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The use of immunostimulants in fish larval aquaculture

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Abstract

The production of fish larvae is often hampered by high mortality rates, and it is believed that most of this economic loss due to infectious diseases is ca. 10% in Western European aquaculture sector. The development of strategies to control the pathogen load and immuno-prophylactic measures must be addressed further to realise the economic "potential" production of marine fish larvae and thus improve the overall production of adult fish.

The innate defence includes both humoral and cellular defence mechanisms such as the complement system and the processes played by granulocytes and macrophages. A set of different substances such as β-glucans, bacterial products, and plant constituents may directly initiate activation of the innate defence mechanisms acting on receptors and triggering intracellular gene activation that may result in production of anti-microbial molecules. These immunostimulants are often obtained from bacterial sources, brown or red algae and terrestrial fungi are also exploited as source of novel potentiating substances.

The use of immunostimulants, as dietary supplements, can improve the innate defence of animals providing resistance to pathogens during periods of high stress, such as grading, reproduction, sea transfer and vaccination. The immunomodulation of larval fish has been proposed as a potential method for improving larval survival by increasing the innate responses of the developing animals until its adaptive immune response is sufficiently developed to mount an effective response to the pathogen. To this end it has been proposed that the delivery of immunostimulants as a dietary supplement to larval fish could be of considerable benefit in boosting the animals innate defences with little detriment to the developing animal. Conversely, there is a school of thought that raises the concern of immunomodulating a neotanous animal before its immune system is fully formed as this may adversely affect the development of a normal immune response.

Keywords: Immunostimulants; Fish larvae; Aquaculture; Toll-like receptors

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1. Introduction

1.1. Immunostimulants in commercial aquaculture

According to Sakai [1] immunostimulants can be divided into several groups depending on their sources: bacterial, algae-derived, animal-derived, nutritional factors as immunostimulants, and hormones/cytokines.

This sub-grouping is independent of their mode of action. A former definition of immunostimulants that restricted the target cells to be mononuclear phagocyte system [2] only should be redefined in view of recent discoveries of the pattern recognition receptors (PRRs). Different leucocytes may possess different PRRs and may bring about different immunological responses dependent on the binding receptor and intracellular signalling events. As such, a new definition must include all elements of the immune system and the definition could be "*An immunostimulant is a naturally occurring compound that modulates the immune system by increasing the host's resistance against diseases that in most circumstances are caused by pathogens*". Although there is evidence for the beneficial use of immunostimulants in aquaculture, current commercial products are rather restricted in their formulation being derived from yeast, as in the case of β1-3, β1-6 glucans and sold under the Macroguard range of products e.g. MacroGard® Immersion Grade, MacroGard® AquaSol or MacroGard® Adjuvant. Another successful commercial immunostimulant is Ergosan which is made from a seaweed-based meal rich in alginates and polysaccharides. A single dose of 1 mg of Ergosan significantly augmented the proportion of neutrophils, increased the degree of phagocytosis, respiratory burst activity and expression of interleukin-1β (IL-1β), interleukin-8 (IL-8) and one of the two known isoforms of trout tumour necrosis factor-alpha (TNF-α) in peritoneal leucocytes at 1 day post-injection [3]. Humoral immune parameters were less responsive to intraperitoneal Ergosan administration with complement stimulation only evident in the 1 mg treated group at 2 days post-injection [3]. The use of immunostimulants in vaccine formulations especially the β1-3, β1-6 glucans, experimentally at least, has given very good antibody responses when used either to replace oil based adjuvants, without the adverse side effects that have been reported for these types of adjuvants, or in addition to them [4–8].

The nucleotide-supplemented diets produced commercially are not strictly immunostimulants but provide a dietary supplement that allows improved resistance to a pathogen insult. Although it does not seem to induce measurable immunostimulatory effects [9–11], there seems to be an up-regulation of several immune genes in turbot fed these diets, which contradicts the earlier claims that these diets are not immunostimulatory [11].

The biological effects of immunostimulants are highly dependent on the receptors on the target cells recognising them as potential high risk molecules and triggering defence pathways. Thus, it is important to increase the knowledge base concerning receptor specificity and the inflammatory processes the different receptors, upon antigen binding, induce. However, many mammalian receptors, that have been reported to bind immunostimulants, have not yet been found in fish. Nevertheless, assuming that fish and mammalian cells contain many similar receptors one may predict the biological outcome of immunostimulants in fish.

1.2. Receptors for immunostimulants

The discovery of the toll-like receptors (TLRs) in many mammalian species has partly shed light on the mechanisms that different immunostimulants induce. Functional analysis of mammalian TLRs has revealed that they recognise specific patterns of microbial components that are conserved among pathogens, but are not found in mammals. In signalling pathways via TLRs, a common adapter MyD88 (myeloid differentiation factor 88) was first characterised as an essential component for the activation of

innate immunity by all TLRs. On the other hand, recent findings have shown that many TLR may induce specific responses facilitated by their own signalling molecules – without MyD88 [12].

