Nutrition and immunity: an update

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Abstract

Immunity encompasses all mechanisms and responses used by the organism to defend itself against bacteria, viruses or parasites. Adequate supply and balance of nutrients are required for proper efficiency of the host defences. Research has identified dietary factors that affect human and animal immune responses like amino acids, fatty acids, minerals and vitamins. Some of these nutrients have been proven to have specific actions on immunity when provided at pharmacological doses. This paper will review these nutrients and their current use in aquaculture. The immune system is an efficient but complex system. Its complexity has made the assessment of the effects of diets difficult. Nevertheless, the standardization of methodology as well as the use of new techniques at the cell or the gene level should help to better understand the mechanisms of immune modulation. This paper will review the major functions of fish and shrimp immune system and the methodologies used. Cellular and humoral functions including cytokines will be discussed in relation to potential means to modulate them and the underlying mechanism. A better understanding of the mechanisms of modulation of the immune functions should help in the discovery of new dietary factors to improve the immune status of the animal, leading to better disease resistance.

Keywords: aquaculture, fish, shrimp, nutrition, immunity, modulation

Introduction

The paper intends to provide an update on the relationships between nutrition and immunity of aquatic animals and more specifically of fish and shrimp. Most recent reviews on the topic of nutrition and immunity or, more generally, health are from Lall (2000) and Verlhac and Viswanath (2004).

The first section reviews fish and shrimp immunity, comparing innate and adaptive immune mechanisms, highlighting the importance of innate immunity in those animals. The second section highlights the key findings in the area of nutrition and immunity, analysing the relationship between immune responses and the different classes of nutrients, extending it to nutraceuticals used as feed additives.

The last section will deal with the evaluation of the immune responses of fish and the methods currently used including cellular and molecular approaches, in line with the study of the nutritional modulation of the immune response. Prophylaxis means and perspectives are discussed in the conclusions.

Fish and shrimp immunity

Being immune corresponds to the capacity of an organism to resist infection through the recognition of a foreign agent, the responses of the system to fight this agent and the memory of this agent, in order to quickly respond to a second aggression. In comparison with mammals, fish have a less specific immune system with a shorter response, a limited immunoglobulin repertoire, a weak memory (which reduces the potential for long-term protection) and a mucosal response (whose importance in comparison with the systemic response is not really known). The immune response of fish is differentiated between humoral- and cellular-mediated systems, with the same type of immune cells such as B and T lymphocytes acting in these responses.

Shrimp as invertebrates are not able to develop a specific response. They have the basal mechanisms

of self- versus non-self recognition, phagocytosis and a system of lectins that can be considered to be antibody-like proteins.

The response of fish to a foreign agent is rather similar to that of mammals, while in shrimp, the response is very rudimentary. All fish pathogens contain antigens: viral particles, bacteria, fungi, toxins and animal parasites.

Distinction between innate and specific immunity

Figure 1 presents the different mechanisms involved in the innate and specific (or adaptive) immunity. Fish have an important first line of defence consisting of epithelial barriers such as skin, scales, mucus membranes (gastro-intestinal tract, secretions of mucus) and physiological barriers like stomach pH, gut microflora and chemical mediators secreted by the mucus (defensins, lysozyme, transferrin, complement system, etc.). Involvement of cells like phagocytes, natural cytotoxic cells (NCC) and inflammatory response through the release of chemical mediators represents a second line of defence that is initiated if the pathogen has been able to pass the epithelial and physiological barriers. The actors of the inflamma-

tory response are interferon (IFN), interleukins (ILs), chemokines and factors like tumour-necrosis factor (TNF-α). Pathogen-associated molecular patterns (PAMP) of recognition have also been discovered as an important element of the innate immunity, involving different receptor types.

The third line of defence consists of the development of a specific immune response with either the proliferation of lymphocytes leading to the production of antibodies specific to the antigen or the development of a T-cell-mediated response via cytotoxic T cells (effector cells) in case of viral infection, for example. These responses are generally also mediated by cytokines, which play an important role in cell-to-cell communication for a rapid expansion of the response to the different parts of the body. The ultimate step of this specific response is the development of a memory, allowing the immune system to maintain a B-cell profile corresponding to a specific pathogen. In case of a second infection, these cells will be recognized and will proliferate quickly to fight the infection. This memory mechanism is much less developed in fish compared with mammals and does not exist in shrimp.

In terms of kinetics of the responses, the innate immunity can act between hours and days, while
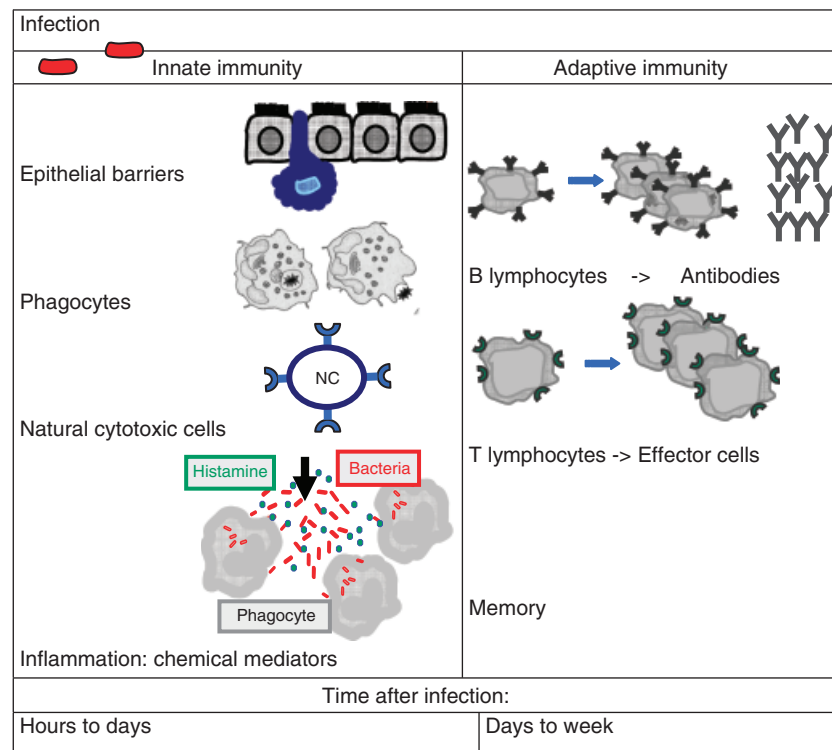

Figure 1 Distinction between innate and specific immunity. Adapted from Abbas, Lichtmann and Pober (1999).

the specific immunity would need weeks to develop depending mainly on the environment (Bowden 2008).

