



Review Article

Nutrition and health of aquaculture fishA Oliva-Teles^{1,2}

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Abstract

Under intensive culture conditions, fish are subject to increased stress owing to environmental (water quality and hypoxia) and health conditions (parasites and infectious diseases). All these factors have negative impacts on fish well-being and overall performance, with consequent economic losses. Though good management practices contribute to reduce stressor effects, stress susceptibility is always high under crowded conditions. Adequate nutrition is essential to avoid deficiency signs, maintain adequate animal performance and sustain normal health. Further, it is becoming evident that diets overfortified with specific nutrients [amino acids, essential fatty acids (FAs), vitamins or minerals] at levels above requirement may improve health condition and disease resistance. Diet supplements are also being evaluated for their antioxidant potential, as fish are potentially at risk of peroxidative attack because of the large quantities of highly unsaturated FAs in both fish tissues and diets. Functional constituents other than essential nutrients (such as probiotics, prebiotics and immunostimulants) are also currently being considered in fish nutrition aiming to improve fish growth and/or feed efficiency, health status, stress tolerance and resistance to diseases. Such products are becoming more and more important for reducing antibiotic utilization in aquafarms, as these have environmental impacts, may accumulate in animal tissues and increase bacterial resistance. This study reviews knowledge

of the effect of diet nutrients on health, welfare and improvement of disease resistance in fish.

Keywords: diet supplements, disease resistance, fish health, fish nutrition, nutrients.

Introduction

With the continuing growth of the aquaculture industry, more attention to fish welfare must be given as it has significant impacts on stress response, health and resistance to diseases, with consequences on the sustainable development of this industry (Ashley 2007). Diets, among other factors, have strong effects on stress tolerance and health, and therefore, for an adequate growth and resistance to stress and disease problems, fish must be fed adequate quantities of diets that meet all their nutrient requirements (Trichet 2010). Feeding animals with diets that do not meet nutrient requirements not only affects growth and feed efficiency but also increases susceptibility to disease and induces the appearance of deficiency signs, including altered behaviour and pathological changes. Unbalanced diets may also induce negative interactions or antagonism among nutrients that provoke signs similar to deficiency of nutrients. At very high levels of nutrient, which are unusual in practical diets, toxicity signs may occur. Several dietary factors, including essential and non-essential nutrients, have also been shown to have specific actions on the immune response when provided at pharmacological doses (Trichet 2010). Therefore, before considering the potential benefits of diet supplementation with any specific nutrient, it is of paramount importance to ensure that fish are fed adequate amounts of balanced diets that meet all nutrient requirements for the specific physiological

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stage of development of the species under consideration.

Though still limited, information is accumulating regarding nutrient requirement of most important aquaculture species (N.R.C. 1993; Halver 2002; Webster & Lim 2002). Basic nutritional data are available to reassure that minimum requirements are met in diet formulation for the majority of exploited species. Data on nutrient bioavailability are, however, more sparse and limited to a few species. Digestion of nutrients in different feedstuffs, metabolic utilization or interactions among the nutrients may differ between species and are related to natural feeding habits of species. For instance, carnivorous and herbivorous fish differ in their capacity to use complex carbohydrates or plant feedstuffs. Diets or feedstuff processing technologies also affect nutrient availability. For example, extrusion applies high temperature and pressure to the feed mixture and has beneficial effects in improving water stability of the pellets, diet pasteurization, starch gelatinization or inactivation of antinutrients, but it may also negatively affect the availability of amino acids such as lysine or increase vitamin losses. Therefore, nutrient deficiencies may still occur in diet formulations owing to insufficient information on bioavailability of nutrients in different feedstuffs and to the diet processing technologies (Hardy 2001). This may induce the appearance of chronic, subclinical deficiencies that negatively affect fish performance and weakens the animals, making them more susceptible to disease problems.

Protein and amino acids

Fish, as all monogastric animals, do not have specific protein requirements, but require the amino acids (AA) that compose proteins (Wilson 2002). Referring to protein requirements is nevertheless usual in fish nutrition as protein includes both indispensable amino acids (IAA) and dispensable amino acids (DAA) that provide the undifferentiated N required for the synthesis of nitrogenous compounds of physiological interest. Protein requirement is not an absolute value but depends on the bioavailability of the protein source, its AA profile and the dietary energy level. Lower protein requirement is achieved with highly digestible protein sources, with well-balanced IAA profiles and adequate digestible protein to energy (DP/DE) levels.

Given balanced diets fish eat to meet their energy requirements (Bureau, Kaushik & Cho 2002). Therefore, with high DP/DE diets, fish will eat more protein than required for growth, and the excess protein will be diverted for energy purposes. This will have negative economic and environmental impacts but except in extreme cases, which may prove to be toxic, will not affect the animal's performance or health status. On the contrary, with low DP/DE diets, fish will stop eating before ingesting an adequate amount of protein, thus compromising growth rate and eventually debilitating the animals. As there is now a trend for increasing dietary energy content for reducing feed intake per unit of growth and decrease feed losses, a reappraisal of dietary nutrient requirements may be required to reassure that adequate amounts of essential nutrients are included in the diets (Hardy 2001; Wilson 2002).

Fish have absolute requirements for 10 AA, which are considered indispensable (N.R.C. 1993; Wilson 2002). Besides these, two other AAs are considered semi-indispensable, cystine and tyrosine, as they may only be synthesized from their precursor IAA, respectively, methionine and phenylalanine. However, inclusion of these semi-indispensable AA in the diets spares part of their precursor IAA. When given IAA-deficient diets, fish display reduced growth and anorexia; gross anatomical signs of IAA deficiency have also been reported under experimental conditions for a few AA (Tacon 1992; Roberts 2002).

On diet formulation, care must be taken to assure that species requirements for the 10 IAA are met and that IAA profile is optimized, IAA-to-DAA ratio is adequate, and that imbalances and antagonism among IAA are not occurring. Antagonism owing to disproportionate levels of specific AA, including leucine/isoleucine, arginine/lysine and methionine/cystine, may arise in farm animals and were also reported in fish for branched-chain AA (Hughes, Rumsey & Nesheim 1984; Robinson, Poe & Wilson 1984), but not for arginine/lysine (Robinson, Wilson & Poe 1981; Robinson *et al.* 1984). Toxic effects of a dietary excess of IAA are not expected to occur in practical diets, but have been reported in fish fed experimental diets with high leucine levels (Choo, Smith, Cho & Ferguson 1991). Care must also be taken in adjusting AA requirements using free AA as fish do not always perform as well with diets including free AA as with practical diets including only whole proteins (Peres & Oliva-Teles 2005).

The sum of estimated IAA requirements of a given fish species usually represents circa 30% of total protein requirements (Cowey 1995), which is not very different from values estimated for terrestrial farm animals. However, fish diets must not have IAA/DAA ratios of 30:70 as this negatively affects growth performance. For adequate performance, IAA/DAA ratio in fish diets must be kept within 50-60/50-40 as either lower or higher ratios negatively affect performance (Cowey 1995; Peres & Oliva-Teles 2006). Although practical diets including whole-protein sources are expected to have IAA/DAA ratios of 50:50, deviation from this ratio may occur in experimental or practical diets including high levels of crystalline AA. In experimental crystalline-AA-based diets, it is also important to consider the DAA mixture used as it may also affect fish performance (Mambrini & Kaushik 1994; Schuhmacher, Munch & Gropp 1995).

Fish meal is still the main protein source in aquafeeds, particularly in feeds for carnivorous fish (Gatlin, Barrows, Brown, Dabrowski, Gaylord, Hardy, Herman, Hu, Krogdahl, Nelson, Overturf, Rust, Sealey, Skonberg, Souza, Stone, Wilson & Wurtele 2007; Tacon & Metian 2008), as it has high protein content, adequate amino acid profile and high palatability; it is also well digested and lacks antinutrients (Gatlin *et al.* 2007). Fish meal is also a source of high-quality lipids, namely essential highly unsaturated fatty acids (HUFA) and of minerals such as phosphorus. However, the limited availability of this commodity in the world market urgently requires that fish meal use in aquafeeds is substantially reduced (Watanabe 2002). However, as fish have high dietary protein requirements, the potential alternative protein sources are restricted to just a few ingredients (Hardy 2008) which mainly fall in three categories: animal rendered by-products, plant feedstuffs (mainly concentrates) and single-cell organisms. Alternative protein sources have several characteristics that make them inferior to fish meal (Hardy 2006; Lim, Webster & Lee 2008b) such as inadequate amino acid profiles, lower digestibility, lower palatability and presence of antinutrients (Gatlin *et al.* 2007). Indeed, if alternative protein sources had a nutritional and economic value similar or even better than fish meal, their use in aquafeeds would be more widespread (Hardy 2006). Besides the problems related to nutritional composition, plant feedstuffs also have several endogenous antinutritional factors that limit their use in aquafeeds (Tacon 1997;

Francis, Makkar & Becker 2001; Gatlin *et al.* 2007). Further, adventitious toxic factors arising from processing or contaminants (biological contaminants and pesticides) within feedstuffs may also raise problems in plant feedstuff use (Tacon 1992; Hendricks 2002).

