The use of immune-stimulants in fish and shellfish feeds.[1]

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ABSTRACT: Immune-stimulants are chemical substances which activate white blood cells (leukocytes). Such substances may also, but not necessarily, render animals more resistant to infectious diseases and reduce the risk of disease outbreaks if administrated prior to situations known to result in stress and impaired general performance (e.g. handling, change of temperature and environment, weaning of larvae to artificial feeds) or prior to expected increase in exposure to pathogenic micro-organisms and parasites (e.g. spring and autumn blooms in the marine environment, high stocking density). In addition, aquaculture may benefit from the use of such immune-stimulants when they are used prior to, and during, developmental phases when the organisms are particularly susceptible to infectious agents (e.g. the larvae phase of shrimp and marine fish, smoltification in salmon, sexual maturation). Immune-stimulants may act in synergy with antibiotics and their effects may be enhanced by nutritional factors (e.g. vit. C, selenium), but there is no nutritional factor which on its own can be defined as an immune-stimulant. Compounds with highly diverse chemical structures have been shown to stimulate white blood cells *in vitro*, but the majority of such compounds have no relevance for practical use, due to high toxicity, obscure mode of action and unpredictable effects under farming conditions. The most promising group of immune-stimulants are the β-1,3/1,6-glucans, because they have a well-defined chemical structure and mode of action on the immune system, described in a great number of scientific papers. In addition, β-1,3/1,6-glucans are non-toxic universal "alarm signals" which activate the immune system by the same basic mechanism in all animal groups, from the simplest invertebrates to man. β-1,3/1,6-Glucans are active not only when injected, but also when administered in the feed, or on mucosal surfaces. The paper will present data on such effects with reference mainly to experience from fish and shellfish farming, and discuss how a high-molecular substance, which is not taken up into the body fluids, can induce a systemic effect.

KEYWORDS: Immunestimulants, fish, shellfish, feeds.

What is an immune-stimulant?

Immune-stimulants are chemical compounds that activate white blood cells (leukocytes) and hence may render animals more resistant to infections by viruses, bacteria, fungi and parasites. Immune-stimulants may be active also against human cancer because they activate the white blood cells which recognise and destroy tumor cells.

During evolution of animals their immune system has developed mechanisms to detect chemical structures which are typical for potentially dangerous micro-organisms and use those structures as «alarm signals» to switch on the defence against infections. In the presence of such chemical signals the immune system will respond as if challenged by a pathogenic microbe. Hence, administration of an immune-stimulant prior to an infection may elevate the defence barriers of the animal and thus provide protection against an otherwise severe or lethal infection.

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Chemical nature of immune-stimulants

Most immune-stimulants are chemical compounds which exist as structural elements of bacteria, mycelial fungi and yeasts. However, there are also a couple of purely synthetic compounds, originally made for other purposes, which have incidentally been found to possess immune-stimulating properties.

The chemical nature and mode of action of a number of different immune-stimulants have been described in a review paper (Raa, 1996). They are grouped as:

a) structural elements of bacteria (*lipopolysaccarides (LPS), lipopeptides, capsular glycoproteins and muramylpeptides);*
b) various β-1,3-glucan products from bacteria (*Curdlan*) and mycelial fungi (*Krestin, Lentinan, Schizophyllan, Scleroglucan, SSG, VitaStim*);
c) β-1,3/1,6-glucans from the cell wall of baker's yeast (*MacroGard, Betafectin*);
d) complex carbohydrate structures (*glycans*) from various biological sources including seaweed;
e) peptides present in extracts of certain animals or made by enzymatic hydrolysis of fish protein;
f) nucleotides, and
g) synthetic products (*Bestatin, muramylpeptides, FK-156, FK-565, Levamisole*).

Immune-stimulants are usually identified by their ability to activate white blood cells in test tube experiments. It is important to be aware, however, that such experiments provide little information regarding the effect of a given immune-stimulant on the whole organism. Experiments with live animals are needed to reveal the overall effect of an immune-stimulant.

Cell wall preparations of bacteria (e.g. *lipopolysaccharides (LPSs), lipopeptides, peptidoglycans* and *muramyl peptides*) are very potent immune-stimulants when tested *in vitro*. However, such products may cause severe inflammations and they may be very toxic at concentrations only slightly above a "safe" dose. Lipo-polysaccharides (LPSs) induce for instance the production of signal molecules (cytokines) which reduce appetite and suppress growth of animals**.** Moreover, bacterial cell wall preparations have chemical structures which can not be defined exactly in chemical terms and their mode of action on the immune system is unspecific and still obscure. Accordingly, such products are therefore not very relevant for use in practice.

