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Prevention of fish photobacteriosis. Comparison of the efficacy of intraperitoneally administered commercial and experimental vaccines

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Abstract

Two commercial multivalent vaccines against vibriosis, caused by *Vibrio anguillarum* serotype(s) and photobacteriosis, caused by *Photobacterium damsela* subsp. *piscicida*, one with oil adjuvant (AJ) and the other, being an aqueous solution (AV), and an experimental monovalent (*Ph. damselae* subsp. *piscicida*) vaccine inactivated with formalin or heat, namely EVF and EVH, were tested in laboratory trials on sea bass (*Dicentrarchus labrax*) with respect to their efficacy against experimentally induced photobacteriosis.

The first trial aiming at high bacterial pressure was carried out 34 days post-vaccination and resulted in 90% mortalities in the control. The relative per cent survival (RPS) of vaccinated fish was calculated at 24, 3.7, 0 and 0 for the AJ, AV, EVF and EVH formulations, respectively. The second trial aiming at medium bacterial pressure was carried out 49 days post-vaccination and resulted in 45% mortalities in the control. The relative per cent survival (RPS) of vaccinated fish was calculated at 100, 92.7, 77.8 and 66.7 for the AJ, EVF, EVH and AV formulations, respectively. Apparently, under both these high and medium bacterial pressure conditions, the commercial vaccine AJ performed better than the commercial vaccine AV, while under medium pressure there was no statistical difference between the performance of EVF and AJ. The measurement of specific antibody titers in sera collected from all fish groups 49 days post-vaccination, showed high levels in the fish vaccinated with the AJ vaccine, almost three times lower levels for the AV and EVF vaccines and even lower levels for the EVH vaccine. Results are discussed with respect to the choices available to mariculture companies in selecting a commercial vaccine against photobacteriosis and possible alternatives, which, if commercially developed, may reduce the cost of vaccination.

Keywords: Multivalent vaccines, efficacy, photobacteriosis, European sea bass.

Introduction

Mariculture of sea bass (*Dicentrarchus labrax*, L.) and sea bream (*Sparus aurata*, L.) faces disease problems of viral, bacterial and parasitic etiology (Athanassopoulou & Bitchava, 2010). The most important bacterial diseases, vibriosis and photobacteriosis, are caused by different serotypes of the Gram negative bacteria *Vibrio anguillarum* (Sorensen & Larsen, 1986; Toranzo & Barja, 1990) and *Photobacterium damselae* subsp. *piscicida* (Bakopoulos *et al.,* 1995), respectively. Cultivated fish, and especially sea bass that reportedly appear to be much more sensitive than sea bream (Athanassopoulou & Bitchava, 2010) to both pathogens, are protected to a certain extend by vaccination and general hygiene measures. Vaccinating millions of individuals per mariculture venture is a copious activity that disrupts the routine on-growing procedure and entails high labour costs, long periods of administration and fish stock losses due to the development of stress and injuries, especially with individually administered vaccines. Due to the latter reasons, there is a tendency to minimize vaccination applications during the life span of cultivated sea bass, which can be from 15 to 18 months, to the expense of better protection through anamnestic administrations. Commercially available vaccines against either vibriosis or photobacteriosis (consisting of inactivated whole bacterial cells) have been available for quite some time with good protection against vibriosis (Woo & Bruno, 1999) and variable results regarding photobacteriosis, especially in the field (Nakai *et al.,* 1992; Le Breton, 1999, 2009; Romalde, 2014). Another issue that stems from the problems associated with intraperitoneal (i.p.) vaccination, in particular, is that although at the beginning companies were vaccinating fish with monovalent or bivalent (two serotypes of *V. anguillarum*) vaccines against vibriosis or photobacteriosis, more recently, multivalent vaccines have been manufactured containing serotypes I & II of *V. anguillarum* and *Ph. damselae* subsp. *piscicida* inactivated bacterial cells. The production of such products was also dictated by the need for a single vaccine application. However, there are no published scientific data on the protection efficacy of these multivalent products. This, and especially the case for protection efficacy against

photobacteriosis, prompted our group to investigate the efficacy of these vaccines.

Thus, the objective of this study was to compare the two commercial and an experimental vaccine (inactivated with formalin or heat) in terms of protection of i.p. vaccinated sea bass against experimental infection with *Ph. damselae* subsp. *piscicida* (hereafter, Phdp).

