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Antibody responses of turbot *Psetta maxima* against various antigen formulations of scuticociliates Ciliophora

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ABSTRACT: The kinetics of the antibody production and the protection at challenge were studied in turbot inoculated with various scuticociliate antigen preparations: live ciliates putatively attenuated through long-term *in vitro* culture (Trial 1) and formalin-killed ciliates without or with GERBU adjuvant in Trials 2, 3, and 4. Antigen used in killed preparations was a mixture of 3 different ciliate isolates (V3) in the case of Trials 2 and 3, whereas in Trial 4, monovalent (V1), trivalent (V3) or pentavalent (V5) antigens were used. A booster injection was administered 28 to 29 d post-priming in all trials. Fish were challenged with virulent live ciliates after the immunization protocol, testing 2 challenge times in Trial 2 (t_1 and t_2). No protection was obtained in Trial 1 with live ciliates, which in turn were not completely attenuated. Using killed-ciliate formulations, protection was high only in Trial 3 when a low dose (50 000 ciliates fish^{-1}) was used for challenge. In Trial 1, heat-inactivated sera of antigen-inoculated fish agglutinated the homologous ciliate, although no specific antibodies were detectable by ELISA. In contrast, high specific antibody levels were detected in antigen-inoculated fish in Trials 2 and 4, and their amount increased progressively, usually peaking after challenge. No advantage was obtained from the use of V5 antigens compared to V1 or V3. No good correlation was observed in most cases between serum antibody levels and protection. Although the use of GERBU adjuvant generally increased the specific immune response, some undesired side effects indicate a need to adjust dosage and/or improve the formulation.

KEY WORDS: Ciliates · Scuticociliatia · Adjuvants · Antibodies · Immunization · Turbot · *Psetta maxima*

INTRODUCTION

Scuticociliates are ciliate protozoans (Ciliophora) occurring abundantly in eutrophic coastal and saprobic maricultural waters. Some of them can behave as opportunistic histophagous parasites causing severe infections in crustaceans and fish. Recently, various scuticociliates have become an important threat for cultured marine fishes such as tuna and various flatfishes. Fatal encephalitis of bluefin tuna was attributed to *Uronema nigricans* (Munday et al. 1997) and, among flatfishes, scuticociliatosis has been reported in Asian Japanese flounder *Paralichthys olivaceus* (Yoshinaga & Nakazoe 1993, Jee et al. 2001). In turbot *Psetta maxima* (= *Scophthalmus maximus*), fatal scuticociliatoses have occurred in Spain and Portugal (Dyková & Figueras 1994, Iglesias et al. 2001, Alvarez-Pellitero et al. 2004, Ramos et al. 2007) and in Norway (Sterud et al. 2000). Morphological studies of different isolates suggested the existence of different species or strains (Alvarez-Pellitero et al. 2004). However, recent DNA-based studies point to a single species involved in flatfish scuticociliatoses (Palenzuela et al. 2005).

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The increasing frequency and severity of scuticociliatosis in turbot culture, with mortality reaching 60% in some infected stocks (authors' unpubl. data), has stressed the need to develop efficacious preventive and control strategies. Since no efficacious treatments are available, the implementation of immunoprophylactic measures seems an attractive option.

Empirical data from field observations in affected farms indicate the acquisition of disease resistance in fish surviving scuticociliate epizootics, stimulating the search for a vaccine. Available information from small, laboratory-scale trials suggests partial protection against a challenge using formalin-killed vaccines in turbot (Iglesias et al. 2003, Lamas et al. 2008, Sitjà-Bobadilla et al. 2008) and olive flounder (Jung et al. 2004). A recent report claimed quite good turbot protection using a killed adjuvanted vaccine (Sanmartín et al. 2008). The search for adjuvants without the toxic side effects of mineral oils (Bowden et al. 2003, Singh & O'Hagan 2003) has led to a different family of products based on lipid microparticles, stabilized in aqueous phase by surfactants and sometimes in association with soluble immunomodulators (such as G-muramyl dipeptide, GMDP; Cox & Coulter 1997). The GERBU preparations used in the present study belong to this group. The presence of specific antibodies against scuticociliates has been demonstrated in turbot both in natural infections (Iglesias et al. 2003) and after inoculation of different vaccine preparations in small-scale (Iglesias et al. 2003, Sitjà-Bobadilla et al. 2008) or medium-scale (Sanmartín et al. 2008) experiments. However, no information is available on the production and kinetics of specific antibodies under different antigen-formulations and/or immunization regimes in medium or large-scale long-term experiments.

In the present work, the kinetics of antibody production in turbot inoculated with live cultured ciliates and different formalin-killed formulations (including GERBU-adjuvanted preparations) were studied in immunization and challenge trials in medium-scale experiments.

MATERIALS AND METHODS

Ciliates and *in vitro* culture. Several isolates of scuticociliates obtained from farmed turbot suffering epizootics (detailed in Alvarez-Pellitero et al. 2004) and 1 isolate (E) from Japanese olive flounder (see Yoshinaga & Nakazoe 1993) were used. The isolates referred to as A-1, A-2 (Cantabric Sea, France), B-2 (Cantabric Sea, NW Spain), C (Atlantic, NW Spain), and D (Atlantic, Portugal) by Alvarez-Pellitero et al. (2004), and the E Japanese isolate were used to prepare the different antigen formulations (see below). The isolates used for challenge were B-2 (Trials 1 and 2) or an additional isolate (A-3) further obtained from the French Cantabric site (Trials 3 and 4). Several of these isolates were also employed to prepare the antigen used in the ELISA assay as described below.

Production of axenic cultures of the ciliate isolates was explained by Alvarez-Pellitero et al. (2004). All isolates have been routinely maintained in 25 cm^2 T flasks, with 7 ml media at 15 or 20°C using L-15 medium (Gibco-Invitrogen) containing 1.28% artificial marine salts (for a final salinity equivalent to approximately 20‰), and antibiotic/antimycotic mixture (PSA) at 1× to 3× final concentration (1× PSA = 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin and 0.25 µg ml^{-1} amphotericin B). The medium was supplemented with 10% heat-inactivated newborn calf serum (NCS), 1× Eagle's Basal Medium (BME) vitamin mixture, and ribonucleic acid from torula yeast (Alvarez-Pellitero et al. 2004). Serial passages and larger-scale cultures were made by harvesting cultures in late stationary phase by centrifugation (500 to 650 × *g*, 15 min) and washing with basal media containing 1× PSA. Parasites were counted in Neubauer chambers and inoculated into fresh media.

Ciliates used for the challenge were harvested, washed, counted and transferred to an appropriate volume of Dulbecco's phosphate buffered saline (D-PBS) plus 0.35% NaCl as a mother, concentrated stock. Within 20 to 24 h, this stock was diluted to the inoculation doses that were injected into the fish, as described below. The active condition of the ciliates in all the treatment doses was verified by observation of a subsample under the microscope.

