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2 **Adjuvants and immunostimulants in fish vaccines: Current knowledge and future**  
3 **perspectives**

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21

22 **Abstract**

23

24         Vaccination is the most adequate method to control infectious diseases that  
25 threaten the aquaculture industry worldwide. Unfortunately, vaccines are usually not  
26 able to confer protection on their own; especially those vaccines based on recombinant  
27 antigens or inactivated pathogens. Therefore, the use of adjuvants or immunostimulants  
28 is often necessary to increase the vaccine efficacy. Traditional adjuvants such as mineral  
29 oils are routinely used in different commercial bacterial vaccines available for fish;  
30 however, important side effects may occur with this type of adjuvants. A search for  
31 alternative molecules or certain combinations of them as adjuvants is desirable in order  
32 to increase animal welfare without reducing protection levels. Especially, combinations  
33 that may target specific cell responses and thus a specific pathogen, with no or minor  
34 side effects, should be explored. Despite this, the oil adjuvants currently used are quite  
35 friendlier with respect to side effects compared with the oil adjuvants previously used.  
36 The great lack of fish antiviral vaccines also evidences the importance of identifying  
37 optimal combinations of a vaccination strategy with the use of a targeting adjuvant,  
38 especially for the promising fish antiviral DNA vaccines. In this review, we summarise  
39 previous studies performed with both traditional adjuvants as well as the most  
40 promising new generation adjuvants such as ligands for Toll receptors or different  
41 cytokines, focusing mostly on their protective efficacies, and also on what is known  
42 concerning their effects on the fish immune system when delivered *in vivo*.

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## 73 1. Introduction

74

75 Disease prevention by vaccination is, on economic, environmental and ethical  
76 grounds the most appropriate method for pathogen control currently available to the  
77 aquaculture sector. Traditionally, vaccines comprise either live-attenuated, replicating  
78 pathogens or non-replicating, inactivated pathogens or their subunits. In many countries,  
79 live vaccines are not approved for use in aquaculture for safety reasons, while  
80 inactivated vaccines based on either killed pathogens or isolated non-replicating  
81 pathogen subunits, are in many cases, weakly immunogenic. Thus, adjuvants or  
82 immunopotentiators, are highly required for the elicitation of immune responses that  
83 may be 100% protective against certain pathogens.

84 During the past, fish vaccines were made by a trial-and-error approach  
85 (conventional vaccine design) including pathogen identification, pathogen cultivation,  
86 and vaccine formulation containing whole cell preparation and oils. Through this  
87 strategy, vaccines based on whole inactivated extracellular bacterial pathogens were  
88 quite efficient; resulting in important reductions in mortalities and antibiotic usage in  
89 the aquaculture industry [1]. However, many of the economically disastrous diseases of  
90 today are due to intracellular pathogens, and for this type of pathogens the production of  
91 effective vaccines has not been an easy task. In this sense, the most promising future  
92 vaccines that induce protection against viruses are DNA vaccines. Intramuscular  
93 injection of a DNA plasmid encoding an immunogenic antigen has proved very  
94 effective in fish, in comparison to the results obtained in other animal models such as  
95 mammals [2]. Because the antigen is produced by the fish cells, it is exposed on the cell  
96 surface both directly or processed in the context of both major histocompatibility  
97 complex (MHC) class I and MHC class II, thus effectively triggering both humoral and

98 cellular immune responses. Although DNA vaccines offer a number of advantages over  
99 conventional vaccines, there are still many aspects that may be optimised with adjuvant  
100 help such as alternative routes of immunisation that allow mass-vaccination. Therefore,  
101 fish vaccine approaches must be subjected to rational vaccine design wherein a  
102 combination of a tailored adjuvant system with the most appropriate antigen is used to  
103 create vaccines that may provide a more effective immune response against a specific  
104 pathogen with minimal side effects.

105         On the other hand, many aspects of fish immunology are still unknown and we  
106 are far from close to understanding on which immune mechanisms the protection  
107 against many of these pathogens resides [3]. Moreover, as we know of today, there are  
108 close to 22000 different fish species, and most of them have their “immune  
109 peculiarities”. Without a doubt the innate defence system of fish is strongly developed  
110 and may cope with many infectious agents, helping the fish to eradicate viruses, bacteria  
111 and even parasites. However, many infectious agents resist innate defence mechanisms,  
112 and then an adaptive immune response, present for the first time in evolution in teleost  
113 fish, must come into play to fight these pathogens, being this adaptive response the  
114 basis for vaccinology. The adaptive immune response provides the vertebrate immune  
115 system with the ability to recognise and remember specific pathogens, to be able to  
116 mount stronger and faster responses each time this pathogen is encountered. In higher  
117 vertebrates, adaptive immunity to extracellular pathogens is generally mediated by  
118 humoral immune responses (antibodies), while immunity to intracellular pathogens  
119 (including viruses) often relies on cellular immune responses (cytotoxic T cells). In fish,  
120 and despite the fact that the main elements for an adaptive immune response are present  
121 in most species, the regulation of these elements greatly differs from mammalian  
122 systems and even among different species. Both immunoglobulin (Ig) or B cell receptor

123 (BCR) and T cell receptor (TCR) genes are known among all lineages of gnathostomes  
124 (jawed vertebrates), but fish Ig are expressed as only as three isotypes (IgM, IgD and  
125 IgT) with no isotype switching and low affinity maturation [4]. Interestingly, there is a  
126 tight link between the innate and adaptive system that has not been much explored in  
127 fish immunology. This link, governed by several innate receptors and signalling  
128 molecules such as cytokines and transcription factors, may provide the key for the  
129 future rational design of vaccine adjuvants, since recent advances in immunology have  
130 shown that the magnitude and specificity of the signals perceived by the innate immune  
131 cells following vaccination shape subsequent adaptive immune responses [5].

132

## 133 **2. Principles of adjuvant actions**

134

135 Adjuvants (from Latin *adjuvare* meaning “to help”) have traditionally been  
136 defined as helper substances that increase the magnitude of an adaptive response to a  
137 vaccine (potency), or ability to prevent infection and death (efficacy). But nowadays  
138 scientists have acknowledged that adjuvants may become more important in the way  
139 adjuvants guide the type of adaptive response against a specific pathogen. Therefore,  
140 adjuvants have been now defined as a group of structurally heterogeneous compounds  
141 able to modulate the intrinsic immunogenicity of an antigen [6]. They can be classed  
142 according to their chemical nature or physical properties, however since even related  
143 compounds can have very different immunomodulating capacities, novel classifications  
144 have focused on the immunological events they induce, even though for many of them  
145 the exact mechanism of action is unknown. At present, the classification of adjuvants  
146 that distinguishes between signal 1 facilitators and signal 2 facilitators has been widely  
147 accepted [7]. According to this two-signal model, both the presentation of an antigen

148 (signal 1) and the additional secondary signals (signal 2) are required for activation of  
149 specific T and B lymphocytes, which form the adaptive arm of the immune system [8].  
150 The signal 1 facilitators influence the fate of the vaccine antigen in time, place, and  
151 concentration, ultimately improving its immune-availability, while signal 2 facilitators  
152 provide the co-stimulation signals during antigen recognition that will provide an  
153 adequate environment for the most adequate antigen-specific immune response.

