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Use of immunostimulants and nucleotides in aquaculture: a review

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Abstract

It is well established that proper nutrition is essential for maintenance of normal growth and health of all animals including various aquatic species. During the last two decades increased attention has focus on immunostimulants and nucleotides to reduce susceptibility to various stressors and diseases, as well as enhance the overall health of fish. The immune response can be modulated by β-glucans and high-M-alginate. β-glucans are glucose polymers that are major structural components of the cell wall of yeast, fungi, and bacteria, but also of cereals like oat and barley. There is much structural variation in the β-glucans from these different sources, which may influence their physiological functions. Alginate is a polysaccharide composed of β-1,4-D-mannuronic acid (M) and α-L-glucuronic acid (G). *In vitro* as well as *in vivo* studies in fish show that especially β-glucans derived from fungi and yeast and alginate have immune modulating properties. Most frequently evaluated are effects on macrophage activation and on lysozyme, respiratory burst and leukocyte activity, which have been suggested to contribute to the increased resistance against infections, after immunostimulant exposure. Although more fish studies are needed, it is tempting to suggest that dietary β-glucans and alginate may be useful tools to prime the host immune system and increase resistance against invading pathogens. As no knowledge is available regarding short versus long-term effects and efficiency, more knowledge is needed on this topic.

Keywords: Fish; Immunostimulants; Nucleotides

Currently there are numerous gaps in existing knowledge about exogenous nucleotide application to fish including various aspects of digestion, absorption, metabolism, and influences on various physiological responses, especially expression of immunogenes and modulation of immunoglobulin production. Additional information is also needed in regard to age/size-related responses and appropriate doses and timing of administration. Thus further research in these areas should be pursued.

Immunostimulants

On a worldwide scale, fisheries landings remain constant at about 90 million tons of fish whereas commercial aquaculture supplies *per se* is approximately 60 million tons and is increasing at a rate of about 8% per annum. Use of immunostimulants (natural immune stimulants) is a unique approach for fish culturists to control disease losses in their facilities and numerous polysaccharides from a variety of sources have the ability to stimulate the immune system, and thus behaving as immunostimulants. Immunostimulants have an architecture consisting of repeating units of single molecular forms such as glucose in β-glucans and (deoxy) riboses in DNA/RNA, fatty acid chains in bacterial lipopolysaccharides (LPS) and certain lipoproteins. Such patterns are abundant in microbial communities of prokaryotes, and can be termed pathogen-associated molecular patterns (PAMPs) if they initiate inflammatory responses. It was turned out that commential microbiota also contain those molecular patterns in them and now scientists tend to use a broader term, microbe-associated molecular pattern (MAMP).

Sakai indicated that immunostimulants could be grouped depending on their sources; bacterial, algae-derived, animal-derived, nutritional factors and hormones/cytokines[1]. This sub-grouping changed the concept that immunostimulants instead are divided according to their modes of actions. Previously, an immunostimulant was defined based upon its activity on mononuclear phagocytic system only[2]. However, due to recent discoveries of the pattern recognition receptors (PPRS) of the phagocytic cells, the former definition of immunostimulant needs to be redefined. Different leucocytes possess different PPRs and the immune cells bring about different immune responses depending on the types of ligands PPRs interact with. To define an immunostimulant, all elements of the immune system must be considered, and according to Bricknell and Dalmo[3] the definition could be; "*An immunostimulant is a naturally occurring compound that modulates the immune system by increasing the host's resistance against diseases that in most circumstances are caused by pathogens*". Immunostimulants have been used as feed additives for several years in aquaculture, and yeast β-glucan may be the one with the longest track record. In nature, β-glucans are widespread and have been characterized in microorganisms, algae, fungi and plants[4]. The chemical structure of β-glucan varies with respect to molecular weight and degree of branching. For example, β-glucan from yeast contains a particular carbohydrate consisting of glucose and mannose residues and is a major constituent in the cell membrane.

Nucleotide-supplemented diets are not strictly immunostimulants by definition but provide a dietary supplement that allows improved resistance to a pathogen insult. Although such diets have been reported not to induce measurable immunostimulatory effects[5-7], they nevertheless seems to be able to up-regulate several immune genes in turbot (*Scopthalamus maximus* L.), which contradicts the earlier claims that these diets are not immunostimulatory [7].

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The biological effects of immunostimulants are highly dependent on the receptors on the target cells recognising them as potential high-risk molecules thus triggering various defence pathways. Thus, it is important to increase the knowledge of receptor specificity and the inflammatory processes the different receptors, upon antigen binding, are induced. However, many mammalian receptors reported to bind immunostimulants such as NLR (NOD-like receptors) have yet to be reported in fish. Nevertheless, assuming that fish and mammalian cells share many similar receptors, one may predict the biological outcome of immunostimulants in fish.

In order to evaluate whether immunostimulants may act in synergy with pro– and prebiotics we recommend this option merits further investigations. Probiotics appear to modulate immunity of the host by improving the barrier properties of mucosa and modulating production of cytokines (protein mediators produced by immune cells and contribute to cell growth, differentiation and defence mechanisms of the host)[8]. Viable live probionts are better than the non-viable heat-killed probionts in inducing higher immune responses in rainbow trout (*Oncorhynchus mykiss* Walbaum), especially enhancing head kidney leucocyte phagocytosis, serum complement activity etc., [9]. In recent years, several *in vivo* and *in vitro* studies have investigated the interaction between dietary probiotics and immunocompetence in humans as well as in fish and aquatic animals[8,10-17]. By increasing the host's adaptive and innate immune mechanisms, lactic acid bacteria (LAB) can protect the host against infection by enteric pathogens and tumour development. Immunological and other mechanisms behind the probiotic action may include;

* Stimulation of antibody secreting cell response [18]
* Enhancement of phagocytosis of pathogens [9,19]
* Modification/enhancement of cytokine production/ natural complement activity [20,21]
* Improvement of the host innate or acquired immune responses, direct effect on other microorganism in the digestive tract, adhesion sites, microbial action or response stemming from microbial products, host products or food components [22]

Consequently, probiotic bacteria may influence both adaptive and innate immune responses. Probiotics may reverse the increased intestinal permeability induced by antigens, but no information is available about long-term effects.

Nutritional factors and the immune response

It is well known that nutrition may affect fish health and immune responses. Nutritional factors are components of the diet essential for normal growth and development of the fish. Many of these undoubtedly have roles in the immune response. Vitamins C, B_6, E and A, and the minerals iron and fluoride have been identified as micronutrients that can affect disease resistance. With respect to the strict definition of immunostimulants; vitamins and minerals are not immunostimulants as they enhance the immune system by providing substrates and serving cofactors necessary for the immune system to work properly, but controversy exists [1,23].

Readers with special interest on the effect of nutritional factors on immune response of fish are referred to the comprehensive reviews published [1,24-37]. To our knowledge no information is available about nutritional balance and the immune response and as a natural consequence this topic merits further investigations.

β-1,3/1,6-glucans

Glucans are high molecular-weight substances composed of glucose as building blocks, usually isolated from cell walls of bacteria, mushrooms, algae, cereal grains, yeast and fungi[38]. Glucans in general comprise a great variety of substances common in nature (such as cellulose, glycogen, and starch), most of which do not interact with the immune system. Pharmacologically, they are classified as biological response modifiers (BRM). The common feature of immunomodulatory glucans is a chain of glucose residues linked by β-1.3-linkages, also called beta-glucans. Of the different β-glucans, the products known as β-1.3/1.6-glucans (read as "beta-one-three-one-six-glucans"), derived from baker's yeast, are suggested to be the most potent immune-system enhancers. β-1.3/1.6-glucans is characterized by side-chains attached to the backbone that radiate outward like branches on a tree.

The primary structure of the β-1,3/1,6-glucan is determinant for its immune-enhancing ability. The frequency and nature of side-chains strongly affect the ability of the glucan to mediate binding to surface receptors on the target cells influencing the effectiveness of the glucan as an immunostimulant.

The effectiveness of MacroGard® in stimulating the immune system combined with improved growth and feed conversion has been proven in some experiments[39,40]. MacroGard® has been suggested to be used in feed for all aquatic organisms and also to enrich live feed such as *Artemia*. Some studies involving shrimp have also suggested that the product contributes to a better growth in crustaceans[40,41].

MacroGard® is an environmentally sound alternative to feed antibiotics and chemotherapeutics for livestock, pets and cultured aquatic organisms. MacroGard® is based on a well-characterized β-1,3/1,6-glucan that has been in use world-wide for almost 25 years as an immune modulating agent in animal husbandry and aquaculture (see Table 1).

When included in feed or administered on mucosal surfaces, MacroGard® modulates immune responses and affects biological functions in a favourable way. The products are extracted and purified from food-grade baker's yeast by patented processes. The use of MacroGard® may be regarded as a natural input that compensates for a possible sub-optimal function of the immune system of farm animals, companion animals and aquatic species in intensive culture. The product has also been suggested to reduce the use of antibiotics in animal husbandry and consequently the concern that developing antibiotics resistance that may undermine the health security effective antibiotics represents. In aquaculture, glucans have been successfully been used to enhance the resistance of fish and crustaceans against bacterial and viral infections and (Table 1) present an overview of papers using glucans as immunostimulants in aquaculture.

In Norway, β-glucan, as prebiotics and nucleotides, has been used in the pancreas disease (PD) diets; to fed fish suffering from PD (Gonzalez Vecino, personal communication). In this case, the β-glucans are a very small part of the diet composition, and the benefits of the diets come mainly from a very specific formulation concentrated to recover pancreas and reduce inflammation in muscle and heart.