Preparation of antigen formulations. *Live ciliates putatively attenuated by prolonged* in vitro *culture (Trial 1):* Preliminary assays carried out in some farms pointed to some virulence loss in ciliates maintained *in vitro* using fish tissues as food source. In order to test this possibility, A-1 ciliates were subcultured until passages 41 and 44, and used alive (LIV) in a vaccination trial, with priming and booster (Bt) injections.

Inactivated (killed) ciliates (Trials 2, 3 and 4): *Antigens:* Ciliates of each isolate were inoculated on 3 consecutive days and cultured in 75 cm^2 T-flasks or in spinner flasks at 20°C, as described above. Cultures were harvested on Day 8 after the first inocula, pooled, and washed with D-PBS. Cells were pelleted (811 × *g*, 10 min) and killed by resuspending in 1% phosphate-buffered formalin. After fixation for 50 min in a slow rocking platform, the ciliates were centrifuged as above and resuspended in fresh D-PBS containing 0.01% thimerosal (D-PBSt) 3 times. Killed and washed ciliates were counted in a Neubauer chamber, and the volume adjusted to a final concentration of 1.2×10^6 ciliates ml^{-1}. A preparation referred to as monovalent antigen (V1) consisted of A-3 isolate alone, at this concentration.

Trivalent (V3) and pentavalent (V5) antigens contained equal numbers of each contributor isolate (A-2, B-2, and D for V3, and A-2, B-2, C, D, and E for V5), which were mixed before adjusting to the same final concentration. V3 was used in Trials 2 and 3, whereas in Trial 4, 3 different preparations (V1, V3, and V5) were tested. In all cases, ciliates were stored at 4°C until used. A crude preparation of killed ciliates was made by sonication using a VC60 Vibra-cell ultrasonic processor (Sonics & Materials). Cells were disrupted in ice at 50% power output, with an effective sonication time of 2 min administered in 2 s pulses separated by 3 s pauses.

Adjuvants and preparation of inocula: The experimental adjuvant GERBU 734 (Gerbu Biotechnik; GER) based on stabilized lipid nanoparticles and the immunopotentiator glycopeptide GMDP was used at doses of 100 µl fish^{-1} (Trials 2 and 3) or 50 µl fish^{-1} (Trial 4). The adjuvant was mixed with 0.1 ml of the antigen preparation (containing 120 000 sonicated ciliates) and/or with D-PBSt as appropriate to produce 0.2 ml individual fish-doses containing the desired formulation in the different experimental groups (see below).

Fish. Juvenile turbot were obtained from a Northwest Spanish farm (Trials 1, 2, 4) and a French Cantabric farm (Trial 3). Following routine farm procedures, fish had been vaccinated (60 s immersion) with a mixed vaccine (GAVA- 3, *Vibrio anguillarum*-FM95, *Flexibacter maritimum*, Laboratorios HIPRA) at 3 mo of age and boosted 1 mo later with the same product and procedure. In all experiments, fish received a flow-through supply of aerated sea water (37.5‰) at natural temperature and photoperiod and were fed daily with a pelleted commercial diet ad libitum.

Experimental procedure. ***General conditions:*** Experimental fish (mean weight ~40 to 50 g) were acclimated for 2 wk to the Centro Tecnológico Gallego de Acuicultura (CETGA) facilities (Trials 1, 2, 4), or a French Cantabric farm (Trial 3), and a sample of 10 fish was taken on Day 0, as an initial control. The remaining fish were randomly allocated into fiberglass tanks of 500 l (50 fish tank^{-1}).

Trial 1: This trial was designed to evaluate the response to live ciliates. Three experimental groups (6 replicate tanks group^{-1}) were established, including 1 control (CTRL) and 2 groups injected intracoellomically (i.c.) with a suspension of live (LIV) ciliates of isolate A1 in 0.2 ml of PBS (LIV-H: high dose, 20 000 ciliates fish^{-1}; LIV-L: low dose, 10 000 ciliates fish^{-1}). Since mortality after this priming injection was higher than expected, the experiment was reoriented to study the kinetics of antibody production. Fish of LIV-H and LIV-L groups and a subgroup of 100 CTRL fish (now named Bt) were boosted (i.c.) on Day 29 post-priming (p.p.) with a lower dose of the live ciliate (5000 ciliates fish^{-1}) in 0.2 ml of PBS, whereas an equal subgroup of CTRL fish received PBS alone. Details of the fish groups, experimental design, water temperature, and sampling schedule can be found in Fig. 1.

Trial 2: The purpose of this trial was to study the response to antigenic formulations containing killed ciliates. Five experimental groups were established, and they received antigen alone (V3), antigen and adjuvant (V3-GER), adjuvant alone (GER), or PBS (CTRL and control-challenged, CTRL-Ch). Two replicates were used for CTRL fish, whereas for the remaining groups, 4 replicates group^{-1} were arranged: two of them to be challenged at Time 1 (t_1) and the other two at Time 2 (t_2). Details on the injection calendar, water temperature, and sampling schedule are provided in Fig. 2.

Trial 3: This trial was designed just for the evaluation of mortality after challenge, and no serum was analyzed. The experimental groups and immunization design were basically the same as in Trial 2 except that a single challenge time was chosen, with the following details: on Day 110 p.p. (82 d post-booster, p.b.), 1 replicate of each group was challenged with 50 000 ciliates fish^{-1} (low dose) and the other replicate received 80 000 ciliates fish^{-1} (high dose; isolate A-3, passage 8 in both cases). Seawater temperature increased during the experiment (July to December 2004) and ranged from 15°C (Day 0) to the following mean values: 15.8°C (Days 0 to 28), 18.5°C (Days 29 to 110), and 18.2°C (Days 110 to 140).

Trial 4: The main purpose of this experiment was to compare the response to different monovalent or polyvalent antigenic formulations. Eight experimental groups (2 replicates group^{-1}) were established. Three groups received antigen alone (V1, V3, V5); 3 groups were injected with the corresponding antigen plus adjuvant (V1-GER, V3-GER, V5-GER); and 1 group received only the adjuvant (GER). The control (CTRL) group was injected with PBS. Details on the injection calendar, water temperature, and sampling schedule are provided in Fig. 3.

General sampling procedure. In all cases, at each sampling point, 10 fish group^{-1} were sacrificed by overexposure to the anesthetic MS-222 (Sigma), and bled from the caudal vein before the necropsy. Fish were weighed and measured, and data on body weight (g) are presented as mean ± SEM. Blood was allowed to clot at 4°C. Serum was obtained after centrifugation at 1500 × *g* for 30 min at 4°C, and stored at –80°C until further analyses were performed.

