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SUMMARY - The alarming evolution which is now taking place in the pathology of aquaculture species, is of great concern to all farmers and the aquaculture industry in general. Today the whole fish/shrimp health picture is far more complex than it was a few years ago. More and more resistant bacterial strains are reported and viral diseases are becoming a major threat to tomorrow's industry viability, especially in shrimp culture. New regulations are decreasing the drugs available to treat pathogens. Vaccine development is rather slow and moreover ineffective in shrimp culture or for the first stages of fish. Therefore, fish/shrimp farmers will have to consider much more than before healthy husbandry procedures and adequate nutrition as the most important tools to ensure their business a secure future. This paper reviews the key nutrients involved in fish/shrimp health and immunology. The effect of vitamin C and E is well documented but other nutrients also play an important role such as astaxanthin, trace minerals (selenium, chromium, zinc, copper) and essential fatty acids. Apart from these keys nutrients, anti-nutritional factors, diet formulation and processing techniques might also affect directly or indirectly the resistance of the species raised. Recently, a great deal of effort has been invested by industry and academia on the development of a new generation of products called immunostimulants. A large number of commercial products have reached the market in a desperate (and sometimes opportunistic) attempt to offer even a partial solution to the spreading of viral and bacterial diseases. However, the results obtained with these substances have not always been consistent and still do not bring a real efficient protection against diseases compared to vaccines. A review of some commercially available substances is presented and the difficulty of their evaluation is discussed.

Key words: Nutrition, health, immunostimulants, micro-nutrients.

RESUME - *"Nutrition et santé". Il est frappant de constater l'évolution préoccupante de la pathologie des espèces élevées en aquaculture. Le nombre d'agents infectieux est beaucoup plus important actuellement qu'il ne l'était il y a quelques années. De plus en plus de résistances sont remarquées chez les bactéries et les maladies virales sont devenues un souci majeur, spécialement pour l'élevage de la crevette péneide. Cette évolution est un défi, non seulement pour les éleveurs, mais aussi pour toute l'industrie de l'aquaculture. De nouvelles mesures réduisent de plus en plus l'utilisation légale d'antibiotiques et le développement de nouveaux vaccins est relativement lent et de plus inefficace dans le traitement des pathologies chez la crevette et les jeunes poissons. Il est donc essentiel que la nutrition et l'hygiène d'élevage soit considérées comme des outils de prévention essentiels pour diminuer les risques de pathologies. Cette article passe en revue les principaux éléments nutritifs jouant un rôle sur l'état de santé des poissons/crevettes. L'effet des vitamines C et E est bien décrit mais d'autres éléments nutritifs tels que l'astaxantine, les minéraux essentiels, les acides gras essentiels jouent aussi un rôle important. Les facteurs anti-nutritionnels, la formulation de l'aliment et les techniques de production peuvent aussi affecter de manière directe ou indirecte la résistance de ces espèces aux maladies. Récemment, beaucoup d'attention a été consacrée à une nouvelle gamme de produits appelés immunostimulants. Un grand nombre de produits commerciaux ont été lancés sur le marché afin de tenter d'apporter une solution (parfois improvisée) à l'étendue de certaines maladies, surtout virales. Malgré cela, les résultats obtenus avec ces substances ne sont pas toujours reproductibles et ne représentent pas une protection aussi efficace telle que celle que* *peuvent offrir les vaccins. Certains de ces produits commerciaux sont présentés et la difficulté de leur évaluation est discutée.*

***Mots-clés** : Nutrition, santé, immunostimulants, éléments nutritifs.*

INTRODUCTION

Nutrition and health are two concepts intimately linked with one another and it is sometimes difficult to identify the boundary between nutrition and health or pathology. All nutritional studies will follow at least two parameters in order to quantify the requirement of a specific nutrient: growth rate and survival. When monitoring survival the nutritionist obviously agrees that nutrition does affect health status.

However, most of the reviews on nutrition (National Research Council, 1993) report on the nutritional requirement without much emphasis given on the possible effect that macro or micro nutrients might have on health. Furthermore, nutrient requirements are almost always determined under standard, well defined and favourable conditions. However, these ideal conditions are not always prevailing in commercial farms; animal often being stressed, confronted with pathogens, and unfavourable environmental conditions. Under these conditions, the requirement for certain nutrients will almost certainly increase due to an increased activity of the immune system. Studies related to the effect of micronutrients on disease resistance have assessed the possible requirement for these nutrients under problematic situation (Blazer, 1992; Lall and Olivier, 1993).

The economical consequences of adapting specific nutrient concentrations in feed is important. The addition of higher levels of micronutrients or immunostimulants will seriously increase the feed cost (+10 to 200%). The final decision will be in the hands of the farmer and the feed formulator: does it pay off? Do additives improve the final output of my commercial operation? The question is probably difficult to answer since the results are highly variable and do not always allow a clear conclusion. However, results from the field are now available and are encouraging. If we take shrimp culture in Latin America for example, it is certain that in the future better formulated, nutrient dense and rich feed will be used more extensively than today.

FEED FORMULATION, FEED PROCESSING AND QUALITY CONTROL

Manufacturing a feed for fish or shrimp involves several steps that all have a defined effect on the quality and performance of the final product. Chronologically, we can roughly classify them as follows: ① selection of raw materials, ② formulation, and ③ processing. Processing essentially shapes a particle and gives the ingredient mash the physical presentation that is required for handling and optimal feeding of the animal. If the production process is done according to basic rules, it will not significantly affect the quality of the diet. On the other hand, selection of raw materials and formulation do have a decisive influence on the performance of the diet. Imbalances in formulation or presence of toxins in the ingredients can severely affect fish health status and can lead to increased sensitivity to diseases.

Although a large number of feed ingredients may be taken into account for feed formulation (Tacon, 1987, 1993, 1994) most of the feed formulations are simply based on a few "universal" ingredients (Table 1): fish meal and fish oil, soya and wheat (or another starch source). In most cases, for "dense" formulas, fish meal and soya (*e.g.* defatted soya 44) will be the most cost effective protein source as can be demonstrated by the use of least-cost software (linear programming).

Table 1. A classical commercial feed formula for salmon, sea bass and shrimp

Ingredients (%)	Salmon	Sea bass	Shrimp
Fish meal	48	40	48
Other marine by-products			10
Defatted Soya 44		23	16
Full Fat Soya	11		
Blood meal		4	
Fish oil	23	6	1.5
Wheat and/or other	16	25	21
Premix	2	2	3.5

Once the formulation is designed, selection of raw material is of prime importance to ensure the quality of the diet and its performance. Bad quality fish meal, inadequately toasted soya, cereals contaminated with mycotoxins can certainly cause reduced growth rate and can lead to increased mortalities.

Imbalances in feed formulation

The effect of formulation on the health status of fish or shrimp is rather indirect. Feeding an inadequately formulated feed to the animal will stress its metabolism and will decrease its resistance to diseases. The same would apply for fish husbandry, handling, feeding procedures, environmental conditions etc. Fish kept in water that is insufficiently oxygenated and containing high metabolite levels will obviously be affected and eventually die due to exposure to unfavourable conditions (Snieszko, 1974).

A study on the carbohydrate content in salmon feed illustrates that concept (Waagbø *et al.*, 1994). It is well known that fish and especially salmonids are considered as "diabetic" (non-insulin-dependent diabetes) and can not utilize large quantities of carbohydrate in their feed (National Research Council, 1993). On the other hand, they can utilize fat fairly well as a source of energy. It has been shown that serum cortisol of salmon fed moist diets with increasing amounts of digestible carbohydrates (from 0 to 30 %), increased as the carbohydrate content increased and that mortality after challenge with live *Aeromonas salmonicida* by intraperitoneal injection was lowest in fish fed 10% carbohydrates (Waagbø *et al.*, 1994).

Reviewing all possible imbalances in feed formulation would be to review nutrition knowledge in its totality. We therefore suppose shrimp or fish feed properly balanced and so we will have a closer look on the effect of ingredient quality or the toxicity of special additives.

Ingredient selection and quality criteria

Oxidation of lipids

Toxicity of rancid and oxidized oils is well described in literature (Tacon, 1985; Cowey, 1986). A study with turbot (Obach and Laurencin, 1992) for example, showed that the presence of oxidized lipid (peroxide value: 300 meq/kg) significantly lowered the chemiluminescent response of head kidney phagocytes and increased mortality rates of the fish when challenged with *Vibrio anguillarum*.

