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Eakapol Wangkahart: Conceptualization, methodology, formal analysis, investigation, resources, data curation, writing-original draft preparation, visualization, project administration, funding acquisition. **Areerat Thongsrisuk**: methodology, formal analysis. **Regis Vialle**: Conceptualization, project administration, funding acquisition. **Sirinya Pholchamat**: methodology, formal analysis. **Phitcharat Sunthamala**: methodology, formal analysis. **Janjira Phudkliang**: methodology, formal analysis. **Prapansak Srisapoome**: writing-review and editing, Advice. **Tiehui Wang**: writing-review and editing, Advice. **Christopher J. Secombes**: writing-review and editing, Advice. All authors have agreed to the published version of this manuscript.

Comparative study of the effects of Montanide™ ISA 763A VG and ISA 763B VG adjuvants on the immune response against *Streptococcus agalactiae* in Nile tilapia (*Oreochromis niloticus*)

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Keywords: Immune Response; Adjuvants; Montanide™ ISA 763A VG; Montanide™ ISA 763B VG; Inactivated Vaccine; Nile tilapia

Abstract

Streptococcus agalactiae is regarded as a major bacterial pathogen of farmed fish, with outbreaks in Nile tilapia causing significant losses. Vaccination is considered the most suitable method for disease control in aquaculture, with the potential to prevent such outbreaks if highly efficacious vaccines are available for use. Several vaccines have been produced to protect against *S. agalactiae* infection in tilapia, including inactivated vaccines, live attenuated vaccines, and subunit vaccines, with variable levels of protection seen. Two commercial adjuvants, Montanide™ ISA 763A VG and ISA 763B VG, have been developed recently and designed to improve the safety and efficacy of oil-based emulsions delivered by intraperitoneal injection. In particular, their mode of action may help identify and stimulate particular immunological pathways linked to the intended protective response, which is an important tool for future vaccine development. Therefore, this study aimed to characterize the potential of two adjuvanted-bacterial vaccines against *S. agalactiae* (SAIV) comparatively, to determine their usefulness for improving protection and to analyse the immune mechanisms involved. Nile tilapia were divided into four groups: 1) fish injected with PBS as a control, 2) fish injected with the SAIV alone, 3) fish injected with the SAIV+Montanide™ ISA 763A VG, and 4) fish injected with the SAIV+Montanide™ ISA 763B VG. Following immunisation selected innate immune parameters were analysed, including serum lysozyme, myeloperoxidase, and bactericidal activity, with significantly increased levels seen after immunization. Cytokines associated with innate and adaptive immunity were also studied, with expression levels of several genes showing significant up-regulation, indicating good induction of cell-mediated immune responses. Additionally, the specific IgM antibody response against *S. agalactiae* was determined and found to be significantly induced post-vaccination, with higher levels seen in the presence of the adjuvants. In comparison to the protection seen with the unadjuvanted vaccine (61.29% RPS), both Montanide™ ISA 763A VG and Montanide™ ISA 763B VG improved the RPS, to 77.42% and 74.19% respectively. In conclusion, Montanide™ ISA 763A VG and Montanide™ ISA 763B VG have shown potential for use as adjuvants for fish vaccines against streptococcosis, as evidenced by the enhanced immunoprotection seen when given in combination with the SAIV vaccine employed in this study.

Keywords: Immune Response; Adjuvants; Montanide™ ISA 763A VG; Montanide™ ISA 763B VG; Inactivated Vaccine; Nile tilapia

1. Introduction

The global aquaculture industry has suffered significant losses as a result of infectious diseases, which has led to mass mortality in many farmed fish species and concomitant extensive economic losses to the industry [1,2]. *Streptococcus agalactiae* is a common pathogenic bacterium and the principal cause of disease in the aquaculture of Nile tilapia, *Oreochromis niloticus* in Thailand and worldwide [3]. To combat this pathogen, chemical treatments and antibiotics are currently used extensively. However, this has led to a number of issues, related to an increase in bacterial drug resistance, environmental pollution, and food safety [4]. Therefore, the establishment of immunoprophylactic treatments is crucial in order to reduce the usage of antimicrobials as well as reducing mortality. Controlling and preventing the outbreak of fish diseases by vaccination is a successful and environmentally friendly strategy [5,6], and is considered the most practical, safe and cost-effective option [7].