The b-*1,3-glucans* found in mycelial fungi and yeast, differ from immune-stimulants of bacterial origin in chemical structure and mode of action. The bacterial products in mode of action, and are highly relevant for use in practice because the overall effects are positive, as a rule. -1,3/1,6-Glucans can be defined quite accurately in chemical terms and their mode of action on the immune system is very specific and has been revealed in great detail, even at the cellular and molecular level. Moreover, it has been shown that β-1,3-glucans may improve health, growth and general performance of many different animal groups, including farmed shrimp, fish and land animals. Since β-1,3-glucans are active also when taken in the feed (or food), there are many actual applications of such products within animal nutrition and health, as well as in human and veterinary medicine, cosmetics and within the rapidly expanding nutriceutical (= "*health promoting food"*) sector.

Immune-stimulating peptides and nucleic acids have in recent years been marketed for the aquaculture sector. However, these products have not yet much support in published research work and efficacy trials, and will therefore not be discussed further in the present paper.

When to use immune-stimulants?

Immune-stimulants may improve health and performance of farm animals, including fish and shrimp in aquaculture, if used prior to:

a) situations known to result in stress and impaired general performance of animals (e.g. handling, change of temperature and environment, weaning of larvae to artificial feeds),
b) expected increased exposure to pathogenic micro-organisms and parasites(e.g. spring and autumn blooms in the marine environment, high stocking density,
c) developmental phases when animals are particularly susceptible to infectious agents (e.g. the larvae phase of shrimp and marine fish, smoltification in salmon, sexual maturation).

Immune-stimulants may act in synergy with antibiotics and thus strengthen the effect of curative medication. However, immune-stimulants are primarily prophylactic agents which should be used to elevate the general defence barrier of the organism and hence to reduce the risk of disease, not as a curative medicine. If used at an advanced stage of a disease development, an immune-stimulant may be recognised by the organism as an «apparent infection» in addition to an existing one, and it may therefore possibly aggravate disease symptoms, at least for a short while.

Benefits

Immune-stimulants are being used today both within the aqua-culture sector and in traditional animal husbandry to reduce mortality due to infections and to improve general performance of animals. Immune-stimulants may provide particular benefits when used in order to:

a) Reduce mortality due to opportunistic pathogens. Opportunistic pathogens cause disease and mortality only when the host is weakened or stressed, for instance by adverse environmental conditions. Such micro-organisms may reduce the general performance of animals even when the environmental conditions and the feed are good, and the animals show no clinical signs of disease. Immune-stimulants may enhance defence mechanisms which counteract opportunistic infections and hence lead to improved performance and growth, and reduced mortality throughout the production period;

b) Prevent virus diseases. Development of vaccines against virus diseases is time consuming and expensive, and it is an unrealistic goal to make efficient products against the great number of different viruses which may affect the very many different cultured species, in particular in aqua-culture. It is therefore a good strategy to reduce the risk of virus diseases by combining good husbandry and good feed with the use of immune-stimulants which enhance the overall disease resistance of the animals.

c) Enhance disease resistance of farmed shrimp. Shrimp and other invertebrates have immune systems that are less well-developed than in fish and warm-blooded animals, e.g. they lack the specialised white blood cells which in higher animals are involved in antibody production and immunological memory (*lymphocytes*). Shrimp are depending on non-specific immune processes for their resistance to infections (Adams, 1991; Söderhäll and Cerenius,1992). Immune-stimulants which stimulate such processes and render shrimp more resistant to disease, are therefore becoming important tools in health management of commercial shrimp farms.

d) Reduce mortality of juvenile fish. Fry and larval fish are particularly susceptible to microbial infections and are often subject to high mortality due to opportunistic pathogens in hatcheries.

