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Aluminum adjuvant potentiates gilthead seabream immune responses but induces toxicity in splenic melanomacrophage centers

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ABSTRACT

A key goal of a successful vaccine formulation is the strong induction of persistent protective immune responses without producing side-effects. Adjuvants have been proved to be successful in several species at inducing increased immune responses against poorly immunogenic antigens. Fish are not the exception and promising results of adjuvanted vaccine formulations in many species are needed. In this study, over a period of 300 days, we characterized the apparent damage and immune response in gilthead seabream immunized by intraperitoneal injection with the model antigen keyhole limpet hemocyanin (KLH) alone or formulated with Montanide ISA water-in-oil (761 or 763), or Imject™ aluminum hydroxide (aluminium), as adjuvants. Throughout the trial, external tissue damage was examined visually, but no change was observed. Internally, severe adhesions, increased fat tissue, and hepatomegaly were recorded, but, without impairing animal health. At 120 days post priming (dpp), histopathological evaluations of head-kidney, spleen and liver revealed the presence of altered melanomacrophage centers (MMC) in HK and spleen, but not in liver. Surprisingly, in all aluminium treated fish, classical stains unmasked a toxic effect on splenic-MMC, unequivocally characterized by a strong cell depletion. Furthermore, at 170 dpp transmission electron microscopy confirmed this data. Paradoxically, at the same time powerful immune responses were recorded in most vaccinated groups, including the aluminium treatment. Whatever the case, despite the observed adhesions and MMC depletion, fish physiology was not affected, and most side-effects were resolved after 300 dpp. Therefore, our data support adjuvant inclusion, but strongly suggest that use of aluminium must be further explored in detail before it might benefit the rational design of new vaccination strategies in aquaculture.

1. Introduction

The extensive use of vaccines on a wide range of species among vertebrates, including fish is recognized as the most effective prophylactic tool against specific diseases [1,2]. In any species, vaccine success relies on the ability of enhancing the immunological memory to respond with greater vigor towards a subsequent infection by the same antigen. To achieve the desired effect, a number of complex signals are required. However, in fish this is not a simple task, due most antigenic preparations contained in vaccines are weakly immunogenic after inactivation, requiring the addition of immunopotentiators. Among them, adjuvants are the choice required for the elicitation of immune responses that may be protective against certain pathogens [3]. Several synthetic and natural substances can be used as adjuvants to improve the efficacy of animal vaccines. Some adjuvants, like many oil based emulsions already have been used in licensed products, whereas others, like Toll-like receptor ligands or cytokines are still experimentally evaluated [4,5]. Whatever the case, several considerations in selecting adjuvants for a particular species are mandatory. Consideration highlights include a proven effectiveness and safety, induction of a long-lasting protective immunity, compliance of human food safety regulation, feasibility for scale-up production, and last but not the least, cost effectiveness. Therefore, finding the appropriate adjuvant or their combinations to meet the previously mentioned criteria is one of the major challenges in animal vaccine development [6].

Despite the significant effect recorded with oil-based adjuvants, still more information is required on the side-effects they produce and the short span of the immune response promoted; both elements are hampering the successful development of efficient animal vaccines [3,5]. Fish aquaculture is a growing industry, but it has many constrains yet,

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among them, fish health due infectious diseases is on the top of the list [7]. Therefore, promising fish vaccine formulations are on-demand in many species [8–10]. So far, effectiveness of many commercially available or experimental fish vaccines has been reported as weakly immunogenic, therefore improvement in the quality of such vaccines through adjuvant addition is recommended to achieve strong and long-lasting protection [8,11–13]. Along many decades, aluminium has been the first not emulsified adjuvant included in commercial vaccines to boost the immune response to specific antigens [14,15]. Paradoxically, the full mechanisms behind its immune stimulating properties are still under debate [16]. Although, there is a general agreement that aluminium adjuvants preferentially promote secretion of cytokines, which stimulates a humoral response directed towards bacteria and parasites [17]. In fish, vaccines containing aluminium have been tested as immune enhancers with variated immune outputs [18,19].

Herein, as part of a series of studies to better understand the potential-side effect of adjuvants in seabream vaccines, we first examined whether the fish health and immune responses were affected throughout a 300 days period. We examined this by injecting fish intraperitoneally (IP) with the model antigen keyhole lymphet hemocyanin (KLH) [20], formulated alone as positive control, or with each of the three selected adjuvants commercially available. Two water-in-oil emulsions (water droplets suspended in a continuous oil phase) and the extended used aluminium salt. Fish external appearance was not affected along trial due to any treatment, but, internal side-effects were recorded. Unexpectedly, during the course of these studies, we observed that melanomacrophage centers (MMC) in the spleen of aluminium treated fish were depleted of their characteristic cellular components. But, despite showing specific MMC damage, cellular and humoral immune responses in this group resulted not affected. Surprisingly, by the end of the trial most side-effects were reverted, and more important, MMC in the aluminium group replenished. Potential mechanisms of damage over the splenic MMC and the application of these findings are discussed.

2. Materials and methods

2.1. Ethics

This study was carried out in strict accordance with the recommendations in the Guide for Care and Use of Laboratory Animals of the European Union Council (86/609/EU). The protocol was approved by the Bioethical Committee of the University of Murcia (Spain). All surgery was performed under tricaine methane sulphonate (MS-222) anesthesia, and all efforts were made to minimize suffering.

2.2. Vaccine formulation and adjuvants

The model antigen Keyhole limpet hemocyanin (KLH) (Sigma-Aldrich, Germany) was injected by IP at 1 μg g^{-1} of fish [21], alone or mixed with each one of the following adjuvants. Aqueous solution of aluminium hydroxide (Imject™ Thermo Sci, USA) (Aluminium), 4 mg $fish^{-1}$ [21]; Water-in-oil emulsions Montanide™ ISA 761VG mineral oil (761) or 763AVG non-mineral oil (763) (Seppic, France), both used at 7 parts of adjuvant per 3 of KLH as aqueous phase following maker specific recommendations. Placebo group was IP provided with phosphate buffer saline (PBS) solution (Control), 100 μl $fish^{-1}$ (Fig. 1A). Italics in parenthesizes denote the name label, used along the trial for each group.

2.3. Animals and experimental design

Healthy gilthead seabream (*Sparus aurata*) juveniles were hatched and stocked at the Spanish Oceanographic Institute (Mazarrón, Murcia). Over the whole experimental period animals were reared in 2 m^3 running seawater aquaria following a natural photoperiod and fed twice daily commercial pellets (Skretting, Burgos, Spain) to satiation. One hundred fish (18.0 ± 2.0 g) in duplicated groups were acclimatized for 10 days prior to vaccination. Vaccination trial consisted on priming by IP 100 μl $fish^{-1}$ of each formulation described above on day 0 and boosted again by IP with the same 15 days post-priming (dpp). Thereafter, samplings for this trial were conducted on day 120, 170 and 300 dpp (Fig. 1B). Priming and booster doses were administered at a water temperature of 14 °C thereafter, temperature variations followed the classic year-round pattern observed at this Mediterranean costal area (Fig. 1C).

2.4. Tissue dissection

On selected days, 10 fish per group were instantly killed with an overdose of MS-222. Immediately after fish dying, head-kidney (HK), liver and spleen, were carefully collected and immersed in Bouin fixative for histological analysis, or in RNAlater (Sigma-Aldrich) and stored at −80 °C for further RNA extraction.