Numerous vaccines have been developed and shown to have great promise against *S. agalactiae* in fish, such as DNA vaccines, inactivated vaccines, subunit vaccines, live attenuated vaccines and a vaccine based on bacterial ghosts [8-14]. For example, a live attenuated injection vaccine was shown to achieve a high Relative Percent Survival (RPS) in tilapia [13], but is difficult to register in all countries due to perceived safety issues. Inactivated vaccines are more conventional and acceptable, and can be delivered by a variety of procedures, such as intraperitoneal (i.p.) injection, immersion or oral vaccination, with injection vaccination known to induce effective and long-lasting immune responses that protect fish against infections [15]. However, it is typically a requirement to combine these killed bacterial suspensions with an adjuvant in order to boost immunogenicity and the persistence of the immune response. These types of vaccines are already widely reported and/or available for use against various fish bacterial pathogens, such as *Yersinia ruckeri* [16], *Vibrio anguillarum* [17], *Aeromonas salmonicida* and *A. sobria* [18], *A. hydrophila* [14], *Flavobacterium psychrophilum* [19], *Edwardsiella tarda* [20], and *S. agalactiae* [21]. In the latter case, a hydrogen peroxide-inactivated (i.p.) vaccine induced modest protection in Nile tilapia (40.7% RPS) but this could be increased by inclusion of aluminium hydroxide or incomplete Freund's adjuvant (IFA) to 59.3% and 77.8% RPS respectively. Whilst such data are encouraging, other adjuvants need to be explored for use in such vaccines.

Adjuvants are used to improve the effectiveness of (fish) vaccines by strengthening and prolonging specific immune responses to antigens [22]. They may also reduce the number of doses (such as boosters) required to be given. Montanide™ adjuvants, developed by SEPPIC, are vaccine adjuvants used in animal vaccines with excellent immune performance, and can significantly improve the immune response to a given vaccine [23]. Two of these commercial adjuvants for injection vaccination in fish are Montanide™ ISA 763A VG and Montanide™ ISA 763B VG. Both are non-mineral oil-based and developed for use in water in oil (w/o) emulsions. They are compatible with inactivated antigens and help promote the immune response, increasing vaccine effectiveness and safety. When compared to IFA, the Montanide™ ISA adjuvants produce stable, low-viscosity emulsions [17]. There have been numerous studies using Montanide™ ISA 763A VG as adjuvant in fish vaccines, and these studies consistently show high vaccine efficiency, as seen in rainbow trout, *Oncorhynchus mykiss* [24-26], turbot, *Scophthalmus maximus* [17,27], Nile tilapia (against francisellosis) [28], gilthead seabream, *Sparus aurata* [29], giant grouper, *Epinephelus lanceolatus* [30], largemouth bass, *Micropterus salmoides* [31] and gibel carp, *Carassius auratus gibelio* [32]. In contrast, the application of Montanide™ ISA 763B VG has been reported only once previously in Nile tilapia, in an *S. agalactiae* bacterial ghost vaccine. The adjuvanted vaccine gave an RPS of 80.8%, vs 73.1% in fish given the unadjuvanted vaccine, and significantly greater levels of specific antibodies compared to fish immunized without adjuvant [22].

Since Montanide™ ISA 763B VG has still to be trialed in a more conventional inactivated (formalin-killed) *S. agalactiae* vaccine, a type of vaccine which is relatively simple and easy to produce, the present study was carried out to assess the immune responses and protection seen following i.p. vaccination of Nile tilapia with this vaccine combination. In addition, a group of fish given an ISA 763A VG adjuvanted vaccine was included, to allow comparison of the relative performance of ISA 763B VG to this relatively well studied adjuvant for fish vaccines. Both of the adjuvanted vaccines were also compared to the responses seen in fish given an unadjuvanted *S. agalactiae* bacterin, as well as to saline injected control fish. Densities of immune cells by immunohistochemical staining of spleen sections from *S. agalactiae* vaccinated fish were also studied. Moreover, analysis of side-effects and safety was included for completeness.

2. Materials and Methods

2.1 Experimental fish and rearing management

Nile tilapia weighing approximately 100 g were purchased from a private commercial fish farm located in Roi Et province, Thailand. Prior to the experiment, fish were acclimatized for 2 weeks in a cement tank ($7 \times 10 \times 1.5$ m^3) at the Division of Fisheries, Mahasarakham University [33]. Fish were fed with a commercial feed containing 32% protein and 4% lipid (Charoen Pokphand Foods; CPF, Thailand) twice per day. Water quality parameters were: water temperature 27±1°C, pH 7.8±0.1, ammonia nitrogen <0.03 mg/L, and dissolved oxygen 6.5±0.3 mg/L. To confirm that fish were not infected by *S. agalactiae*, five were chosen at random for bacteriological testing and verified negative.