154 Another important aspect of the immune response against many adjuvants is the  
155 recognition of microbes through the detection of conserved molecular patterns,  
156 designated as pathogen-associated microbial patterns (PAMPs), through pathogen  
157 recognition receptors (PRRs) that include Toll-like receptors (TLRs), NOD-like  
158 receptors, dectin-1 or RIG-like helicases which are predominantly found on cells of the  
159 innate immune system. Nowadays, this recognition is considered critical in signal 2  
160 induction and downstream activation of distinct T helper cell subsets; however, other  
161 authors make a distinction and refer to adjuvants that trigger PRRs as signal 0  
162 adjuvants. In fact, recent work on adjuvants has especially focused on different PRR  
163 ligands including different PAMPs, and as well as other endogenous TLR ligands  
164 (Damage-associated molecular pattern molecules or DAMPs) such as heat-shock  
165 proteins (hsp), studying their ability to induce targeted Th responses. Once there is a  
166 production and expression of IL-2 (T cell growth factor) and its alpha-subunit of the IL-  
167 2 receptors (CD25) during e.g. activation of naïve Th cells into Th0 cells, proliferation  
168 of Th cells starts. Th0 cells will differentiate to Th1 or Th2 cells depending on the  
169 cytokine environment, wherein IFN- $\gamma$  drives Th1 cells while IL-4 induces Th2 cell  
170 production/differentiation [9]. Additionally, after many cell generations, the Th cells  
171 progenitors differentiate to effector Th cells, memory Th cells and regulatory Th cells.  
172 Different vaccine adjuvants that are in use in veterinary and human medicine aid

173 differentiation of Th cells into several T cell lineages – such as Th1, Th2, Th9 and Th17  
174 [10, 11]. In Table 1, we describe known adjuvant actions by both commercial and  
175 experimental adjuvants, used mainly in human medicine.

176

### 177 **3. Signal 1 adjuvants used in fish vaccinology**

178

#### 179 *3.1. Oil emulsions*

180 To increase the immunogenicity of an antigen, a slow release is often achieved  
181 through the introduction of the antigen in the context of an emulsion. An emulsion is  
182 defined as a dispersion of a liquid, called the dispersed phase, in a second liquid, called  
183 the continuous phase in which the first one is not miscible. In vaccine formulations,  
184 these phases are water (antigenic media) and oil. In order to stabilise the emulsions,  
185 surfactants are added. A surfactant is a compound containing a polar group that is  
186 hydrophilic and a non-polar group that is hydrophobic and often composed of a fatty  
187 chain. Surfactants can be defined by their hydrophilic: lipophilic balance (HLB) value  
188 that gives information on their relative affinity for both phases. According to the HLB  
189 value of the surfactant, different kind of emulsions can be achieved [12]. Those having a  
190 low HLB value have a high affinity for oily phases and render W/O emulsions, whereas  
191 those with a high HLB value have a high affinity for the aqueous phase and render O/W  
192 emulsions, which are well tolerated but induce a shorter term immune response. With  
193 certain specific surfactant systems, when the HLB value is intermediate, W/O/W  
194 emulsions can be achieved. In this case, the continuous phase is aqueous and the  
195 dispersed phase is oil. But inside the oil droplets, an entrapped aqueous phase is found.  
196 This type of emulsions has shown to generate long-term immune responses with various  
197 antigens.



198

### 199 3.1.1. Freund's complete adjuvant

200 The most widely used and most effective adjuvant for experimental purposes has  
201 been Freund's complete adjuvant (FCA). FCA is composed of heat-killed *Mycobacteria*  
202 and a mineral oil with surfactant [13]. Before injection, the antigen in an aqueous  
203 solution is mixed with the FCA producing a stable W/O emulsion. Immunisation with  
204 FCA and antigens results in strong Th1 and Th17 responses predominantly via the  
205 MyD88 pathway. Unfortunately, the use of FCA has been associated with a variety of  
206 severe side effects including injection site granuloma; therefore, the use of FCA has  
207 been limited to research on animals including fish for establishing an effective immune  
208 response. Furthermore, the use of FCA in fish has not always resulted in an increase in  
209 immunogenicity or protection.

210 Pasteurellosis, caused by *Pasteurella piscicida*, also named *Photobacterium*  
211 *damsela* subsp. *piscicida* is one of the major diseases in many species of wild and  
212 farmed fish in Asia, USA and Europe. In yellowtail (*Seriola quinqueradiata*), a  
213 susceptible species, vaccination against pasteurellosis has been assayed with a  
214 lipopolysaccharide (LPS)-mixed chloroform-killed bacterin which resulted in protection  
215 against challenge with the virulent bacterium. In this case, the inclusion of FCA in the  
216 vaccine did not significantly enhance the protective effect [14].

217 *Streptococcus iniae* is a Gram positive bacterium associated with disease in  
218 several commercial species including tilapia (*Oreochromis aureus* and *O. niloticus*),  
219 yellowtail, hybrid striped bass (*Morone saxatilis*), turbot (*Scophthalmus maximus*), and  
220 rainbow trout (*Oncorhynchus mykiss*). Vaccination of rainbow trout with a formalin-  
221 killed culture of *S. iniae* resulted in good protection against experimental challenge that  
222 was not significantly potentiated in the presence of FCA [15].

223 *Aeromonas hydrophila* is a Gram-negative bacterium known to cause motile  
224 aeromonas septicemia (MAS) in freshwater fish farming. The major adhesin of *A.*  
225 *hydrophila*, a 43 kDa outer membrane protein, was cloned, expressed and emulsified in  
226 FCA to be used in a vaccine for the blue gourami (*Trichogaster trichopterus*) [16]. The  
227 vaccine was intraperitoneally (i.p.) injected and after three weeks a booster was given  
228 without FCA. Two weeks after the booster, the fish were challenged with two strains of  
229 *A. hydrophila*. The recombinant adhesin protected against challenge with both the  
230 homologous strain of *A. hydrophila*, and the heterologous strain, providing the same  
231 immune protection as the native adhesin [16].

232 *Aeromonas salmonicida* is the etiological agent for furunculosis. In a study in  
233 coho salmon (*Oncorhynchus kisutch*), formalin-killed *A. salmonicida* was i.p. injected  
234 in the absence or presence of FCA. In this model, the best protection was found for the  
235 FCA adjuvanted vaccine. Interestingly, fish injected with FCA (without antigen) gave  
236 some protection even 90 days after challenge [17]. Injection of inactivated *M. bovis* may  
237 induce innate defence mechanisms that may result a certain degree of protection to a  
238 heterologous pathogen, as shown by Kato *et al.* [18] where Japanese flounder  
239 (*Paralichthys olivaceus*) were partially protected against nocardiosis with FCA. In a  
240 recent study, Zheng *et al.* [19] compared naturally occurring adjuvants (astragalus  
241 polysaccharide and propolis) with FCA used in pentavalent vaccines. In that study, FCA  
242 outcompeted the other adjuvants although the natural adjuvants induced some  
243 immunostimulant activities.

244 It has generally been difficult to develop effective vaccines against *A.*  
245 *hydrophila* most probably because of the high degree of antigenic variation [17, 21, 22],  
246 this is in contrast to vaccines against Gram-negative pathogens of salmonids like  
247 *Aliivibrio salmonicida*, *Vibrio anguillarum*, *Yersinia ruckerii* and *A. salmonicida* –

248 where vaccines show up to 100% efficiency. Recently, a vaccine against *A. hydrophila*  
249 giving protection in rainbow trout was prepared [20]. LaPatra and co-workers developed  
250 a new challenge model in rainbow trout with *A. hydrophila* by injection into the dorsal  
251 sinus to determine the efficacy of a bacterial lysate. The vaccine was shown to give  
252 protection after i.p administration, and this protection could be potentiated in the  
253 presence of FCA [20]. Also, fish that survived an *A. hydrophila* challenge were very  
254 resistant to re-infection.

255 *Flavobacterium psychrophilum* is a widespread Gram-negative pathogen in  
256 freshwater causing rainbow trout fry syndrome (RTFS) and bacterial cold water disease  
257 (BCWD) [23]. In addition to rainbow trout, coho salmon is the most susceptible species  
258 together with other non-salmonid species that are also affected. Injection of a low  
259 molecular weight fraction emulsified in FCA resulted in an enhanced level of protection  
260 for rainbow trout [23].

261 *Flavobacterium columnare* is a Gram-negative bacterium responsible for  
262 columnaris disease. The disease was first described in 1917 in several warm water fish  
263 species from the Mississippi river, and since has been isolated from freshwater fish  
264 species worldwide [24]. Specific antibodies were found in tilapia plasma and mucus  
265 following i.p. injection of formalin-killed sonicated or whole cells of *F. columnare* in  
266 FCA within 2 weeks. After a secondary immunisation, the antibody response increased  
267 and at 10 weeks post-immunisation the titre remained elevated. Also, antibodies were  
268 observed in cutaneous mucus in fish i.p. immunised with formalin-killed sonicated cells  
269 (ultrasound disrupted cells) in FCA 6 and 8 weeks post-immunisation [24].

