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Vaccination of European sea bass *Dicentrarchus labrax* with avirulent *Mycobacterium marinum* (*iipA::kan* mutant)

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ABSTRACT

Mycobacteriosis is a chronic progressive disease affecting teleost fishes all over the world. No vaccine is commercially available against its main etiological agent, *Mycobacterium marinum*. The mycobacterial gene responsible for invasion and intracellular persistence, *iipA*, is known to modulate *M. marinum* pathology. The innate and adaptive immune responses in sea bass (*Dicentrarchus labrax*) vaccinated with *M. marinum iipA::kan* mutant with (and without) the use of adjuvant, with (and without) a booster vaccination were monitored. The adjuvanted vaccine induced enhanced immune responses. TNF-α transcription levels were extremely high in spleen of the fish vaccinated with the addition of adjuvant in both fish vaccinated once and twice, followed by an IgM response highly specific for *M. marinum*. Also, histologically, granulomas started appearing in spleen and head-kidney tissues (but with no visible bacteria) within a month after vaccination, mainly with the adjuvanted vaccine. This was followed by reduction in pathology, as demonstrated by the lower number of granulomas (with visible bacteria), indicating that even heat-killed bacteria were able to elicit granulomatous formations. Adhesion of the internal organs and moderate pigmentation were observed in the perivisceral adipose tissue of nearly all vaccinated fish. Although the adjuvanted heat-killed avirulent *iipA::kan* mutant clearly induced a strong humoral and adaptive immune response, the booster treatment did not seem to have produced a significantly higher degree of protection from the disease compared to fish that received a single vaccination.

1. Introduction

Mycobacteriosis, or “fish tuberculosis”, is a chronic progressive disease affecting teleost fishes all over the world. The responsible agents, mycobacteria, are weakly gram-positive, aerobic, acid-fast and non-motile rods. *Mycobacterium marinum* is perhaps the most commonly isolated species known to infect fish in freshwater, brackish water and saltwater environments [1–5]. In culture, most fish species eventually develop skin ulcers, enlarged visceral organs, with spleen and kidney most severely affected, which renders the infected fish unmarketable [6,7]. The effects of chemotherapeutic agents are limited by the ability of mycobacteria to survive and even multiply within the host's macrophages [8,9]. There are currently no FDA (US Food and Drug Administration) approved drugs for the treatment of fish mycobacteriosis, often leaving depopulation and thorough disinfection of the rearing facilities as the only managerial option. Mycobacteriosis is a serious and costly issue in fish culture and advancements in treatment alternatives are warranted [10].

To date, vaccines have been shown to be critical for the successful culture of various fish species [11,12]. In general, empirically developed vaccines based on inactivated bacterial pathogens have proven to be relatively efficacious in fish [13]. However, despite the many studies conducted using a large variety of antigens [14–17], none of the experimental vaccines against *M. marinum* has ever stepped beyond the laboratory threshold and no vaccine is commercially available today. Vaccination with attenuated or avirulent mycobacteria, including attenuated *M. marinum* and BCG (Bacillus Calmette–Guérin), as well as with selected DNA-encoding mycobacterial antigens, has offered fish various - but generally unsatisfactory - degrees of protection from experimental mycobacteriosis [14,15,18,19].

In *M. marinum*, the genes responsible for the invasion and intracellular persistence (*iipA* and *iipB*) are homologous to Rv1477 and Rv1478 of *M. tuberculosis*, conferring antibiotic resistance and protection from lysozomal enzymes by maintaining bacterial cell wall structural integrity [20]. Both genes are highly conserved within the *Mycobacterium* genus, including - in addition to *M. marinum* and *M. tuberculosis* - *M. bovis*, *M. leprae*, *M. avium*, and *M. smegmatis* [20]. When mortalities of zebrafish experimentally infected with a virulent *M. marinum* and the *iipA::kan* mutant were compared, the virulent *M. marinum* caused 100% mortality within 3–5 weeks, whereas a 100%

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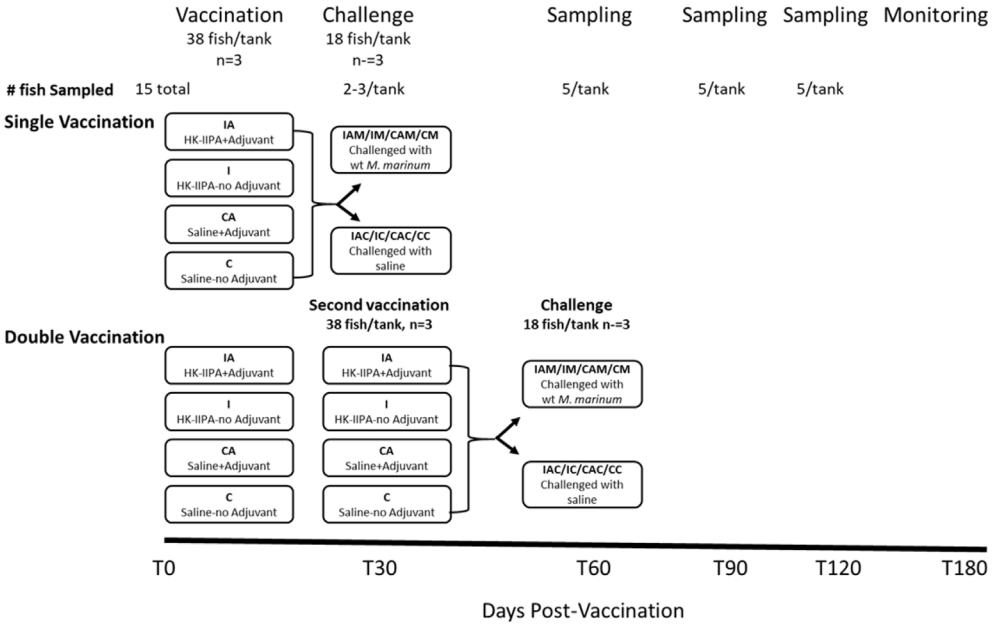

Fig. 1. Vaccination experimental design conducted with European sea bass (T0 = before vaccination. T30-T180 = days post-vaccination (dpv). Single vaccination - challenged 1 month after vaccination (T30), Double vaccination – vaccinated again on 30 dpv (T30) and challenged 60 dpv (T60). Vaccinations were carried out using heat-killed *M. marinum iipA::kan* mutant injected intraperitoneally (IP) with and without adjuvant (IA, I respectively). Controls were sham-vaccinated with saline with and without adjuvant (CA, C respectively). Challenges were carried out by IP-injection of live virulent Eilat Wild Type (WT) *M. marinum* (IM, IAM, CM, CAM) or sham-challenge with saline with and without adjuvant (IC, IAC, CC, CAC). Sampling: tissues (head-kidney, spleen and blood) were collected randomly from 2 to 5 fish from each replicate (n = 3), for histological, serological and molecular analyses.

fish survival during the entire 16-week long experiment was obtained with the *iipA::kan* mutant [20].

Acting on the same lines as Gao and collaborators' (2006) [20], a heat-killed avirulent *M. marinum iipA::kan* mutant strain was used to vaccinate sea bass (*Dicentrarchus labrax*). The fish were then challenged with a virulent live strain of *M. marinum* and the pathogenesis of the disease was monitored [21]. The *iipA::kan* mutant induced a strong immune response accompanied by only modest tissue disruption [21]. A high survival rate, high specific IgM response and increase in cytokine TNF-α mRNA expression levels were observed in all vaccinated fish [21]. In the light of these encouraging results, we explored the feasibility of potentiating the immune response in the European sea bass (*Dicentrarchus labrax*) by coupling the heat-killed *iipA::kan* mutant with an adjuvant and a booster treatment. In fish, oil-based adjuvants are known to induce long-term protection [22] to a high degree [23]. Oil-adjuvants are also known to induce side effects such as intra-abdominal adhesions, inflammation, granulomatous peritonitis and pigmentation [24–26]. Despite these drawbacks, in vaccines that do not elicit strong immune responses or in lower vertebrates in which the adaptive immunity is comparatively less evolved (such as fish), the addition of improved adjuvants could boost the level of protection towards a desired level [27].