Young fish do not possess a mature specific immune system (Ellis,1988) and depend on the non-specific cellular defence mechanisms to be resistant to microbial infections (Trust, 1986). The use of immune-stimulants to reduce mortality of larval and juvenile fish in hatcheries is therefore another important application.

e) Enhance the efficacy of anti-microbial substances. It has been shown that immune-stimulants may act in synergy with antibiotics in preventing infections in human patients (Smets *et al*. 1988; de Felippe, *et al*. 1993) and in fish (Thompson *et al*. 1993). There is a sound biological basis for such a synergy; the aggressiveness of the infectious bacterium is lowered by the anti-microbial agent while the antibacterial mechanisms of the body itself are being stimulated. It is therefore worth consideration to use immune-stimulants in combination with curative anti-microbial agents at an early phase of disease development, or prior to anticipated disease outbreak.

f) Enhance the resistance to parasites. The development of parasites which attach to skin and mucosal membranes are inhibited by complement factors, enzymes and enzyme inhibitors. These chemical defence weapons are produced by specialised cells in the surface tissues, and are continuously secreted. Certain immune-stimulants, e.g. β-1,3/1,6-glucans, stimulate these cells and thereby the defence against surface parasites. It has been reported that immune-stimulants are indeed effective against sea-lice on farmed salmon (Fish Farming International, March 1998, Volume 25, No 3, p.26).

g) Enhance the efficacy of vaccines. Immune-stimulants are used as helper substances (*adjuvant*s) in vaccines to activate antigen presenting cells (e.g. *macrophages*) and to stimulate these cells to produce more of the signal molecules *(cytokines*) which activate the group of lymphocytes (*B-cells* in warm-blooded animals) which produce specific antibodies. It is a discovery of great practical significance that a β-1,3/1,6-glucan acts as a true adjuvant which enhances antibody production, not only when injected together with the vaccine antigens, but also when administrated in the feed and the vaccine antigens by injection (Nicoletti *et al*. 1992; Raa *et al*. 1992; Verlhac *et al*. 1998). Such an adjuvant effect of orally administered β-1,3/1,6-glucan on injected vaccines, and mucosal vaccines, has been demonstrated with fish, pigs and mice. The practical significance of this observation is that feeding of fish and farm animals with β-1,3/1,6-glucan may enhance the production of antibodies against pathogens present in the local environment and thus help to build up an efficient specific resistance against such pathogens, in addition to enhancing the non-specific cellular defence (Raa *et al*. 1992; Raa, 1996).

Improved health and growth of different animal groups: a few examples

A series of slides will be presented to illustrate that β-1,3/1,6-glucan enhances disease resistance and improves growth of many different animal groups, hereunder that:

a) immersion of halibut larvae resulted in a marked increase in survival rate during a very critical phase of their development (Ottesen, Lunde and Engstad, 1999),
b) immersion of shrimp post-larvae in β-1,3/1,6-glucan increased their survival (Supamattaya and Pongmaneerat, 1998),
c) feeding of shrimp with β-1,3/1,6-glucan resulted in faster growth, reduced mortality and better feed utilisation (Sung *et al.* 1994),
d) injection of β-1,3/1,6-glucan into fish produces very effective resistance to several bacterial diseases (Robertsen *et al.* 1990), and enhanced efficacy of vaccines (Rørstad, Aasjord and Robertsen, 1993),

e) feeding of various fish species with β-1,3/1,6-glucan results in improved growth and reduced mortality,
f) β-1,3/1,6-glucan in the feed enhances the resistance to bacterial diseases (Raa *et al.* 1990), and the efficacy of vaccines (Raa *et al*., 1990), also when the vaccine was injected (Verlhac *et al*. 1998),
g) β-1,3/1,6-glucan in the feed acts in synergy with antibiotics (Thompson, Cachos and Inglis, 1993) and vitamin C (Verlhac *et al.*1998)

Besides aquaculture, basic research and efficacy studies with warm-blooded animals have produced a strong foundation for the use of immune-stimulating β-1,3/1,6-glucans in animal veterinary and human medicine, and in traditional animal farming.

The reason why β-1,3/1,6-glucans can be used for the same purpose with so many different groups of organisms, is that this immune-stimulant is acting at a very basic level of the immune system: - the β-1,3/1,6-glucan molecule "recognises" a specific receptor present on white blood cells in all animal groups, from the simplest invertebrates to man, and switches on their most basic defence mechanisms.

Mode of action of β-1,3/1,6-glucans

β-1,3/1,6-Glucans bind specifically to a "receptor molecule" on the surface of phagocytes (Engstad and Robertsen, 1994). The receptor for β-1,3/1,6-glucan has been retained during evolution and is found in all animal groups from invertebrates, such as shrimp, to man. This is why β-1,3/1,6-glucans have the same basic biological effect within the whole animal kingdom. When the receptor is engaged by β-1,3/1,6-glucan, the cells become more active in engulfing, killing and digesting bacteria and at the same time they secrete signal molecules (*cytokine*s) which stimulate the formation of new white blood cells. In animals which have the specific immune mechanisms in addition to non-specific defence (fishes and higher up in evolution), the activated phagocytes produce cytokines which also activate antibody-producing white blood cells (B- and T-cells). Therefore β-1,3/1,6-glucan enhances also the efficacy of vaccines. Due to the very basic mode of action of β-1,3/1,6-glucans, products in this category affect a number of different biological processes, including not only disease resistance, but also growth, wound healing, repair of cells damaged by ultraviolet light, skin care, arthritis symptoms etc. (Raa, 1999)