270

271 3.1.2. Freund's incomplete adjuvant

272           Because of its high toxicity, the use of FCA has been widely replaced by  
273 Freund's incomplete adjuvant (FIA) that lacks the mycobacterial components of the  
274 emulsion, being therefore just a W/O emulsion. This adjuvant is still highly effective in  
275 vaccination with a significant reduction of toxicity, however, some important side  
276 effects are still present, effects very well detailed for Atlantic cod (*Gadus morhua*) in a  
277 very recent paper [25].

278           *Edwardsiella tarda* is a Gram negative intracellular bacterium that can infect  
279 both marine and freshwater fish, including Japanese flounder. In order to develop  
280 effective vaccines against this pathogen, fish were i.p. injected with a vaccine  
281 containing a major antigenic protein of *E. tarda* in the absence or presence of FIA [26].  
282 Protection against experimental challenge achieved by the vaccine without adjuvant  
283 resulted in a relative per cent survival (RPS) of 34% that was increased to 81% in the  
284 presence of FIA. Moreover, vaccination with the oil-adjuvanted antigen stimulated the  
285 expression of a series of genes like complement component 3 (C3), MHC class I and  
286 MHC class II, CD8 $\alpha$ , CD40, Mx, interferon  $\gamma$  (IFN- $\gamma$ ), tumour necrosis factor  $\alpha$  (TNF-  
287  $\alpha$ ) and interleukin 6 (IL-6), whereas vaccination with the antigen alone resulted in  
288 increased expression of just IgM, MHC class I and class II, and Mx [26].

289           *Nocardia seriolae* is a Gram-positive acid-fast bacterium that causes nocardiosis  
290 in cultured marine and freshwater fish in Taiwan, Japan and China. Although the  
291 disease results in considerable economic loss, no commercial vaccines are available.  
292 Very recently, an oil-adjuvanted vaccine was developed and tested on protection against  
293 challenge with a virulent strain [27]. Formalin-inactivated whole cell antigen was used  
294 as a vaccine with or without FIA, however, and even though antibody levels increased,  
295 no protective effects were found.

296 Another Gram-positive bacterium that causes disease (lactococcosis) and  
297 mortality in rainbow trout is *Lactococcus garvieae*. Recently a vaccine was prepared  
298 based on formalin inactivated bacterin or bacterin together with FIA. Fish were given  
299 i.p. injections and challenged by exposure to virulent bacteria 30, 75, and 125 days after  
300 vaccination [28]. A hundred and twenty five days after vaccination the RPS in fish  
301 vaccinated with bacterin only was 54% and whereas it was 85% in fish vaccinated with  
302 bacterin together with FIA.

303 *Tenacibaculum maritimum* is a marine bacterium that causes flexibacteriosis  
304 worldwide. In Australia (Tasmania), Atlantic salmon (*Salmo salar*) and rainbow trout  
305 are the most heavily affected species, and due to the lack of vaccines, so far the disease  
306 has been treated with trimethoprim and oxytetracycline with the subsequent negative  
307 impact on the environment [29]. Salmon injected with formalin inactivated bacteria  
308 mixed with FIA provided protection against challenge with *T. maritimum* while the  
309 vaccine without the adjuvant could not provide sufficient protection against a moderate  
310 challenge of *T. maritimum*.

311 Infection with fungi oomycetes such as *Aphanomyces invadans* may cause heavy  
312 mortalities of fresh water and estuarine fish species as a result of granulomatous  
313 inflammation. In catla (*Catla catla* Hamilton), fungal extract combined with FIA  
314 showed to increase both the survival rate during experimental challenge with *A.*  
315 *invadans* and the antibody response compared to non-adjuvanted vaccines [30].

316

### 317 3.1.3. Montanide

318 Mineral oil adjuvants registered under the trademark of Montanide have been  
319 optimised in order to improve efficacy and stability of vaccine formulations and to

320 reduce side effects. These adjuvants are based on either mineral oil, non-mineral oil or a  
321 mixture of both, as well as those made from specific surfactant chemistry using  
322 mannitol oleate and may be used to manufacture different type of emulsions, W/O, O/W  
323 or W/O/W, for use in both mammals and fish [31, 32].

324 *Philasterides dicentrarchi* is a scuticociliate parasite that causes mortalities and  
325 significant economic losses in cultured turbot [33]. An important attempt to optimise a  
326 vaccine against this parasite was performed on the basis of antigenic dose, concentration  
327 of inactivating agent (formalin) and proportion of the adjuvant Montanide ISA763A  
328 (W/O, non-mineral oil) in the emulsion. The results of the study showed that a high  
329 concentration of antigen, 0.2 % formalin and 50 % adjuvant generated the longest time  
330 of survival after challenge 30 days after the second injection, and the highest levels of  
331 antibodies in the vaccinated fish [33].

332 *Pseudomonas plecoglossicida* is a bacterium causing bacterial hemorrhagic  
333 ascites of cultured ayu (*Plecoglossus altivelis*). To develop a vaccine against the  
334 disease, formalin-killed *P. plecoglossicida* bacterin was emulsified with Montanide and  
335 injected i.p. The fish were challenged with an i.p injection of virulent *P. plecoglossicida*  
336 22 and 52 days after vaccination [34]. The RPS of vaccinated fish was 17-58% without  
337 adjuvant, 57-92% with Montanide ISA711 and 65-86% with Montanide ISA763A.  
338 Another study on the same disease and adjuvant (Montanide ISA 763A) concluded that  
339 there is a good correlation between antibody levels and protection against disease in a  
340 challenge test [35].

341 To study the efficacy of different adjuvants in Atlantic halibut (*Hippoglossus*  
342 *hippoglossus*), fish were injected i.p. with a model vaccine of human gamma globulin  
343 with either FCA or Montanide ISA711 as adjuvants [36]. Antibody responses and

344 intraperitoneal adhesions were examined every month for up to 12 months. FCA  
345 produced the highest and fastest antibody response, since in the group injected with the  
346 Montanide adjuvant only 4 of 47 fish reached a titre of 1:1000 (month 6) compared to  
347 27 of 48 fish in the FCA group (after 2 months), however, FCA also induced the fastest  
348 intraperitoneal adhesions [36].

349         In a very recent study in carp (*Cyprinus carpio*), a recombinant S-layer protein  
350 of *A. hydrophila* was used to assess the ability to protect fish against six virulent isolates  
351 of *A. hydrophila*. The recombinant S-layer protein of *A. hydrophila* was produced,  
352 diluted in phosphate buffered saline and mixed with a Montanide adjuvant at a ratio of  
353 30:70. Common carp were i.p. injected with the emulsion, and after 35 days the fish  
354 were challenged with six different isolates of *A. hydrophila* [37]. The RPS values varied  
355 between the different challenge isolates (40-75%), but they suggested that the S-layer  
356 protein together with Montanide adjuvant is a good candidate for an efficacious vaccine.

357         Furthermore, Montanide ISA-763 has also been used as an adjuvant in  
358 experimental bivalent vaccine for *L. garvieae* and *A. hydrophila* with high degree of  
359 efficacy in rainbow trout [38].

360

#### 361 3.1.4. Other mineral oil adjuvants

362         *Moritella viscosa* is the causative agent of winter ulcers in farmed fish like  
363 Atlantic salmon and Atlantic cod. Vaccination of Atlantic salmon against *M. viscosa* is  
364 performed with oil-adjuvanted polyvalent injection vaccines based on formalin-  
365 inactivated bacterial cultures, using an AJ-oil (Alpha Ject 5200) used in some vaccines  
366 commercialised by Pharmaq [39]. However, a multivalent commercial salmon vaccine  
367 containing *M. viscosa* as one of five bacteria mixed in a mineral oil adjuvant (Alpha Ject

368 5200) did not protect turbot against challenge [40], whereas moderate intra-abdominal  
369 adhesions were detected in vaccinated fish.