However, commercial feeds or feedstuffs having a peroxide value higher than 10 meq/kg (acceptable level) are still frequently found. Feedstuffs of marine origin such as fish meal or fish oil, are particularly prone to oxidation due to their high content of highly unsaturated fatty acids. Adequate levels of antioxidants (*e.g.* 200-400 ppm ethoxiquin) should be added. Moreover, if these ingredients are not to be used immediately, their oxidation level should be checked regularly and eventually more antioxidant might be added.

Toxicity of oxidized fish oil seems to be neutralised by the addition of adequate levels of vitamins E and C. Levels of 100 to 150 ppm are commonly used in commercial fish and shrimp feeds. However, for salmon diets containing high levels of unsaturated fat (up to 35 %), levels of 350 ppm or more are used. A level of 40 ppm vitamin E was insufficient to compensate the toxicity of oxidized fish oil in European sea bass (Stephan *et al.*, 1991), while a level of 300 ppm prevented pathological and clinical signs. Vitamin E (100 ppm) and ethoxiquin were efficient in preventing mortalities in *Penaeus vannamei* (Ricque-Marie *et al.*, 1995; San Martin Del Angel *et al.*, 1996).

Freshness of animal proteins

Microbial degradation of feedstuffs, especially fish-based, results in the possible production of toxic metabolites (Ruiter, 1995). Biogenic amines are probably the most problematic substances (*e.g.* tyramine, histamine, phenylethylamine) as they can affect the metabolism of the animal (*e.g.* increasing blood pressure). Moreover, histamine may bind with lysine and form the toxin gizzerosine during processing under pressure or at high temperatures. Gizzerosine is known to provoke gizzard erosion in chickens, but can also cause mortalities in fish and shrimp (Cruz-Suarez *et al.*, 1994). Levels of histamine lower than 500 ppm and levels of gizzerosine lower 1-1.4 ppm are recommended.

Contamination with mycotoxins

If not properly dried, or when kept under humid conditions for long periods, feedstuffs, especially from vegetable origin, will be subjected to fungal growth. Some feedstuffs like tapioca, which is mostly sun dried, are often contaminated with aflatoxins. Some of these fungi (*e.g.*, *Aspergillus flavus*) will produce mycotoxins (aflatoxins, zearaleone, fumonisin, ochratoxin, etc.) of which aflatoxin B1 is probably the most toxic. Levels of aflatoxin B1 of 50 to 400 ppb are already affecting feed consumption, growth and provoke an increased sensibility to diseases (Hussain *et al.*, 1993; Van Gulick, 1993; Ostrowski-Meissner *et al.*, 1995). *Fusarium moniliforme* (a corn mould) has been responsible for decreased weight gain and reduced resistance to bacterial infection in channel catfish (Lovell *et al.*, 1994). Levels as low as 20 ppm of fumonisin are toxic.

Antinutritional factors from vegetable feedstuffs

Antinutritional factors (ANF) in vegetable ingredients are rather complex as there is a vast array of toxic substances (Tacon, 1985, 1987). Insoluble fibers, soluble fibers, enzyme inhibitors, saponins, lectins, tannins, phytic acid, gossypol are the most important antinutritional factors acting in the gut of the animal and therefore transmitted via the feed (Krogdahl, 1989). The presence of these antinutritional factors limits the use of vegetable feedstuffs in aquaculture. In practice, soybean remains the main ingredient from vegetable origin used in formulations because most of its ANF are heat labile and the processing of soybean (toasting, extrusion) is rather well controlled.

Toxicity of some additives

Certain additives are toxic when used at high concentrations. Errors in formulation or in ingredient proportioning have caused severe mortalities in animal farming. Most of commercial feed companies probably experienced these unfortunate situations. The implementation of strict production and control procedures, to avoid this “human factor”, is mandatory.

<u>Fat soluble vitamins</u>: Vitamins A, E and D_3 are liposoluble and can be toxic when used at levels exceeding the requirements (Tacon, 1985; National Research Council, 1993).

- Vitamin A will lower growth and cause anaemia at levels exceeding 100 times the requirements.
- Vitamin D_3 toxicity is probably linked with presence of calcium in water and in the feed. Feeding 50.000 IU/kg Vitamin D_3 (10-20 times the requirements) will depress growth and cause hypercalcemia.
- High concentrations of Vitamin E (5000 ppm) have shown to cause reduced concentration of erythrocytes in blood and levels higher than 2500 ppm depressed the killing ability of macrophages (Lall and Olivier, 1993).

<u>Trace minerals</u>: Although all trace minerals will be toxic when used at high concentrations, selenium is certainly the most toxic of all and is responsible for a number of “accidents”. The dietary requirements for selenium are in the order of 0.1 to 1 ppm. Although fish meal is a good source of selenium, almost all mineral complements do contain selenium, mostly as NaSe. Dosage of higher than 10 ppm will already cause mortalities in most animals (Hamilton *et al.*, 1990) and levels of 50 ppm would certainly be lethal (Hilton *et al.* 1980; Tacon,1985).

Antibiotics: Antibiotics have been of invaluable help for the aquaculture industry. However, their use is now controversial because of the increased resistance (Brown, 1989; Dixon, 1990). Moreover, antibiotics like oxytetracycline can depress the immune system (Anonymous, 1994). The DNA synthesis of mitogen-stimulated pronephric cells was reduced to about 50% at therapeutic doses of oxytetracycline. Levels of 10 ppm were sufficient to reduce the activity of both the specific and non specific immune system in salmonids, whereas oxolinic acid did not cause this immunosuppression (Siwicki *et al.*, 1989).

Transmission of diseases through the feed

Transmission of diseases through the feed is of course of importance and is a major concern for the farmer and the feed producer. For years now, *Salmonella* has been carefully monitored in feed ingredients to avoid contamination through the feed. Serious losses in poultry farming have been attributed to *Salmonella* infection. It has been shown recently (Mortensen, 1993) that Infectious Pancreatic Necrosis Virus (IPNV) can be transmitted to trout via feed pellets with a virus titer of 10^6 $TCID_{50}$ g^{-1}.

Some machine manufacturers are advertising special conditioners for pellet mills that kill *Salmonella* thanks to a long exposure of feed ingredients (30-60 seconds) to high temperatures (90-100°C). Extrusion is a technology that ensures the production of a sterile feed, at least when the feed leaves the die of the extruder. Good sanitary procedures in the feed plant will limit the contamination occurring afterwards during drying, cooling, coating, transport and packaging.

Salmon feed manufacturers avoid the use of salmon fish meal (by product from filleting industry) to reduce the risk of cross contamination. A salmon feed manufacturer in Puerto Montt, Chile purchases fish meal from the north of Chile to be sure that this fish meal will be made with fish that has never been in contact with the salmon industry located in the south.

More and more shrimp feed producers in South-East Asia and Latin America are now reconsidering the use of shrimp head meal in their formulation since there is not sufficient evidence that viruses such as Yellow head virus, White spot virus or Taura Syndrome Virus can not be transmitted through the feed via the use of processed shrimp heads originating from contaminated shrimp ponds.

HOW TO MODULATE DISEASE RESISTANCE OR INCREASED IMMUNITY?

Through micronutrients

Nutritional studies are assessing the requirement for a defined nutrient under optimal culture conditions. If feeds are formulated according to these data, it would underlie that fish or shrimp would always be raised under the same optimal conditions in commercial operations. However, some problems may arise and place the animal in a different situation (oxygen, temperature or crowding stress, presence of pathogens, etc...) in which the requirements for macro and/or micronutrients are likely to be different. Numerous studies have been carried out assessing the requirements for micronutrients under conditions such as high immunological activity, aggression by a pathogen or unfavourable environmental conditions. It is essential for the nutritionist to consider this information because the fish/shrimp are likely to face these adverse conditions during their rearing cycle.

Vitamin C

Vitamin C or ascorbic acid is the major water soluble antioxidant. Several roles are attributed to vitamin C (Landolt, 1989; Anderson, 1992; Blazer, 1992; Lall and Olivier, 1993): it acts a biological reducing agent for hydrogen transport, in synergy with vitamin E and selenium to maintain activity of glutathione peroxidase and superoxide dismutase. Vitamin C is a necessary factor in the regulation of steroid and collagen synthesis (epithelial barrier). In homeotherms, it has been shown to be important in iron metabolism, antibody response and other immune functions. Phagocytic cells generate superoxide anions and hydrogen peroxide that might cause oxidative damage to the host and the white cells in general; vitamin C protects the host against these damages and would enhance phagocytic activity of macrophages.