2.5. Serum and skin mucus collection

Ten fish per group were anesthetized with MS-222, and blood was collected with syringe, processed to obtain serum and stored as described [22]. With the aid of a cell-scrapper, seabream skin mucus was gently scraped from the skin surface, transferred into an Eppendorf tube, vigorously vortexed, and centrifuged at 400 × g for 10 min at 4 °C to remove fish cells. To separate skin bacteria from mucus, the cell-free supernatant was thereafter centrifuged at 10,000 × g for 10 min. The resulting supernatant containing the skin mucus was harvested, filtered with a 0.45-μm syringe filter (Millipore, USA), and stored at −80 °C [23].

2.6. Light microscopy and immunohistochemistry

Bouins-fixed tissue fragments from 10 fish per group were processed for routine paraffin axially embedding, sectioned at a thickness of 5 μm, deparaffinized following standard procedures and stained by hematoxilin and eosin technique [13]. This procedure enabled the analysis of liver, HK and spleen serial sections. Bright field histological images were taken on a Zeiss Axiolab (Carl Zeiss, Germany) with CoolSNAP image capture program (Roper Sci. Photometrics). For each specimen, at least five sections were analyzed. The coefficient of variation was < 10% among individuals.

2.7. Transmission electron microscopy

For transmission electron microscopy (TEM), spleen tissue samples of six fish per group were fixed for 1 h in 1.5% glutaraldehyde and 0.1 M sodium cacodylate buffer (pH 7.4), then treated with 1% osmium tetroxide, and embedded in Epon after dehydration. Thin sections stained with uranyl acetate and lead citrate were observed under a TECNAI 12 Phillips transmission electron microscope (TMS) at 80 kV.

2.8. Analysis of gene expression

Total RNA was extracted from tissues of ten fish per group with TRIzol Reagent (Invitrogen) following the manufacturer's instructions and treated with DNase I, amplification grade (1 U mg^{-1} RNA; Invitrogen). Quantity of extracted RNA was measured using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc). The SuperScript III RNase H_ reverse transcriptase (Invitrogen) was used to synthesize the first strand of cDNA with an oligo (dT)18 primer from 1 μg of total RNA at 50 ^{a}C for 50 min. Quantitative real-time PCR (qPCR) was performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR core reagents (Applied Biosystems). Reaction mixtures were incubated for 10 min at 95 °C, followed by 40

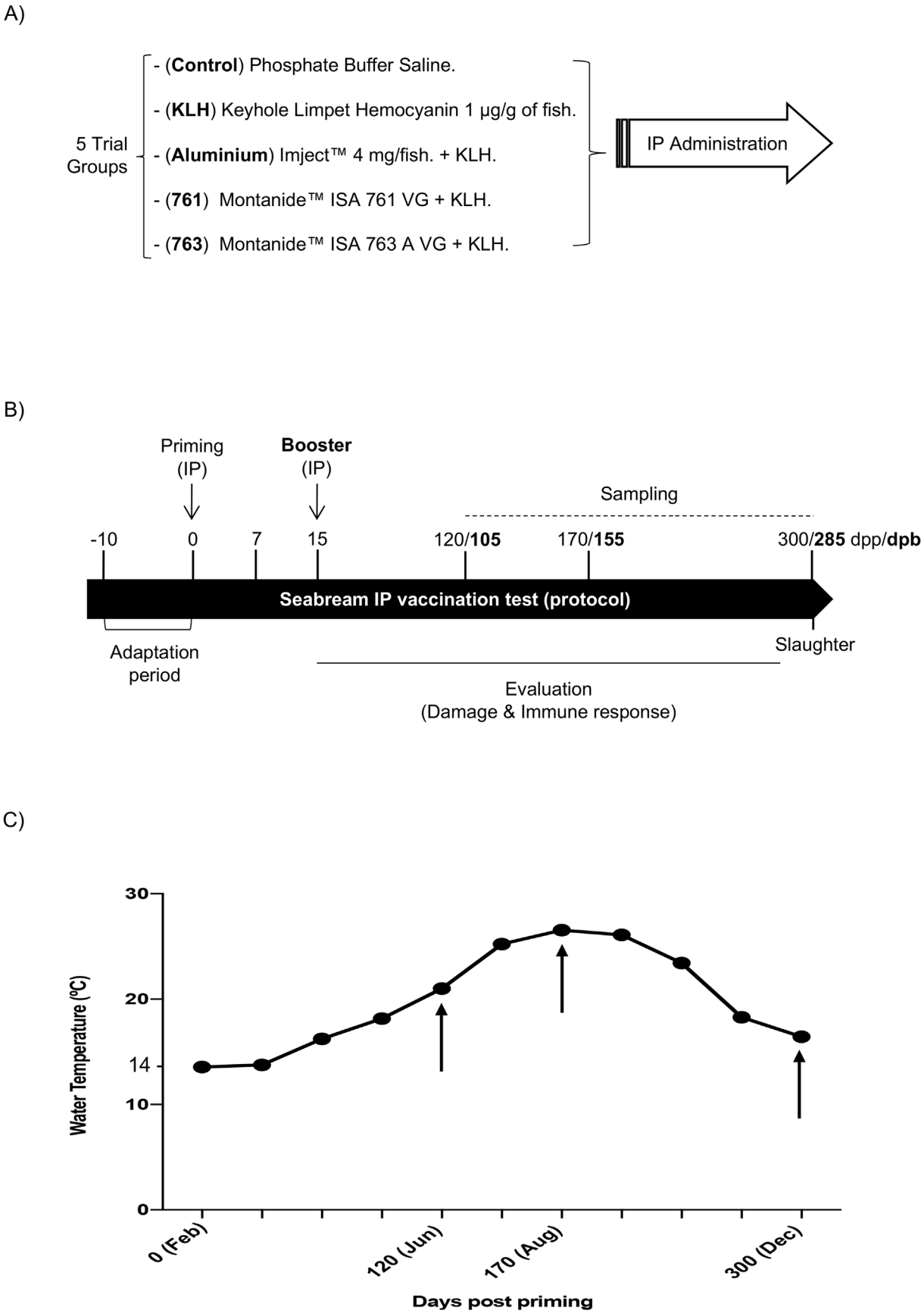


Fig. 1. Simplified experimental settings along the vaccination trial conducted with juvenile seabream. A) Trial groups, their abbreviations and amount used of each preparation administered by intra-peritoneal (IP) injection. B) Trial setup, fish followed an adaptation period of 10 days before receiving priming and booster administrations by IP on 0 and 15 dpp, respectively. Bold letters denote the days post booster (dpb) by IP. Biological samplings were conducted on days 120, 170 and 300 dpp. C) Monthly average temperatures (ºC) during the course of the experiment. Priming and booster doses were administered at 14 °C. Arrows indicates the three sampling points post priming vaccination.

Table 1
Primers used for expression analysis by Quantitative Real-Time PCR (qPCR).