Cellular actors of innate immunity

Phagocytes and NCC are the main cellular elements of the innate immunity. As shown in Fig. 2, phagocytes are able to develop different microbicidal mechanisms to try to definitely eliminate pathogens. Enzymes, reactive oxygen species and nitric oxide (NO) act in concert to provide the best chances to limit the response to the second line of defences described earlier. The production of reactive oxygen species, either internally or externally, constitutes the oxidative burst mechanism. The extracellular response is more pronounced in fish compared with the intracellular one.

Natural cytotoxic cells are involved in innate anti-viral immunity. Natural cytotoxic cells possess receptors that recognize proteins expressed at the surface of virus-infected cells. Through a mechanism of perforin granules released externally, the membrane of the virus-infected cells will be degraded and subsequently subjected to an osmotic shock, leading to their complete lysis and death (Fig. 3).

Humoral factors of innate immunity

Interferon is also involved in anti-viral innate immunity (Fig. 4). Following viral infection, type I IFN is released and recognized by specific receptors on the membrane of non-infected cells. The binding of IFN to membrane receptors initiates the secretion of enzymes like lysozyme, which will then block the replication of the virus and will induce an anti-viral state.

The inflammatory response consists of the release of chemical mediators: histamine, prostaglandins (PG), complement and cytokines (IL1β, 6, 8, 10, 12, TNF-α). Interleukin 1β has been characterized in teleost fish (Randelli, Buonocore & Scapigliati 2008). Interleukin 6 have been identified at the molecular
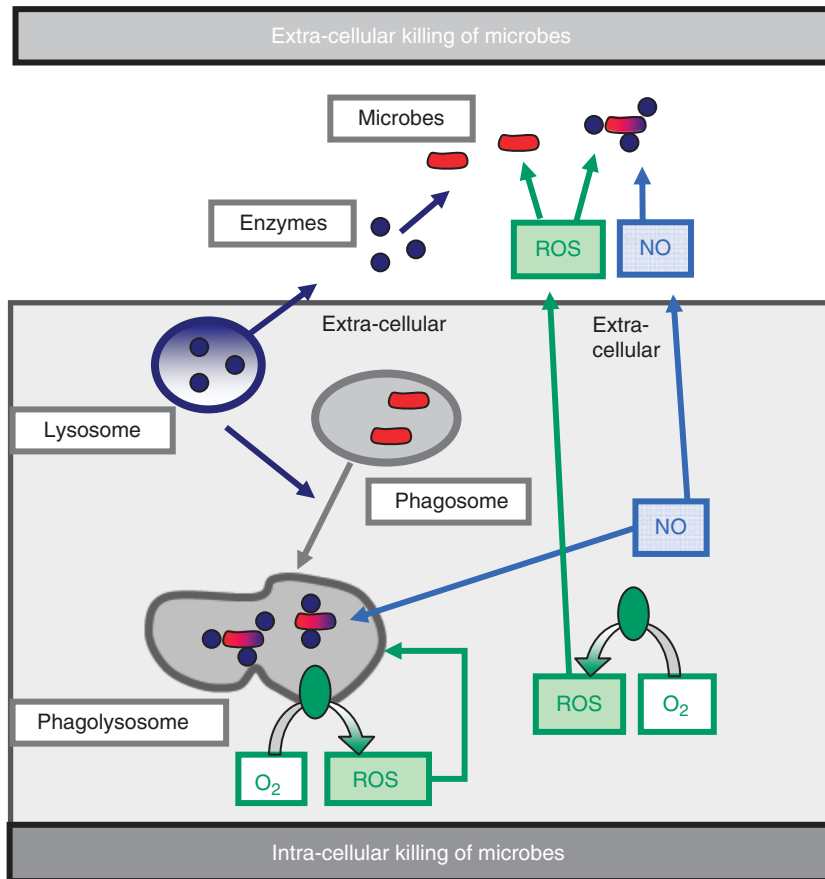

Figure 2 Microbicidal mechanisms of phagocytosis. Adapted from Abbas *et al.* (1999).
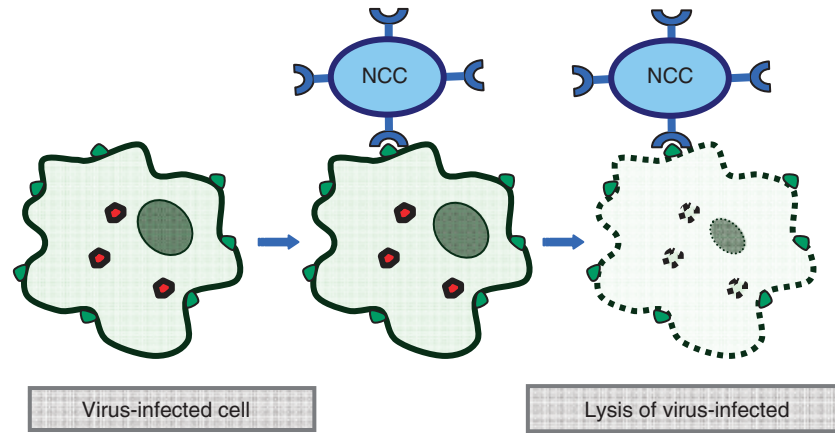
Figure 3 Natural cytotoxicity cells in anti-viral innate immunity. Adapted from Abbas *et al.* (1999).
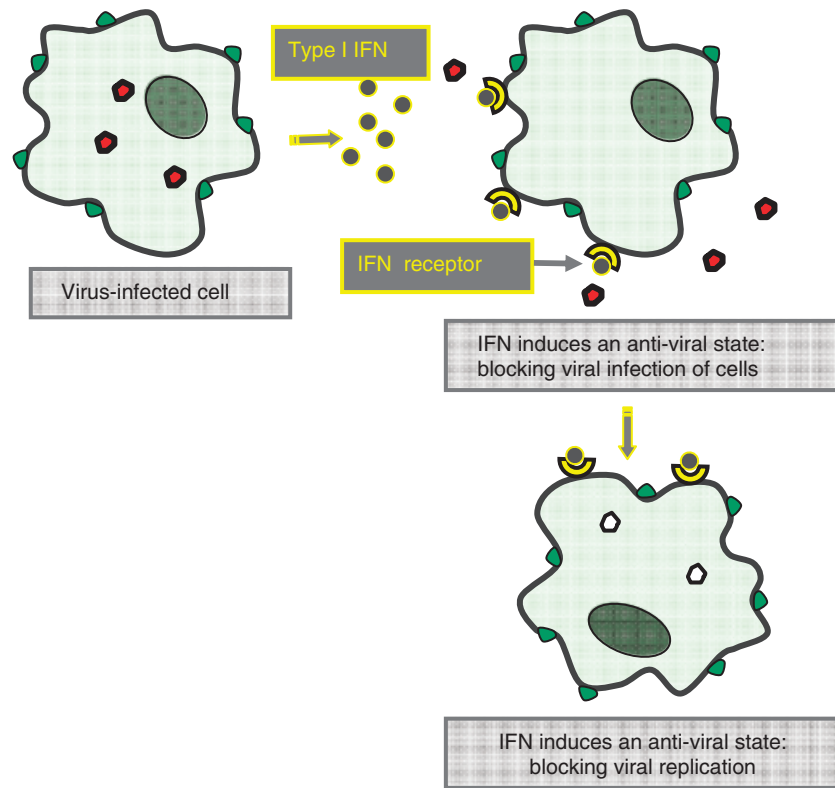

Figure 4 Interferon in anti-viral innate immunity. Adapted from Abbas *et al.* (1999).

level in sea bream by Castellana, Iliev, Sepulcre, MacKenzie, Goetz, Mulero and Planas (2008).

