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**UNIVERSIDADE DO ALGARVE**

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Immunological and histological changes of gilthead sea bream (*Sparus aurata*) to Alum, when used as an adjuvant in *Photobacterium damselae* ssp *piscicida* vaccine.

A thesis submitted in partial fulfilment of the requirements for the degree of Master of Science in European Masters of Science in Aquaculture and Fisheries

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**Abstract**

Pseudotuberculosis or pasteurellosis is caused by the microorganism *Photobacterium damselae* ssp *piscicida* (formely *Pasteurella piscicida*). Vaccines are one of the most efficient methods for this control. Together with this, adjuvants are enhancers of fish immune response.

The aim of this research is to understand the effects of the Aluminium Potassium Sulfate (Alum) when used as an adjuvant of fish vaccines by immersion, namely in the sea bream (*Sparus aurata*) histological and specific immunological response.

**Key-words:** *Photobacterium damselae* ssp. *piscicida;* Alum; *Sparus aurata*;

vaccine; immersion;


**Resumo**

A pseudotuberculose ou pasteurelose é uma doença causada pela bactéria *Photobacterium damselae* ssp *piscicida* (anteriormente designada por *Pasteurella piscicida*). A medida profilática mais eficaz contra bacterioses são as vacinas; estas, juntamente com adjuvantes, potenciam a resposta imunitária dos peixes.

O objectivo desta investigação foi determinar e compreender os efeitos a nível histológico e imunológico do Sulfato de potássio e aluminio (alumen) quando aplicado como adjuvante em vacinas por imersão, em douradas – *Sparus aurata*.

**Palavras-chave:** *Photobacterium damselae* ssp. *piscicida;* Alumen; *Sparus aurata*; vacina; imersão;

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*INTRODUCTION*

Since 1970, aquaculture has grown around 8.9 % per year, and thus has become the fastest growing sector of food production in the world (FAO, 2004; Subasinghe, 2005). It is estimated that the aquatic products generated from aquaculture by the year 2050 will be between 177.9 and 209.5 million tons per year (Wijkström, 2003).

Gilthead sea bream (*Sparus aurata*, Linnaeus 1758) is one of the most important marine fish cultured in the Mediterranean countries (Balebona *et al*., 1998; Cuesta *et al*., 2005). The intensive farming of *Sparus aurata* achieved commercial status in the beginning of the eighties when the last technical barriers restricting their full scale profitable culture were overcome (Le Breton, 1999). According to FAO, in 2004 the global production is still growing (Fig I), and particularly in Portugal the culture of gilthead sea bream was 53% of the total production of marine fishes.
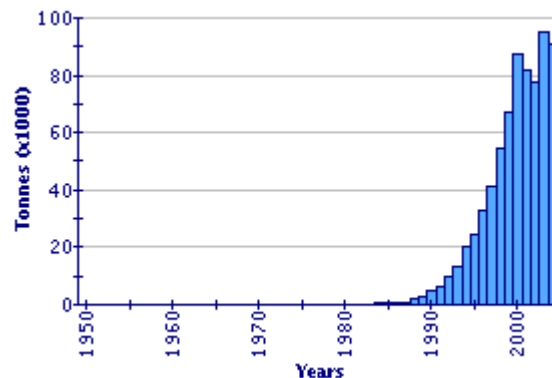


**Figure I:** Global Aquaculture production for *Sparus aurata* (FAO Fishery Statistic, 2004).

Despite the encouraging trends, several constraints may still have some negative impact on the growth of aquaculture (Subasinghe, 2003; FAO, 2004). As in other animal industries, the progress of marine fish production depends on three dominant limiting factors: nutrition/feeding optimisation, genetic improvement and pathology (Le Breton, 1999).

The main goal of aquaculture production is the maximum production, within a short period, having all the resources hoarded, with a profitable marketing strategy (Le Breton, 2003a; Pereira, 2004). In the other hand, intensification of animal production leads to increased stress levels, and consequently higher risk of pathologies (Evensen, 2003; Pereira, 2004), acting as a limitation for aquaculture (FAO, 2004). Although global economic losses from aquaculture diseases have not been compiled, disease reports from many regions of the world have been increasing with advances in the live aquatic animal trade (Le Breton, 1996; Balebona *et al.*, 1998; Subasinghe, 2005).

The most common pathologies in gilthead sea bream are parasitic and bacterial. The main parasites that affect this species are Protozoa (e.g. *Myxidium leei*), Monogenea *(*e.g. *Microcotyle* sp.) and Crustacea (e.g. *Anilocra physodes*) (Le Breton, 1996). If the parasitic pathologies are easy to treat, bacterial pathologies are a problem, due to bacterial antibiotic resistance and changes in fish behaviour (e.g. appetite) (Pereira, 2004). The major bacterial infections in sea bream involve pathogens such as *Mycobacterium marinum*, *Aeromonas hydrophila*, *Pseudomonas anguilliseptica,* several *Vibrionaceae,* and also *Photobacterium damselae* ssp. *piscicida* (Le Breton, 1999; Pereira, 2004).

There are many problems concerning the use of antibiotics in fish intended for human consumption, since residues of antibacterial treatment have an indirect impact on human health as well as a bad environmental impact on aquatic species (Subasinghe, 2003). The number of chemicals indexed for use in aquaculture has decreased and is very limited in our days (Subasinghe, 2005), and moreover, for some infectious diseases there are no treatments.

The future of pathology in aquaculture lies in the prevention of diseases and not in their treatment (Le Breton, 2003b). Prevention is essential, and has proven to be economically profitable, and can be achieved by applying two different approaches: (a) hygienic procedures (including cleaning and disinfection) and (b) prophylactic programmes (immunostimulants and vaccines) (Le Breton, 2003a). Vaccination strategies covering the fish life cycle need to be developed further together with programs of preventive treatments (Le Breton, 1999; Toranzo *et al.*, 2003). Pharmaceutical companies have performed a considerable amount of research on fish vaccines, however, limited information is available in scientific publications (Sommerset *et al.*, 2005).

## I. FISH IMMUNE SYSTEM

Fish health depends on three important factors: environment, pathogen nature and issues intrinsic to fish (as host) (Schreck, 1996); this concept was first illustrated in the famous diagram "epidemiological triad" of Snieszko (1974) (Fig II).
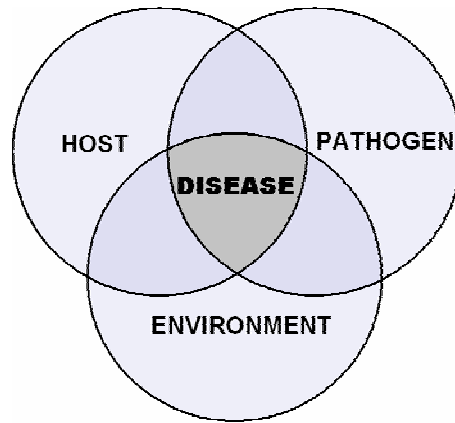
**Figure II:** The epidemiological triad (Snieszko, 1974)

All animals possess defence mechanisms that enable survival in hostile environments (Evensen, 2003). The defensive mechanisms are directed against pathogens, such as bacteria and virus, as well as against malignant cells (Anderson, 1996; Ellis, 1999). Fish immune system have many humoral and cellular mechanisms, which are non-specific (innate) or specific (adaptive), as presented in Table I (Ellis, 1999).

**Table I:** Defence mechanisms in fish (modified from Ellis, 1999).

|  | Humoral | Cellular |
|---|---|---|
| Non-Specific/ Innate | Inhibitors (transferrin, Interferons, antiproteases, lectins, antibacterial peptides) <br> Lysins (proteases, lysozyme, complement, C-Reactive Protein) | Non-specific cytotoxic cells <br> Neutrophils (respiratory burst, hypohalite ions, lysozyme) <br> Macrophages (hydrolytic enzymes, respiratory burst, nitric oxide) <br> Macrophage/neutrophil cooperation |
| Specific/ Adaptive | Antibody (anti-adhesins, anti-toxins, anti-invasins, activates classical complement pathway) | Activated macrophages (specific T lymphocytes and antigens, cytokines) |

The innate immune system of fish consists of physical barriers including scales, mucus, gills and epidermis; immunocytes (cellular mechanism) include phagocytic cells (e.g. neutrophils and macrophages), non-specific cytotoxic cells and endothelial cells; innate humoral components concern inhibitors and lysins (Magnadóttir, 2005). The adaptive immune responses are dependent on the activities of B and T lymphocytes, which work as in specific immunoglobulin production, cytotoxic activities, immunomodulation via cytokines and complement pathways (Shoemaker *et al.*, 2001).

The complement system comprises serum proteins that are activated using either classical or alternative pathways. These defense systems are vital to the ability of fish to respond to invasion (Ellis, 1999).

To start an infection, pathogenic bacteria must invade the host, adhere to the epithelium surface, spread throughout the fluids, and be able to survive within the host. Antibodies that react with bacterial cell wall structures may prevent pathogens from gaining access to host cells. This acts like opsonins, activating complement. Then, the bacterial cell can be lysed, phagocytosis is enhanced and inflammation starts (Kaattari & Piganelli, 1996; Ellis, 1999; Shoemaker *et al.*, 2001; Magnadóttir, 2005).

The antibody response of fish to disease has been one of the most studied aspects of fish immunology. Antibodies are among the most structurally complex biological molecules (Kaattari & Piganelli, 1996). Although IgD has recently been described, IgM is the main immunoglobulin present in teleosts where, it is thought, different forms of IgM and its structural flexibility may compensate for a lack of diversity (Cuesta *et al.*, 2004). Teleost IgM appear

primarily as a tetramer, composed of four subunits, each containing two "heavy" protein chains, and two "light" chains (Kaattari & Piganelli, 1996), and circulating IgM levels reflect the immune system status without exposing the fish to a specific antigen (Cuesta *et al.*, 2006).

Pathogenic bacteria (e.g. *Photobacterium damselae* ssp. *piscicida*) have many ways of subverting fish defense mechanisms, including surface layers, capsules and elongated lipopolysaccharide (LPS) O-antigens that protect the pathogen against complement (Ellis, 1999; Gudding *et al.*, 1999).

The ability of fish to resist pathogens and respond to stress, and the interactive effects of stress and disease resistance capacity of fish, change seasonally and through ontogeny (Schreck, 1996; Hanif *et al.*, 2005).

### a. Immunomodelators

Aquaculturists are interested in substances that benefit or stimulate immune system in order to prevent diseases (Anderson, 1996).

A better understanding about the effect of different immunomodelators on fish immune system is an important research topic, especially in farmed fish. Once a stressor is perceived, a cascade of neuroendocrine events generally leads to elevation of cortisol in circulation, where it affects lymphocytes and antibody production (Schreck, 1996). The physiological stress response and the immune system are under genetic influence (Schreck, 1996). In addition, the fish's past and present rearing environment affect both the physiological response to stress and the ability to resist pathogens.

Numerous environmental variables have been shown to affect the physiological stress response in teleost (Schreck, 1996). However, the effects of environmental factors in fish immune system, such as temperature or salinity are not well documented despite their importance in fish biology (Cuesta *et al.*, 2005).

It is known that the physiological effects of stressors in fish depend upon duration and intensity of such stressors, and two categories of responses can be established: acute and chronic stress response. Although the impact of these two categories of stress on fish immune system have been the object of much study in recent years there are no available results on the effect of stressors on fish serum IgM levels (Cuesta *et al.*, 2004).

A difficulty often arises when work has to be done with low levels of heavy metals in determining toxicity and immunomodulation effects (Anderson, 1996).

A good example of an immunomodelator is Aluminium. It is one of the heavy metals that affect fish health (Anderson, 1996). Deposition of Al onto gills is water quality dependent, and increased Al accumulation in fish gills is reported to enhance physiological stress symptoms, such as elevated levels of glucose in blood (Teien *et al.*, 2004). Low levels of heavy metals are sometimes immunostimulatory, perhaps because of stress subsequent hyperactivity. Indeed, in protection tests, the metals may affect the pathogen's physiology (Anderson, 1996).

## II.    FISH VACCINATION

Vaccination is the most efficient prophylactic treatment. The purpose of vaccination is to induce protective immunity that should be specific and long lasting, thus reducing the level of fish infections (Ellis, 1999). The ability of fish vaccines to induce a protective immunity is based on experimental challenge studies and/or field experiments. Laboratory research focused on determining which antigens are the most immunogenic in fish is very scarce, and it is very important to identify the individual components involved in inducing protection (Arijo *et al.*, 2004).

The fish development stage and ontogeny is an important factor to have in mind, since early vaccination (when the major lymphoid organs are not completely developed) may give an undesired effect of immunosuppression. The right age for vaccination and the immunisation route have to be estimated for different fish species like sea bream (77 days of age) (Toranzo *et al.*, 2003; Hanif *et al.*, 2005).

The practice of vaccinating fish has yielded good results on reduction of antibiotic use and thus avoiding perceived risks to the environment and to human health (Midtlyng, 2001).