2. Material and methods

2.1. Fish and their maintenance

A stock of 912 European seabass (*Dicentrarchus labrax*) (average weight of 50.2 ± 1.5 g), cultured in the facilities of Israel Oceanographic and Limnological Research, National Center for Mariculture (IOLR-NCM) in Eilat and with no clinical signs of mycobacterial infections (confirmed by random samples examined by histological and molecular means) was randomly distributed, in batches of 38 individuals, into 24 100 L tanks with filtered and UV-treated flow-through seawater (27.5 ± 0.5 °C; 40 ppt, pH 8.13). Fifteen additional fish were randomly sacrificed for Time 0 analyses. The water exchange rate in the tank was 50%/hour and the oxygen level never dropped below 5 mg/l. Fish were fed a commercial diet (Raanan Fish Feed Ltd., Israel) throughout the experimental period. All fish were mildly sedated with clove oil (100 mg/L) before any handling.

2.2. Bacterial vaccination

The non-virulent *M. marinum iipA::kan* mutant was developed at the Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, MD [20]. The Israeli *Mycobacterium marinum* (eilaticum, “Eilat strain”, termed in the present work as virulent “wild type” or WT) was originally isolated from a diseased white grouper, *Epinephelus aeneus*, cultured at IOLR-NCM (Eilat, Red Sea). Mycobacteria were cultured on BBL™ Löwenstein-Jensen Medium (BD, Becton, Dickinson and Company, MD) and incubated at 24 ± 0.5 °C. Subcultures were made every three months using the same medium.

For the vaccination trials, the non-virulent *M. marinum iipA::kan* mutant was heat-killed (75°C, 60 min) (as per Gao et al., 2002) [28], homogenized and diluted in a 0.2 µm filtered saline solution to which 0.3% Tween 80 had been added to minimize clumping. Bacteria concentration was determined by optical density at a wavelength of 600 nm (OD_{600} 1.0 = 7.7×10^7/ml) (as per Solomon et al., 2003) [29]. Inactivation was confirmed by plating the respective suspensions on the aforementioned culture media and monitoring the lack of growth for three months. The vaccine contained 0.77×10^8 bacteria/ml, with or without 70% (W/W) ISA 760 VG SEPPIC, Montanide™, a commercial adjuvant, added according to the manufacturer's instructions (https://www.seppic.com/montanide-isa-w-o-w). The fish were injected intraperitoneally (IP) with 100 µl of vaccine. The same volume of sterile saline with 0.3% Tween 80 was used for sham-vaccination of the control groups. Fish were vaccinated either once or twice (in the latter case, a booster injection was done 30 days after the first vaccination). For the challenge, live WT “Eilat strain” was used at a concentration of 3.5×10^7 bacteria/ml in the fish vaccinated once, and at a concentration of 6×10^7 bacteria/ml the second time in the fish vaccinated twice.

2.3. Experimental design

The experimental design and schedule are summarized in Fig. 1. Following a ten-day acclimation period, on day 0 (T0, i.e. before vaccination), 15 fish were randomly sacrificed, and other groups of 38 fish each were respectively vaccinated intraperitoneally (IP) with one of the various vaccine types: IA (heat-killed (HK) *iipA::kan* mutant with adjuvant), I (HK *iipA::kan* mutant without adjuvant), CA (control, saline with adjuvant), and C (control, saline without adjuvant). Six replicates were carried out for each treatment. On day 30 (T30) post-vaccination

(dpv), 15 fish from each treatment (2–3 from each replicate) were randomly sampled for serological, histopathological and molecular analyses. The experiment was then split into 2 parallel lines. In the first line, 18 fish from each treatment were challenged by IP-injection with live virulent WT isolate and the other 18 were sham-challenged with saline (3 replicates for each treatment). This trial was referred to as "single vaccination". In the second line, fish from each treatment, were revaccinated with the same vaccine types. This trial was referred to as "double vaccination". Sixty dpv (T60), 5 fish from each treatment of the single vaccination and 15 fish from each treatment (2–3 from each replicate) of the double vaccination, were randomly sampled. From each treatment of the double vaccination, 18 fish were then challenged by IP-injection with live virulent WT isolate and the other 18 were sham-challenged with saline (3 replicates for each treatment). Five fish from each replicate were randomly sampled every month for two additional months (T90, T120). The fish were monitored daily during 180 dpv and mortalities recorded. The survival function calculation took into account the number of fish remaining in the tank after each sample point.

2.4. Sampling procedure

Anesthetized fish were weighed, blood was drawn from the caudal vein for serological analyses and left to clot at 4 °C overnight. The fish were then euthanized by decapitation and spleen and head-kidney were collected aseptically and immediately frozen at −80 °C for later molecular analyses.

2.5. Histopathology

Samples of spleen and head-kidney tissue were fixed in buffered neutral formalin (BNF), embedded in paraffin for standard histological process and the sections were stained using the Ziehl-Neelsen acid fast procedure [30]. The severity of infection was assessed by quantitative analysis of the presence of granulomas in the 15 fish sacrificed from each group. Granulomas were counted from an entire, randomly selected field area at x10 magnification. In addition, granulomas with visible, acid-fast bacteria and granulomas with no visible acid-fast bacteria were compared and severity of infection was estimated by percentages.

2.6. DNA and RNA extraction

For detection of *Mycobacterium*, DNA was extracted from the spleen (5–100 mg) ground with 300 µl of grinding buffer (100 mM Tris-HCl pH 9, 100 mM EDTA, 1% SDS) by FastPrep-24™ 5G (MP Biomedicals LLC, Santa Ana, CA) and incubated at 70 °C for 30 min. The homogenate was then placed on crushed ice for 30 min with 42 µl of 8 M potassium acetate and centrifuged twice at 12 000 g (15 min and 5 min at 4 °C). DNA was precipitated with 1 volume of isopropanol for 15 min at room temperature (RT), washed twice with 70% ethanol and air-dried. Pelleted DNA was dissolved in 50 µl ddH_2O. Total RNA was extracted from spleen and head-kidney (50–100 mg) target tissues using Bio-Tri RNA Reagent (Bio Lab Ltd., Jerusalem, Israel) according to the manufacturer's instructions and ground by FastPrep-24™ 5G (MP Biomedicals LLC). DNA and RNA quantity and purity (260/280 ratio) were estimated in a NanoDrop™ One Microvolume UV–Vis Spectrophotometer (Thermo Fisher Scientific, Madison, WI). Samples were stored at −80 °C until further analysis. DNA residues from the extracted RNA were removed by using DNase I (RNase-Free)® kit (Ambion) according to the manufacturer's instructions.

2.7. Molecular detection by PCR

The presence of *Mycobacterium* in spleen tissues was examined using PCR analysis followed by nested PCR (ns-PCR). For the 16S rDNA gene, the specific primers 246 (forward), and 266 (reverse) [31] (amplicon size: 612 bp long) were used. Nested PCR was performed with internal primers My1 (forward) [5], and 414 (reverse) [31] (amplicon size: 155 bp long). The kit GoTaq® Green Master Mix (Promega) was used in a total volume of 10 µl, and PCR assays were performed with the Eppendorf Mastercycler gradient (Eppendorf). To rule out possible contaminations, a negative control sample was applied in every reaction.