Tumour necrosis factor-α is conserved in all vertebrate classes and has been identified in teleost fish. Roca, Mulero, López-muñoz, Sepulcre, Renshaw, Meseguer and Mulero (2008) have demonstrated that the main proinflammatory effects of fish TNF-α are mediated through the activation of endothelial cells,

leading to the recruitment and activation of phagocytes but they also found implications of TNF-α in different aspects as compared with mammals like increased susceptibility to viral infection and increased virus replication.

All this work on cytokines and chemokines is still, for a great part, carried out at the molecular level and therefore there is a need to demonstrate the biological

activities of these different factors in comparison with mammalian ones.

The complement system plays an essential role in providing an alert signal of the presence of pathogens, in eliminating pathogens by accelerating phagocytosis process (opsonization), and it also plays an important role in the specific immunity through its classical pathway of activation, being involved in the formation of the antigen–antibody complex. The complement system of teleost fish has been reviewed by Boshra and Sunyer (2006).

Receptors of the innate immune system

Activation of the innate immunity is dependent on the recognition of structural motifs expressed only by pathogens; the so-called PAMP that have specificity for structures shared by different classes of pathogens. These motifs are recognized by the pattern recognition receptor, notably the Toll-like receptors (TLR) known in mammals. TLR constitute a family of transmembrane receptors, whose activation is not only linked to innate immunity but is also involved in shaping the adaptive immune response resulting in antigen-specific immunity. Their main role is to provide an alert signal to the organism about the presence of pathogens. Their existence seems to be limited to vertebrates and many different ones are known in mammals (Roach, Glusman, Rowen, Kaur, Purcell, Smith, Hood & Aderem 2005). Some TLR also recognize other structures like elements of heat shock proteins. Toll-like receptors have been described in teleost fish (Magnadottir 2006; Purcell, Smith, Hood, Winton & Roach 2006). Whether they have similar functions in fish as in higher vertebrates is unknown.

Mannose receptors have also been identified as receptors of innate immunity, different microbes being recognized by the same mannose receptor (Underhill & Gantner 2004).

Shrimp immunity: cellular and humoral defences

The primary defense cells in shrimp are called haemocytes. Table 1 presents the cellular and humoral defences that compose the immune response of shrimp. There are different types of haemocytes and some have the ability to phagocyte foreign cells, and others to encapsulate and to inactivate foreign agents. The main functions of these cells are there-

Table 1 Cellular and humoral defences of shrimp

Cell types	Antibacterial peptides
Large granular haemocytes	Penaeidins
Small granular haemocytes	
Agranular haemocytes (hyaline cells)	
Functions	*Plasma proteins*
Phagocytosis, encapsulation,	Lectins
Nodular aggregation	
Oxidative burst	α2 macroglobulin (clotting)
Wound healing (cell clump,	Lipopolysaccharide-
Coagulation initiation)	Binding protein
Synthesis and discharge of lectins	β-glucan-binding protein
Phenoloxidase system	Lysozyme

fore phagocytosis, oxidative burst, coagulation process and then secretion of lectins. There is also a phenoloxidase system (PO), specific to shrimp haemocytes. The PO system is responsible for the menalization process in arthropods and results from the activation of the prophenoloxidase (proPO) system in large granular haemocytes and hyaline cells (Rodriguez & Le Moullac 2000).

Humoral factors include lectins (glycoproteins), which bind with the sugar portion of foreign molecules and have a broad specificity. Viruses and other infectious agents can be recognized by their surface glycoproteins. The mechanism of action of lectins is agglutination and induction of rapid initiation of the phagocytic process.

Along with lectins, shrimp have lysozyme and other plasma proteins such as α-2 macroglobulin responsible for clotting, lipopolysaccharide (LPS)-binding protein and β-glucan-binding protein (BGBP). Specific antibacterial peptides classified as penaeidins have been identified in shrimp (Bachère 2003).

In general, the defence mechanisms of shrimp are more primitive and singular in their ability to control infection in comparison with fish.

Key findings in the area of nutrition and immunity

Disease or stress can be identified as a break in immune competence. One can assume that a nutritional intervention could potentially get the immune system back on track in order to resist and even prevent disease or stress. Both macronutrients and micronutrients influence the resistance of fish and shrimp to pathogens like viruses, bacteria, parasites and fungi.

Proteo-energetic malnutrition is a major cause of immune depression, leading to an alteration in cellular immunity and more specifically depletion in

lymphocytes and alteration of their functions. Elucidating cellular targets and molecular mechanisms affected by dietary restrictions may aid the development of nutrition-based strategies for growth and immune system development.

Major nutrients like proteins (including amino acids and nucleotides), lipids and fish oil substitution, carbohydrates (including starch, β-glucans, carrageenans, peptidoglycan and chitosan), antioxidant vitamins, carotenoids and minerals are known to participate, in different ways, in nutritional modulation of the immune responses. Plant-derived feed additives, although not necessarily nutrients, are also capable of modulating the immune responses of fish and shrimp.

Feeding rate and starvation

The influence of feeding rate and starvation on immune competence has been demonstrated in several studies and highlights the importance of good feed management and the consequences for undernourished animals on their capacity to fight infections.

First of all, does the ration level affect the immunity of fish? Alcorn, Pascho, Murray and Shearer (2003) have studied the relationship in chinook salmon. They found that the salmon immune system can handle huge changes with regard to available metabolic energy, while it seems that cellular functions like phagocytosis, whose response was inversely correlated with the feed ration, may be affected by the ration. The impact of starvation was studied on the immune response of shrimp by Pascual, Sánchez, Zenteno, Cuzon, Gaxiola, Brito, Gelabert, Hidalgo and Rosas (2006). A reduction in all the physiological and immune parameters tested was observed after a 3-week starvation. Before the starvation period, shrimp were fed either a low (5%)-protein or a high (40%)-protein diet. The protein level fed before starvation had a significant effect. The authors concluded that shrimp can withstand a deprivation period without affecting their immunocompetence and that the nutritional status of the animals before starvation has a strong influence.