In all TLRs found in mammalian species, the extracellular portions of the TLRs are structurally different, whereas the cytoplasmic domain consists of a Toll/IL-1 receptor domain called TIR. The downstream signalling events, due to ligand binding, are very well documented and reviewed [12,13]. Genes for several TLRs have been identified in zebrafish and pufferfish. To date 19 putative TLR variants are found in zebrafish [14,15], and 10 different TLR genes in pufferfish [16,17]. TLR genes have also been discovered in the Japanese flounder and goldfish [18,19]. Recently, putative TLR3 gene has been found in carp (GeneBank accession no AY162178.1) and TLR5 genes have been sequenced from rainbow trout (AB062504.1 and AB091105.1) and Atlantic salmon (AY628755.1), and both TLR3 and TLR5 have been characterised in channel catfish [20]. The genes for the intracellular adaptor proteins MyD88, SARM, MAL, TRIF and TRAM that also contain TIR domains have been found in zebrafish [14]. Thus, it seems that fish have the TLR and intracellular signalling batteries to facilitate similar responses as observed in many mammalian species.

1.2.1. Toll-like receptor 1

It has been suggested that bacterial triacetylated lipopeptides are preferentially recognised by a heterodimer between TLR1 and TLR2. On the other hand, a heterodimer resulting from the cytoplasmic assemblance of TLR2 and TLR6 has been shown to bind diacetylated mycoplasmal lipopeptide. It has been suggested that compounds with different degree of acylation may bind different receptor complexes and this may result in different gene activation [13]. Triacetylated lipopeptide has been shown to act as an adjuvant, initiating a specific immune response in chickens resulting in increased antibody titres against the immunising antigens [21]. Zebrafish and pufferfish TLR1 equivalents have been reported [14–16] and are expressed at low levels in zebrafish muscle and heart. After experimental infections with *Mycobacterium marinum*, the TLR1, TLR2, TLR5 and TLR9 genes were expressed at higher levels in infected fish compared with controls [15]. However, no functional studies have been carried out using triacetylated peptides to evaluate the immunological significance of fish TLR1/TLR2 or TLR1/TLR6 heterodimers.

1.2.2. Toll-like receptor 2

Compared to other TLRs, the TLR2 recognises a vast number of different ligands [22], either alone, or in most circumstances as heterodimers between TLR1 and TLR4 [23]. To make the picture even more complex, the TLR2 may also form heterodimers with TLR6 [24]. Among a high number of compounds, substances such as zymosan, BCG cell walls, heat shock protein 60, polymannuronic acid polymers and LPS from *Leptrospira interrogans*, *Porphyromonas gingivalis* and *Legionella pneumohila* have been shown to be ligands for TLR2 [22]. From an immunostimulatory point of view, zymosan has been extensively used as a reference compound monitoring immune functions in fish and as a non-specific complement component C3 activator [4,25,26]. Zymosan is isolated from *Saccharomyces cerevisiae* and is composed of β-glucans and mannan with minor lipid and protein contents [27]. Due to this fact it is possible that zymosan can bind both mannose receptors and TLRs that recognise e.g. mannan and β-glucans, respectively. The binding of β-glucans of fungal origin to Dectin-1 [28] and TLR2 has recently been shown [29,30]. Dectin-1 expression increases TLR-mediated activation of nuclear factor κB by glucan-containing particles and these molecules act synergistically with each other mediating production of cytokines such as IL-12 and TNF-α [30]. Dectin-1 is a type II transmembrane receptor containing one lectin-like carbohydrate recognition domain which binds β1,3- and β1,6-linked glucopyranose residues either as pure polymers or as polysaccharides in cell walls. This receptor may also recognise an endogenous ligand on T cells [28]. The receptor is expressed at high levels on macrophages and neutrophils, and to a lesser extent in dendritic cells and a subpopulation of T cells [31]. Using gene knockout technology, MyD88, the adaptor protein that binds the TLR cytoplasmic domain and

is vital for downstream signalling, and TLR2 have been shown to be responsible together with Dectin-1 to induce production of TNF-α. This production was abolished in the MyD88 and TLR2 knockout mice [29]. Furthermore, it has been shown that Dectin-1 binding of zymosan triggered the production of reactive oxygen species (ROS). By the co-operation with TLRs β-glucan may promote responses associated with Th1 immunity including production of IL-12. Such responses are critical for defence against many pathogens. TLR2 gene sequences have been found in zebrafish, pufferfish and Japanese flounder [14–16,18]. In zebrafish it has been found expressed in blood, skin, brain, liver, spleen and ovaries [14,15]. TLR2 expression was found in developing embryo, but the significance of this expression was, however, not discussed. Expressions of TLR2 and TLR4 have been found in prenatal mice liver but not in the lungs, the authors speculated that deficient expressions during the prenatal stage was consistent with an immature alveolar immune system [32]. The immunological effects of β-glucans have extensively been reviewed elsewhere [1,33–37]. There is no established explanation of why the β-glucans have positive immunological effects in fish. By unravelling the molecular targets for β-glucans (e.g. Dectin-1 and TLRs), the downstream signalling events and the gene activation that results in cytokine production, it may be possible to establish under what circumstances the β-glucan group of immunostimulants should be used.