Following challenge, the presence of ciliates was evaluated in the coelomic cavity, blood and cerebral tissue of moribund or dead fish, and in the fish sacrificed in the last sampling. Fish were considered positive for ciliatosis when active ciliates were found. The relative percentage survival (RPS) was calculated using the formula: RPS = 1 – (% mortality in vaccinated fish / % mortality in control fish) × 100.

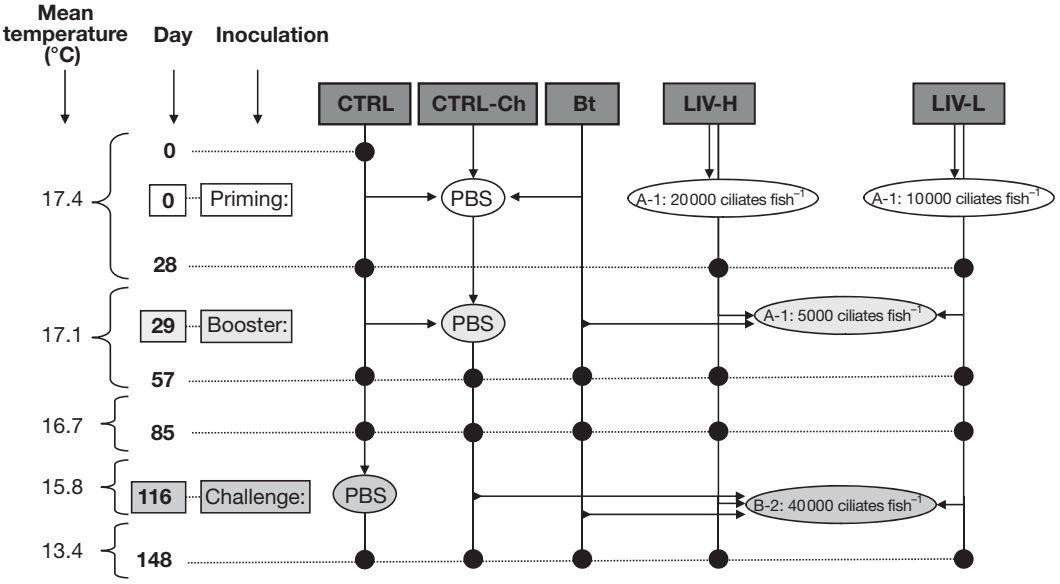

Fig. 1. Experimental procedure in Trial 1, indicating the experimental groups (grey boxes), the doses inoculated at priming, booster, and challenge (ellipses), the sampling points (black spots), and the mean water temperature in each indicated period. Day column indicates the time at which fish were sampled (10 fish per sampling) or inoculated (numbers in boxes). CTRL and CTRL-Ch were considered as a homogeneous group until challenge. A-1 and B-2 are the ciliate isolates used for immunization and challenge, respectively. Arrows point to the type of inoculation received by each group at each inoculation time. CTRL: control; CTRL-Ch: challenged control; Bt: booster-control; LIV-H, LIV-L: inoculated with the high or low dose of live ciliate, respectively

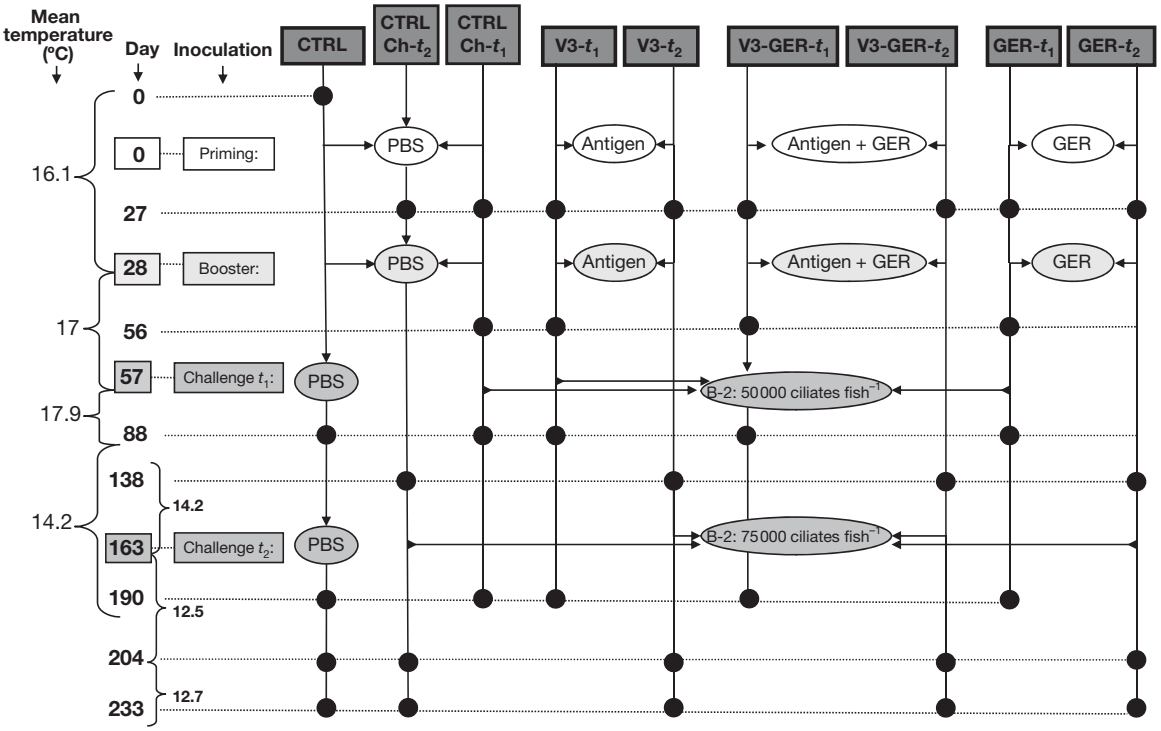

Fig. 2. As in Fig. 1, but for Trial 2. The antigen inoculated at priming and booster was a mixture of 3 ciliate isolates (V3). Each group was challenged (Ch) at 2 different times (t_1 and t_2), with the B-2 isolate. CTRL, CTRL-Ch-t_1 and CTRL-Ch-t_2 were considered as a homogeneous group until challenge at t_1. Arrows point to the type of inoculation received by each group at each inoculation time. CTRL: control; CTRL-Ch: challenged control; V3: fish inoculated with V3 antigen; V3-GER: fish inoculated with V3 antigen plus adjuvant (GER); GER: fish inoculated with adjuvant alone; -t_1 and -t_2 indicate that fish were challenged at Times t_1 (57 d) and t_2 (163 d), respectively