Although the vitamin C requirement for normal growth and survival might be quite limited (10-20 ppm) (Sandnes *et al.*, 1992), higher levels of vitamin C have proven to improve immunological defensives, resistance to oxygen or salinity stress, etc. Therefore, the definition of the requirement for vitamin C is related to the objective that one will follow.

Merchie *et al.* (1996) demonstrated for turbot larvae an improved resistance to salinity stress and survival in a challenge test with *V. anguillarum* (Fig. 1), and when fed vitamin C-enriched *Artemia* nauplii (1500 and 2500 ppm). However, variability among replicates was rather high (Fig. 2); a typical feature during this kind of evaluations. Kontara *et al.* (1996) showed that 40 ppm of vitamin C was sufficient for optimal growth of *Penaeus monodon* postlarvae, whereas 2000 ppm gave 100% survival (Fig. 3) during the rearing period and during a salinity stress test. Waagbø *et al.* (1993) tested graded levels of vitamin C (40, 400, 2000, 4000 ppm) in Atlantic salmon and obtained increased survival after challenge with *Aeromonas salmonicida*, and increased lysozyme and complement activity with the treatment containing 4000 ppm vitamin C. In another study serum lysozyme and spleen cell phagocytic activity were markedly increased in turbot fed 2000 ppm vitamin C (Roberts *et al.*, 1995).

Roughly, the following hypothesis could be stated (Tacon and Kurmaly, 1996): dietary vitamin C levels lower than 100 ppm would avoid deficiencies, maximum growth would be attained with levels of 100-150 ppm, improved immunity would be achieved with levels of 200-1000 ppm, and maximum immunity would be reached with levels of 2000-4000 ppm. However, a level of 4000 ppm vitamin C would cost between 30 and 50 % of the total ingredient cost of the feed!

Vitamin E

Vitamin E is an essential fat soluble vitamin primarily functioning as an antioxidant. It is the major lipid soluble antioxidant in membranes where it protects unsaturated fatty acids. It has been shown to affect phagocytosis and humoral and cellular immune responses, and to enhance proliferation, chemotaxis and bactericidal activity of phagocytes. However, excessive doses may reduce intracellular killing ability if that killing depends on peroxidic damage to engulfed organisms. Vitamin E stimulates cell proliferation in immunopoietic organs, increases the number of antibody-producing plasma cells and stimulates T helper lymphocytes. It also modulates prostaglandin, thromboxane and leukotriene biosynthesis (Bendich, 1992; Blazer, 1992; Lall and Olivier, 1993).

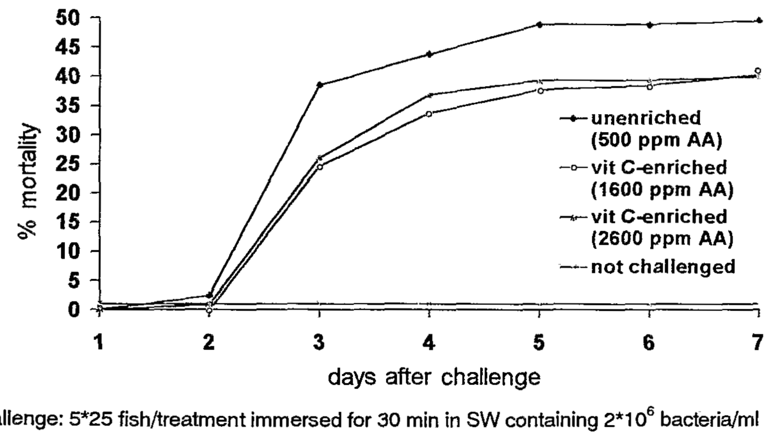


Fig. 1. Cumulative mortality of turbot larvae (35d) fed different dietary ascorbic acid levels (After MERCHIE *et al.*, J. Fish Biol., in press).

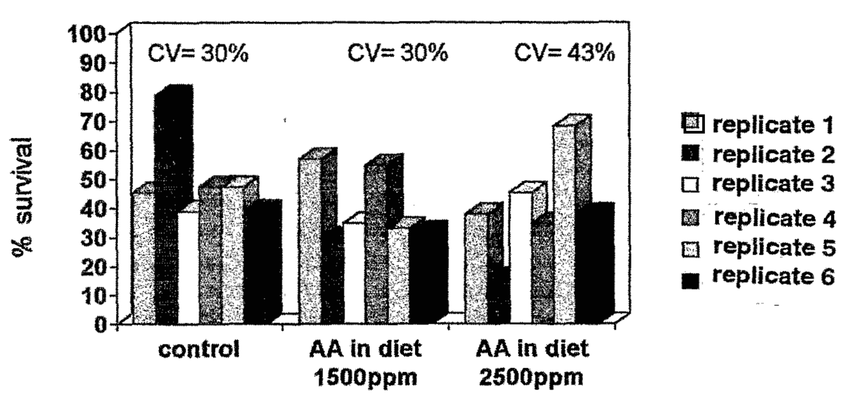
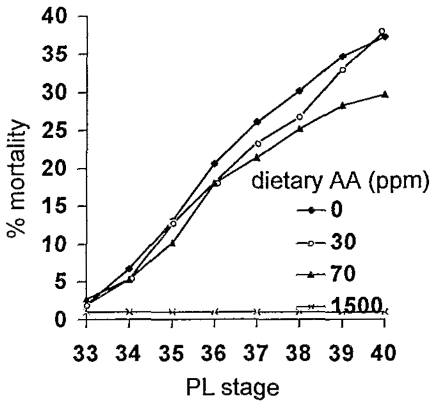


Fig. 2. Variability among replicates - Challenge: turbot fed various ascorbic acid levels (After MERCHIE *et al.*, J. Fish Biol., in press).



Vibrio harveyi infection in culture tanks

Cumulative stress index (2h, 0ppt)

0	63 ± 8
30	41 ± 9
70	35 ± 1
1500	3 ± 3

Fig. 3. Cumulative mortality and stress resistance of *P. vannamei* PL fed various vitamin C levels (After KONTARA *et al.*, Aquaculture Int., in press).

Blazer and Wolke (1984) demonstrated that vitamin E-deficient rainbow trout had significantly reduced humoral immunity. Atlantic salmon fed graded levels of vit E (7, 86, 326, 800 ppm) showed decreased resistance to the pathogen *A. salmonicida* and decreased complement activity when fed 7 ppm vitamin E. Humoral immunity reached a maximum with 326 ppm vitamin E. On the other hand salmon fed 800 ppm vitamin E displayed a lower lysozyme activity and CH_{50} (Hardie *et al.*, 1990). Levels of 300-400 ppm are described to provide maximum protection and higher levels might be detrimental by reducing the oxygen burst of macrophages (Blazer, 1992; Lall and Olivier, 1993).

Vitamin A

Vitamin A is a fat soluble vitamin important in maintaining the integrity of epithelial and mucosal surfaces. Vitamin A influences in homeotherms haematopoiesis of phagocytes and lymphocytes. Phagocytosis and intracellular killing are increased in animals given supplemental vitamin A. It restores normal humoral and cellular activity in steroid-treated animals. Retinoids and carotenoids increase proliferation of T helper cells, the induction of cytotoxic T cells and expression of IL-2 receptors on natural killer cells. Vitamin A is a relatively weak anti-oxidant (Bendich, 1992; Blazer, 1992; Lall and Olivier, 1993).

Carotenoids

It has been illustrated that astaxanthin has an anti-cancer effect and increases the number of communicating cells. Carotenoids (ß-carotene and canthaxanthin) are potent antioxidants, enhance proliferative responses of T and B lymphocytes to mitogens, increase cytotoxic T-cell and macrophage tumor-killing activities and stimulate the secretion of tumor necrosis factor-α and simultaneously lower the tumor burden. Canthaxanthin lacks vitamin A activity while ß-carotene is a precursor of vitamin A. (Bendich, 1989, 1992; Burton, 1989; Blazer, 1992; Lall and Olivier, 1993; Chew, 1995).

Different doses of astaxanthin (0, 50, 400 ppm) were evaluated in rainbow trout (Verlhac *et al.*, 1996). Astaxanthin improved phagocytosis and complement activity between 50 and 400 ppm, although not significantly. Tacon and Kurmaly (1996) reported that wild shrimp contain more astaxanthin (50-80 ppm) than cultured shrimp (10 ppm). Best growth rates have been obtained in *P. japonicus* fed 200 ppm astaxanthin.