1.2.3. Toll-like receptor 3

Viral double stranded RNA and poly I:C have been shown to bind TLR3. TLR3 deficient mice macrophages were shown to have greatly reduced responses to poly I:C [38]. Although many studies have been performed to characterise the biochemical pathways activated by poly I:C, the role of TLR3 in viral infection in vivo remains a key avenue of research to be explored [39]. Poly I:C has been extensively studied in fish systems where its induction of antiviral activities in vivo and in vitro have been shown [40–45]. The antiviral activity generated after ligand binding and intracellular signalling has been addressed to the production of type I interferons and Mx proteins. Human natural killer cells (NK cells) have been reported to express TLR3, to up-regulate TLR3 mRNA and cytotoxic activity following poly I:C stimulation [46]. The NK cells also produced higher amounts of IL-6, IL-8 and IFN-γ after TLR3 stimulation. Taken together, there is substantial evidence that the use of dsRNA could be helpful to prevent viral diseases. Whether such use is applicable in sustainable fish farming remains to be investigated. Lockhart et al. [43] observed severe pathological changes in Atlantic salmon post-smolt livers resulting from apoptosis and necrosis of hepatocytes after poly I:C treatment. Also dose-dependent mortalities were recorded. A high level of TLR3 mRNA expression was found during embryonic development of zebrafish and a high number of organs and tissues were shown to constitutively express the TLR3 at relatively high levels [14,15]. In channel catfish there was an up-regulation of TLR3 following experimental challenge with *Edwardsiella ictaluri* up to 21 days post-infection in kidney [20]. Conversely, no up-regulation of TLR3 was observed in zebrafish infected by *M. marinum* [15]. Whether the up-regulation of TLR3 in the channel catfish was a result of overall and general immunostimulation caused by bacterial products was not addressed. Following this, whether a "mild" challenge of non-pathogenic bacteria would confer protection of fish against viral pathogens should be addressed. If the TLR3 is central in mounting antiviral mechanisms a high expression level together with wide tissue distribution would imply that fish have a high potential for antiviral defence, especially against dsRNA viruses, if the proper signalling events is present.

1.2.4. Toll-like receptor 4

Lipopolysaccharide (LPS) is an integral component of the outer membrane of Gram-negative bacteria, and has been used in experimental systems for several decades as a potent immunostimulant. Several proteins may capture circulating LPS in bloodstream, one of them is lipopolysaccharide binding protein (LBP) [47]. Assemblance of LBP, CD14 (a glycosylphosphatidyl inositol (GPI)-anchored cell surface glycoprotein that recognises a variety of polysaccharides) and LPS complexes on the cell surface may

trigger activation of several members of mitogen-activated protein kinase family and NF-κB. Indeed, the LBP/CD14/LPS complex has affinity to the TLR4 that results in cell activation. A high number of other ligands have been reported to bind TLR4 such as several heat shock proteins, fibrinogen, beta-defensins and polymannuronic acid [22]. The formation of CD14/LPS complex significantly reduces (100 to 1000-fold) the concentration of LPS required for activation of macrophages as compared with LPS alone. Myeloid differentiation protein-2 (MD-2) is also crucial for LPS responsiveness. MD-2 is a small protein that lacks a transmembrane domain and is reported to bind to the extracellular part of TLR4. This protein has been shown to regulate the intracellular distribution of TLR4, which, in MD-2 deficient cells, is primarily localised to the Golgi apparatus. This has been shown to be the case in intestinal epithelial cells where there is a lack of LPS responsiveness [48].