Baker's yeast, *Saccharomyces cerevisiae*, is the 2nd major by-product from brewing industry and contains various immunostimulating compounds such as β-glucans (the cell walls are constructed almost entirely by β-1,3-D-glucan, β-1,6-D-glucan, mannoproteins and chitin bound together by covalent linkages), nucleic acids and oligosaccharides [42] and it has the capacity to enhance growth and

Immunostimulant	Species	Administration and dose	Length of administ-ration	Mechanism of action/results	References
β-1,3 glucans	Carp	i.p; 2-10 mg kg^{-1} BW	12 days	↑ phagocytic activity of kidney leucocytes	[152]
	Channel catfish	Injected; 50 and 70 µg $fish^{-1}$ (100 g BW) suspended in 0.2 ml PBS	2 weeks	↑ phagocytic, bacterial activity and serum antibody concentration	[153]
	Marron	0.08, 0.1, 0.2, 0.4 and 0.8 % supplementations	12 weeks	↑ haemocyte count → on physiological parameters	[154]
β-glucan	Atlantic halibut (larvae)	Immersion (25 mg l^{-1})	5 days	Absorbed laminaran	[155]
	Carp	i.p (10 mg kg^{-1})	15 days	↑ superoxide dismutase and catalase activities	[156]
	Coho	i.p (5 and 15 mg kg^{-1})	36 days	→ immune response	[157]
	Trout	Oral	4 weeks	↑ stress resistance	[158]
	Turbot	Oral (2 g kg^{-1})	5 weeks	↑ leukocyte number	[159]
	Trout	i.p	18 days	↑ resistance to IHNV	[39]
	Trout	Oral (0, 0.2 and 0.4 %)	37 days	↓ expression of genes involved In acute inflammation reaction	[160]
	Sea bass	Oral (2% wet body weight)	2 weeks	↑ complement activation	[161]
	Sea bass	Oral (0.1%)	60 days	↑ serum complement activity, serum lysozyme, gill and liver HSP	[162]
	Sea bass	Oral (250, 500, 1,000 ppm)	25 days	↑ respiratory burst activity of head kidney macrophages	[163]
	Snapper	Oral (10g kg^{-1})	84 days	↑ macrophage superoxide Anion production and growth → complement activity	[164]
	Tilapia and Japanese eels	i.p (10 mg kg^{-1})	2 days	↑ lysozyme activity, phagocytic activities in anterior kidney and peripheral blood phagocytes and classical complement path-way	[165]
	Nile tilapia	Oral	6 weeks	↑ stress resistance	[166]
	Red drum	Oral (2% of diet)	6 weeks	→ on stress resistance	[124]
	Flounder	Oral (3 g kg^{-1})	n.i	↑ respiratory burst activity	[167]
	Channel catfish	Oral (1g/kg)	4 and 6 weeks	→ growth performance hematology or immune function Some improvement in stress resistance	[168]
	Climbing perch	B-glucan suspension (0, 5, 10 15 mg l^{-1})	7 days	↑ lysozyme and bactericidal activities and survival of spawns immersed in 15 mg l^{-1} when challenged with *A. hydrophila*	[169]
	American White shrimp	Immersed	120 hours	↑ total haemocyte counts and soluble haemocyte protein after 48-120 hours	[170]
	Shrimp	Oral (0.2% w/w)	7 days	↑ prophenoloxidase and reactive oxygen intern-mediate activity	[171]
	White shrimp	Oral (2000 mg kg^{-1})	6 weeks	↑ total haemocyte counts, phenoloxidase, superoxide anion and superoxide dismu-tase until day 27	[172]
	Carpet shell clam	i.v (0.05, 0.1, 0.5 and 1.0 mg ml^{-1})	Different sampling times	↑ nitric oxide production Hemolymph treated with β-glucan inhibited growth of *Vibrio algynolyticus*, *Vibrio splendidus* and *Escherichia coli*	[173]
	Scallop	i.p	7 days	↑six enzymes in haemocytes. β-glucan-induced activation was stronger at 15˚C than 25˚C .	[174]
β-glucan + mannose	Snapper	Oral (0.1-1.0% w/w)	n.i *in vitro* study	↑ macrophage activation	[175]

Immunostimulant	Species	Administration and dose	Length of administ-ration	Mechanism of action/results	References
β-glucan + LPS	Salmon	10 µg ml^{-1V}	n.i *in vitro* study	↑ lysozyme activity	[176]
β-1,3 and β-1,6 glucans	Sea urchin eggs	0.01, 0.05, 0.1, 0.25, 0.5 and 1.0 mg ml^{-1}	Incubation	↑ survival of embryos	[177]
β-1,3 and β-1,6 inked yeast glucan (M-glucan)	Salmon	Injected i.p (1 ml of 0.5% (w/v) M-glucan in 0.9% saline)	22 days	↑ macrophage and neutrophil numbers, head kidney macrophages and ability to kill *A. salmonicida*	[178]
	Salmon	0, 500 and 1000 mg kg^{-1}	70 days	→ no detrimental effect ↓lice- infected fish when the fish were fed 14 % soybean meal + 14 % sunflower and yeast extract	[179]
	Trout	Injected i.p 1 ml of 1% M-glucan suspension	3 weeks	↑macrophages ability to kill *A. salmonicida* and serum lysozyme activity	[180]
β-1,3 glucan from *Schizophyllum commune*	Black tiger shrimp	Oral (0 and 2 g kg^{-1})	40 days	↑ survival, haemocyte activity, cell adhesion and superoxide anion production	[181]
Glucan (barley extract (Sigma)	Trout	Injected i.p. 100 µg glucan dissolved in PBS. Immersed the concentration of 100 µg ml^{-1} glucan for 30 min	10 days	↑ phagocytic activity and numbers of circulatory glass-adherent cells	[182]
Yeast glucan	Salmon	i.p	20 days	↑ resistance to *V. anguillarum*, *V. salmonicida* and *Y. rückeri*	[183]
	Salmon	i.p	4 weeks	↑ complement and lysozyme activity	[184]
	Salmon	i.p (0.5 mg/fish)	43 weeks	↑ survival against *A. salmonicida* infection	[185]
	Salmon	i.p	7 weeks	↑ antibody → resistance to *A. salmonicida*	[186]
	Salmon	Oral and anal (150 mg kg^{-1})	2 days	↑ acid phosphatase	[187]
	Trout	i.p	n.i	↑ lysozyme activity	[188]
	Trout	Diet (0, 0.125 and 0.25 g k^{-1})	4 + 4 weeks	4 weeks ↑ survival and growth 4 – 8 weeks ↑ survival (0.25 g k^{-1}) → feed conversion	[189]
	Indian major carp		15 + 30 days	↑ superoxide anion, *in vitro* phagocytic activity and nitrite production of leucytes (10 days) → heamatocrit	[190]
	Indian major carp and rohu	Diet (0 and 5 g kg^{-1})	15 + 20 days	↑ phagocytosis and prolifer-ation of lymphocytes and oxidative radical and nitrate production	[191]
	Pacu	Diet (2.5 and 5 %)	86 days	↑ feed efficiency → plasma glucose and cortisol	[192]
	Fathead minnows	Diet (10 g kg^{-1})	14 days	↑ degranulation of primary granules in fish neutrophils	[193]
	Shrimp	immersion	43 days	↑ growth at 0.5; 1 and 2 mg ml^{-1}, but not at 0.25 mg ml^{-1} ↑ phenoloxidase activity and resistance to *V. vulnificus*	[194]
	Black tiger shrimp	Oral (0.2 %)	3 days	↑ phenoloxidase, no. of haemocytes and bacterial killing activity against *Vibrio harveyi*	[195]
	Pacific white shrimp	Oral (0, 1 and 2 g kg^{-1})	4 weeks	↑ total haemocyte – and granular haemocyte counts → growth	[196]

Immunostimulant	Species	Administration and dose	Length of administ-ration	Mechanism of action/results	References
Yeast glucan + Vitamin C	Trout	Diets containing yeast glucan and vitamin C at 150, 1,000 and 4,000 p.p.m	4 weeks	↑ alternative pathway of complement – and macrophage activity and specific Ab response following vaccination with *Y. ruckeri*	[197]
Saccharomyces cerevisiae	Sea bass	Diet (0, 10, 20, 30 and 40 %)	12 weeks	↑ feed conversion (10, 20 and 30 %), N retention (% N intake) → growth and energy retention (% E intake)	[198]
	Sea bream	0 and 10g kg^{-1}	6 weeks	↑ serum peroxidases and complement activity	[199]
	Sea bream	*In vitro* experiment, head kidney leucocytes	0 – 30 min	↑ degranulation	[200]
	Sea bream	0, 1, 5 and 10g kg^{-1}	6 weeks	↑ total serum IgM level	[201]
	Sea bream	*In vitro* experiment, blood leucocytes	30 min	↑ inhibition of the phagocytic ability	[202]
	Sea bream	0, 11.5 and 23 % (Y2)	10 weeks	↑ growth, feed intake, lipid content (Y2) and arginase activity → body composition	[203]
	Gilthead seabream	Lyophilised *S. cerevisiae* (0, 1, 5 or 10g kg^{-1})	4 weeks	↑ cellular parameters	[204]
	Gilthead seabream	*S. cerevisiae* (0, 10, and 20 %) (BDY protein) instead of fish meal	12 weeks	→ growth, alkaline phospha-tase, blood urea Nitrogen, serum protein, cholesterol, triglyceride, albumin and amylase ↓ plasma glucose (10 % BDY)	[205]
	Nile tilapia	Diet (0 and 0.1 %)	9 weeks	↑growth and feed efficiency	[206]
	Nile tilapia	*S. cerevisiae* (0, 25, 50, 75 and 100 %) (BDY protein) instead of soy bean meal (SBM) protein	6 weeks + 5 days	→ growth and feed utilization when 50 % BDY protein instead of SBM protein	[207]
	Nile tilapia	*S. cerevisiae* (0, 25, 50, 75 and 100 %) (BDY protein) instead of soy bean meal (SBM) protein	122 days	→ growth and feed utilization when 25 % BDY protein instead of SBM protein	[208]
	Hybrid striped bass	Diet (0, 1, 2 and 4 %)	8 weeks	↑growth and feed efficiency	[209]
	Hybrid striped bass	Diet (0, 1 and 2 %)	7 weeks (trial 1) and 4 weeks (trial 2)	→ growth (trial 1) Trial 2 ↑ resistance against *Streptococcus iniae* and extra-cellular superoxide anion → growth and feed efficiency	[210]
	Hybrid striped bass	Diet (0, 1 and 2 %)	16 and 21 weeks	↑growth, feed efficiency, serum peroxidase and extracellular superoxide anion of head kidney macrophages (16 weeks) → resistance against mycobacterial infection	[211]
	Pacu	*S. cerevisiae* (0, 30, 35, 50, 70 and 100 %) (BDY protein) instead of fish meal	54 days	↑growth and feed utilization (until 50 % replacement). Protein retention was higher in fish fed 35 and 50 5 replace-ment → protein digestibility	[212]
	Galilee tilapia	0 and 10g kg^{-1}	6 weeks	↑growth, feed utilization and resistance against waterborne Cu toxicity	[213]
	Beluga	Diet (0, 1 and 2 %)	6 weeks	2 % inclusion ↑final weight, weight gain, SGR and FCR ↑ autochthonous LAB levels →survival rate, haematological and serum biochemical parameters resist	[97]