There are several commercial vaccines available for bacterial diseases, with satisfactory results, but little is yet understood about the nature of the protective mechanisms involved. In addition, for some economically important fish diseases (e.g. pasteurellosis) there aren't any effective vaccines (Barnes *et al.*, 2005). With such information, more directed approaches to the production

and presentation of pertinent antigens may permit further development of effective vaccination against many economically important fish diseases (Ellis, 1999).

Vaccines can be found as:

- inactivated (or bacterins)
- live (attenuated)
- genetic (DNA/RNA)

So far, most commercial vaccines in use are inactivated vaccines (Gudding *et al.*, 1999; Toranzo *et al.*, 2003).

Inactivated vaccines are usually formulated by inactivating the whole pathogen or portions of it, by fermentation and subsequent formalin inactivation or by heat inactivation (Gudding *et al.*, 1999; Toranzo *et al.*, 2003).

Live vaccines have great potentialities/advantages in aquaculture, either by potential protection or for economic reasons. In fact, live vaccines are an infection (attenuated strain), with effective dissimilation of the antigen in the fish population over an extended time period (Toranzo *et al.*, 2003). However, live vaccines for fish have the great risk of reversion of virulence and uncontrolled environmental spreading. For this reason, live/attenuated vaccines have so far only been allowed for field trial purposes (Gudding *et al.*, 1999).

The basis of genetic vaccines (nucleic acids) is the delivery of a gene encoding for a protective vaccine antigen. The so-called naked DNA/RNA vaccines have already some results with characterization of fish immunization as well as high clinical efficacy (Midtlyng, 2001; Evensen, 2003), and in these

vaccines the possibility of reversion to virulence does not exist (Gudding *et al.*, 1999).

Before applying these vaccines in aquaculture commercial enterprises, fish, environment and consumer safety have to be addressed (Heppell & Davis, 2000; Evensen, 2003).

Fish vaccines are commonly administered by injection, orally or by immersion (Table II). Injection is widely used and gives the best and most long-lasting protection, and can be easily combined with adjuvants (Evensen, 2003). Injection has also the advantage of delivering the right dose of vaccine antigen. The major disadvantages are intensive labouring and costs, and is only feasible if applied in fish with *c.* 10g onwards (Evensen, 2003). The vaccine can be applied by injecting the fish either intra peritoneal (i.p) or intramuscularly (i.m) (Ellis, 1999). In i.p. injection reduction/depress in growth rate and qualities of farmed fish species are sometimes observed (Midtlyng, 1997; 2001).

Oral vaccination is an option, but still largely in an experimental stage. Research in this field focuses on protection of the vaccine from digestion in the early digestive system through encapsulation. On the other hand, a large quantity of antigen is necessary and the protection achieved is generally weak and of short duration (Sommerset *et al.*, 2005).

Immersion vaccination is an established practice in aquaculture, because it is useful for mass vaccination, and has a particularly low cost/effect relation method of administration in small fish (Huising *et al.*, 2003).

**Table II:** Comparison of vaccine delivery methods [adapted from (Evensen, 2003)].

| Delivery method | Injection | Immersion | Oral |
|---|---|---|---|
| **Efficiency** | + | ± | - |
| **Amount of Vaccine** | - | - | + |
| **Labour** | + | - | - |
| **Handling Stress** | + | ± | - |
| **Small fish vaccination** | - | + | + |
| **Use of Adjuvant** | + | ±/- | ±/- |
| Operator safety | - | + | + |

**+:** high/possible; **±:** moderate; **-:** low/not possible

### a. Immersion

Vaccine delivery in immersion vaccination may vary. Vaccine uptake is thought to occur largely along fish mucosal surfaces, i.e. the gills and skin. Lateral line and intestine are also implicated as sites of antigen uptake. An additional advantage of immersion is that vaccine delivery is done through the same route used by many pathogens, generating specific mucosal immunity (Huising *et al.*, 2003). The efficacy of immersion vaccination is mostly evaluated by antibody production and/or survival upon challenge, and may depend on the amount of antigen absorbed (Midtlyng, 2001). Immersion vaccination induces detectable systemic and mucosal antibody responses, confers protection upon challenge, or both (Tatner, 1996; Huising *et al.*, 2003). However, some reports indicate a transient humoral response (Whittington *et al.*, 1994), indicating that the exact mechanisms of protection are sometimes enigmatic. Immune responses following immersion vaccination are generally less robust and of shorter duration than those obtained from injection (Huising *et al.*, 2003).

Hyperosmotic immersion increases the uptake of vaccine and enhances the efficacy by which vaccine components are processed and presented by the innate immune system, dually enhancing the mucosal immune response (Huising *et al.*, 2003). In the gilthead sea bream the ability to maintain constant ion concentration and osmolarity of body fluids appears early in the development (Guerreiro *et al.*, 2004; Laiz-Carrion *et al.*, 2005). Fish in seawater tend to gain ions such as sodium and chloride through diffusion and to lose water by osmosis. The acquisition of the capacity to control water balance in relation to external salinity through drinking by fish is, therefore, fundamental for osmoregulation (Jensen *et al.*, 1998; Guerreiro *et al.*, 2004; Laiz-Carrion *et al.*, 2005).

Although the inherent difficulty of adding adjuvants in vaccines through this delivery method (immersion), some research focusing the antigen presentation by absorption phenomena and mucosal irritation is being performed nowadays (Ascarateil & Dupuis, 2003).

### b. Adjuvants

Adjuvants (from the latin *adjuvare*, "to help") are defined as a group of structurally heterogenous additives (Evensen *et al.*, 2005). When these are added to an antigen enhance or modulate the immunogenicity of the poorly immunogenic vaccine proteins or peptides, resulting in an effective prolonged protection (Evensen *et al.*, 2005; Schijns & Tangeras, 2005). The use of adjuvants also reduces the amount of purified antigen required for successful

immunization, thus making vaccine production more economical and more feasible (Anderson, 1997). Nevertheless, the type and role of adjuvant becomes very important to achieve an acceptable immune response (Stills, 2005).

The role of innate immunity in stimulating adaptive immune responses is the basis of the action of adjuvants. Among the mechanisms used by adjuvants to enhance the immune response are the "depot" effect, antigen delivery (to Antigen Presenting Cells – APC's), immune activation/modulation, and cytotoxic lymphocyte induction (Mulvey *et al.*, 1995; Evensen *et al.*, 2005).

The effects produced by adjuvants in the immune system encloses an enhancement of processing and presentation of antigen, an increased cellular infiltration, induction of cytokine release, enhancement of speed, magnitude and duration of the immune response, modulation of antibody avidity and affinity, and stimulation of cell-mediated immunity and non-specifically lymphocyte proliferation (Midtlyng, 1997; Evensen *et al.*, 2005).

Usually adjuvants are delivered in fish by injection, and in many cases adjuvanted vaccines tend to elicit different intra abdominal lesions at the site of injection (Cossarini-Dunier, 1985). Because of these side-effects, researchers are studying adjuvants that augment protective immune responses with or without minimal side-effects (Gupta *et al.*, 1993; Anderson, 1997; Midtlyng, 1997; Evensen, 2003; Evensen *et al.*, 2005).

There are different types of adjuvants that have been used in commercial fish vaccines (Table III) (Anderson, 1997).

Aluminium adjuvants are the most widely used adjuvants in both human and veterinary vaccines (Lindblad, 2004).

**Table III:** Most common types of adjuvants and their mode of action.

| TYPE | ADJUVANT | MODE OF ACTION |
|---|---|---|
| **Aluminium Salts** | Aluminium Phosphate<br>Aluminium Hydroxide<br>Alum | **Slow release<br>Antigen depot** |
| **Water-in-oil emulsions** | Freund's Incomplete Adjuvant (FIA)<br>Muramyl Dipeptide | **Slow release<br>Antigen depot** |
| **Complex carbohydrates** | Freund's Complete Adjuvant (FCA) | **Slow release<br>Antigen depot** |
| **Bacterial fractions** | Lipopolysaccaride (LPS)<br>Saponin | **Macrophage stimulator** |
| Surface-active agents | **Glucans<br>Dextran Sulfate** | **Macrophage stimulator** |

## III. Aluminium

Potassium aluminium salts (alums) are one category of surface-active antigens that have long been studied, and are still used extensively in the production of bacterins. But there have been few studies on the use of alum-adjuvanted bacterins in fish (Mulvey *et al.*, 1995). When evaluating the effect on fish growth, divergent results have been obtained after use of vaccines prepared with aluminium salt adjuvants (Horne *et al.*, 1984; Mulvey *et al.*, 1995; Midtlyng, 1997). The adjuvant effect of aluminium compounds is generally more significant in primary than in secondary responses (HogenEsh, 2002). Thus, aluminium compounds can further enhance the immune response by direct or indirect stimulation of dendritic cells, activation of complement and by inducing the release of chemokines. The relative importance of these mechanisms remains to be determined (HogenEsh, 2002).

## IV. *Photobacterium damselae* ssp. *piscicida* (*Phdp*)

Pasteurellosis, also described as photobacteriosis (due to the change in the taxonomic position), is caused by the halophilic bacterium *Photobacterium damselae* ssp. *piscicida* – *Phdp* - (formerly *Pasteurella piscicida*). It was first isolated in mortalities occurring in natural populations of white perch (*Morone americanus*) and striped bass (*M. saxatilis*) in 1963 in Chesapeake Bay, USA (Snieszko *et al.*, 1964). Since 1969, this disease has been one of the most important in Japan, affecting mainly yellowtail (*Seriola quinqueradiata*) (Kusuda & Yamaoka, 1972). From 1990 it has caused economic losses in different European countries including France (Baudin-Laurencin *et al.*, 1991), Italy (Ceschia *et al.*, 1991), Spain (Toranzo *et al.*, 1991), Greece (Bakopoulos *et al.*, 1995), Turkey (Canand *et al.*, 1996), Portugal (Baptista *et al.*, 1996) and Malta (Bakopoulos *et al.*, 1997). Gilthead sea bream (*Sparus aurata*), seabass (*Dicentrarchus labrax*) and sole (*Solea* spp.) are the most affected species in Europe Mediterranean countries, as well as hybrid striped bass (*M. saxatilis* x *M. chrysops*) in the USA. However, the natural hosts of the pathogen are a wide variety of marine fish (Romalde & Magariños, 1997).

It is presently the most devastating pathology in sea bream production. Severe mortalities, rising from 50 to 100% of the affected batches, are observed as soon as larvae reach 35 days old (Le Breton, 1999). At this stage, antibiotic treatments have a very limited efficacy and antibiotic resistance can occur

within a week. Juvenile sea bream up to 80g are also sensitive to the disease. In adults, the disease assumes a chronic form with low sporadic mortalities (Romalde, 2002; Barnes *et al.*, 2005). This pathology is temperature dependent and occurs usually when water temperatures rise above 18-20°C. Below this temperature, fish can harbour the pathogen as subclinical infection and become carriers for long time periods (Romalde, 2002).

Pastereullosis is also known as pseudotubercullosis because it is characterized by the presence, in the chronic form of the disease, of creamy-white granulomatous nodules or whitish tubercules in several internal organs, composed of masses of bacterial cells, epithelial cells, and fibroblasts. The nodules are most prominent in internal viscera, particularly kidney and spleen, and the infection is accompanied by widespread internal necrosis (Evelyn, 1996; Romalde, 2002; Barnes *et al.*, 2005). Anorexia with darkening of the skin as well as focused necrosis of the gills are the only external clinical signs often observed. These lesions are generally missing in the acute form. The disease is difficult to erradicate with antibiotic treatments, and there is evidence that carriers under stressful conditions could suffer from reinfection (Le Breton, 1999).

The presumptive identification of the pathogen is based on standard biochemical tests. In addition, although *Photobacterium damselae* subsp. *piscicida* is not included in the API-20E code index, this miniaturised system can also be useful for its identification, since all strains display the same profile (2005004). Slide agglutination test using specific antiserum is needed for a confirmative identification of the microorganism (Romalde, 2002).

The pathogenesis of *Phdp* is poorly understood (Lopez-Doriga *et al.*, 2000). However, the major strategy of *Phdp* is the induction of extensive apoptosis of fish macrophages and neutrophils that results in lysis of these phagocytes by post-apoptotic secondary necrosis (Barnes *et al.*, 2005; do Vale *et al.*, 2005). Other strategy is the capacity of intracellular survival in non-phagocytic cells in order to avoid phagocyte bactericidal mechanism (Ellis, 1999; Lopez-Doriga *et al.*, 2000; Barnes *et al.*, 2005). The virulence of the pathogen implies the production of polysaccharide capsular layer, and extracellular products, and is also depending on iron availability (Lopez-Doriga *et al.*, 2000). The bacteria spreads via infected phagocytes, mainly macrophages. This spread can be rapid, and lethal effects may occur within a few days of challenge, affecting tissues containing large numbers of the pathogens (Evelyn, 1996).