2.8. Gene expression by quantitative real-time PCR (RT-qPCR)

TNF-α mRNA expression was analyzed in the spleen and head-kidney from healthy fish and, in parallel, from the experimentally infected fish (n = 9, 3 fish from each replicates for each treatment). TNF-α mRNA transcript (163bp) was amplified using specific primers and probe designed by Ziklo et al. (2018) [21].

RNA samples were treated with DNse-I (DNse-I RNse-Free®, Ambion Inc, Foster City, CA) according to the manufacture's protocol. cDNA from the RNA samples was synthesized by using qScript™ cDNA Synthesis Kit (Quanta BioSciences, Inc., Gaithersburg, MD) according to the manufacturer's protocol. The reaction mix (final volume of 20 µl) consisted of 4 µl qScript Reaction Mix (5X), 1 µl qScript RT, 10 µl Nuclease-free water, 4 µg RNA template.

The TNF-α transcription level was determined by RT-qPCR, using PerfeCta® qPCR ToughMix™, Low ROX™ (Quanta BioSciences), in 10 µl reaction volume with duplicates. Mix preparation and cycling parameters were set according to the manufacturer's protocol. To rule out possible contaminations, in every reaction a negative control sample containing nuclease-free water was loaded. A constant mixed sample which had shown medium transcription level was also added as positive control. The results were analyzed by 7500 Fast Real-Time PCR system software (Applied Biosystems, Thermo Fisher Scientific, version 2.0.6). Relative quantification levels were calculated by $RQ = 2^{-\Delta\Delta ct}$, Ct-Threshold cycle.

In order to verify efficiency (90–110%) in the expression of both endogenous and target genes, a standard curve was applied for each target tissue (spleen and head-kidney), in 6 different serial dilutions (for endogenous gene 18S, 10^{-1}-10^{-6}, 175-1.75x10^{-4} ng/µl; for TNF-α gene, 1-4^{-6}, 175-0.04 ng/µl), with 3 technical replicates for each sample.

Transcription levels were normalized according to the endogenous reference gene 18S. The results were analyzed by 7500 Fast Real-Time PCR system software (Applied Biosystems version 2.0.6).

2.9. Serological analyses and ELISA

Blood samples were refrigerated, left to clot at 4 °C overnight, centrifuged (3500 g, 30 min) and the serum was subsequently stored at −20 °C until further analysis. To quantify the *M. marinum* specific IgM titers, non-competitive ELISA serological analyses were performed. A 96-well microplate was coated with 50µl/well, 0.05% (w/v) poly-L-lysine in coating buffer (carbonate-bicarbonate solution) and incubated at RT for 1 h. Fluids were discarded and the plate was rinsed (2 × 5 min) with 250 µl/well of washing buffer (PBS-T). The plate was then coated with 100µl/well heat-killed, suspended bacteria (*M. marinum* eilaticum WT) in saline to which 0.3% Tween 80 had been added to a final concentration of 1. x10^8 bacteria/ml and incubated (4 °C, overnight). The day after, 50 µl 0.05% (v/v) glutaraldehyde in PBS was added to each well and incubated for 20 min at RT. Fluids were discarded and the plate was rinsed (2 × 5 min), saturated with 250 µl/well of blocking solution (1% gelatin in PBS), incubated for 2 h at RT, to which a single brief wash followed. Then, 100 µl of fish serum (diluted 2×10^{-3} in PBS) was added to each well and incubated for 1 h at RT. Fluids were discarded and the plate was rinsed (3 × 5 min). 100 µl of mouse anti-European sea bass IgM monoclonal antibodies (Aquatic Diagnostics, Ltd., Stirling, U.K.) suspended the day before in 1 ml PBS, then diluted again 2×10^{-3} (Normal Goat Serum Gibco®, Applied Biosystems, Thermo Fisher Scientific) in PBS was added in all the wells

and incubated for 1 h at RT. The fluids were discarded and the plate was rinsed (3 × 5 min). 100 µl of Goat Anti-mouse IgG-HRP conjugate (Bio-Rad Laboratories, Hercules, CA), diluted 10^{-3} in 1% NGS, was added to the entire plate and incubated for 1 h at RT. Fluids were discarded and the plate was rinsed (3 × 5 min). 100 µl of SureBlue™ TMB Microwell Peroxidase substrate (Thermo Fisher Scientific) was added to all the wells and the plate, wrapped in aluminum foil, and allowed to react for 10 min at RT. Finally, 50 µl/well of stop solution (2 M H_2SO_4) was added. Plates were read at 450 nm absorbance in microplate spectrophotometer (Bio-Tek® reader GeneQuant-Pro, Amersham, Cambridge, U.K.). Antibody titers in fish serum were quantified and expressed as fold-change approach, while dividing OD measurements in the baseline response of unvaccinated control group according to the fold induction method. For controls, each sample was tested with the same procedure, only without coating the well with bacteria, in order to eliminate "background noise" as a result of non-specific IgM in the fish serum. The background noise was deducted from the actual results. Also, a negative control sample (PBS) and a positive control sample (serum pooled from *M. marinum* infected fish) were added to each plate.

2.10. Statistical analysis

Data analyses were performed using the Statistical Package for Social Sciences, version 20 (IBM SPSS Statistics, Chicago, IL). Data were transformed when needed to fulfill the assumptions of normality or equal of variance. Unless otherwise specified, the significance of differences between group means was determined by analysis of variance (ANOVA) followed by Tukey's post hoc tests. Differences in mortality were tested for statistical significance by the Chi-square contingency table test.

3. Results

3.1. Survival

Fish grew 2.3 times on average during the 4-month trial (to an average weight of 113.7 ± 1.8 g). Differences in weight gain among treatments were not statistically substantial. Uninfected fish (IAC, IC, CAC, CC) of both immunization regimens (single and double) and fish that were vaccinated once with any of the various vaccine types, and challenged (IAM, IM, CAM, CM), did not suffer any major mortalities (0–7.2%) during the experimental period (not shown). However, in fish that were vaccinated twice and challenged, significant mortalities started 30 days post-challenge (dpc) in the sham-vaccinated fish (CAM, CM). In 120 dpc, 29% and 30% mortality occurred in fish vaccinated with saline (CM) and saline plus adjuvant (CAM), respectively, whereas in 120 dpc, mortalities of vaccinated challenged fish (IAM, IM) were only 15% and 9% respectively (Fig. 2).

3.2. Pathology

External clinical signs of *M. marinum* infection revealed skin erythema and ulceration, displayed only in the unvaccinated challenged fish (Fig. 3a). In several vaccinated fish, a moderate pigmentation was found on the surface of perivisceral adipose tissue and a moderate visceral adhesion was observed (Fig. 3b).

At T0 prior to vaccination, tissues appeared to be healthy and no granulomas were observed (Fig. 4a and b). A month after vaccination before infection (T30), numerous areas of bacteria-free granulation tissue appeared in the histological sections of spleen and head-kidney stained Ziehl-Neelsen (Fig. 4c and d) both in fish vaccinated with and without adjuvant (IA, I). No granulomas were observed in the sham-vaccinated fish (CA, C). One, two and three months after challenge (T60, T90, T120), the normal tissue appeared in all groups to be displaced by numerous granulomatous lesions. Inside these nodules, large concentrations of mycobacteria were visible (Fig. 4e–h).