Fish meals and animal by-products are rich sources of taurine (Gaylord, Teague & Barrows 2006), an amino acid that although not being incorporated in proteins has important physiological roles. Taurine can be synthesized from cysteine, but the rate of synthesis may be inadequate to fulfil the requirements in animals fed diets without animal proteins (Gaylord, Barrows, Teague, Johansen, Overturf & Shepherd 2007). In such cases, a pathological condition called green liver symptom may develop (Sakaguchi & Hamaguchi 1979; Watanabe, Aoki, Shimamoto, Hadzuma, Maita, Yamagata, Kiron & Satoh 1998; Goto, Takagi, Ichiki, Sakai, Endo, Yoshida, Ukawa & Murata 2001; Takagi, Murata, Goto, Endo, Yamashita & Ukawa 2008; Takagi, Murata, Goto, Hatate, Endo, Yamashita, Miyatake & Ukawa 2010). Therefore, depending on species or physiological status, supplementation of animal protein-free diets with taurine may improve fish performance (Takagi, Murata, Goto, Ichiki, Endo, Hatate, Yoshida, Sakai, Yamashita & Ukawa 2006; Chatzifotis, Polemitou, Divanach & Antonopouliou 2008; Matsunari, Furuuta, Yamamoto, Kim, Sakakura & Takeuchi 2008).

Partial replacement of fish meal by alternative protein sources has been achieved successfully at different replacement levels in several species. However, fish-meal-free diets or almost fish-meal-free diets that promote similar performance to diets including fish meal are more rarely achieved, particularly in carnivorous species (Takagi, Hosokawa, Shimeno & Ukawa 2000; Lee, Dabrowski, Blom, Bai & Stromberg 2002; Kaushik, Coves, Dutto & Blanc 2004; Kissil & Lupatsch 2004). The effect of partial or total replacement of fish meal by mixtures of plant protein sources on non-specific defence mechanisms has been very rarely assayed in fish. In gilthead sea bream, for instance, Sitja-Bobadilla, Pena-Llopis, Gomez-Requeni, Medale, Kaushik & Perez-Sanchez (2005) observed that in fish fed a 100% plant protein diet, there were alterations in the gut histology, namely increased lipid vacuoles and/or deposition of protein droplets in the enterocytes and hypertrophic intestinal submucosa, which was infiltrated with eosinophilic

granular cells. Plasma lysozyme levels were not affected by fish meal replacement level but respiratory burst of head kidney leucocytes was significantly increased in the 75% plant protein diet. On the other hand, complement significantly increased in the 50% plant protein diet but decreased in the 75% and 100% plant protein diets. Though the interpretation of the results is complex, overall, they indicate that replacement of fish meal by plant protein decreased one of the immune defence mechanisms at above the 75% level.

Effect of protein and amino acids on health condition

Protein and AA deficiencies have long been recognized to impair immune function and increase the susceptibility of animals to infectious diseases, as protein malnutrition reduces the concentration of most plasma AA, and these have an important role in the immune response (Li, Gatlin & Neill 2007b; Li, Yin, Li, Kim & Wu 2007a). However, available data on the effects of protein and AA in health and disease resistance are relatively scarce in fish.

In adult rainbow trout, *Oncorhynchus mykiss* (Walbaum), dietary protein level did not affect antibody production against *Aeromonas salmonicida* in a challenge test, although the survival of fry of the same species challenged against infectious haematopoietic necrosis virus was related to dietary protein level (Kiron, Fukuda, Takeuchi & Watanabe 1993). Also, in Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum) (Hardy, Halver, Brannon & Tiews 1979), and in channel catfish, *Ictalurus punctatus* (Rafinesque) (Lim, Yildirim-Aksoy & Klesius 2008a), serum antibody in vaccinated fish was not affected by dietary protein level. Kiron, Watanabe, Fukuda, Okamoto & Takeuchi (1995b) further observed in rainbow trout that, although antibody production was not affected by dietary protein level in protein-deficient fish (10% protein), lysozyme activity and C-reactive proteins were reduced, thus negatively affecting non-specific defence mechanisms. It was thus concluded that adequate protein level is required to maintain non-specific defence mechanisms while the humoral-specific immune system seems to be independent of dietary protein level.

Nitric oxide (NO) produced by fish macrophages plays an important role in macrophage killing of microorganisms (Buentello & Gatlin 1999). As the sole precursor of NO is arginine, these authors

investigated the effect of increasing dietary levels of arginine in the induction of NO synthesis in channel catfish macrophages. Although *in vitro* dietary arginine did not correlate significantly with the amount of NO produced, it was suggested that *in vivo* plasma arginine may contribute to prolong the macrophage production of NO by regulating the intracellular availability of arginine and in this way playing a major role in the ability of macrophages to produce NO. This was indeed confirmed later by the same authors in the same species (Buentello & Gatlin 2001) in a challenge test against *Edwardsiella ictaluri*. In that study, maximum survival was observed in fish fed diets with increased levels of arginine.

Lipids and essential fatty acids

Lipids are the main conventional energy sources in fish diets as carbohydrate utilization is not very efficient, particularly in carnivorous species. Within limits, increasing dietary lipid level spares protein utilization for plastic purposes (Sargent, Tocher & Bell 2002). Although there is now a trend for using high-energy nutrient dense diets in fish aquaculture, there are great differences among species in their ability to use high dietary lipid levels. Therefore, there are limits on the maximum lipid levels that can be incorporated in the diets without affecting fish growth performance or body composition. For instance, while Atlantic salmon, *Salmo salar* L., performed better with diets including 38% or 47% lipids than 31% lipids (Hemre & Sandnes 1999) in European sea bass, *Dicentrarchus labrax* L., no growth differences were observed with diets including 12–30% lipids (Peres & Oliva-Teles 1999), although at the highest lipid level, protein and energy utilization efficiency were reduced compared to the other diets.

Dietary lipids are also a source of essential fatty acids (EFA). Fish, as other vertebrates, have dietary requirements of n-3 and n-6 polyunsaturated fatty acids (PUFA) but specific EFA requirements are different in marine and freshwater species (Sargent *et al.* 2002). Two signs of EFA deficiency in fish are poor growth and feed efficiency (Sargent, Henderson & Tocher 1989); besides, these ubiquitous signs other signs occur that are more species specific (Sargent *et al.* 1989; Tacon 1992). Rather than being fixed values, EFA requirements are related to dietary lipid level and increase with dietary lipid level (Takeuchi, Shiina & Watanabe 1991, 1992a;

Takeuchi, Shiina, Watanabe, Sekiya & Imaizumi 1992b). EFA requirement may also differ with stage of development and with EFA source, with HUFA usually having a higher EFA value than PUFA (Watanabe 1982; Izquierdo 2005). Accurate definition of EFA requirement of a given species also involves establishing the optimal balance between n-3 and n-6 series (Sargent *et al.* 2002). Excess EFA may also be a problem, as dietary inclusion levels exceeding that of requirements by several times depress growth (Yu & Sinnhuber 1976; Takeuchi & Watanabe 1979).