Because of its broad host range, ubiquitous distribution, widespread antibiotic resistance and lack of effective vaccines, *Phdp* remains a major concern for the world aquaculture and vaccine companies (Barnes *et al.*, 2005).

The main objective of this research is to test a hydro soluble adjuvant – alum (aluminum and potassium sulfate dodecahydrate) - in immersion vaccination allowing it to be used earlier in fish life, in order to prevent pasteurellosis. Histological and immunological effects of the adjuvant were evaluated, as well quantification of aluminium. The accumulation or non-accumulation of this substance can provide remarkable information for its potential use in commercial aquaculture vaccines. From this research, it is also possible to check for the aluminium immunostimulant role that some authors defend.

The finding of this study will increase the existing knowledge of immunoprophylaxis in gilthead sea bream against pasteurellosis. Furthermore, the findings are also expected to assist vaccine industry and interested companies to make effective pseudotubercullosis vaccines for gilthead sea bream.

## *MATERIAL AND METHODS*

This chapter provides detailed information about the material and methods used to run this experimental study.

### I.  Fish

Gilthead sea bream (*Sparus aurata* L.) were obtained from a fish farm (Timar) located in the south of Portugal. They were placed in IPIMAR (Olhão, Portugal) in 1000L tanks (open system), at a maximum density of 0.5 Kg.m$^{-3}$. Fish were fed *ad libitum* with a commercial diet. Prior to their use in experiments, fish were transported to PRODEP – LEOA (Universidade do Algarve, Portugal), and acclimatized in 40L aquaria (recirculation system), with a maximum density of 2 Kg.m$^{-3}$.

### II.  Hyperosmotic Shock

Hyperosmotic shock was performed by placing the fishes in a solution of 55 PSU for 96 hours according to the method used by Guerreiro *et al.* (2004) and Cuesta *et al.* (2004). The 55 PSU solution was prepared adding salt (CORAL REEF) *q.b.* to seawater, and the salinity was checked every 3-4 hours, using a salinometer.

### III. Aluminium Exposure

98 fish with 4.08±2.31g (μ±σ) body weight were separated in two groups, with the first group going through the osmotic shock and the second group maintained in normal seawater (35 PSU). After 96 hours, an immersion in a solution of alum (Aluminium and Potassium Sulphate Dodecahydrated, ) with 1% final concentration (Mulvey *et al.*, 1995) was performed to both groups for a period of 30 seconds (standard time for immersion vaccines).

Seven fish of each group were randomly sampled at 0, 3, 6, 14, 48, 672 and 840 hours, euthanized with excess of anaesthetic (Ethyleneglycol mono phenyl ether also known as Phenoxy-2-ethanol) and immediately processed for aluminium detection (Fig III).
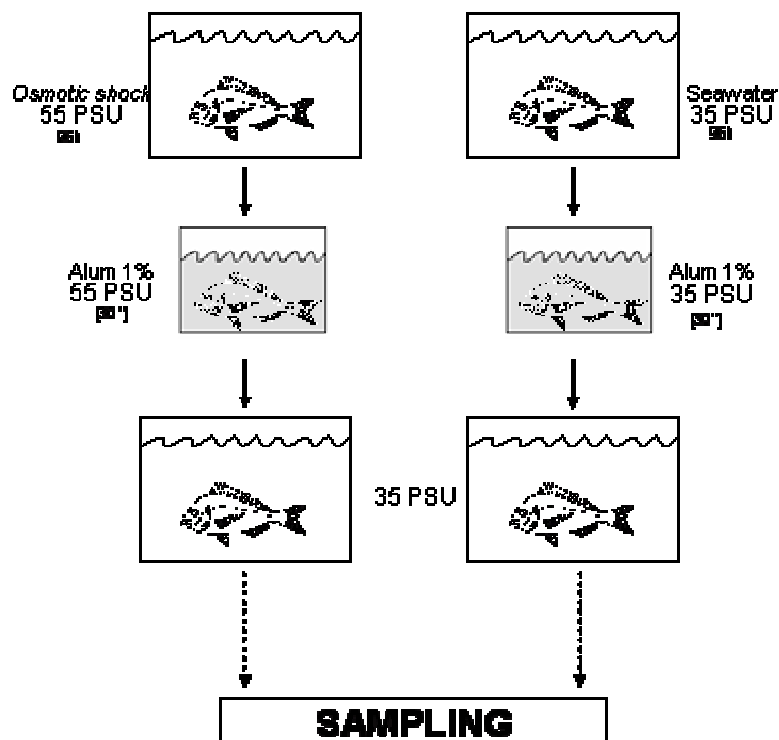


**Figure III**: Design of the aluminium exposure.

## IV. Al detection

Two methods were used in order to detect aluminium in fish: Graphite Furnace Atomic Absorption Spectroscopy (GFAAS) for the whole body aluminium concentration, and histological analysis for deposition observation.

### a. GFAAS

Because aluminium concentration in fish is usually very small (Türkmen *et al.*, 2005), Graphite Furnace Atomic Absorption Spectroscopy (GFAAS) was used to perform this determination for its sensitivity and accuracy (Bouman *et al.*, 1986; Exley, 1996). At each time point, 5 fish of each group were euthanized, and whole fish were frozen at -20ºC until processing. Processing and pre-treatment of samples were done according to (Bouman *et al.*, 1986). After weighting (wet weight), individual fish were dried to a constant weight in a drying oven at 105ºC (24-36h) and then incinerated in a muffle furnace (DINKO) at 550ºC (12 h). To ensure a constant humidity of the samples and to cool them, a desiccator was used. The ashes were weighted and acidified with concentrated $HNO_3$ () to a final concentration of 5%. Total aluminium in each digest was determined by GFAAS (Soolar M AA, Unicam), using the wavelength 309,3 nm, at temperatures of ashing and atomization of 1500ºC and 2700ºC, respectively. The detection limit of the method was 0.005 $\mu g.g^{-1}$ of dry

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weight. These analysis were performed in the Autoridade da Segurança Alimentar e Económica (ASAE) laboratory, in Lisbon (Portugal).

### b. Histological analysis

At each time point, fish were euthanized and samples of gills, liver, spleen and head-kidney were collected from two fish of each group. Histological sections of these organs were performed in order to detect pathological effects and aluminium deposition. The organs were immediately fixed in a buffered formalin (10%) at pH 7,0 (Panreac), processed in alcohol series, mounted in wax and 5 µm tissue sections were prepared. This sections were stained using Haematoxylin-Eosin stain (HE) (Luna, 1968), and a Modified Haematoxylin stain for aluminium detection (MH) (Havas, 1986). MH was prepared in pure water, with haematoxylin (0.2%) and sodium iodate (0.02%), obtaining an amber-orange solution which gives a vivid deep purple colour when it reacts with Aluminium (Al). Histological work was performed in ITTUCA (Universidade do Algarve, Portugal), and expert identification and critical observations were given by Doctor Christopher Exley (Keele University, UK).
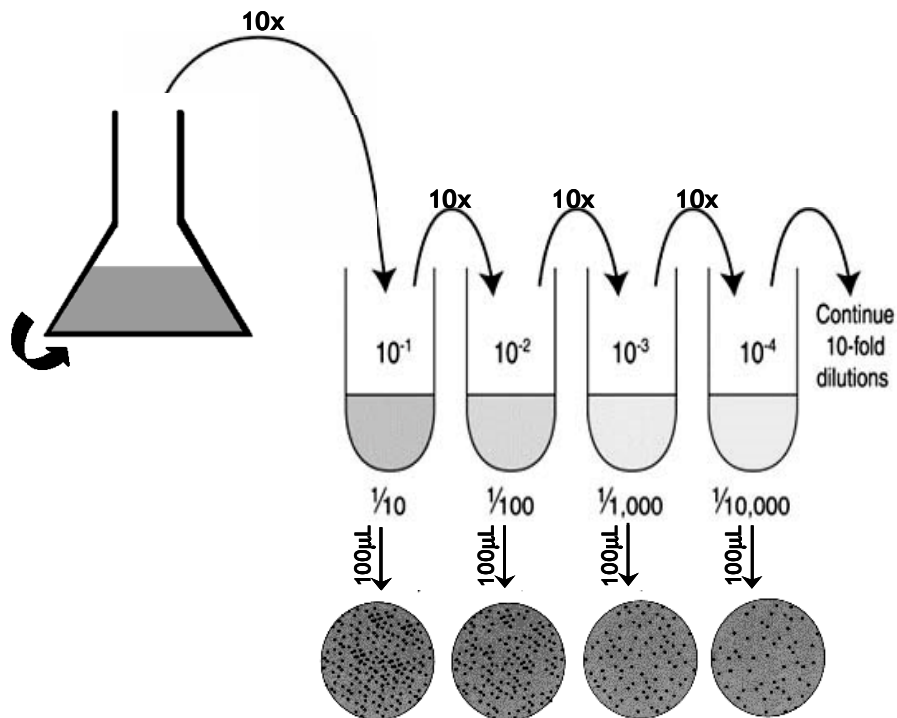
### V. Bacteria

*Photobacterium damselae* ssp. *piscicida* (*Phdp*) strain PTAVSA95 was gently provided by Doctor Nuno Santos (Fish Immunology and Vaccinology group, Institute for Molecular and Cellular Biology - IBMC, Porto, Portugal). The

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bacteria were kept at -80ºC in stock solutions of Tryptic Soy Broth (TSB, Difco) or Brain Heart Infusion Broth (BHIB, Merck), both supplemented with NaCl (Merck) at 1% final concentration (TSB-1 or BHIB-1) and 15% (v/v) of Glycerol (Panreac). Prior to use, bacteria were unfrozen and plated in Tryptic Soy Agar (TSA, Difco) or Brain Heart Infusion Agar (BHIA, Merck), both supplemented with NaCl for a final concentration of 1% (TSA-1 or BHIA-1). After colonies formation on plates, a swab was used to transfer bacteria to TSB-1 (or BHIB-1), and were kept overnight in a shaker (KS 501 digital KIKA Labortechnik) at room temperature ($\pm$25ºC).

### a. Calibration Curve

A calibration curve was performed in order to have a correlation between the Absorbance at 600nm ($Abs_{600\ nm}$) (ThermoLabsystems Multiskan RC) and the Colony Forming Units ($CFU.mL^{-1}$) of the bacterial solution (do Vale et al., 2005). At 0, 4, 6, 12 and 24 hours, $Abs_{600\ nm}$ was measured and serial dilutions of the solution were plated into TSA-1 (or BHIA-1) (Fig IV) for CFU determination. Colony counts were performed within the range of 30 to 300 colonies per plate. All the microbiology experiments were performed at the Laboratório de Microbiologia (Faculdade de Ciências do Mar e Ambiente, Universidade do Algarve).

**Figure IV:** Ten fold dilutions to determine CFU's of bacterial solution.

### b. Bacteria Identification

Morphological and biochemical tests were often performed in order to confirm bacteria purity. Morphological tests included microscopical observation to detect form and mobility, whereas biochemical tests as gram staining, capsules staining and API 20E (Biomériaux-Vitek) were performed to detect the biochemical profile of *Phdp*.

## VI. LD$_{50}$ (Lethal Dose 50) Experiment

Bacteria were unfrozen, plated in TSA-1 (or BHIA-1), incubated 48h at 25ºC, transferred to TSB-1 (or BHIB-1) and shaked overnight at room temperature (±25ºC). When Abs$_{600nm}$ value was between 0.400 and 0.600, the shaker was stopped and CFU determined.

60 fish with 2.22±1.16 g ($\mu \pm \sigma$) body weight were slightly anaesthised and i.p. (intraperitoneal) injected with 100µL of *Phdp,* using 1 mL syringe and 26 G needles. Fish were divided in 6 groups, regarding the dosis from 10$^3$ to 10$^8$, and mortalities were registered for a period of 7 days.

