authors were able to demonstrate the interference on the adhesion of the pathogen in the gills by the probiotics as this mode of action was deemed important in the elimination of pathogens from mucosal surfaces [29]. Using gill mucus as a model substrate, two host-derived probiotics interfered the adhesion of

Listonella anguillarum by means of either exclusion, competition or displacement in gilthead seabream [75].

6.4. Microflora of the mucosal surfaces

The commensal microflora cannot be considered as part of the mucosa-associated lymphoid tissues in its strictest anatomical sense. However, the importance of these microbial associations in the development and normal functioning of MALT makes them indispensable when studying the relationship between mucosal immunity and probiotics. The diversity and functions of the commensal microbiota of the mucosal surfaces of fish is dependent on several factors. Besides the direct influence of probiotics to the local/mucosal immunity, it could also influence to some extent the commensal microflora at the mucosal surfaces. There are three possible mechanisms on how probiotics influence the commensal microflora of fish: *i*) they could change the microbial community structure by promoting the dominance of beneficial population of the commensals; *ii*) they could colonize the mucosal surfaces and alter the microbial ecology by dominating the population of the microbiota; and *iii*) they could repress the population of pathogenic population, colonize the mucosal surfaces and maintain homeostasis with beneficial commensal consortia. These mechanisms were typified by the documented observations enumerated below.

The extent of probiotics-related changes in the microbial community structure of the gut is influenced by the strategy of dietary administration. For instance, administration either by single species or multispecies probiotic combinations had differing results. It was shown that the gut microbiota of group fed with multispecies combination of probiotics had higher number of operational taxonomic units while those fed with individual probionts had high Shannon index (H′) or diversity index [110]. Probiotics could also affect the microbial community structure by altering the bacterial population into a well-defined cluster that mainly foster the population of beneficial bacteria instead of increasing the richness of microbial diversity [91]. This was observed in juvenile grouper fed with *Bacillus pumilus* wherein feeding favored the colonization of specific bacteria without altering the overall microbial diversity [111]. It was shown that probiotic supplementation facilitated the increase of potentially beneficial bacteria and decreased the population of the harmful microbial population in the gut [112]. Similarly, the population of viable LAB increased rapidly in probiotics-fed rainbow trout [113] and tilapia [89]. The length of administration and the concentration of bacterial inclusion are also significant factors on the probiotics-mediated changes in microbial diversity of the gut. As shown in hybrid tilapia fed with *Bacillus subtilis*, low inclusion level of probiotics required longer period of administration in order for the bacteria to colonize the gut. This dietary inclusion of the probiotics altered the autochthonous bacterial communities in the gut by favoring the dominance of adhesive viable bacterial population [97]. In a study in rainbow trout, the proportion of the administered probiotics in the total intestinal microflora increased significantly concomitant to the duration of feeding [18]. Successive administration is necessary for some probiotics to confer beneficial effects to the host as demonstrated in rainbow trout [17] and brown trout, *Salmo trutta* [114]. Inclusion of probiotics in the diets results in alteration of the microbial community structure in the gut of the host and could be region-dependent. As shown in grouper, *Epinephelus coioides* the foregut samples formed an independent cluster of bacterial population, which is distinctly different from the midgut and the hind gut, and the increase of species richness and Shannon index in the last two gut segments suggested that the probiotics, *Enterococcus faecium* MM4 could elevate the autochthonous microbial diversity in these gut sections [111].

Positive and negative synergistic effects were observed with the commensal microbiota following simultaneous application of probiotics and prebiotics. The capacity of *L. lactis* to colonize the hind gut of juvenile Siberian sturgeon, *Acipenser baerii* was improved following administration of a prebiotics, arabinoxylan-oligosaccharides (AXOS). This synergistic effect changed the microbial structure of the hind gut by specifically favoring the richness of *L. lactis* [115]. This regional difference on the changes of microbial community richness following synbiotic feeding was also observed in Atlantic salmon fed with *P. acidilactici* and short chain fructooligosaccharides (scFOS) [103]. On the other hand, no positive synergistic effect was observed in gilthead seabream [101]. The dietary administration of synbiotics (inulin and *Bacillus subtilis*) revealed negative alteration of the intestinal microbiota by lowering the bacterial diversity.