Unlike lipopolysaccharides (LPSs) and peptidoglycans (Pgs) from bacteria, which by definition are immune-stimulants, purified β-1,3/1,6-glucan do not induce any antibody production against itself. This is a significant advantage because the immune system will not be "cheated" to waste antibody producing capacity on the immune-stimulant itself.

Stimulation of the gut-associated immune system

It is not generally recognised that the immune system is one of the largest "organs" in the body of higher animals; in man it constitutes 3 % of the body weight. This organ is distributed all over the body and represents its "armed forces". The largest division of this defence is localised in the gut tissues, where specialised lymphoid organs (*Peyer's patches*), lymphoid nodules, and the whole range of different white blood cells are found. The gut is also equipped with special gateways (*M-cells*) for entrance of small particles and bacteria. This major lymphoid organ of the body may be activated or down-regulated by food and feed ingredients, by micro-organisms and by immune-stimulants. Since signals are transmitted from the lymphoid organs in the gut to the rest of the body,

the well-being of an animal, or human, is very sensitive to changes in the processes which go on in the digestive tract.

In accordance with this general understanding of how the immune system in surface tissues of warm-blooded animals are functioning, it has been shown that β-1.3/1.6-glucans induce significant disease resistance when administrated as a nasal spray or orally, and enhance systemic immunity (Nicoletti *et al*. 1992; Maeda *et al.* 1994; Shoenherr *et al.* 1994; Raa *et al*. unpublished).

Fish, on the other hand, have not developed histologically distinct and specialised lymphoid tissues such as the Peyer's patches of warm blooded animals, and special gateways for absorption of particles, like the M-cells. However, all the cell types responsible for a local immune response are present in the intestinal wall of fish (e.g. carp and tilapia), including immunoglobulin positive lymphocytes (B-cells), immunoglobulin negative lymphocytes (T-cells), and antigen presenting macrophages (Davina *et al*. 1980; Doggett and Harris,1987; Rombout *et al*.1989; 1993). Like in warm blooded animals, the gut associated immune system of fishes seems to function in the absorption of macro-molecules from the intestine and in the production of antibodies in response to antigens (Hart *et al*. 1988). In other words, immune cells in the gut of fish may function like mammalian M-cells in sampling macromolecules. By this assumption one may explain the fact that β-1.3/1.6-glucans, also when administered in the feed of fish, enhance their disease resistance, growth performance and the efficacy of injected vaccines.

Immune-stimulants in shrimp farming

Shrimp have white blood cells with the same biological properties and functions as the macrophages, granulocytes and natural killer cells in higher animals. These cells, which are called *hemocytes*, are found in the tissues around the digestive organ and in other tissues, as well as in blood. They produce anti-microbial products of importance for defence and they carry out phagocytosis and remove foreign particles and infectious agents from tissues and body fluids. Exposure of shrimp to live pathogenic bacteria in high numbers, results in uptake of such bacteria into the body fluids, followed by efficient clearance (Sung *et al*. 1996). The capacity to clear the tissue from invasive bacteria is probably of great importance for shrimp to stay healthy.

Three types of circulating hemocytes can be isolated from the hemolymph of crustaceans; these are the *hyaline*, the *semi-granular,* and the *granular* hemocytes. The hyaline hemocytes have phagocytic activity and attach and spread on glass surfaces like macrophages of fish and warm-blooded animals. The semi-granular hemocytes are characterized by a number of small granules and thus resemble the granulocytes of vertebrates. This cell type has receptors for ß-1.3-glucans (Smith and Söderhäll, 1983; Johansson and Söderhäll, 1985). The granular hemocytes are filled with large granules. They do not have phagocytic activity and their ability to encapsulate foreign particles is limited. The main function of the granular hemocytes, is to store the pro-phenol oxidase enzyme which plays a key role in the defence reactions of crustaceans. The granular hemocytes can be triggered to undergo exocytosis and release of this pro-enzyme. The pro-phenol oxidase system may be activated by ß-1.3-glucans, peptido-glycans and lipo-polysaccharides (Söderhäll *et al*. 1994; Barracco *et al*. 1991). The activation by ß-1.3-glucans is very specific. In its active form the phenol oxidase catalyses the oxidation of phenols to semi-quinones and quinones which due to their high reactivity, will kill microbes.