## VII. Challenge Test

### a. Immunization

In order to do this experiment, 170 fish with 1.76±0.61 ($\mu \pm \sigma$) body weight were separated in two groups. The first group went through osmotic shock (55 PSU) for 96 h, and the second group was maintained in seawater (35 PSU).The design of this experiment is shown in Fig V. After 96h (4 days), immunization by immersion for 30 seconds was performed and the fish received the following treatments (20 fish each treatment):

- **Control (CTRL):** *placebo* bath in seawater at 55 PSU for the HSW group and seawater at 35 PSU for the SW group;

- **Bacterin (B):** *Phdp* bacterin (*Phdp* MT1415 inactivated, supplied by Doctor Nuno Santos, IBMC, UP) diluted at 1:10 [(v/v) bacterin:seawater at 55 PSU for the HSW group and bacterin:seawater 35 PSU for the SW group];

- **Bacterin&Alum (B&A)**: *Phdp* bacterin diluted 1:10 (v/v), with addition of 1% of alum to water (final concentration);

- **Alum (A)**: bath with 1% of alum (final concentration) in seawater at 55 PSU for the HSW group and seawater at 35 PSU for the SW group.
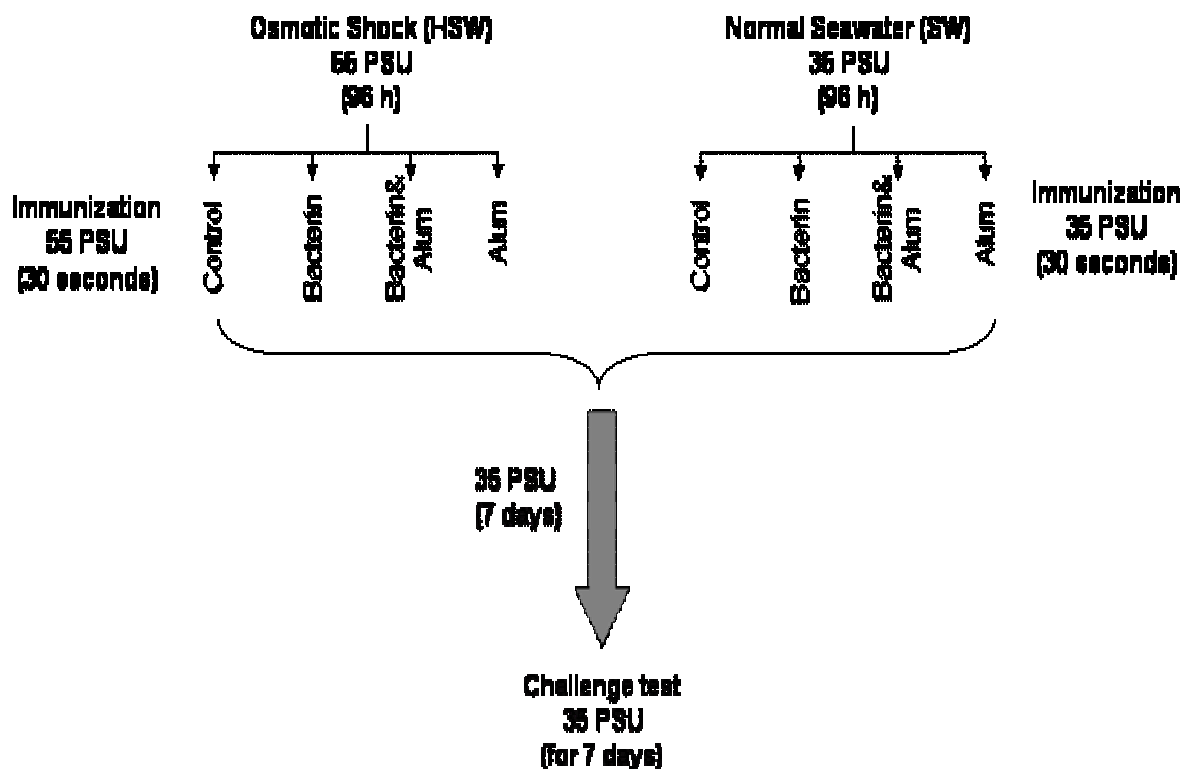


**Figure V:** Design of the experiment: osmotic shock, immunization and challenge.

### b. Challenge experiment

One week post immunization (Fig V), the challenge with bacteria was performed. 10 fish from each treatment were anaesthetised with Phenoxy-2-ethanol and i.p. injected with 100 µL of *Phdp*. The *Phdp* dosis used was $2.7 \times 10^7$ CFU/fish (determined previously). Mortality in each group was recorded daily, and samples of moribund and dead fish were taken in order to confirm the cause of death. Samples of liver, spleen and head kidney were plated in TSA-1 and incubated at 25°C for 24-48 hours. Morphological and biochemical tests were then performed to confirm that the cause of death was *Phdp* (explained previously in section **V-b**).

Cumulative mortality was registered and RPS% (Relative Percent Survival) was recorded at the end of the experiment (day 7 after challenge), according to the method used by Amend (1981):

$$RPS\% = 1 - \left( \frac{mortality\ in\ vaccinated\ group\%}{mortality\ in\ control\ group\%} \right) \times 100$$

### VIII. Immune Response

#### a. Blood collection and serum separation

5 fish of each experimental group were pooled for evaluation of antibody (IgM) level at 11 and 18 days. The t=0 represents a single pooled group (5 fish) sampled at the beginning of the experiment. The t=4 represents samples from fish (5) in both SW and HSW groups, 96 hours (4 days) after the osmotic shock,

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and immediately before immunization. The fish were killed with excess of anaesthetic (Phenoxy-2-ethanol), and blood was immediately collected by severing caudal peduncle and using capillary tubes. The blood samples were kept at room temperature for 60 minutes and stored at 4°C overnight for complete blood clotting. The samples were then centrifuged at 1700 rpm for 30 minutes, and sera was separated and stored at –80°C until use.

### b. ELISA of serum IgM quantification

The antibody responses of *Sparus aurata* against *Phdp* were measured by indirect Enzyme Linked Immunosorbent Assay (ELISA).

All protocols were followed as a recommendation from AquaticDiagnostics Ltd, except for the chromogen/substrate use that followed Sigma Aldrich recommendation product.

### b.1. Antigen Coating

*Phdp* was incubated overnight in TSB-1 at room temperature with agitation, as previously described. When solution reached the desired concentration indicated by the manufacturer's instructions ($10^9$ CFU.mL$^{-1}$), 1 mL of this solution was resuspended in 9 mL (dilution 1:10) of Phosphate Buffered Saline (PBS: 0.02M Phosphate and 0.15M NaCl), and 100µL of this solution was added to each well of a 96-well ELISA plate (NUNC, Flat-bottom, MaxiSorp). The plate was covered and incubated overnight at 4°C. After this

incubation period, 50µL of a solution of 0.05% (v/v) of gluteraldehyde was added. Finally, the plate was incubated for 20 minutes at room temperature.

## b.2. Block non-specific binding sites

After the last antigen coating incubation period, three rinses with Low Salt Buffer (LSB: 2.42% Trisma base, 22,22% NaCl, 0.1% Merthiolate and 0.5% Tween 20, pH 7.3) were performed. The wells were then post-coated/blocked for 2h at room temperature with blocking/conjugate buffer [1% (w/v) of Bovine Serum Albumin (BSA) in LSB] followed by three rinses with LSB.

## b.3. Serum samples and negative control

Double dilutions of the fish serum samples were prepared in PBS, ranging from ½ to ¼, and PBS was used as negative control. 100µL of each sample dilution and negative control were then added to the plate, and incubation took place for 3h at room temperature.

The plate was then washed 5 times with High Salt Buffer (HSB: 2.42% Trisma base, 29,22% NaCl, 0.1% Merthiolate and 1% Tween 20, pH 7.3) and incubated for 5 minutes on the last wash.

### b.4. IgM monoclonal Antibody labbeled with HRP

After HSB washes and incubation, 100 µL of the reconstituted anti-gilthead sea bream IgM monoclonal antibody-HorseRadish Peroxidase (Aquatic Diagnostics Ltd.) was added to each well. The plate was then incubated for 1h at room temperature. Rinse took again place with the HSB (5x), performed as previously.

### b.5. Chromogen/substrate & Readings

After the incubation of the anti-IgM labbeled with HRP, 200 µL of Chromogen (OPD: o-Phenyldiamine dihydrochoride), with the respective substrate buffer (SIGMA FAST tablets) were added to each well. The reaction was allowed to proceed for 30 minutes and stopped by the addition of 50 µL of 2M $H_2SO_4$. Afterwards Optical Density was measured at 450 nm (O.D.$_{450nm}$) in a ELISA reader (ThermoLabsystems Multiskan RC).

Negative control consisted of wells without serum (i.e. PBS), whose OD value was deducted from each value of the samples. The results are expressed as (a) Stimulation Index ($\mu \pm \sigma$), which is obtained by dividing each sample value by its respective control value, and (b) Circulating IgM level (OD$_{450nm}$).

## IX. Statistical Method of Analysis

The SIGMA-STAT 3.11.0 of Systat software was used in GFAAS, Stimulation Index and Circulating IgM level statistical analysis performing the two way ANOVA, with subsequent Tukey corrections for multiple comparisons. The significant difference was considered if $p < 0.05$.

For the statistical analysis of Survival Percentages, the same software was used, although performing the Kaplan-Meier Survival Analysis - Gehan-Breslow, with subsequent Holm-Sidak corrections for multiple comparisons. The significant difference was considered if $p < 0.05$.

## I. Aluminium Detection

### a. GFAAS

Total aluminium values in the whole fish body were carried out at 0, 3, 6, 14, 48, 672 and 840 hours. Fig VI shows the results of HSW (Hyperosmotic shock) and SW (normal sea water) groups.
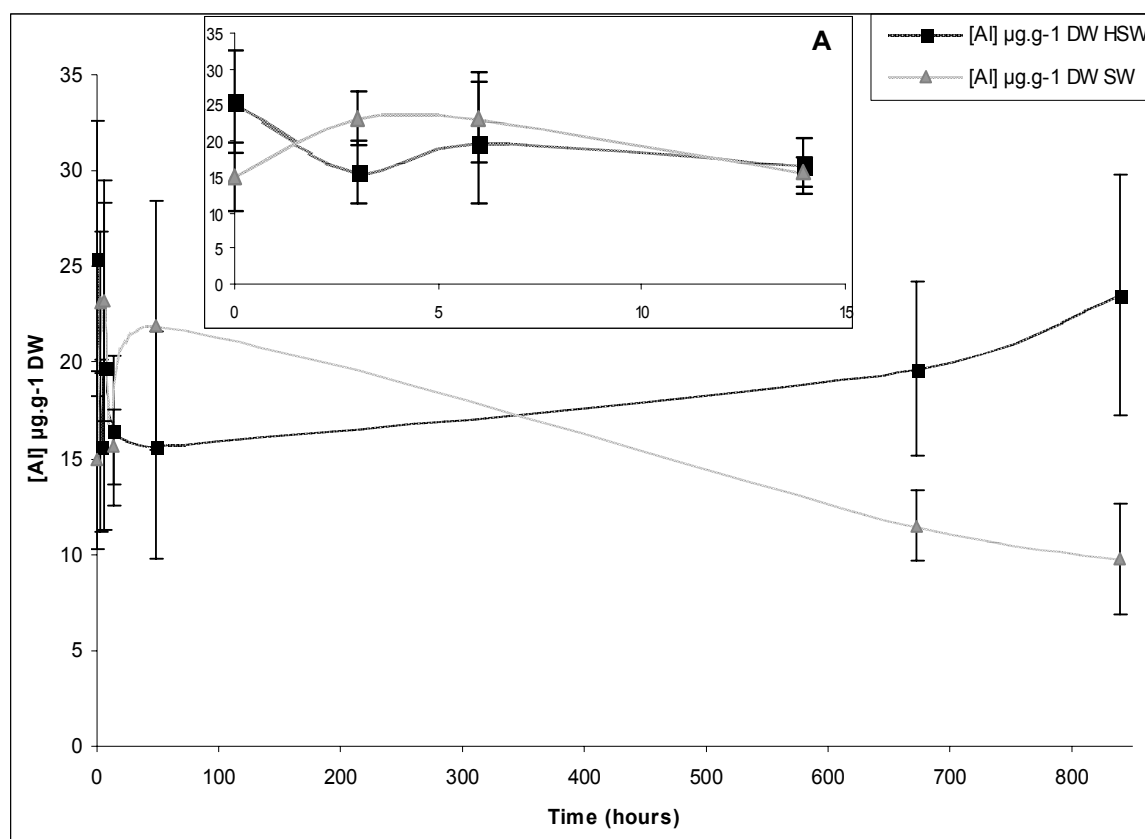


**Figure VI:** Distribution of Aluminium (expressed in $\mu g.g^{-1}$ DW of the all sea bream body) at different time points in Hyperosmotic Shock (■HSW) and Normal Salinity (▲SW) groups. **A** represents the aluminium concentration within the first 14 hours. Values are expressed as mean±SD (n≥4).

HSW group presented a decrease on aluminium concentration from 25.46±7.17 µg.g$^{-1}$ DW (0 hours) to 15.67±4.46 µg.g$^{-1}$ DW (3 hours) after the immersion treatment. From 3 hours onwards, the HSW group showed small variations in the aluminium concentrations.

The SW group had a small increase on the aluminium content from 14.94±4.65 µg.g$^{-1}$ DW (0 hours) until 23.23±6.28 µg.g$^{-1}$ DW (6 hours). At 14 hours, a decrease was observed (15.62±1.93 µg.g$^{-1}$ DW), after witch there was a new raise at 48h (21.93±6.48 µg.g$^{-1}$ DW). There was again a decrease at 840h (9.76±2.90 µg.g$^{-1}$ DW).

There where no statistically significant differences between SW and HSW groups (p=0.173).