Materials and Methods

Bacteria

A Phdp strain isolated in Greece in North Evoia during a natural photobacteriosis outbreak affecting sea bass in the summer of 2012 was used throughout this study. The isolate was a kind donation of Dr L. Papanna, Nireus S.A. The isolate was kept at -85°C on cryobeads (Protect, Heywood, Lancs., UK). For the initiation of culture, a microbead was added directly to a small volume of growth medium.

Both on arrival and every time we were initiating a culture from the freezer, bacterial colonies were tested with the API 20E system of Biomérieux, following the instructions of the manufacturer but using 2% NaCl as diluent for the bacterial colonies.

Concentrations of bacteria in solution were determined by measuring the OD_{605}. The optical reading was compared against a standard curve that we had previously constructed (OD_{605} versus bacterial cells/ ml) using plate counts of bacterial colonies of various dilutions of bacterial suspensions with known OD_{605}.

Media and culture conditions

For maintenance purposes or the initiation of culture, the isolate was cultured in brain heart infusion broth (BHIB) (Oxoid, Hampshire, UK) supplemented with NaCl at a final concentration of 2%.

For the preparation of the experimental vaccine, the Phdp isolate was grown in the liquid media described by Bakopoulos *et al.* (2003) for 48h at 21 ± 0.5°C. All chemicals were supplied by Sigma Chemical Company, St Louis, MD, USA, unless otherwise stated.

Commercial and experimental vaccine

Two commercially available vaccines against photobacteriosis were utilized in this study. According to the label attached to each of the commercial vaccines, they had the following characteristics:

AJ, is a vaccine formulation for i.p. injection; it contains inactivated cultures of the bacteria *V. anguillarum* (serotype I), Phdp and a non-mineral oil adjuvant. This vaccine is in the form of water-in-oil emulsion.

AV, is a vaccine formulation that can be used in both immersion and i.p. injection; it contains formalin-inactivated cultures of the bacteria *V. anguillarum* 785KID and MSC275 (serotype I & II) and Phdp, and is an aqueous solution without adjuvant.

The experimental vaccine was prepared as described by Bakopoulos *et al.* (2003) but modified so that the culture liquid was not separated from bacterial cells. The bacterial cells concentration in the vaccine mixture was 10^9 bacterial cells/ mL. A sample was then taken for the measurement of protein and carbohydrate content in the supernatants. Part of the bacterial mixture was then formalin-inactivated as described by Bakopoulos *et al.* (2003) and this experimental vaccine mixture is referred to as EVF, hereafter. The other part of the bacterial mixture was heat inactivated at 57°C for 30min. This experimental vaccine mixture is referred to as EVH, hereafter.

Measurement of protein and carbohydrate concentration

Samples collected from the mixture of the experimental vaccine were analyzed for protein and carbohydrate content as described previously (Bakopoulos *et al.,* 2003).

Fish, tank and aquarium system

Seven hundred and fifty (750) non-vaccinated, healthy sea bass juveniles weighing approximately 1g, were kindly donated by Selonda S.A., Loutra facility, Lesvos. Fish were transferred under sedation (0.01% phenoxyethanol) to the wet laboratory facilities of the Department of Marine Sciences, University of The Aegean, Lesvos, Greece and were placed in five separate tanks. Fish were fed twice daily at 4% /kg body weight/day , with commercial feed of the appropriate size until reaching an average weight of 10g (at about 8 weeks), in order to be vaccinated. One day before and one day after any handling, the fish were fasted, while handling was always performed after anaesthesia (0.1% phenoxyethanol).

The health status of fish during this period was assessed daily by observation (normal activity, feeding activity, reaction to stimuli, recording of any external disease signs or mortality) and found to be normal. A microbiological sampling was performed prior to the initiation of vaccination and prior to experimental infection to confirm that the fish were indeed not infected by an infectious agent. These tests were all negative for the presence of bacteria.

Growing and vaccinated fish were kept in a closed marine water recirculating system comprising 8 circular tanks with a conical bottom and a holding capacity of $2m^3$ each, i.e. a total of $16m^3$, a sand filter, a 110W UV water sterilization unit, a biological filter, a marine water pump with $9m^3$/h water flow capacity and air pumps distributing air to all the tanks through air stones. The tank system water was partially (1/3) renewed every 4 months.

Infected fish were kept in a static plastic aquaria system, comprising 15 aquaria of 50L capacity and air pumps distributing air to all the aquaria through air stones. Water in the aquaria system was partially (1/2) renewed daily.