Invertebrates, including farmed shrimp, are not equipped with cells that are analoguous to antibody producing lymphocytes in fish and warm-blooded animals. Shrimp are therefore apparently entirely dependant on non-specific immune mechanisms to resist infections, and they lack the same kind of

specific immunological “memory” as that found in fish and warm-blooded animals. Accordingly, it does not seem to make sense to vaccinate a shrimp against a specific disease by administration of vaccine preparations of the disease causing micro-organisms. Nevertheless, shrimp farmers, both in Asia and Latin America, have experienced that after an initial period of high mortality due to a new virus disease in a region, shrimp in contaminated farms acquire a high degree of specific resistance to that particular virus. Since brood stock animals are collected from the wild, this resistance can not be due to genetic selection, but be the result of an acquired and specific immune mechanism, not based on immunoglobulins. It is an exciting and important field of research, apparently ignored by trend-setters in this field, to explore the biological mechanisms involved in this kind of specific immunological memory. Eventual “shrimp vaccines” based on this yet unknown mechanism belongs to the future, although farmers have already made practical use of the phenomenon. It seems that shrimp which become carriers of a given virus in very early life stages, develop a state of tolerance and specific immunity to that virus.

β-1,3/1,6-Glucans have proved to enhance the biological activity of shrimp hemocytes and to improve growth, survival rate and feed conversion efficacy under experimental conditions (Sung *et al*. 1994; Song and Hsieh, 1994; Sung *et al*. 1996; Sung *et al*. 1998; Song *et al.* 1997; Supamattaya *et al.* 1998) and in practical shrimp farming. The enhanced resistance to bacterial infection (*Vibrio vulnificus*) lasted for 2-3 weeks after one single administration of β-1,3/1,6-glucan to *post-larvae* shrimp, or about the life span of the hemocytes. To maintain an elevated level of resistance, the immune-stimulant has to be administered on a continuous basis, or in periods with 2-3 weeks intervals.

Immune-stimulation by environmental micro-organisms

It may be argued that the natural environment of a commercial shrimp farm in itself is such a rich source of microbial immune-stimulants that any artificial supplementation should not be necessary. However, a high number of live algae and bacteria does not necessarily elicit the right response for improved resistance, and eventual immune-stimulants present as cellular constituents inside microbial cell walls are not necessarily exposed in active form. It has for instance been shown that β-1,3/1,6-glucan enhances the anti-bacterial activity of cell free plasma of shrimp, whereas Vibrio bacteria, even in high numbers (10 million bacteria per ml tank water), do not (Sung *et al*. 1996). Also other cellular processes of shrimp hemocytes, related to disease resistance (ability to produce super-oxide radicals and level of phenol oxidase), were affected differently by live bacteria and a β-1,3/1,6-glucan, the latter being more potent (Sung *et al*. 1996).

Nutrients

Improvements in the health status of fish can certainly be achieved by balancing the diets with regard to nutritional factors, in particular lipids and antioxidative vitamins, but this is primarily a result of an input of substrates and cofactors in a complex metabolic system. This is unlike immune-stimulants, which interact directly with the cells of the immune system and make them more active. Nevertheless, some nutritional factors are so intimately interwoven with the biochemical processes of the immune system that significant health benefits can be obtained by adjusting the concentration of such factors beyond the concentration range sufficient to avoid deficiency symptoms or below a certain concentration range. This is in the focus of what has been designated nutritional immunology and which has been studied also in fish (Blazer, 1992; Waagbö, 1994).

Imbalances in the nutrient composition of the diet will affect growth and general performance of an animal and, most likely, also the biochemical processes of the immune system. It has been

demonstrated that diet composition may affect the ability of leukocytes to phagocytose and kill bacteria (Blazer and Wolke, 1984a) and that trout maintained on a diet deficient in α-tocopherol for 12-17 weeks had significantly reduced responses in a number of assays for immune functions (Blazer and Wolke, 1984b).