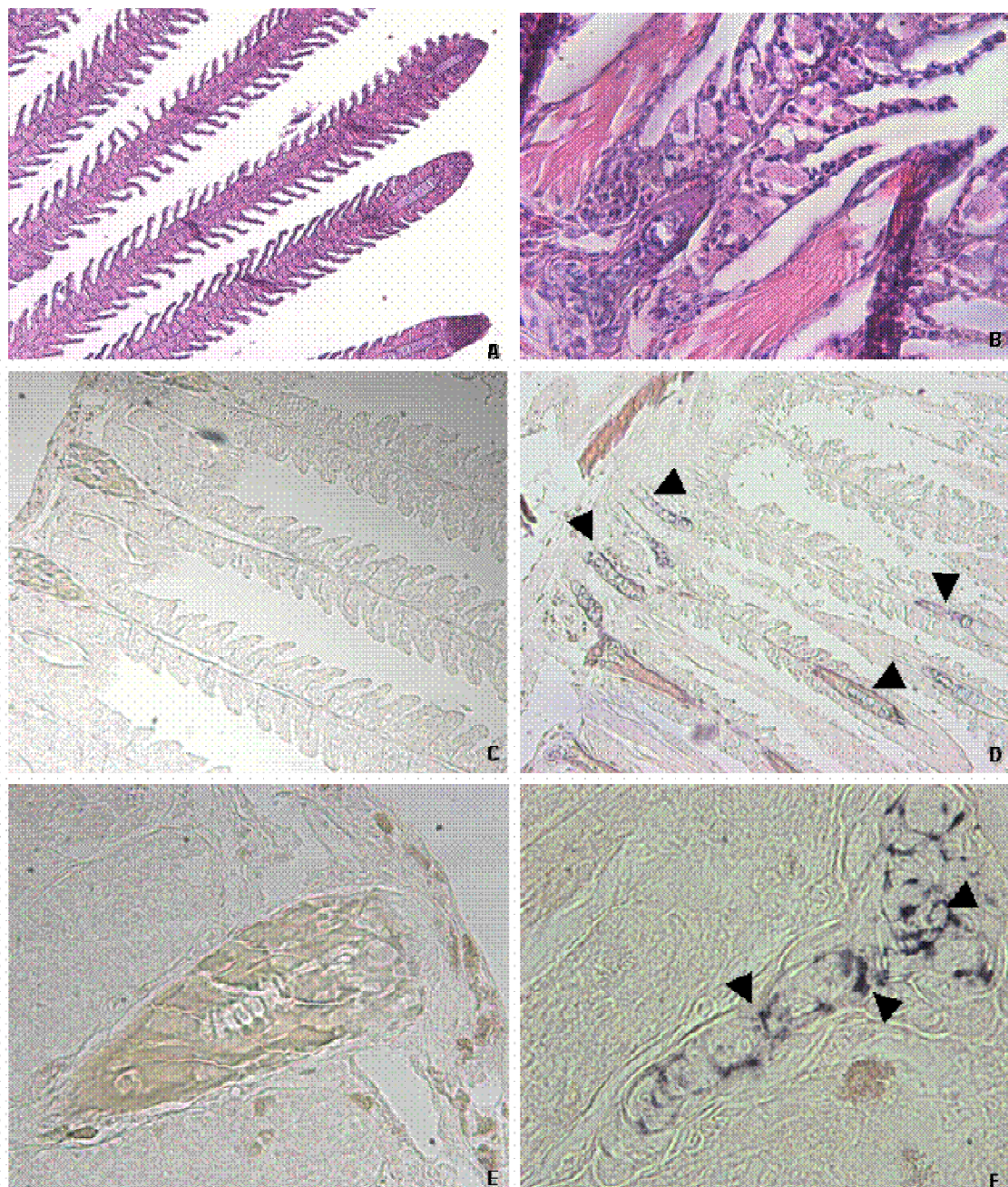### b. Histological analysis

#### b.1. Gills

Histological sections of gills stained with Haematoxylin-Eosin (HE) (**A** and **B**) and Modified Haematoxylin (MH) (**C - F**) are shown in Fig VII. No pathological effects were observed in this tissue, either in control group or in groups exposed to aluminium.

In order to observe aluminium deposits, MH stain was performed. Here (**C - F**) is possible to see gills from a fish of the control group (**C** and **E**), where no purple deposits were found. **D** (HSW group, 48h after exposure) and **F** (SW group, 3h after exposure) show abnormal deposits of aluminium mainly in the branquial cartilage, where the purple stain from the MH reaction appeared in the gill's

primary lamellae. All groups that were exposed to aluminium presented these abnormal deposits.



**Figure VII:** Histological sections of gills stained with Haematoxylin Eosin (**A** and **B)** and with Modified Haematoxylin stain (**C-F)**. **A** SW at 3h (125x); **B** HSW at 48h (600x); **C** control (125x); **D** SW at 48h (125x); **E** control (975x); **F** SW at 3h (975x). ► aluminium deposits stained purple.
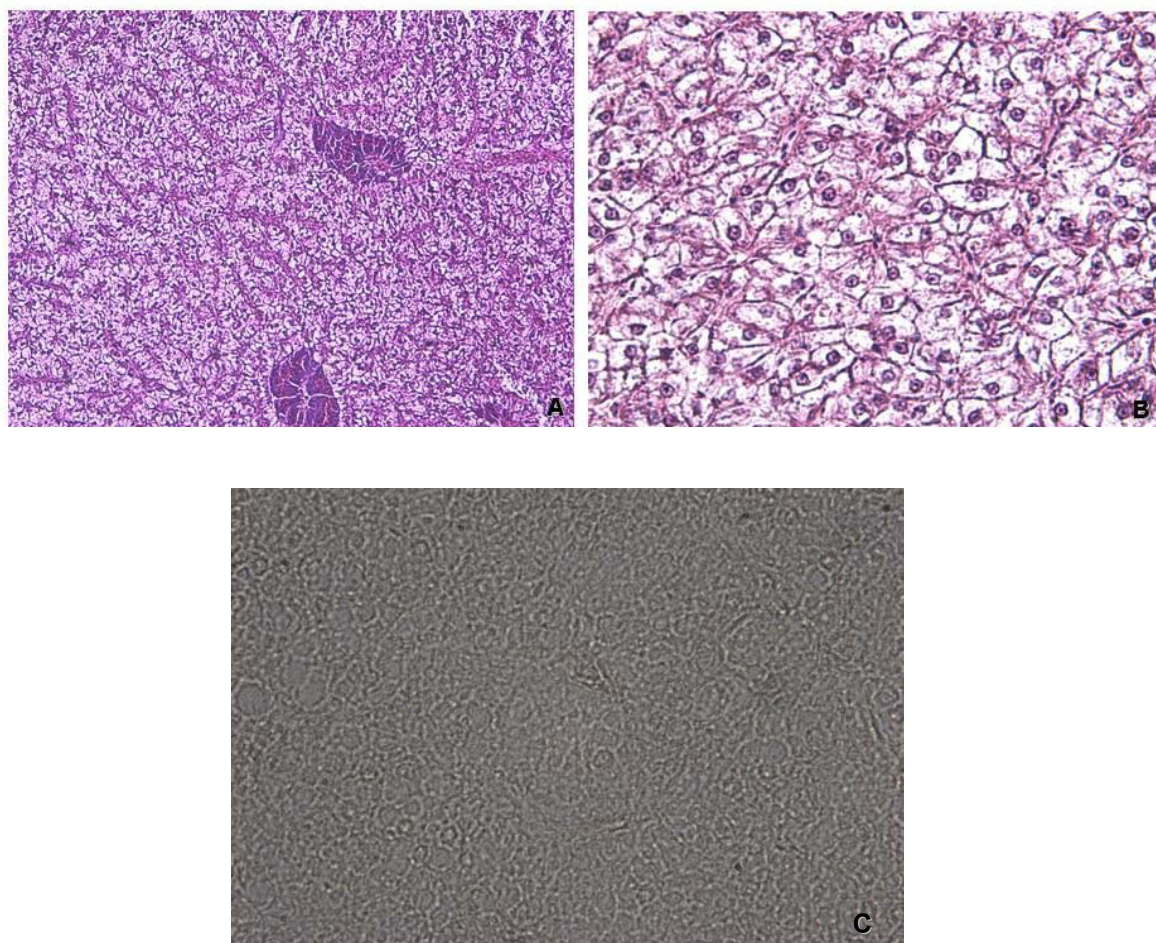
Liver sections stained with Haematoxylin-Eosin (**A** and **B)** and Modified Haematoxylin stain (**C**) are showed on Fig VIII. The **A** and **B** section histological sections present a normal liver from SW group after 14 hours of exposure to aluminium. **B** illustrates structure of liver, with well defined hepatocytes. No pathological effects were observed.

In the Modified Haematoxylin stain of liver sections there was no aluminium deposition in slides observed (all groups) (**C,** HSW at 672h).
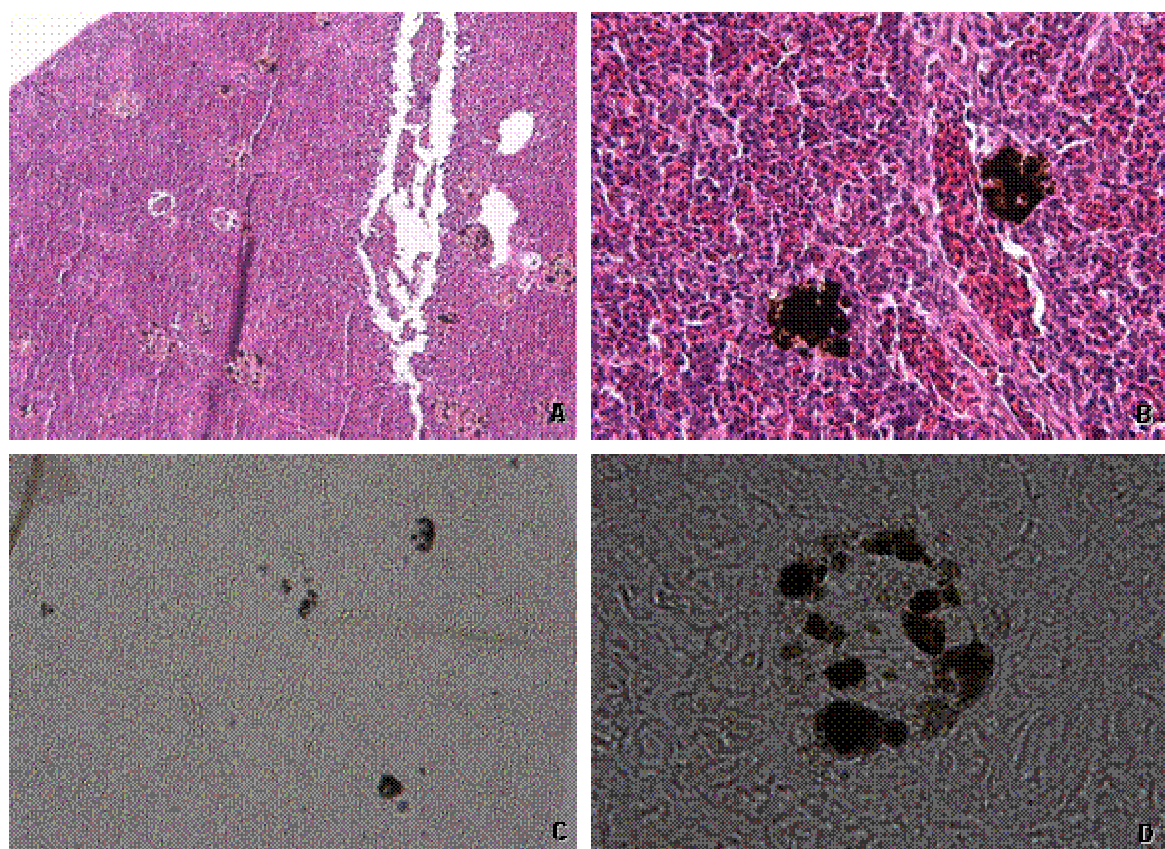


**Figure VIII**: Histological sections of liver stained with Haematoxylin-Eosin (**A** and **B**) and with Modified Haematoxylin stain (**C**). **A** 125x; **B** 600x; **C** 975x.

## b.3. Spleen

Spleen sections stained with Haematoxylin-Eosin (**A** and **B**) and Modified Haematoxylin stain (**C** and **D**) are showed in Fig IX, (HSW fish after 6 hours of aluminium exposure). Melanomacrophagic Centers (MMC) can be observed in **B** with a magnification of 600x. No pathological effects were observed in the tissue sections (in all groups).