Both systems were cleaned by fish excrements and uneaten food, and water parameters were monitored,

daily. Temperature ranged between 15.2°C in early spring and 24.05°C in mid-summer. Oxygen concentration ranged between 8.5mg/L in early spring and 5.7mg/L in mid-summer. Total ammonia concentration never exceeded 0.025mg/L throughout the experimental period.

Vaccination

Five groups of 10g sea bass comprising 150 fish/group were vaccinated i.p. with 50μL of each vaccine, with one group serving as control receiving the same volume of sterile 2% NaCl. The concentration of antigens in both the experimental vaccines per volume injected was $0.5x10^8$ bacterial cells of Phdp, 1.9μg supernatants protein, 11.2μg supernatants carbohydrate without adjuvant, while both commercial vaccines contained the same number of bacterial cells. Further information cannot be provided because it is of proprietary nature.

After vaccination, the fish were placed in their respective tanks. Toxicity of the various vaccines was monitored throughout the experimental period and recorded. Randomly sampled, moribund and recently dead fish resulting from the efficacy studies were dissected and macroscopically observed for the presence of pathological signs caused by vaccination in the abdominal cavity. Tissue samples were also processed for microscopic observation using routine procedures and stained with hematoxylin-eosin.

Assessment of efficacy

The efficacy of the four vaccine formulations was assessed with two separate experimental infection trials, one resulting in heavy bacterial pressure and the other in milder bacterial pressure. Assessment of efficacy was initiated after 28 days post-vaccination to allow for full development of immune response, according to the suggestion of one of the manufacturers of the commercial vaccines (AV).

The bacterial doses required to achieve high and mild bacterial pressure were determined prior to the initiation of the efficacy assessment by performing i.p. experimental infections of controls using various concentrations of bacterial cells.

Thirty four days post-vaccination, 60 fish from each group were infected with an i.p. injection of 50μL of the Phdp isolate used in this study, grown in BHIB 2% NaCl for 48h at 21 ± 0.5°C. The dose of bacteria received by each individual fish was $1.75x10^5$ cells. After infection, the 60 fish of each group were randomly divided into three groups of 20 fish and placed in separate aquaria. Mortalities were monitored and recorded daily and the experiment was stopped when no mortalities were recorded for two consecutive days.

The 2nd infection trial was performed 49 days post-vaccination but in this case individual fish received $3.25x10^4$ bacterial cells. The rest of the experiment was performed as described above.

Protection conferred by the different commercial and experimental vaccine formulations was assessed as described by Amend (1981) by calculation of the relative per cent survival (RPS) using the following formula:

$$RPS = [1 - \frac{\%\ \text{mortality of immunized fish}}{\%\ \text{mortality of non immunized fish}}] * 100$$

Moribund and recently deceased fish were sampled with routine procedures for microbiological investigation in order to determine the cause of disease/death. Microbiological samples taken from the spleen and kidney were used to inoculate the surface of tryptone soy agar supplied with 2% NaCl, in petri dishes. Developed bacterial colonies were identified using the API 20E system (Biomérieux) using 2% NaCl as diluent.

Measurement of specific immunoglobulins (IgMs) in sea bass serum samples

Blood samples collected on day 49 (2nd trial) from all experimental groups were processed for the isolation of serum. Sera from each group were pooled and these samples were utilized in ELISA analysis (Bakopoulos *et al.,* 1997) for the determination of specific IgM levels against Phdp. Results were read on a MR-96A microplate reader (MINDRAY).

Statistical analysis

Results were processed using the Kaplan-Meier survival curves (Kaplan & Meier, 1958).

In order to compare the survival distribution for all the different vaccine treatments and the two experiments as well as pairwise, the Log-rank test (Mantel, 1966) was employed, setting statistically different results at $p<0.05$. All statistical analyses were conducted with the use of the R software for Statistical Computing (R Core Team, 2013) and the Survival Package (Therneau, 2013).

Results

Toxicity-pathology evaluation

No mortalities in any group or vaccine formulation were recorded post-vaccination throughout the experimental period.

The macroscopic observation of internal organs in the abdominal cavity of vaccinated fish revealed none or mild reactions (localized erythema, mild inflammation at the point of injection) of fish vaccinated with the aqueous vaccine solutions (AV, EVF, EVH and 2% NaCl) and more severe reactions in fish vaccinated with the AJ vaccine (macroscopic data not shown).