Vitamin C, polyunsaturated fatty acids and vitamin E have been the most studied factors relating to disease resistance in animals, including fish. There are reports of a positive effect of high ascorbic acid doses on disease resistance in fish (Hardie *et al.* 1983; Navarre and Halver, 1989; Satyabudhy *et al*. 1989; Hardie *et al*. 1991; Waagbö *et al*. 1993) and on the ability of salmon to produce antibodies as a response to vaccination (Navarre and Halver, 1989; Erdal *et al*. 1991; Waagbö *et al*. 1993). But there are also reports showing that antibody production is not affected by the concentration of ascorbic acid in the diet up to 2 g/kg (Lall *et al*. 1989). This apparent discrepancy may be due to differences in the level of other feed components which interact physiologically with ascorbic acid. One such factor may be iron, which may increase the susceptibility to disease if present in fish at concentrations above the binding capacity of transferrin (Rørvik *et al*. 1991).

There is apparently a further biochemical link between the antioxidant function of ascorbic acid, the prooxidative effect of iron, the level of polyunsaturated fatty acids, and the concentration of the antioxidant vitamin E in the diet (Waagbö, 1994). High levels of n-3 polyunsaturated fatty acids have been proposed to increase disease resistance in fish (Salte *et al*. 1988) and it has been demonstrated that cell membranes of salmon feeding on such diets become stronger and more resistant to lysis (Erdal *et al*. 1991). Since the fluidity of the membranes is a function of the fatty acid composition of the membrane lipids, it is to be expected that leukocyte functions may also be affected by dietary lipids and environmental temperature. However, the interactions are complex and not easy to predict; high levels of n-3 long chain fatty acids in the diet of Atlantic salmon increased the strength of the erythrocyte membranes, but at the same time such diets had an immunosuppressive effect and resulted in reduced survival rates in challenge tests with *Vibrio salmonicida* and *Yersinia ruckeri* (Erdal *et al.* 1991).

There is a current trend to look for feed ingredients which may contribute to better health by interferring with colonisation and microbial growth in the gut. Mannose-rich proteins from yeast belong to the category of compounds which adhere to receptors which are used by pathogenic microbes as the first step of colonisation of the gut. Another group of substances are glutamine/glutamic acid/a-ketoglutaric acid which are known to be energy substrates for intestinal cells and which contribute to a ”healthy gut”. A third group are peptides which in some way interfere with the regulation of the enzyme secretion/digestion processes. In addition there are much exploratory research on prophylactic microbes and selective anti-microbial agents which contribute to maintaining a healthy commensal microbial flora in the intestinal environment.

REFERENCES

Adams, A., 1991. Response of penaeid shrimp to exposure to Vibrio species. Fish and Shellfish Immunology, 1, 59-70.

Barracco, M.A., Duvic, B., Söderhäll, K., 1991. The β- glucan-binding protein from the crayfish, *Pacifastacus leniusculus* when reacted with a β-1,3-glucan, induces spreading and degranulation of crayfish granular cells. Cell Tissue Res., *266, 491-497.*

Blazer, V. S., 1992. Nutrition and disease resistance in fish. Annual Review of Fish Diseases 2, 309-323.

Blazer, V. S., Wolke, R. E., 1984a. Effect of diet on the immune response of rainbow trout (*Salmo gairdneri*). Canadian Journal of Fisheries and Aquatic Science, 41, 1244-1247.

Blazer, V. S., Wolke, R. E., 1984b. The effects of α-tocopherol on the immune response and non-specific resistance factors of rainbow trout (*Salmo gairdneri* Richardson). Aquaculture, 37, 1-9.

de Felippe, J., da Rocha e Silva, M., Maciel, F. M. B., de Macedo Soares, A., Mendes, N. F., 1993. Infection prevention in patients with severe multiple trauma with the immunomodulator beta 1-3 polyglucose (Glucan). Surgery, Gynecology and Obstetrics, 177, 383-388.

Davina, J. H. M., Rijkers, G. T., Rombout, J. H. W. M., Timmermans, L. P. M., van Muiswinkel, W. B., 1980. Lymphoid and non-lymphoid cells in the intestine of cyprinid fish, p. 129-140. In*:* Development and differentiation of vertebrate lymphocytes, (J. D. Horton, ed.). Elsevier/North-Holland Biomedical Press, Amsterdam.

Doggett, T. A., Harris, J. E., 1987. The ontogeny of gut-associated lymphoid tissue in *Orechromis mossambicus*. J. Fish Biol. 31, suppl. A, 23-27.

Ellis, A. E., 1988. Ontogeny of the immune system in teleost fish. pp. 20-31. In: Fish vaccination, (A. E. Ellis, ed.). Academic Press, London.