Protein, amino acids and nucleotides

Animals need to synthesize new proteins to defend themselves. The impact of single nutrients on protein synthesis is therefore an important component of every aspect of immunity.

A series of studies have been performed in shrimp in order to elucidate the influence of dietary protein levels on their immune response (Pascual, Zenteno, Cuzon, Suárez, Sánchez, Gaxiola, Taboada, Suárez, Maldonado & Rosas 2004). Shrimp fed 5% or 15% protein had compromised immune capacities in comparison with the group fed a 40% protein diet. A reduction in haemocyte counts occurred when shrimp were fed sub-optimal dietary protein levels. Haemocyte functions like oxidative burst were also affected by a low dietary protein level.

One could think that the use of vegetable proteins as an alternative protein source in the diet of carnivorous fish could affect immunocompetence, mainly due to the presence of antinutritional factors in these protein sources. Sitjà-bobadilla, Peña-llopis, Gómez-requeni, Médale, Kaushik and Pérez-sánchez (2005) have demonstrated that the replacement of fish meal by graded levels of plant protein mixtures from 50% up to 100% in sea bream induced changes in innate immunity. The results showed that the oxidative burst of phagocytes was enhanced with a 75% replacement level. No changes were observed in humoral factors like lysozyme, while an increase in the alternative pathway of complement activation was observed at 50% and a strong decrease at the 100% replacement level. Barros, Lim and Klesius (2002) had studied the replacement of soybean meal by cottonseed meal in a catfish diet at 27.5% and 55%. The authors observed both an enhanced macrophage chemotaxis and higher agglutination antibodies.

Proteins *per se* are antigenic. Animals and humans cope with this ambiguity by having immune tolerance mechanisms at the level of the intestinal mucosa. This means that not all proteins are considered to be a foreign agent that has to be eliminated. This system works perfectly in higher vertebrates but it is possible that fish, whose gastro-intestinal immune response is not very well known and established, do not have a strong enough immune tolerance to cope with these drastic changes. On the other hand, further processing of some of the vegetable protein sources into protein concentrates will solve part of the problem by eliminating several of the anti-nutritional factors they originally contain.

Amino acids are essential components of the cells and tissues of an efficient immune system. Their role in immunity has been extensively studied in mammals. Several papers deal with the relationship between human nutrition and immunity, especially using glutamine as a dietary supplement for patients in critical conditions. This means that most of the

effects of arginine are shown under abnormal conditions.

The main role of arginine is due to its catabolism into NO, which has an important cytotoxic function in macrophages and mediates inflammation. Fuchs and Bode (2006) have also shown that arginine could act as a stress and apoptotic modulator at the cellular level. The beneficial effect of arginine could be due to the fact that glutamine is an essential amino acid for cells like monocytes/macrophages or lymphocytes. For example, monocytes can consume large quantities of arginine but they are not able to synthesize it. They are therefore entirely dependent on plasma levels.

Glutamine is, in addition to glucose, a major energy substrate of activated immune cells or cells with a high turnover like enterocytes, fibroblasts or tumour cells. It has an important role in maintaining intestinal trophicity and therefore reducing the risk of pathogen translocation. Monocytes consume large quantities of glutamine and they depend on plasma levels. Pérez-bárcena, Regueiro, Mares, Raurich, Rodríguez, Ibáñez, De Lorenzo Mateos and Bengoechea (2008) have studied the influence of glutamine on TLR expression in critical care human patients and found no modulation of the expression by glutamine.

Both arginine and glutamine can modulate cell-mediated immunity. The molecular targets for these amino acids in terms of immune and cell modulation have been reviewed by Roth (2007).

Very few studies have been conducted in fish on these aspects of immune modulation. Nevertheless, the influence of dietary arginine on NO production in activated macrophages from channel catfish has been demonstrated by Buentello and Gatlin (1999). Ganassin, Barlow and Bols (1998) studied the influence of glutamine on phytohaemagglutinin-stimulated mitogenesis of leucocytes from the rainbow trout head kidney. These studies tended to show a comparable role of the amino acids in terms of modulation of the cellular immune response in fish and mammals.

Nucleotides are low-molecular-weight compounds that are important for many biological processes. They are particularly important in new cell production as they are part of the building blocks of nucleic acids. Nucleotides have two biosynthetic pathways, either, *de novo*, from small precursors or through a salvage pathway using products from nucleotide breakdown. Immune cells such as lymphocytes, monocytes/macrophages and granulocytes as well as intestine cells are not able to synthesize them. An exogenous source of nucleotides not only provides nucleotides to these cells but it also saves energy expenses for their synthesis. The rate of nucleotide generation by the organism is most probably not sufficient to meet the requirement at certain life stages and during times of stress and disease. At such times, dietary delivery of nucleotides is probably essential. Holen and Jonsson (2004) have studied the modulation of the growth of intestinal cell lines by dietary nucleotides from different sources such as DNA from fish soft roe, RNA from Baker's yeast and various deoxy-mononucleotides. The results show that although some suppressing effects have been observed in the case of DNA or RNA, all nucleotide sources provide beneficial effects on growth depending on the intestinal cell line investigated. This also tends to show that free nucleotides could be more efficient because they are more readily available.

Li and Gatlin (2006) have reviewed the nucleotide nutrition in fish including the modulation of the innate and adaptive immune responses. From that review, clear benefits on selected immune parameters and on disease resistance were observable. Burrells, Williams and Forno (2001) and Burrells, Williams, Southgate and Wadsworth (2001) first investigated the influence of dietary nucleotides on immune response, vaccination and disease resistance in Atlantic salmon. Li, Lewis and Gatlin (2004) have demonstrated that dietary nucleotides from yeast RNA influence the immune responses and resistance of hybrid striped bass to *Streptococcus iniae* infection.

Low, Wadsworth, Burrells and Secombes (2003) have demonstrated that the expression of immune genes in turbot fed a nucleotide-supplemented diet was enhanced, especially immunoglobulin, transferrin, lysozyme and cytokine genes, among others.