7. Probiogenomics in aquaculture

The emergence of **omics** science has become one of the significant events that revolutionized most contemporary researches including in aquaculture. The availability of modern omics technologies makes it easier for the researchers to understand biological phenomena in a holistic perspective such as in the collective context of genomics, transcriptomics, proteomics or/and metabolomics.

The exponential increase in the utilization of probiotics for human use under the mentioned scientific dimensions gave rise to a special area called probiogenomics. First coined by Ventura et al. [116], probiogenomics is a genomic-based studies of probiotic bacteria. The genome of a number of probiotic bacteria predominantly from the genera *Lactobacilli* and *Bifidobacteria* has been sequenced, including *Bifidobacterium longum* NCC2705 [117], *Lactobacillus johnsonii* NCC 533 [118], *Lactobacillus plantarum* WCFS1 [119] and *Sporolactobacillus vineae* SL153T [120]. The genomic information that was generated from sequencing these probiotic bacteria provided clear understanding on the inherent probiotic properties especially regarding their adaptation to the harsh conditions of the gastrointestinal tract, nutrient synthesis related to essential amino acid utilization, cell-surface repertoire relevant to adhesion and regulatory molecules crucial to cell-to-cell communication and intestinal crosstalk.

In aquaculture, the concept of probiogenomics is not yet widely recognized. To our knowledge, this is the first paper that has raised the relevance of this contemporary perspective in fish. Some probiotic bacteria with published genome had been extensively used in fish such as *L. plantarum* [4,22,121,122] but fish-derived probiotic bacteria are yet to be sequenced. The traditional dependence of aquaculture to terrestrial probiotics could be attributed as a main factor why no fish probiotic bacteria have been sequenced to date. However with the recent advances particularly on the utilization of host-derived probiotics in fish and the availability and accessibility of sequencing technologies, there is a great possibility that the genome of some candidate host-derived probiotics might be published soon.

The importance of omics science in probiotics research goes beyond the understanding of the genetic repertoire of the probiotic bacteria. The integration of transcriptomics, proteomics and metabolomics in unraveling the responses of the host and the properties of the probionts could validate the concept that probiotic action is a multi-network phenomenon capable of orchestrating different biological pathways and simply not a one dimensional action-reaction event. These holistic approaches on evaluating the probiotic impacts on the host have been demonstrated in mammals [123–126]. For example, whole genome transcriptional profiling and mass-spectrometric analysis revealed that

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the presence of *Bifidobacterium longum* elicits an expansion in the diversity of polysaccharides targeted for degradation by *Bacteroides thetaiotaomicron* (*e.g.*, mannose- and xylose-containing glycans), and induced host genes involved in innate immunity [123]. The reports on the application of available *omics* technologies in fish probiotics are scant because evaluation strategies are mainly dependent on conventional technologies such RT-PCR, qPCR, enzyme assays, western blotting, SDS-PAGE to name a few. Though these techniques are not considered obsolete, the present perspective presents that they should be employed together with the available omics technologies. This scientific integration will provide multi-dimensional discussion on the impacts and beneficial consequences of probiotic applications.

The dynamic nature of probiotics research in aquaculture is acknowledged [2] and it is only apt that research approaches should be progressing as well according to the present trend. The field of probiogenomics is an emerging field and its relevance in aquaculture is yet to be established.

8. Synthesis and future perspectives

Researches on the application of probiotics in fish have come a long way and the dynamism is very evident on the approaches that have been developed through the years. Scrutiny of the available published papers on probiotics provides solid and clear manifestations that *i*) application of probiotics is a viable and sustainable disease control strategy; *ii*) there is continuous search for new probiotics candidates; *iii*) probiotic actions have diversified, thus application is no longer limited to its use as a disease control agent.