Symbols represent an increase (↑) in the specified response; no change (→); decrease (↓); n.i – no information given.
Salmon – Atlantic salmon; Trout – rainbow trout; Coho – Coho salmon (*Oncorhynchus kisutch*), Turbot – *Scophthalmus maximus*; Sea bass - *Dicentrachus labrax*, Snapper – pink snapper (*Pagrus auratus*); Flounder – Japanese flounder (*Paralychthis olivaceus*), Nile tilapia - *Oreochromis niloticus*; Red drum - *Sciaenops ocellatus*; Channel catfish - *Ictalurus punctatus*
ROS – reactive oxygen species; IHNV – infectious hematopoietic necrosis virus

Table 1: Glucans as immunostimulants in fish, shrimp and scallop.

increase both humoral (myeloperoxidase and antibody titer) and cellular (phagocytosis, respiratory burst and cytotoxicity) immune responses, and to increase or confer resistance against pathogenic bacteria in various fish species (Table 1).

Bioactive alginate (high-M alginate) and Ergosan

The adaptive immune system is poorly developed in early developmental stages of fish [43,44], which is why immunostimulants and probiotics have been used in an attempt to increase survival of larvae against microbial pathogens [45-47]. In this respect, alginate has been proposed as a potential candidate. Alginate is a polysaccharide composed of β-1,4-D-mannuronic acid (M) and C5-epimer α-L-glucuronic acid (G) [48]. The monomers are usually arranged in M-blocks, G-blocks and alternating MG-blocks [49]. Alginate also binds various cations found in the seawater such as Mg^{2+}, Sr^{2+}, Ba^{2+}, and Na^{+}. Commercial alginates are extracted from three species of brown algae; *Laminaria hyperborean*, *Ascophyllum nodosum* and *Macrocystis pyrifera*, in which alginate comprises up to 40% of the dry weight [50]. Furthermore, bacterial alginates, not commercial products, have also been isolated from *Azotobacter vinelandii* and several *Pseudomonas* species [51].

Commercially available alginates today have M-content ranging between 30 and 70%. Alginates with even higher M-content, typically higher than 80%, have also been shown to be potent stimulators of immune cells such as human monocytes [52]. High-M alginate has also been used as an immunostimulant for enhancement of innate immune resistance in fish larvae and fry[53-56].

Ergosan is an algal based product and contains 1 % alginic acid extracted from *Laminaria digitata*. The first study on Ergosan in aquaculture, to the author's knowledge, was reported by Miles and co-authors on striped snakehead (*Channa striata*) [57]. In a study with rainbow trout, Peddie et al. reported that a single injection of 1 mg of Ergosan significantly augmented the proportion of neutrophils in the peritoneal wall, increased the degree of phagocytosis, respiratory burst activity and expression of interleukin-1β (IL-1β), interleukin-8 (IL-8) and one of the two known isoforms of tumour necrosis factor-alpha (TNF-α) in peritoneal leucocytes one day post-injection [58]. However, humoral immune parameters were less responsive to intraperitoneal alginate administration with complement stimulation only evident in the 1 mg-treated group at 2 days post-injection. (Table 2) presents an overview of reports using alginates and Ergosan in fish studies.

Plant extracts

Some immunostimulants cannot be used because of various disadvantages, such as high cost and limited effectiveness upon parenteral administration. Numerous plants have on the other hand long been used in traditional medicine for the treatment and control of several diseases[59]. As herbs have little side effects and are easily degradable and abundantly available in farm areas, numerous investigations have investigated the effect of plant products on innate and adaptive immune response and to prevent and control fish and shellfish diseases [60].

Dügenci and co-authors investigated the effects of mistletoe, nettle and ginger on dietary intake of rainbow trout [61]. The diets contained lyophilized extracts of these plants at two inclusions levels, 0.1 and 1%, in a 3 week experiment. At the end of the experimental period, various parameters of innate defence mechanisms, including extracellular and intracellular respiratory burst activities, phagocytosis in blood leukocytes, total plasma protein level, specific growth rates and condition factors were examined. Inclusion of the plant materials increased the extracellular respiratory burst activity ($P<0.001$) compared to control. Furthermore, fish fed the diet containing 1% ginger roots exhibited a significant innate immune response. Phagocytosis and extracellular burst activities of blood leukocytes were significantly higher in this group compared to the control group. All plant extracts increased plasma protein level except for the 0.1% ginger supplemented diet. The highest level of plasma proteins was observed in the group fed with 1% ginger extract.

Oral administration of the medical plant, *Eclipta alba*, on the non-specific immune responses and disease resistance of tilapia (*Oreochromis mossambicus*) has been investigated[62]. The results indicated that *E. alba* administrated in the diet significantly enhanced the non-specific immune parameters tested. Furthermore, when tilapia was challenged with *Aeromonas hydrophila* mortality was significantly reduced in *E. alba* treated fish.

Lectins are proteins or glycoprotein substances, usually of plant origin, and they are sugar-binding proteins which are highly specific for their sugar moieties. Lectins are also known to play important roles in the immune system by recognizing carbohydrates that are found exclusively on pathogenic bacteria, or that are inaccessible to host cells [63]. Examples are the lectin complement activation pathway, and mannose binding lectin (MBL), also named mannose- or mannan-binding protein (MBP). MBL recognizes carbohydrate patterns, found on the surface of a large number of pathogenic micro-organisms, including bacteria, viruses, protozoa and fungi. Readers with special interest in lectins and immune response are referred to the recent comprehensive reviews [64-65].

In a recent paper, Galina and co-authors reviewed studies currently being carried out on herbs and herbal extracts that have been shown to modulate the immune system in fish and special attention was given to; *Astragalus*, *Ganoderma*, *Lonicera* and Chinese and Indian herbs [66]. Based on the results available *per se* Galina and co-authors suggested that herb extracts might have a potential application as an immunostimulant is fish, due to that they can easily be obtained, are as not expensive and act against a broad spectrum of pathogenic bacteria, and could be alternatives to vaccines, antibiotics or chemotherapeutic agents. In a more recent study, Soosean and co-authors reported that mangosteen (*Garcinia mangostana*) extracts as feed additive to African catfish (*Clarias gariepinus*) had no significant effect on growth parameters, feed conversion ratio and haemoglobin content. However, significantly higher red blood cells and white blood cell counts were recorded in fish fed the shoot extract [67]. In their study on kelp grouper (*Epinephelus bruneus*), Harikrishnan and co-authors evaluated the efficacy of dietary doses of *Lactuca indica* extracts on immunological parameters and disease resistance against *Streptococcus iniae* infection [68]. Based on their results, the authors suggested that supplementation of *L. indica* enhanced the immune system and the disease resistance against pathogenic infection. Enhanced immune response of tiger shrimp (*Penaeus monodon*) by the medical herb black nightshade (*Solanum nigrum*) and disease resistance against *Vibrio harveyi* were investigated by Harikrishnan and co-authors [69]. In this study the authors displayed improved immune response including respiratory bursts, PO-, SOD – and GPx activities and disease resistance by feeding tiger shrimp black nightshade at 0.1 and 1 % doses. This herb has also been shown to have inhibitory *in vitro* growth effect on *A. hydrophila* and improved survival and increased haematological parameters of spotted snakehead (*Channa punctatus*) infected with *A. hydrophila* [70].