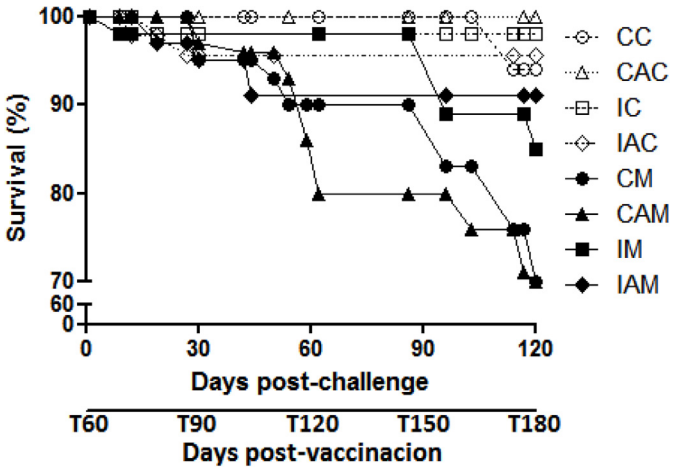

Fig. 2. Survival percentage within the double vaccinated challenged groups during the 4-month period post-challenge. Vaccinations were carried out using heat-killed *M. marinum iipA::kan* mutant with and without adjuvant. Controls were sham-vaccinated with saline with and without adjuvant. Challenges were carried out with live virulent Eilat WT *M. marinum*. Controls were sham-challenged with saline. CC (○), control fish; CAC (Δ), control fish with adjuvant; IC (□), vaccinated fish; IAC (◊), vaccinated fish with adjuvant; CM (●), control fish challenged; CAM (▲), control fish with adjuvant challenged; IM (■), vaccinated fish challenged; IAM (♦), vaccinated fish with adjuvant challenged; T30-T150 days after first vaccination.

The results of the quantitative analyses of granuloma formation in spleen and head-kidney and the consequent estimation of the percentage of fish diseased by comparison of granulomas with visible (and presumably active) *M. marinum* vs. non-visible (and presumably bacteria-free) are summarized in Figs. 5 and 6, respectively. In the singly vaccinated fish at T30, granulomas with no apparent bacterial presence evolved in fish vaccinated with the mutant mainly with adjuvant supplementation (IA) in both spleen and head-kidney tissues (Fig. 5, T30). About 3.5 times more granulomas developed in the spleen than in the head-kidney (17.6 ± 4.2 vs. 4.7 ± 1.15 per field area). A similar result was obtained in the fish vaccinated twice, one month after the vaccination was repeated (Fig. 5, T60), but with an increase in the number of granulomas in the head-kidney (11 ± 4.3 per field area). In the singly vaccinated fish, a month after challenge (Fig. 5, T60), granulomas developed in all the fish infected. However, more granulomas developed in the spleen of the fish vaccinated with the addition of the adjuvant (IAC 19.3 ± 3.6 vs. IAM 28.7 ± 6 per field area) (Fig. 5, T60). Two and three months after infection (T90, T120), no significant differences in the number of granulomas between treatments and between the two organs were observed (Fig. 5, single vaccination, T90, T120). With the development of pathology, a lower number of granulomas was observed within the challenged fish. In the fish vaccinated twice, a month after challenge (Fig. 5, double vaccination, T90), more granulomas were counted in the non-vaccinated group in both spleen and head-kidney (CM 33,7 ± 3.1 vs. 27.6 ± 2.8, CAM 44.2 ± 3.2 vs. 32 ± 2.9) compared to those a month after challenge in the fish vaccinated once (Fig. 5, single vaccination T60) (CM 7.4 ± 1.9 vs. 4.6 ± 1.3 respectively, CAM 6.1 ± 2.4, 1.5 ± 0.9 respectively). At T120 no differences were found between the treatments, but the average number of granulomas was higher in the fish vaccinated twice (Fig. 5, double vaccination T120) than in the fish vaccinated once (Fig. 5, single vaccination T90 and T120).

When using the bacteria dyeing test for the estimation the percentage of diseased fish, significant differences were found in the challenged fish that had been vaccinated once (Fig. 6, T90) between the vaccinated fish (IM 33%, IAM 22%) and the sham-vaccinated ones (CAM 89%, CM 100%). Those differences continued to be observed 2–3 months (T90-T120) post-infection (Fig. 6, single vaccination T90-

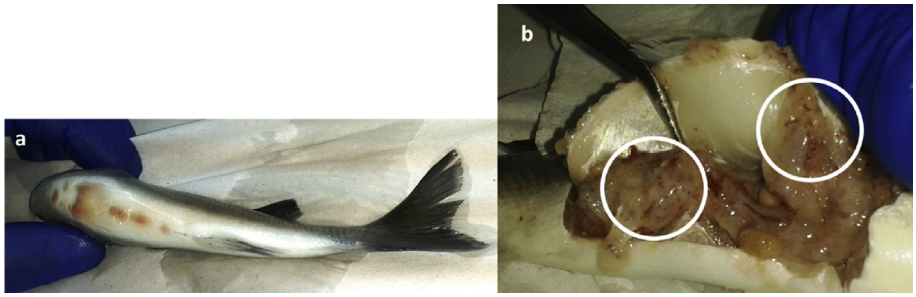

Fig. 3. Clinical signs of *M. marinum* infection. (a) Skin erythema and ulceration in the unvaccinated challenged fish. (b) Moderate pigmentation on the surface of perivisceral adipose tissue (white circles) and moderate visceral adhesion of the internal organs in vaccinated fish.

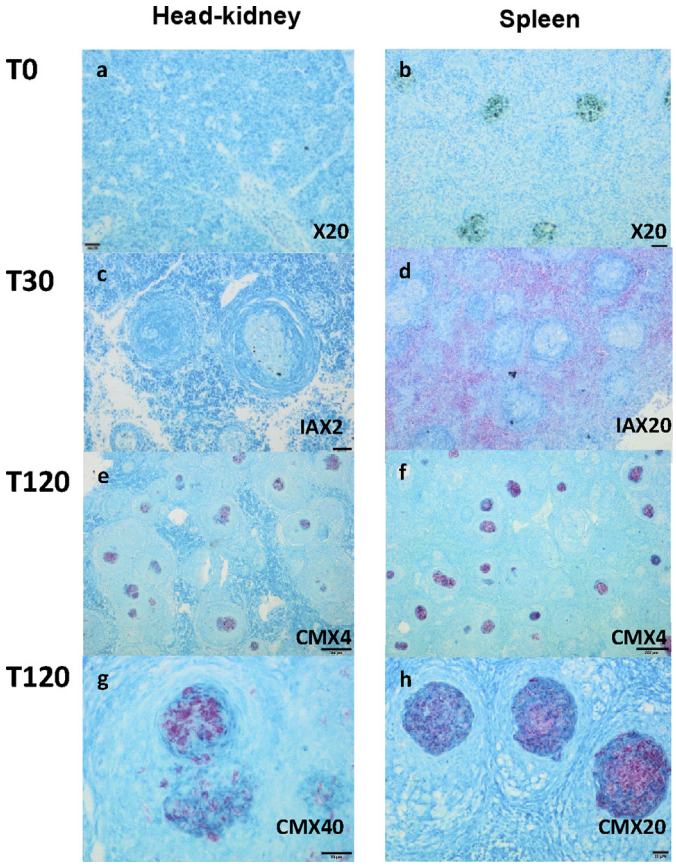

Fig. 4. Histological sections of sea bass (*Dicentrarchus labrax*) spleen and head-kidney tissues stained Ziehl-Neelsen for the detection of *M. marinun*.
At T0, prior to vaccination, healthy tissues were observed free of any granulomas (a,b, x20 magnification). At T30, a month after vaccination with heat-killed avirulent *iipA::kan* mutant and adjuvant, before infection (IA), numerous areas of bacteria-free granulation tissue appeared in the spleen and head-kidney (c, d, x20 magnification). At T120, non-vaccinated control fish (CM) 3 months after infection: numerous granulomatous lesions, with large concentrations of visible mycobacteria (e,f x4 magnification, g,h x40, x20 magnification, respectively).