However, even if effective, the practical use of astaxanthin is restricted by its price: 400 ppm represent 200-250% of the total ingredient cost of a formula.

Trace minerals

In iron-deficient animals, antibody production and NK cell activity are severely impaired. Recently, the iron binding proteins transferrin, lactoferrin and ferritin have been shown to affect immunoregulatory properties. It is suggested that transferrin binds circulating iron making it unavailable to the invading organism, hence overloading serum with iron may overwhelm the iron binding ability and increase susceptibility of the host. Iron also influences the production of interferon and prostaglandins (Blazer, 1992).

It has been reported recently that commercial feeds commonly contain 200-350 ppm of iron and that these levels may be detrimental to fish health (Blazer, 1992). The occurrence of winter ulcers and infestation by salmon lice was significantly lower in salmon fed 120 ppm than in the other groups (220, 295, 430 ppm). In addition, mortality after an experimental challenge with *V. anguillarum* increased linearly with the dietary iron content (Blazer, 1992).

The multi-level defence system to protect cells against chain reaction initiated by free radicals comprises superoxide dismutases (SOD) that are Cu, Zn and Mn dependant. The activities of both the cytosolic Cu-Zn SOD and the michondrial Mn SOD in the liver of rainbow trout were significantly altered in response to changes in dietary intake of these minerals (Cowey, 1986).

Levels of 8 and 16 ppm of fluoride were effective in reducing mortalities (± 3-5 %) of rainbow trout to Bacterial Kidney Disease whereas 0, 1 or 2 ppm were not effective (± 20 % mortality) (Blazer, 1992).

Through probiotics

Probiotics are very interesting and promising tools to control fish or shrimp diseases. They have been widely and successfully used in agriculture (based on living yeast, lactobacillus, etc.) and their use has resulted in a sharp decrease in antibiotic consumption (Fuller, 1989; Wolter, 1990; Castaldo, 1991; Smith, 1991; Sainsbury, 1993).

Some studies have shown that the use of probiotics from agriculture (*Bacillus, Lactobacillus, Streptococcus*) can also be useful for aquaculture (Gatesoupe *et al.*, 1989; Gatesoupe, 1991). However, these organisms are not endemic in sea water and have only a limited effect. They need to be supplied in large amounts and on a regular basis to provide any beneficial influence. Most of commercial products today on the market, if not all of them, are not designed for aquaculture but "repackaged" from existing products sold for the agriculture market.

The current use of isolated marine bacteria in shrimp hatcheries in Ecuador is a remarkable achievement. *V. alginolyticus* and other bacteria were selected from successful shrimp larval tanks and are now cultured massively as probiotics (Austin *et al.*, 1995; Griffith, 1995). Their use brought a solution to the disease known as "las bolitas" caused by *V. parahaemolyticus*, cut the use of antibiotics by 95 %, increased production volume by 35% and reduced the down time in hatcheries to 7 days a year (previously 21).

Similar work was initiated on turbot (Westerdahl *et al.*, 1991; Olsson *et al.*, 1992). From 400 bacterial strains isolated from turbot gut, 89 were found to inhibit the growth of *V. anguillarum*. The application of these findings to commercial operations can be very useful for the aquaculture industry.

Through immunostimulants

By definition, an immunostimulant is a chemical, drug, stressor, or action that elevates the non-specific defence mechanism or the specific immune response (Anderson, 1992). This definition describes the complexity of the interaction between the immune system and its environment. The immune system can be found in any possible state between the normal state and the high "alert" state. Each individual is also likely to present a different state. Any environmental stress (Fevolden *et al.*, 1994), any substance present in the food will have an effect. This also explains the wide variety of immunostimulants in use today (Table 2) (Anderson, 1992; Secombes, 1994).

The sole injection of a phosphate buffer will already trigger some defence mechanism. On the other extreme, the injection of a vaccine or bacterin together with an immunostimulant will probably give the highest response. A number of studies have dealt with the evaluation of well known immunostimulants such as polysaccharides, lipoproteins, lipopolysaccharides, etc. mainly derived from the cell wall of microorganisms.

Yeast

For a long time yeast has been known for its effect on health and disease resistance. Thus yeast was often fed to exotic animals before or after their transfer to zoos to decrease the mortality caused by the transport and the related adverse conditions. Experimental studies with monkeys showed that the oral administration of brewer's yeast could increase their resistance to infection (Sinai *et al.*, 1974). A two weeks lag was necessary between the initiation of the yeast treatment and the expression of the resistance. The effect was attributed to the *in vivo* stimulation of phagocytosis.

Table 2. Immunostimulants, adjuvants and vaccine carriers used routinely and experimentally in fish and other animals (after Anderson, 1992)

Reservoirs and depots:	B cell stimulators:
Freund's adjuvant	lipopolysaccharides
liposomes	Cell membrane modifiers:
mineral oil	detergents
lanolin	sodium dodecyl sulfate
parafin oil	quaternary ammonium compounds
dextran sulfate	saponins (plant extracts): quil
ethylene-vinyl acetate	Animal extracts:
Carriers and vehicles:	*Ecteinascidia turbinata* extract
bentonite	*Haliotis discus* extract
kaolin	fish extract
latex beads	Mitogens:
sheep red blood cells	pokeweed mitogen
dimethylsulfoxide	phytohemagglutinin
Inflammatory agents:	concanavalin A
silica particles	Nutritional factors:
carbon particles	vitamin C
T cell stimulators:	vitamin E
Mycobacterium sp.	Cytokines:
muramyl dipeptide	interleukins (leukotriene B_4,
glucans (yeast extracts)	interferon)
metallic salts	Heavy metals:
levamisole	cadmium
bacille calmette guerin	germanium
Corynebacterium parvum	
polynucleotides	

Yeast is also favoured by feed formulators since it is cheap (inclusion costs 1 to 3% of total ingredient cost), contains B vitamins, is a good protein source (when properly plamolysed) and is known as an anti-stress factor. It is often used as a cheap additive (when sales prices permit its inclusion), as the "magic touch" of the formulator or as carrier for some premixes.

Glucans

Glucans have probably attracted the attention of researchers in aquaculture more than any other immunostimulant. Their availability and "reasonable" price (still 8-16% of total ingredient cost) is probably a reason for that situation.

Glucans, as all immunostimulants, are used either by direct injection into the body (parenteral injection) or by oral administration via the food (*per os*). The results obtained by one way of administration can not be extrapolated to the other; they can differ radically. Although the effect of glucans via parenteral injection is well described (Yano *et al.*, 1989; Chen and Ainsworth, 1992; Jørgensen *et al.*, 1993; *Rørstad et al.*, 1993) and often used to potentiate the reaction on vaccination, the effect of glucans *per os* is still controversial.

An aminated ß1-3 polyglucose (^{3}H-labelled) has been tested on Atlantic salmon (Ingebrigtsen *et al.*, 1993) via parenteral injection and oral administration. Parenteral injection resulted in high levels of radioactivity in organs with large amounts of lymphoid tissues, such as spleen, pronephros, gills and wall of the posterior intestine and the radioactivity persisted at high levels throughout the experimental period (96 h). However, substantial levels of radiolabelled substances following the oral administration was only located in the wall of the posterior intestine, whereas only traces were found in other tissues.

Another study on the effect of laminaran (glucan from *Laminaria hyperborea*) was carried on Atlantic salmon (Dalmo *et al.*, 1994) revealing that the form of the glucan (sulphated or not) had an important incidence on the absorption of the compound by the intestine. This substance was concluded to have a potential as immunomodulatory feed additive. A later study confirmed the presence of specific ß-1,3-glucan receptor on macrophages from Atlantic salmon (Engstad and Robertsen, 1994).

In conclusion, the type of glucan, its presentation and chemical structure seem to be of definite importance on its potential effect as an immunostimulant. Oral administration of glucans has proved to enhance NBT positive cells in African catfish (Yoshida *et al.*, 1995). The effect was increasing during the first two-three weeks of feeding. After that period, the count of NBT positive cells returned to normal. This can be explained by the habituation of the organism to this external compound (antigen) and/or by the exhaustion of micronutrients needed to keep the immune system in an alert stage for a long period of time. Most companies selling glucans do not recommend the continuous use of these substances. Periods of rest should be installed to enable the immune system to recover and return to normality.

Others

Other chemical or natural substances have been evaluated on fish immunology.