Immunostimulant	Fish	Administration and dose	Length of administration	Mechanism of action/results	References
High-M alginate	Atlantic halibut	Immersion	n.i	↑ survival	[214]
	Atlantic halibut	Bioencapsulated in *Artemia*	Different periods during initial feeding	↑ growth	[215]
	Atlantic cod and spotted wolffish	Diet	59 days 55 days	↑ growth Uptake of 125 I-labelled molecule in the stomach and intestine	[56]
	Turbot	Enriched in *Artemia*	1 week	↑ survival	[53]
Alginate	Atlantic salmon	Macrophage culture	n.i	↑ phagocytic activity and respiratory burst	Rokstad *et al*. (unpublished data) cited in Vadstein [54]
	Atlantic salmon	Diet	Six months	↑ lysozyme activity → survival, colour, taste consistency and grading	[216]
	Turbot	Diet	13 days	↑ protein synthesis and protein turnover → survival and larval size	[217]
Ergosan	Striped snakehead	i.p	14 days	↑ inhibition index of macrophages and serum on *in vitro* growth of *Aphanomyces invidans*	[57]
	Rainbow trout	i.p	1 day	↑ neutrophils and respiratory burst	[58]
	Sea bass	Diet (0.5 %)	60 days	↑ serum complement activity, serum lysozyme, gill and liver HSP	[162]
AquaVac Ergosan	Rainbow trout	Diet (0.5 %)	Three cycles (95 days in total)	↑ growth (95 days) and the growth related gene expression of IGFI, TRαmRNA and TRβ, and gene expression of IL-1β, IL 8 and TLR3 in spleen ↓ cortisol, HSP70 gene expression in liver, MYO gene expression	[218]

AquaVac Ergosan – extracts of *Laminaria digitata* and *Ascophyyllum nodosum*.
Symbols represent an increase (↑) in the specified response; no change (→); decrease (↓).
n.i – no information given

Table 2: Bioactive alginate (high-M alginate) and Ergosan as immunostimulants in fish.

Readers with special interest in the topic plant products on innate and adaptive immune response of fish and shellfish are referred to the recent review of Harikrishnan et al. [60].

Mechanisms of action

Phagocytic activity of kidney leucocytes

Phagocytic activity is the principle function of kidney leucocytes of teleosts. Monocytes, macrophages, dendritic cells, and neutrophils belong to the phagocytic leucocytes called phagocytes. In host defense, phagocytes respond to microbes in a sequential manner: active recruitment of the cells to the sites of infection through inflammatory responses, ingestion of microbes by the process of phagocytosis, and destruction of the ingested microbes. Phagocytosis is an active, energy-requiring engulfment process of large particles (>0.5 μm in diameter). The first step in phagocytosis is the recognition of microbe by the specific receptors expressed on the phagocyte such as TLRs (toll-like receptors). The bound microbes are ingested into vesicles forming phagosomes inside of the cells and the phagosomes are then fused with lysosomes that contain many different proteases. The internalized microbes will be killed there through proteolytic processes [71].

Macrophage activation

Macrophage activation is initiated upon binding of the microbe to the cell. The recognition of microbes by phagocytes such as macrophages, neutrophils, and dendritic cells is selective and mediated by receptors on phagocytic cells. Upon microbe binding, the receptors cooperatively activate the cells through a receptor-mediated signal transduction to kill ingested microbes. The receptors responsible for microbe recognition include TLR (toll-like receptors), G protein-coupled receptors, antibody Fc and complement C3 receptors, and receptors for cytokines, mainly IFN-γ. Activated macrophages kill phagocytosed microbes by the action of microbicidal molecules in phagolysosomes of phagocytes. In addition, activated macrophages secrete cytokines such as TNF, IL-1, and IL-12, which cause inflammation and bridge to activate the adaptive immune system.

Respiratory burst activity

Respiratory burst (sometimes called oxidative burst) is the rapid release process of reactive oxygen species (ROS) such as superoxide anion, hydroxyl radical and hydrogen peroxide from activated macrophages and neutrophils. The respiratory burst, releasing important bactericidal ROS molecules, plays a crucial role to remove microbes from the body. The respiratory burst is mediated by the enzyme phagocyte oxidase, which converts molecular oxygen to ROS and is usually triggered by bacterial products or by inflammatory mediators. The respiratory burst activity is an indicator of the status of macrophage and neutrophil activation [71].

Acid phosphatase

Acid phosphatase is a ubiquitous enzyme that removes phosphate groups from other molecules. The acid phosphatase is one of the lysosomal hydrolases which exist in phagolysosomes of macrophages (lysosomal acid phosphatases). When the macrophage is activated, the inside of phagosome becomes acidic. In an acidic environment, lysosomal hydrolases including acid phosphatase are activated to kill microbes. Acid phosphatase activity is an indicator of the microbicidal activity of macrophages and a high concentration of acid phosphatase in the blood is a sign of a chronic infection [71,72]. Recently, acid phosphatase activity tests are used for the diagnosis of prostate cancer and breast cancer in human.

Serum complement activity

The complement system is one of the major effector mechanisms of the humoral immunity as well as the adaptive immunity, which involves the clearing of pathogens from an organism. The complement system consists of serum and cell surface proteins that are activated by microbes (the alternative pathway) and antibodies (the classical pathway). Activation of the complement system involves the sequential proteolysis of proteins to generate new enzyme complexes with proteolytic activity, which is a characteristic of a proteolytic enzyme cascade. The products of the complement activation become covalently attached to microbes, antibodies bound to microbes, and to other antigens. The binding of complement products to the target molecules cause lysis of the microbe, facilitation of opsonization (the process of attaching IgG or complement fragments to microbial surfaces for phagocytosis) and phagocytosis, and inflammation [71].

Phenoloxidase activity

Phenoloxidases are known to be involved in the innate immune response of invertebrates such as shrimps and crabs. Phenoloxidases are composed of tyrosinases, catecholases and laccases which are the terminal components of the prophenoloxidase (proPO) system, the modified complement system of several phyla of invertebrates[73]. The proPO system is present in the blood of a wide range of marine invertebrates, especially crustaceans. Phenoloxidases play a role in bactericidal activity and the enhancement of phagocytosis in crustaceans [74-76].

Serum antibody concentration

B lymphocytes recognize antigens such as microbes using their receptors (membrane-bound antibody molecules). The engagement of antigen receptors and other signals trigger the lymphocytes to expand their numbers and to differentiate into antibody-secreting mast cells, which secrete different classes of antibodies with distinct functions. The functionally different antibodies have the same antigen specificity, through which the antibodies recognize the same microbes despite their different classes. Antibodies bind to microbes and prevent them from infecting cells (neutralization). The binding of antibodies to microbes also promotes opsonization and phagocytosis of microbes to kill and mediate antibody-dependent cellular cytotoxicity (ADCC). In addition, antibodies trigger activation of the complement system (the classical pathway), which in turn causes to increase phagocytosis of microbes opsonized with complement fragments and inflammation. Serum antibody concentration is an indicator of B lymphocyte activation against specific microbes [71].

Leukocyte number

Leukocytes (white blood cells) are immune cells involved in defending the body against both infectious microbes and foreign materials. Leucocytes consist of neutrophil, eosinophil, basophil, monocyte, and lymphocyte. They are all derived from a hematopoietic stem cell in the bone marrow. Neutrophils and monocytes are involved in the innate immune response as the first line of the defense system. Effector cells of the innate immune system circulate in the blood and migrate into tissues and kill microbes through phagocytosis. The innate immune cells also play a role in the inflammatory response by releasing cytokines. Lymphocytes including T cells and B cells are the only cells in the body capable of specifically recognizing and distinguishing different antigenic determinants (epitopes). When these adaptive immune effector cells recognize the microbes, the number of lymphocytes is significantly increased as a part of their effector functions. Although the number of the innate immune cells are also expanded upon activation, the level of increase is much lower than that of the adaptive immune cells. The number of leukocytes in the blood is often an indicator of the activation of the immune system or of leukemia [71].

Lysozyme activity

Lysozyme is a cationic enzyme that attacks the β-1,4 glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine in the peptidoglycan of bacterial cell walls. This activity causes the lysozyme to lyse certain gram-positive bacteria and even some gram-negative bacteria. Lysozyme activity has been detected in mucus, serum, organs and eggs of several fish species [77]. Lysozyme is produced mainly by macrophages and its production is induced in response to microbial components such as microbial lipopolysaccharide (LPS) [78] and many other immunostimulants.

Do immunostimulants raise the level of protection against disease?

A large number of reviews have been published during the last 20 years concerning the advantages of immunostimulants in fish and the immune system [1,3,23,28,30,32,79-90]. In this section an attempt has been made to compile the recent developments in the field, mainly over the last five years, besides the earlier work done in the field of disease resistance in fish with exposure to immunostimulants.

Several types of β-glucan have been successfully used to enhance resistance of fish and crustacean against bacterial and viral infections. An overview is presented in (Table 3). Most pathogens used in these challenge experiments are bacteria reported in commercial aquaculture and include; *A. hydrophila*, *Aeromonas salmonicida*, *Aphanomyces invadans*, *Edwardsiella ictaluri*, *Edwardsiella tarda*, *Photobacterium damselae* ssp. *piscicida*, *Vibrio* (*Listonella*) *anguillarum*, *Vibrio harvei*, *Yersinia ruckeri*, *Lactococcus garvieae*, *S.iniae* and *Streptococcus sp.* as well as infectious hematopoietic necrosis virus (IHNV), white spot syndrome virus (WSSV) and parasites. Readers with special interest in the topic immune response and aquatic viruses are referred to the recent reviews [91,92].

Recognition of invasive pathogens is an essential step for the activation of the immune system. This is accomplished by binding of PRRs to PAMPs. The PRRs and PAMPs activate appropriate immune responses [93]. β-glucan binding proteins (β-GBP) are known as one of the most important PRRs in invertebrates [94] and fish [95]. The presence of these proteins in invertebrates and fish may indicate that the defence systems against β-glucan containing microorganisms are crucial for these organisms.