The signalling events after CD14/MD-2/LPS/TLR4 association are either dependent or independent on MyD88. MyD88 is an adaptor protein that has two binding sites, one that binds to TIR domain of TLR and IL-1R, and the other binds the N-terminal death domain of IL-1 receptor-associated kinase (IRAK) and recruits it to the receptor complex [48]. These two binding events lead eventually to the production of TNF-α, IL-6 and IL-1β. The MyD88 independent signalling results in IFN-inducible gene expression by interferon regulatory factor 3 (IRF3). The MyD88 independent LPS cell activation has also been shown to induce iNOS expression [49]. Interestingly, it is evidenced that only TLR4, TLR3 and TLR7-9 can activate IRF3 leading to production of IFN-β [50]. It is proposed that CD11/CD18 β2 integrins, a family of heterodimeric glycoproteins expressed on leucocytes, also participate in the binding of LPS, where the individual receptors (e.g. TLR4, CD14, CD11/CD18) are brought together to elicit a complex pattern of signalling and gene activation [51]. Several zebrafish TLR4 orthologs have been described [14], where zebrafish TLR4.2 only is expressed at a high level in the blood. The expression of zebrafish TLR4.1 mRNA was relatively high during the early stage of embryonic development, whereas the expression of TLR4.2 increased during embryonic development [14,15]. This could be related to the development of the whole range of blood cells. The use of LPS is widely acknowledged in studying fish immunological responses [1,33,35,52]. However, the functional assessments of TLR4, CD14, MD-2 have not yet been studied in fish. The closest being the study of Seternes et al. [53] that discovered that LPS was selectively bound to scavenger receptors in cod heart endothelial cells. However, to differentiate the affinity of TLR4 complex towards LPS from the scavenger receptor binding of LPS is a major challenge in order to identify possible biding sites for LPS on fish cells. Polymannuronic acid polymers (PM), an alginate rich in mannuronic acids, have been reported to bind TLR4-MD-2/TLR2 heterodimers [54] on human monocytes, and this polysaccharide has been used as an immunostimulant in fish [55,56]. Increased specific daily growth of spotted wolffish and Atlantic cod larvae, that were fed formulated feed containing PM, has been observed (R.A. Dalmo; unpublished data). Atlantic halibut, repeatedly fed *Artemia* enriched with PM for short periods, showed increased disease resistance to *Vibrio anguillarum* O2 compared with fish fed control feed [57]. Whether fish TLR4 receptor complex recognises PM is yet unknown.

1.2.5. Toll-like receptor 5

The bacterial protein, flagellin, is recognised by TLR5 [58]. The ligand binding induces MyD88/IRAK/TRAF6 dependent NF-κB activation and production of pro-inflammatory cytokines. TLR5 genes have been discovered in zebrafish where their expression was found to be low during ontogeny. The tissue expression of TLR5 was also found to be low [14]. However, a peak up-regulation of TLR5 at days 5–8 was observed in the adult channel catfish liver after *E. ictaluri* challenge [20], this increased expression was indicative of activation of innate defence responses.

1.2.6. Toll-like receptor 6

The TLR6 forms heterodimers with TLR2. The ligands, besides those mentioned under TLR2, for this heterodimer are e.g. modulin, soluble tuberculosis factor, group B streptococci soluble factor and necrotic

cells [22]. Peptidoglycan, that is especially abundant in Gram positive bacteria, has been reported to increase innate defence mechanisms in rainbow trout, and following 24-day oral administration disease resistance against *V. anguillarum* was observed. The disease resistance of 56-day fed fish was, however, not different compared to the controls [59]. Besides TLR2/TLR6 heterodimers, several proteins have been reported to bind peptidoglycan such as CD14, peptidoglycan-binding proteins (PGBPs) and Nod (nucleotide-binding oligomerization domain) proteins [60] and are proposed to be central in disease resistance against Gram positive bacterial infections. Nods are a family of cytoplasmic proteins with structural homology to a large family of plant resistance proteins. They can recognise intracellular bacteria and their products such as peptidoglycan.

1.2.7. Toll-like receptor 7

Antiviral and antitumor substances such as imiquimod, R-848, loxoribine and bropirime are members of imidazoquinolinamines and are all TLR7 ligands [13] that upon receptor binding with TLR7 induce NF-κB translocation and production of IFN-α, TNF-α, IL-6 and IL-12. The resulting CD4+ T cell activation and Th1 immune response is vital for the hosts antiviral defence. The natural ligand for TLR7 remains unclear. The expression of TLR7 gene was found in many organs and tissues in zebrafish, the expressed gene was also found in zebrafish during embryonic development [14].

1.2.8. Toll-like receptor 8

In zebrafish two TLR8 genes have been found [14,15] where TLR8.1 is expressed in blood, digestive organs, testis, skin, liver, ovaries and heart whereas TLR8.2 is expressed in blood, testis, muscle, skin and heart [14]. The analysis of the ontogenic appearance of TLR8 revealed that TLR8.2 expression is absent prior to 5 days post-fertilisation, and high expressions of TLR8.1 were found throughout ontogenic development [14]. It has been suggested that TLR7 and TLR8 confer antiviral activity after receptor binding by single-stranded RNA (ssRNA) [61].