- Via injection *e.g.* saponin mixed with aluminium hydroxide, levamisole, complete Freund's adjuvant, ovalbumin, muramyldipeptide, formylpeptides, chitin, etc. (Cossarini-Dunier, 1985; Nikl *et al.*, 1991; Anderson, 1992; Secombes, 1994; Whittington *et al.*, 1994).
- Via oral administration e.g. bacterin from *Clostridium butyricum* (Sakai *et al.*, 1995). Its oral administration enhanced the resistance to vibriosis in rainbow trout, showing clearly that the oral administration of bacteria stimulates the non-specific immunity.

The wide variety of substances is rather confusing for the farmer of the feed formulator. Which one to use? Which is the most cost-effective? Which supplier should be trusted ? The next section presents and discusses some products available on the market today.

COMMERCIAL IMMUNOSTIMULANTS CURRENTLY AVAILABLE FOR THE INDUSTRY

Table 3 gives an overview of commercially available immunostimulants.

Immunostimulants for larval and juvenile stages

Little information is available on the effect of immunostimulants on fish or shrimp larvae. The immune system of fish and shrimp larvae is generally believed to be less developed than that of adult animals. The specific immune system of fish starts to develop when the fish reach more or less 1 g size. Before that, fish rely on the non-specific defence system only and possibly on antibodies inherited from the mother via the yolk sac. Lacking a specific immune system, shrimp will essentially if not totally rely on the non-specific immune system.

Artificial diet for shrimp larvae

Fig. 4 shows an experiment carried out on *P. indicus* larvae fed the artificial diet Lansy® ZM (INVE, Belgium) supplemented or not with a blend of selected immunostimulants. Although survival was not significantly better by the end of the rearing trial (zoea 2 until PL 1), it is clearly improved 48 h after the shrimp postlarvae had been challenged with *V. harveyi* (54% *versus* 13% for the control). A high variability was also noticed and is inherent to this type of experiments in which a challenge with a pathogen is involved.

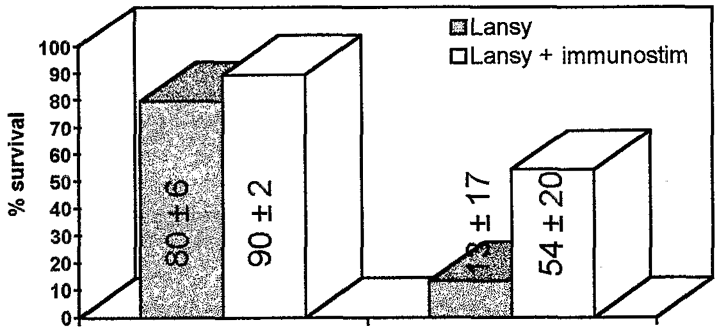


Fig. 4. Survival of *P. indicus* larvae fed Lansy® ZM + immunostimulants (INVE AQUACULTURE, Internal Report)

Table 3. Some commercial immunostimulants currently available on the market

Name	Company	Type	Mode of action	Instruction for use	Specific supporting literature	Recommended Level of inclusion (kg/ton)
Immustim	Immudyne, USA	ß(1,6) branched ß(1,3) glucan from yeast	activates macrophages	4 days - yes 3 days - no	General on similar products	0.5 - 12.5
Macroguard	Biotec-Mackzymal, Norway	ß(1,6) branched ß(1,3) glucan from yeast	activates macrophages	1. With vaccines in injection 2. in feed < 6-8 weeks cont.	Yes, on salmonids and tiger shrimp	1.0
Vitastim	Taito & Company, Japan	ß(1,6) branched ß(1,3) glucan from fungi	activates macrophages	As feed ingredient	Yes, on shrimp and fish	1.0 50 mg/kg body weight
Aqua-Mune	Park Tonks	ß(1,6) branched ß(1,3) glucan from baker's yeast	activates macrophages	As feed ingredient	No	1.0
Penstim	AURUM Aquaculture Ltd, USA	ß glucan (?)	activates macrophages	Immersion for larvae and feed ingredient for larger animals	?	2.0 to 5.0 in feed
Laminaran	Pronova, Norway	ß(1,6) branched ß(1,3) glucan from brown algae Laminariae.	activates macrophages	As feed ingredient	Yes	?
Calcium spirulan	Kelly Moorhead, Hawaii	Sulfated polysaccharide from spirulina	Inhibit viral envelope replication, inhibits virus penetration in host cell	Not known	Yes, on mammals	Not known
SP 604	Alltech Inc USA	Premix of Mannan (Biomos), Cr and Se yeast, probiotics	Multiple: activates macrophages, source of trace minerals, ...			
Agrimos	Agrimerica, USA Santel, France	Mannan based oligosaccharide from ?	Good substrate for lactic bacteria, occupies binding sites of pathogens in gut, stimulates immune response	As feed ingredient	Yes, on mammals	1.0

Table 3. Some commercial immunostimulants currently available on the market (continued)

Name	Company	Type	Mode of action	Instruction for use	Specific supporting literature	Recommended Level of inclusion (kg/ton)
Elorisan	BUGICO, Switzerland	Organic silicon	Lower lipid permeability, protection of nervous tissues	intraperitoneal injection	Yes	0.1 ml of 1% solution
DS 1999	International Aquaculture Biotechnolo-gies Ltd	Bacterin ?	activates macrophage	Add directly in culture medium (larval culture)	Yes - field trails	0.5 in larval diets
Levamisole	Janssen Pharmaceutica, Belgium	Tetrahydro-6-phenylimidazothiazole hydrochloride	(Drug) activates macrophages	As feed ingredient In bath	Yes, on mammals and fish	5-10 mg//kg body weight
Lysozyme Hydochlori-de	Belovo, Belgium	Lysosyme from hen's eggs	Kills/lyses bacterias	As feed ingredient	Yes, mammals (cheese)	?
Lactoferrin	DMV International, The Netherlands	Lactoferrin from bovine milk	Binds Iron, makes it unavailable to pathogens	As feed ingredient	Yes, on mammals	?
Selenium yeast	Alko, Finland	Selenium Yeast	Acts a yeast (activates macrophages) and source of selenium	As feed ingredient	?	1.0
Polypeptides fish hydrolysates	Tepual, Chile Sopropêche, France	Short peptides	Activates macrophages	As feed ingredients	?	?
Blood plasma	Harimex, The Netherlands	Blood serum (immunoglobulins, glycoproteins)	Activates macrophages, Binds on gut bacteria receptors	As feed ingredient	Yes, on mammals	50-100
Fish oils	Fish oil producer	ω3 fatty acids	Lower prostaglandin E-2 production (immunodepressor), Increase membrane fluidity.	As feed ingredient	Yes, on mammals	10-100

Complement diet for fish larvae, juveniles and shrimp post larvae

A fish and shrimp larval diet (Lansy® Dynamic; INVE, Belgium) has been especially developed for improving the survival of the animals being exposed to stress conditions. The diet is formulated with large amounts of micronutrients together with immunostimulants, using the synergy that these two groups of substances can exert together.

The transfer of shrimp postlarvae from the hatchery (clean and controlled conditions) to the grow-out pond (natural environment) is often accompanied by important losses. Moreover, the presence of pathogens in the pond (Taura Syndrome Virus, and others) are always striking young animals. The farmer has little control on the grow-out pond during the first month because the animals are small and will almost only feed on benthos and plankton. An interesting solution for the farmer is to increase the resistance of the postlarvae before transfer to the ponds. Similarly, in a fish hatchery, animal grading and transfer are stressful conditions that are always accompanied by mortalities.

Figs 5 and 6 show the results of the use of Lansy® Dynamic as a partial replacement of a complete diet in European sea bass and *P. indicus*. Survival of European sea bass (Fig. 5) after challenge with *V. anguillarum* was significantly increased in the treatment fed 50% of Lansy® Dynamic. The first 15 days of shrimp postlarvae are always associated with mortalities, especially when they are exclusively fed artificial diets. The use of Lansy® Dynamic shows a correlation between the supplementation of Lansy® Dynamic and the survival during these first difficult days.