Effect of immunostimulants on gut microbiota

Resistance to pathogen	Agent	Species	Route of exposure	Length of administration	Mechanism of action/results	References
Aeromonas hydrophila	Yeast glucan	Tilapia and grass carp	i.p	14 days	↑ protection in both species and number of NTB-positive staining cells	[219]
	Glucan	Rohu	Oral	7 days	↑ phagocytotic activity, bactericidal activity, antibody titre and agglutinin level	[220]
	Glucan	Rohu	i.p	28 days	↑ phagocytotic activity, lysozyme activity, bactericidal activity, complement activity and resistance	[221]
	Garlic	Rohi	i.p	10 days	↑superoxide production, lysozyme activity and resistance	[222]
	Glucan	Catla	i.p	30 days	↑ antibody titre and macrophage activating factor	[223]
	Glucan from *S. cerevisiae*	Carp (25-30 g)	i.p (0, 100, 500 and 1000 µg fish^{-1})	14 days	↑ total leukocyte count and neutrophil and monocytes, increase survival in a challenge experiment with *Aeromonas hydrophila*	[224]
	Glucan from *S. cerevisiae*	Carp (25-30 g)	i.p (0, 100, 500 and 1000 µg fish^{-1})	14 days	↑ total leukocyte count and neutrophil and monocytes, superoxide anion production by kidney macrophages and resistance (500 µg) against *A. hydrophila*	[225]
	β-glucan in combination with LPS	Carp (25-30 g)	i.p, bathing and oral ad-ministration	14	↑ total blood leukocyte count and neutrophil and monocytes and resistance against *A. hydrophila* when i.p and oral administration were used	[226]
	Levamisole	carp	Diet (0, 125, 250 and 500 mg kg^{-1})	70 days	↑ resistance and erythrocyte count, haemoglobin content, haematocrit, total serum protein, albumin and globulin of fish fed 250 mg kg^{-1}	[227]
	β-glucan and *S. uvarum*	Carp	i.p	15 days	↑ resistance and serum lysozyme activity	[228]
	Glucan	Asian catfish	Oral	30 days	↑ antibody titre and protection	[229]
	Glucan	Blue gourami	i.p (5, 10, 20 and 40 mg kg^{-1})	22 days	↑ chemiluminescent response of head-kidney phagocytic cell isolated from fish i.p with a dose of 20 mg kg^{-1} and resistance	[230]
	Glucan	Indian major carp	i.p	1 week	↑superoxide production and phagocytic activity resistance unclear	[231]
	S. cerevisiae	zebrafish	i.p (0.5, 2 and 5 mg ml^{-1})	6 + 4 days	↑ resistance (5 mg), myelomonocytic cells in kidney and enhanced the ability of kidney cells to kill the pathogen	[232]
	S. cerevisiae	Nile tilapia	Oral (0, 0.25, 0.5, 1, 2 and 5 g kg^{-1}) and i.p	12 weeks + 10 days	12 weeks ↑ growth, feed utilization and protein turn-over (1-5 g kg^{-1}) and resistance (5 g kg^{-1})	[233]
	S. cerevisiae	Nile tilapia	Oral (2 % autolyzed yeast; 0.3 % cell wall)	70 days	↑thrombocytes and nonspecific acute inflammatory response ↓neutrophils, macrophages an lymphocytes	[234]
	Green tea (*Camellia sinensis*)	Nile tilapia	Oral (0, 0.125, 0.25, 0.5, 1 and 2 g kg^{-1})	12 weeks + 10 days (i.p challenge)	↑ growth and feed utilization (highest at 0.5 G kg-1), haematological and biochemical parameters (0.25-2 g kg^{-1}) and resistance with increasing inclusion levels	[235]
	Debaryomyces hansenii	Leopard grouper	Oral (10^6 yeast g^{-1})	5 weeks	↑ resistance and growth → immunological parameters after 4 weeks	[236]

Resistance to pathogen	Agent	Species	Route of exposure	Length of administration	Mechanism of action/results	References
	Macrogard	Tench	Oral (0, 0.5, 1 and 2 g kg^{-1})	1 month	↑ resistance and phagocytic activity (1 and 2 g kg^{-1})	[237]
	Schizophyllan	Two ornamental fish species	i.p (10^{8} cells ml^{-1})	24 days	↑ resistance against both pathogens	[238]
A.hydrophila and *Pseudomonas fluorescens*	Glucan	Brook trout	i.p and 30 min immersion	4 weeks	↑ resistance (up to 7 days). By day 14 no resistance was reduced	[239]
Aeromonas salmonicida	Macrogard	Trout	Oral	1 week	↑ MPO activity, phagocytic activity, superoxide production and Ig level	[240]
Aeromonas salmonicida and Vibrio salmonicida	Laminaran	Atlantic salmon	i.p (23.6 mg kg^{-1})	35 days	↑resistance against *V. salmonicida* → resistance against *A. salmonicida*	[241]
Aphanomyces invadans	Ergosan	Striped snakehead	Injected intramuscularly	2 weeks	↑ inhibitory effect of Serum and macrophage activating factor	[57]
Edwardsiella ictaluri	Yeast glucan	Channel catfish	i.p	2 weeks	↑ resistance and phagocytic activity	[153]
	Glucan	Channel catfish	Oral	2 weeks	↑ macrophage and neutrophil migration → resistance	[242]
Edwardsiella tarda	Glucan	Carp	i.p	12 days	↑ resistance	[152]
	Glucan	Rohu	i.p	10 days	↑ bactericidal activity ↑resistance	[243]
	Glucan	Rohu	i.p	28 days	↑ phagocytotic activity, lysozyme activity, bactericidal activity, complement activity and resistance	[221]
	Glucan - injections	Rohu	i.p	28 days	↑ phagocytotic activity, lysozyme activity, bactericidal activity, complement activity and resistance	[222]
	Yeast glucan	Tilapia and grass carp	i.p	14 days	↑ protection in both species and number of NTB-positive staining cells	[219]
Pasteurella piscicida	Glucan	Yellowtail	i.p	10 days	→ resistance	[244]
Photobacterium damselae ssp. *piscicida*	Glucan	Gilthead sea bream	Immersion (10^{7} cells l^{-1})	10 days	↑ resistance (10 g kg^{-1}) → resistance (5 g kg^{-1}) and phagocytic activity	[245]
Vibrio alginolyticus	Glucan	White shrimp	Oral	120 hours	↑ resistance, phenol-oxidase and respiratory burst	[246]
	Glucan	Fresh-water prawn	Bath exposure with glucan.	4 hours + 1 week immersion with the pathogen	↑ haemocyte lysate supernatant, lysozyme and phosphatase active-ties and increased resistance → total protein content	[247]
Vibrio anguillarum	Yeast glucan	Salmon	i.p	20 days	↑ resistance	[183]
	High-M alginate	Turbot	Immersion (10^{5} cells ml^{-1})	1 week	↑ resistance	[53]
	High-M alginate	Atlantic halibut	Immersion (5x10^{5} or 1.4x10^{7} cells ml^{-1})	Different periods during initial feeding	↑ resistance at the highest dose → resistance at lowest dose	[215]
	Cationic cod milt protein	Atlantic cod (5 g)	Bath challenge (5x10^{6} cells ml^{-1})	4 weeks	↑ resistance	[248]
Vibrio anguill-arum and *Vibrio campbellii*	Glucan from *S. cerevisiae*	Gnoto-biotic *Artemia*	Immersion (5x10^{6} cells ml^{-1})	6 days	↑ resistance against both pathogens	[249]
Vibrio campbellii	Glucan from *S. cerevisiae*	*Artemia*	Immersion (5x10^{6} cells ml^{-1})	5 days	Protection against *Vibrio campbellii* when supplied in advance of the challenge	[250]

Resistance to pathogen	Agent	Species	Route of exposure	Length of administration	Mechanism of action/results	References
	Glucan from *S. cerevisiae*	*Artemia*	Immersion (5x10^6 cells ml^{-1})	6 days	↑ survival and resistance against *V. campbellii*	[251]
Vibrio damsela	Yeast glucan	Turbot	Yeast glucan was used as adjuvant	42 days	↑ activities of the immune parameters when glucan was injected after the bacterin	[252]
Vibrio harvei	Glucan	Croaker	i.p	8 weeks	↑ lysozyme activity, phagocytic activity and resistance (0.09%) → resistance (0.18%)	[253]
Vibrio vulnificus	Glucan	Tiger shrimp	Immerse	43 days	↑ resistance in shrimp treated with 0.5 and 1 mg/ ml glucan but no in groups treated with 0.25 and 2 mg/ ml glucan	[194]
Vibrio sp. 90-69B3	Yeast Culture	Pacific white shrimp	Oral	i.p after 4 weeks	↑ resistance	[254]
Yersinia ruckeri	Yeast glucan	Salmon	i.p	20 days	↑ resistance	[183]
	S. cerevisiae	Rainbow trout	i.p	14 days	↑ resistance, growth, lysozyme and complement activities	[255]
Lactococcus garviae	Alginate micro-particles	Trout	i.p	3 weeks	→ resistance	[256]
Streptococcus sp.	Glucan	Yellowtail	i.p	10 days	↑ resistance and serum complement ↑ lysozyme activity	[244]
	Sodium alginate	Grouper	i.p	6 days	↑ lysozyme activity, respiratory bursts, phagocytic activity and resistance	[257]
Streptococcus iniae	Glucan	Hybrid striped bass	Oral	3 weeks	→ resistance	[258]
	Glucan	Nile tilapia	Oral	14 weeks	↑ resistance when fish were fed 100 and 200 mg glucan	[259]
	Glucan	Shark	Oral	2 weeks	↑ resistance	[260]
Virus						
IHNV	Glucan	Rainbow trout	i.p	18 days	↑ resistance → respiratory bursts and TNF-α-expression	[39]
WSSV	β-1,3-glucan	*Penaeus monodon*	Oral (0 and 2g kg^{-1})	15 days	↑ resistance	[261]
	β-1,3-glucan	*Penaeus monodon*	i.p	20 days	↑ resistance and the Immune system	[262]
	Marine yeast (*Candida sake*)	White prawn	Oral (10 %)	40 + 10 days	↑ immunity index and resistance	[263]
	Marine yeast glucan	*Penaeus monodon*	Oral	21 days	↑ resistance, but higher molecular weight and lower degree of branching acts better	[264]
Parasite						
Loma morhua	Glucan	Atlantic cod	Oral	n.i	↑ lymphocyte density	[265]
Loma salmonae	β-1,3/1,6 glucan	Rainbow trout	i.p (0, 50, 100, 500 and 1000 µg)	10 weeks	During week 8-9 post exposure a significant reduction in no. of xenoma-positive fish was noticed.	[266]

Symbols represent an increase (↑) in the specified response; no change (→); decrease (↓); n.i – no information given.
i.p – intraperitoneal injection
n.i – no information given
IHNV – infectious hematopoietic necrosis virus
IHNV – infectious hematopoietic necrosis virus
WSSV - White spot syndrome virus

Table 3: Immunostimulants and disease susceptibility in fish and shrimps.