Localized aseptic lesions caused by an inflammatory response (concluded after microbiological examination) to the latter vaccine were observed in all the specimens examined. These lesions were round, raised, off-white to brownish in colour, their diameter ranged between 0.3 and 1.2cm, they were located on the surface of the fatty tissue surrounding the gut and a vessel reticulum was clearly

evident around them. Microscopically, these lesions were characterized as aseptic granulomatous lesions (Fig. 1).

As evidenced in Figure 1A, consisting of granulomatous tissue at various developmental stages, the lesions have a core area of caseous necrosis tissue, mainly composed by oil (vacuoles), necrotic cells and debris (areas with dark colour). Externally, a layer of fibroblast-like cells was present (arrowhead in Fig. 1A and letter F in insert B), with large amounts of associated collagen fibres (arrow in Fig. 1A and letter C in insert B). These findings are consistent with granulomatous peritonitis.

Efficacy of vaccines

Moribund and recently dead fish, due to the experimental infections that were microbiologically sampled, were all found positive for Phdp.

High Infection Pressure Experiment

The high infection pressure experiment resulted in 90% cumulative mortality in the controls The experiment lasted nine days and the final cumulative mortalities and RPS achieved for all the groups of fish is shown in Table 1.

The experimental EVH and EVF vaccines conferred no protection to the respective vaccinated fish groups. In contrast, an RPS value of 24% and 3.7% was calculated for the AJ and the AV commercial vaccines, respectively. Apparently, the protection efficacy of the AJ vaccine was superior by far compared to the AV commercial vaccine, under these experimental conditions.

The Kaplan-Meier survival curves for the LD_{90} experiment are presented in Figure 2.

Evidently, survival rates dropped steeply for all vaccine groups except the AJ group.

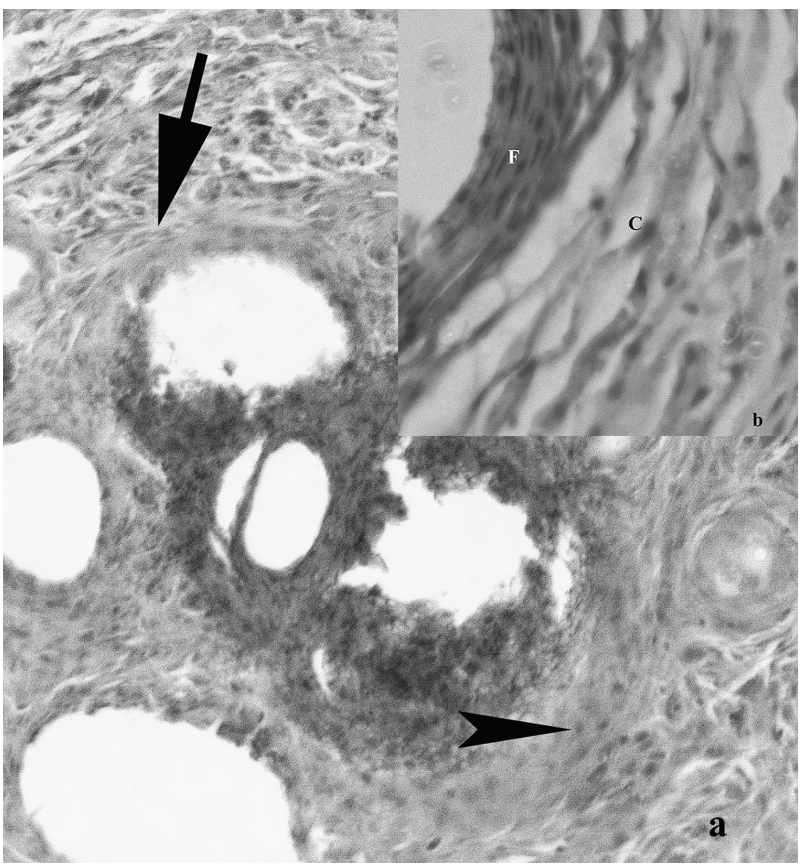

Fig. 1: A: Granulomatous tissue at various stages of development (10x magnification). Insert B: Detail of a formed granuloma (40x magnification). Haematoxylin-eosin.

Table 1. Cumulative mortalities and RPS values for all groups in both trials. LD_{90} bacterial dose, 9 days post-challenge, 34 days post-vaccination & LD_{45} bacterial dose, 15 days post-challenge, 49 days post-vaccination.