370 Other commercial oil-adjuvanted vaccines have been shown to give protection in  
371 Atlantic salmon against bacterial diseases like vibriosis, coldwater vibriosis and  
372 furunculosis for a long time. However, side effects and retardation in growth have been  
373 clearly demonstrated [41, 42]. Mutoloki and co-workers investigated the intraperitoneal  
374 lesions induced by an oil-adjuvanted vaccine against infection with *A. salmonicida* and  
375 *M. viscosa* in Atlantic salmon [43]. The cellular composition was typical of granulomas  
376 containing large macrophages, eosinophilic granular cells, lymphocytes and  
377 multinucleated cells.

378 Oil-adjuvanted vaccines are also used to control sea bass (*Dicentrarchus labrax*)  
379 against bacterial diseases like vibriosis and pasteurellosis. Sea bass is one of the most  
380 explored fish species in the Mediterranean area, and suffers from infection by *V.*  
381 *anguillarum* and *Photobacterium damsela* subsp. *piscicida*. Oil-adjuvanted vaccines  
382 against these diseases have been prepared and injected i.p., but despite their  
383 effectiveness, granulomatous peritonitis was also recognised [44].

384 The major bacterial disease of farmed Atlantic cod is classical vibriosis [45].  
385 Cod vaccinated by injection with mineral oil adjuvanted vaccines against both *V.*  
386 *anguillarum* and atypical *A. salmonicida* were very well protected against homologous  
387 challenges [46]. In this model, even without adjuvant the fish were protected against *V.*  
388 *anguillarum*, but not against atypical *A. salmonicida* challenge.

389



### 390 3.2. *Nano/ microparticles as adjuvants*

391            Microparticles offer a promising option to oil emulsions, and their beneficial  
392 use as carriers for vaccine delivery has been widely discussed [47]. An association  
393 or/and encapsulation of antigen(s) with/in microparticles can be achieved by covalent  
394 linkage or physical entrapment. Compared to the latter technique, where the antigen is  
395 non-covalently and physically incorporated in the interior of the microparticle, covalent  
396 coupling offers distinct advantages: fewer amount of antigen is required; processing and  
397 presentation by antigen-presenting cells is more efficient; a higher stability during  
398 storage is obtained and any excess of (valuable) material can easily be regained. With  
399 the use of microparticles, a very low dose of antigen can give rise to an optimal humoral  
400 response.

401            The structure and the properties of microparticles may change markedly with  
402 slight alterations in production conditions, but nanoparticles can be prepared in a  
403 physico-chemically reproducible manner within narrow size limits. For this reason,  
404 adjuvants on the basis of these submicron polymeric particles were developed and have  
405 also been suggested for use as potent adjuvants in mammalian systems [48].

406

#### 407 3.2.1. *PLGA particles*

408            Encapsulation of vaccines in biocompatible and biodegradable Poly-(lactide-co-  
409 glycolide) (PLGA) polymers has been studied for over twenty years. Antigen is released  
410 from the microspheres by diffusion through matrix pores and by matrix degradation.  
411 Biodegradation rates can be regulated by alterations in polymer composition and

412 molecular weights. In addition, there is often instant release of surface associated  
413 antigens that may be beneficial to aid a rapid response.

414         So far, a few studies have been carried out on fish with regard to uptake and  
415 degradation of PLGA particles and the immune response obtained. For the most part,  
416 these studies have been on oral administration [49-53]. A recent article appeared on  
417 parenteral immunisation of Indian major carp, rohu (*Labeo rohita*) with PLGA  
418 encapsulated antigen [54]. Outer membrane proteins (OMP) of *A. hydrophila* were  
419 encapsulated in PLGA microparticles and mixed with FIA in an emulsion or  
420 administered alone by i.p. injection in rohu. Twenty-one and 42 days after  
421 immunisation, the antibody titres were significantly higher in the PLGA-encapsulated  
422 antigen group containing FIA [54].

423         A dose-dependent transient increase of antibody response following i.p injection  
424 of PLGA particles containing human gamma globulin (HGG) has been shown by  
425 Fredriksen and Grip [55] where it was shown that microparticle carriers were superior  
426 compared to nanoparticles. Furthermore, when the formulation of PLGA entrapped  
427 HGG was performed with  $\beta$ -glucan or oil, it resulted in a continuous increase of  
428 antibodies over time (over 120 days).

429         Oral vaccines encapsulated in PLGA have been also used in Japanese flounder  
430 [51, 53] and salmonids like rainbow trout [50, 52, 56] or Atlantic salmon [49]. In the  
431 case of Japanese flounder, a plasmid encoding the major capsid protein of lymphocystis  
432 disease virus (LCDV) was constructed and encapsulated in PLGA. Controls were naked  
433 plasmid vaccine and blank PLGA particles [53]. The fish were orally intubated, and 28  
434 days post vaccination the fish were challenged by intramuscular injection with LCDV.  
435 Vaccine-effects were evaluated by observing the presence of lymphocystis nodules. The

436 cumulative percentage of Japanese flounder with nodules after challenge was greatly  
437 reduced in the group receiving the plasmid coding for the LCDV protein in PLGA  
438 particles in the period of 15 to 120 days post-immunisation [53]. In addition, the levels  
439 of antibody in sera of fish vaccinated with PLGA microcapsules increased for up to nine  
440 weeks; although from this point it started to decrease [51].

441 In rainbow trout, oral vaccination (as a feed additive) against lactococcosis was  
442 attempted with antigens encapsulated in PLGA particles [52]. RPS of the PLGA-  
443 vaccine amounted to 63 % and booster vaccination with oral administration of the  
444 PLGA-vaccine gave a RPS of more than 60 % 120 days after the first vaccination. Also  
445 in rainbow trout, human gamma globulin (HGG) was microencapsulated in PLGA [50].  
446 Specific antibodies were detected in the intestinal mucus of fish that were administered  
447 with the microencapsulated antigen after boosting with soluble HGG, but not in fish that  
448 were primed with the soluble antigen. The fate of orally administered HGG in Atlantic  
449 salmon was determined, demonstrating that fifteen minutes after administration, the  
450 HGG-PLGA was found in the intestine resembling the observation for free HGG [49].  
451 The results from this study indicate that orally delivered HGG-PLGA had higher levels  
452 and greater persistence of HGG systemically than free HGG.

453 Finally, feeding of rainbow trout with feed containing plasmid DNA encoding  
454 IHNV G protein induced slightly higher amount of neutralising antibodies against  
455 IHNV but no increased survival after experimental challenge with IHNV [56].

456

### 457 3.2.2. *ISCOMs*

458 Immune-stimulating complexes (ISCOMs) were conceived to co-formulate antigen  
459 and adjuvant in a particle [57]. ISCOMs represent an interesting approach to stimulation

460 of the humoral and cell-mediated immune response towards amphipathic antigens. They  
461 are relatively stable but non-covalently-bound complex of approximately 40 nm  
462 diameter of saponin adjuvant Quil-A (saponin extracted from the cortex of the South  
463 American tree *Quillaja saponaria molina*), cholesterol and amphipathic antigen in a  
464 molar ratio of approximately 1:1:1. ISCOMs produced through the patented Matrix™  
465 technology by Isconova have been widely studied in combination with different  
466 veterinary vaccines, and are currently incorporated in a number of commercialized  
467 animal vaccines. At this moment, Pharmaq is studying the introduction of these  
468 adjuvants in commercialised fish vaccines.

469

470

#### 471 **4. Signal 2 facilitators and TLR ligands as adjuvants or immunostimulants**

472

473 A large number of adjuvants that have been investigated do not directly affect  
474 the concentration and distribution of antigen between injection site and presentation site  
475 (this has not been established in fish yet). This category of vaccine adjuvant has  
476 dominated the literature on vaccine research in the last decade, and comprises the  
477 category of signal 2 facilitators, which include stranger and danger molecules, as well as  
478 inflammatory cytokines.