It is also important to mention that nucleotides involved in the nutritional modulation of immunity as exogenous sources are mainly of yeast origin and are different from the synthetic CpG oligodeoxynucleotides (CpG ODN) investigated in some studies like Pedersen, Johansen, Olsen and Jørgensen (2006), who studied the stimulation of type I IFN activity in Atlantic salmon leukocytes by synergistic effects of cationic proteins and CpG ODN. CpG motifs are common on bacteria and oligodeoxynucleotides containing CpG motifs are recognized as PAMP of viruses and bacteria and can modulate both innate and adaptive immune responses. The potential applications of synthetic CpG oligodeoxynucleotides as immune stimulants for fish have been reviewed by Tassakka and Sakai (2005). The mode of action of
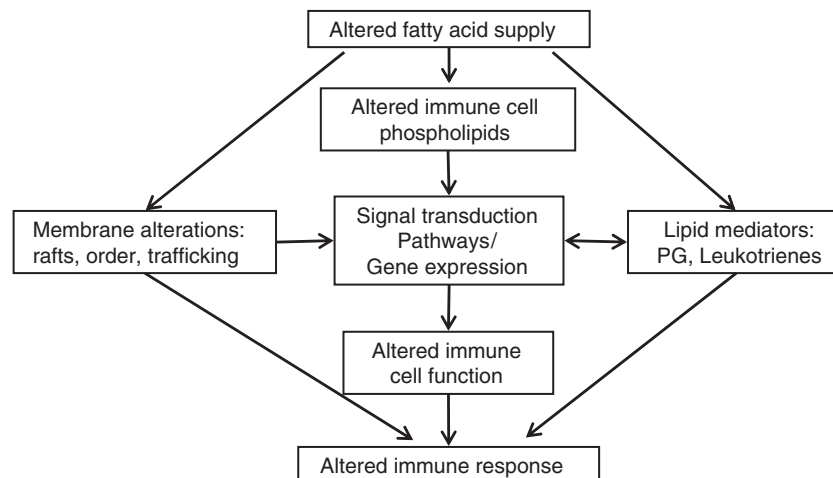
Figure 5 Altered fatty acid supply. Adapted from Calder (2007).

dietary nucleotides is assumed to be, on the one hand, linked to the incorporation of nucleotides into the cells and their subsequent higher proliferation rate. Holen and Jonsson (2004) also suggested that in line with the discovery of the recognition of immunostimulatory CpG motifs by TLR9, one could assume a potential nucleotide signalling through receptors of innate immunity.

Fatty acids

They are unique among the major macronutrients in that they survive digestion intact, remodelling for example the composition of the cell membranes.

The role of lipids in immunity has been reviewed by Watkins, German, Shoenfeld and Gerschwin (2001). Lall, Milley, Higgs and Balfry (2002) have reviewed the dietary lipids and their impact on immune function and pathogenesis of disease in fish. More recently, Calder (2007) discussed in a paper the immune modulation by ω-3 fatty acids. Modifying the composition of the diet leads to altered fatty acid composition of the immune cells, with increased appearance of the fatty acids in which the diet has been enriched. Fatty acids aid energy generation for the cells. The fatty acids that comprise the membrane phospholipids contribute to the physical and functional properties of the membrane. Fatty acids could also act as covalent modifiers of protein structure, influencing the location and the function of proteins. They can also regulate gene expression through effects on receptor, intracellular signal transduction mechanisms or transcription factor activation, with

subsequent modification of gene expression. Fatty acids also play a role as precursors of PG and leukotrienes. The flow chart from Calder (2007) presented in Fig. 5 summarizes the mechanisms by which an altered supply of fatty acids could affect the immune responses described above. In mammals, several studies have demonstrated specific roles of fatty acids in immune responses like the modulation of T-cell signalling by non-esterified fatty acids studied by Brassard, Larbi, Grenier, Frisch, Fortin, Carpentier and Fülöp (2007). Lin, Hsu, Yeh, Yeh, Chang, Lee and Chen (2007), studied the effects of ω-3 fatty acids on leucocyte T-helper (Th) type 1/Th2 cytokine and integrin expression in rats. Székely, Kitajka, Panyi, Márián, Gáspár and Krasznai (2007) have shown that certain fatty acids differently modify the membrane composition, especially kinetics of transport across the membrane.

Chiu, Tsou, Yeh, Hou and Yeh (2008) have shown that the ω-3 fatty acids can affect inflammatory mediators and splenocyte cytokine mRNA expressions in rats. Increased expression of IL2 and 10 mRNA was observed when soybean oil was replaced by fish oil.

It is important to know these mechanisms at the cellular level especially in relation to the trend of replacing oil of fish origin by vegetable oils in the diets for carnivorous fish. Montero, Kalinowski, Obach, Robaina, Tort, Caballero and Izquierdo (2003) have studied the influence of using vegetable lipid sources on the health of gilthead sea bream in 2003. Kiron, Puangkaew, Ishizaka, Satoh and Watanabe (2004) have investigated the antioxidant status and nonspecific immune responses in rainbow trout fed two levels of vitamin E along with three lipid sources

(pollock liver oil, linseed oil and safflower oil). No statistical differences were observed in the immune responses investigated while performance of the fish was affected.

In a more recent study, Montero, Grasso, Izquierdo, Ganga, Real, Tort, Caballero and Acosta (2008) have observed the consequences of total substitution of fish oil by vegetable oils in gilthead sea bream diets with a special focus on the effects on hepatic Mx expression and some immune parameters like phagocytosis and complement activity. Phagocytosis and complement activity were decreased in case of total substitution of fish by vegetable oil (linseed and/or soybean oil). An up-regulation of the expression of the Mx protein involved in anti-viral immunity was observed. The effects were not observed on using a blend of vegetable oils.

In conclusion, it is of major importance to look at the consequences of changing the fatty acid profile on defence mechanisms where phospholipids of the membrane are major actors in cell communications.

The beneficial effects of polyunsaturated fatty acids (PUFA) particularly of the n-3 series have been well documented in fish and other animals. However, PUFA are extremely prone to oxidation. At the cellular level, several nutrients act in concert to prevent oxidative damage. Antioxidant vitamins and carotenoids are particularly important.

Antioxidant vitamins, carotenoids and minerals

Vitamin E plays an important role in protecting lipid from peroxidation. Dietary vitamin E is partly used to protect the cellular lipids (including those of the immune cells) from peroxidation. The vitamin E concentration in the immune cells is also related to its dietary intake. It is also dependent on the lipid level in the diet. Vitamin E is present in the membranes and is important for their fluidity and the receptor-based functioning of the immune response. In fish, most recent studies on the effect of dietary vitamin E have been performed in groups by Lin and Shiau (2005). For 8 weeks, fish were fed graded levels of vitamin E from 0 to 800 ppm at either 4% or 9% lipids. A positive correlation was found with almost all immune responses investigated in relation to increasing dietary dose of vitamin E.

Ascorbic acid plays a major role by protecting the cells from oxidative damage. High concentrations of ascorbic acid were measured in phagocytes and lymphocytes. Ascorbic acid is taken up by the cells and stored in the cytoplasm. A positive correlation between the cellular ascorbic acid concentration and the activity of phagocytes has also been established in rainbow trout in our laboratory (Fig. 6). Mulero, Esteban and Meseguer (1998) have investigated the effects of *in vitro* addition of exogenous vitamin C and vitamin E on gilthead seabream phagocytes. The authors observed a modulation of phagocyte functions as well as ultrastructural changes characteristic of cell activation in the presence of the two vitamins. Sealey and Gatlin (2002) studied *in vitro* manipulations of vitamin C and vitamin E concentrations after intracellular O_2^- production of hybrid striped bass head-kidney cells and demonstrated a vitamin E- and vitamin C-sparing mechanism in the phagocytes of hybrid striped bass.