T120). In the fish vaccinated twice, a month after infection (T90), the percentage of diseased fish was much higher than those that were vaccinated once (Fig. 6, T60), although fewer diseased individuals were found within those vaccinated once (IM, IAM). Two months after infection (T120), almost 100% of these fish appeared to be diseased.

Granulomas with visible acid-fast bacteria and granulomas with no visible acid-fast bacteria were counted from histological sections of 15 fish sacrificed from each group treatment. Counts were performed from the entire field area at x10 magnification during 60–120 days post-vaccination (T60-T120) of both vaccine regime (single and double). Severity of infection was estimated by percentages. CM – challenged, non-vaccinated control fish. CAM - challenged non-vaccinated control fish with adjuvant. IM – challenged, vaccinated fish. IAM – challenged, vaccinated fish with adjuvant. Different letters indicate significant differences ($P < 0.05$, one-way ANOVA and Tukey) from one another ($n_{(fish/treatment)} = 15$).

3.3. Detection of *M. marinum* by PCR

All fish from the control groups, whether vaccinated or sham-vaccinated, were found to be negative for *Mycobacterium* by PCR methods (not shown). In the singly vaccinated challenged fish at T60, *Mycobacterium* was detected in 33–44% of fish only by ns-PCR (Table 1). It was not detected in IAM treatment by either method (PCR or ns-PCR) up to 60 and 90 dpv. On T120 post-infection, it was detected in IAM only by ns-PCR (Table 1). In the doubly vaccinated challenged fish, *Mycobacterium* was diagnosed by both methods, although ns-PCR was much more sensitive (100% compared to 44–89% by PCR).

3.4. ELISA

Specific IgM response for *M. marinum* was detected in fish serum by commercial anti-sea bass (*D. labrax*) IgM antibodies. One month post-vaccination (T30), before the challenge, only the fish vaccinated with the addition of adjuvant (IA) elicited a high antibody titer, in comparison to non-adjuvant group or controls (I, C, CA) (Fig. 7, T30). However, in the double vaccination, a month after the second vaccination (T60), an increase in the specific antibody titer in the non-adjuvant group (I) was also observed (Fig. 7, T60).

Antibody titers in fish serum were quantified and expressed as fold-change approach, while dividing OD measurements in the baseline response (OD) of unvaccinated control group, according to the fold induction method. For controls, each sample was tested with the same procedure, only without coating the well with bacteria. A negative control sample (PBS) and positive control sample (pool of *M. marinum* infected fish) was added to each plate as well.

Vaccinations were carried out using heat-killed *M. marinum iipA::kan* mutant with and without adjuvant (IA, I respectively) and sham-challenged with saline with and without adjuvant (CA, C respectively) of both vaccine regime (single and double). Challenges were carried out with live virulent Eilat WT *M. marinum* (IM, IAM, CM, CAM). Control fish were sham-challenged with saline with and without adjuvant (IC, IAC, CC, CAC). Blood was collected randomly from 2 to 5 fish from each replicate (n = 3). T0 = before vaccination; T30 = 1 month after vaccination before challenge; T60 = single vaccine - 1 month after challenge, double vaccine −1 month after second vaccination: T90 = single vaccine - 2 months after challenge, double vaccine – 1 month after challenge: T120 = single vaccine - 3 months after challenge, double vaccine – 2 months after challenge. Different letters indicate significant differences ($P < 0.05$, one-way ANOVA and Tukey) from one another ($n_{(fish/treatment)} = 9–15$).

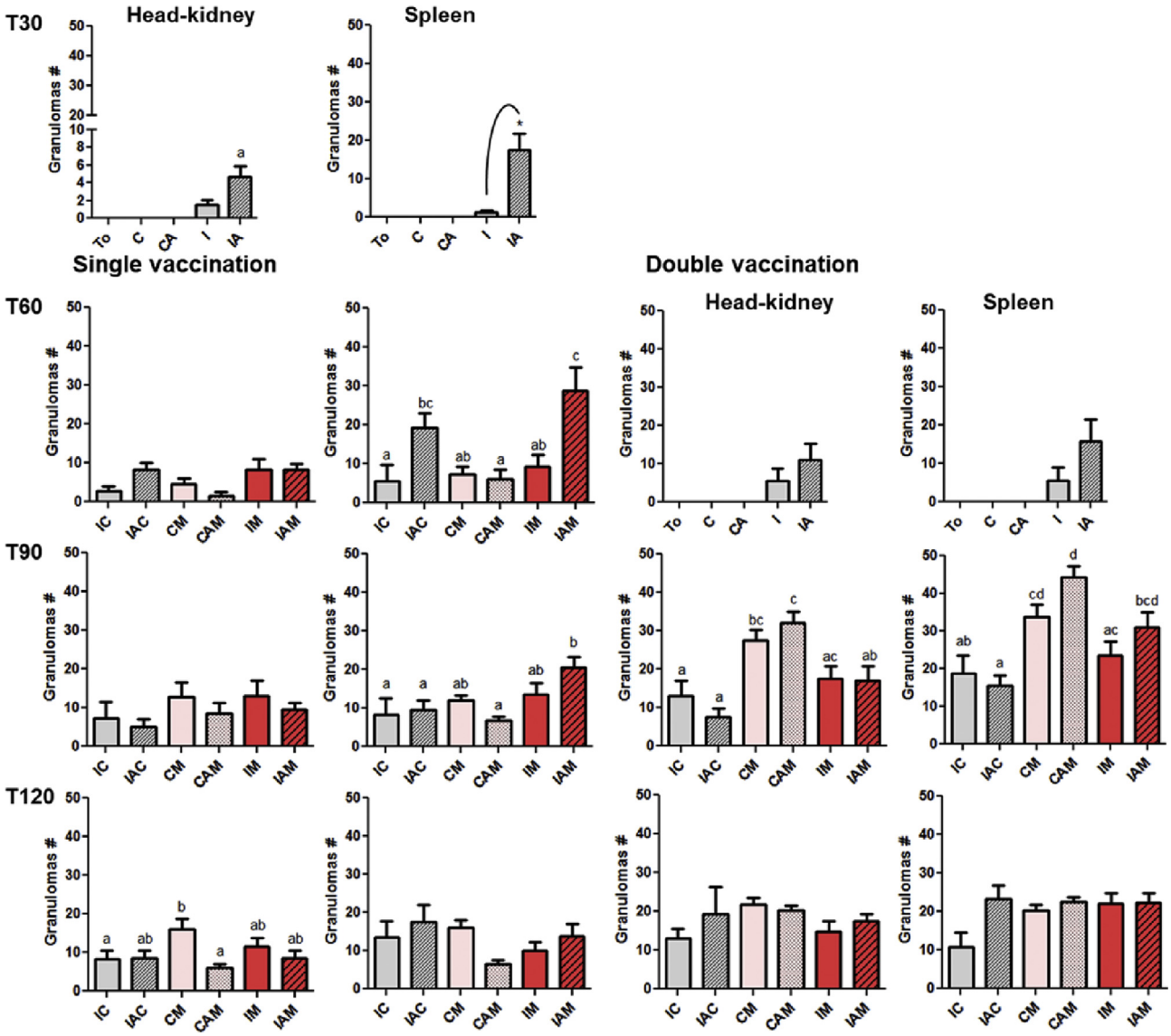

Fig. 5. Quantitative analysis of infection level by a numerical score based on the number of granulomas formed in the spleen and head-kidney. Granulomas counts were made from histological sections of 15 fish sacrificed from each group treatment. Counts were performed from the entire field area at x10 magnification during 30–120 days post-vaccination (T30-T120) of both vaccine regimes (single and double). T0 - before vaccination. Vaccinations were carried out using heat-killed *M. marinum iipA::kan* mutant with and without adjuvant (IA, I respectively) and sham-vaccinated control fish with saline with and without adjuvant (CA, C respectively). Challenges were carried out with live virulent Eilat WT *M. marinum* (IM, IAM, CM, CAM). Control fish were sham-challenged with saline with and without adjuvant (IC, IAC, CC, CAC). Different letters indicate significant differences ($P < 0.05$, one-way ANOVA and Tukey) from one another. The asterisk indicates significantly different means ($P < 0.05$, Student's t-test). ($n_{(fish/treatment)} = 15$).