Engstad, R. E., Robertsen, B., 1994. Specificity of a β-glucan receptor on macrophages from Atlantic salmon (*Salmo salar* L.). Developmental and Comparative Immunology 18, 5, 397-408.

Erdal, J. I., Evensen, Ø., Kaurstad, O. K., Lillehaug, A., Solbakken, R., Thorud, K., 1991. Relationship between diet and immune response in Atlantic salmon (*Salmo salar* L.) after feeding various levels of ascorbic acid and omega-3-fatty-acids. Aquaculture 98, 363-379.

Hardie, L. J., Fletcher, T. C., Secombes, C. J., 1991. The effect of vitamin E on the immune response of the Atlantic salmon (*Salmo salar* L.). Aquaculture, 87, 1-13.

Hardie, L. J., Marsden, M. J., Fletcher, T. C., Secombes, C. J., 1983. *In vitro* addition of vitamin C affects rainbow trout lymphocyte responses. Fish & Shellfish Immunology, 3, 207-219.

Hart, S., Wrathmell, A. B., Harris, J. E., Grayson, T. H., 1988. Gut immunology in fish: A review. Dev. Comp. Immunol., 12, 453-480.

Johansson, M.W., K. Söderhäll. 1985. Exocytosis of the prophenoloxidase activating system from crayfish haemocytes. J. Comp. Physiol., 156, 175-181.

Lall, S. P., Olivier, G., Weerakoon, D. E. M., Hines, J. A., 1989. The effect of vitamin C deficiency and excess on immune response in Atlantic salmon (*Salmo salar*). Proceedings of the Tird International Symposium on Feeding and Nutrition in Fish, pp. 427-441, Toba, Japan.

Maeda, Y.,Yonekawa, H., Chihara, G., 1994. Application of Lentinan as Cytokine Inducer and Host Defense Potentiator in Immunotherapy of Infectious Diseases. In: Immunotherapy of Infections. Ed. Noel Masihi. Nato copyMarcel Dekker, Inc., New York, Basel, Hong Kong

Navarre, O., Halver J. E., 1989. Disease resistance and humoral antibody production in rainbow trout fed high levels of vitamin C. Aquaculture, 79, 207-221.

Nicoletti, A., Nicoletti, G., Ferraro, G., Palmieri, G., Mattaboni, P., Germogli, R., 1992. Preliminary Evaluation of Immunoadjuvant Activity of an Orally Administered Glucan Extracted from *Candida albicans.* Arzneim - Forsch./Drug Res. 42(II). Nr. 10

Robertsen, B., Rørstad, G., Engstad, R., Raa, J., 1990. Enhancement of non-specific resistance in Atlantic salmon, *Salmo salar* L., by a glucan from *Saccharomyces cerevisiae* cell walls. J.Fish Diseases, 13, 391-400.

Rombout, J. H. W. M., Bot. H.E., Taverne-Thiele, J.J., 1989. Immunologic importance of the second gut segment of carp. II. Characterization of mucosal leucocytes. J. Fish Biol., 35, 167-178.

Rombout, J. H. W. M., Taverne-Thiele, J.J., Villena, M.I., 1993. The gut-associated lymphoid tissue (GALT) of carp (*Cyprinus carpio* L.): An immunocytochemical analysis. Dev. Comp. Immunol. 17, 55-66.

Rørstad, G., Aasjord, P.M., Robertsen, B., 1993. Adjuvant effects of yeast glucan in vaccines against furunculosis in Atlantic salmon (Salmo salar L.) Fish Shellfish Immunol., *3, 179-190*

Rørvik, K. A., Salte, R., Thomassen, M., 1991. Effects of dietary iron and n-3 unsaturated fatty acids (omega-3) on health and immunological parameters in farmed salmon. Proceedings from the European Association of Fish Pathologists, p. 71, Budapest, Hungary.

Raa, J., 1996. The Use of Immunostimulatory Substances in Fish and Shellfish Farming. Reviews in Fisheries Science, 4(3), 229-288. CRC Press.

Raa, J., Rørstad, G., Engstad, R., Robertsen, B., 1992. The use of immunostimulants to increase resistance of aquatic organisms to microbial infections. In: Diseases in Asian Aquaculture (I. M. Shariff, R. P. Subasinghe and J. R. Arthus, eds.), p. 39-50. Fish Health Section, Asian Fisheries Society, Manila, Philippines.

Raa, J., 1999. US patent (not disclosed).