The biological active forms of EFA are C20 and C22 fatty acids (FAs) derived from the C18 PUFA, 18:2n-3 and 18:3n-3 (Sargent *et al.* 2002). Freshwater fish can convert C18 PUFA to C20 or C22 HUFA by a series of chain elongation and desaturation reactions; thus, their EFA requirements are met by PUFA (18:3n-3 and 18:2n-6). On the other hand, marine fish cannot perform such conversion as they lack or have reduced expression of delta-5 desaturase enzyme (Mourente & Tocher 1993) or have limited capability of C18 to C20 elongation (Ghioni, Tocher, Bell, Dick & Sargent 1999). Therefore, marine fish have a specific requirement for n-3 HUFA (20:5n-3 and/or 22:6n-3). EFA are precursors of eicosanoids, a group of highly biologically active compounds that comprise prostaglandins, prostacyclins and thromboxanes, which are hormone-like compounds produced by the cells and that have a wide range of physiological functions, including immune and inflammatory responses (Sargent *et al.* 2002; Wall, Ross, Fitzgerald & Stanton 2010). Eicosanoid production is associated with stressful situations, with excess production occurring under pathological conditions. Arachidonic acid (AA, 20:4n-6) is the major precursor of highly active eicosanoids in mammals while EPA (20:5n-3) competitively interferes with eicosanoid production from AA and produces much less active eicosanoids (Bell & Sargent 2003; Wall *et al.* 2010). Thus, dietary intake of n-3 and n-6 PUFA affects eicosanoid production and activity with effects on health status, as high n-6 derived eicosanoids are associated with cardiovascular and inflammatory problems (Sargent *et al.* 2002; Wall *et al.* 2010). AA-derived prostaglandins (PGE2) are associated with the modulation of immune function, and although a low concentration of PGE2 is required for normal immune function, high concentrations are immunosuppressive (Bell & Sargent 2003). Diet FA composition influences immune

response by determining which eicosanoid precursors are present in the cell membranes, with n-6 PUFA-rich diets enhancing immune response and n-3 PUFA-rich diets being immunosuppressive. However, the type of eicosanoids produced and the ultimate impact on the immune response are very complex (Balfry & Higgs 2001), depending on factors such as competition for FA metabolism, cell types involved and form and source of dietary FA.

Besides EFA, inclusion of phospholipids (PL) in the diets for larvae and small fry of various fish species may improve growth performance, survival and stress resistance (Tocher, Bendiksen, Campbell & Bell 2008). This apparent PL requirement in the early stages of ontogeny is possibly due to limited capacity of PL synthesis in these fast growing stages, as no PL requirement has been demonstrated in fish bigger than 5 g.

Fish oil is the main lipid source in aquafeeds for most species, as it is an excellent source of n-3 EFA and does not affect lipid composition and organoleptic characteristics of the fish carcass. Fish oil is the only commercial source of HUFA, which are required for marine fish (Sargent *et al.* 2002). However, it is estimated that in 2006, the aquaculture sector already used 88.5% of total fish oil production (Tacon & Metian 2008). Thus, at the expected rates of aquaculture increase, actual levels of fish oil incorporation in aquafeeds will not be economically sustainable and fish oil will need to be partially replaced by vegetable oils (Turchini, Torstensen & Ng 2009). However, while fish oil is a very rich source of HUFA (DEA and EPA), vegetable oils do not contain these FA. Among vegetable oils, only linseed oil is a rich source of n-3 PUFA (linolenic acid) (Turchini *et al.* 2009). Vegetable oils may also contain minor amounts of phytosterols, which are known for their cholesterol lowering properties, thus having a potential effect on health. On the other hand, fish oil is also a good source of vitamins A and E, but may be contaminated with dioxins. Indeed, fish oil is considered the main source of persistent organic pollutants in farmed fish (Jacobs, Covaci & Schepens 2002; Turchini *et al.* 2009).

Replacing fish oil by vegetable oils in fish diets has effects on dietary FA composition and ratio of n-3/n-6 HUFA, and this may affect fish health status and resistance to diseases. Analysis of health effects is complex as it is related to numerous factors, including the species EFA requirements and the balance between dietary n-3 and n-6 FA. For

instance, Atlantic salmon fed on diets with high sunflower oil (rich in n-6 PUFA) may present cardiovascular disorders which are attributed to the low n-3/n-6 FA ratio (Bell, McVicar, Park & Sargent 1991; Bell, Dick, McVicar, Sargent & Thompson 1993). Though no apparent differences were noticed in the non-specific immune parameters measured, resistance of Atlantic salmon to bacterial challenge was higher when fed fish oil (high n-3/n-6 ratio) than vegetable oil (low n-3/n-6 ratio)-based diets (Thompson, Tatner & Henderson 1996), suggesting that fish fed diets with low n-3/n-6 PUFA may be less resistant to infection. Similar results were also observed in channel catfish by Sheldon & Blazer (1991). In rainbow trout, a fish oil diet was more chemoattractive as head kidney supernatants promoted a higher *in vitro* locomotion of neutrophils than supernatants obtained from a sunflower oil diet (Ashton, Clements, Barrow, Secombes & Rowley 1994). In contrast, Waagbo, Sandnes, Lie & Nilsen (1993b) observed lower antibody levels in Atlantic salmon fed fish oil (high n-3 HUFA) than soybean oil (high linolenic acid, n-6). Waagbo, Sandnes, Joergensen, Engstad, Glette & Lie (1993c) further analysed the effect of dietary oil source on the non-specific immune response of Atlantic salmon and concluded that it was complexly related to diet FA composition and water temperature. Such an effect of water temperature and FA source was also observed in catfish by Lingenfeller, Blazer & Gay (1995) but not by Sheldon & Blazer (1991). In channel catfish, Fracalossi & Lovell (1994) and Li, Wise, Johnson & Robinson (1994) further observed reduced disease resistance in fish fed fish oil (rich in n-3 PUFA) rather than corn oil, offal oil or beef tallow, particularly at high temperature. Fracalossi & Lovell (1994) attributed these results to possible competitive inhibition of arachidonic acid metabolism by n-3 FA. Overall, the results suggest that channel catfish is more susceptible to infection by bacteria when fed fish oil and that mixtures of fish and animal oils or just animal oils in the diets are advisable to provide a more adequate n-3/n-6 balance. In gilthead sea bream, replacing 60% fish oil by either soybean oil, rapeseed oil or linseed oil affected fish health in terms of immunosuppression or stress resistance, while a blend of vegetable oils instead of individual oils did not affect fish health (Montero, Kalinowski, Obach, Robaina, Tort, Caballero & Izquierdo 2003). Similarly, in European sea bass, the number of circulating leucocytes

and macrophage respiratory burst was also negatively affected by replacing fish oil by individual vegetable oils (Mourete, Dick, Bell & Tocher 2005) but replacing it by blends of vegetable oils did not compromise non-specific immune function (Mourete, Diaz Salvago, Tocher & Bell 2000), owing to a more correct n-3/n-6 FA ratio.

EFA in health condition

Reports on the effect of EFA on immune response are still conflicting (Lall 2000; Balfry & Higgs 2001). Groupers, *Epinephelus malabaricus* (Bloch & Schneider), fed 12% or 16% lipids (fish oil/corn oil, 1:1) showed higher plasma lysozyme and alternative complement activities than fish fed 4% and 8% lipid diets, respectively (Lin & Shiau 2003). Also, fish fed diets including lipids showed higher white blood cell count and leucocyte respiratory burst than fish fed a lipid-free diet. This enhancement of immune response in lipid-supplemented diets was mainly because of the EFA. Indeed, in rainbow trout, it was shown that EFA enhances immunocompetence while EFA deficiency compromises *in vitro* killing of bacteria by macrophages and antibody production (Kiron, Fukuda, Takeuchi & Watanabe 1995a). EFA deficiency also decreases complement activity, haemolytic and agglutination activity in gilthead sea bream, *Sparus aurata* L. (Tort, Gómez, Montero & Sunyer 1996; Montero, Tort, Izquierdo, Robaina & Vergara 1998). In juvenile Japanese seabass, *Lateolabrax japonicus* (Cuvier), serum lysozyme, alternative complement pathway and superoxide dismutase activity were enhanced by the supplementation of diets with ARA up to moderate levels, but no further improvements were observed at higher levels (Xu, Ai, Mai, Xu, Wang, Ma, Zhang, Wang & Liufu 2010). On the other hand, excessive EFA levels can also inhibit the immune response. For example, in Atlantic salmon, excess EFA reduced survival and antibody levels after challenge with *Yersinia ruckeri* (Erdal, Evensen, Kaurstad, Lillehaug, Solbakken & Thorud 1991) whilst in channel catfish high n-3HUFA diets decreased survival, phagocytic capacity and killing activity after bacterial challenge (Fracalossi & Lovell 1994; Li *et al.* 1994). In Atlantic salmon, it was shown that diets with low n-3/n-6 ratios may cause changes in FA metabolism that are deleterious to the animal health, owing to severe heart lesions (Bell *et al.* 1991).