1.2.9. Toll-like receptor 9

Synthetic oligonucleotides, containing CpG islands, have been shown to bind the TLR9 inducing production of antiviral IFN-α [62]. The earliest trial in fish was conducted in 1999 where synthetic ODN was shown to be inefficient to increase antibody production towards co-administered β-Gal in goldfish [63]. The authors concluded that the action of CpG was dependent on phyla. Since then, several other authors have challenged this view. Following this, Atlantic salmon leucocytes were shown to produce antiviral activities and IL-1 [42,64,65]. Studies in catfish have suggested that natural cytotoxic cells (NCC) recognise bacterial nonmethylated DNA [66]. In carp, the synthetic oligodeoxynucleotides (ODNs) containing unmethylated CpG were evaluated for their ability to enhance the innate immune response. Intraperitoneal injection of CpG-ODNs to carp daily for 3 days, resulted in increased phagocytic activity and increased respiratory burst activities in kidney phagocytic cells. The serum lysozyme activity also increased in fish treated with CpG-ODNs [67]. Another study showed that CpG had immunomodulatory effects on grass carp macrophages by increasing the levels of superoxide anion, hydrogen peroxide, acid phosphatase and by increasing the bactericidal activity [68]. Similar results have also been shown in olive flounder [69]. Induction of antiviral activity in vivo by the use of TLR9 ligands remains to be shown. TLR9 genes are found both in pufferfish and in zebrafish [14–16]. In zebrafish, the expression of TLR9 is increased after experimental infection with *M. marinum* [15], and has been shown to increase during embryonic development [14]. The highest expression of TLR9 was found in blood of the zebrafish [14].

1.3. Immunological responses following immunostimulation

The pro-inflammatory cytokines TNF-α, IL-1 and IL-6 up-regulate the acute phase response with the production of complement components, C-reactive protein, ceruloplasmin, metallothionine etc. These cytokines also increase the epithelial response to pathogens and induce expression of adhesion molecules on vascular endothelium enhancing the diapedesis of leucocytes. The pattern of host responses is determined ultimately by the pathogen and the innate receptors it triggers. The triggering leads to a local cytokine/chemokine milieu that varies and can affect the direction of the acquired response i.e. Th1 versus Th2 response. Th1 effector cells may activate macrophages, induce B cells to produce opsonising antibodies (cell-mediated immunity), whereas Th2 cells are efficient to activate B cells to make neutralising antibodies, and have various effects on macrophages (humoral immunity). The Th1 response includes the production of IL-1, IFN-γ and TNF-α, whereas the Th2 response produces IL-4, IL-5 and IL-13 amongst others. The central cytokines helping to direct the Th polarisation are IFN-γ and IL-4. IFN-γ induces "reductive" macrophages that produce IL-12 and IL-18, but have deficient capacity for production of IL-6 and IL-10. IL-4 induces "oxidative" macrophages with high levels of IL-6 and IL-10, but low levels of IL-12. The activation of "reductive" macrophages may lead to a Th1 response, and a Th2 response is facilitated by induction of "oxidative" macrophages [70]. Whether newly hatched fish larvae have the capacity to mount both cell-mediated and humoral immunity has not yet been addressed and it is certain there will be considerable inter-species variation relating to this issue.

Assuming that, at a certain developmental point, fish larvae will have developed the full compliment of leucocytes and a comprehensive cytokine network needed for cellular and humoral immune responses, one may, broadly speaking, apply different immunostimulants to selectively direct immune responses towards a cell-mediated or humoral immunity. It has been proposed that ligand binding to TLR1, TLR2, TLR3, TLR4 (the MyD88 independent pathway) and TLR7, together with IRF3 translocation, are important in inducing antiviral activities [39]. However, since much of the intracellular events leading to the antiviral "state" are not fully understood, one must be cautious in concluding that only these four TLRs are "antiviral" receptors [39]. Correspondingly, it is proposed that mannose receptors, TLR2 and TLR4, and Dectin-1 may co-operationally induce fungicidal responses [71] although there much work remains to prove this relationship. It could be argued that larval fish have not yet developed the full range of cellular and molecular properties, compared to juveniles and adult fish. This is important when immunostimulation is the issue. As such, immunostimulation of fish larvae could simply be a waste of time and money. It is clear more fundamental studies, with respect to immune defence mechanisms and responses, are indeed needed to make the use of immunostimulants feasible for fish larval aquaculture. However, there are several studies conducted on fish larvae that have revealed some beneficial effects of immunostimulants in terms of survival and growth. The next question to solve is to find the molecular differences caused by such immunostimulants.

1.4. Benefits and "non-benefits" using immunostimulants in fish larval aquaculture

The perceived benefits of using immunostimulants in larval culture are numerous, but mainly theoretical. Immunostimulants should up-regulate the innate defences of a larval fish putting it in a more prepared state to meet and overcome an invading pathogen. This may be induced by an increase of known defensive proteins such as complement (zymosan induced) or interferon or the activation of cellular defences such as macrophages [1]. However, much of the perceived benefit remains theoretical based on their known function in mammals.