Salte, R., Thomassen, M. S., Wold, K., 1988. Do high levels of dietary polyunsaturated fatty acids (EPA/DHA) prevent diseases associated with membrane degeneration in farmed Atlantic salmon at low water temperatures? Bulletin of the European Association of Fish Pathology, 8, 63-65.

Satyabudhy, A. M. A., Grant, B. F., Halver, J. E., 1989. Effects of L-ascorbyl phosphates (AsPP) on growth and immunoresistance of rainbow trout (*Oncorhynchus mykiss*) to infectious hematopoietic necrosis (IHN) virus. Proceedings from the Third International Symposium on Feeding and Nutrition in Fish, pp. 411-426. Toba, Japan.

Shoenherr, W. D., Pollman, D. S., Coalson, J. A., 1994. Titration of MacroGard on growth performance of nursery pigs. J. Anim. Sci. 72: Suppl. 1.

Smets, P., Zalisz, R., Boisnic, F., 1988. Biostim ® (RU 41740), immune functions and infection. Advances in the Biosciences, 68, 85-102.

Smith, V.J., Söderhäll, K., 1983. β- 1,3- glucan activation of crustacean hemocytes *in vitro* and *in vivo*. Biol. Bull., 164, 299-314.

Song, Y-L., Hsieh, Y-T., 1994. Immunostimulation of tiger shrimp (*Penaeus monodon*) hemocytes for generation of microbicidal substances: analysis of reactive oxygen species. Development and Comparative Immunology, 18 (3), 201-209.

Söderhäll,K., Cerenius, L., 1992. Crustacean immunity. In: Annual Rev. of Fish Diseases, pp 3-23; Vol 2. Pergamon Press, Oxford.

Sung, H. H., Kou, G. H., Song, Y. L., 1994. Vibriosis resistance induced by glucan treatment in tiger shrimp (*Penaeus monodon*). Fish Pathology, 29(1), 11-17.

Song, YL, Liu, J.J., Chan, L.C., Sung, H.H., 1997. Glucan induced diseases resistance in tiger shrimp (Penaeus monodon). In: Gudding, R., Lillehaug, A., Midtlyng, P.J., Brown, F., eds. Fish Vaccinology. pp. 413 – 421. Krager

Söderhäll, K., Cerenius, L., Johansson, M.W., 1994. The prophenoloxidase activating system and its role in vertebrate defense. Primordial immunity: foundations for the vertebrate immune system. 712 of the Ann. N.Y. Acad. Sci.: 712:155-161.

Sung, H.H., Yang, Y.L., Song, Y.L., 1996. Enhancement of microbicidal activity in the tiger shrimp *(Penaeus monodon)* via immunstimulation. Journal of Crustacean Biology 16(2), 278-284.

Sung, H.H., Chang, H.-J., Her, C.-H., Chang, J.-C., Song, Y.-L., 1998. Phenoloxidase activity of hemocytes derived from *Penaeus monodon and Macrobrachium rosenberghii.* J. of Invertbrate Pathology 71, 26-33.

Supamattaya., Pongmaneerat., 1998. The effect of MacroGard® on growth performance and health condition of black tiger shrimp. Scientific report, Princ of Songkhla University.

Thompson, A., White, A., Fletcher, T. C., Houlihan, D. F., Secombes, C. J., 1993. The effect of stress on the immune response of Atlantic salmon (*Salmo salar* L.) fed diets containing different amounts of vitamin C. Aquaculture, 114, 1-18.

Thompson, K. D., Cachos, A., Inglis, V., 1993. Immunomodulating effects of glucans in the presence of oxytetracycline in rainbow trout, *Oncorhynchus mykiss*, pp. 31. Second Symposium on Diseases in Asian Aquaculture, Phuket, Thailand.

Trust, T. J., 1986. Pathogenesis of infectious diseases of fish. Ann. Rev. Microbiol. 40: 479-502.

Verlhac, V., Obach, A. Gabaudan, J., Schuep, W., Hole. R., 1998. Immunomodulation of dietary vitamin C and glucan in rainbow trout (*Onchorhynchus mykiss*). Fish and Shellfish Immunology, 8, 409-424.

Waagbø, R., 1994. The impact of nutritional factors on the immune system in Atlantic salmon, *Salmo salar* L.; a review. Aquaculture and Fisheries Management, 25, 175-197.

Waagbø, R., Glette, J., Nilsen, E. R., Sandnes, K., 1993. Dietary vitamin C, immunity and disease resistance in Atlantic salmon (*Salmo salar*). Journal of Fish Physiology and Biochemistry 12: 61-73.