The capability of probiotics in modulating host immunity has been the subject of intensive research. This expansion is the product of the interminable drive to further the use of these beneficial microorganisms. The effects in systemic immunity have been proven without doubt and convincing results were shown in commercially important fish species including the salmonids, carp and tilapia. The mucosal immunity of the fish is fascinating and intriguing; however, research efforts on the influences of probiotics on these mucosal immune tissues generated little interest in comparison to studies done in systemic immunity. The gut, skin and gills are three of the most-studied mucosal immune tissues in teleosts and research undertakings on the effects of probiotics on these mucosal tissues are largely focused on the gut. Evidences, though in modest amount, proved that immune responses of the other mucosal surfaces namely the skin and gills, could also be modulated by probiotic treatment whether by dietary inclusion or direct addition to the rearing water. The control of mucosal pathogens requires targeted immunotherapies that specifically protect local mucosal sites [33] therefore, it is aptly encouraged that the effects of probiotics on mucosal surfaces should not be limited to gut alone but their impacts on the skin and gills must also be extensively explored. The mucosal commensal microbiota, which serves as the first line of defense and an immunological reinforcement could also be modulated by the probiotics. Similarly, the current knowledge is too focused on the gut microbiota and the influence on the commensal microbiota of the skin and gills are left unexplored. Our understanding of mucosal immunology in teleost escalated to greater heights in the past years and recent findings will facilitate future studies on the immunomodulatory properties of probiotics particularly in mucosal surfaces.

In conclusion, our understanding of the relationship of mucosal immunity and probiotics is limited, hence more research is necessary to unravel the network existing between these two scientific domains (Fig. 2). Probiotics research in fish should be prepared to keep up with the developments and advancements by broadening the current outlook particularly on employing more discriminating techniques and new scientific approaches. Above all, understanding this immune-related beneficial aspect of probiotics will solidify
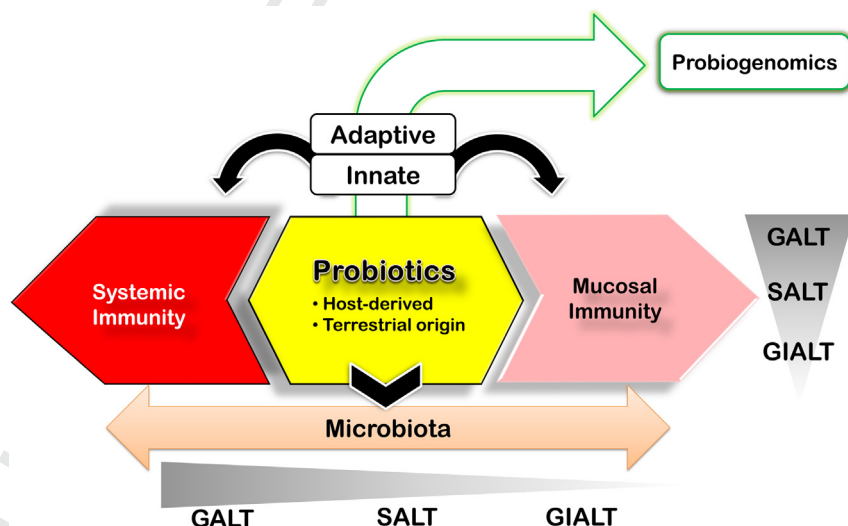

Fig. 2. Paradigm of the present and future of immunological studies in fish probiotics. This is a pictorial representation of the current status and future directions of immunomodulation through probiotics in teleosts. Probiotics that are used in fish are either host-derived or of terrestrial origin, wherein the latter is the most popular choice. They are known to modulate both the innate and adaptive immunity of fish. The approaches in the study of the immunomodulatory properties of probiotics in fish can be divided into two: *i*) influences on the systemic immunity; and *ii*) influences on mucosal immunity. The intensity in the background shade represents the quantity of research that has been done in this area: the greater is the color intensity means the more researches have been published, and vice versa. The inverted triangle bearing the name of the three main mucosal tissues in fish represents the researches discussing the influence of probiotics in these mucosal surfaces. The intensity and area covered exhibit the quantity of information to date: the more intense/greater the area means the more information is available in this mucosal surface. Probiotics could also manipulate the microbiota of fish and the effects of manipulation have been shown to have influence in the systemic and mucosal immunity of teleost thus the diagram bridges the two domains. The current understanding the effects of probiotics on the commensals of the mucosal surfaces is presented by the horizontal triangle below the two-headed arrow. The implication of the diagram is the same as mentioned above. The future of probiotics research is also represented by the emergence of "probiogenomics" and could facilitate a holistic insight on the immunomodulatory properties of probiotics both at the systemic and mucosal levels. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

their importance as viable and sustainable biological agents in teleost particularly those of aquaculture relevance.

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