Härtel, Strunk, Bucsky and Schultz (2004) have observed some influence of vitamin C on intracytoplasmic cytokine production in human whole blood
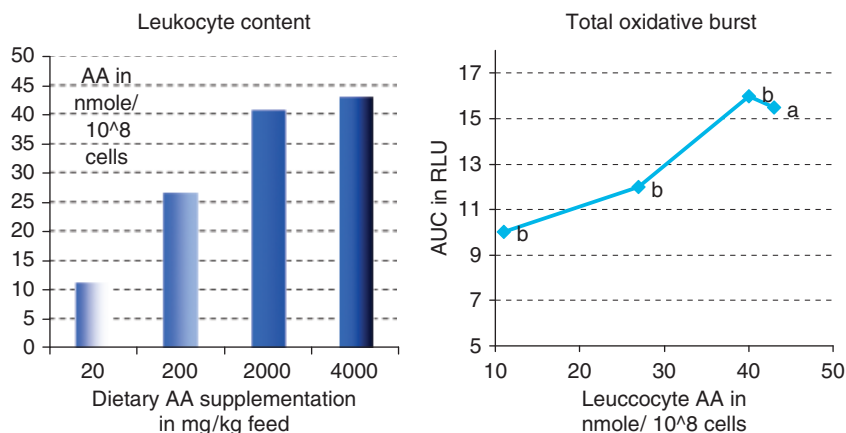

Figure 6 Ascorbic acid level in the phagocyte and extracellular oxidative burst.

monocytes and lymphocytes. The results of this study showed that vitamin C can inhibit, in a dose-dependent manner, the number of monocytes producing IL-6 and TNF-α after induction of inflammation in the presence of LPS.

A number of studies have demonstrated enhanced immune response of fish after dietary supplementation with vitamin C, which is generally regarded as a short-term booster of the immune response. Verlhac and Gabaudan (2005) have reviewed the effect of vitamin C on fish health including the different aspects of the immune response. Sakai (1999) has performed a review of immune stimulants, which includes the work performed on vitamin E.

Carotenoid pigments are potent antioxidants. They also play an important role in intercellular communication, which is vital for the coordination of all biological activities in tissues. Amar, Kiron, Satoh and Watanabe (2004) demonstrated an enhancement in innate immunity in rainbow trout associated with the dietary intake of carotenoids. Supamattaya, Kiriratnikom, Boonyaratpalin and Borowitzka (2005) studied the effect of a *Dunaliella* extract on growth performance, health condition, immune response and disease resistance in black tiger shrimp. A beneficial effect was observed on resistance to viral infection but it was difficult to correlate with an enhancement in the immune response.

Figure 7 shows the localization of the different vitamins at the membrane level. Astaxanthin crosses the membrane and vitamin E is located in the external phospholipid bilayer. Vitamin C is stored in the cytoplasm. Thus, the combination of these vitamins and carotenoids provides the cell with an efficient antioxidant system.

Zinc, selenium and iron are all important for the cell proliferation and functioning of immune cells like lymphocytes, macrophages and NCC. The immune response is modulated both in case of deficiency and supplementation in these minerals. Considerable work has been carried out in mammals showing the importance of zinc in the proliferation of lymphocytes in antioxidant defences (Prasad 2002).

In fish, Hung, Tu and Wang (2007a, b) have studied the *in vitro* and *in vivo* effects of singular or combined anti-oxidative vitamins (A, C and E) and/or minerals (Se, Zn, Cu, Mn and Fe) on the immune functions of tilapia monocytes/macrophages. Negative effects have been shown using very high doses *in vitro* and *in vivo* but these doses could have strongly affected the chemical composition of the medium in the case of *in vitro* studies. In the cases of *in vivo* studies, for some minerals tested, the highest dose tested is extremely high in comparison with the recommended dose for this micronutrient and might not be optimal. Lall (2002) has also addressed the potential role of some minerals in relation to health.

Carbohydrates, oligosaccharides and β-glucans

Dietary carbohydrates play a role in the immune responses through their interactions with the intestinal bacterial flora and the gut-associated lymphoid
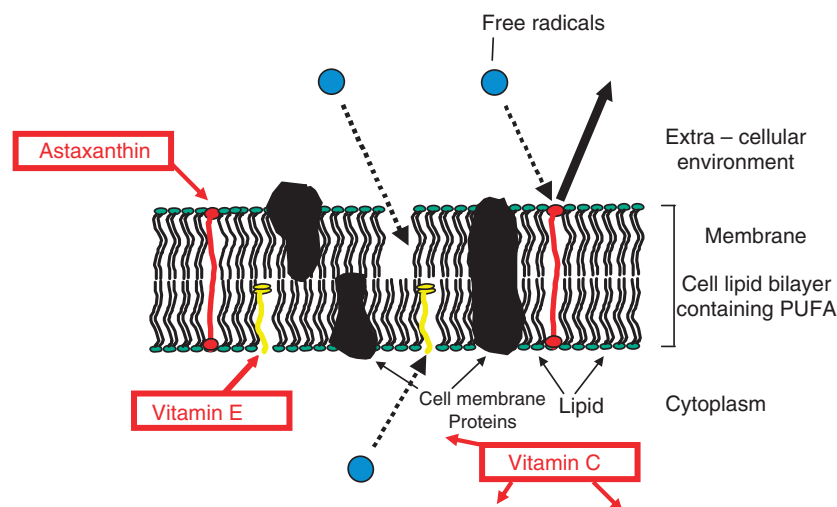

Figure 7 Antioxidant vitamins and carotenoids at the membrane level.

tissue in fish. The insoluble dietary fibres can trap pathogenic bacteria and prevent their access to gut mucosa. Recent attempts in fish include the use of mannanoligosaccharides, inulin and fructooligosaccharides as a prebiotic, in combination with probiotics to achieve long-term health benefits via the gastro-intestinal immune system. Probiotics and prebiotics are not a topic in this paper. The effects of starch, β-glucans, carrageenans, peptidoglycan and chitosan as immune stimulants will be reviewed in this section.