Gene	Forward (5′ to 3′)	Reverse (5′ to 3′)	Accession Number
rps18	AGGGTGTTGGCAGACGTTAC	CTTCTGCCTGTTGAGGAACC	AM490061
il1b	GGGCTGAACAACAGCACTCTC	TTAACACTCTCCACCCTCCA	AJ277166
il10	TGGAGGGCTTTCCTGTCAGA	TGCTTCGTAGAAGTCTCGGATGT	JX976621.1

cycles of 15 s at 95 °C, 1 min at 60 °C, and finally 15 s at 95 °C, 1 min at 60 °C, and 15 s at 95 °C. For each mRNA, the gene expression was normalized to the ribosomal protein S18 content in each sample using the Pfaffl method [24]. The sequences of primers used, and the GeneBank accession number of the sequence the primers were designed against are listed for each gene in (Table 1). In all cases, each qPCR was performed with triplicate samples and was repeated with at least three independent samples.

2.9. Enzyme-linked immunosorbent assay (ELISA)

One hundred µl of serum or skin mucus samples from 10 fish per group, obtained at different time points were two-fold serially diluted and assessed with ELISA [13]. Briefly, polystyrene microtiter flat wells were sensitized and coated with KLH, to assess specific antibody production. Each assay run included a negative control, and a monoclonal antibody against seabream IgM (Aquatic Diagnostics Ltd.) which was further applied according to manufacturer instructions. Absorbance was read using a luminometer FLUOstar Optima (BGM, Lab Technologies) set at 450 nm.

2.10. Lysozyme activity assay

Serum and mucus lysozyme activity was carried out using ten fish per group [25]. Briefly, fifty microliters of undiluted serum were placed in triplicate into each well of a 96-well microtiter plate, and 150 µl of Micrococcus lysodeikticus (Sigma, USA) suspension in 0.1 M phosphate buffer (pH 6.2) was added to each well. After rapid mixing, the decrease in absorbance per min was recorded at 530 nm at room temperature.

2.11. Spleno-somatic index (SSI)

After 300 days of trial, remaining animals in each tank were sacrificed by means of an overdose of MS-222 to preserve fish welfare at the uttermost. Verified the death, each animal was wet-weighed in an electronic scale, and the full spleen was immediately isolated and carefully cleaned of debris. The spleno-somatic index (SSI) was calculated by the formula SSI = spleen weight (g) body weight $(g)^{-1} \times 100$ [26].

2.12. Statistical analysis

Statistics were performed with GraphPad Prism 7 software by one-way ANOVA and one of the following post hoc tests: unpaired Student t-test to determine differences between treated and control groups; or Tukey test to determine differences among groups in weight and length at 300 dpp.

3. Results and discussion

Vaccination is the most viable method to control infectious diseases. However, a big constrain is that most vaccines are usually not able to confer protection on their own, and generally need adjuvants of different types to boost their effectiveness [3,13]. In aquaculture facilities, fish are provided with vaccines by a variety of routes depending on their size, desired type of effect or antigen availability. Among them, parenteral administration typically produces the highest bioavailability of antigen because this method, in most cases avoids digestive processes and complex metabolic steps. However, substances parenterally injected into the peritoneum may induce strong reactions, producing subsequent side-effects that affect fish commercial qualities [27,28]. Thus, in this study we took the challenge to test the hypothesis that adjuvants are safe and ultimately required to improve seabream IP vaccines on inducing a strong expression of immunological memory upon a considerable time period without producing major side-effects, in order to provide the fish aquaculture industry with an invaluable knowledge on the risk/benefit associated to the use of this type of adjuvants in vaccines.

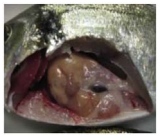
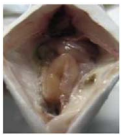
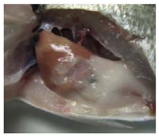
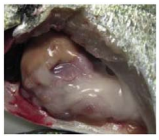
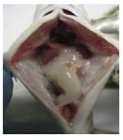
3.1. Vaccine safety on IP vaccinated seabream

Firstly, to determine possible changes on fish wellness (represented by external signs of disease, lack of appetite or natural mortality), juvenile seabream in the five groups, including controls were periodically monitored during the course of the trial after receiving priming and booster IP injections, following the setting described on (Fig. 1A). Since long ago, several types of substances have been employed as adjuvant systems in animal vaccines. In particular, use of oil based adjuvants has demonstrated effectiveness for disease control in aquaculture, but invariantly they produce side-effects [29]. Here, in an effort to identify and characterize such adverse reactions in seabream, we assessed the effect of two different water-in-oil emulsions and contrasted against the well-recognized adjuvant action of aluminium salt. Outcomes from the present study indicate that after continuous gross examination, the only obvious external lesion recorded was a slight tissue damage at the site of injection. This lesion was expressed as a transitory inflammation, without infection which did not last more than one week (Data not-shown). However, removal of fish due a major safety issue on any stock group was never required along the 300 dpp of experimentation. These results are in agreement with the observations of Noia et al. (2014), who reported good appetite, no mortality or signs of disease, but, observed some structural tissue change resulting from injecting fish in the wrong direction along a turbot trial vaccinated by IP with different adjuvanted preparations [30]. Therefore, our safety data at a glance suggest a good efficiency of all adjuvants tested, without inducing any external major side-effect along the trial.

3.2. Internal side-effects are produced by the adjuvanted IP vaccine injection in seabream

To assess whether internal side-effects exists in seabream after an extended period, at 120 dpp naked abdominal cavity gross macroscopic observations and detailed histopathological analyses of target organs were conducted. Gross observations determined by means of the newly provided intraperitoneal damage score for IP vaccinated seabream (Table 2), revealed that fish vaccinated with KLH, alone or in the presence of adjuvants had marked intra-abdominal lesions compared to the control group. Among them, high level of peritoneal adhesions between the liver, intestine or adipose tissue, and the abdominal wall were recorded. These results are consistent with previous studies where some internal lesions caused by oil-based adjuvants or aluminium, contained in vaccines parenterally administered have been observed [30–33]. However, seabream physiology contrasts with those observations, because granulomas or melanisation were seldom observed, but excessive

Table 2
Intraperitoneal damage score for IP vaccinated sea bream.

Damage	Score	Dorsal	Ventral
No visible changes in abdominal cavity	0		
Mild presence of connective tissue filaments surrounding the injection site. No tearing after detachment of filaments.	1		
Adhesions are clearly observed. Mild presence of fat tissue surrounding organs and/or the intestine.	2		
Adhesions firmly attached to the walls of abdominal cavity. Abundant fat tissue. Mild liver damage.	3		
Severe adhesions which may rip fillets when detached. Excessive fat tissue surrounding must organs. Organs fusion with severe lesions.	4		

adipose tissue was present in all vaccinated fish. Interestingly, the increase in adipose tissue has been recently proposed as a major contributor of vaccine side-effects in rainbow trout [10]. And, the exposure to potential toxic substances, like antigens or adjuvants has been observed to result in severe liver physiological problems, ultimately affecting disease resistance and may lead to fish death [34,35]. In this study, the liver in all KLH treated fish, with special emphasize on the aluminium batch, presented a compromised aspect which was characterized by hepatomegaly, pale color and swollen aspect when compared to control (Fig. 2A). This behavior was somewhat expected due to the liver dual essential function on mediating the blood supply from systemic and local immune regulation, while detoxify it from the action of exogenous material [36]. Perhaps, in our study the common toxic element was the T-cell-dependent antigen KLH, but, presence of both water-in-oil and aluminium adjuvants induced an obvious synergy potentiation. Therefore, these findings prompted us to conduct a detailed histopathological characterization of immune relevant tissues, namely liver, HK and spleen.