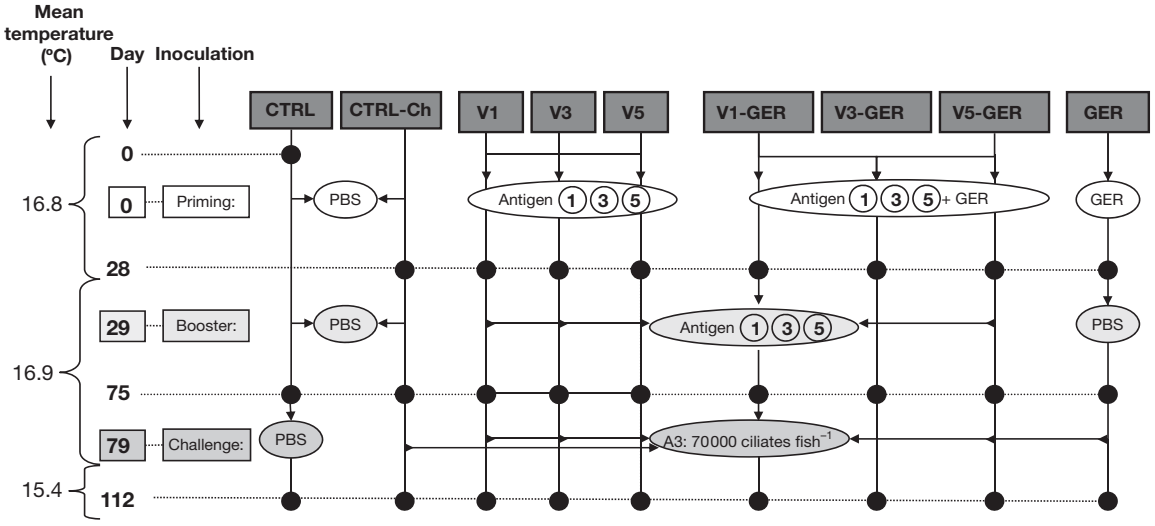

Fig. 3. As in Fig. 1, but for Trial 4. Three types of antigens were inoculated at priming and booster: V1 (1 isolate), V3 (a mixture of 3 isolates), and V5 (a mixture of 5 isolates), with or without the adjuvant Gerbu (GER). Fish were challenged with A-3 isolate. CTRL and CTRL-Ch were considered as a homogeneous group until challenge. Arrows point to the type of inoculation received by each group at each inoculation time. CTRL: control; CTRL-Ch: challenged control; V1, V3, V5: fish inoculated with mono-, tri-, or pentavalent antigens, respectively; V1-GER, V3-GER, V5-GER: fish inoculated with V1, V3, or V5 antigens plus adjuvant (GER), respectively: GER: fish inoculated with adjuvant alone

Agglutination assays. *In vitro* cultured ciliates from the isolates A-1, B-2, and D were harvested and washed 3 times in iHBSS (HBSS plus 0.35% NaCl) at room temperature, and the final concentration adjusted to 10^4 ciliates ml^{-1}. The sera from all tested groups in Trial 1 were heat-inactivated (45°C for 30 min), serially diluted in iHBSS, and dispensed in 96 well flat-bottom plates (100 µl well^{-1}; Nunc) in duplicates. After addition of the ciliates to the wells (100 µl well^{-1}), the plates were incubated at 20°C and monitored every 10 min for 2 h for immobilization/agglutination/lytic responses, using an Olympus IX71 inverted microscope. In all assays, control wells without fish serum were included. The immobilization titer was defined as the highest dilution value at which parasites were immobilized, agglutinated, or lysed.

Determination of specific antibodies. An enzyme-linked immunosorbent assay (ELISA) was developed and optimized to determine the presence of specific antibodies to the ciliate. The procedures for antigen preparation and for the application of the assay are fully described by Sitjà-Bobadilla et al. (2008). Briefly, whole cell lysates of ciliates from the different *in vitro* cultured isolates were used as antigens. After incubation with test fish serum (diluted 1:8), a monoclonal antibody anti-turbot IgM (Estévez et al. 1994) diluted 1:1000 was used as a second antibody, followed by a goat anti-mouse IgG (H + L) (third antibody) horseradish peroxidase conjugate (BioRad) diluted 1:2000 as the third antibody. In all assays, previously selected positive and negative fish were used as appropriate controls. Specific antibodies were determined against A-1 and B-2 isolates in Trial 1. In Trial 2, antigens of each isolate used in the V3 formulation (A-2, B-2, and D) were tested individually or in a mixture of the 3 in equal numbers. In Trial 4, the isolate A-3 used for challenge and included in V1, V3, and V5 formulations was used. The working protein concentration of the antigen was set at 24 µg ml^{-1}.

Statistical analysis. A 1-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls method was used to compare the biometrical, hematological, and antibody levels of the experimental groups at each sampling point, and also within each group along the sampling times, in Trials 1, 2, and 4. When necessary, a Kruskal-Wallis 1-way ANOVA on ranks followed by Dunn's method was applied instead. All statistical analyses were performed using Sigma Stat software (SPSS), and the minimum significance level was set at $p < 0.05$.

RESULTS

Trial 1

Fish weight. All fish grew as expected during the experimental period, and no significant differences were found between groups except at the last sampling, when LIV-H fish had a significantly lower weight (233.1 ± 19.2 g) than CTRL fish (297.1 ± 24.1 g).

Agglutination assays. No agglutination or lysis of ciliates used for vaccination (A-1) was observed with

inactivated sera of CTRL fish. Among vaccinated fish, individuals of some groups showed agglutination, although no lysis or escape of agglutinated ciliates was detected. Both the percentage of positive fish and the agglutinating titer increased in LIV-H and LIV-L fish on Day 28 p.p. Boosting increased this response in both groups over the short term (Day 57 p.p. = 28 p.b.), but a decrease was detected at the subsequent sampling (Day 85 p.p. = 56 p.b.; Fig. 4). After challenge, an increase occurred in all groups in the percentage of positive fish, except in CTRL-Ch fish, in which no agglutination was detected. The maximum titer obtained was 1/32.

The agglutinating ability of inactivated fish serum against the ciliate used for the challenge (B-2) was evaluated before (57 d p.p.) and after (148 d p.p.) the challenge. No agglutinating effect was detected before the challenge, and it was found only in 20% LIV-L and 10% CTRL-Ch after challenge. In addition, on Day 57 p.p., inactivated sera were tested against a different isolate (D), and agglutination was detected only in 10% of LIV-L fish.

Antibody levels. Specific antibodies (determined by ELISA) against the isolate used for vaccination (A-1) and for challenge (B-2) were below the threshold level in most groups. Only LIV-H fish exhibited clearly positive values on Day 28 p.p., but differences with respect to the CTRL group were not significant (data not shown).