Carbohydrates

Natural fish food usually does not include high dietary carbohydrate levels, particularly in carnivorous species. Fish do not have specific dietary carbohydrate requirements and use diets with no carbohydrates as efficiently as those including carbohydrates (Hemre, Lambertsen & Lie 1991; Peres & Oliva-Teles 2002; Sa, Pousao-Ferreira & Oliva-Teles 2007; Enes, Panserat, Kaushik & Oliva-Teles 2009). Carbohydrate utilization in fish is species related, with carnivorous species tolerating lower levels of dietary carbohydrates than omnivorous or herbivorous species. It is also related to carbohydrate source, molecular complexity of the molecule, processing treatments and dietary inclusion level (Wilson 1994; Stone 2003; Krogdahl, Hemre & Mommsen 2005; Enes *et al.* 2009).

Dietary carbohydrate may affect fish disease and stress tolerance. For example, in Atlantic salmon, varying dietary carbohydrate level affected immunity and resistance to bacterial infections to a minor extent (Waagbo, Glette, Sandnes & Hemre 1994). Fish fed moist diets with increasing digestible dietary carbohydrate (wheat starch) ranging from 0 to 30% had decreased blood haemoglobin concentration, serum cortisol and serum haemolytic activity, while humoral immune response after vaccination with *Vibrio salmonicida* was not affected by diet, although mortality after challenge with *A. salmonicida* was lowest in fish fed 10% carbohydrates (Waagbo *et al.* 1994). On the other hand, long-term feeding a high carbohydrate diet in rainbow trout had no substantial effect on non-specific immunity measured as pronephros lysozyme activity and macrophage superoxide production (Page, Hayworth, Wade, Harris & Bureau 1999). In cod, *Gadus morhua* L. plasma glucose response after handling stress was significantly more affected in fish fed a carbohydrate diet than a carbohydrate-free diet (Hemre *et al.* 1991); the authors thus suggested that a change of diet in advance of handling and transportation could reduce losses as a result of stress. However, in European whitefish, *Coregonus lavaretus* L., plasma glucose did not differ significantly between fish fed a carbohydrate-free and a 33% corn starch diet after rapid water cooling-induced stress (Vielma, Koskela, Ruohonen, Jokinen & Kettunen 2003). In the same study, it was shown during a 10-week feeding period that liver glycogen and plasma glucose increased while plasma IgM decreased with increasing dietary

carbohydrate levels. Starch gelatinization ratio also affected immune response of rohu (Kumar, Sahu, Pal, Choudhury, Yengkokpam & Mukherjee 2005; Kumar, Sahu, Pal & Kumar 2007).

Low digestibility diets may provide selective media for the growth of different bacterial species, thus inducing changes in bacterial metabolism and virulence mechanisms (Lim *et al.* 2008a). Dietary fibre is a physiologically inert material with bulk and laxative properties (Shiau 1989) and may affect gut microbiota. Dietary fibre can trap pathogenic bacteria and prevent their access to gut mucosa (Trichet 2010). Feeding high fibre diets to rainbow trout increased feed consumption, gastric evacuation time and decreased dry matter ADC (Hilton, Atkinson & Slinger 1982) but did not affect haemoglobin, haematocrit, plasma glucose or plasma protein levels.

Chitin, a polymer of glucosamine, is a major component of crustacean exoskeleton (Nakagawa 2007), an important food for fish, particularly during larval stages. Dietary chitin stimulates the innate immune response in gilthead sea bream (Esteban, Cuesta, Ortuno & Meseguer 2001) by increasing complement activity, cytotoxic activity, respiratory burst and phagocyte activity, but not lysozyme activity. Chitin in fish diets interferes with bacteriolytic activity of lysozyme in trout stomach (Lindsay 1984). Thus, chitin may be of interest as an immunostimulant (Esteban *et al.* 2001).

Vitamins

Vitamins are organic compounds required in trace amounts from an exogenous source for normal growth, reproduction and health (N.R.C. 1993). A few vitamins can be partially synthesized from other essential nutrients if these are present in sufficient amounts. For example, niacin can be synthesized from tryptophan and choline from methyl donors such as methionine (Wilson & Poe 1988), although this hardly occurs in practical conditions. A part of water-soluble vitamins may be derived from gut microbiota in warm-water fish although in carnivorous coldwater fish, gut microbiota is not a significant source of vitamins (N.R.C. 1993).

Although vitamin requirement data are only available for a limited number of fish species and for a limited number of vitamins (Gouillou-Coustans & Kaushik 2001; Halver 2002), comparison between phylogenetically distant species such as rainbow trout, channel catfish, chick and pig

indicates that vitamin requirements are very similar between species (Woodward 1994). Thus, it can be hypothesized that differences in vitamin requirements, particularly of water-soluble vitamin requirements, are negligible within fish species. Indeed, in a study to verify whether dietary vitamin supply as detailed by N.R.C. (1993) was sufficient for fish species as diverse as rainbow trout, Chinook salmon or European sea bass, Kaushik, Gouillou-Coustans & Cho (1998) concluded that such supply was indeed adequate in practical diets but not in semi-purified diets. In such diets, a safety margin of < 50% was required to achieve good growth performances.

Although vitamins, together with mineral deficiencies, are easy to avoid in practical fish diets, these are the most common category of deficiencies observed in commercial aquaculture (Hardy 2001). Deficiencies may result, among other factors, from incorrect dietary supplementation or antagonistic interactions with other dietary compounds. According to Hardy (2001), correctly identifying a vitamin deficiency based upon primary clinical signs or upon time of onset of primary deficiency signs is not difficult in commercial aquaculture, provided that only one vitamin deficiency exists at a given time. However, in practice, diets are rarely deficient in only one specific micronutrient, and usually the clinical signs and histopathological features are not particularly specific (Roberts 2002). Detailed signs of vitamin deficiency or excess were recently reviewed by Halver (2002).

In dietary vitamin supplementation, care must be taken to take account of vitamin losses during diet processing or storage conditions (Tacon 1992; Jobling 2008). Indeed, vitamins are prone to be lost because of exposure to adverse environmental conditions such as high moisture, temperature or light, and water-soluble vitamins may also be lost through leaching in water. In contrast to water-soluble vitamins, that are excreted when fed in excess, fish accumulate fat-soluble vitamins when dietary intake exceeds metabolic demand. Under such conditions, hypervitaminosis may be observed although it is unlikely to occur under practical feeding conditions (Tacon 1992; Halver 2002).

Vitamins and health condition

Research on the influence of vitamins on immune response and disease resistance remains limited (Lim *et al.* 2008a). Nevertheless, considerable data

have accumulated showing that diet oversupplementation with certain vitamins, particularly vitamins C and E, improved stress tolerance, immunological response and disease resistance in fish (Koshio 2007; Lim *et al.* 2008a). Both vitamins have antioxidant properties, besides other distinct metabolic functions (Blazer 1992; Gatlin 2002; Halver 2002) and have been shown to affect complement and antibody production, and macrophage function, including respiratory burst and intracellular killing (Blazer 1992; Gatlin 2002). The dietary vitamin level required for promoting such health benefits is, however, generally much greater (10–100 times) than that required for normal growth (Sealey & Gatlin 2001; Koshio 2007).