What remains an enigma is the impact immunostimulants have on the developing immune system of a larval fish. Some researchers maintain that the effect is minimal and immunostimulants can be fed to

larval fish as soon as the animal can be weaned onto an artificial diet. The other school of thought is that administering potentially powerful immunomodulating compound to an animal that is still to undergo major developmental milestones such as thymus maturation (see Bowden et al., this issue) is detrimental. Although not tested, this hypothesis is based on the assumption that a larval fish has a finite number of immunologically or potentially immunologically mature cells and that the presentation of a molecule that fools the immune system into mounting an immune response will adversely impact these populations of cells by either skewing the developing immune system to be primed, possibly irreversibly, to respond to these stimuli or to down-regulate this response if tolerance is induced.

1.5. Risks and benefits using immunostimulants in aquaculture

In aquaculture, immunostimulants are more widely applied than in larval culture and there are many examples of the successful use of immunostimulants to improve fish welfare, health and production. One of the earliest applications of immunostimulants in aquaculture was the use of glucans in salmon diets. These diets were considered to be effective in managing disease outbreaks after stressful events such as grading and there was believed to be some benefit in reducing sea lice settlement; allowing the stock to go longer between anti-sea lice treatments. Certainly, the use of in-diet immunomodulators has become widely accepted in both salmonid and non-salmonid aquaculture with commercially available diets supplemented with nucleotides which have been demonstrated to reduce sea lice settlement and provide better protection against *Aeromonas salmonicida* and *V. anguillarum* infection [9,10].

In non-salmonid species, the state of the art may be considered to be more advance than in salmonid culture with the application of immunostimulants in sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) culture being more widely applied than in salmonid culture. For example, long-term use of glucans in sea bass has been reported [72]. Such benefits have also been reported for sturgeon [73] and turbot [11]. Novel immunostimulants such as propolis and *Mucor circinelloides* cell walls on the immune system of gilt head sea bream [74–75] and alginates have been investigated in halibut [57] and nisin in turbot [76].

Salmonid research seems to be concentrating on the use of immunostimulants in managing viral diseases possibly due to the serious impact IPNV is having on newly transferred smolts in Northern Europe [77–80]. Here, immunostimulants such as poly I:C [43,82,83] have been used to evaluate the potential of Mx induction to protect against IPNV infection, with variable results. However, it does seem that there is a potential to improve post-smolt survival by the application of immunostimulants.

Naturally, there is a risk that the use of immunostimulants in aquaculture may cause unforeseen problems. The most obvious contra indication, as it would be in larval fish, is tolerance. Tolerance has been reported in some fish species [84–86] and antigen competition has been observed in Atlantic salmon suggesting that the mechanisms for the induction of tolerance may exist [87] but are poorly understood. It is felt that a fundamental study on the mechanisms for induction of immune tolerance in fish should be a high research priority. Other problems that may occur with the use of immunostimulants in aquaculture are less well known. These may include palatability problems, problems with hierarchies where dominate fish receive much more of the immunostimulants than subordinate individuals and the timing of the delivery of the immunostimulants.

1.6. Delivery of immunostimulants to larval fish

The feed delivery methods are well established and used by most commercial feed manufactures to incorporate compounds such as MacroGard® into their diets. However, there is little agreement on the strategy to be adopted in weaning and crumb diets for the delivery of immunostimulants. Some strategies

suggest continual delivery, others a pulsed delivery [88]. These issues need to be resolved before dietary delivery of immunostimulants to larval fish is widely attempted.

Bath delivery is less frequently used for the commercial delivery of immunostimulants as there are several drawbacks although commercial products do exist such as MacroGard® Aquasol™. In flow-through systems where the tank water is continually replaced, the dose of immunostimulant will constantly need to be replaced to maintain a therapeutic dose. This may make the immunostimulant impractical or too expensive to use. In recirculation systems, the addition of an organic compound such as an immunostimulant may adversely affect the functioning of the filters due to the increased organic load. This in turn may lead to an increase in ammonia, nitrite and nitrate and an associated decline in water quality. However, bath delivery is the only option available to immunostimulate yolk sac animals that have yet to open their buccal cavity or develop a functional digestive tract. In some species this may be desirable but, as discussed later, if larval fish have a finite number of immunologically or potentially immunologically mature cells, the presentation of an immunostimulant may adversely impact these populations of cells.

Given the concern over the actual benefit of immunostimulating larval fish, these issues need to be resolved before dietary or bath delivery of immunostimulants to larval fish is widely attempted.

1.7. The use of immunostimulants in immunologically mature and immunologically immature (larval) fish

It is generally accepted that the feeding of immunostimulants to immunologically mature fish is beneficial, providing improved protection against bacterial [36,89] and to a lesser extent viral diseases [9,90]. In addition, the inclusion of an immunostimulant in experimental vaccine preparations has been shown to improve the duration of protection and increase the magnitude of the immune response, especially following intraperitoneal injection of the vaccine preparation [4–7,91,92]. Conversely, there are several reports that the use of immunostimulants has conferred no beneficial effects [4,7,93,94]. Indeed some of these preparations have caused toxicity [93,95] to the animals following oral or injection administration of the immunostimulant.