Immunostimulants seem to be valuable for the control of fish diseases. However, knowledge regarding the ability of immunostimulants to modulate the gut microbiota in a healthier way and decrease the infection pressure by improving the gut function is scarce. To our knowledge only two studies, Gildberg and Mikkelsen [46] and Skjermo et al. [96] have been done on the topic; immunostimulants and modulation of the gut microbiota. In their study on Atlantic cod (*Gadus morhua*) fry, Gildberg and Mikkelsen evaluated the effect of supplementation of LAB either alone or in combination with immunostimulating peptides [46]. After three weeks of feeding, the fish were challenged by bath exposure to *V. anguillarum* (10^7 ml^{-1}; 1hour). Twelve days after infection significantly ($p<0.05$) reduced cumulative mortality was recorded in fish fed diet supplemented with LAB, originally isolated from Atlantic salmon (*Salmo salar*) and with immunostimulating peptides. No synergistic or cumulative effects were achieved by combining LAB and immunostimulating peptides.

Four weeks after infection similar cumulative mortality (80–84%) was observed in all groups. LAB seems to colonize the internal mucus layer of the cod fry pyloric caeca, and a significant number of the bacteria survived the passage of the whole gastrointestinal tract.

In a study with Atlantic cod larvae, Skjermo and co-authors [96] evaluated the effect of β-(1→3, 1→6)-glucans (chrysolaminaran) from the marine diatom *Chaetoceros mülleri*, a commercial yeast-glucan product and high-M alginate (high content of mannuronic acid isolated from *Durvillaea antarctica*), on the microbial community in larval gut and water. Total colony forming units (CFU) were evaluated on marine agar, and *Vibrio* - and *Pseudomonas* - like species on selective agars (TCBS and marine *Pseudomonas* agar with CFC - supplement). The larvae were rapidly colonised after hatching, but no or weak effects of the stimulants were observed on the bacterial colonisation rates or the bacterial community. The total CFU varied from 10^1 to 10^2 CFU per µg larva after initiation of the first feeding. Bacteria belonging to *Pseudomonas* appeared to increase throughout the period, whereas the level of *Vibrio*-like bacteria was low and stable. However, in this investigation the authors only focused on characterisation of *Pseudomonas*- and *Vibrio*-like bacteria and did not use molecular methods to evaluate the entire bacterial community.

Hoseinifar and co-authors evaluated the effect of brewer's yeast (*S.cerevisiae* var. *ellipsoideus*) on gut microbiota of juvenile beluga (*Huso huso*) and displayed that juveniles fed 2 % *S. cerevisiae* var. *ellipsoideus* increased the levels of autochthonous LAB [97]. The authors suggested that this interesting finding might occur as a result of the provision of enzymes, RNA and free nucleotides, B-complex vitamins and/ or amino acids by the dietary yeast. Whether supplementation of *S. cerevisiae* var. *ellipsoideus* improved disease resistance against pathogens was not investigated and merits further evaluation.

Based on the fact that no information is available about the effect of immunostimulants on the "*good*" gut microbiota with antagonistic activity against fish pathogenic bacteria, this should be a topic of further research as the gastrointestinal tract in fish is a potential port of entry for pathogenic bacteria [98-106]. The importance of the topic is illustrated by a study showing that intraperitoneal injection of immunostimulatory substances affects the allochthonous (transit) gut microbiota including LAB of Atlantic salmon [107].

Nucleotides

Dietary nucleotides have attracted attention as key ingredients missing from nutritional formulae for many years. They are the building blocks of tissue RNA and DNA and of ATP, and their presence in breast milk has stimulated research in babies which has indicated that supplementation of infant formula milk leads to improved growth and reduced susceptibility to infection [108-110]. Nucleotide fortification of breast milk substitutes has been recommended to the U.S. Food and Drug Administration for approval [111]. There is increasing evidence that nucleotides administered intravenously or in the diet are capable of modifying immune responsiveness and recovery of organs that have undergone a metabolic or inflammatory insult.

It is generally accepted that nucleotides have essential physiological and biochemical functions including encoding and deciphering genetic information, mediating energy metabolism and cell signalling as well as serving as components of coenzymes, allosteric effectors and cellular agonists[112,113]. However, controversy has existed for many years over the roles of nucleotides administered exogenously. As neither overriding biochemical malfunctions nor classical signs of deficiency are developed in endothermic animal models, nucleotides have traditionally been considered to be non-essential nutrients. However, this opinion has been challenged by several research publications during the last decade which suggest that dietary nucleotide deficiency may impair liver, heart, intestine and immune functions[114]. The modulatory effects of dietary nucleotides on lymphocyte maturation, activation and proliferation, macrophage phagocytosis, immunoglobulin responses, gut microbiota as well as genetic expression of certain cytokines have been reported in endothermic animals[115,116]. Nucleotide supplementation has been one important aspect of research on clinical nutrition and functional food development for humans[109,110].

Although initial efforts in evaluation of dietary supplementation of nucleotides for fishes could be traced to the early 1970s, research at that time mainly focused on the possible chemo-attractive effects of these compounds [117-119]. However, the pioneer investigations by Burrels and co-authors resulted in increased attention on nucleotide supplementation for fishes as their studies indicated that dietary supplementation of nucleotides enhanced resistance of salmonids to viral, bacterial and parasitic infections as well as improved efficacy of vaccination and osmoregulation capacity[5,6]. To date, research related to nucleotide nutrition in fishes has to some extent showed consistent and encouraging beneficial results in fish health management (Table 4), although most of the suggested explanations put forward by the authors remain hypothetical. Systematic research on fishes is therefore needed.

Because increasing concerns of antibiotic use have resulted in a ban on subtherapeutic antibiotic usage in Europe and the potential for a ban in the US and other countries[120], research on immunonutrition for aquatic animals is becoming increasingly important[121]. Research on nucleotide nutrition in fish and shrimp is needed to provide insights concerning interactions between nutrition and physiological responses as well as provide practical solutions to reduce basic risks from infectious diseases for the aquaculture industry. Devresse hypothesized that nucleotides are a key nutrient for the shrimp immune system and supplementation of nucleotides or other nucleic acid-rich ingredients such as yeast or yeast extract may enhance disease resistance and growth of shrimp [122]. Although yeast products have been used in shrimp diet formulations, the role of yeast nucleotides remains largely unanswered. In their comprehensive review, Li and Gatlin summarized and evaluated knowledge of nucleotide nutrition in fishes as compared with that of terrestrial animals[123].

The roles of nucleotides and metabolites in fish diets have been sparingly studied for nearly 20 years. Beside possible involvement in diet palatability, fish feeding behaviour and biosynthesis of non-essential amino acids, exogenous nucleotides have shown promise most recently as dietary supplements to enhance immunity and disease resistance of fish produced in aquaculture. Research on dietary nucleotides in fishes has shown they may improve growth in early stages of development, enhance larval quality via broodstock fortification, alter intestinal structure, increase stress tolerance as well as modulate innate and adaptive immune responses. Fishes fed nucleotide-supplemented diets generally have shown enhanced resistance to viral, bacterial and parasitic infection. Despite occasional inconsistency in physiological responses, dietary supplementation of nucleotides has shown rather consistent beneficial influences on various fish species. Although nucleotide nutrition research in fishes is in its infancy and many fundamental questions remain unanswered, observations thus far support the contention that nucleotides are conditionally or semi-essential nutrients for fishes, and further exploration of dietary