Starch, whose influence on the immune response of *Labeo rohita,* was studied in relation to the gelatinization level by Kumar, Sahu, Pal and Kumar (2007). The authors found reduced immune parameters tested with a high gelatinization level while a ratio of 80:20 of non-gelatinized:gelatinized starch promoted protective immunity. In another study, Kumar, Sahu, Pal, Choudury Yengkokpam and Mukherjee (2005) found that 46% of non-gelatinized carbohydrates in combination with amylase stimulated the immune system of *Labeo rohita,* while supplementation of amylase to the gelatinized carbohydrates had no stimulating effect. The same group also studied the influence of the gelatinization level of starch and PUFA (n-3 PUFA) on immunocompetence and resistance to disease of *Labeo rohita.* Non-gelatinized carbohydrate supplemented with 1.0% n-3 PUFA is found to be optimum to enhance the immunity in *Labeo rohita* juveniles (Misra, Sahu, Pal, Xavier, Kumar & Mukherjee 2006).

On the other hand, no effect was observed on evaluating the influence of carbohydrate stress on immune parameters of the European whitefish by Vielma, Koskela, Ruhonen, Jokinen and Kettunen (2003). There was no difference between low- and high-starch diets but fish could not adapt to high dietary carbohydrate levels.

Immunomodulation by carrageenans was studied in shrimp (Yeh & Chen 2008). The main component of carrageenans is 1,3-α-D-galactose and 1,4-3-anhydro-β-D-galactose, with differences in the galactose subunits depending on the type of carrageenans. Stimulation of the immune response of shrimp was observed with all types of carrageenans while resistance to *Vibrio* was only effective with the λ-type.

Chitosan and not chitin administered via feed was found to enhance the innate immune system and the survival to *Aeromonas hydrophila* infection of common carp (Gopalakannan & Arul 2006).

The effects of dietary supplementation of A3α-peptidoglycan on innate immune responses and defence activity of Japanese flounder were demonstrated by Zhou, Song, Huang and Wang (2006).

β-glucans are polysaccharides extracted from yeast cell walls. Their immunostimulating function is linked to the presence of specific receptors at the surface of macrophages and other phagocytic cells of fish. In shrimp, it is linked to the presence of β-1,3 glucan-binding proteins, which specifically recognize this molecule. The oral delivery of β-1,3/1,6 glucan leads to a significant increase in resistance to diseases in both fish and shrimp. Several studies have been performed on the effect of β-glucans on the immune response of fish and shrimp, showing effects on the different aspects of the immune response, either innate or adaptive. Very recently, Dalmo and Bøgwald (2008) have reviewed the work performed in fish and shrimp. As an example, β-glucan supplemented to the diet had no effect on the antibody response and resistance of Nile tilapia to *Streptococcus iniae* challenge (Whittington, Lim & Klesius 2005). Kumari and Sahoo (2006) have compared several immune stimulants including β-glucan on the non-specific immune response of healthy and immunocompromised Asian catfish. Most of the immune parameters of the non-specific response were enhanced by the different immunostimulants.

Regarding shrimp, the combination of β-1,3/1,6 glucans and probiotics in larviculture modifies the immune response of *Penaeus vannamei* juveniles and the natural survival in ponds and the better resistance to white spot syndrome virus challenge (Rodriguez, Espinosa, Echeverria, Cárdenas Román & Stern 2007).

Table 2 presents the structure of β-glucans of various origins. β-1,3/1,6 glucan from yeast and fungi have been proven to have immuno-modulating properties in fish and shrimp. β-1,3 glucan from bacteria can modulate the immune response of shrimp (Volman, Ramakers & Plat 2008).

In vertebrates, the specific receptor for β-1,3/1,6 glucan expressed by phagocytes is named Dectin-1 (Brown, Herre, Williams, Willment, Marshall & Gordon 2003). It consists of an N-terminal carbohydrate

Table 2 Structure of β-glucans from various origins

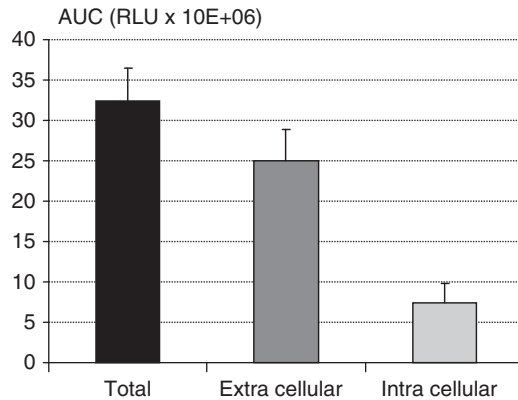| β-glucan origin	Structure	Molecule type
Bacterial	Linear	β-1,3 glucan
Fungi	Short branched	β-1,3/1,6 glucan
Yeast	Long branched	β-1,3/1,6 glucan
Cereal	Linear	β-1,3/1,4 glucan

AUC (RLU x 10E+06)

Figure 8 Activation of rainbow trout phagocyte oxidative burst by β-1,3/1,6 glucan *in vitro*. Results are expressed as the area under the kinetics curve of development of the response in relative luminescence units (RLU × 10^6). Total = response in the presence of luminol − extracellular = response in the presence of isoluminol − intracellular = total − extracellular response.

recognition domain that is external to membrane and an immunoreceptor tyrosine-based activation motif that is positioned through the internal face of the membrane. Two functions have been identified in relation to the binding of β-1,3/1,6 glucan to this receptor: (1) Recognition of β-1,3/1,6 glucan particles by macrophages and subsequent cell activation. (2) Dectin-1 + TLR leads to an induction of a proinflammatory response.

Figure 8 shows the oxidative burst response of rainbow trout phagocytes to activation by β-1,3/1,6 glucan. The extracellular response is more pronounced than the intracellular one, demonstrating the importance of extracellular killing of pathogens in lower vertebrates.

In shrimp, β-1,3/1,6 glucan binds to BGBP and induces degranulation of haemocytes and activation of proPO.

β-glucans can directly activate defensive cellular functions. It is possible that β-glucans contained in the diet would contribute to the prevention of binding of pathogens and therefore blocking might prevent their attachment to the intestinal epithelial membrane.

Plant-derived micronutrients as additives

Algae (spirulina, alginates, seaweed extract), herbs, plant extracts rich in polyphenols, green tea extracts like epigallate–catechin–gallate, fucoidans and flavonoids, essential oils are used as nutraceuticals in hu-

mans and are developing in aquaculture for immune enhancement and improved disease resistance.

Immunostimulant effects of dietary *Spirulina platensis* were demonstrated in common carp (Watanuki, Ota, Tassakka, Kato & Sakai 2006). Dietary sodium alginate administration enhanced the non-specific immune responses and disease resistance of the juvenile grouper (Chiu, Tsai, Hsu, Liu & Cheng 2008). Cheng, Chen and Chen (2008) have shown an enhancement in the innate immune response and resistance to *Vibrio* infection after dietary administration of sodium alginate and κ-carrageenan.