Most of the available data on the effect of diet overfortification with vitamin C were obtained in a limited number of species. Therefore, more studies are required for a better understanding of vitamin C effects on immune enhancement and disease resistance in fish. Overall, available evidence shows that increased survival to infectious disease of fish fed diets overfortified with vitamin C is most likely due to the effects on non-specific resistance mechanisms rather than on specific immune responses (Blazer 1992). Published data appear to indicate that vitamin C deficiency is immunosuppressive, and fish fed vitamin C-deficient diets are more prone to infectious diseases than fish fed vitamin C-sufficient diets (Lim, Shoemaker & Klesius 2001a). On the other hand, the beneficial effect of diet overfortification with vitamin C in improving immune response and disease resistance in fish is not consistent (Lim *et al.* 2001a; Koshio 2007). Several studies showed that health and disease resistance responses are enhanced by dietary supplementation with vitamin C (Durve & Lovell 1982; Li & Lovell 1985; Anggawati-Satyabudhy, Grant & Halver 1989; Liu, Plumb & Lovell 1989; Navarre & Halver 1989; Erdal *et al.* 1991; Hardie, Fletcher & Secombes 1991; Verlhac, N' Doye, Gabaudan, Troutaud & Deschaux 1993; Waagbo, Glette, Raa Nilsen & Sandnes 1993a; Waagbo *et al.* 1993b,c; Verlhac & Gabaudan 1994; Roberts, Davies & Pulsford 1995; Verlhac, Obach, Gabaudan, Schuep & Hole 1998; Ortuno, Esteban & Meseguer 1999; Sobhana, Mohan & Shankar 2002; Ai, Mai, Zhang, Xu, Duan, Tan & Liufu 2004; Lin & Shiao 2005a,b; Ai, Mai, Tan, Xu, Zhang, Ma & Liufu 2006; Cruz de Menezes, Tavares-Dias, Ono, Alves de Andrade, Brasil, Roubach, Urbinati, Marcon & Affonso 2006; de Andrade, Ono, de Menezes,

Brasil, Roubach, Urbinati, Tavares-Dias, Marcon & Affonso 2007; Misra, Das, Mukherjee & Pradhan 2007; Pen, Koshio, Ishikawa, Yokoyama, Micheal, Uyan & Tung 2007; Ren, Koshio, Uyan, Komilus, Yokoyama, Ishikawa & Abdul 2008; Tewary & Patra 2008; Ibrahim, Fathi, Mesalhy & Abd El-Aty 2010) although other studies failed to find evidence of any positive effect (Lall, Olivier, Weerakoon & Hines 1989; Sandnes, Hansen, Killie & Waagbo 1990; Johnson & Ainsworth 1991; Li, Johnson & Robinson 1993; Merchie, Lavens, Storch, Ubel, Nelis, Deleenheer & Sorgeloos 1996; Nitzan, Angeoni & Gur 1996; Li, Wise & Robinson 1998; Eo & Lee 2008).

Similarly, diet overfortification with vitamin E was shown to improve immune response and disease resistance in fish in several studies (Blazer & Wolke 1984; Hardie, Fletcher & Secombes 1990; Furones, Alderman, Bucke, Fletcher, Knox & White 1992; Verlhac *et al.* 1993; Ortuno, Esteban & Meseguer 2000; Clerton, Troutaud, Verlhac, Gabaudan & Deschaux 2001; Cuesta, Esteban, Ortuno & Meseguer 2001; Sahoo & Mukherjee 2002; Lin & Shiau 2005c), although other studies were not able to clearly establish benefits of dietary vitamin E supplementation at levels above requirement (Thorarinsson, Landolt, Elliott, Pascho & Hardy 1994; Lygren, Hjeltnes & Waagbo 2001; Pearce, Harris & Davies 2003; Puangkaew, Kiron, Somamoto, Okamoto, Satoh, Takeuchi & Watanabe 2004; Cruz de Menezes *et al.* 2006; de Andrade *et al.* 2007). Wang, Mai, Liufu, Ma, Xu, Ai, Zhang, Tan & Wang (2006) observed that although high dietary intake of vitamin E improved non-specific immune responses and disease resistance in Japanese flounder, *Paralichthys olivaceus* (Temminck and Schlegel), dietary vitamin E and n-3 HUFA had a synergistic effect on that response. This may help to explain the lack of clear positive response owing to vitamin E overfortification in some of the studies mentioned earlier. In Nile tilapia, *Oreochromis niloticus* (L.), even though some immune parameters (serum protein, lysozyme or alternative complement activity) were affected by dietary lipid and vitamin E levels, these nutrients had no effect on fish resistance to *Streptococcus iniae* infection or on antibody titre against that bacterial infection (Lim, Yildirim-Aksoy, Li, Welker & Klesius 2009).

A synergistic effect of high doses of vitamin C and vitamin E in enhancing immune response and disease resistance was also demonstrated in some

studies (Wise, Tomasso, Gatlin, Bai & Blazer 1993a; Wise, Tomasso, Schwedler, Blazer & Gatlin 1993b; Hamre, Waagbo, Berge & Lie 1997; Wahli, Verlhac, Gabaudan, Schueep & Meier 1998; Ortuno, Cuesta, Angeles Esteban & Meseguer 2001; Chen, Lochmann, Goodwin, Praveen, Dabrowski & Lee 2004) but not in others (Kim, Wang, Choi, Park, Koo & Bai 2003; Cruz de Menezes *et al.* 2006; Yildirim-Aksoy, Lim, Li & Klesius 2008). According to Sealey & Gatlin (2002a), dietary vitamin C and vitamin E interacted to influence growth and body composition of hybrid striped bass, *Morone chrysops* female \times *Morone saxatilis* male, but had limited effects on immune response (Sealey & Gatlin 2002a) and disease resistance (Sealey & Gatlin 2002b). The interactions between vitamins C and E may be related to the ability of vitamin C to regenerate vitamin E to its functional form, although it may also be due to the ability of vitamin E to spare vitamin C (Sealey & Gatlin 2002a; Lim, Yildirim-Aksoy, Welker, Klesius & Li 2010a). However, according to Lim *et al.* (2010a), in Nile tilapia, excessive levels of dietary vitamin C or E appear to be of little or no benefit in improving the immune response. Dietary levels of these vitamins adequate for growth and survival seem to be enough to sustain normal immune response and challenge against *S. iniae*. More research is therefore needed to assess the benefits of using high dietary levels of vitamin E as an immunoactivator in fish. Particularly, it is necessary to evaluate better the interactions of vitamin E with other nutrients such as selenium and HUFA (Blazer 1992).

Besides vitamins C and E, limited studies are also available on vitamin A (which also has antioxidant properties) effect on the immune system and disease resistance (Thompson, Fletcher, Houlihan & Secombes 1994; Cuesta, Ortuño, Rodriguez, Esteban & Meseguer 2002). Carotenoids are a source of vitamin A and also play a role in improving the defence mechanisms in fish (Christiansen, Glette, Lie, Torrissen & Waagbo 1995; Amar, Kiron, Satoh, Okamoto & Watanabe 2000; Amar, Kiron, Satoh & Watanabe 2004). In the presence of vitamins A, C and E, carotenoids exerted a greater influence on the bio-defence mechanisms of rainbow trout (Amar, Kiron, Satoh & Watanabe 2001).

A few other vitamins were also shown in a limited number of studies to improve health status in fish. Pyridoxine supplementation to diets

improved disease resistance in Chinook salmon (Hardy *et al.* 1979) and in Jian carp, *Cyprinus carpio var. Jian* (Feng, He, Jiang, Liu & Zhou 2010) but not in Atlantic salmon (Albrektsen, Sandnes, Glette & Waagbo 1995). High dietary levels of folic acid improved disease resistance in channel catfish (Duncan & Lovell 1994). Diet overfortification with choline chloride or Ca-pantothenate increased complement activity in red sea bream, *Pagrus major* (Temminck & Schlegel) (Yano, Nakao, Furuichi & Yone 1988). Also in Jian carp, disease resistance and both non-specific and specific immune response were enhanced by diet supplementation with pantothenic acid (Wen, Feng, Jiang, Liu & Zhou 2010). Diet supplementation with inositol had, however, no effect on the resistance of channel catfish to bacterial infection (Peres, Lim & Klesius 2004), although in Jian carp, both non-specific (phagocytic activity of leucocytes, haemagglutination titre and lysozyme activity) and specific (anti-*Aeromonas hydrophila* antibody titre and immunoglobulin M) activities were enhanced in fish fed myoinositol-supplemented diets (Jiang, Feng, Liu, Jiang, Hu, Li & Zhou 2010). Vitamin D3 (cholecalciferol) supplementation of diets improved innate immune defence in gilthead sea bream (Cerezuela, Cuesta, Meseguer & Esteban 2009), with the immunostimulant effect being higher in cellular than in humoral innate immune parameters analysed.