Nucleotide form	Dose and/or feeding regime	Length of administration	Species	Initial size	Effect[a]	Authors
Ascogen S	2 and 5 g kg^{-1} diet	16 weeks	Hybrid tilapia	21 days old	↑growth and survival	[267]
Ascogen P	5 g kg^{-1} diet, fixed ration approaching satiation daily	7 weeks	Hybrid striped bass	7.1; 9.1 g	↑ neutrophil oxidative radical production and survival after challenge with *S. iniae*	[268-269]
Ascogen	5 g kg^{-1} diet	120 days	Hybrid tilapia	30 days old	↑ antibody titer after vaccination and mitogenic response of lymphocyte	[270]
	0.62, 2.5 and 5 g kg^{-1} diet at 1% bw day^{-1}	37 days	Rainbow trout	163.4–169.7 g $fish^{-1}$	↑ growth	[134]
Nucleic acid	5.8 and 11.5 %	10 weeks	Sea bream	12.7 g	↑ growth, ornithine carbamyltransferase activity → body composition, hepatic glutamate dehydrogenase and uricase activities	[203]
Optimûn	2 g kg^{-1} diet, containing 0.03% NT, 2% bw day^{-1}	3 weeks	Rainbow trout	217 ± 62 g	↑ survival after challenge with *V. anguillarum*	[5]
	2 g kg^{-1} diet, containing 0.03% NT, 1% bw day^{-1}	2 weeks	Rainbow trout	53–55 g	↑ survival after challenge with infectious salmon anaemia virus	[5]
	2 g kg^{-1} diet, containing 0.03% NT, 2% bw day^{-1}	3 weeks	Coho salmon	100 g	↑ survival after challenge with *Piscirickettsia salmonis*	[5]
	2 g kg^{-1} diet, containing 0.03% NT, 2% bw day^{-1}	3 weeks	Atlantic salmon	60 g	↓ sea lice infection	[5]
	2 g kg^{-1} diet, containing 0.03% NT at 1.5% bw day^{-1}	3 weeks before vaccination and 5 weeks post-vaccination	Atlantic salmon	34.7 ± 9.6 g	↑ antibody titer ↓ mortality	[6]
	2 g kg^{-1} diet, containing 0.03% NT at 1.5% bw day^{-1}	8 weeks	Atlantic salmon	43 ± 3.0 g	↑ growth ↓ plasma chloride	[6]
	2 g kg^{-1} diet, containing 0.03% NT	10 weeks	Atlantic salmon	205 g	↑ intestinal fold	[6]
	NA	120 days	"all-female" rainbow trout	80–100 g	↑ B lymphocytes and resistance to IPN virus ↓ plasma cortisol	[271]
	2 g kg^{-1} diet, containing 0.03% NT to hand satiation daily	15 weeks	Turbot	120.9 ± 5.1 g	Altered immunogene expression in various tissues	[7]
	2 g kg^{-1} diet	6 weeks	Red drum	1 g	→ Growth, whole body composition and *in situ* challenge with *Amyloodinium ocellatum*	[126]
Ribonuclease-digested yeast RNA	15 mg $fish^{-1}$, by intubation	3 days	Common carp	100 g	↑ phagocytosis, respiratory burst, complement and lysozyme ↓ *A. hydrophila* infection	[273]
Aquagen	Oral	2 weeks	Shark	1.4 ± 0.2 g	↑ resistance after challenge with *S. iniae*	[260]
Nucleotide mixture	4 g kg^{-1} diet	5 weeks	Pacific white shrimp	ca. 0.83 g	↑ growth	[274]
	4 g kg^{-1} diet	4 weeks	Red drum	10.2 ± 0.2 g	↑ growth, neutrophil oxidative radical production and survival after challenge with *V. harveyi*	[275]

[a] - symbols represent an increase (↑), decrease (↓) or no change (→) in the specified response.

Table 4: Research on dietary supplementation of nucleotides on fishes.

supplementation of nucleotides for application in fish culture is warranted. Hypothesized reasons associated with these beneficial effects include dietary provision of physiologically required levels of nucleotides due to limited synthetic capacity of certain tissues (e.g. lymphoid), inadequate energetic expenditure for *de novo* synthesis, immunoendocrine interactions and modulation of gene expression patterns.

The numbers of scientific publications dealing with dietary nucleotides in fish has been relatively limited since the comprehensive review of Li and Gatlin[123] was published. The section below summarises the dietary effects of nucleotides on a number of fish species such as red drum (*Sciaenops ocellatus*), red-tail black shark (*Epalzeorhynchos bicolor*), Asian carp (*Catla catla*), barramundi (*Lates calcarifer*), cobia (*Rachycentron canadum*), grouper (*Epinephelus malabaricus*) and salmonids such as rainbow trout and Atlantic salmon published post 2006.

The use of dietary nucleotides in red drum *Sciaenops ocellatus* has also been evaluated on growth and feed utilisation[124]. Juvenile fish (ca. 122g) were fed purified a commercial nucleotide product 0.2% (Optimûn, Chemoforma, August, Switzerland) or nucleotides mixtures, which contained equal amounts of CMP (cytidine monophosphate), UMP (uridine monophosphate), AMP (adenosine monophosphate), IMP (inosine monophosphate) and GMP (guanosine monophosphate), at 0.03%, 0.1% and 0.3% of the diet for a period of four weeks[125]. Fish fed dietary nucleotides had showed a clear trend towards increased growth, measured as SGR at the end of the trial. Growth improvements

ranged from 5-6% up to 8% higher than the control diet for the fish fed the nucleotide mixtures and commercial product respectively. A similar trend towards improved feed efficiencies was observed in all the groups fed diets containing added nucleotides. No diet effect on body composition was observed in contrast to previous observations. A clear trend of diet effect on neutrophil oxidative radical production was also observed that could suggest that excessive dose could inhibit immune responses. Fish were challenged against *V.harveyi* at the end of the four-week feeding period but no differences were observed in terms of survival when compared to the control diet. The authors reported on unexpected high mortalities within the 3 days post-challenge in contrast to previous experiments so it was concluded that further studies are needed in order to evaluate potential effect of dietary nucleotides on red drum resistance against *V. harveyi* [126].

Red drum was also the fish species selected by Cheng and co-authors to assess the effects of dietary nucleotides on intestinal morphology and immune responses [127]. The authors used around 7g fish to compare a commercial nucleotide product, Ascogen P® (Canadian Byosystems Inc., Calgary, Alberta, Canada), at 0.5% and 1% inclusion levels against a control diet not enriched with nucleotides. Evaluation of immune response after six weeks of feeding showed increased extracellular superoxide anion production at the highest dose. In general intestinal morphology, measured as fold height, enterocytes height and microvillus height, in the pyloric caeca, proximal, mid- and distal intestine, was positively influenced by dietary nucleotides. Thus, fold height in the proximal intestine was increased by nucleotides as previously reported for Atlantic salmon [5,6]. Enterocyte height was increased in the pyloric caeca, proximal and distal intestine, not in the mid intestine, by nucleotides. The microvillus height was also increased in the pyloric caeca, proximal, mid and distal intestine.

Russo et al. examined the effects of commercial beta-glucans (Macroguard®, Biotec Pharmacon ASA, Tromsø, Norway) and nucleotide product (Aquagen™, NOVARTIS-Aqua Health Ltd., Charlottetown, Canada) on disease resistance of red-tail black shark (*Epalzeorhynchos bicolor*) against *S.iniae* infection [128]. Two trials were conducted using 1.4g fish feeding control, beta-glucan (0.1% inclusion) or nucleotide diet (0.2% product inclusion) for 24 days. In the first trial both vaccinated and non-vaccinated fish were used while in the second experiment nucleotide- or beta-glucan-enriched diets were fed only to vaccinated fish and results compared against a non-vaccinated fish fed control diet. Results of the first trial showed that both commercial products were able to reduce mortalities compared to the fish fed control diet in both vaccinated and non-vaccinated fish. In the second experiment vaccinated fish fed beta-glucans and nucleotide suffered lower mortalities than the vaccinated fish fed control diet and despite slightly lower mortality in the beta-glucan group compared to nucleotide group no statistical difference could be claimed. Growth performance was influenced significantly by dietary treatment in any of the trials conducted.

Jha et al. tested yeast nucleotides, in the form of RNA (Sisco Research Laboratories, India), at 0.4% and 0.8% inclusion levels on *Catla catla* fingerlings (ca. 8g start weight) to assess potential changes in haemato-immunological responses followed by a challenge trial against *A. hydrophila* after 60 days feeding [129]. Feeding dietary nucleotides increased leucocyte counts, total protein, globulins, and albumin: globulin ratio, lysozyme activity and respiratory burst activity compared to controls. When comparing differences between nucleotide doses only the respiratory burst activity was increased by augmenting the dietary nucleotides from 0.4% to 0.8% dose. Conversely, the highest survival was observed in the fish fed the highest dose of nucleotides. Fish fed 0.4% nucleotides also showed significantly higher survival than control fish.

At 30˚C barramundi fed diets supplemented with nucleotides (Optimûn, Chemoforma, August, Switzerland) grew significantly better and had lower FCR than those fed the reference diet with no additional nucleotides added[130]. In the same study it is noted that the supplementation of dietary nucleotide at 30˚C significantly improved the retention efficiencies of protein, energy, lysine and histidine compared to the reference diet. However at 37˚C , under heat-stress conditions, despite dietary nucleotides reduced FCR an improvement in growth was not observed.

Salze et al. supplemented cobia diets with a nucleotide-rich yeast extract (Nupro, Alltech, Nicholasville, KY, USA) part of a zero-fishmeal feed[131]. The 104g fish fed the zero-fish meal diet, which was based on soy protein concentrate, worm meal and mannan-oligosaccharides, had similar growth as a control diet with 25.3% fish meal. However the experimental design tested was insufficient to separate the effects of the different raw materials and to evaluate whether cobia benefited from nucleotide supplementation.

Lin et al. reported the benefits of supplementing dietary nucleotides on growth and immune responses of grouper (*Epinephelus malabaricus*) juveniles[132]. The first experiment assessed different inclusion levels of a nucleotide mixture containing equal amounts of IMP, AMP, GMP, UMP and CMP. Weight gain and feed efficiency were the highest in the group of grouper fed 0.15% of the nucleotide mixture; with head kidney leucocyte superoxide anion production ratio and plasma total immunoglobulin concentration also being higher than the fish fed the control diet. In the second experiment a diet containing 0.15% nucleotide mixture was compared against a control diet and also diets containing 0.15% of IMP, AMP, GMP, UMP or CMP. From the second experiment it was concluded that juvenile grouper diets containing 0.15% AMP seemed to have better effects than other diets supplemented with different single-nucleotides. Overall conclusion showed that both growth and immune responses of juvenile grouper were enhanced in grouper diet with 0.15% nucleotide mixture.