479 A number of so-called toll-like receptors (TLR) ligands (agonists) may induce  
480 strong innate responses that may be decisive for the outcome of acquired responses.  
481 Teleost fish species may possess close to twice the number of different TLR compared  
482 to mammalian species presumably due to an ancient genome duplication event. Many  
483 similarities between mammalian and fish with respect to intracellular and downstream  
484 signaling events exist, but there are dissimilarities that warrant focus. In this issue, a

485 detailed review authored by Aoki and Robertsen has been included, giving an excellent  
486 overview of the current knowledge on fish TLR (technical editor: Check whether this  
487 review is included in the special issue, and give reference). Another up-to-date review  
488 on immune relevant genes including TLR-like receptors in fish is also authored by Zhu  
489 *et al.* [58]. In general, those TLRs that, after ligand binding, induce the production of  
490 IL-12 favour a Th1 response (TLR 3, 4, 5, 7, 8, 9 and 11) and in addition, the activation  
491 of these TLRs may induce cross-presentation of antigens facilitating a cytotoxic T cell  
492 response under certain conditions [59]. It should be mentioned that ligand binding to  
493 TLRs 3 and 4, 7 and 9 may also induce type I IFN responses via interferon regulating  
494 factors. Within this group of signal 2 facilitators, we have also included alum, as it has  
495 been recently discovered that this adjuvant directly interacts with dendritic cells in a  
496 similar way to that of danger signals [60].

497

#### 498 *4.1. Aluminium containing adjuvants*

499 The adjuvant property of aluminium salts was discovered in 1926 [61].  
500 Aluminium compounds, especially aluminium phosphate and aluminium hydroxide, are  
501 some of the few adjuvants that have been allowed and considered safe to use in human  
502 vaccines. Aluminium adjuvants have been shown to induce Th2 responses almost  
503 exclusively [26], thus they have been used as adjuvants with great success, being  
504 particularly effective at promoting protective humoral immunity. However, alum is not  
505 optimally effective for diseases where cell-mediated immunity is required for  
506 protection. It was believed that alum activates NLRP3 inflammasome and induces  
507 necrotic cell deaths that release the danger signal uric acid [62]. However, very recently,  
508 it has been discovered that being a crystal, alum binds dendritic cell plasma membrane  
509 lipids with substantial force, independent of inflammasome and membrane proteins

510 [60]. The subsequent lipid sorting activates an abortive phagocytic response that leads  
511 to antigen uptake. Such activated dendritic cells, without further association with alum,  
512 show high affinity and stable binding with CD4<sup>+</sup> T cells via the adhesion molecules  
513 intercellular adhesion molecule-1 (ICAM-1) and lymphocyte function-associated  
514 antigen-1 (LFA-1). Only a few studies have been performed with aluminium adjuvants  
515 in the optimization of fish vaccines.

516         Fifteen years ago a vaccine against *A. salmonicida* mixed with potassium  
517 aluminium sulphate (alum) as an adjuvant was tested in Atlantic salmon [63]. Alum  
518 appeared to enhance the protection against challenge, but not at a statistically significant  
519 level. In another study, an *Escherichia coli* mutant was used for vaccination against  
520 *Edwardsiella ictaluri*-induced enteric septicaemia of catfish (*Ictalurus punctatus*).  
521 Killed *E. coli* bacteria with or without alum were administered i.p to catfish and the fish  
522 were challenged with virulent *E. ictaluri* bacteria [64]. Fish given *E. coli* in alum  
523 showed an enhanced survival (92 %) compared with the fish for which *E. coli* was  
524 administered alone (54%) or fish given saline (56 %).

525         Recently, an aluminium hydroxide adjuvanted *E. tarda* vaccine was prepared  
526 and injected i.p in Japanese flounder. The RPS was found to be 69 % [26] while  
527 immunisation with the antigen alone followed by an experimental challenge gave a RPS  
528 of 34, however, the FIA coupled vaccine showed a RPS of 81%.

529         Another experiment has been recently carried out by Fan *et al.* [65], in which  
530 formalin-inactivated reddish body iridovirus (TRBIV) were mixed with alum and either  
531 injected or bath administered twice in turbot. The resulting RPS calculated was 83.3%  
532 and 90.5%, respectively.

533

534 4.2.  $\beta$ -glucans – ligands for dectin-1

535  $\beta$ -glucans are known to stimulate the non-specific immune response of both  
536 mammals and fish where dectin-1 may be involved [66, 67]. To obtain protective effects  
537 against diseases the glucan is injected i.p., and there seems to be a dosage-dependent  
538 and short-lived protection. In addition, there are some reports on the adjuvant effect of  
539  $\beta$ -glucans [41, 42, 68-75].

540 DeBaulney and co-workers prepared an oral vaccine against vibriosis for use in  
541 turbot, and after feeding the vaccine for 5 days the fish were challenged 28 days  
542 thereafter. Fish given the vaccine alone resulted in a RPS of 52 %, while a combination  
543 of the vaccine and the  $\beta$ -glucan gave a RPS on 61 %, higher protection levels but not  
544 statistically different from the vaccine alone [71]. In 1998, an attempt to establish  
545 immunisation protocols to obtain the highest immune response against *V. damsela* was  
546 performed in Spain [72]. In this study they i.p. injected the O-antigen of *V. damsela* in  
547 combination with  $\beta$ -glucan. As a correlate to vaccine efficacy, the phagocytic index of  
548 head kidney macrophages was evaluated. The obtained results were as follows: the  
549 enhancement of the phagocytic index lasted longer in fish injected with  $\beta$ -glucan at the  
550 same time or after being injected with the antigen when compared with fish injected  
551 with  $\beta$ -glucan before the antigen. Similar results were obtained with regard to antibody  
552 titres [72].

553 Yeast glucan (mainly a  $\beta$ -1,3-D glucan) was included in a furunculosis vaccine  
554 that consisted in a formalin-killed culture of *A. salmonicida* and *V. salmonicida* [70].  
555 The vaccine, either with or without  $\beta$ -glucan, was injected i.p. and salmon challenged 3-  
556 46 weeks after vaccination. Vaccines supplemented with  $\beta$ -glucan induced significantly  
557 higher protection against furunculosis than vaccines without this adjuvant [70], but  $\beta$ -  
558 glucan alone did not result in protection after 11 weeks. In another study,  $\beta$ -glucan-

559 adjuvanted vaccines against furunculosis seemed to give protection at an early time-  
560 point after vaccination (6 weeks), but no protection was seen after 3 and 6 months [41].  
561 As a side effect, the average weight of the  $\beta$ -glucan-adjuvanted group was significantly  
562 lower compared to the controls, but the weight of fish given oil-adjuvant was also  
563 significantly lower than the  $\beta$ -glucan-adjuvanted group [42]. In a further study  
564 performed in coho salmon, Nikl *et al.* evaluated the potentiating effect of seven  
565 substances on the protection after vaccination with formalin-treated *A. salmonicida*  
566 bacterin [68]. Statistically significant improvement in survival over the group receiving  
567 bacterin alone was noted in fish groups that also received  $\beta$ -glucans like Vitastim-Taito  
568 and lentinan. However, agglutinin levels were significantly elevated in all cases where  
569 the bacterin was injected, and no significant elevation in agglutinin titer occurred as a  
570 result of combining an immunostimulant with the bacterin [68].

571         Catla is one of the major Indian carp species often affected with *A. hydrophila*,  
572 thus a formalin-inactivated *A. hydrophila* vaccine was developed and protection was  
573 studied in the absence and presence of a  $\beta$ -glucan adjuvant [74]. A reduction in  
574 mortality was found in the presence of  $\beta$ -glucan compared to the vaccine itself, although  
575 the differences were not statistically significant (RPS of 67.7 % and 58.0% with and  
576 without the adjuvant, respectively). In carp, a vaccine against *A. hydrophila* showed a  
577 higher antibody titer when  $\beta$ -glucan was i.p. injected prior to vaccination, while bath  
578 and oral administration of  $\beta$ -glucan before vaccination did not result in enhanced  
579 antibody response [75]. In a further study by Selvaraj and coworkers, carp were  
580 vaccinated against *A. hydrophila* with LPS from a virulent strain of the bacterium in the  
581 presence of different concentrations of  $\beta$ -glucan and administered through various  
582 routes such as i.p, oral or bath [76]. The RPS was significantly higher in i.p. injected  
583 groups even at the lowest concentration of  $\beta$ -glucan and fish given a mixture of LPS and



584  $\beta$ -glucan orally obtained a higher RPS compared to controls. The administration of the  
585 LPS-glucan by bath did not result in increased survival, and antibodies were never  
586 detected in fish vaccinated either orally or by bath. However, no possible analysis of the  
587 contribution of  $\beta$ -glucan in the vaccine efficacy could be established because an obvious  
588 control group in this study was missing, namely the protective effect of LPS without  
589 adjuvant [76].