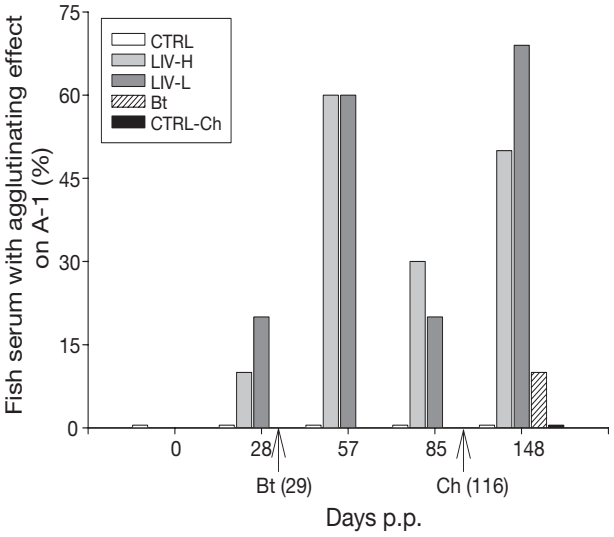

Fig. 4. *Psetta maxima.* Percentage of fish whose inactivated fish serum had an agglutinating effect on A-1 ciliates in Trial 1, for each experimental group at each sampling time post priming (p.p.). Arrows indicate the days for booster (Bt) and challenge (Ch)

Trial 2

Fish weight. In fish challenged at Time 1, the weight was lower in all vaccinated groups than in CTRL fish. These differences were significant on Day 88 p.p., when GER fish had the lowest values (123.8 ± 9.79 versus 204 ± 16.37 in CTRL). In fish challenged at Time 2, vaccinated fish presented lower weights than CTRL, but differences were significant only for GER fish at 138 d p.p. (190.8 ± 16.49 versus 277.2 ± 14.66 in CTRL), and for CTRL-Ch fish at 204 d p.p. (361.99 ± 37.65 versus 467.35 ± 31.15 in CTRL).

Antibody production kinetics. The production of specific antibodies against the isolate used for challenge (B-2) was monitored for all groups and sampling times (Fig. 5). At the first sampling (Day 27 p.p.) positive fish belonged to V3-GER and V3 groups (although V3 was slightly above the threshold value), with values significantly higher than in CTRL and GER groups. On Day 56 p.p (28 d p.b.), V3- t_1 and V3-GER-t_1 values were also significantly higher than in unvaccinated groups (Fig. 5A). In the case of groups challenged at t_2, such difference was maintained at 138 d p.p. (111 d p.b.), but it was significant only for the V3-GER-t_2 group (Fig. 5B). At the first sampling after challenge (88 and 204 d p.p., respectively, for t_1 and t_2), a significant increase in the antibody levels with respect to the previous sampling was detected in all challenged groups, regardless of the challenge time. In both cases, the highest values were found in the V3-GER group, being significantly different from the remaining samples. In addition, V3-t_1 and V3-t_2 values were positive and significantly higher than CTRL. At the last sampling (190 and 233 d p.p., respectively, for t_1 and t_2), a drop in the antibody levels was observed in most groups, regardless of the challenge time. However, at t_1, all challenged groups were still positive and with values significantly higher than CTRL (the GER group even increased compared to the previous sampling), whereas at t_2, only vaccinated groups were positive, with values significantly higher than CTRL and GER fish.

To further study the kinetics of antibody production against the ciliates, the sera from the group with the highest antibody levels against B-2 (V3-GER), obtained before and after challenge, were tested against the trivalent antigen included in the formulations (V3) and also against each of its components separately (A-2, B-2, and D; Fig. 6). Interestingly, regardless of the challenge time, antibody levels against A-2 and D antigens were clearly and significantly higher than against B-2 and V3, before and after the challenge, with the anti-V3 antibodies having the lowest values. However, at the t_1-challenge, a clear and significant increase in antibody levels against all antigens occurred after challenge, whereas at the t_2-challenge, such increase was significantly higher only for anti-B-2 and anti-V3 antibody levels.

Trial 3

In fish receiving the low challenge dose, cumulative mortality was clearly lower in all fish inoculated with any antigen or adjuvant preparation with respect to CTRL fish (RPS = 94–95). The situation was completely different in fish receiving the high challenge dose, as mortality was similar to that of CTRL fish (V and V-GER fish, RPS = 0 and 4, respectively) or even higher (GER group, RPS = –32). The interval of mortality was very long in all fish groups challenged with the high dose, particularly CTRL and GER (up to 83 and 96 d, respectively).

Trial 4

Fish weight. Most fish groups grew as expected during the experimental period with a 4-fold increase in their weight, although some groups inoculated with adjuvant exhibited the lowest values. Weight was significantly lower with respect to CTRL group in GER fish at 28 d p.p. (46.34 ± 2.03 g versus 62.86 ± 2.45 g in CTRL) and in V5-GER fish at 75 d p.p. (169.2 ± 16.83 g versus 216.4 ± 12.37 g in CTRL).

Antibody production kinetics. The kinetics of antibody production against V3 antigen was studied in all groups (Fig. 7). At the first sampling (28 d p.p.) all groups receiving V1 and V3 formulations (either alone or adjuvanted) were positive, and most of them had significantly higher antibody levels than non-vaccinated fish. A subsequent change in the pattern occurred after booster, as only those groups receiving adjuvanted formulations were positive for antibodies. After challenge, a further increase in the antibody levels occurred in all challenged groups, all of them having significantly higher values than CTRL fish (non-challenged). Most adjuvant-vaccinated groups presented the highest antibody levels, but they did not differ significantly from CTRL-Ch. The analysis of antibody kinetics within each group during the experiment demonstrated a progressive and significant increase in V5 and V5-GER groups, whereas the remaining vaccinated fish showed a decrease at p.b. with respect to p.p. sampling (V1 and V3), or very similar values (V1-GER, V3-GER). A clear and significant increase occurred after challenge in all groups.

The percentage of positive fish was variable between groups. At 28 d p.p., the number of positive fish was higher for V1 and V3 fish than for the remaining vaccinated fish, V5 being the group with fewer positive fish. At p.b. sampling (75 d p.p.), only V1-GER fish reached 100% of positive fish, and an increase with respect to the previous sampling occurred in most groups. At p.c. sampling, 100% of fish were positive in all groups, except GER (Fig. 7).

Relationship between antibody levels and protection at challenge

No good correlation was observed in most trials between serum antibody levels and protection at challenge, as summarized in Table 1.