Due to its economical impact as a high-value species, research on salmonids species and nucleotides has received lots of attention in the last years. Tahmasebi-Kohyani and co-authors conducted a dose response trial on rainbow trout fingerlings (ca. 23g start weight) in order to evaluate their effects on growth, humoral immune responses and resistance against *S.iniae*[133]. A basal diet was supplemented with Optimûn (Chemoforma, August, Switzerland) at 0.05%, 0.1%, 0.15% and 0.2% inclusion levels and fish were fed for eight weeks. Results showed that 0.05% dose was too low and thereafter increased growth, FCR, alternative complement activity, lysozyme activity and IgM as nucleotide dose was augmented, reaching highest values for each of them at the highest nucleotide dose. Similar results were observed at the end of the challenge trial and increased survival was observed with increasing dietary level of nucleotides from 0.05% to 0.2%. This is in agreement with previous work on rainbow trout by Adamek and co-authors that used a wider range of concentrations of the same commercial product, from 0% up to 0.5% and reported approximately 0.2% as the optimal dose (Figure 1)[134].

The efficacy of dietary nucleotides (Optimûn, Chemoforma, August, Switzerland) increasing survival (+39%) against *Piscirickettsia salmonis* was first reported by Burrells and co-authors on 100g coho salmon (*Oncorhynchus kisutch*) after feeding for three weeks before

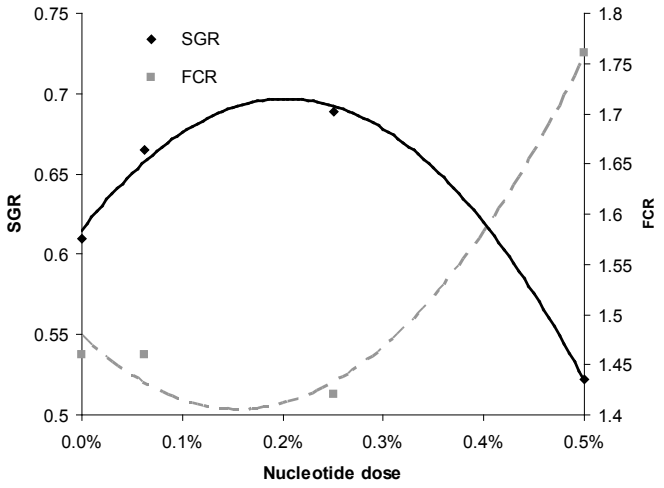

Figure 1: Growth and FCR data of rainbow trout fed diets with increasing doses of nucleotides. Adaption of data reported by Adamek et al. [135].

challenge[5]. The efficacy of dietary nucleotides against *P. salmonis* has also been confirmed in other salmonid species such as Atlantic salmon. González Vecino et al. reported increased protection against *P. salmonis* when Atlantic salmon were fed diets containing a combination of dietary nucleotides with a bacterial cell-wall extract (Sanictum®, Chemoforma, Augst, Switzerland)[135]. Thus, a synergistic effect of the combination of nucleotides and Sanictum® was observed since survival was significantly better than feeding nucleotides or bacterial cell-wall extract separately.

Dietary nucleotides in broodstock diets

Fish reproduction, and egg and larval quality are affected by broodstock nutrition. Nucleotides, the building blocks of nucleic acids, are now considered as semiessential nutrients during periods of food deficiency, stress, rapid growth and immunological stress. González Vecino reported that since oogenesis is a process of intensive cell division with high nucleic acid formation and a concomitant high requirement for nucleotides, supplementation of broodstock diets with nucleotides was beneficial for fish[136]. The results showed that Atlantic halibut (*Hippoglossus hippoglossus* L.) and haddock (*Melanogrammus aeglefinus* L.), two cold water species with different life histories, had improved fecundity, egg quality, larval quality and survival when broodstock diets were supplemented with nucleotides. In addition it was observed that haddock larvae from broodfish fed nucleotides had a significantly better developed gut and first feeding success that those from broodstock fed diets not supplemented with nucleotides. Similar beneficial effects have been observed in salmon broodstock diets supplemented with nucleotides (González Vecino, unpublished data).

Dietary nucleotides against sea lice

Immune modulation of salmon can significantly affect the infection intensity of sea lice both *Caligus* spp. and *Lepeophtheirus salmonis*. Salmon with reduced or compromised immunity have higher infection with sea lice [137-139]. In addition it is known that during the attachment period sea lice release immunosupressive compounds, including prostaglandin E2 (PGE_2) and leukotriene B4 (LTB_4) (cytokines) creating a local immunosuppression at the infestation site allowing the lice to attach unhindered [139-144]. Salmon with enhanced immunity have lower infection with sea lice, thus a range of products such as alginates, ß-glucans and nucleotides have shown to reduce sea lice infections. Burrells et al. [5] reported a 38% reduction in sea lice (*L. salmonis*) infestation on 60g Atlantic salmon fed diets containing nucleotides for three weeks. This was in agreement with reports of 38% reduction on attached stages of *L. salmonis* on 700g Atlantic salmon after feeding nucleotides for 3 weeks [145]. In the same study Burrells et al. demonstrated that the combination of dietary nucleotides with anti-sea lice bath treatments increased the efficacy of the treatment by 53% [145]. Later work on *Caligus rogercresseyi* also reported increased efficacy of deltamethrin (bath treatment) and emamectin benzoate (oral treatment) when applied in combination with diets containing nucleotides Optimûn and bacterial cell-wall extract Sanictum® (Chemoforma, August, Switzerland) [146].

Sea lice are well adapted to develop resistance against medicines. Even within a fully sensitive population a number of sea lice will survive treatment. Wadsworth et al. reported that the use of pyrethroids at the recommended dose showed a survival of 20% of attached stages in a naïve population [147]. Although these lice survived the sub-lethal toxicity of the compound and were able to complete their life cycle, the pyrethroid still had a significant, negative effect upon the rate of development, dramatically increasing the time required to reach pre adult I stage. Increasing the time the lice takes to reach pre adults will expose them to the host immunity during this extended period. In their weakened state, it is unlikely the lice are deploying the full range of immune suppressive compounds as effectively as untreated lice. Therefore, it is recommended that immune modulating compounds such as dietary nucleotides are deployed against lice during treatments with anti-sea lice medicines. It is essential that attached lice surviving treatments are targeted to prevent the development of resistant populations.

Furthermore a sea lice infestation represents a substantial stress to the infected animal. An anti-sea lice bath treatment is in its nature a severe multi stress event including starvation, handling, crowding, hypoxia and exposure to a toxic pharmaceutical all within a short period of time. Even a presumably soft action such as feeding diets containing emamectin benzoate has been shown to trigger stress. Thus, Olsvik and co-authors reported that dietary treatment with Slice® (Schering-Plough Animal Health, Boxmeer, The Netherlands) in salmon triggers the expression of stress coding genes in the fish due to sheer metabolisation and degrading of emamectin benzoate in the liver [148]. Since dietary nucleotides have been shown to reduce levels of cortisol its use could also be suggested with emamectin benzoate treatment.

The use of dietary nucleotides as an immune modulating tool against sea lice should be utilised in an integrated pest management programme with rotation of compounds, resistance monitoring, co-ordinated treatments, cleaner fish as well as effective monitoring and control of other diseases.

Conclusions and Recommendations

Immunostimulants

It is generally accepted that immunostimulants used in fish experiments induce beneficial effects such as disease protection due to increased cellular and humoral responses. However, precautions have to be taken regarding issues such as tolerance, non-wanted side effects such as immunosuppression using too high doses of immunostimulants or non-desirable effects caused by a prolonged use of such compounds.

In future, it is hoped that following the development of genomic and proteomic tools for several fish species, many issues with special attention to immune response polarisation after receptor binding of immunostimulants will be unveiled.

Possible effect of immunostimulants on potential probiotics ability to adhere to intestinal mucus should be given high priority in future studies. New and vital information on this topic is needed as the gastrointestinal tract is a potential port of entry for pathogenic bacteria.

Both prebiotics Bio-MOS® and β-1.3 glucan are two commercially available products, derived from the cell wall of *S.cerevisiae*, in which the use of Bio-MOS® in aquaculture has increased during the last years [149]. However, as less information per se in available on the effect of Bio-MOS® and β-1.3 glucan in aquatic animals [150], this topic should be given high priority in future studies.

Nucleotides

Based on available knowledge and experience within nucleotides, demonstrating that nucleotide supplementation improves the composition of the gut microbiota in formula-fed infants should encourage bacteriologists working with fish to investigate interaction between nucleotides and gut microbiota. Furthermore, we recommend that the following topics should be given high priority in future; (1) in broodstock during oogenesis to improve fecundity, egg and larval quality, (2) during sea water transfer: feeding pre- and post- seawater transfer provides fish with a better osmoregulation and adaptation to marine environment, (3) combination with vaccines: feeding nucleotides during (pre- and post-) the vaccination period modulates the immune response and reduces the negative side effects caused by vaccines (e.g. growth suppression), (4) improved immune response and protection against pathogens such as; *V. anguillarum*, *A. salmonicida,* IPN, ISA, rickettsia-like bacteria, *Flavobacterium*, *Moritella viscosus* and (5) protection against sea lice and improved (higher) efficacy of pyrethroids when used combined with nucleotide feed.

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