The immunomodulation of larval fish has been proposed as a potential method for improving larval survival by increasing the innate responses of the developing animals until its adaptive immune response is sufficiently developed to mount an effective response to the pathogen. To this end it has been proposed that the delivery of immunostimulants as a dietary supplement to larval fish is of considerable benefit, boosting the animals innate defences with little detriment to the developing animal. Conversely, there is a school of thought that raises the concern of immunomodulating a neotanous animal before its immune system is fully formed as this may adversely affect the development of a normal immune response. Following this, the leukocyte profiles in carp and sea bass larvae that were fed formulated feed containing bioactive alginate and LPS were different from fish that were fed control feed. This probably would infer changes to the innate and acquired defence of these fish larvae but the significance of these finding is not clear with regards to disease resistance (G. Scapigliati, H. Huttenhuis and J.H.W.M. Rombout, personal communication). However, both of these hypotheses have been poorly tested. Feed manufactures have often included immunostimulants in their larval feeds and live food enrichment products for some time, often with no apparent success. However, there is little published in the literature that demonstrates an adverse affect of the immunostimulants on the developing immune system of larval fish.

The range of potential immunostimulants for larval fish is quite limited and a short review can be found in Table 1. There are several studies that have shown the induction of tolerance in mammals if inappropriate strategies for the delivery of immunostimulants are adopted [96–101] and a single study in the shrimp *Penaeus monodon* [102]. So the field of immunostimulation in larval fish is clearly lacking in appropriate studies and little is known concerning the impact the immunostimulant has on the developing immune system.

Table 1
The immunostimulants used in experimental fish larval aquaculture

Species	Immunostimulant used	Results	Reference
Atlantic cod (*Gadus morhua*)	Cod milt proteins	Better survival post challenge	[109]
Turbot (*Scophthalmus maximus*)	Alginate	Better overall larval survival and growth	[56]
Common snook (*Centropomus undecimalis*)	Unclear implemented at bacterial cell wall components	Better overall larval survival and growth	[108]
Common dentex (*Dentex dentex*)	β1-3, β1-6 glucans	Better overall larval survival and growth	[103]

However, it should be stressed that the use of immunostimulants in larval fish must be approached with caution. There is no conclusive evidence that feeding an immunostimulant to an animal with an immature or developing immune system, such as that present in a larval fish up to and around metamorphosis, may be detrimental to the optimal development of later immune responses. However, there is great potential to improve larval survival against bacterial and viral pathogens by the judicious use of immunostimulants.

1.8. Theoretical benefits

The theoretical benefit of immunostimulants is considerable. They have the potential to elevate the innate defence mechanisms of fish prior to exposure to a pathogen, or improve survival following exposure to a specific pathogen when treated with an immunostimulant. Table 2 summarises the main findings of the experimental studies in fish, whereas the following immunostimulants have been found to be effective against the following groups of pathogens

- Bacterial infections; improved resistance obtained with β-glucans, FK-565 (synthetic acyltripeptide), MDP (muramyl dipeptide) and peptidoglycan.
- Viral infections; improved resistance obtained with levamisole, CpG, ssRNA, MDP, β-glucans and LPS
- Parasitic infections; improved resistance obtained with β-glucans, MDP and levamisole.

Table 2
The major responses seen in fish treated with immunostimulants

In vivo effect	In vitro effect
Increased survival after challenges with bacteria	Increased *macrophage* activity including:
Anti-parasitic effects including reduced settlement of sea lice	• Phagocytosis
Improved resistance to viral infection and increased interferon levels	• Free radical production
	• Enzyme activity
	• Migration activity
	• Production of cytokines
	• NO production
	• Bacterial killing
Growth enhancement	Increased cytotoxicity
Increased antibody production following vaccination	Increased lysozyme activity
Increased lysozyme levels	Increased cytokine induction
	Increased oxygen radical induction
	Increased cell proliferation

1.9. Perceived benefits of immunostimulants

The perceived benefits of immunostimulants are many and varied. Initially immunostimulants are often naturally occurring molecules that can be obtained from a natural source in large amounts, e.g. glucans from yeast [5,6,72,103–106] or chitosan [5,89,93,107] from arthropods such as shrimps shell meal. Potentially they can make cost effective dietary supplements due to the relatively low cost of their source ingredients.

The use of immunostimulants, given as dietary supplements, can improve the innate defence of the animal providing resistance to pathogens during periods of high stress, such as grading, sea transfer and vaccination [35,56,72,103,108].

The use of immunostimulants in vaccine formulations especially the β1-3, β1-6 glucans, experimentally at least, has given very good antibody responses when used either to replace oil based adjuvants, without the adverse side effects that have been reported for these types of adjuvants, or in addition to them [4–8].