Histological sections of spleen stained with Modified Haematoxylin stain are showed in **C** and **D**, where no aluminium deposits were observed. Melanomacrophagic centres (MMC) appear brown, as in Haematoxylin-Eosin stain. Illustrations **C** show a fish from SW group at 6 hours and **D** a fish from HSW group also at 6 hours.
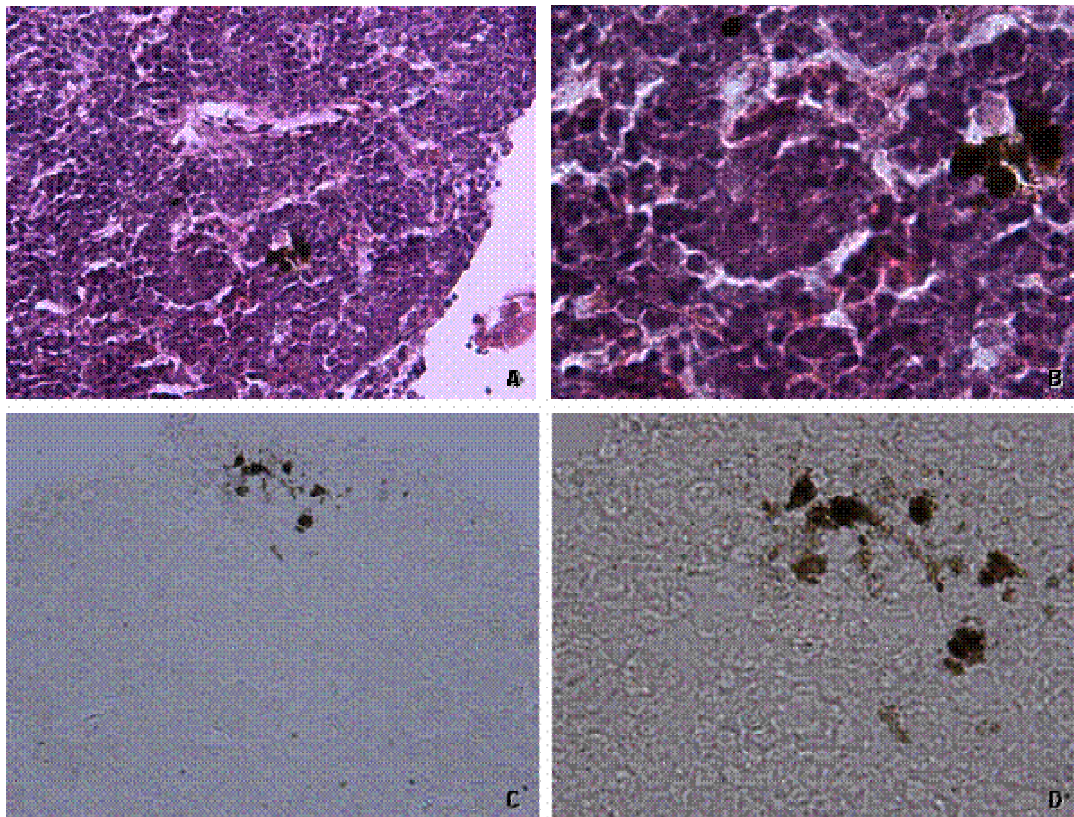


**Figure IX:** Histological sections of spleen stained with Hematoxylin-Eosin (**A** and **B)** and with Modified Haematoxylin stain. **A** 125x; **B** 600x; **C** 125x; **D** 1250x.

### b.4. Head-kidney

Fig X present histological sections of head-kidney stained with Haematoxylin-Eosin (**A** and **B**) and Modified Haematoxylin stain (**C** and **D**). HSW group after 3 hours of aluminium exposure). **B** shows a magnification of a MMC that is distinguished by the brown characteristic colour. After analysing the head-kidney histological sections, from all groups, no pathological effects were observed.

In Modified Haematoxylin stain, MMC appear brown, as it can be seen in spleen sections. This Fig is illustrating a fish from the group HSW after 840 hours of aluminium exposure. No aluminium deposits were found in the histological sections of the head-kidney.



**Figure X:** Histological sections of head-kidney stained with Haematoxylin-Eosin (**A** and **B**) and with Modified Haematoxylin stain (**C** and **D**). **A** 125x; **B** 1250x; **C** 600x and **D** 1250x.

## II. Bacteria

### II.a. Calibration Curve

After performing the Calibration curve, the following equation was obtained: $y=5.4561x+7.5961$ (Fig XI), where y represents log $CFU.mL^{-1}$ and x the $Abs_{600nm}$. The $r^2$ value obtained was 0.7675.
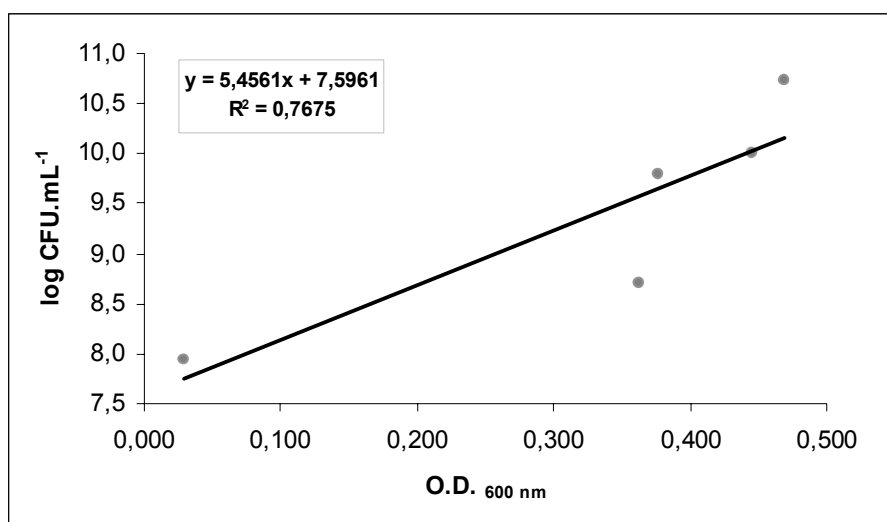


**Figure XI:** Representation of the calibration curve, where bacterial growth is presented relating the optical density at 600 nm with the logarithm of the $CFU.mL^{-1}$.

### II.b. Bacteria Identification

Morphologically, the bacteria was a rod shaped cell, with no motility.

Results of biochemical tests performed to the bacteria can be seen in Fig XII and Fig XIII.

When Gram staining was performed (Fig XII-A), it was perceptible the bipolar staining characteristic of *Phdp*. With capsular stain (Fig XII-B), it is possible

to see a hale (transparent) around the bacteria (blue-purple), which is also characteristic of *Phdp*.

The result obtained in the API-20E (2005004) was coincident with the profile already obtained by some authors, which described similar pattern for all *Phdp* strains.
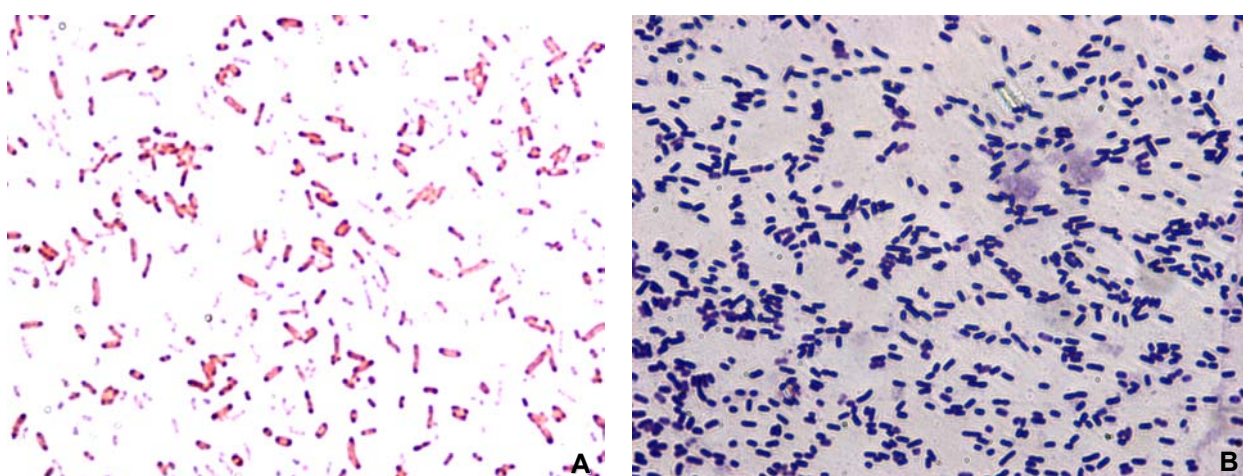


**Figure XII**: (**A**) Gram negative result; (**B**) capsular layer. Both images with a 1250x magnification.



**Figure XIII**: Biochemical profile of *Phdp;* gallery API 20E after 24 hours of incubation and biochemical result.

## V.    LD$_{50}$

The value of the Lethal Dose 50 obtained was $2.7 \times 10^7$ CFU.mL$^{-1}$, calculated according to the method of Muench and Reed (1938).

## VI.    Challenge-test

### VI.a. Challenge experiment

Challenge-test provided the survival curves presented in Fig XIV (SW group) and Fig XV (HSW group). By analysing the curve of SW group, it is possible to see that the treatment with B&A had the highest survival (90%), and that the lower value of survival corresponds to Alum treatment (30%). On the other hand, in the HSW group curve, Alum treatment presented the highest survival (60%) when compared with the other treatments of this group. The lowest value correspond to the B&A treatment (20%).

The mortalities obtained in the HSW group were higher than in the SW group.
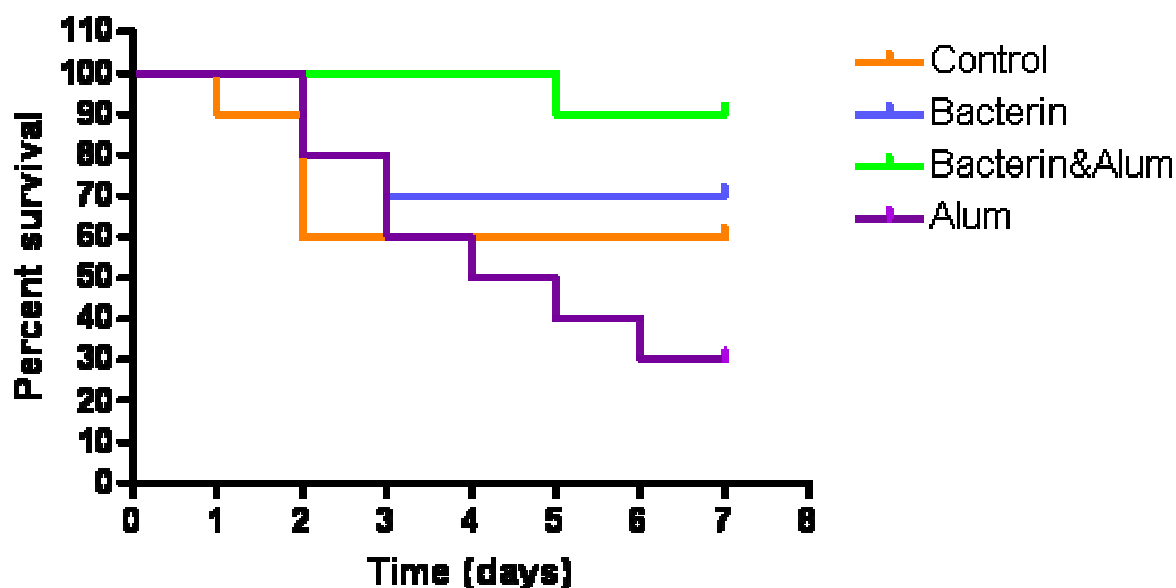
**Figure XIV:** Survival curves of sea bream challenged with *Phdp* in the SW group.
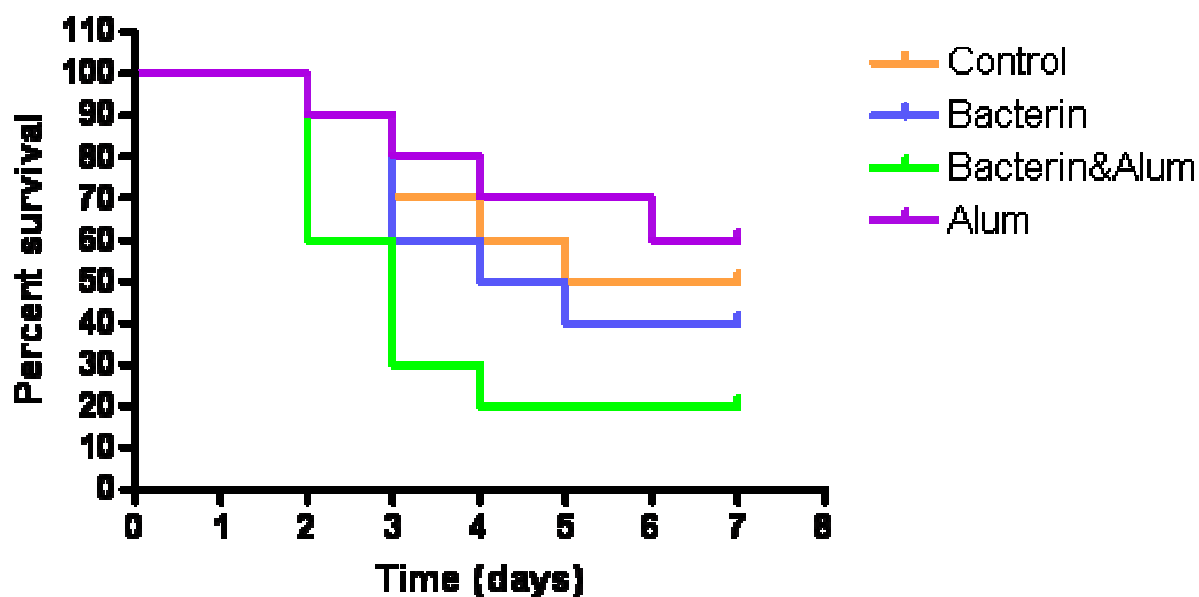


**Figure XV:** Survival curves of sea bream challenged with *Phdp* in the HSW group.

### VI.b. RPS (%)

Relative Percent Survival (RPS %) values for the different group treatments can be seen in Table IV. There are negative RPS%, which means that the

mortalities in those treatments are higher than the respective control. The best RPS% found was in the B&A treatment of the SW group (75%), and the worst protection was with Alum in the same group. Is also necessary refer that B&A in the HSW group had the opposite effect than the same treatment in SW group, with -60% and 75%, respectively.