In the first month post-challenge, a strong specific immune response against *M. marinum* was observed in the singly vaccinated fish as well as in the non-challenged fish injected with adjuvanted vaccine (IM, IAM, IAC) (Fig. 7, T60). Antibody titer remained high for two to three months (T90-T120) after challenge, in the fish vaccinated in combination with the adjuvant (Fig. 7, T60-120).

In the doubly vaccinated challenged fish, the highest antibody titer was observed in the group that has been vaccinated in combination with the adjuvant (IAM) for two to three months (T60-T120) after infection (Fig. 7, T60-T120). In addition, a high response though with lower antibodies titer, was observed in the challenged, non-vaccinated groups (CM, CAM) and the non-adjuvant vaccinated groups (IM).

In addition, antibody titer remained high during 60–120 dpv in the vaccinated non-infected fish combined with the adjuvant in both vaccine regimes (IAC) (Fig. 7). Unchallenged control fish maintained basal antibody expression throughout the whole experiment.

3.5. TNF-α mRNA expression

One month post-vaccination (T30), significant ($P < 0.05$) induction in expression level was observed in both spleen and head-kidney tissue of the fish vaccinated with the addition of adjuvant (IA) and also in the head-kidney of the fish vaccinated without the addition of adjuvant (I) (Fig. 8, T30). In the double vaccinated fish, a month after the second vaccination (T60), high expression level was observed only in the spleen of fish vaccinated with the addition of adjuvant (IA) (Fig. 8, T60).

In the singly vaccinated challenged fish, 1–3 months post-challenge (T60-T120), expression was not up-regulated only in the head-kidney and spleen of the control groups, with or without the addition of adjuvant (CC, CAC). The highest levels were observed 120 dpv in the sham-vaccinated challenged groups with or without the addition of adjuvant (CM, CAM) (Fig. 8, T120). In the spleen, expression levels were higher than in the head-kidney.

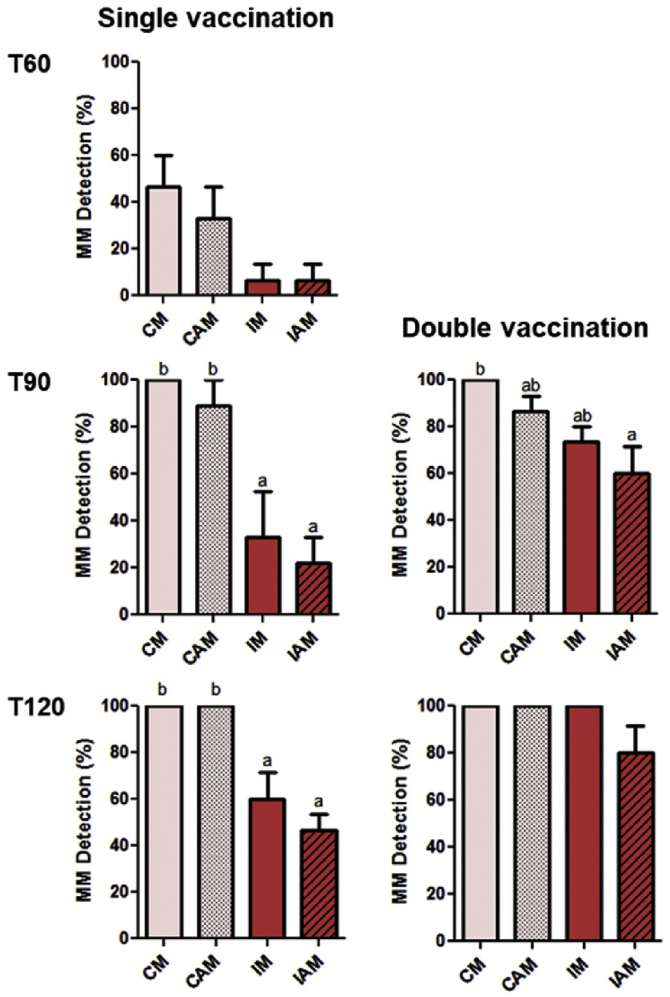

Fig. 6. Percentage of *M. marinun* diseased fish as highlighted by Ziehl-Neelsen stain.

In the doubly vaccinated challenged fish 1–2 months post-challenge (T90-T120), expression was up-regulated only in the head-kidney and spleen of the challenged groups (vaccinated and non-vaccinated, CM, CAM, IM, IAM) (Fig. 8, T90, T120). In the spleen, expression levels were higher than in the head-kidney. One month post-challenge (T90), the highest levels were observed in the non-vaccinated challenged groups with and without the addition of adjuvant (CM, CAM). Two month post-challenge (T120), the highest levels were observed in all the challenged groups with and without the addition of adjuvant (CM, CAM, IM, IAM) (Fig. 8, T120).

Vaccinations were carried out using heat-killed *M. marinum iipA::kan* mutant with and without adjuvant (IA, I respectively). Control fish were sham-challenged with saline with and without adjuvant (CA, C respectively) of both vaccine regime (single and double). Challenges were carried out with live virulent Eilat WT *M. marinum* (IM, IAM, CM, CAM). Control fish were sham-challenged with saline with and without adjuvant (IC, IAC, CC, CAC). Head-kidney and spleen tissues were collected randomly from 2 to 5 fish from each replicate (n = 3), for molecular analyses. T0 = before vaccination. T30 = 1 month after vaccination before challenge. T60 = single vaccine - 1 month after challenge, double vaccine – 1 month after second vaccination. T90 = single vaccine - 2 months after challenge, double vaccine – 1 month after challenge. T120 = single vaccine - 3 months after challenge, double vaccine – 2 months after challenge. Different letters indicate significant differences ($P < 0.05$, one-way ANOVA and Tukey) from one another ($n_{(fish/treatment)} = 9$).

4. Discussion

It has been previously shown that it is possible to obtain partial protection from mycobacterial infection in the European sea bass (*D. labrax*) immunized with a high dose of the heat-killed *M. marinum iipA::kan* mutant [21]. In the present study, we further analyzed the innate and adaptive immune responses in sea bass vaccinated with the same *M. marinum iipA::kan* mutant, with (and without) the use of a synthetic lipophilic polymer (ISA 760 VG SEPPIC) as adjuvant, with (and without) a booster treatment.

While no significant difference was recorded in the fish growth between immunized and non-immunized fish for the entire duration of the experiment, adhesion of the internal organs and moderate pigmentation were nevertheless observed in the perivisceral adipose tissue of nearly all vaccinated fish. On the other hand, the adjuvanted vaccine induced enhanced immune responses. TNF-α transcription levels were extremely high in spleen of the fish vaccinated with the addition of adjuvant (IA) in both singly and doubly vaccinated fish, followed by a highly specific IgM response for *M. marinum*. Also, granulomas (but with no apparent presence of bacteria) formed in fish vaccinated with the mutant (mainly when the vaccine was supplemented with the adjuvant). As a likely consequence, more granulomas appeared to have developed in the spleens of the challenged fish vaccinated with the addition of the adjuvant.

In the single vaccination trial, fish did not suffer major mortalities

Table 1
Percentage of *Mycobacterium* present in spleen tissues in challenged fish, using PCR analysis, followed by nested PCR (ns-PCR).