Vitamins and stress response

Diet overfortification with vitamins may also contribute to reducing stress that occurs under culture conditions, thus improving health and welfare of animals. Vitamin C is the more studied regarding its benefits in reducing stress effects in fish (Li & Robinson 2001). Several authors confirmed the reduction in several stress-induced parameters owing to the fortification of diets with vitamin C, i.e. stress induced by hypoxia (Ishibashi, Kato, Ikeda, Murata, Nasu & Kumai 1992; Henrique, Gomes, Gouillou-Coustans, Oliva-Teles & Davies 1998; Dabrowski, Lee, Guz, Verlhac & Gabaudan 2004; Chagas & Val 2006), low temperature (Falcon, Barros, Pezzato, Sampaio & Hisano 2007), osmotic shock (Lim, Dhert, Chew, Dermiaux, Nelis & Sorgeloos 2002), chronic high ammonia level in water (Liu, Xie, Zhu, Lei, Han & Yang 2008) or wound healing (Wahli, Verlhac, Girling, Gabaudan & Aebischer 2003), although

such benefits were not proven in other studies (Mazik, Tomasso & Brandt 1987; Thompson, White, Fletcher, Houlihan & Secombes 1993; Li *et al.* 1998; Henrique, Gouillou-Coustans & Gomes 2002).

Vitamin E was also shown to reduce stress susceptibility under crowding conditions (Montero, Marrero, Izquierdo, Robaina, Vergara & Tort 1999; Montero, Tort, Robaina, Vergara & Izquierdo 2001; Trenzado, de la Higuera & Morales 2007) or repetitive chasing (Montero *et al.* 2001). According to Montero *et al.* (1999), it seems to have a more protective role against stress than vitamin C in gilthead sea bream under crowding stress. On the other hand, no effects of high vitamin E levels on improving oxidative stress resistance under moderate hyperoxic conditions were observed in Atlantic salmon (Lygren *et al.* 2001). Vitamin E was also shown to improve the oxidant stress defence mechanisms in different fish species fed diets containing oxidized oil (Mourente, Diaz-Salvago, Bell & Tocher 2002; Tocher, Mourente, Van der Eecken, Evjemo, Diaz, Bell, Geurden, Lavens & Olsen 2002; Tocher, Mourente, Van der Eecken, Evjemo, Diaz, Wille, Bell & Olsen 2003) but not in halibut, *Hippoglossus hippoglossus* (L.) (Tocher *et al.* 2003). Furthermore, halibut fed moderately oxidized dietary lipids (peroxide value up to 15 meqkg⁻¹) were able to cope with temperature stress regardless of dietary vitamin E content (Martins, Afonso, Hosoya, Lewis-McCrea, Valente & Lall 2007).

Pyridoxine effect in stress mitigation and immunomodulatory response was also analysed in rohu, *Labeo rohita* (Hamilton), fingerlings submitted to endosulfan-induced stress (Akhtar, Pal, Sahu, Alexander, Gupta, Choudhary, Jha & Rajan 2010). The authors concluded that diet supplementation with pyridoxine reduced the stress while triggering the immune response in fish exposed to endosulfan.

Vitamins and lipid oxidation

Unsaturated FAs are prone to oxidation, and this may cause problems both with the diet and in the fish (Hardy 2001). Lipid degeneration may induce pathological problems associated with aldehydes, ketones and free radicals produced during the peroxidation process (Bell & Cowey 1985). One of these pathological problems is known as pansteatitis or yellow fat or lipid liver disease and has been observed in fish fed highly unsaturated rancid oils in

diets with low levels of vitamin E (Murai & Andrews 1974; Roberts, Richards & Bullock 1979; Moccia, Hung, Slinger & Ferguson 1984; Begg, Bruno & McVicar 2000; Goodwin 2006). Vitamin E is the main soluble lipid antioxidant in animals; therefore, to prevent oxidative damage, an adequate supply of antioxidants, such as vitamin E, is required (Martinez-Alvarez, Morales & Sanz 2005). High-energy diets contain high lipid levels and PUFA and therefore increase vitamin E requirement (Watanabe, Takeuchi, Matsui, Ogino & Kawabata 1977; Watanabe, Takeuchi & Wada 1981a; Watanabe, Takeuchi, Wada & Uehara 1981b; Stephan, Guillaume & Lamour 1995). However, above a minimum threshold, dietary vitamin E does not seem to significantly improve antioxidant defences (Martinez-Alvarez *et al.* 2005). Indeed, several studies on dietary PUFA and vitamin E levels failed to show an induction of antioxidant defences (Olsen, Løvaas & Lie 1999) or immune responses (Kiron, Puangkaew, Ishizaka, Satoh & Watanabe 2004) in fish fed high PUFA diets.

Vitamin C has the ability to regenerate vitamin E; therefore, both vitamins have a synergistic effect against oxidation (Hilton 1989; Moreau, Dabrowski, Czesny & Cihla 1999; Shiau & Hsu 2002). Owing to their antioxidant role, dietary levels of vitamin C and E may decrease during storage to values that do not meet requirements (Hardy 2001). Therefore, diets are usually supplemented with protected forms of these vitamins to prevent their oxidation during diet storage, although this also prevents their use as natural antioxidants in the diets (Hardy 2001).

The synthetic antioxidant ethoxyquin scavenges free radicals formed during lipid oxidation and has been used as an antioxidant in feeds to spare natural antioxidants such as vitamins C and E. However, it was recently shown that ethoxyquin has an adverse effect in Nile tilapia immunity and that continuous administration of this synthetic antioxidant to feeds may decrease disease resistance and therefore potentiate outbreaks of fish disease (Yamashita, Katagiri, Pirarat, Futami, Endo & Maita 2009).

Besides vitamins, trace elements such as Mn, Cu, Zn and Se are also involved in prevention of lipid peroxidation. The first three elements are present in the enzyme superoxide dismutase, which combines with a proton to yield a hydroperoxide radical, and Se is present in the enzyme glutathione reductase,

which disposes the hydrogen peroxide into water (Cowey 1986). Both Se and vitamin E act synergistically in the prevention of lipid peroxidation (Bell & Cowey 1985; Hilton 1989). Further details may be found in the next section.

Minerals

Fish mineral requirements are still poorly studied, mainly due to the difficulties involved in their study (Lall 2002). Fish may obtain minerals both from the diet and the surrounding water, and it is difficult to provide mineral-free water for mineral requirements studies (N.R.C. 1993). Moreover, dietary mineral supplementation is quite inexpensive, and therefore, it is easy to avoid mineral deficiency problems in aquaculture (Hardy 2001). Detailed signs of mineral deficiencies were recently reviewed by Lall (2002). As a portion of mineral requirements of fish may be met by the surrounding water, and most minerals are also present in adequate amounts in dietary ingredients, the list of minerals that are likely to be deficient in aquafeeds is relatively short: P, Zn, I, Cu and Se; thus, studies have been mainly focused in these specific minerals (Hardy 2001; Lall 2002). Fish regulate the body concentration of several minerals by absorbing or excreting them from the water; however, some minerals such as Pb, Cd, Cu or Hg are poorly regulated and may accumulate in the body, eventually becoming toxic (N.R.C. 1993; Lall 2002). Therefore, attention must be paid to levels of these minerals in the diets and within body stores to avoid health problems. Availability and utilization of minerals by fish is dependent on dietary source and level, concentration in the surrounding water, body stores and interactions with other minerals or other nutrients (Tacon 1992; Lall 2002). Several mineral/mineral and mineral/vitamin antagonistic or synergistic interactions have been reported (Hilton 1989) and must be taken into consideration when defining dietary nutrient levels. For instance, Ca, P and phytate are well-known inhibitors of Zn availability (Satoh 2007). Therefore, diets including high levels of fish meal (rich source of Ca and P) or phytate require higher Zn levels.

Phosphorus has received particular attention in fish nutrition as its content is low both in fresh water and sea water, and therefore it is required in diets in high quantities. Phosphorus-related pathology in fish was recently reviewed by Sugiura, Hardy & Roberts (2004). Availability of P is dependent on

dietary source, with inorganic and animal P sources being more available to fish than plant feedstuff sources (Lall 1991; Pimentel-Rodrigues & Oliva-Teles 2007). This is because a high proportion of plant feedstuffs P is stored as phytate which is not available to animals as they lack the enzyme phytase (Oliva-Teles, Pereira, Gouveia & Gomes 1998). When using fish meal as the main dietary protein source, no P deficiencies are to be expected as fish meal has a high P content which is readily available to fish (Lall 1991; Pimentel-Rodrigues & Oliva-Teles 2007). However, the need to reduce dietary fish meal level, replacing it by plant feedstuffs, may cause P deficiencies owing to the lower availability of plant P. Therefore, when using plant feedstuffs, more attention must be paid to dietary available P to avoid deficiency problems.