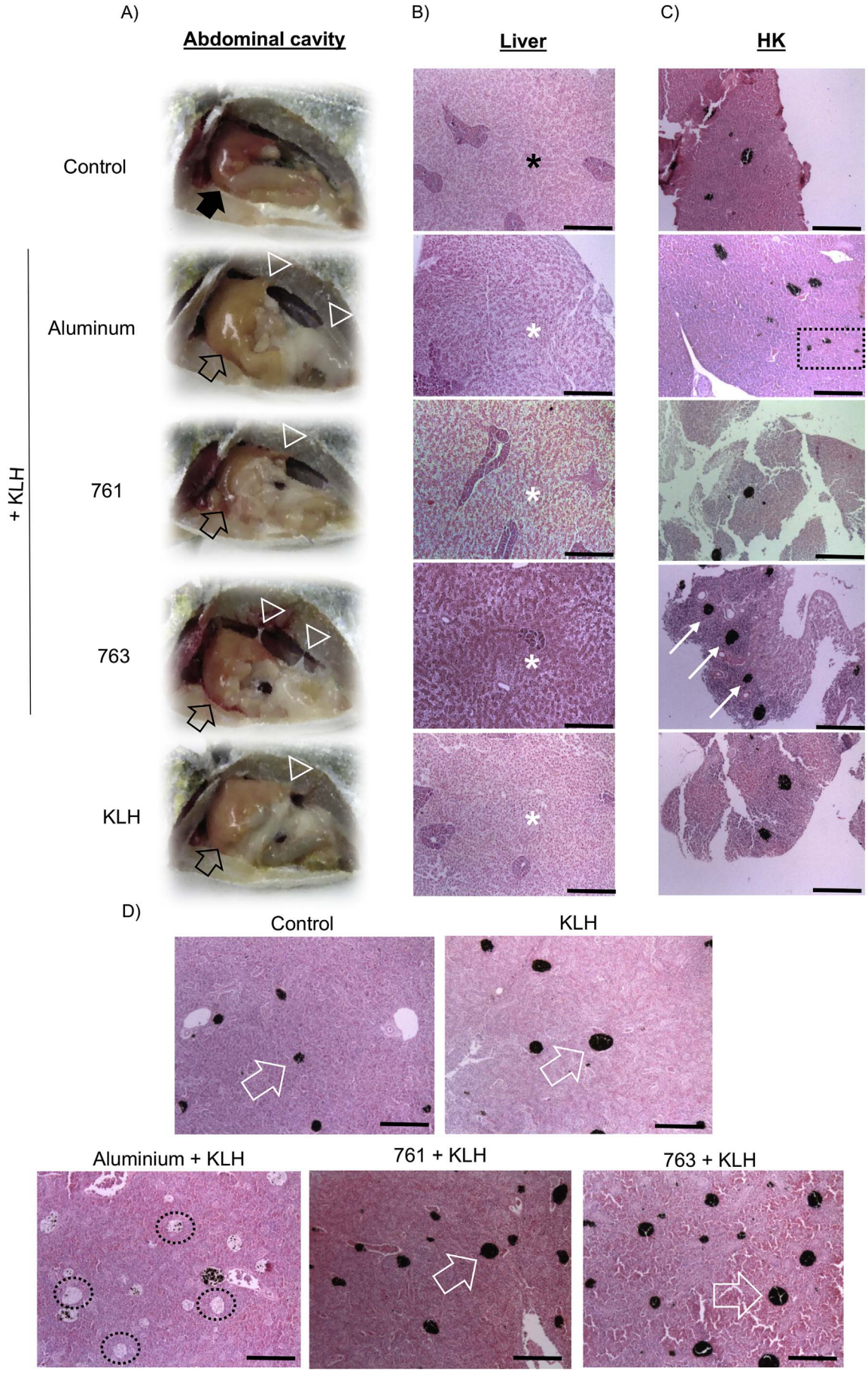
Histopathological examination, confirmed the hepatic damage previously recorded. Damage followed a consistent pattern, characterized by an altered parenchymal architecture and vacuolization of hepatocytes, compared to the control group. Therefore, indicating a fatty degeneration in the hepatocytes mainly associated with the periacinar zone of the liver, which is the primarily responsible for detoxification. Vacuolization was present in all KLH treatments, but much increased in the group where the mineral oil formula, Montanide™ ISA 761VG was applied (Fig. 2B). This was contrary to what expected, because most Montanide™ oil adjuvants administered by IP are generally considered to be well tolerated in several species [3]. Nevertheless, our data are in line with the recent report of Veenstra et al. (2017), who observed a gradual increase on damages, scored in rainbow trout according to the Spielberg scale, until day 53 after receiving *Aeromonas salmonicida* vaccine by IP, containing Montanide™ ISA 761VG [10]. But, the same did not happen on those trout receiving the 763 adjuvanted group which kept a low damage profile in the same period. Fish liver has been basically associated to metabolic functions. However, just recently it has been clearly put forward as a central immunological organ [37]. Due multiple hepatic lesions recorded, we next questioned if also primary hematopoietic organs may present affections hampering normal immune functions performance in seabream.

The tissues of primary hematopoietic organs, HK and spleen studied here in vaccinated seabream, roughly presented a similar conformation in the five treatments. But, in HK the major histopathology clearly observed was a shift in number, shape and size of MMC, with an increased response on the 763-treated fish, suggesting that the seabream extended immune response can be safely, selectively elicited and potentiated by IP injection of adjuvanted vaccine with non-mineral oil (Fig. 2C). These observations seem to be in agreement with previous data where different changes on MMC has been demonstrated following immunization or infection [38], stressful episodes [39], aging [40], and the active phagocytosis of heterogeneous materials, such as pollutants [41] or cell debris [42]. Their primary function in fish, like other macrophages is phagocytosis. But, contrary to melanocytes, melanomacophages lack of the classical melanin synthesis capacity. Especially, on relation to the nature of their dopa-oxidase, which has properties more akin to a peroxidase than a tyrosinase [42]. Thus, to obtain their pigmented granules, mainly they eliminate exhausted or senescent melanocytes-like cells [43], and erythrocytes [44], to further recycle the pigments they contain. However, the presence of these pigments varies between different species [45]. In our setting, seabream liver did not present MMC. Therefore, our results confirm previous reports where lack or very limited presence of MMC aggregates in the liver of several species are reported [19,45]. Interestingly, Zuasti et al. (1990) after a comparative study using three commercial fish species (*Sparus auratus*, *Mugil cephalus*, and *Dicertranchus labrax*), reported a scanty pigment accumulation in seabream liver due the presence of macrophages without cytoplasmic melanosomes [43]. In our study, splenic-MMC were conspicuously increased in shape and number in all treated groups. However, in the aluminium treatment additionally to the enhanced phenotype, we found a puzzling and unexpected issue, most of the splenic MMC were deployed from their conforming melanomacrophages (Fig. 2D). Several reports exist showing histochemical changes in the spleen of fish after vaccination or infection [46–48]. But, to the best of our knowledge, a side-effect like the one observed here, at the spleen of aluminium treated fish has never been reported before.