In Trial 1, the immunization of fish with the presumptively attenuated ciliates and/or the boosting with a lower dose of the same isolate did not produce protection

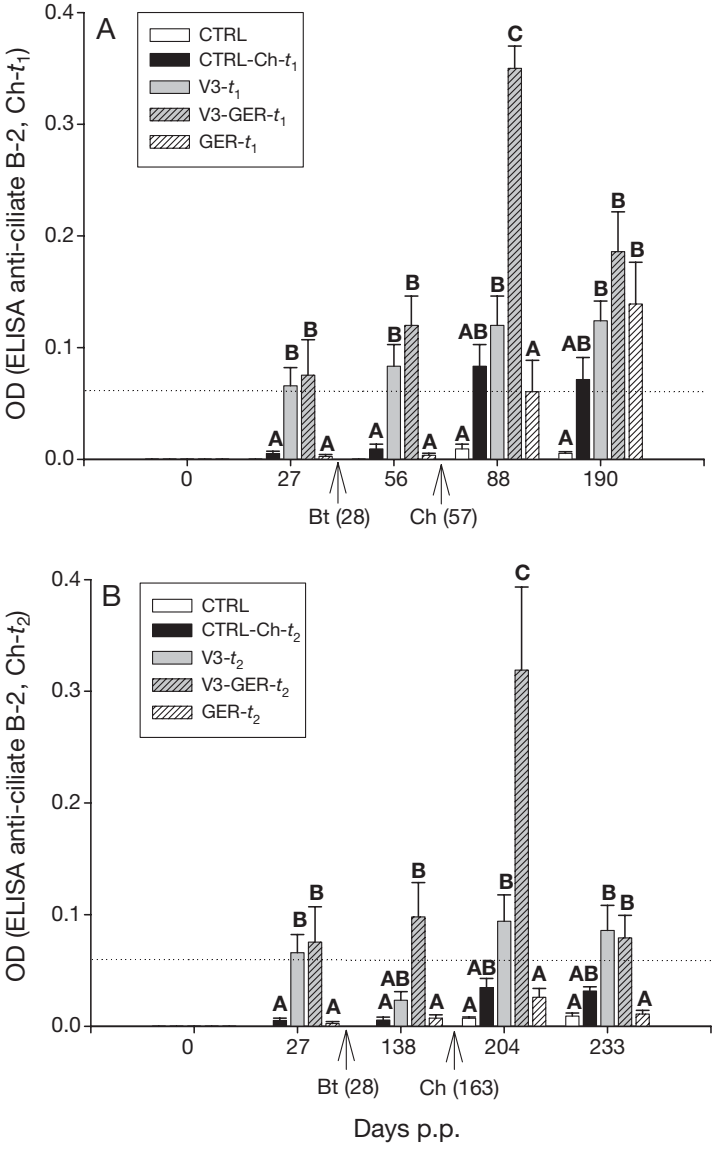

Fig. 5. *Psetta maxima*. Serum antibodies against the ciliate B-2 inoculated in the challenge (Ch) at Times (A) 1 (t_1) and (B) 2 (t_2) in Trial 2. Each bar represents the mean plus the SEM of the optical density (OD; ELISA). Different letters above bars indicate significant differences ($p < 0.05$) between groups at each sampling time after priming (p.p.). Dotted line: threshold level. Data on the first sampling (Day 27 p.p.) are common to t_1 and t_2. Arrows indicate the days for booster (Bt) and challenge (Ch)

at challenge, as mortality was higher in all vaccinated groups than in CTRL (mortality value 8.9%, unexpectedly low in this control group). Among the immunized fish, the highest mortality (24.1%) was registered in fish receiving only the booster injection (Bt-Ch) followed by the group primed with the highest dose of ciliate (LIV-H: 17.4%). Antibody levels in immunized fish were not significantly higher than in CTRL fish.

In Trial 2, cumulative mortality was relatively low in CTRL-Ch fish (32.9%) challenged at Time 1. Certain protection was only observed in V3-t_1 fish (21.7%; RPS = 36), as all groups receiving adjuvant (alone or combined with antigen) showed higher mortality than CTRL fish. The mortality interval was delayed in all inoculated fish, regardless of the preparation (5 to 27 d), with respect to CTRL fish (3 to 12 d). For fish challenged at Time 2, cumulative mortality was very low in all groups, but V fish again showed a lower value than CTRL group (RPS = 56). Both groups inoculated with adjuvant had mortality somewhat higher (9.8% in V3-GER-t_2 and 13.2% in GER-t_2) than CTRL fish (8.5%). The mortality intervals were very similar in most groups (17 to 33 d), but in GER-injected fish, mortality started somewhat earlier (Day 13). Only in the cases of V3-t_1 and V3-t_2 were the antibody levels related to protection.

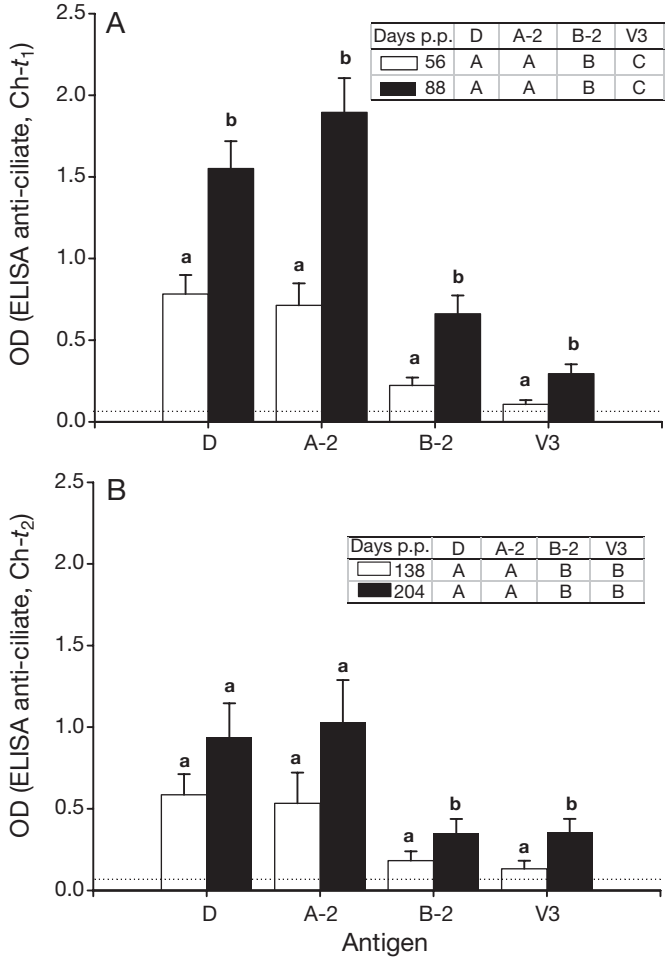

Fig. 6. *Psetta maxima.* Serum antibodies against the different types of ciliate antigen used in the antigen formulation (V3) in Trial 2 before and after the challenge (Ch) at Times (A) 1 (t_1) and (B) 2 (t_2). Each bar represents the mean plus the SEM of the optical density (OD; ELISA). Different lower case letters above bars indicate significant differences ($p < 0.05$) between sampling times for each antigen. Different upper case letters in each graph inset indicate significant differences ($p < 0.05$) between antigens at each sampling day after priming (p.p.). Dotted line: threshold level

In Trial 3, good results in terms of protection at challenge were obtained with the low dose of ciliate (RPS = 94–95). However, serum samples were not analyzed in this experiment and thus the relationship between antibody levels and protection could not be determined.