Fish Groups	No of fish		Specific loss		% fish mortality		RPS value	
	$^{*}LD_{90}$ Exp.	$^{**}LD_{45}$ Exp.	LD_{90} Exp.	LD_{45} Exp.	LD_{90} Exp.	LD_{45} Exp.	LD_{90} Exp.	LD_{45} Exp.
Control	3x20	3x20	54	27	90	45	-	-
AJ	3x20	3x20	41	0	68.4	0	24	100
AV	3x20	3x20	52	9	86.7	15	3.7	66.7
EVF	3x20	3x20	58	2	96.7	3.3	0	92.7
EVH	3x20	3x20	60	6	100	10	0	77.8

In order to compare the survival distribution for the all different vaccine treatments as well as pairwise, the Log-rank test (Mantel, 1966) was employed. Comparisons of the differences between the five different treatments (controls and vaccines) in the experiments were found statistically significant ($p < 0.05$). Most pairwise comparisons of differences were statistically significant ($p < 0.05$). The non-significant differences observed in the LD_{90} experiment were between the EVF vaccine and the control ($p = 0.756$).

Medium Infection Pressure Experiment

The medium infection pressure experiment resulted in 45% cumulative mortality in the controls. The experiment lasted fifteen days and the final cumulative mortalities and RPS achieved for all the groups of fish is shown in Table 1.

Fish vaccinated with the commercial AJ vaccine were 100% protected, as evidenced by the respective RPS value that was calculated. The performance of the experimental EVF vaccine conferring 92.7% RPS, followed by the EVH vaccine with 77.8% RPS, was also notable, while lower performance was observed by the AV commercial vaccine achieving a 66.7% RPS value.

The Kaplan-Meier survival curves for the LD_{45} experiment are presented in Figure 3.

Evidently, survival rates dropped comparatively steeply for the control fish groups over a long period, which was longer for the AV groups but with a milder drop in survival rates, while, these rates for the EVH and EVF groups, after a steep and short drop, were stabilized.

The application of the Log-rank test for these results showed that the comparison of the differences between the five different treatments (controls and vaccines) in the experiments were found statistically significant ($p < 0.05$). Most pairwise comparisons of differences were statistically significant ($p < 0.05$). The non-significant differences observed for the LD_{45} experiment were between the AV and EVH vaccine ($p = 0.48$), AJ and EVF vaccine ($p = 0.156$) and EVH and EVF vaccines ($p = 0.138$).

The RPS results obtained from both high and medium bacterial pressure experimental infection trials for the vaccines used are visualized in Figure 4 along with fitted linear regression lines. RPSs were related to the different bacterial doses achieving the LD_{45} and LD_{90} effect. These values were log10 transformed.

Comparing these data it is expected that both the AJ and the EVF vaccines would perform better with respect to the RPS achieved up to a certain bacterial pressure and as this pressure is closer to causing 90% mortality in the controls, EVF is replaced by the AV vaccine in terms of the RPS achieved.

Levels of specific anti-Phdp IgM in sea bass sera

The levels of specific antibody titers against Phdp from all the vaccinated groups and controls on the start day of the 2nd trial (day 49 post-vaccination) measured as absorbance at 450nm with ELISA analysis, are presented in Figure 5. Obviously, specific antibody levels against Phdp for the fish vaccinated with the AJ vaccine were very high, followed by the commercial AV vaccine and the experimental EVF vaccine that showed similar levels. The lowest antibody levels were measured for the EVH formulation.

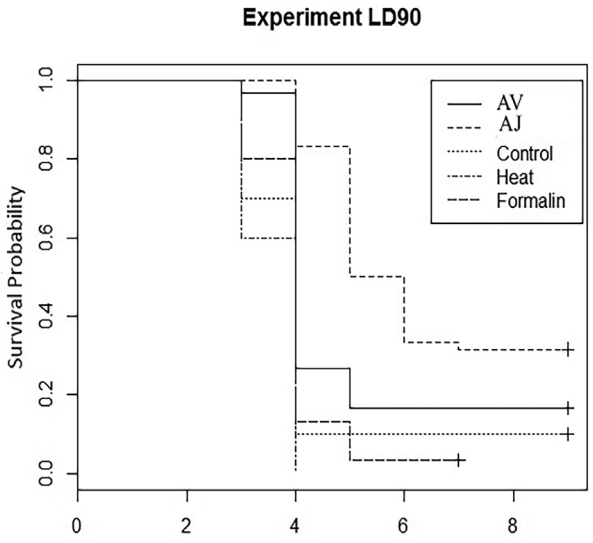

Fig. 2: Kaplan-Meier survival curves for the LD_{90} experiment. Plus signs indicate right censored data.