Minerals and health

Diet supplementation with certain minerals at levels above requirements for normal growth and below those causing toxicity may enhance immune function and disease resistance in fish, although such effects are not always evident (Gatlin 2002; Lim *et al.* 2008a,b).

The effect of P on disease resistance is very poorly known, and many of the presumptive observed effects may be indirect effects of P deficiency, secondary to anorexia or increased body fat in fish fed P-deficient diets (Lim, Klesius & Webster 2001b; Sugiura *et al.* 2004). For instance, in channel catfish fed graded levels of P, it was shown that antibody production and disease resistance were negatively affected by low P intake, although dietary P required for maximal growth was sufficient for maximum resistance against challenge with *E. ictaluri* (Eya & Lovell 1998). Similarly, in European whitefish fed low P diets, plasma IgM levels were lower in fish fed the P-unsupplemented diet than in the P-fortified diets, but lysozyme activity did not differ between groups (Jokinen, Vielma, Aaltonen & Koskela 2003). The authors concluded that P deficiency had only minor effects on the immune responses of European whitefish and that diets with P content sufficient for normal growth do not compromise the immune functions of the species. Recently, Baruah, Pal, Sahu, Deb-nath, Yengkokpam, Norouzitalab & Sorgeloos (2009) demonstrated that in rohu fed a suboptimal protein diet microbial phytase and citric acid had a haemato-immune-enhancing effect.

There are two possibilities regarding the effect of iron supplementation of diets (Lall 2000). On the other hand, Fe deficiency may protect against infection by limiting the amount of Fe available to bacteria, while on the other hand, Fe deficiency predisposes the animal to infection and Fe supplementation would increase disease resistance. Fe deficiency in fish does not, however, seem desirable as animals become more susceptible to infectious agents (Lall 2000). Diet supplementation with Fe had no effect on haematology, antioxidant status or non-specific immune parameters in Atlantic salmon (Andersen, Lygren, Maage & Waagboe 1998) except for a small but significant increase in catalase activity in head kidney. In channel catfish, it was found that diet supplementation with various Fe levels either provided as FeS or as Fe-methionine did not affect antibody production, although chemotactic migration by macrophages was depressed and mortality owing to enteric septicaemia was increased in fish fed Fe-deficient diets (Sealey, Lim & Klesius 1997). Similar results were also noticed in the same species an Fe-deficient diet and subjected to *E. ictaluri* challenge (Lim & Klesius 1997). In this last study, the Fe-sufficient diet did not protect fish against mortality owing to bacterial challenge, but the onset of mortality was earlier in fish fed an Fe-deficient diet. Later, Lim, Klesius, Li & Robinson (2000) also showed that macrophage migration in either the absence or presence of *E. ictaluri* exoantigen was higher in fish fed Fe-supplemented diets, while either dietary levels of Fe or vitamin C or their interaction affected survival of channel catfish juveniles in a post-challenge with *E. ictaluri*. Barros, Lim & Klesius (2002) further observed in channel catfish that dietary Fe supplementation had no effect on mortality post-challenge with *E. ictaluri*.

In rainbow trout, Mn and Zn deficiencies depressed natural killer activity of leucocytes and activity could be restored to normal levels by feeding diets with sufficient levels of these trace elements (Inoue, Satoh, Maita, Kiron & Okamoto 1998). In sockeye salmon, *Oncorhynchus nerka* (Walbaum), diet supplementation with both Mn and Zn did not improve fish resistance against bacterial kidney disease (Bell, Higgs & Traxler 1984) while survival was related to dietary vitamin C level (supplied as ascorbate-2-sulphate) only when diets contained low levels of Zn and Mn. In Atlantic salmon fry increasing dietary Mn levels also did not affect mortality after a challenge test with

Vibrio anguillarum as the minimum diet supplementation level used was enough to saturate hepatic superoxide dismutase activity (Maage, Lygren & El-Mowafi 2000).

In channel catfish, Zn deficiency and Ca excess decreased mortality of non-immunized fish after intraperitoneal injection with *A. hydrophila*, but Zn supplementation above requirement did not enhance fish resistance (Scarpa, Gatlin & Lewis 1992). This contrasts with the results in the same species obtained by Paripatananont & Lovell (1995) that indicated that all fish fed diets without Zn died following *E. ictaluri* challenge, whereas fish fed Zn-sufficient diets showed low mortality. In this study, it was also shown that Zn-methionine was 3–6 times more potent than ZnS (zinc sulphate heptahydrate), in protecting the animals against the pathogen. Similar results were also obtained in the same species by Lim, Klesius & Duncan (1996). In this last study, neither the source nor the level of dietary Zn provided protection against *E. ictaluri*, although chemotactic responses of macrophages were higher for fish fed Zn-methionine-supplemented diets or the higher level (60 mg Znkg⁻¹) of ZnSO₄ diet.

Selenium is a component of Se-dependent glutathione peroxidase, an enzyme that acts along with vitamin E as a biological antioxidant, protecting cell membranes from oxidative damage and therefore playing an important role in maintaining normal immune response (N.R.C. 1993; Lim *et al.* 2008a,b). In channel catfish, intracellular superoxide anion production by macrophages was higher with Se- and vitamin E-fortified diets than with diets fortified with one or the other nutrient alone (Wise *et al.* 1993a). Results also indicate that Se and vitamin E did not complement each other nor that one nutrient compensated for a deficiency of the other and that higher than recommended levels of one or both nutrients might enhance macrophage function. In Nile tilapia, Kim *et al.* (2003) also found no synergistic effects of diet supplementation with ascorbic acid, alpha-tocopherol and Se on disease resistance to *Edwardsiella tarda* challenge. In channel catfish, Wang & Lovell (1997) found that source and concentration of dietary Se significantly affected growth and immune response of juveniles. Though dietary Se concentration for growth and survival of *E. ictaluri* challenge was identical, antibody production increased as dietary Se level increased. In rainbow trout, it was observed that Se supplementation to a basal diet had no effect on

oxidative status, immune competence or haematological and growth parameters (Rider, Davies, Jha, Fisher, Knight & Sweetman 2009). However, physical chronic stress conditions elevated Se utilization, and thus, diet supplementation may be required to maintain Se reserves in these circumstances. In Chinook salmon, diet supplementation with vitamin E and Se had no definite effect on prevalence and severity of a natural outbreak of bacterial kidney disease, although fish fed a diet supplemented with both vitamin E and Se showed no mortality, in contrast to what was observed in fish fed diets supplemented with either vitamin E or Se alone (Thorarinsson *et al.* 1994).

Information on the health benefit of other minerals is even more scarce. Compared with a commercial diet, diets supplemented with high levels of F and I significantly reduced prevalence of natural infection of bacterial kidney disease in Atlantic salmon (Lall, Paterson, Hines & Adams 1985). Cr was shown to modulate immune response of rainbow trout, affecting serum lysozyme activity, respiratory burst of head kidney macrophages and phagocytosis by macrophages (Gatta, Thompson, Smullen, Piva, Testi & Adams 2001), the effect appearing to be both dose and time dependent. An absence of dietary Mg affected resistance of juvenile channel catfish to *E. ictaluri* (Lim & Klesius 2003). However, graded levels of dietary Mg did not affect lysozyme activity, complement haemolytic activity or blood haemoglobin in Atlantic salmon (El-Mowafi, Waagbo & Maage 1997).

Non-nutritive dietary compounds

Diets may contribute to increased stress tolerance and disease resistance of animals by judicious inclusion of certain feedstuffs or functional constituents other than essential nutrients (Nakano 2007). These functional foods may be defined as foods that target functions in the body that improve health and well-being of the animals or decrease risk of disease (Parracho, Saulnier, McCartney & Gibson 2008) and include probiotics, prebiotics and immunostimulants.