Plant extracts rich in polyphenols are efficient antioxidants to prevent lipid peroxidation in plasma lipids from animals fed n-3 PUFA supplemental diets (Gladine, Morand, Rock, Bauchart & Durand 2007). Ergosan-containing alginic acid, fed to rainbow trout, influences the expression of cytokine genes in the liver of juvenile rainbow trout exposed to enteric redmouth vaccine (Gioacchini, Smith & Carnevali 2008). Water-soluble seaweed extracts modulate the respiratory burst activity of turbot phagocytes (Castro, Zarra & Lamas 2004). Resveratrol modulates innate and inflammatory responses in fish leucocytes (Castro, Lamas, Morais, Sanmartín, Orallo & Leiro 2008). Chinese herbs (*Astragalus membranaceus* and *Lonicera japonica*) enhance the non-specific immune response of Nile tilapia and resistance against *Aeromonas hydrophila* (Ardó, Yin, Xu, Váradi, Szigeti, Jeney & Jeney 2008). Essential oils are purified plant extracts obtained by distillation. Some essential oils like Origanum essential oil, for example, have been proven to reduce mortality in fish farming. Karagouni, Athanassopoulou, Lytra, Komis and Dotsika (2005) have studied the antiparasitic and immunomodulatory effect of *Origanum* essential oil treatments against *Myxobolus* sp. infection in *Diplodus puntazzo* in comparison with other drug treatments (salinomycin + amprolium and fumagillin). While resistance to parasitical infection was significantly improved with all treatments with a much stronger effect of salinomycin + amprolium, the authors were not able to demonstrate the restoration of immune functions with *Origanum* after parasitical infection.

Evaluation of nutritional modulation of the immune response

The immune responses are complex and involve a broad range of molecules, receptors and cells. Assessing immune modulation in relation to nutrition by

analysing the response of one cellular function or one cytokine may induce an overestimation of the potential of the immune stimulant in terms of disease prevention efficacy, which is the ultimate goal of the nutritional modulation of the immune response.

The evaluation of an immune stimulant should first consider the potential target cell or molecule for this immune stimulant as a primary investigation in order to understand the mechanism underlying the modulation of the immune response. This first investigation could be performed *in vitro* and confirmed *in vivo* after dietary supplementation.

In a second step, it is important to measure various aspects of the responses in order to evaluate the consequences of the application of this immune stimulant in terms of development of the innate and adaptive immune responses.

Table 3 presents the cellular and humoral functions that should be investigated when assessing the effect of an immune stimulant: phagocyte, lymphocyte and NCC functions should be evaluated as well as complement, acute-phase proteins, antibody response and cytokines. Depending on the potential target area for the immune stimulant, different parameters could be chosen within these categories.

There is a good correlation between immune modulation and resistance to disease when immunity has been investigated in broad aspects.

Regarding the nutritional aspect, the formulation of the diet as well as the feeding rate and also the zootechnical conditions of the trial (water temperature, water quality, fish age, initial fish health status) could also largely impact the results of the evaluation.

Indeed, it is quite important to ensure that the basal diet, which will be supplemented with the immune stimulant, satisfies the nutrient and micronutrient requirements of the fish in order to better evaluate the effect of the immune stimulant itself.

Table 3 Immune functions investigated

Cellular functions	Humoral functions
Phagocytes	*Complement*
Phagocytosis	Alternative or classical pathway
Oxidative burst	Opsonization
Nitric oxide	*Acute phase proteins*
Bacterial killing	Lysozyme
Lymphocytes	*Antibody production*
Proliferation	
Natural cytotoxic cells	*Cytokines*
Natural cytotoxicity	Interleukins
	Proinflammatory cytokines

Rearing conditions at optimum temperature of the fish species studied should also be preferred unless the influence of temperature is assessed.

Measuring the immune stimulant in the feed and the target fish tissues whenever possible would also ensure a better evaluation of the results of the immune stimulation.

All these aspects could strongly influence the results of the studies.

It is also possible that variability in results could also arise from the fish species investigated. Indeed, some aspects of the immune response are different among fish species but the general responses proposed for investigation are rather similar. Certainly, temperature has a major influence on the development of the immune response as well on the physiology of the animal and on its nutritional requirements. This should be taken into consideration when evaluating an immune stimulant.

In terms of methodology for the investigation of the immune response, flow cytometry nowadays offers a great potential to analyse some of these immune responses. Flow cytometry is mostly used in human medicine for diagnostic purposes using cell markers. In recent years, a number of fluorescent probes and molecules have been developed to serve the purpose of analysing the functionality of immune cells. Phagocytosis, lymphocyte proliferation and natural cytotoxicity are examples of immune functions that can be determined by flow cytometry analysis. Another important advantage of this technique is that the subpopulation of immune cells targeted can be identified in most cases, which yields a more accurate analysis of the response.

The development of 'immuno-genomics' is another important step for a better understanding of nutritional modulation of the immune response. These techniques provide more possibilities for investigation of the immune response, especially regarding humoral factors like cytokines, which have only been rarely characterized in fish, while gene expression could be assessed for most of them.

Considerable work is being performed in fish genomics and also 'immuno-genomics' (Thorgaard, Bailey, Williams, Buhler, Kaattari, Ristow, Hansen, Winton, Bartholomew, Nagler, Walsh, Vijayan, Devlin, Hardy, Overturf, Young, Robison, Rexroad & Palti 2002; Chaves-pozo, Liarte, Fernández-Alacid, Abellán, Meseguer, Mulero & García-Ayala 2008).

However, it is important to demonstrate to which extent the up-regulation of a gene is linked to a modulation of the function. Wang, Chang and Chen

(2008) have performed a study in which they have first demonstrated that dietary β-1,3 glucan up-regulates BGBP. They have demonstrated that the up-regulation is correlated with the appearance of the modulation of phagocytosis and oxidative burst. Indeed, up-regulation started 7 days after feeding but was not maximized at this time. The optimal gene regulation was observed at the same time the immune functions studied were enhanced. This is very important if using 'immuno-genomics' as a tool for assessing immune stimulants.

Prophylaxis and perspectives

Good management practices in intensive production, adequate nutrition and prophylaxis measures are essential for a better control of diseases in aquaculture. The proper functioning of the barriers is vital for the prevention of disease. An early supply of nutrients will preserve immune functions and reduce the secondary risk of infection. The focus should be on effects on the primary elements of the response as it is more and more evident that the competence of innate immunity is still the most essential element in the control of disease. Indeed, natural cytotoxicity and cytokines are early events in the development of an immune response while T-cell-mediated immunity as well as antibody response are later events.

It is necessary to reinforce prevention measures and to develop nutrition-based strategies that support growth and immune system development. A broader view of the nutritional modulation of immunity through the life cycle might help to find new strategies for disease prevention through nutrition. Increasing knowledge of the molecular aspects of nutrition and immunity is also of major importance.

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