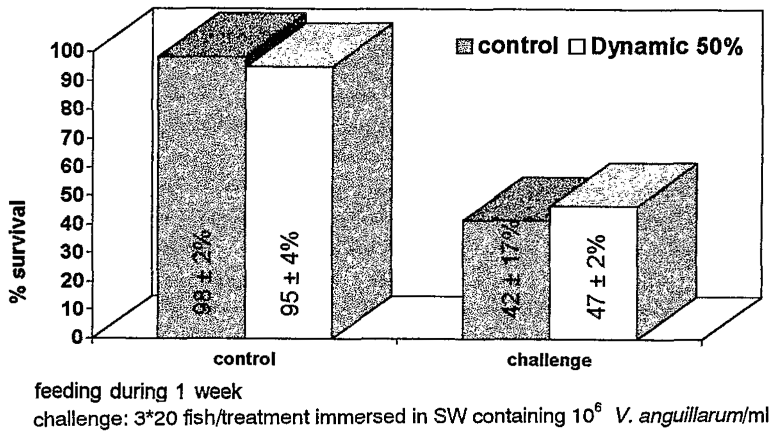


Fig. 5. Survival of European sea bass fry (70d) fed Epac-a partially replaced by Lansy® Dynamic (INVE AQUACULTURE, Internal Report).

The use of immunostimulant-boosted microparticles is an interesting tool for improving the quality of shrimp postlarvae and fish juveniles.

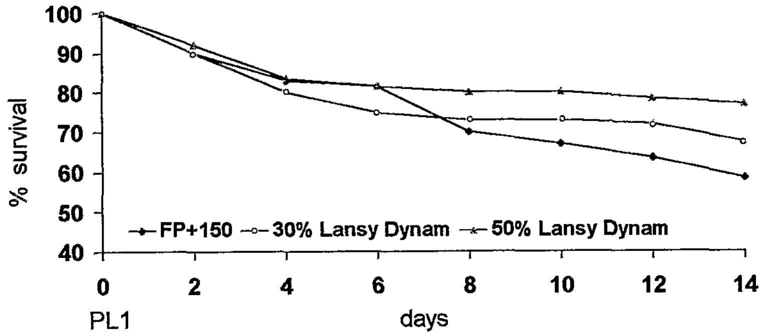
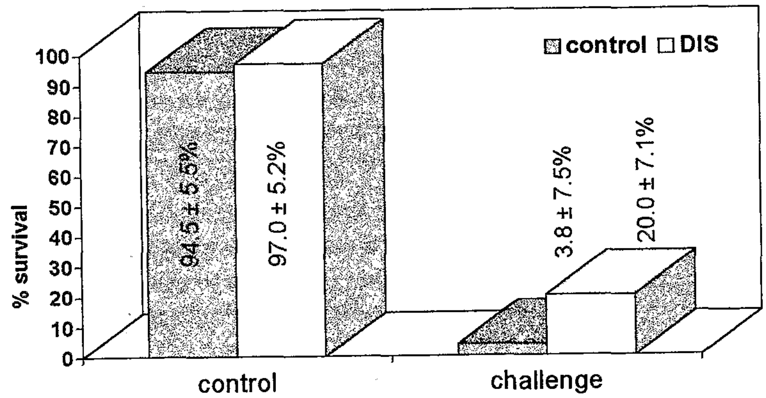


Fig. 6. Survival of *P. indicus* fed Frippak PL+150 Ultra partially replaced by Lansy® Dynamic (INVE AQUACULTURE, Internal Report).

Immunostimulant boosted enrichment product

For animals that are too young to prey on artificial diets, a new microencapsulated booster (Dry Immune Selco, INVE Belgium) has been developed to increase resistance to diseases and stress conditions through the use of rotifers or *Artemia*. Dry Immune Selco (DIS) is fed to *Artemia* Instar II for 12 or 24 h at 2x 300 ppm. The enriched *Artemia* is then fed to shrimp or fish larvae still feeding on natural plankton.

Fig. 7 shows the drastic reduction in mortality (3-20% instead of 94-97% for the control) gained through the use of DIS with turbot larvae challenged with *V. anguillarum*. The same product has been evaluated on *P. monodon* postlarvae; feeding DIS-enriched *Artemia* reducing mortality during a salinity stress test to ± 10% (as compared to 90% in the non-enriched control). The effect of the product was already noted 24 hours after first feeding (Fig. 8). However, a positive control enriched with Selco only gave intermediate results.



feeding during 10 days
challenge: 4*20 fish/treatment immersed in SW containing 10^5 *Vibrio anguillarum*/ml

Fig. 7. Survival of turbot fry (40d) fed DIS-enriched *Artemia* (7 days after challenge) (INVE AQUACULTURE, Internal Report).

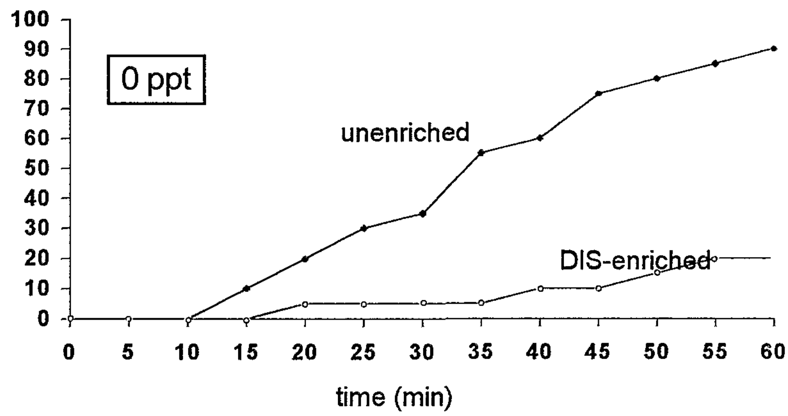


Fig. 8. Cumulative mortality of *P. monodon* (PL12) fed DIS-enriched *Artemia* (INVE AQUACULTURE, Internal Report).

EVALUATION OF COMMERCIAL IMMUNOSTIMULANTS - WHICH ONE TO CHOOSE?

The data presented in Table 3 shows the difficulty that a feed formulator will have in choosing which immunostimulant to use, when and how. Some large feed companies have developed their own research programs on this subject and will have certainly realized the complexity of the task. However, small feed companies cannot normally afford to carry out such work themselves or sub-contract research to outside institutions because they are limited in financial resources for research. Although some information has been published, the information has generally dealt with one single commercial product (Raa, 1992) or have dealt with experimental substances that have limited or no practical application in aquaculture.

The next section presents the results of some studies carried out comparing commercial substances on Atlantic salmon, rainbow trout and European sea bass. The aim of study was to select the best substances and to provide them in the form of formulated premixes.

Trials with European sea bass

European sea bass juveniles were fed three commercial immunostimulants based on yeast *versus* a control. After the rearing period (10 weeks), the fish were challenged with *V. anguillarum* and subsequent survival was recorded over 6 days (Fig. 9). Lysozyme activity from blood neutrophiles was measured (Fig. 10). The results showed that differences in survival between treatments were limited and that only one commercial product (INVE) proved to be better than the other three treatments tested. Fig. 10 shows the lysozyme activity for the same treatment and reveals that there is no evident correlation between survival after challenge and lysozyme activity. The INVE product that was giving higher survival presented the highest lysozyme activity. On the other hand, the product named Immunostim A gave the poorest survival together with highest lysozyme activity. Fig. 11 shows the variability obtained from the measures of lyzozyme activity.

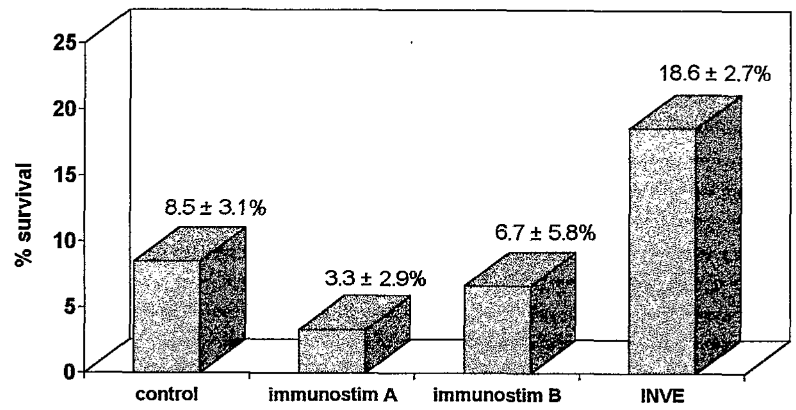


Fig. 9. Survival of European sea bass fry 6 days after challenge (INVE AQUACULTURE, Internal Report).