590 In another study, the i.p. injection of  $\beta$ -glucan on days 1 and 3 followed by two  
591 i.p. immunisations of *E. ictaluri* on days 7 and 14 performed in channel catfish resulted  
592 in higher serum antibody levels relative to catfish receiving PBS instead of  $\beta$ -glucan  
593 before administration of *E. ictaluri* [69]. Serum antibody levels were determined on day  
594 7 (day 21) after the last immunisation, reaching with  $\beta$ -glucan antibody titers that were  
595 typically two-fold higher than those of fish without  $\beta$ -glucan.

596 In order to investigate possible treatments against *A. hydrophila* in blue gourami,  
597 laminaran, a  $\beta$ -1,3-D-glucan, was injected i.p. in the absence and presence of formalin-  
598 killed *A. hydrophila* bacteria [77]. A single i.p. injection of 20 mg kg<sup>-1</sup> laminaran alone  
599 was sufficient to protect the fish against infection by a virulent strain of *A. hydrophila*  
600 up until 29 days after injection in correlation with an increased phagocytic activity of  
601 head kidney phagocytes. Despite this, the addition of 20 mg kg<sup>-1</sup> laminaran to a  
602 formalin-killed *A. hydrophila* did not significantly improve the protection [77].

603

#### 604 4.3. Saponins

605 Saponins are natural glycosides of steroid or triterpene which have been widely  
606 explored as adjuvants in different mammalian systems due to their capacity to stimulate  
607 both Th1 and Th2 responses [78]. The most widely used saponins are Quil A

608 (component of ISCOMs) and their derivatives, however, due to their high cytotoxicity  
609 and instability in aqueous phase, the use of different kinds of saponins is being  
610 explored.

611 In Japanese flounder, formalin-killed *E. tarda* cells were administered to fish by  
612 feeding in the absence or presence of curdlan or curdlan together with Quil A saponin.  
613 Although the incorporation of curdlan gave higher survival rates, only the group in  
614 which the vaccine was administered with both curdlan and Quil A showed significantly  
615 better survival [73].

616

#### 617 4.4. Poly I:C – toll-like receptor 3 agonist

618 Polyinosinic polycytidylic acid (poly I:C) is a double stranded  
619 polyribonucleotide, that mimics a viral infection and therefore has been widely used to  
620 induce a type I IFN in many species including fish [79-81]. IFNs are cytokines with a  
621 major role in the early defence against viral infections, thus Poly I:C induces a non-  
622 specific antiviral state after its binding to TLR3 and the subsequent activation of  
623 intracellular signalling events. This non-specific antiviral activity of Poly I:C has been  
624 recently tested in rainbow trout infected with infectious hematopoietic necrosis virus  
625 (IHNV) [82]. Fish pre-injected with Poly I:C were protected against IHNV challenge 2  
626 days later and IHNV-specific antibodies were detected in survivors. The survivors  
627 showed a 100% survival rate following re-challenge with IHNV both 21 and 49 days  
628 after the primary IHNV challenge [82], demonstrating that the fact that fish were at an  
629 antiviral state during the encounter of a virus, gave them an important advantage for  
630 posterior specific antibody production. A similar study was performed in the sevenband  
631 grouper *Epinephelus septemfasciatus* in which fish were immunised against the

632 nodavirus red-spotted grouper nervous necrosis virus (RGNNV) [83]. Fish injected with  
633 50 mg Poly I:C fish<sup>-1</sup> or more intramuscularly (i.m.) and challenged i.m. with RGNNV  
634 2 days post-injection showed more than 90% survival rate. When surviving fish were re-  
635 challenged with RGNNV 3 weeks after the primary challenge, no mortalities were  
636 detected in the group that had been previously exposed to Poly I:C, since upon RGNNV  
637 challenge the antibodies against RGNNV were higher in these fish. All survivors that  
638 were re-challenged with RGNNV showed even higher levels of specific antibodies. In  
639 addition, the RGNNV titres in brain tissues of the survivors in the Poly I:C-RGNNV-  
640 RGNNV group were all under the detection limit [83]. Following up this work, this  
641 research group conducted a field trial exploring the vaccine efficacy of a RGNNV  
642 vaccine followed by Poly I:C injection. The Poly I:C-adjuvanted vaccine showed  
643 reasonable efficacy, but a one-shot Poly I:C injection in sevenband grouper 20 days  
644 after a natural RGNNV outbreak also induced a high survival rate (93.7%) compared to  
645 non-treated fish (9.8%) [84].

646 A prophylactic strategy using poly I:C was also used by Takami and co-workers  
647 in Japanese flounder experimentally infected with viral haemorrhagic septicaemia virus  
648 (VHSV) [85]. The survival rate in Japanese flounder after VHSV challenge following  
649 Poly I:C administration was 100%, while all untreated fish died within 9 days. Survival  
650 rates of the fish given a secondary challenge VHSV were 100% in the Poly I:C-VHSV  
651 group (Poly I:C-VHSV-VHSV group), while non-immunized fish showed a 0%  
652 survival.

653

654 4.5. *Lipopeptides*

655 Lipoproteins and lipopeptides have been found in a large number of  
656 microorganisms, the most prominent being mycobacteria and mycoplasmas. These  
657 molecules have been found to exhibit both a strong innate (inflammatory) response and  
658 a long-lasting adaptive immune response in mammals. Very few studies have been  
659 performed on lipopeptides in fish. The adjuvant effect of polar glycopeptidolipids in  
660 experimental vaccines against *A. salmonicida* was investigated [86]. Polar  
661 glycopeptidolipids (pGPL-*Mc*) were extracted from *Mycobacterium chelonae*, which is  
662 one of three mycobacteria species that are fish-pathogenic. At 12 weeks post  
663 vaccination, the antibody response of fish given 0.25 mg kg<sup>-1</sup> pGPL-*Mc* in combination  
664 with an *A. salmonicida* bacterin was significantly higher than that induced by a non-  
665 adjuvanted bacterin. Increased doses of pGPL-*Mc* suppressed the antibody response,  
666 while no significant side effects were observed in the peritoneal cavity after use of this  
667 adjuvant [86].

668

669 4.6. *Flagellin – toll-like receptor 5 agonist*

670 The structural protein of Gram-negative flagella is called flagellin. Flagellin is a  
671 potent activator of a broad range of cell types within the innate and adaptive immune  
672 system. Several studies have demonstrated the ability of flagellin as an adjuvant,  
673 promoting cytokine production [87]. Flagellin is known to induce immune responses via  
674 the TLR5 signalling resulting in a mixed Th1 and Th2 response, although it has also  
675 been reported that inflammasomes containing NLRC4/IPAF may bind cytosolically  
676 located flagellin [62]. During the last decade, the adjuvant effect of flagellin has widely  
677 been studied in vertebrates and during the last couple of years also in fish [88-90]

678 Piscirickettsiosis is a severe disease reported in salmonids that has caused  
679 especially great problems for the Chilean aquaculture industry. In 1989, the bacterium  
680 *Piscirickettsia salmonis* was isolated from a moribund coho salmon and was found to be  
681 the etiological agent of this disease. The pathogen is a Gram-negative obligate  
682 intracellular bacterium. The disease has also been reported to affect Atlantic salmon,  
683 rainbow trout and other farmed salmonid species [88]. A recombinant subunit vaccine  
684 was developed in order to control the disease due to poor responses after treatment by  
685 antibiotics. Three experimental formulations were prepared containing two or three  
686 recombinant proteins of the bacterium, and the formulations were emulsified with one  
687 volume of FIA [88]. The highest protective response was obtained with a vaccine  
688 formulation containing the subunit of the flagellum and chaperonins Hsp60 and Hsp70  
689 of *P. salmonis*, suggesting that the use of more than one recombinant protein antigen is  
690 needed to obtain a good protective effect against this disease.