Single Vaccination						
	T60		T90		T120	
	PCR	ns-PCR	PCR	ns-PCR	PCR	ns-PCR
CM	0	44.44 ± 11.1	77.78 ± 22.2	88.89 ± 11.1	88.89 ± 11.1	100
CAM	0	44.44 ± 29.4	66.67 ± 19.2	100	88.89 ± 11.1	100
IM	0	33.33 ± 19.2	0	33.33 ± 19.2	66.67 ± 19.2	100
IAM	0	0	0	0	0	100
Double Vaccination						
			T90		**T120**	
			PCR	**ns-PCR**	**PCR**	**ns-PCR**
CM			88.89 ± 11.1	100	88.89	100
CAM			88.89 ± 11.1	100	88.89	100
IM			44.44 ± 22.2	100	77.78 ± 11.1	100
IAM			66.67	66.67 ± 19.2	66.67 ± 19.2	100

CM = control fish challenged; CAM = control fish with adjuvant challenged; IM = vaccinated fish challenged; IAM = vaccinated fish with adjuvant challenged. T60-T120 = days after first vaccination.

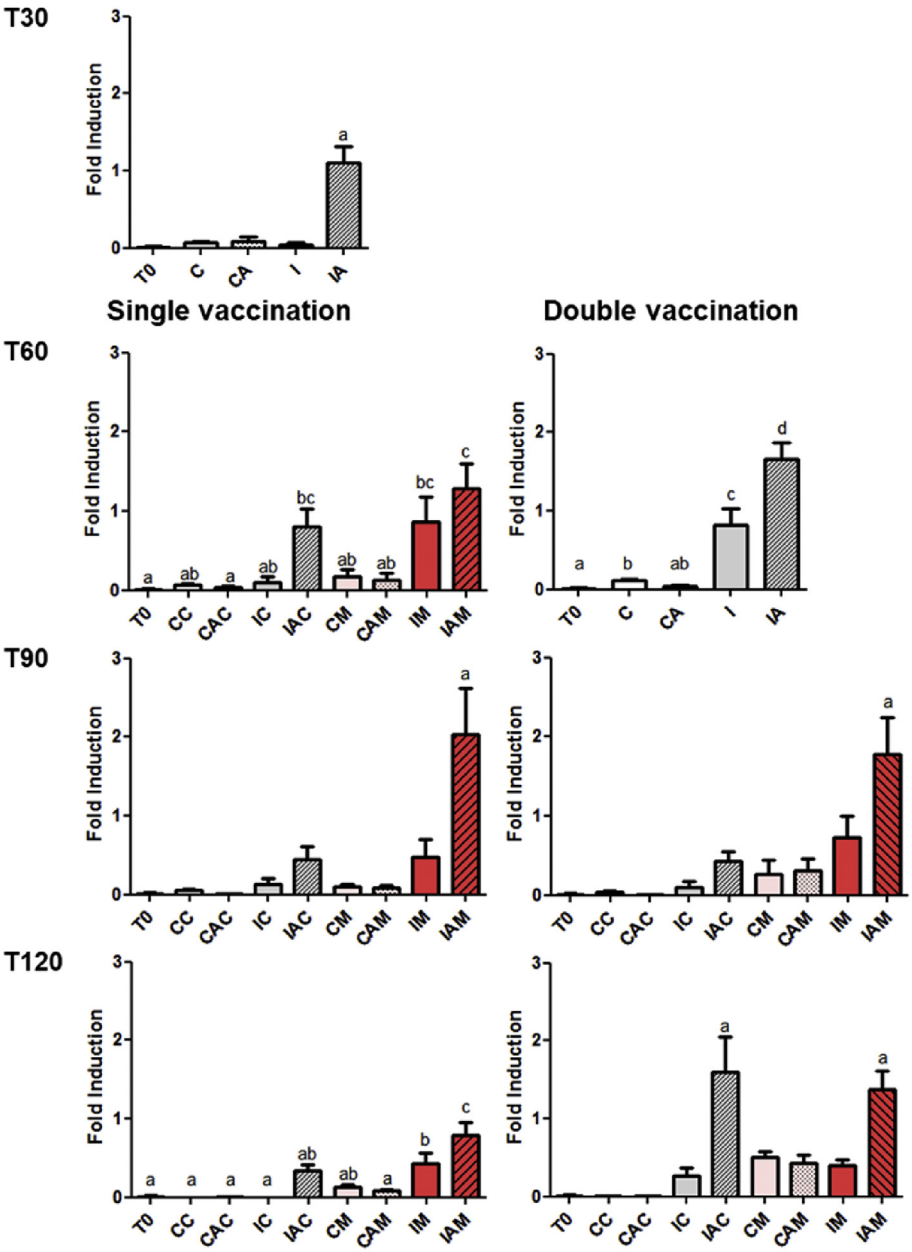

Fig. 7. Specific antibody response to *M. marinum* in fish serum.

whether the fish were challenged or left unchallenged. Conversely, in the double vaccination trial, in the aftermath of the challenge (120 dpc), non-vaccinated fish suffered up to 30% mortality (CM, CAM) compared to only 9–15% in the vaccinated ones (IM, IAM). These results could be explained by the possibility that cultures of WT mycobacteria closer to their stationary or even decline phase were used for the challenge in the single vaccination trial, whereas cultures of WT mycobacteria closer to their exponential phase of growth – and presumably at their peak of virulence - were used for the challenge in the double vaccination trial. Being *M. marinum* a slow grower, determining when its log phase ends and its stationary or even decline phase – and presumably reduced virulence – begins is tricky.

Histologically, granulomas started appearing in spleen and head-kidney tissues within a month after vaccination, mainly in the adjuvanted vaccine, indicating that even heat-killed bacteria were able to elicit granulomatous formations, although no bacteria were actually visible in these lesions.

The cell wall of *Mycobacterium tuberculosis* consists of a lipid layer enriched with lipopolysaccharide: lipoarabinomannan (LAM), mycolylarabinogalactan-peptidoglycan (mAGP) and the glycolipids trehalose-6, 6-dimycolate (TDM), glucose-6-monomycolate (GMM) and phthiocerol dimycocerosates (PDIM) [32]. TDM in particular induces granuloma formation because it is a very strong inducer of host innate immunity [33]. *M. marinum* is phylogenetically relatively close to *M. tuberculosis* [34]. The phenomenon of granulomas formed in response to the vaccination with heat-killed bacteria supports the assumption that the heating process in our work did not destroy the cell wall lipopolysaccharides and our vaccine stimulated the immune system. Heat-inactivated *Mycobacterium bovis* vaccine (*M. bovis* IV) administered by immersion protected zebrafish against mycobacteriosis caused by *M. marinum* by reducing mycobacterial infection, the number of mycobacteria per granuloma and the number of granulomas per fish. The protective mechanism elicited by *M. bovis* IV was based on the activation of the innate immune response way [35,36].

In addition, in the single vaccination, a lower number of infected granulomas formed in the vaccinated challenged fish during 120 dpc.