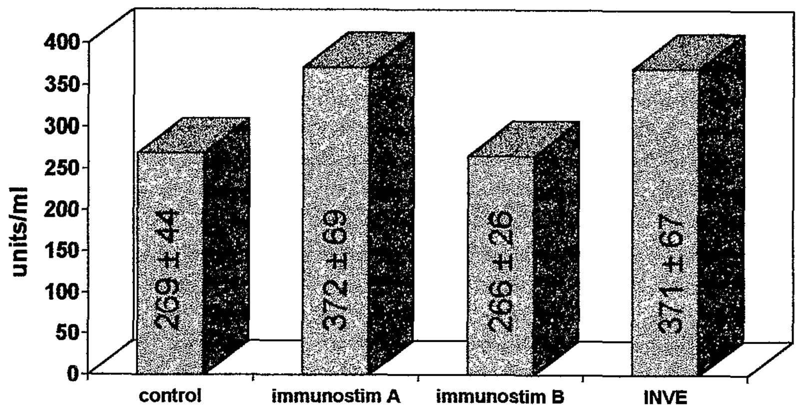


Fig. 10. Effect of different immunostimulants on the lysozym activity of European sea bass fry (INVE AQUACULTURE, Internal Report).

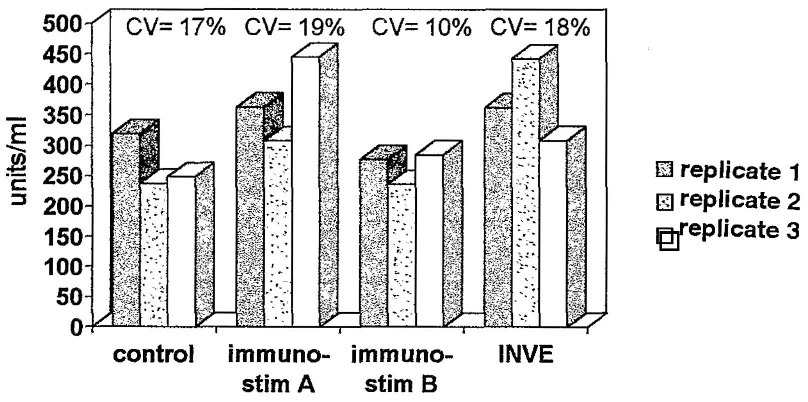


Fig. 11. Variability among replicates - Lysozym activity: European sea bass fed various immunostimulants (INVE AQUACULTURE, Internal Report).

This first test brought the following remarks or conclusions:

- Different commercial products give a different degree of protection against pathogens
- There is no absolute relation between humoral parameters such lysozyme activity and resistance to pathogen during a challenge test
- There is high variability probably due to inter-individual heterogeneity
- More tests should be performed before any final conclusion can be drawn.

Trials with Atlantic salmon

Atlantic salmon fry were fed different diets (22 in total) containing commercial immunostimulants during 6 weeks. After the rearing period, salmon juveniles were injected with *V. anguillarum* and survival recorded over 7 days. Some of these treatments are presented in Figs 12 and 13.

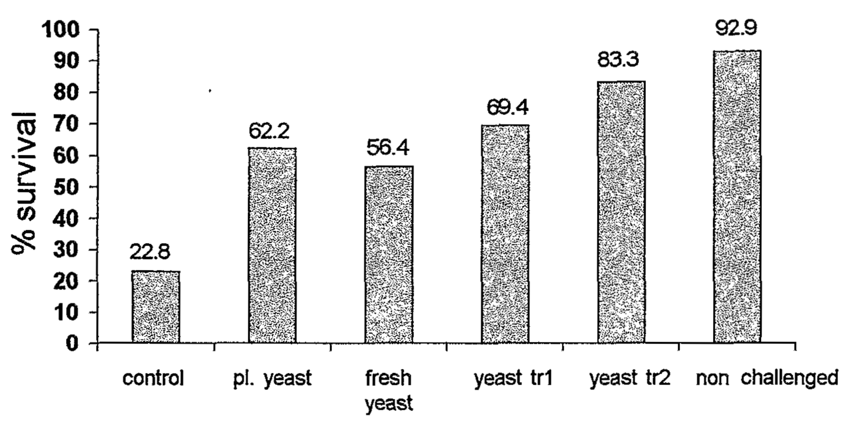


Fig. 12. Survival of Atlantic salmon fry fed different yeast products (1 week after challenge) (INVE AQUACULTURE, Internal Report).

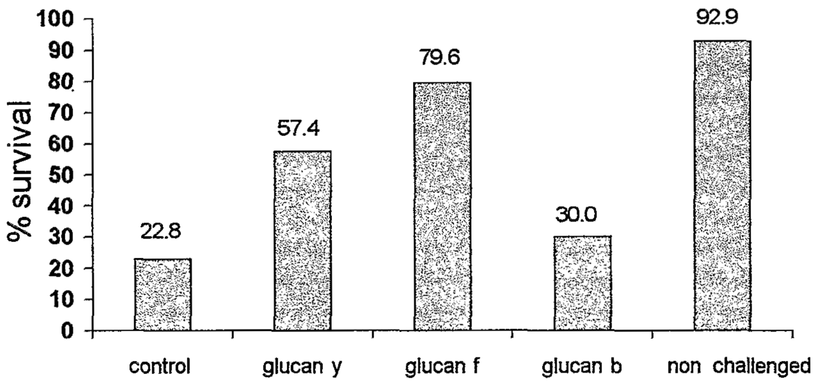


Fig. 13. Survival of Atlantic salmon fry fed different glucans (1 week after challenge) (INVE AQUACULTURE, Internal Report).

Fig. 12 presents the results obtained with different yeast-based products. Fresh yeast improved survival significantly (56.4% *versus* 22.8% for the control). The same results were also obtained with animals fed commercial dried yeast (pl. yeast 62.2%). Two other treatments with treated yeast have been evaluated chemically and the results show an improvement of the performance of the product. The data show that yeast as a sole product (treated or not) significantly improved survival. The fact that this ingredient is relatively cheap should encourage its use in formulation or its inclusion in standard premixes.

Fig. 13 shows the results obtained with different glucans originating from yeast, fungi and barley. Results differed greatly from one another, with glucan from fungi performing better and glucan from barley displaying the poorest results. Engstad *et al.* (1994) reported that glucan from barley lacks the ß-1,3 structure that can be recognized by salmon macrophages.

This second test brought the following remarks or conclusions:

- Not all glucans give the same degree of protection against pathogens
- Simple yeast products are performing reasonably well compared to glucans.

Trials with rainbow trout

Rainbow trout (250-300 g) were fed diets containing different commercial immunostimulants over a four week period. The products were only evaluated through a blood test. Every trout was individually marked and its blood was sampled (1.5 ml) every 15 days. It was decided to sample individually marked fish so as to control inter-individual variability and to follow how individuals reacted to the treatment. The results are presented in Figs 14 to 18.

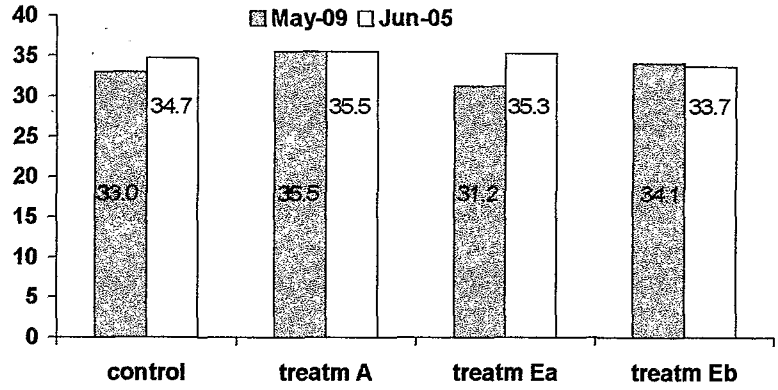


Fig. 14. Haematocrit levels of rainbow trout fed different glucans (INVE AQUACULTURE, Internal Report).

Fig. 14 presents the haematocrit value (in percent) for each treatment at the start of the test (May-09) and at the end of the test (Jun-05). This value was measured to determine the influence of repeated blood sampling on fish blood cell pool and immune system. Values are fairly constant indicating that fish possessed the ability to regenerate their red blood cell pool very quickly; a 1.5-ml blood sampling representing more than 20% of the total fish blood reserves (7 ml).