Among vertebrates, aluminium salts remain as the most widely used and effective adjuvant in vaccination and immunotherapy [5,49]. In fish, few studies exist on the action of aluminium on the same. But, contrary to what we have found so far, aluminium potential has been reported as immune enhancer in this phylogenetic group, without causing serious side-effects [19,30]. Although, a detailed understanding of aluminium action over extended periods has only started to be revealed. Whatever the case, our findings at this stage demonstrate that cells loaded with KLH, alone or together with selected adjuvants were migrating from the peritoneal cavity to key immune-related organs, and stress that adjuvants, particularly aluminium in fish produce strong internal changes, suggesting the possibility that they exert toxicity and systemic immune alterations among their side-effects.

3.3. Adjuvants effectively induce cellular and humoral immune responses in vaccinated seabream

In fish, there had been evidence that antigen retention is conducted at the ellipsoids capillary sheaths and adjacent MMC, which are populated by numerous melanomacrophages [38]. Several authors have noted that antigen, perhaps in complex with antibody are retained on or proximal to melanomacrophages, who present a phagocytic nature similar to that of the tingible body macrophages in mammals. Interestingly, Castro et al. provided concluding evidences of the striking diverse responses of B and T cell in the spleen of rainbow trout, where immunoglobulins show clonal expansion by expressing secreted isoforms



(caption on next page)

Fig. 2. Anatomical and histological overview of damages observed after 120 dpp in seabream vaccinated by IP injection. A) Naked abdominal cavity of fish (28.55 ± 2.1 g) showing adhesions and increased production of fat tissue (arrow heads), liver damage manifested as hepatomegaly and weak pigmentation in all treated fish (solid arrows) compared to control (clear arrow). (B, C) Histopathological changes at key organs, B) liver presented an altered parenchymal architecture and vacuolization of hepatocytes at different degrees in all treated groups (white asterisks), but not in control (black asterisk). Liver preparations stained with PAS. C) Head-kidney showing moderate diffusion (spotted-black rectangle) and expansion (arrows) of MMC in all treated groups. D) Major changes were observed in spleen. A high diffusion and proliferation of MMC is observed in all treated groups compared with control tissue (white arrows). Unexpectedly, in the aluminium vaccinated group MMC were depleted of melanocytes (spotted-black circles). HK and spleen preparations stained with H&E respectively. Scale bars equals 50 μm at 20× magnification.

[50]. However, an efficient affinity maturation of fish B cells is still not fully appreciated.

Next, we evaluated if the side-effects observed on splenic MMC were inducing any systemic immune related disorders at an extended time point. After 170 dpp, we analyzed by RT-qPCR the expression on HK, spleen and liver, of the genes encoding the two major pro- and anti-inflammatory cytokine markers, the IL-1β and IL-10 respectively. These two genes have an essential role as potent molecules of the innate immune system, but also act directly on T helper (Th) cells to assist certain adaptive immune responses of diverse immune cells, such as B cells and macrophages [51–53]. In this trial, similar responses were observed on both analyzed genes at lymphomyeloid organs. IL-1β activity, was significantly dominated in HK by KLH alone or followed by the two water-in-oil (761 & 763) adjuvanted treatments (Fig. 3A). In consonance with our results, Mutoloki et al. (2006) after an IP trial with two water-in-oil vaccines, reported that changes on pro-inflammatory genes expression in salmon HK are most likely linked to the severity of side-effects [54]. In our setting, we observed HK-MMC activation, but without strong side-effects. In the spleen, the significant IL-1β enhanced response was switched between the 763 and aluminium, with the former group evoking the strongest induction (Fig. 3C). However, IL-10 followed the same fashion in both organs, showing significant increases only in the aluminium and 761 adjuvanted groups, stressing that adjuvants function was still active (Fig. 3B–D). Therefore, our results are in agreement with other studies in fish reporting similar increased expression levels of this anti-inflammatory gene [55]. Note that in the liver, despite the observed damages, no significant changes were detected in the mRNA levels of the IL-1β (Fig. 3E). In contrast, IL-10 presented a marked inhibitory tendency in all treated groups compared with control, with the strongest significant effect recorded in the KLH group (Fig. 3F). Therefore, all damages in the liver described here, perhaps are due a restricted IL-10 response which contributed to an uncontrolled inflammatory process on the early days after immunization. Nevertheless, further studies are required to clarify this issue.

Next, in order to get insights on the humoral secreted immunity IgM titers and the lytic activity of lysozyme were assessed in serum and skin mucus. Despite the side-effects previously mentioned, IP vaccination using the KLH alone did not induce any change associated with higher IgM antibody levels neither in serum nor in skin mucus at 170 dpp. However, the three adjuvanted vaccines induced significant increased IgM antibody induction in serum (Fig. 4A). In contrast, skin mucus IgM levels at the same time point showed no significant change among control and any of the treated groups (Fig. 4B). These results reinforce the idea that most antigens require of adjuvants to boost their power. And, it is noteworthy that injection of KLH alone did not produce any change in serum or skin mucus antibody titer. This suggest that in seabream, the positive effect on potentiating the adaptive immune response, after an extended period of 170 dpp is directly associated to the presence and positive activity exerted by the two water-in-oil and aluminium adjuvants. Moreover, our results are in total agreement with recent observations by Jaafar et al. (2015) who reported a significant increase in IgM antibody titers when compared Ag alone or in presence of adjuvant 763, 90 days after vaccination of rainbow trout by IP [56].

The ability of adjuvants on potentiating the immune response depends in part on recruiting and activating myeloid cell functions. Therefore, we tested the activity of the antimicrobial enzyme lysozyme. Lysozyme activity in serum at 170 dpp showed significant differences between all immunized groups and the control. Among them, lysozyme activity was highest in the 761 group (Fig. 4C). In contrast, at the same time point, skin mucus lysozyme activity followed a non-significant fashion when compared to the control (Fig. 4D). These results are in contrast to what has been reported in Nile tilapia, where after 10 weeks of immunization with an experimental vaccine against *Aeromonas hydrophila*, serum lysozyme activity increased compared to control, but not following a significant fashion. Perhaps, this lack of activity would have been simply improved by the addition of any effective adjuvant in the basal vaccine formulation. Together, our results demonstrate that even though tested adjuvants induce internal major side-effects in seabream, they do not compromise the immune system capacities. Moreover, they induce a proper and high stimulation of B-cells leading to an increased production of IgM titers.

3.4. Histopathology and transmission electron microscopy at 170 dpp

Our histochemical analyses after 120 dpp revealed a strong depletion of splenic-MMC in the aluminium treated group. To extend such previous observations, we then visualized this specific side-effect at 170 dpp using light microscopy and TEM, respectively. Unexpectedly, routine staining revealed an outstanding finding, discrete melanocytes started to repopulate previously depleted MMC, suggesting a partial recovery of the affected structure in fish vaccinated in presence of aluminium (Fig. 5A). Several researchers have proposed that once melanomacrophages have accumulated some amount of biological extra- or intra-lysosomal “garbage”, formed due metal-catalyzed oxidation of autophagocyted or heterophagocyted material [57], they simply aggregate or move into pre-existing splenic MMC [41,58].