Probiotic and prebiotic use in aquafeeds may result in better health condition, improved disease resistance, improved growth performance, reduced malformations and improved gastrointestinal morphology and microbiota balance (Merrifield, Dimitroglou, Foey, Davies, Baker, Bogwald, Castex &

Ringo 2010). Probiotics are live microbial organisms, components of microbial cells or products from microbes (Gatesoupe 1999; Nakano 2007; Weese, Sharif & Rodriguez-Palacios 2008) that provide protection by establishing an inadequate environment for pathogen proliferation, by competing for essential nutrients, reducing gut pH and adhesion sites, releasing chemicals with bactericidal or bacteriostatic effects on other microbial populations or improving the immune response (Balcazar, de Blas, Ruiz-Zarzuola, Cunningham, Vendrell & Muzquiz 2006a; Balcazar, Decamp, Vendrell, De Blas & Ruiz-Zarzuola 2006b; Nakano 2007; Kesarcodi-Watson, Kaspar, Lategan & Gibson 2008). A comprehensive review of the immunomodulatory activity of probiotics and the factors that induce immune response in fish has been recently published (Nayak 2010a). Although it is important to use selected organisms of the normal dominant gut microbiota of the species under concern as probiotics, it has been difficult to detect beneficial and specific microorganisms in the fish gut (Nakano 2007), and this complicates the selection of microbiota to be used as effective probiotics.

A wide range of microalgae, yeasts and bacteria have been evaluated as probiotics (Irianto & Austin 2002), although most probiotics used in aquaculture are bacteria (Verschuere, Rombaut, Sorgeloos & Verstraete 2000; Irianto & Austin 2002; Burr, Gatlin & Ricke 2005; Nakano 2007; Merrifield *et al.* 2010; Nayak 2010b). Lactic acid bacteria have received considerable attention as probiotics in fish, and their effects on disease resistance were recently reviewed (Burr *et al.* 2005; Ringo, Lovmo, Kristiansen, Bakken, Salinas, Myklebust, Olsen & Mayhew 2010a). A variety of fish ingest algae in nature, and addition of small amounts of algae (macro or microalgae) to fish diets is expected to improve physiological condition and disease resistance (Nakagawa & Montgomery 2007). Seaweeds, for instance, are known to produce biological active compounds with cytostatic, antiviral, antifungal and antibacterial activities that might be useful against fish pathogens (Bansemir, Blume, Schroder & Lindequist 2006).

Prebiotics are non-digestible feed ingredients, including oligosaccharides and dietary fibre that promote growth of beneficial gut microbes and depress the proliferation of harmful microbes (Burr *et al.* 2005; Nakano 2007; Ringo, Olsen, Gifstad, Dalmo, Amlund, Hemre & Bakke 2010b), or

enhance intestinal immunity (Delzenne 2003). While health benefits of probiotics are relatively well documented, that of prebiotics is more limited (Ringo *et al.* 2010b). Recently, the effect of dietary inclusion of different prebiotics such as inulin, fructo-, galacto-, xylo- and isomalto-oligosaccharides and of mannan-oligosaccharides (Ringo *et al.* 2010b; Sweetman, Torrecillas, Dimitroglou, Rider, Davies & Izquierdo 2010) on performance, immune status and gut morphology was reviewed in aquaculture fish. Although the wisdom of introducing non-digestible plant extracts in diets can be questionable (Gatesoupe 2005), one advantage of prebiotics over probiotics is that they are natural feed ingredients (Gatlin, Li, Wang, Burr, Castille & Lawrence 2006), and it seems wise to try to understand eventual benefits of their incorporation in the diets. Choice of immunomodulator based on potential activity and cost are also of concern. For instance, recently, Ibrahim *et al.* (2010) concluded that a high dose of vitamin C could be a less expensive and promising diet supplementation than inulin in terms of its effect on growth, haematology, innate immunity and disease resistance of Nile tilapia.

The influence of probiotics and prebiotics on fish is still largely unknown, and this also applies to the action of mixtures of probiotics or prebiotics and possible interaction between these functional foods (Nakano 2007). Indeed, so far most studies on probiotics analysed single microorganisms, and this can be less effective than using probiotic mixtures (Verschuere *et al.* 2000). Such mixtures may be called 'synbiotics' and represent a very new concept in aquaculture (Gatlin *et al.* 2006; Ringo *et al.* 2010b). It is expected that such mixtures may improve the survival and implantation of live health-promoting microbials in the gut that contribute to the host welfare.

Dietary supplements can also act as immunostimulants, improving the innate defence of animals, providing resistance to pathogens during stress periods and therefore contributing to reduce antibiotic use in farmed fish (Galina, Yin, Ardo & Jeney 2009; Bergh & Nerland 2010; Trichet 2010). Immunostimulants improve resistance to disease not by increasing specific immune responses but by enhancing non-specific defence mechanisms (Anderson 1992; Galeotti 1998; Sakai 1999; Galindo-Villegas & Hosokawa 2004). As fish depend more on non-specific defence mechanisms than mammals, the action of immunostimulants may

prove to be more effective in fish than in higher vertebrates (Raa 2001). Immunostimulants may, however, contribute to improve the specific immune response acting as adjuvants when used in conjunction with an antigen in fish vaccines (Anderson 1992; Raa 2001; Gatlin 2002).

A number of molecules have immunostimulant effects, such as beta-glucans, chitin, lactoferrin, levamisole (Sakai 1999) and nucleotides (Li & Gatlin 2006). Most immunostimulants used in fish diets are polysaccharides derived from bacteria, fungi or yeast and may consist of the cells themselves or preparations from the cell walls containing beta-glucans (Gannam & Schrock 2001). Yeasts, such as baker's yeast, besides being considered a probiotic, are an important source of beta-glucans and nucleotides (Oliva-Teles & Gonçalves 2001; Gatlin & Li 2007). According to Rumsey, Winfree & Hughes (1992), nucleotides correspond to 12–20% of total N in yeasts and nucleotides are known to increase innate defence mechanisms and disease resistance in fish (Burrells, Williams & Forno 2001; Sakai, Taniguchi, Mamoto, Ogawa & Tabata 2001; Low, Wadsworth, Burrells & Secombes 2003; Rodriguez, Cuesta, Ortuno, Esteban & Meseguer 2003; Li, Lewis & Gatlin 2004), although such an effect was not always evident (Li *et al.* 2007b). Recently, Li & Gatlin (2006) reviewed the effect of nucleotides on fish nutrition, namely their effects on innate and adaptive immunity, stress responses and resistance to infectious diseases. Nucleotides involved in nutritional modulation of immunity are mainly of yeast origin and are different from the synthetic oligodeoxynucleotides also evaluated in a few studies (Trichet 2010). In grouper, it was further observed that although growth and immune function were enhanced with diet supplementation with a nucleotide mixture, AMP seems to have more beneficial effect on the immune responses than other nucleotides (Lin, Wang & Shiau 2009). The effect of long-term use of immunostimulants should also not be neglected. For instance, although diet supplementation with levamisole for 15 days increased leucocyte production in juvenile pacu, *Piaractus mesopotamicus* (Holmberg), long-term administration was toxic to lymphopoietic tissues (Sado, Bicudo & Cyrino 2010).

Thus far, beta-glucans, which are potent activators of macrophages, lysozyme and complement activation or oxidative capacity of phagocytic cells,

appear to be most promising as immunostimulant molecules for use in aquaculture (Gannam & Schrock 2001; Raa 2001; Gatlin 2002), and their positive effects have been shown in numerous studies (Yano, Mangindaan & Matsuyama 1989; Robertsen, Rrstad, Engstad & Raa 1990; Nikl, Albright & Evelyn 1991; Chen & Ainsworth 1992; Matsuyama, Mangindaan & Yano 1992; Jorgensen, Lunde & Robertsen 1993; Siwicki, Anderson & Rumsey 1994; Dalmo, Bogwald, Ingebrigtsen & Seljelid 1996; Efthimiou 1996; Jeney, Galeotti, Volpatti, Jeney & Anderson 1997; Santarém, Novoa & Figueras 1997; Gopalakannan & Arul 2010). The effect of beta-glucan administration on fish growth performance, immune response and potential to increase survival to disease was recently reviewed by Dalmo & Bogwald (2008).

Feeding animals with immunostimulants prior to an infection or in situations known to result in stress will elevate defences and thus provide protection against otherwise potentially severe or lethal conditions (Anderson 1992; Raa 2001; Galindo-Villegas & Hosokawa 2004). The use of immunostimulants as feed additives is very recent, and results have not shown consistent positive effects; therefore, caution must be used when evaluating immunostimulatory effects at production scale (Gannam & Schrock 2001). Indeed, the most effective method of administration of immunostimulants to fish seems to be by injection, as the efficacy of oral or immersion methods seem to decrease with long-term administration (Sakai 1999). However, the use of immunostimulants as dietary supplements, especially those that are of non-nutritional value, should be a good choice to enhance a transitory disease resistance in fish (Galindo-Villegas & Hosokawa 2004).

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