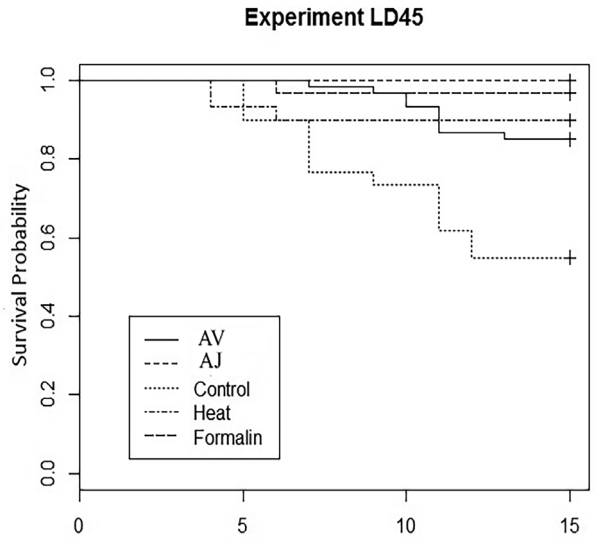

Fig. 3: Kaplan-Meier survival curves for the LD_{45} experiment. Plus signs indicate right censored data.

Discussion

Although there are previous studies on the protection efficacy achieved post-vaccination with monovalent (and bivalent with serotypes I and II) vaccines either against *V. anguillarum* (Viale *et al.,* 2006; Galeotti *et al.,* 2013) or against Phdp (Magarinos *et al.,* 1994; Magarinos *et al.,* 1994a), information on the protection efficacy when fish are vaccinated with bivalent vaccines against both pathogens