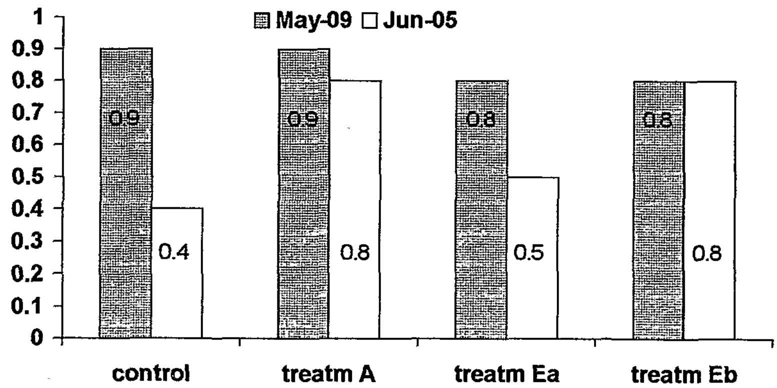


Fig. 15. Leucocrit levels of rainbow trout fed different glucans (INVE AQUACULTURE, Internal Report).

Fig. 15 presents the leucocrit level (in percent) for each treatment. There was a significant decrease in white blood cells for all treatment except for treatment Eb indicating that fish could not re-build their white blood cell pool. The fact that the same individuals were sampled hampered the immune system and questions the validity of comparing different immunostimulants. However, treatment Eb seemed to stimulate the synthesis of white blood cells.

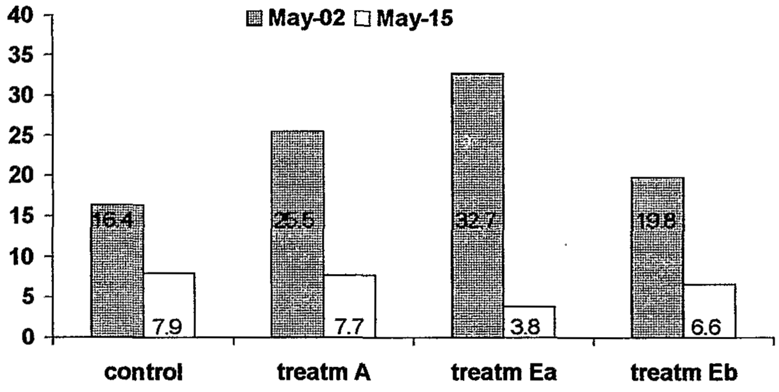


Fig. 16. Ratio lymphocytes:neutrophiles of rainbow trout fed different glucans (INVE AQUACULTURE, Internal Report).

Fig. 16 presents the neutrophile:lymphocyte ratio for each treatment. The results showed a sharp decrease the in neutrophile:lymphocyte ratio for all treatments. This was probably due to an induced lymphoponemia. Stressed fish will have increased concentration of cortisol that will affect the ratio neutrophile:lymphocyte. These results confirm the results presented in Fig.16 where the influence of the blood sampling on trout was noticed.

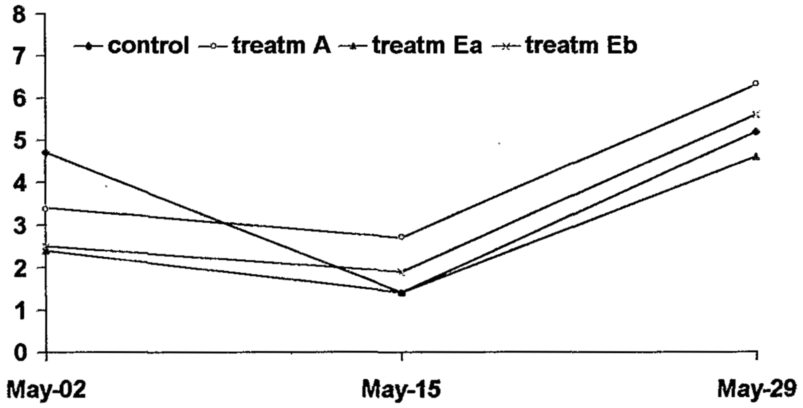


Fig. 17. NBT values of rainbow trout fed different glucans (INVE AQUACULTURE, Internal Report).

Fig. 17 shows the observed NBT values from blood neutrophiles for each treatment. The decrease in NBT value on May-15 was correlated with the breakdown of a water pump during the experiment. This also shows how sensitive a fish is, and how easily it can be stressed. The stress has a profound influence on the immune system and questions the possible results obtained at evaluating different treatments. However, different NBT values are recorded for each treatment and the sampling of May-15 is in accordance with the sampling of May-29. The values presented in Fig. 17 are the average values and Fig. 19 shows the individual values for the same treatment, different replicates; a significant variability is noticed.

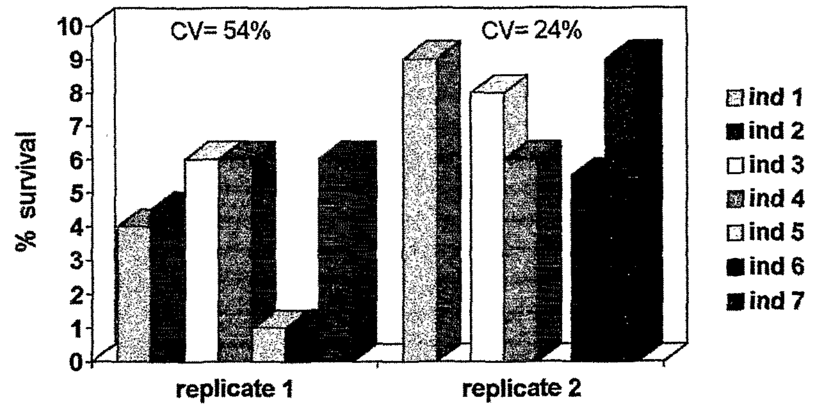


Fig. 18. Variability among individuals - NBT values: rainbow trout fed immunostimulant Eb (INVE AQUACULTURE, Internal Report).

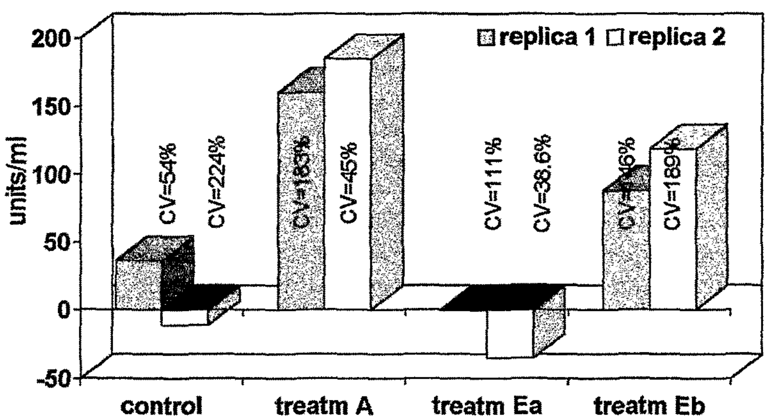


Fig. 19. Variability among replicates - Lysozym activity: rainbow trout fed various immunostimulants (INVE AQUACULTURE, Internal Report).

Fig.19 presents the lysozyme activity for the different treatments. The values reported are expressed as the percentual difference of lysozyme activity (unit/ml) between the start and the end of the test. More consistency and less variability was noticed in lysozyme activity. The lysozyme activity of the control remains rather stable showing that the immune system (concerning this parameter) remained at its original level. Treatment A and Eb showed a sharp increase in lysozyme activity (100 to 200 % of its original value).

This last test results in the following remarks or conclusions:

- Fish are stressed very easily and the stress has a profound effect on the immune system (NBT values, neutrophile:lymphocyte ratio)
- Sampling blood from the same fish affects its immune system and interferes with the effect of the treatment (immunostimulant)
- Fish can re-build their red blood cell pool rather fast but white blood cell synthesis is slower
- Lysozyme activity remains a good criterion
- A challenge test will always be necessary to confirm the analytical tests.

CONCLUSIONS

It is clear that nutrition and health are intimately related. Feed formulation and selection of ingredients should be carried out carefully so as to avoid any nutritional disorder that could increase the sensibility to diseases.

To improve the disease resistance of the fish of shrimp, the feed formulator can use a number of additives that have shown a positive influence. These additives can be roughly classified into micronutrients and immunostimulants. There is almost certainly a synergy between these two classes of compounds: glucans will stimulate the immune system of the animal and this increased immunological activity will probably change the requirements for micronutrients like vitamin C or vitamin E. Adding these micronutrients together with glucans will optimize the immune reaction and will prevent eventual deficiencies resulting from (for example) a vitamin C exhaustion.

The large number of commercial immunostimulants on the market clearly reflects the interest of the sector for the development of alternative tools to treat diseases. However, their selection is not easy and the high variability observed in biological trials, often limits the conclusions which can be drawn. Clearly considerable further research is required in this critical area.

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