691 Jiao and co-workers have been studying different vaccine concepts against *E.*  
692 *tarda* in the Japanese flounder to obtain effective protective formulations, based on both  
693 recombinant proteins and DNA vaccine constructs [89, 90]. The most promising  
694 vaccine concept was the one consisting in a chimeric DNA vaccine coding for the *E.*  
695 *tarda* proteins Eta6 fused in-frame to FliC, the flagellin for *E. tarda*. Although they  
696 found that *E. tarda* FliC induced low protective immunity by itself, it could function as  
697 a molecular adjuvant and potentiate the specific immune response induced by the *E.*  
698 *tarda* antigen Eta6. Fish immunised with pEta6 and FliC produced specific serum  
699 antibodies and exhibited enhanced expression of genes that are involved in both innate  
700 and adaptive immune responses (IL-1 $\beta$ , IFN, Mx, CD8 $\alpha$ , MHC-I $\alpha$ , MHC-II $\alpha$ , IgM)  
701 [89, 90]. Such up regulation following immunisation with flagellin has also been  
702 described by Hynes *et al.* [91], where TNF- $\alpha$ , IL-6, IL-8 and IL-1 $\beta$  were significantly

703 up regulated compared to non-adjuvanted controls. In this study, however, there was no  
704 induction of specific antibody response against flagellin or the model antigen *Limulus*  
705 *polyhemus* hemolymph (LPH) in the Atlantic salmon.

706

#### 707 4.7. CpG – toll-like receptor 9 agonist

708 Bacterial DNA and synthetic oligodeoxynucleotides (ODNs) expressing  
709 unmethylated CpG motifs trigger an immunostimulatory cascade that culminates in the  
710 maturation, differentiation and proliferation of multiple immune cells, including B and  
711 T lymphocytes, NK cells, monocytes, macrophages and dendritic cells. CpG motifs are  
712 approximately 20 times less common in mammalian than microbial DNA, and therefore  
713 are recognised as a danger signal by cells that express TLR9. In mammals, it has been  
714 widely demonstrated that CpG ODNs function as adjuvants when co-administered with  
715 vaccines, being able to both accelerate and magnify the immune response [92]. In fish,  
716 although many studies have been carried out on the immunomodulatory effects of CpGs  
717 [93-96], only few studies have focused on the adjuvant effect of these molecules.

718 Chinook salmon (*O. tshawytscha*) reared in the Pacific Northwest of the United  
719 States often suffers from infection with *Renibacterium salmoninarum*, the causative  
720 agent of bacterial kidney disease (BKD). The conclusion from a study in which whole  
721 cell vaccines with or without CpG adjuvants were used, was that either the vaccine  
722 alone or that with CpG provided protection against i.p. challenge with *R. salmoninarum*  
723 [93]. However, a combination of a commercial *R. salmoninarum* vaccine (Renogen)  
724 with a CpG adjuvant significantly reduced the level of bacterial antigens in the kidney  
725 of naturally infected fish [93].

726 In a study in rainbow trout, four groups were i.m. injected with a commercially  
727 available, non-adjuvanted aqueous vaccine against furunculosis containing inactivated  
728 cultures of *A. salmonicida* (Aquavac Furovac 5) alone, or together with CpG ODN  
729 1982, CpG ODNs 2133 or ODN2143. The fish were challenged with i.p. injection of a  
730 pathogenic strain of *A. salmonicida* 7 weeks after injection and the only group that  
731 showed a significantly lower mortality compared to those injected with Furovac alone  
732 (mortality of 52%) was the group injected with Furovac and the CpG ODN 2143 in  
733 which only a 21% of the fish died [94].

734 The protective effect of CpG motifs was also studied by Liu and co-workers in  
735 turbot and Japanese flounder [95, 96]. Sixteen CpG ODNs were synthesized and  
736 examined for the ability to inhibit bacterial dissemination in Japanese flounder blood.  
737 Four ODNs with the strongest inhibitory effects were selected and a plasmid pCN6 was  
738 constructed containing the sequences of the 4 selected ODNs. Japanese flounder were  
739 injected i.m. with plasmids pCN6 and pCN3 (control) and PBS. Four weeks post-  
740 vaccination the fish were challenged with *A. hydrophila* and mortality was monitored  
741 over a period of 20 days. Accumulated mortalities were 30%, 66.7% and 63.3% in  
742 pCN6-, pCN3, and PBS-immunised flounder, respectively [96]. Fish were also  
743 vaccinated as above and challenged with *E. tarda* 4 weeks after vaccination and the  
744 mortalities were 53.3, 90%, and 93.3% respectively. Therefore, the pCN6 plasmid  
745 provided a nonspecific protection against both *A. hydrophila* and *E. tarda* infections.  
746 This nonspecific protective effects have also been observed in fish parasitic infections  
747 since certain CpGs (e.g. CpG-ODN 1668 and CpG-ODN 2359) have also proved to  
748 have effects protecting fish against *Miamiensis avidus* [97]. Following on, a salmonid  
749 alphavirus (SAV) vaccine containing antigen plus CpG and Poly I:C as adjuvants  
750 induced a significant production of neutralizing antibodies and conferred some level of

751 protection – as evaluated by percentage of SAV positive fish compared to controls [98] .  
752 The authors reported that the adjuvanted vaccines induced prominent IFN type I  
753 expression – that is crucial in antiviral response.

754 To analyse the adjuvant effect of CpGs in turbot, fish were vaccinated with a  
755 *Vibrio harveyi* recombinant subunit vaccine, DegQ, in combination with a CpG that had  
756 been shown to provide anti-bacterial effects in the host species after injection. Fish were  
757 vaccinated by i.p. injection including all the appropriate controls and twenty-eight days  
758 after vaccination, the fish were challenged by a virulent strain of *V. harveyi*, and  
759 accumulated mortalities were recorded [95]. The only vaccine formulation that induced  
760 a significant protection was DegQ in combination with this pCN5 CpG. The duration of  
761 the adjuvant effect was found to last at least 50 days. 07/02/2013

762 One of the unique features of DNA vaccines is the ability to stimulate both  
763 cellular and humoral immune responses through the administration of a bacterial  
764 plasmid coding for a protective antigen [99]. Thus, these DNA vaccines possess  
765 intrinsic immunostimulatory capacity due to the presence of CpG motifs in the bacterial  
766 plasmid backbone. Therefore, the inclusion of additional CpG motifs in the vaccine  
767 plasmid would provide us with an intrinsic adjuvant within the same construct, being an  
768 easy method to increase the immunogenicity. In this sense, a recent work by Martinez-  
769 Alonso *et al.* [100] explored the possibility of increasing the immunogenicity of a  
770 VHSV DNA vaccine though the introduction of several copies (either two or four) of a  
771 fragment containing multiple CpG sequences of known immunostimulatory effects into  
772 the DNA vaccine. The addition of these CpG motifs significantly increased the titre of  
773 neutralising antibodies in serum and increased the levels of transcription of several  
774 immune genes such as Mx or MHC-I, demonstrating for the first time that additional  
775 CpG motifs may also be used to increase the immunogenicity of these DNA vaccines.



776

777 *4.8. Cytokines*

778 In the past years, a great number of cytokine genes have been identified in many  
779 fish species, however, and despite the fact that the use of cytokines as adjuvants has  
780 been widely explored in mammals, not many studies have focused on the possible use  
781 of cytokine genes as vaccine adjuvants in fish. This may be due to the fact that for the  
782 majority of these molecules, many details concerning their immunological role are still  
783 lacking, and until we know what immune processes they are regulating, their use would  
784 be a mere trial and error process. In any case, some attempts to explore their potential  
785 have been made in some fish species.