In Trial 4, the comparison of RPS between groups is not possible, as no mortality was observed in the challenged CTRL fish (CTRL-Ch). Mortality was very low in all fish inoculated with antigen alone (1.25 to 5%), although somewhat higher in fish receiving adjuvant (10 to 21.8%). Thus, no protection was obtained, despite the significantly higher antibody levels in most immunized groups with respect to CTRL.

DISCUSSION

Most antigen formulations used in this study induced the production of specific antibodies, especially following inoculation with adjuvanted ciliates. However, the protection against challenge with live ciliates was variable and did not correlate with serum antibody levels. Overall, the administration of antigen preparations (even when adjuvanted) had slight or no effects on fish weight.

Although we have previously observed some attenuation of virulence after serial passages *in vitro* (Alvarez-Pellitero et al. 2004), some mortality was caused in Trial 1 by using passage 41 of A-1 isolate as a live vaccine candidate. Moreover, the injection of live parasites did not protect fish against challenge with a virulent isolate (see Alvarez-Pellitero et al. 2004). Thus the live ciliate was disregarded as a potential vaccine, at least under the conditions assayed, and further trials were performed using killed ciliates. In contrast with the current results, a better protection of fish against other ciliates, namely *Ichthyophthirius multifiliis*, has been reported when using live ciliates with respect to killed parasites (Burkart et al. 1990, Alishahi & Buchmann 2006).

Among the immunization trials using formalin-killed ciliates, the best protection was obtained in Trial 3,

against a low dose-challenge. However, fish challenged with the high dose were not protected equivalently. In Trial 2, partial protection was obtained with the trivalent antigen alone (group V3-t_1), but the mortality in groups receiving adjuvanted preparations was higher than in CTRL. In Trial 4, no mortality was observed in CTRL fish, and thus comparison of relative protection was not possible. However, in this trial, mortality in groups receiving antigen alone (V1, V3, and V5) was also very low and cannot be considered significant.

The low mortality generally observed in the challenged CTRL fish in all trials is remarkable and complicates the evaluation of protection in vaccinated groups. Although a partial attenuation of the passages used for challenge could have occurred (see Alvarez-Pellitero et al. 2004), other factors related to fish condition and temperature cannot be disregarded. Virulence of these organisms seems to be higher at their optimal growth temperature (18 to 20°C), although higher temperatures induce low mortality values (Sitjà-Bobadilla et al. 2008). In this study, the cumulative mortality in CTRL fish was generally higher, and the mortality periods were shorter, after challenges at warmer temperatures. The current results confirmed the high variability in the virulence of this ciliate, which is extremely influenced by environmental and fish-related factors. Such circumstances complicate the experimental designs and the interpretation of results.

The injection of live ciliates (Trial 1) was capable of inducing agglutinating activity in serum but did not induce significant levels of specific antibodies measured by ELISA. The absence of a correlation between ELISA results and immobilization assays has also been reported for turbot inoculated with *Philasterides dicentrarchi* (Iglesias et al. 2003). Since heat-inactivated sera were used in the current work, the involvement of complement or other thermolabile components should be disregarded and other factors could be involved in agglutination, such as thermostable lectins and/or immobilization antigens (i-antigens) undetectable by ELISA. The absence of both lysis and further escape of agglutinated ciliates seem to indicate a change in i-antigens after agglu-

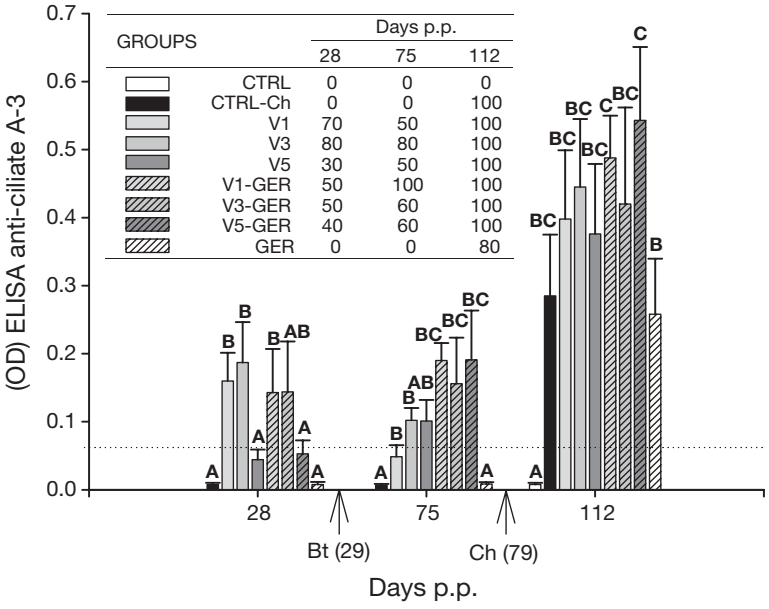

Fig. 7. *Psetta maxima.* Serum antibodies against the ciliate B-2 inoculated in the challenge (Ch) in Trial 4. Each bar represents the mean plus the SEM of the optical density (OD; ELISA). Different letters above bars indicate significant differences ($p < 0.05$) between groups at each sampling time after priming (p.p.). Arrows indicate the days for booster (Bt) and challenge (Ch). Dotted line: threshold level. Table: percentage of fish positive for antibodies in each group and sampling time

Table 1. *Psetta maxima.* Relationship between the serum antibody levels found in turbot injected with the different types of inocula (antigen, antigen + adjuvant [GER], or adjuvant alone) and the protection when challenged with a pathogenic scuticociliate strain. 'Raised' refers to significantly increased optical density (OD) in ELISA with respect to CTRL-Ch fish at the post booster (p.b.) and post challenge (p.c.) samplings. Detailed data on specific antibodies for Trials 1, 2, and 4 can be found in Figs. 5, 6, and 7, respectively. 'Protection' indicates whether inoculated fish did (YES) or did not (NO) have lower cumulative mortality than CTRL fish after challenge. Relative percentage survival (RPS) was not determined (nd) in Trial 4, as no CTRL fish died. Abbreviations for inocula are given in legends to Figs. 1 to 3

Trial	Type of inoculum	Antibody levels raised p.b.	p.c.	Protection	RPS
1	LIV-H	NO	NO	NO	−34
	LIV-L	NO	NO	NO	−95
2	V3-t_1	YES	YES	YES	36
	V3-GER-t_1	YES	YES	NO	−64
	GER-t_1	NO	YES	NO	−74
	V3-t_2	NO	YES	YES	56
	V3-GER-t_2	YES	YES	NO	−77
	GER-t_2	NO	NO	NO	−55
4	V1	YES	YES	NO	nd
	V3	YES	YES	NO	nd
	V5	NO	YES	NO	nd
	V1-GER	YES	YES	NO	nd
	V3-GER	YES	YES	NO	nd
	V5-GER	YES	YES	NO	nd
	GER	NO	YES	NO	nd

tination, as previously suggested for *P. dicentrarchi* (Iglesias et al. 2003, Lee & Kim 2008). Lee & Kim (2008) observed that *P. dicentrarchi* expressed different i-antigens, and a lower dilution of immune sera was needed to kill the ciliates when obtained from infected fish than when coming from cultures, suggesting the presence of other surface antigens besides the i-antigens.