**Table IV:** Relative Percent Survival of the SW and HSW groups, exposed to different treatments of immunization – Bacterin (B), Bacterin and Alum (BA) and Alum (A).

| | Bacterin (B) | Bacterin&Alum (BA) | Alum (A) |
|---|---|---|---|
| SW | 25 | 75 | (-75) |
| HSW | (-20) | (-60) | 20 |

## VII. Immune Response

### VII.a. Stimulation Index

Values of SI obtained in both SW and HSW groups are showed in Fig XVI and XVII.

In both groups, the dash line represents the control value of SI (SI=1). The vertical bars (6), represent the value of SI obtained in different treatments (B, B&A and A), sampled at days 11 and 18.

At day 11, in the SW group (Fig XVI) the only increase in SI was in the Bacterin treatment. At day 18, B and A treatments showed no variation in the SI values, while B&A treatment presented a remarkable SI increase.

Significant statistical differences were found between SW and HSW groups (p=0.003), mainly because of contribution day 18 of the the B&A (SW group) treatment.

HSW group (Fig XVII) points up to a general stimulation in all treatments, having all the values been higher than the control. With exception of the Bacterin treatment, there was a certain depression of the values from day 11 to day 18 of sampling.
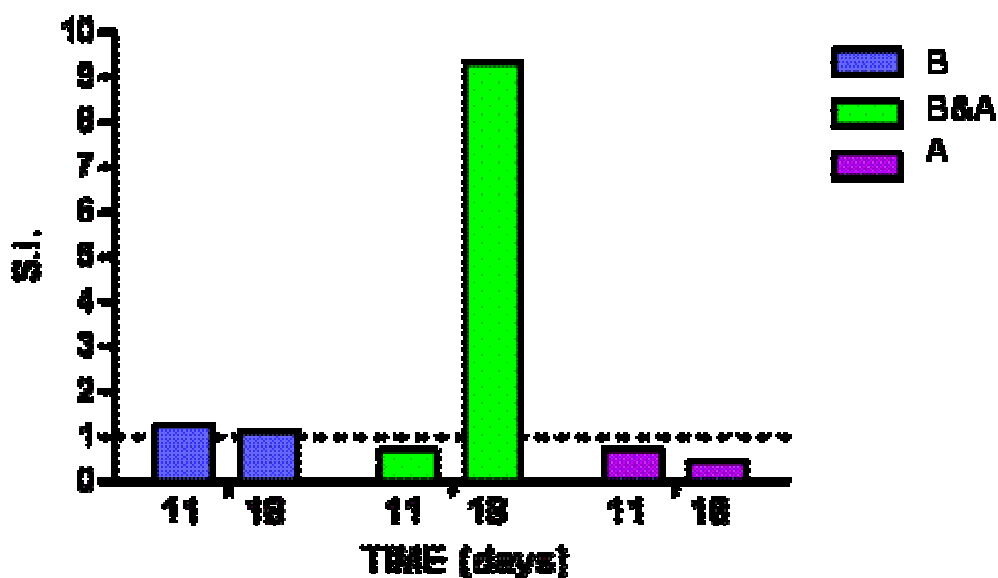


**Figure XVI:** Stimulation Index (S.I.) of SW group. The control value is 1 (dot line) and higher values express an increase of serum content IgM.
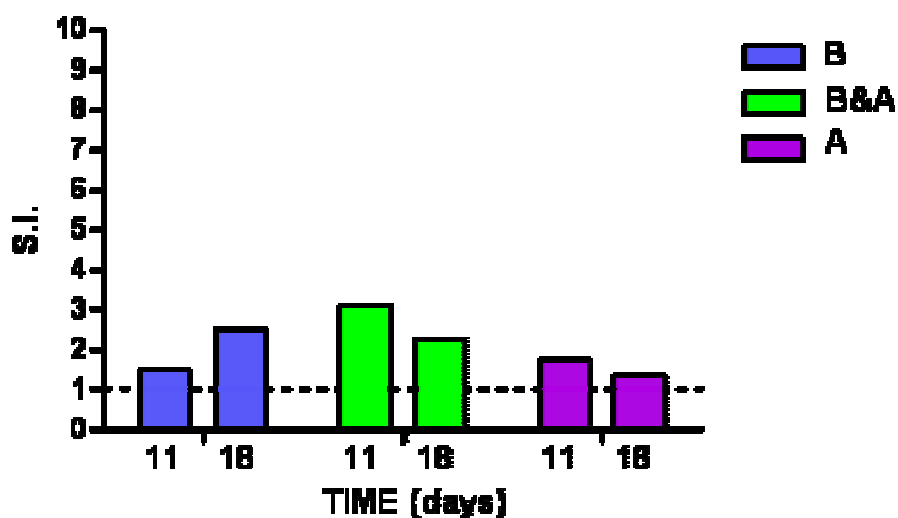
**Figure XVII:** Stimulation Index (S.I.) of HSW group. The control value is 1 (dot line) and higher values express an increase of serum content IgM.

### VII.b. Circulating IgM level

Circulating IgM level in both SW and HSW groups represented by O.D.$_{450nm}$ can be observed in Fig XVIII and Fig XIX, respectively.

The O.D.$_{450nm}$ value of the control (t=0), was 0,0504±0,0332.

At day 4, the O.D.$_{450nm}$ values in the SW group and HSW group were similar (0,0575±0,0470 and 0,0634±0,0407, respectively). There is no evidence that this values are different from t=0 (p=0.363).

At day 4 immunizations were performed.

Statistical significant differences were found between SW and HSW (p<0.0001), because of the B&A treatment (SW group) contribution at day 18.
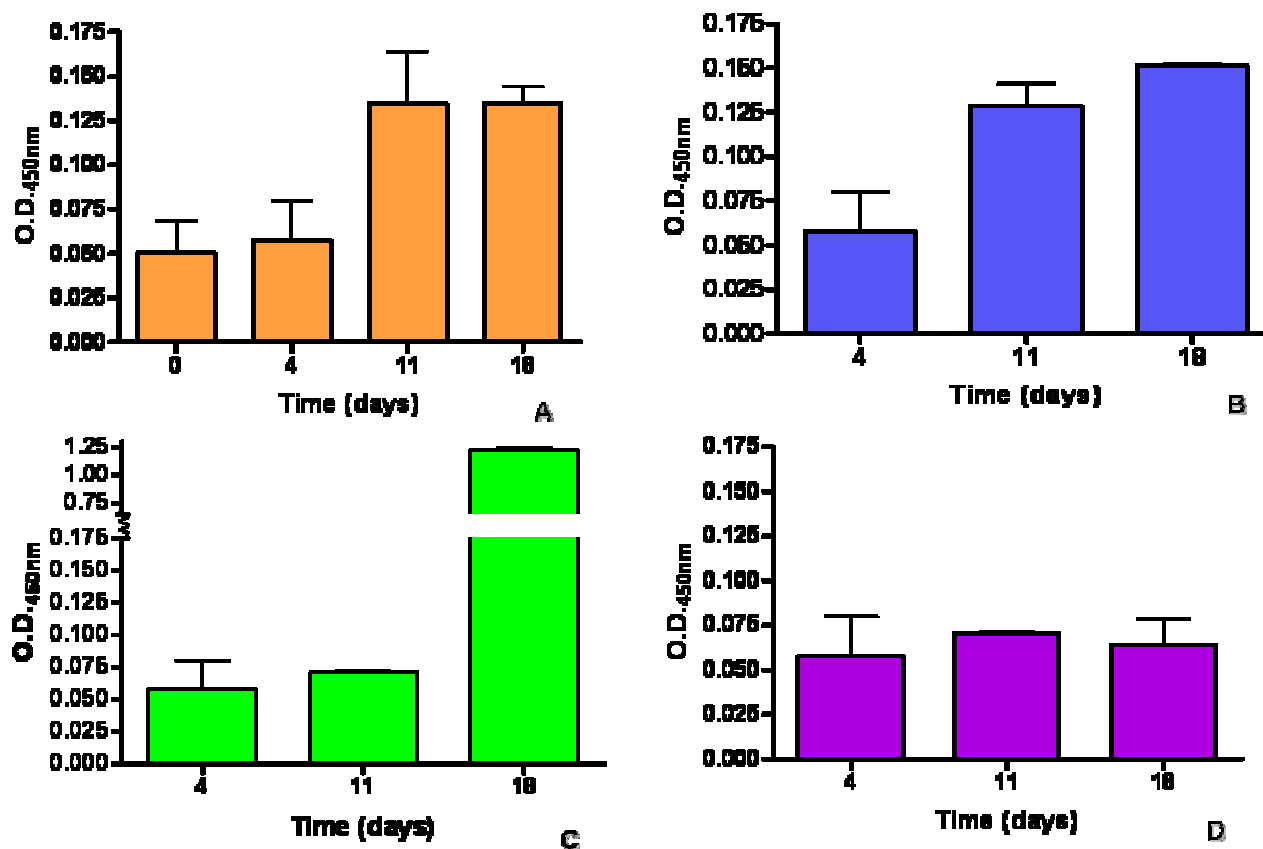
**Figure XVIII:** Circulating IgM level, expressed as Optical density at 450 nm, in serum of gilthead sea bream in SW group. Data represent the different treatments that include Control (**A**), Bacterin (**B**), Bacterin&Alum (**C**) and Alum (**D**). In **C**, y axis is different from the others, being in two segments.
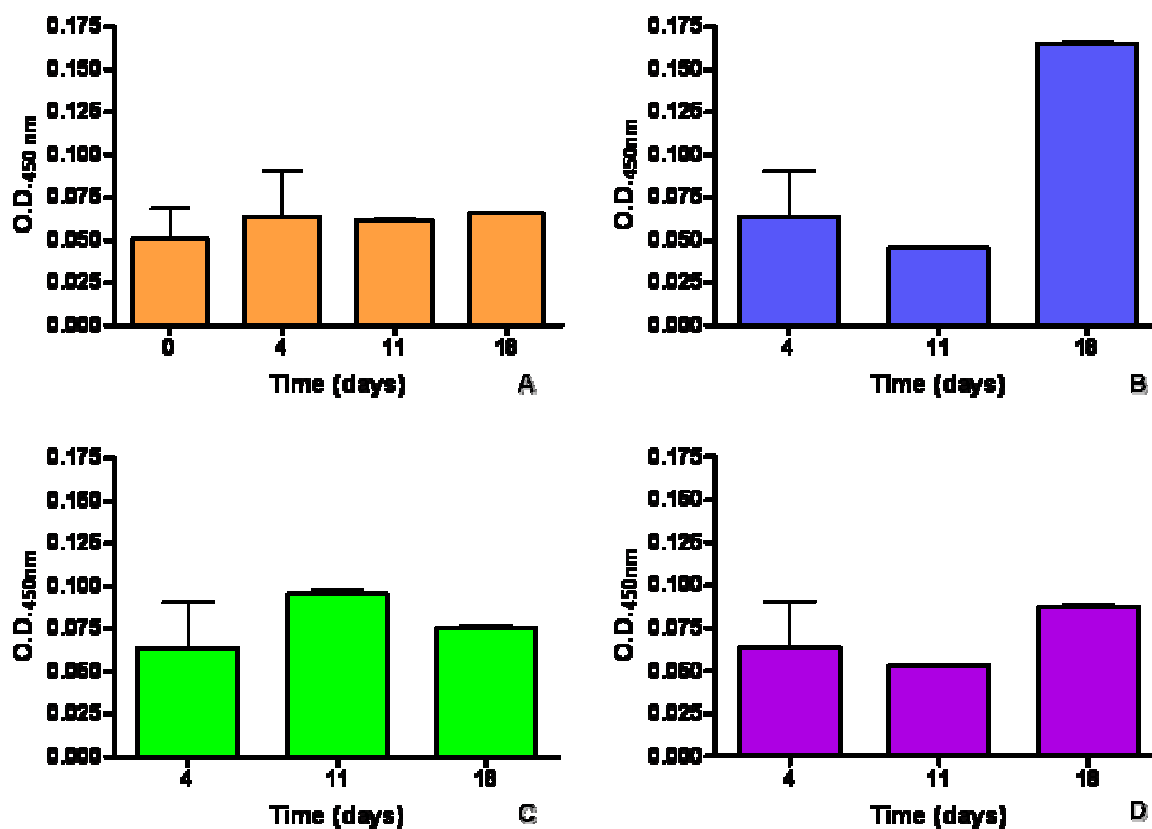
**Figure XIX:** Circulating IgM level, expressed as Optical density at 450 nm, in serum of gilthead sea bream in HSW group. Data represent the different treatments that include Control (**A**), Bacterin (**B**), Bacterin&Alum (**C**) and Alum (**D**).