1.10. Strategies for the use of immunostimulants in immunologically mature fish

In fish that are immunologically mature, i.e. those fish that have undergone metamorphosis and have a fully developed range of immune responses, there are only two effective strategies that will be of use. These are continual feeding of the immunostimulant, or, pulse feeding of the immunostimulant. Bathing the fish in a solution of immunostimulant is a third strategy but does not seem to have been studied for adult fish. This may be due to the problems in maintaining a constant dose if a flow to waste system or bio-degradation in recirculatory systems.

Continual feeding of immunostimulants has generally been abandoned, in adult fish, in favour of pulse feeding. There are two possible outcomes of continuous feeding of an immunostimulant. Firstly, the immunostimulant up-regulates the immune system to heightened levels and this is maintained until the immunostimulant is withdrawn (Fig. 1). However, this is a very rare occurrence. Secondly, continual

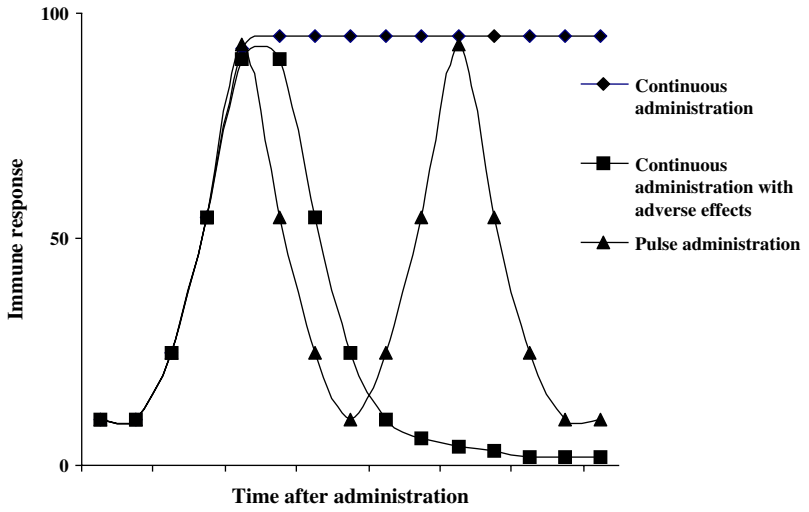

Fig. 1. Possible outcomes after administration of immunostimulants to fish. Continuous administration of an immunostimulant may up-regulate the immune system to heightened level and this is maintained until the immunostimulant is withdrawn (line designated by rombic boxes), whereas continuous administration may cause adverse effects such as tolerance or immunosuppression (line designated by squares). Conversely, pulse administration may oscillate the immune response from a resting level to an enhanced response then back to resting again (line designated by triangles).

exposure to an immunostimulant can induce tolerance. This is caused by the immune system of the host becoming de-sensitised to the immunostimulant and the immunostimulant response is lost. In extreme circumstances the continued expose to an immunostimulant causes the immune response to become suppressed, giving a lower level of innate defences whilst exposure to that particular immunostimulant is maintained (Fig. 1, line designated by squares).

To overcome this, most immunostimulation strategies involve pulse feeding the immunostimulant for a short period, usually 4–6 weeks, to up-regulate the immune response. The immunostimulant is then withdrawn for a similar period of time and the level of immunostimulation falls back towards the resting level before another dose of immunostimulant is then given. This tends to cause the host immune system to oscillate from the resting level to an enhanced response then back to resting again (Fig. 1, line designated by triangles). Obviously, such a strategy offers immense flexibility in fish farming as the immunostimulant can be fed during periods of increased disease risk. Such periods of risk include: spring and autumn ambient temperature changes; for batch spawning fish such as cod or halibut, just prior to the breeding season; or to smolts just before sea transfer.

1.11. Strategies for the use of immunostimulants in immunologically immature fish

Theoretically, larval fish can be bathed in a solution of an immunostimulant or it can be incorporated into their diet to provide improved protection through crisis periods such as end of yolk sac/first feeding or metamorphosis. However, the majority of studies concern delivery of immunostimulant in the diet [56,103,109] and only Kennedy et al. [108] have opted for an environmental approach to the delivery of immunostimulants.

2. Conclusions

With the discoveries of pattern recognition receptors the biological effects of a few immunostimulants have been studied. Although the output gene activation and the resulting production of pro-inflammatory cytokines have been studied there remains much research to systematise the effects of immunostimulants with regard to structure–activity relationship, and the global effects the immunostimulants induce. In most cases, many intracellular signalling events that lead to e.g. disease protection caused by a certain immunostimulant have to be defined properly. Inevitably, many immunostimulants used in fish experiments induce beneficial effects such as disease protection due to increased cellular and humoral responses. However, cautions have to be taken regarding issues such as tolerance, non-wanted side effects such as immunosuppression using too high doses of immunostimulants or non-desirable effects caused by a prolonged use of such compounds. It is hoped that following the development of genomic and proteomic tools for several fish species, many issues with special attention to immune response polarisation after receptor binding of immunostimulants will be unveiled.

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