However, reverse migration of MMC following specific signals, or if their intimate structures were damaged at a high extent has been never investigated. Therefore, we next performed an ultrastructural analysis of splenic MMC. TEM observations revealed the presence of cells with empty cytoplasm, plenty cellular debris and loose melanin granules spread in the MMC of the vaccinated fish with aluminium (Fig. 5B). But, some cells inside the splenic MMC were starting to replenish their content. In contrast, any of these damages or behavior was observed on the positive (KLH) or negative (control) groups, whose morphology was mostly similar and clearly delimited by an intact membrane and the cytoplasm full of the usual spherical electron-dense granules conforming the splenic melanomacrophages (Fig. 5C). Our comparative microscopic analyses in seabream seem to give full support to the affirmation that damage recorded at the aluminium group was probably due a toxic effect by the presence of aluminium hydroxide which was provided on the vaccine formula administered by IP. Therefore, with the few evidences we got so far, we cannot determine the exact depletion mechanisms operating over the splenic melanomacrophages treated with aluminium. But, we speculate that an increased iron retention diminishing autophagic capacity, sensitize cells to oxidative stress or a high catabolic activity exerted by phagocyted erythrocytes, and resolve it as possible mechanisms associated with depletion.

3.5. Indicators of recovery at 300 dpp

Since microscopic analyses showed a partial recovery of splenic MMC damage in the aluminium treated fish after 170 dpp, we next investigated whether seabream have the capacity to naturally restore macro and micro damages after a long-time period without receiving any external treatment. Interestingly, most macro side-effects were

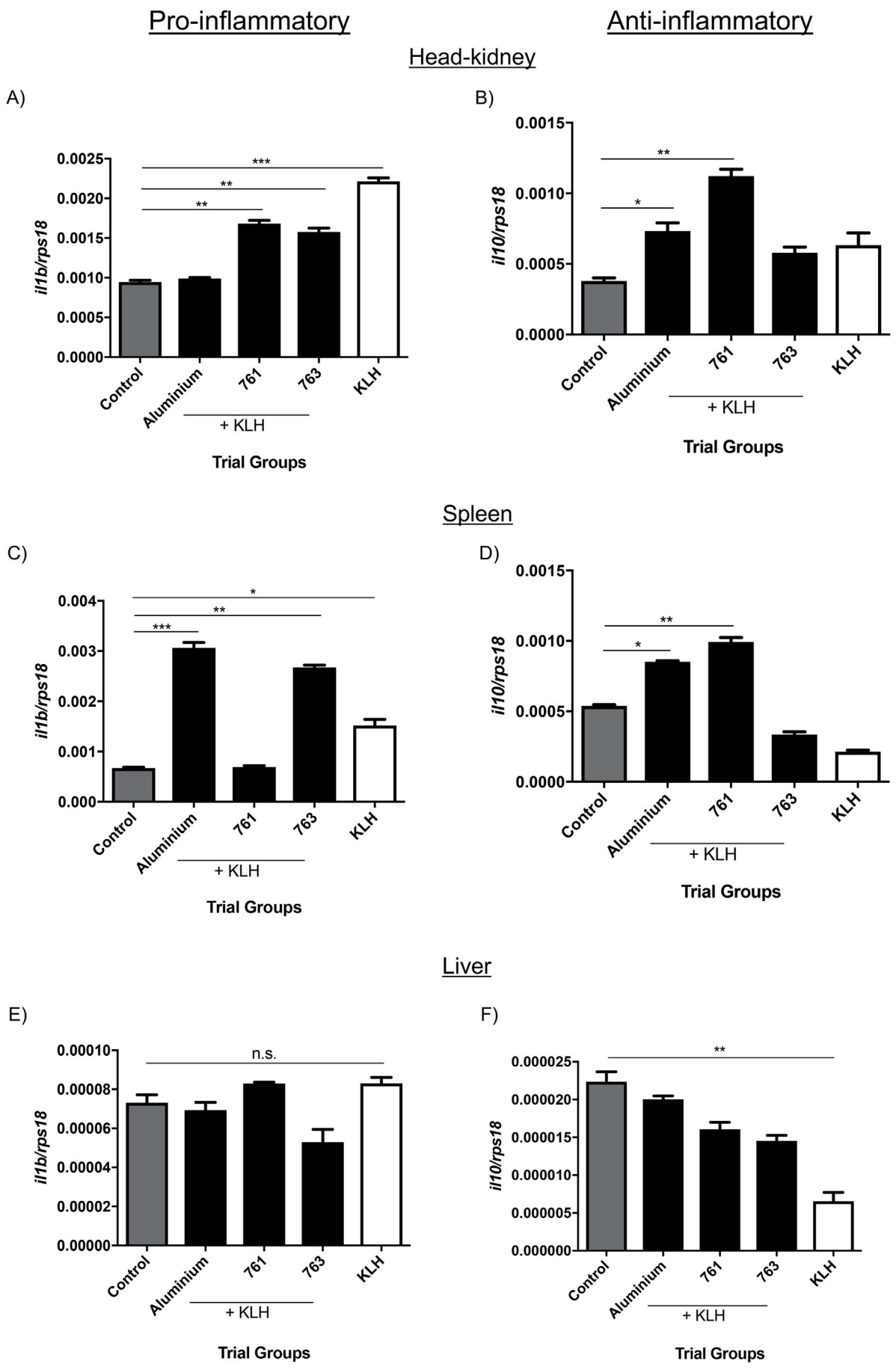


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Fig. 3. RT-qPCR of cell mediated immune marker genes in relevant tissues of vaccinated seabream after 170 dpp. Two marker genes, il1b (A, C, E) and il10 (B, D, F) representing the pro- and anti-inflammatory cytokine milieu release, respectively, in head-kidney, spleen and liver. The mRNA level of each cytokine gene was normalized to that of ribosomal protein rps18. Mean and SD (of 5 pools). ANOVA (effect of treatments): $P < 0.001$. Significant differences between control and each treatment were detected by Student's t-test $P < 0.05$ (n.s., not significant).

restored at a high degree. Among them, hepatomegaly and adhesions were not more highlighted between the aluminium treated and control groups (Fig. 6A). Histologically, KLH group display a similar morphology as the aluminium group, although reduced melanin is still evident (Fig. 6B). These data were encouraging, thus, to verify the overall fish condition we followed several approaches.

Splenosomatic index (SSI) was determined for each fish remaining in the five treatment tanks in duplicate after 300 dpp. Interestingly, we were not able to find a significant difference in SSI among groups (Fig. 6C). These results indicate that whether fish become affected by the action of aluminium, or the different vaccine formulations, the spleen has a strong homeostatic capacity, and following a long rest period, without any external stimuli they present a similar condition as the control.