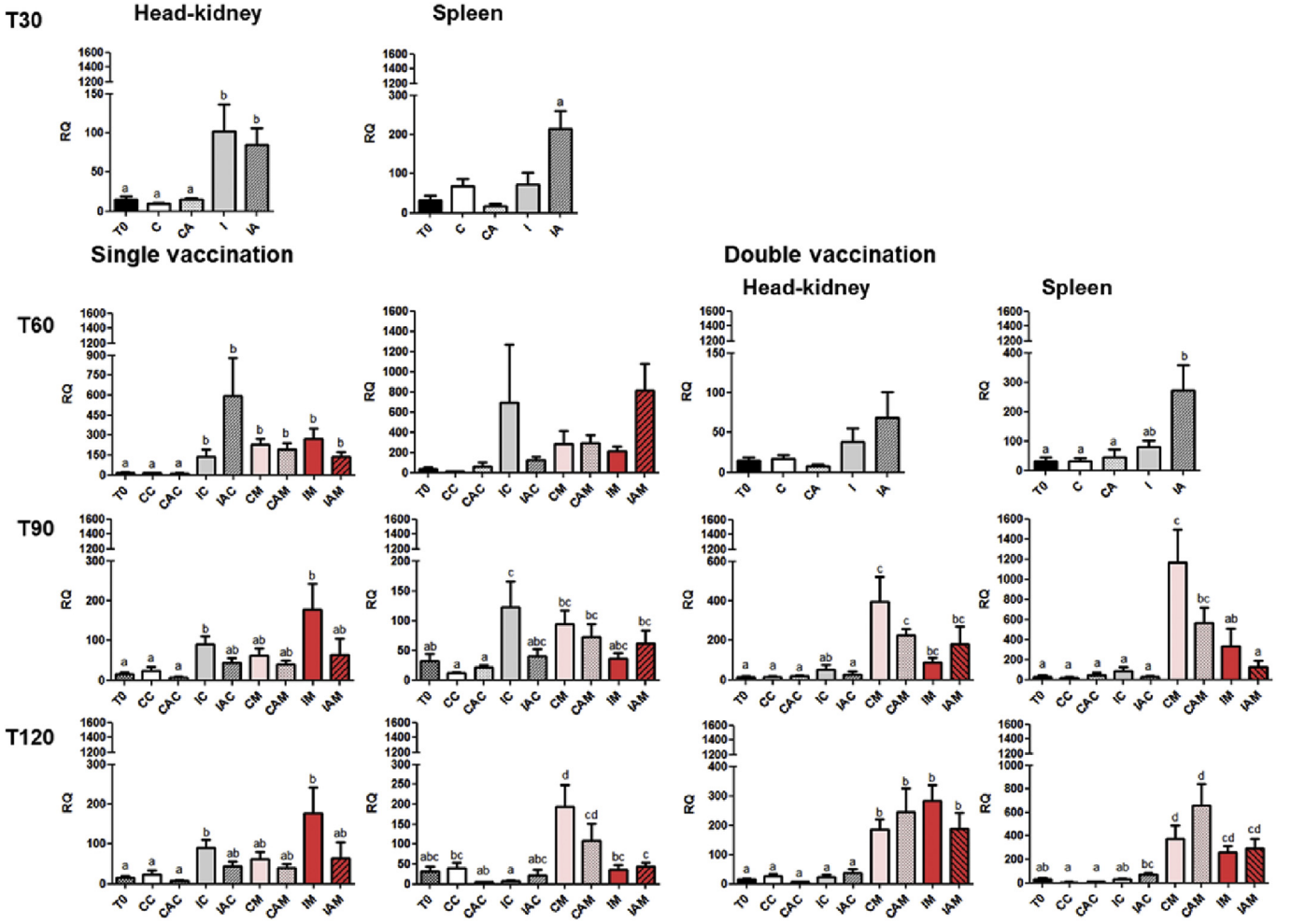

Fig. 8. TNF-α mRNA gene expression in head-kidney and spleen tissues.

These results correlate with the presence of mycobacteria in spleen tissues as detected by PCR and nested PCR. A lower percentage of mycobacteria was detected in the vaccinated challenged fish. Conversely, in the doubly vaccinated challenged fish, 90 dpc, the percentage of infected granulomas was much higher whether the fish were vaccinated or not. These results seem also to depend on the WT bacteria vitality, as theorized above. In addition, with the increase in the severity of the infection, there was nevertheless a slight decrease in the actual number of granulomas, which could be explained by assuming that the increased diameter of the infected granulomas brought about a decrease in their number measured per area.

Tumor necrosis factor (TNF) is one of the first effector molecules that was found to have a key role in the host protective response against tuberculosis in mice [37]. In humans, TNF is thought to exert protection by participating in the formation of granulomas, and loss of TNF signaling causes progression of tuberculosis [38]. TNF-α was proposed by Lam and collaborators (2011) [39] to be used as a biomarker to monitor the effectiveness of vaccination in fish. We found that one month post-vaccination before challenge, TNF-α expression levels were significantly higher in vaccinated fish. The highest expression was in spleens of fish vaccinated with the addition of the adjuvant (IA) in both fish vaccinated once and twice. After challenging, higher expression was also recorded in the unvaccinated fish that were exposed to the live virulent WT bacteria, mainly in the doubly vaccinated fish. Our results support the effectiveness of our vaccine as a stimulant to the fish immune system. TNF was demonstrated as essential for the maintenance of granuloma structures and for reducing bacterial growth within infected macrophages [40]. Bernut and collaborators (2016) [41] demonstrated the crucial role of TNF signaling in a continuum of effects in zebrafish embryos. TNF modulated the engagement of neutrophils to the infection site and their subsequent recruitment to granulomas, which are essential for control of the early and later stages of *Mycobacterium abscessus* infection, respectively. Also, up-regulation of TNFα was maintained in BCG vaccinated Japanese flounder (*Paralichthys olivaceus*) until 7 days post-vaccination, indicating that the host may have induced a cell-mediated immune response against *Mycobacterium bovis* [18]. Vaccination with attenuated or avirulent mycobacteria, including attenuated *M. marinum* and BCG as well as with selected DNA-encoding mycobacterial antigens, has offered zebrafish various degrees of protection from experimental mycobacteriosis [14,15,19].

Macrophages activated by inflammatory cytokines differentiate into epithelioid cells, and infected cells were separated off by formation of fibroblasts [42]. Granuloma formation by *M. marinum* is linked to virulence [43]. Mycobacteria virulence is associated with these bacteria's ability to reside within host cells and evade the microbicidal mechanisms of macrophages [44].

Fish vaccinated with adjuvant induced significantly high specific antibody response one and two months post-vaccination (in fish vaccinated once and twice, respectively), indicating a strong humoral immune response to the adjuvanted vaccine. Significant antibody response against *M. marinum* continued during 30–90 dpc in the single vaccination and 30–60 dpc in the double vaccination in all adjuvanted vaccinated challenged fish. Apparently, the live virulent bacteria alone were not sufficient to induce a strong humoral immune response. Only

vaccinated fish showed a high specific immune response against mycobacteria infection, which suggests that heat-killed mycobacteria indeed triggered a valid specific immune response against *M. marinum* and enhanced the fish immune-competence.

Our results are in agreement with those of Matsumoto et al. (2018) [45] who demonstrated that glycolipids isolated from a *Mycobacterium* sp. activated host innate and adaptive immunity, especially cell-mediated immunity (CMI), and may act as a CMI-inducible adjuvant. Yamasaki and collaborators (2015) [46] claim, however, that conventional inactivated vaccines, although inducing a humoral immunity in the host, are not effective against intracellular bacteria.

5. Conclusions

Although the booster treatments did not seem to have produced a significantly higher degree of protection from the disease compared to fish that received a single vaccination, we were able to demonstrate that the adjuvanted heat-killed avirulent *iipA::kan* mutant induced a strong humoral and adaptive immune response in European sea bass (*Dicentrarchus labrax*). The enhanced protective responses from mycobacteriosis caused by *M. marinum* was expressed by: reduction in fish mortality, reduction in mycobacterial infection as demonstrated by the lower number of granulomas with visible bacteria, and reduction in mycobacterial concentration as measured by PCR. The strong specific antibody response against *M. marinum* was accompanied by up-regulation of TNF-α and lasted as long as 4 months post-vaccination. Our results point to adjuvanted avirulent *M. marinum* mutants as the most appropriate model for the development of a protective vaccine against fish mycobacteriosis.

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