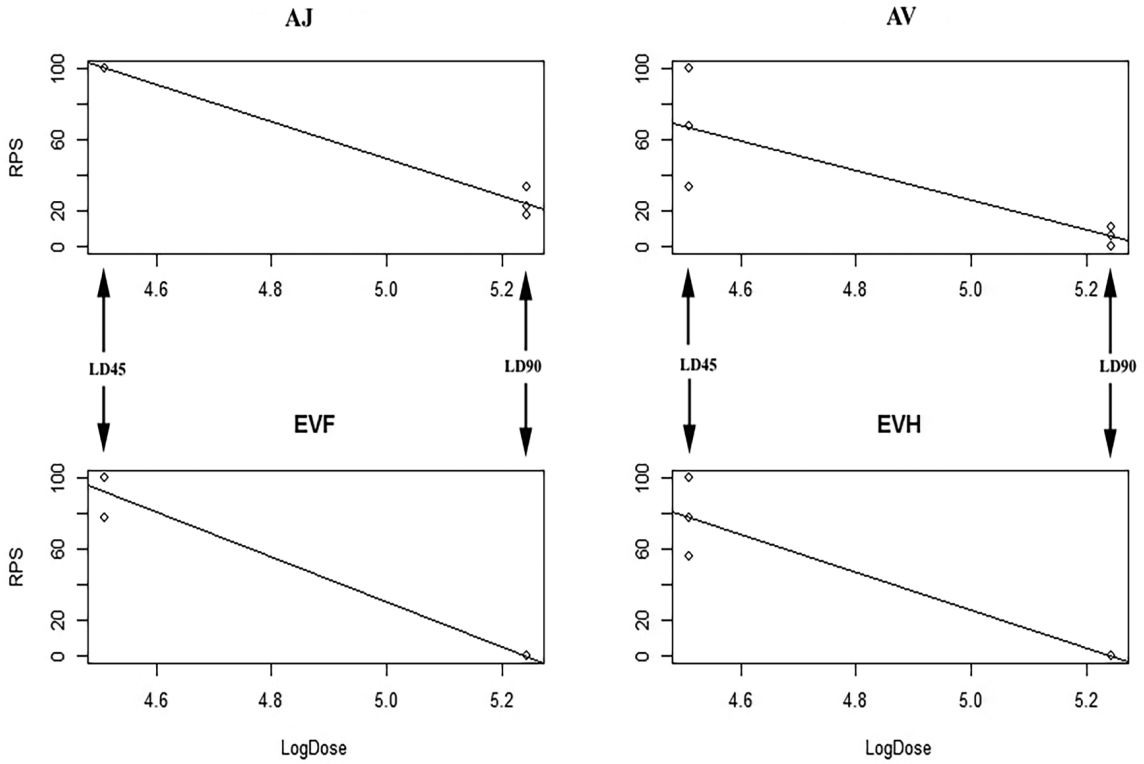

Fig. 4: Relative percentage of survival (RPS) for different lethal doses (values transformed Log_{10}) and different vaccines. Linear regression equations as follows: AJ: y = -103.7x + 567.6, R^2 = 0.98, p < 0.0001, AV: y = -83.2x + 442, R^2 = 0.71, p = 0.0358, EVF: y = -126.4x + 662.8, R^2 = 0.98, p = 0.0002, EVH: y = -106.4x + 557.9, R^2 = 0.9, p = 0.0035.

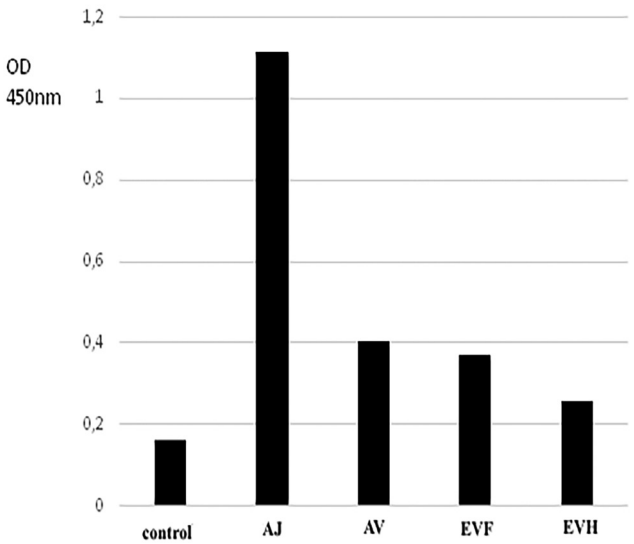

Fig. 5: Specific sea bass serum antibody titers against Phdp 49 days post-vaccination with the various vaccines**.**

is scarce [only the inconclusive article of Gravningen *et al.* (1998), regarding photobacteriosis]. Protection conferred post-vaccination against *V. anguillarum* is reportedly effective (Athanassopoulou & Bitchava, 2010; Galeotti *et al.,* 2013). In contrast, protection conferred post-vaccination against Phdp is variable (Nakai *et al.,* 1992; Le Breton, 1999, 2009; Romalde, 2014). For the former and latter reasons, in this study it was decided to compare two commercially available vaccines and an experimental vaccine against fish photobacteriosis in terms of protection of intraperitoneally (i.p.) vaccinated sea bass against experimental infections with Phdp, designed to exert extreme and average bacterial pressures.

Neither the commercial or the experimental vaccines caused mortality or other noticeable effects on the wellbeing of vaccinated fish. Internally, however, the fish groups that were vaccinated with the AJ vaccine (water-in-oil emulsion) showed abdominal adhesions and developed granulomatous peritonitis. These findings are consistent with the findings of Afonso *et al.* (2005) and Poppe & Koppang (2014). The extent to which these aseptic inflammatory responses can influence the functioning of the digestive system remains to be investigated. The other groups showed none to very mild adhesions.

In order to evaluate how the various groups of vaccinated fish respond to high and medium bacterial pressure, two separate challenges were performed. To our knowledge this is the first time that performance of the same vaccinated groups is evaluated under different challenge pressures, performed a short period apart.

Results showed that, in both challenges, the groups that were vaccinated with the adjuvanted commercial AJ vaccine responded better compared to all the other commercial or experimental vaccines, achieving RPS values of 24% and 100%, for the LD_{90} and LD_{45} challenges, respectively. In contrast, fish groups vaccinated with the aqueous commercial vaccine AV, achieved an RPS value of only 3.7% in the

LD_{90} challenge, and both the experimental vaccines failed to confer any protection at all. The latter result was very interesting because it contradicts the very good protection that was described in the Bakopoulos *et al.* (2003) study for the experimental vaccines that were assessed here. The differences between the two experiments were that in this study a higher cumulative mortality was achieved, the entire bacterial culture was used as the vaccine without prior isolation of bacterial cells and ECPs, and the challenge was performed with Phdp grown in BHIB 2% NaCl. Despite the fact that the culture medium plays a very important role in the antigens that a pathogen synthesizes and virulence (Bakopoulos *et al.,* 2003a; Bakopoulos *et al.,* 2004), the differences in challenge severity and the modification of the preparation method applied for the experimental vaccine, may account for the contradictory results obtained in this study. Unfortunately, protection of vaccinated sea bass against photobacteriosis under such extreme conditions cannot be compared with previous studies.