Finally, we analyzed whether essential morphometric measurements (body length and weight) correlated with the observed internal recovery. Interestingly, from the six sampling records obtained for these two parameters, only a slight dispersion from the mean was recorded among the five groups at 300 dpp. Despite the observed dispersion, there was no significant difference between the three groups of fish treated with adjuvant preparations or both controls (Fig. 6D–E). Thus, together these data illustrate that an important aquaculture fish species

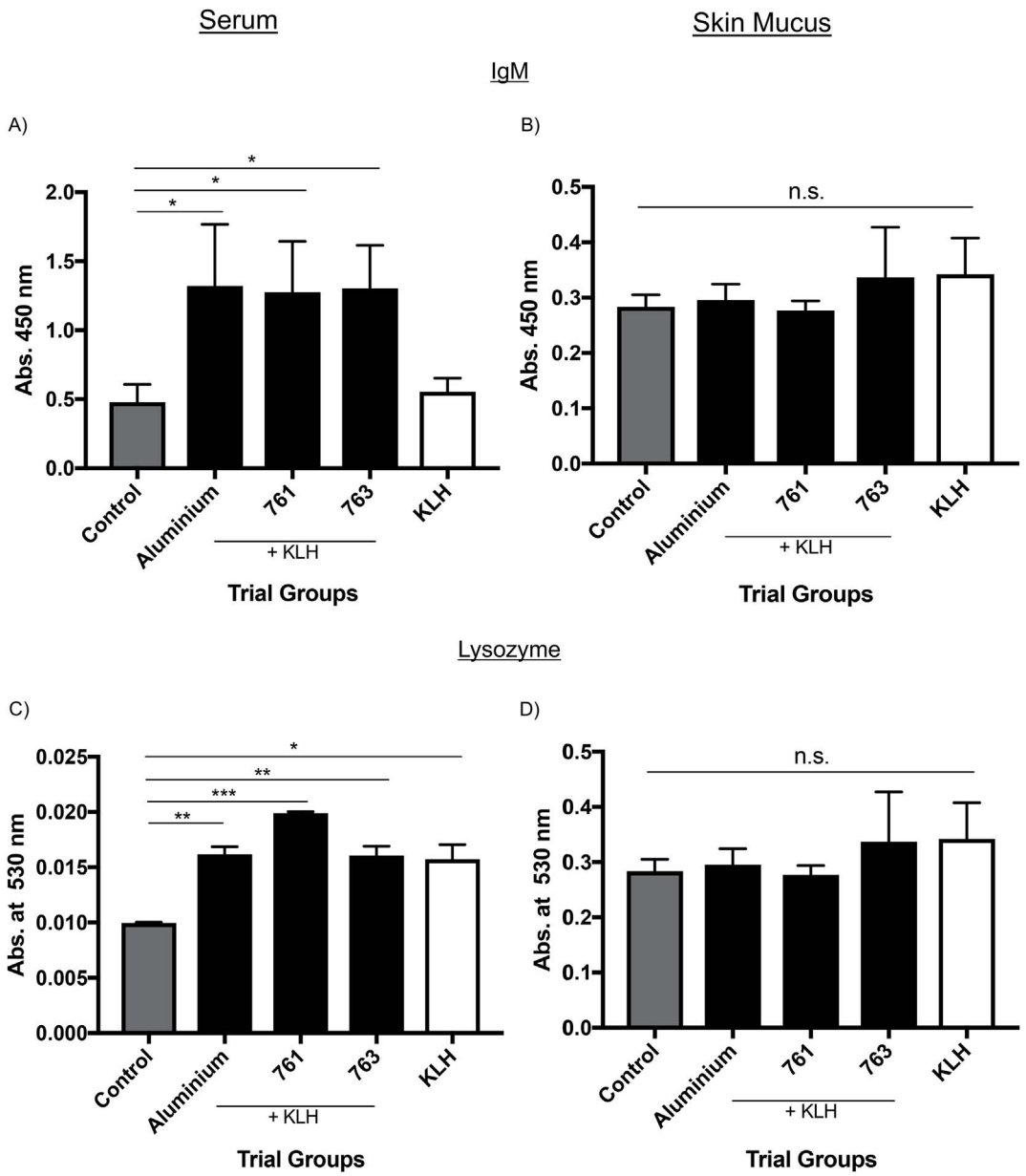

Fig. 4. Humoral immunity expressed as IgM titer and lysozyme activity in vaccinated seabream after 170 dpp. Specific IgM quantification in the five-experimental fish treated groups against KLH was conducted through indirect ELISA of anti-KLH in serum (A) and skin mucus (B). Panels showing the activity of lysozyme, in serum (C) and skin mucus (D) required to lysate live cells of Micrococcus lysodeikticus was quantified in triplicate as the decrease in absorbance per minute at 530 nm. Statistical differences between control and each of the different treatments were detected by Student's t-test $P < 0.05$ (n.s., not significant).