## DISCUSSION

Aquaculture is the fastest growing sector of food production industry in the world. Because this sector growth, maximum profitable production tend to be required, thus leading to higher densities. This enhanced culture conditions tend to increase pathological problems. Prophylactic measures, such as vaccines, are among several possibilities of fish pathology control.

Adjuvants are substances that potentiate efficacy of vaccines. Current information show that adjuvants are commonly used in injected vaccines. Nevertheless, information about the use of adjuvants by immersion is still scarce (Ascarateil & Dupuis, 2003).

The aim of this research was to test the efficacy of alum, when used as an adjuvant in immersion vaccine against *Photobacterium damselae* ssp. *piscicida* in gilthead sea bream (*Sparus aurata).*

Mechanisms of vaccine absorption in fish are not clearly described (Ascarateil & Dupuis, 2003; Huising *et al.*, 2003), but adsorption of the vaccine can be enhanced by the use of hyperosmotic shock (Huising *et al.*, 2003). At the same time, the hyperosmotic shock increases ion influx (Guerreiro *et al.*, 2004; Laiz-Carrion *et al.*, 2005), when fish were placed in 55 PSU water.

Our first objective was to test aluminium absorption in sea bream. For that we used the standard immersion time of 30 seconds (recommendation of fish vaccines producers), applied together with an hyperosmotic shock of 55 PSU, in order to see the influence of the salinity in metal (Al) absorption.

The hyperosmotic shock lasted for 4 days, because there are indications that this period is needed to augment absorption of the ionic influx (Jensen *et al.*, 1998; Guerreiro *et al.*, 2004; Laiz-Carrion *et al.*, 2005).

Although aluminium adjuvants have been used for a long time, surprisingly little is known about mechanisms by which they enhance the immune response (Lindblad, 2004). The two most commonly cited mechanisms are formation of an antigen depot and immunostimulation (HogenEsh, 2002). Aluminium adjuvants are the only adjuvants approved for use in human and veterinary vaccines (HogenEsh, 2002; Lindblad, 2004), but there have been few studies on the use of the alum adjuvanted bacterins in fish (Horne *et al.*, 1984; Mulvey *et al.*, 1995). In injected vaccines, the alum concentration tested was of 1% (Mulvey *et al.*, 1995) and 2.5% (Horne *et al.*, 1984). There are evidences that aluminium can be toxic to fish (Horne *et al.*, 1984; Exley, 1998), thus we decided to use alum at 1% final concentration. The choice of alum use was because of its solubility in water (W.H.O., 1997). The presence of aluminium in fish was measured by two methods: GFAAS for aluminium quantification and histology for aluminium deposition. There are no data on whole body aluminium concentration, thus we cannot compare our results with other works. Concentrations of aluminium in fish can be mainly found in muscle and gills (Exley, 1996 and 1998; Tein *et al.,* 2004; Türkmen *et al.,* 2005; Tein *et al.* 2006).

In studies regarding the augment of ion absorption with hyperosmotic shock have already been published (Jensen *et al.*, 1998; Huising *et al.*, 2003; Guerreiro *et al.*, 2004; Laiz-Carrion *et al.*, 2005). Our results didn't support

those statements, since there were no differences in aluminium concentration between SW and HSW groups.

Modified Haematoxylin technique (Havas, 1986) is used by several authors (Exley, 1996; 1998; Peuranen *et al.*, 2003; Vuorinen *et al.*, 2003) to evidenciate aluminium in histological sections. According to Exley (1996), the gill is the main site of accumulation during acute exposure to aluminium. Our observations showed the same pattern of deposition in gills, while liver, spleen and head-kidney didn't show any signs of aluminium presence. Several authors (Exley, 1996; Peuranen *et al.*, 2003; Vuorinen *et al.*, 2003; Teien *et al.*, 2006) indicated that aluminium binds to the gill epithelium, where it is internalized to secondary lamellae (Peuranen *et al.*, 2003; Vuorinen *et al.*, 2003). In our study, the main site of deposition was the cartilage of primary lamellae, where the aluminium could play a role in replacing Calcium from bone matrix (Verbost *et al.*, 1992; Goyer, 1997).

MMC (cells filled with dark brown pigment granules) are normally present in spleen and head-kidney of fish (Rodrigues & Pereira, 2004), although it has been also reported in the liver of some teleost (Manera *et al.*, 2000; Leknes, 2001). Sea bream didn't showed this MMC structures in the liver, which is similar to the findings in sea bass (Rodrigues & Pereira, 2004). These macrophage aggregates can deposit iron (Rodrigues & Pereira, 2004). In circulation, iron is captured by transferrin and transported to several organs (Rodrigues & Pereira, 2004). This plasma binder, transferrin, is able to bind aluminium, since aluminium behaves like iron in many biological systems

(Goyer, 1997). In this way, it could be expected to find aluminium deposits in MMC, however MH didn't show aluminium deposits in sea bream MMC.

In addition, there were no indications of pathological problems in all the tissues analyzed, in spite some results indicate gill damage, mainly hypertrophy and necrosis (Peuranen *et al.*, 2003; Vuorinen *et al.*, 2003).

These experiments showed that the hyperosmotic shock didn't influence aluminium absorption, indicating that alum can be used as an adjuvant in fish immersion vaccines, without side effects.

*Photobacterium damselae* ssp, *piscicida* is the causative agent of pasteurellosis (or pseudotubercullosis) in fish, associated to big mortalities in fish farms all over the world (Romalde & Magariños, 1997; Le Breton, 1999; Romalde, 2002; Barnes *et al.*, 2005). Pahology and histopathology have been widely described and reviewed (Barnes *et al.*, 2005). Susceptibility to pasteurellosis differs in some fish species on the basis of fish age; moreover, the fact that this bacteria is highly pathogenic indicates that it must have strong virulence mechanisms (Le Breton, 1999; Romalde, 2002).

Our results showed morphological and biochemical similitudes between PTAVSA95 and other *Phdp* strains (Romalde, 2002). Thus, the result obtained in the API-20E (2005004) sustain author indications on API-20E usefulness for the presumptive identification of *Phdp* (Romalde, 2002)*.*

In order to determine the virulence of *Phdp* strain PTAVSA95 in $2.22\pm1.16$ g sea bream, the $LD_{50}$ experiment was performed. Several studies indicate that *Phdp* $LD_{50}$ range for sea bream is between $10^3$ and $\geq 10^8$ (Magariños *et al.*, 1992; Magariños *et al.*, 1996; Balebona *et al.*, 1998; do Vale

*et al.*, 2005). Our $LD_{50}$ result was $2.7 \times 10^7$ CFU/fish which is within this range. In addition this result is similar with the result obtained by do Vale *et al.*, (2005) when used PTAVSA95 in sea bass. Thus we can consider PTAVSA95 as a low virulent strain (Magariños *et al.*, 1992; Magariños *et al.*, 1996).

Throughout the last 25 years, there have been a variety of studies analysing the effectiveness of immunization in preventing pasteurellosis (Romalde, 2002). The ability of fish vaccines to induce a protective immunity is for the most part based on experimental studies and/or field experiments, but is critical to identify the individual components involved in inducing protection (Arijo *et al.,* 2004).

Immunological status of fish exposed to different treatments (B, B&A and A) are represented by SI and circulating IgM level. The ratio obtained by $IgM_{sample}/IgM_{control}$ is the SI value, which means that the SI value obtained is already a comparison with the control. SI values below the control were found in some treatments in SW group, namely in A (11 and 18 days) and B&A (11 days) treatments. Cuesta *et al.* (2004) found negative SI values when exposed sea bream (100-200g WW) to 1.6 mM 2-phenoxyetanol (anaesthetic) for 1 hour, and when applied the immunostimulant levamisole at 0.3g/kg diet, after 3 and 6 weeks.

Few studies have evaluated the salinity effects on fish immune parameters (Cuesta *et al.*, 2005). According to Huising *et al.* (2003) hyperosmotic immersion acts as an adjuvant in the way that it enhances vaccine uptake and stimulates the activation of the innate as well as the acquired immune system. Results from Cuesta *et al.* (2005) show that

hyperosmotic acclimatization has a beneficial (or no) effect on sea bream (100-150g WW) humoral immune parameters. Our results are in agreement with those obtained by Cuesta *et al.* (2005), having been achieved positive SI values in all treatments of HSW group. However, statistical analysis show that SW group had a general better Stimulation Index, because of the contribution of the B&A treatment at day 18.

Circulating IgM molecules are affected by immunomodulators (Cuesta *et al.*, 2004), however there is no evidence of which mechanisms influence the fish immune system after salinity disturbances (Cuesta *et al.*, 2005). Our results show that after 4 days of acclimatization (HSW or SW), values of circulating IgM were not significantly different (p=0.924). Thus, it is possible to consider that sampling days 11 and 18 correspond to 7 and 14, respectively. Cuesta *et al.* (2005) found higher circulating IgM level at day 14 in response to hyperosmotic shock. In opposition, our results at day 14 show a significant higher circulating IgM level in the SW group, because of contribution of B&A treatment (p<0.001). According to Huising *et al.* (2003), HSW leads initially to a profound acute inflammatory response (as the antigen uptake increases, intracellular uptake by leucocytes also increase) and later to an enhanced specific mucosal response. Thus, a lower circulating IgM level obtained can be justified, for the short sampling time (14 days).

Antibodies in the circulation constitute an important part of resistance against pathogenic microorganisms (Arijo *et al.*, 2004). To correlate serum antibodies (circulating IgM level) with protection offered by the different treatments, a challenge test with *Phdp* was performed. After sea bream

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infection with *Phdp,* survival curves (Fig XIV and Fig XV) along 7 days were made. The challenge results showed statistically significant differences (p=0.003) between SW and HSW group, again because of the contribution of B&A treatment. Mulvey *et al.* (1995) results indicate lower mortalities in B&A, followed by Bacterin, Control and Alum, respectively. RPS% values obtained from this work depended upon challenge time, since the authors used 30, 60 and 90 days post-vaccination to perform challenges. In these challenges, RPS% values in average were $100\pm0.00\%$ for B&A, $86.37\pm8.20\%$ for B and finally $-8.30\pm13.95\%$ for A treatment. Considering the survival percentage of the SW group, our results are coincident with Mulvey *et al.* (1995). On the other hand, survival rate results from HSW group, showed an opposite response, having the B&A obtained the higher mortalities, followed by Bacterin, Control and alum, respectively.

Our RPS% values indicated a 75% protection in the B&A treatment. The other treatments showed RPS% values lower than 70%, meaning that those treatments are not so effective. Bakopoulos *et al.* (2003) tested several vaccines, and obtained better RPS% values with vaccine mixtures (94.5-100%) than with a commercial Bacterin (25%) in sea bass (*c.* 20 g). Our result is similar in what concerns Bacterin treatment (SW group), where we obtained 25% of RPS.

## CONCLUSION

The aim of this study was to investigate a hydro soluble adjuvant – alum (aluminum and potassium sulfate dodecahydrate) - in immersion vaccine against *Photobacterium damselae* ssp. *piscicida.* The target species of fish was gilthead sea bream.

The results obtained showed that an osmotic shock (55 PSU for 96 hours) didn't allow enhance absorption of aluminium, in addition hyperosmotic shock didn't confer additional protection to fish.

No pathological effects were found in the tissue samples of the fish treated with alum. The aluminium deposits were only detected at the branchial cartilage of the primary lamellae.

Specific antibody responses and stimulation index demonstrated that although the fishes from the HSW group presented higher antibody responses, the best protection was in SW group.

The challenge test showed that the best protection is given by a treatment with Bacterin and Alum, thus could be applied for a commercial vaccine.

Further research should be done with longer challenge tests, in order to test efficacy as well as in other species, with the intention of evaluate similar results.

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