Very different results were obtained when the milder LD_{45} challenge was performed. In this case, the RPS values obtained were in descending order, 92.7%, 77.8% and 66.7% for the fish groups vaccinated with the EVF, EVH and commercial AV vaccine, respectively, while the RPS value achieved by the AJ reached 100%. In this case, there was no statistical difference between the commercial AJ and the experimental EVF vaccines and between the experimental EVH and the commercial Aqua Vac vaccines. Furthermore, as it is evident from this challenge, heat inactivation of the experimental vaccine (EVH) had a deleterious effect on the protection conferred against photobacteriosis compared to formalin inactivation (EVF), despite the fact that no statistical difference was calculated. This is probably due to higher antigen destruction by heating as demonstrated in earlier studies (Levings, 1984; Samoylova *et al.,* 2012). The results obtained in this study cannot be directly compared to the previous study of Bakopoulos *et al.* (2003) because of the different conditions between the two experiments; however, results for the experimental EVF vaccine are comparable. Vaccines containing ECPs seem to perform better compared to vaccines composed of bacterial cells only (Magarinos *et al.,* 1994), as is the case for the commercial vaccines tested here.

The analysis of sea bass sera collected on the start day of the 2nd efficacy assessment trial, as regards the levels of specific anti-Phdp antibodies, correspond very well with the performance of the commercial AJ vaccine only, for which high antibody levels were measured. Interestingly, there was no correlation between the levels of specific anti-Phdp antibodies in the sea bass sera collected from the other experimental groups and the performance of the respective vaccines. The adjuvant included in the AJ vaccine acts in a beneficial way, increasing the levels of antibodies even at 49 days post-vaccination in contrast to the other formulations that did not contain an adjuvant where antibody levels were indisputably much lower. Protection against the infection, however, is not a matter of quantity but rather of quality as discussed below.

Apparently, the results obtained from this study suggest that the commercial AJ vaccine acts better in comparison to the commercial AV vaccine as regards protection against the experimental challenge with Phdp, under the described conditions. This better performance was demonstrated under both extreme and medium bacterial pressure. The differences between the two product formulations, based on the information provided by the producers, consist mainly in the inclusion of a non-mineral adjuvant in the AJ vaccine and the fact that the bacterial cells included in the AV vaccine were grown in both normal and iron-limited media. Each of these differences is beneficial for different reasons. Adjuvants cause intense attraction of various types of leukocytes at the location of the vaccine and may have a prolonged effect because of slower antigen release (Afonso *et al.,* 2005). These may have a positive quantitative stimulation effect on the immune response. Bacterial growth under iron limitation conditions leads to the expression of antigens that may be involved in virulence (Magarinos *et al.,* 1994b; Bakopoulos *et al.,* 1997a) and, thus, may exert a positive qualitative effect on immune response and protection.

Regarding the experimental vaccine formulations, no protection was conferred under extreme conditions of infection, but a completely different case was revealed under medium infection pressure. In these conditions, the EVF vaccine conferred protection against Phdp infection in a similar manner to the commercial AJ vaccine, despite the fact that it does not contain adjuvants. It seems that the qualitative characteristics of the antigens included (Bakopoulos *et al.,* 2003a) have an "added-value" in terms of protection, comparable to an adjuvanted formulation. The EVH formulation was inferior to the EVF but apparently performed somewhat better than the AV vaccine. At practical level, this study suggests that the adjuvanted AJ vaccine needs to be used in areas where a high Phdp stress is foreseen, whereas under milder pressure the aqueous AV or an improved formulation, such as the EVF formulation without any adjuvant could be the alternative if commercially developed. This could reduce both the cost of the vaccine formulation and the effects of the adjuvant on the abdominal cavity.

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Conflict of Interest: No conflict of interest exists between this research and any other companies providing the commercial products.

Ethics: The work presented in the article has been carried out in an ethical way and according to Directive 2010/63/EU on the protection of animals used for scientific purposes.

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