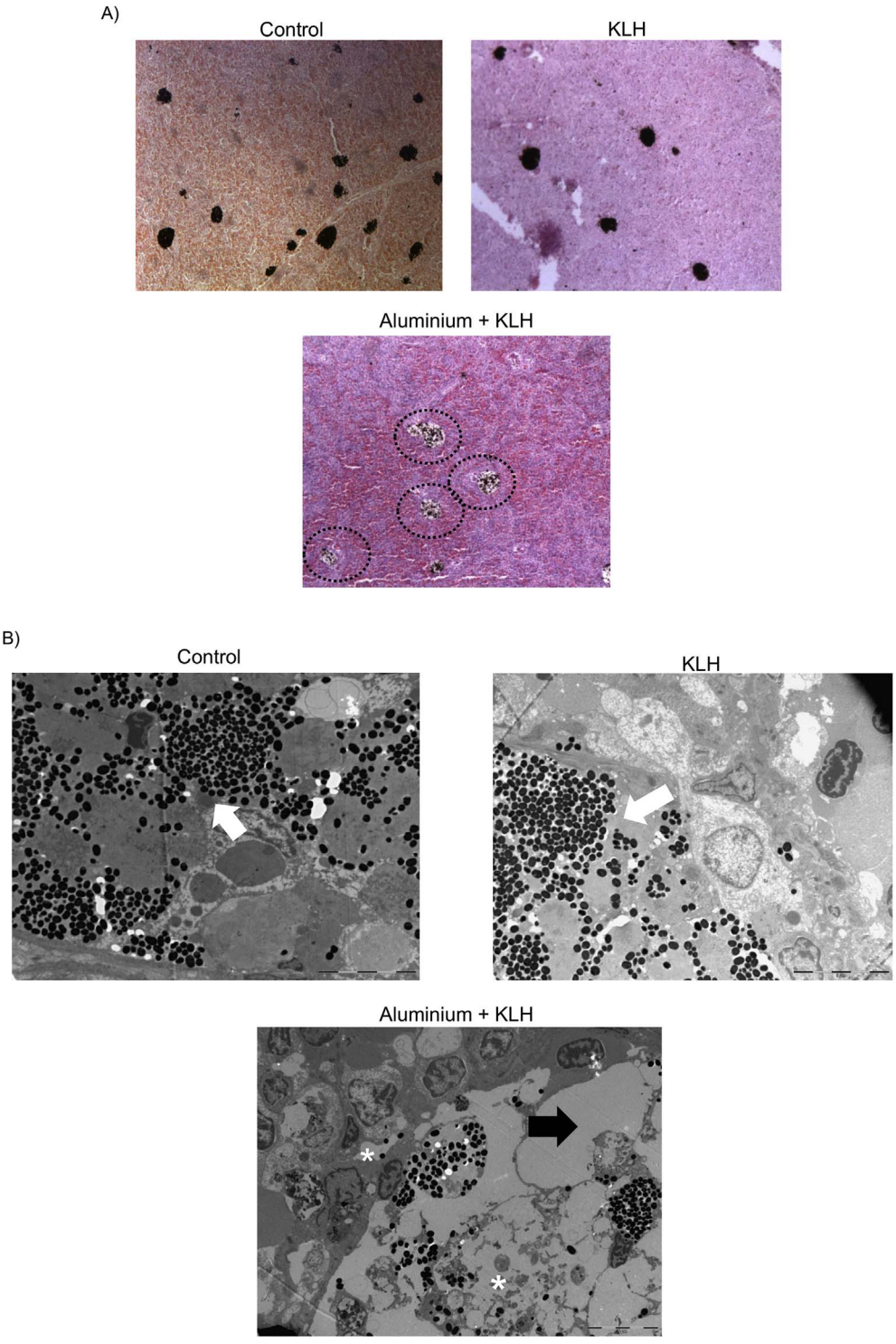

Fig. 5. Light and TEM analysis of MMC in seabream after 170 dpp of aluminium challenge. A) The histopathological phenotype observed previously in the splenic MMC of fish vaccinated with aluminium, after 170 dpp start to revert (spotted-black circles). Control and KLH serial sections were used as negative and positive controls respectively. All preparations stained with H &E. Scale bars equals 50 μm at 20× magnification. B) Representative TEM micrographs of MMC in the spleen of IP vaccinated fish with aluminium or the negative and positive controls after 170 dpp. The white arrows indicate cellular aggregates at the MMC in the control and KLH groups, characterized by the presence of mature cells of different shape and size, with the cytoplasm full of melanin granules and electron densities. However, the aluminium + KLH treated MMC presented cells with empty cytoplasm (black arrow), free melanin granules spread among dying cells of different types and a high amount of debris (asterisks). Resin-sectioned (100 nm sections), magnification X 80 K, scale bars equal 5 μm. Transmission electron microscopy (TEM).

like the seabream, efficiently can modulate their physiological functions to resolve the damage caused by the aluminium toxicity at a key lymphomyeloid tissue, namely the spleen.

4. Conclusion

Herein, we characterize the presence of side-effects, with particular attention on the phenotype of splenic MMC resulting from administering three different vaccine adjuvants by IP in seabream. We demonstrate that most fish, within the different groups examined, maintained their regular external phenotype and a normal behavior along the time. Also, our results prove that each adjuvant elicited at some extent cellular or humoral immune responses, acting as enhancer or facilitator accordingly to their origin. Even though they produce some internal side-effects, these do not compromise fish physiology or phenotype at the time of slaughter, suggesting that our findings are extremely relevant to a full fish culture cycle. An interesting observation from these studies is that fish treated with aluminium displayed a period of splenic MMC cell depletion, while the effect on the same in the remaining KLH-treated groups just observed a normal activated state. However, any of these phenotypes do not compromise fish immune integrity. Together, our results suggest that cell deploy effect in

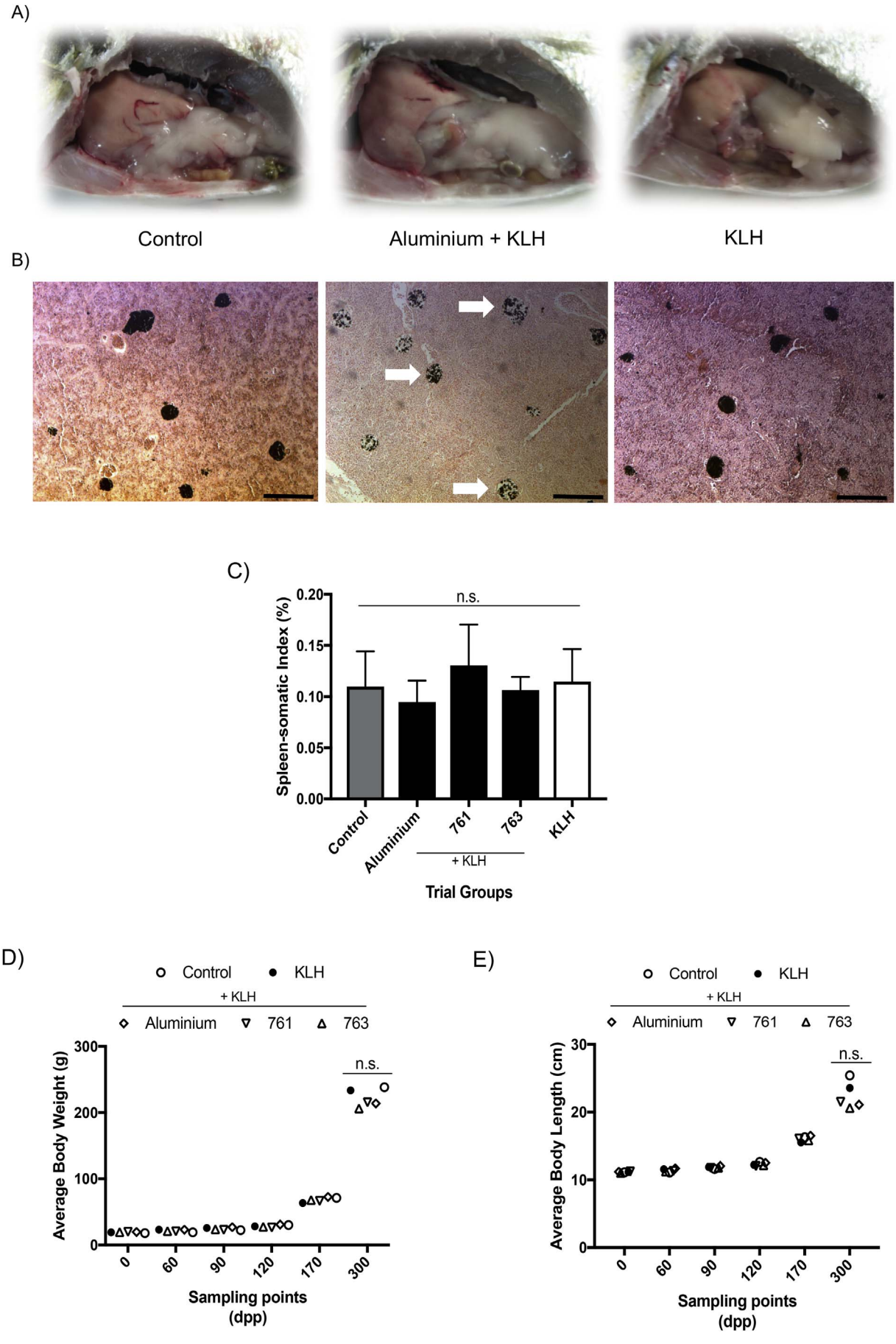

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Fig. 6. Evolution of external and internal damages of vaccinated seabream with aluminium after 300 dpp. A) Gross overview of the peritoneal cavity showing similar conditions with reminiscent abnormalities among the aluminium treated group and both controls. B) Histopathological analyses showing the phenotype rescue of MMC in the aluminium treated group (White arrows). MMC are replenished with melanomacrophages and resemble the normal condition observed in both control groups. All preparations stained with H&E. Scale bars equals 50 μm at 20× magnification. C) Spleen somatic index data by cross showing not significant (n.s.) differences among the 5 groups used in this study determined by ANOVA with Tukey's multiple comparison post hoc test ($p < 0.05$). D, E) Average body length and weight from remaining fish in each tank along the trial. Until 170 dpp growth was homogeneous among groups. But, at 300 dpp fish in the different groups start to spread from the mean but without causing any statistical difference among them.

the spleen of aluminium treated fish was strictly attributed to the presence of aluminium. Although, a number studies have demonstrated positive effects of this substance as seabream vaccine adjuvant without major side-effects [21,59], to the best of our knowledge, this is the first report correlating a physical trait due the use of aluminium hydroxide as vaccine adjuvant in a teleost fish, but without compromising a normal antibody production.

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