Homologous agglutination was mostly observed in the current work, as B-2 isolate was agglutinated only with serum obtained after challenge with this isolate. In addition, only serum from a single low-dose fish (LIV-L) was able to agglutinate a heterologous D isolate. Xu et al. (2006) and Swennes et al. (2007) found no heterologous immobilization of different live *Ichthyophthirius multifilis* serotypes, although serum antibodies reacted with both serotypes in ELISA and cross-protection was observed in some cases. In the current work, live A-1 ciliates used in Trial 1 did not induce heterologous protection against challenge with B-2 isolate, nor were specific antibodies against these isolates detectable by ELISA. However, turbot surviving natural scuticociliatosis have been reported to present relatively high antibody levels (Iglesias et al. 2003) and they seem to resist further infections (empirical observations in the farms). Under these natural infections, it can be presumed that fish are exposed to the parasite repeatedly and for longer periods and new challenges are probably due to ciliates with similar serotypes. Thus, it seems that persistent and repeated contact is necessary to mount a response that protects fish against new scuticociliate challenges. In addition, in natural infections, the routes of entry of the parasite could involve contact with mucosal immune tissues and elicit more effective responses than when using the i.c. route, as has been described for *Cryptocaryon irrritans* (Luo et al. 2007).

In contrast to the results obtained with live ciliates in the current study, a specific antibody response was clearly detected by ELISA when using formalin-killed ciliate formulations. In both Trials 2 and 4, a progressive increase in serum antibodies specific against the ciliates used for the challenge was observed in vaccinated fish, from p.p. sampling and onwards, and usually peaking at the first sampling after challenge. In Trial 2, specific antibody levels against individual isolates A-2 and D were significantly higher than against B-2 (the isolate used for challenge) or the trivalent antigen (V3). In a previous immunization experiment using the same V3 antigen, the specific response tested by ELISA was stronger against the isolate inoculated at challenge than against V3 (Sitjà-Bobadilla et al. 2008). These differences further suggest the existence of variable immunogenicity among ciliate isolates.

The comparison of monovalent and polyvalent antigen formulations (Trial 4) demonstrated higher antibody titer induction with V1 and V3 than with V5. Polyvalent vaccines have been used for some bacterial diseases in fish, although inhibition of the specific responses can occur (Nikoskelainen et al. 2007). The absence of protection against some bacteria included in polyvalent turbot vaccines has also been reported (Björnsdóttir et al. 2004, 2005). In the present work, no advantage was obtained using the V5 ciliate preparation. Antigenic competition, induction of tolerance, and other factors could be involved, as previously suggested (Nikoskelainen et al. 2007).

According to our results, the administration of antigen plus adjuvant produced a higher specific antibody response than when using antigen alone. Interestingly, an immunopotentiating effect of the adjuvant alone, which was able to enhance the production of specific antibodies after challenge, was observed in Trial 2. The elucidation of the mechanisms involved in the effect of the different adjuvanted formulations requires further investigation. Some antigen-independent mechanisms could have been stimulated, including the polyclonal activation, as described for mammals (Minoprio 2001, Traggiai et al. 2003). In the GERBU 734 formulation used in this study, lipid microparticles stimulate Th2 cell responses, and GMDP increases the Th1 cell activity (Guy 2007).

Despite the good antibody response elicited by the assayed preparations, the antibody levels did not correlate with protection, as this was low or inexistent with the antigen preparations used in Trials 2 and 4. A similar absence of correlation between protection and unequivocal specific responses has been reported for *Ichthyophthirius multifiliis* (Sigh 2004). By contrast, Luo et al. (2007) reported good correlation in fish immunized with live *Cryptocaryon irritan*s. According to Alishahi & Buchmann (2006), other immune effectors could be involved in the response and protection against *I. multifilis*, such as lysozyme (whose levels rise after immunization) or other non-specific elements. In a previous short-scale experiment (to evaluate the effect of the same V3 antigen and the adjuvant Montanide ISA 763 on turbot) we demonstrated the stimulation of lysozyme after immunization (Sitjà-Bobadilla et al. 2008), although a significant increase was detected only after challenge, when complement levels were also increased in all groups. In the same experiment, preliminary information on the effect of GER adjuvant on turbot was also obtained, and high peroxidase levels observed in fish inoculated with V3 antigen + GER indicated a profound effect of this adjuvant in turbot immune response, which is confirmed by the high antibody levels obtained in the current study. However, GER adjuvant was not adequate in the con-

ditions and doses assayed. The failure to obtain better protection might be due to an excessive stimulation by its components, which can lead to an exhaustion of the response and also contribute to undesired side effects. GERBU 734 is a complex experimental formula intended to enhance both cellular and humoral immune responses, and no previous information on its use in fish was available before our experiments. Small lipid nanoparticles (average ∅ = 0.1 µm) were included in the formula as promoters of the humoral response. However, as larger particles (5 to 10 µm) seem to be more effective, and L121 (used as an emulsifier but with its own immunopotentiating effect) is often counterproductive, the manufacturers have forsaken this particular experimental approach (N. Grubhofer pers. comm.). Thus, an improvement of the formulation and an adjustment of the adequate dose would be necessary for its use in turbot.

Since certain but limited protection was observed using killed ciliates, the use of an appropriate adjuvant should improve these results. Promising protection results have been recently reported in small-scale (Sitjà-Bobadilla et al. 2008) or medium-scale (Lamas et al. 2008, Sanmartín et al. 2008) vaccination experiments using Montanide ISA 763, and data of the current work suggest the potential of GER or other complex adjuvants combining easiness of delivery (oil-in-water emulsions or stabilized-lipid microparticle suspensions) and immunostimulating compounds. Thus, further studies should address large-scale experiments to compare formulations, insisting on optimum adjuvants, dosages, and polyvalent antigens (including A and D isolates), in order to obtain a combined effect on different immune response pathways without induction of tolerance. However, it must be stressed that standardization of the challenge procedure is needed in order to evaluate protection accurately. Since the development of turbot scuticociliatosis seems a rather complex process in which environmental, host, and parasite-related factors affect significantly, this standardization is currently among the main bottlenecks in the evaluation of vaccine formulas.

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