786 Interferon regulatory factors (IRFs) form a large family of transcription factors.  
787 IRF-1 has been shown to have a role in cytokine signalling and host defence against  
788 pathogens. For example, IRF-1 is up-regulated in response to virus infection in fish  
789 cells, inducing an antiviral state [101]. In a recent study, the potential use of IRF-1 as a  
790 vaccine adjuvant was investigated in Japanese flounder. The co-injection of IRF-1 with  
791 a DNA vaccine encoding the major capsid protein (MCP) gene of red sea bream  
792 iridovirus (RSIV) resulted in elevated serum neutralisation antibodies but was not  
793 significantly different from that in the fish vaccinated with the DNA vaccine alone  
794 [102]. Despite the moderate effect in protection, in this study, IRF-1 was responsible for  
795 the up-regulation of antiviral substances like nitric oxide (NO), interferon  $\beta$  (IFN  $\beta$ ) and  
796 interferon inducible genes such as Mx.

797 Interleukin 8 (IL-8) is a CXC chemokine produced by many cell types in  
798 mammals like macrophages, monocytes, epithelial cells, neutrophils and fibroblasts  
799 upon infection, or stimulated by cytokines like IL-1 $\beta$  and tumor necrosis factor  $\alpha$  (TNF-

800  $\alpha$ ). In mammals, chemokines have been widely used as adjuvants in vaccines against  
801 viral infections, since not only they attract more cells to the site of inflammation, but  
802 they also regulate the immune functions of the recruited cells. In fish, IL-8 has been  
803 characterised in rainbow trout among other species, and its chemo attractant properties  
804 established [103]. In this species, a vaccine plasmid coding for the glycoprotein gene of  
805 VHSV was co-injected with a plasmid coding for rainbow trout IL-8 to explore its  
806 potential adjuvant effect [104, 105]. When the plasmid coding of IL-8 (pIL-8+) was  
807 administered together with the VHSV vaccine, an increase of IL-1 $\beta$  in the spleen was  
808 found together with a greater cellular infiltration at the site of inoculation. Furthermore,  
809 fish injected with pIL-8+ alone showed a significantly higher expression of TNF- $\alpha$ , IL-  
810 11, TGF- $\beta$  and IL-18 in the spleen [104]. In a further study, the transcription of different  
811 inducible CC chemokines were studied in rainbow trout in response to both the VHSV  
812 DNA vaccine and/or pIL8+, demonstrating that when IL-8 is used as an adjuvant, the  
813 expression of other chemokines such as CK5A, CK6, CK7 and CK5B is also modulated  
814 [105]. All these results showed that IL-8 was able to modulate the early immune  
815 response and could be a potent vaccine adjuvant in fish against viral infections.

816 Administration of IL-1 $\beta$ -derived peptides to rainbow trout by i.p. injection  
817 induced reduced mortality of fish when exposed to VHSV after 2 days [106]. The  
818 peptides also induced leukocyte migration into the peritoneal cavity 1-3 days post-  
819 injection, however its possible use as adjuvant was not further explored. The role of IL-  
820 1 $\beta$  as an adjuvant was investigated in carp after i.p. injection of killed *A. hydrophila* in  
821 the absence and presence of recombinant C-terminal peptide of carp IL-1 $\beta$ . It was found  
822 that the agglutinating antibody titre was significantly higher in the fish injected with  
823 killed bacteria plus recombinant IL-1 $\beta$  peptide compared with killed bacteria alone 3  
824 weeks after vaccination [107].

825

## 8266. **Conclusive remarks and perspectives**

827

828           The development of effective vaccines should be approached by combining the  
829 search for protective antigens together with the application of specific, and targeting,  
830 adjuvants that maximise the immunogenicity with a desired immune response. These  
831 vaccine-specific adjuvants may be able to trigger specific immunological processes,  
832 without producing a generalised response with strong side effects. However, an obvious  
833 consequence for the lack of detailed knowledge on vaccine potency and efficacy using  
834 novel adjuvants such as the TLR ligands or cytokines - is that the vaccine producers  
835 may use oil-adjuvants instead for simplicity reason. The oil adjuvants, being able to  
836 induce very strong and durable immune responses may “overshadow” significant  
837 protective mechanisms that have been overlooked up till now. Thus, the search for real  
838 molecular correlates of protection should be pursued with strong efforts. In future  
839 vaccine research, the immunostimulatory potential of a given substance followed by  
840 vaccine potency and efficacy studies should be unequivocally established in the context  
841 of vaccination. Only then, we will be able to convince the pharmaceutical industry to  
842 move from traditional adjuvants to more sophisticated adjuvants that specifically trigger  
843 adequate immune responses that may be optimised for a specific pathogen.

844

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846

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851

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1179 Table 1. Adjuvants, central components, receptors/process and principal immunological  
 1180 responses elicited by licensed and experimental adjuvants mainly for human medicine.  
 1181 Adapted from Coffman *et al.* [57].  
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Adjuvant	Central immunostimulatory component(s)	PPR/Process	Principal immune response elicited
<b>Alum</b> <b>MF59 and AS03</b>	Aluminum salts	NLRP3 (?)	Ab, Th2 (+Th1 in humans)
	Squalene in water emulsions	Tissue inflammation	Ab, Th1 and Th2
<b>AS04</b>	MPL + Alum	TLR4 and NLRP3(?)	Ab and Th1
<b>Adjuvants in experimental use or in late stage clinical development</b>			
<b>Poly I:C</b> <b>MPL, and in diff. formulations</b>	Synthetic dsRNA		Ab, Th1, CTL
			Ab, Th1
<b>Flagellin, flagellin-Ag fusion proteins</b>	Recombinant flagellin from bacteria	TLR5	Ab, Th1 + Th2
<b>Imiquimods</b>	Imidazoquinoline derivatives	TLR7, TLR8 and both	Ab, Th1, CTL (when conjugated)
<b>CpG, and in different formulations</b>	Synthetic phosphothioate-linked DNA oligonucleotides with optimized CpG motifs	TLR9	Ab, Th1, CTL (when conjugated)
<b>ISCOMS</b> <b>IFA (and montanide formulations)</b>	Saponins	Not defined	Ab, Th1 + Th2, CTL
	Mineral or paraffin oil + surfactant	Not defined	Ab, TH1 + Th2
<b>CFA</b>	IFA + peptidoglycan, trehalose dimycolate	NLR, TLR?	Ab, Th1, Th17

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1184 Abbreviations and descriptions: MF59 (Novartis proprietary adjuvant MF59 containing  
 1185 squalene, polyoxyethylene sorbitan monooleate and sorbitan trioleate), AS03  
 1186 (GlaxoSmithKline) contains squalene, DL- $\alpha$ -tocopherol, polysorbate), AS04 (Aluminum  
 1187 hydroxide and monophosphoryl lipid A (MPL), ISCOMs (immune stimulating complex;  
 1188 nanostructure of cholesterol, phospholipids and Quil-A saponin), IFA (incomplete Freund's  
 1189 adjuvants). Ab: antibodies.

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1193 Table 2. Adjuvants currently used in fish vaccines commercialised by the main fish  
 1194 vaccine manufacturers.

Company	Vaccine name	Pathogen	Adjuvant	Immunization route
<b>PHARMAQ</b>	Alpha Ject and Alpha Marine vaccines	Different bacterial and viral pathogens	Mineral oil	i.p.
	Alpha Dip		No adjuvant	Immersion
<b>MSD Animal Health</b>	AquaVac	<i>A. salmonicida</i> , <i>Y. ruckeri</i> , <i>Vibrio</i>	No adjuvant	i.p., immersion
	AquaVac FNMPlus	<i>A. salmonicida</i>	Montanide ISA711	i.p.
	Norvax Compact PD	Salmonid alphavirus (SAV1 and SAV3)	Montanide ISA763A	i.p.
	AquaVac ERM Oral	<i>Y. ruckeri</i> , <i>Vibrio</i>	No adjuvant	oral
<b>Novartis</b>	Birnagen Forte As	<i>A. salmonicida</i> and infectious pancreatic necrosis virus (IPNV)	Mineral oil (Drakeol 6VR)	i.p.
	Ermogen	<i>Y. ruckeri</i>	No adjuvant	Immersion
	Apex®-IHN	IHNV	No adjuvant	i.m.

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