2.2 Bacterial strain and vaccine preparation

For inactivated vaccine preparation, *S. agalactiae* was isolated from the spleen and kidney of diseased Nile tilapia in the northeastern region of Thailand [22]. *S. agalactiae* were inoculated into Brain Heart Infusion (BHI) Broth with shaking at 180 rpm at 30°C for 12 h. After that, the bacterial culture was inactivated by adding 2% formalin solution (v/v) at 4°C for 48 h, and the death of bacteria determined by the absence of growth on BHI agar plates after 48 h of incubation at 30°C. The inactivated cells were centrifuged (5,000 rpm for 10 min at 4 °C), and washed 3 times with phosphate-buffered saline (PBS). The inactivated cells were resuspended in PBS and adjusted to a final concentration of 1×10^9 colony forming units (CFU)/mL, using a spectrophotometer at 600 nm [34].

2.3 Adjuvants and vaccine formulation

The *S. agalactiae* inactivated whole-cell vaccine (SAIV) was mixed with the non-mineral oil Montanide^{TM} ISA 763A VG or ISA 763B VG according to the method in Wangkahart et al. [22]. In brief, the two oil-adjuvanted vaccines were prepared at a ratio (v/v) of 73:27 of adjuvant and SAIV using a T25 easy clean digital high shear mixer (IKA, Germany). A similar procedure was used to prepare the SAIV alone, which was diluted in PBS at a ratio of 27:73 (v/v). As a result, each vaccine prepared contained the same quantity of SAIV at 1×10^8 cells/mL.

2.4 Vaccines adjuvant safety test

Potential acute toxicity of the adjuvants was tested in Nile tilapia. Ten fish per group were injected intraperitoneally (i.p.) with 200 μL of each adjuvanted vaccine and monitored for 14 days post injection to determine whether any acute side effects were seen *in vivo* caused by these vaccines. Prior to injection, fish were anesthetized with 0.5% 2-phenoxyethanol (Sigma, UK). Feeding, body color and abnormalities, lesions near the injection site, and mortality were recorded. Remaining fish were killed at 5 weeks post vaccination (w.p.v.) and necropsied to check (macroscopically) for symptoms of long-lasting side effects such as internal lesions, adhesions or vaccine residues in the peritoneal cavity [35].

2.5 Fish immunization and blood sample collection

Fish were randomly divided into 4 groups (60 fish per group) as detailed in Table 1. The fish were anaesthetized, as above, and vaccinated i.p. with each vaccine formulation. Group 1: CTRL, fish injected i.p. with 100 μL PBS; Group 2: SAIV, fish injected i.p. with 100 μL SAIV vaccine alone; Group 3, SAIV+763A, fish injected i.p. with 100 μL vaccine containing Montanide™ ISA 763A VG; and Group 4: SAIV+763B, fish injected i.p. with 100 μL vaccine containing Montanide™ ISA 763B VG. Fish were fasted for 24 h before blood sampling. Whole blood was collected at 1, 2, 3, 4, and 5 w.p.v. for study of the innate and adaptive immune response from 8 fish per group. Fish serum was obtained by centrifugation at 4,000 rpm for 10 min at 4°C.

2.6 Investigation of innate immune parameters

The innate immune responses studied in the Nile tilapia sera included lysozyme (LZM), myeloperoxidase (MPO), and serum bactericidal activity described previously [34]. The activity of LZM (U/mL) was determined by measuring the decrease in turbidity after the lysis of the Gram-positive bacterium *Micrococcus lysodeikticus* at the absorbance at 450 nm. The MPO activity was measured spectrophotometrically at 450 nm based on the use of 3,3′–5,5′-tetramethyl benzidine hydrochloride hydrate (TMB. 2HCl.xH_2O) as substrate. The antibacterial activity (%) was analysed by the growth of bacteria onto BHI agar. The number of colonies that appeared on the plates was enumerated after cultivation for 24 h in serum from the vaccinated groups and control

group following equation: antibacterial activity (%) = (number of colonies in treatment samples/number of colonies in control "BHI only" samples) × 100.

2.7 Enzyme-linked immunosorbent assay (ELISA) for IgM antibody levels

Specific IgM antibody levels in Nile tilapia sera against *S. agalactiae* were determined by Enzyme-linked immunosorbent assay (ELISA), as described previously [36]. Briefly, 96-well microplates (Thermo scientific) were coated with 1.0×10^8 CFU/mL *S. agalactiae* in 50 µL/well coating buffer (pH 9.0, 100 mM $NaHCO_3$, 12 mM Na_2CO_3) at 37°C for 2 h. Plates were washed with PBS plus 0.05% Tween-20 (PBST) and then blocked with 5% (w/v) skimmed milk in wash buffer at 37°C for 2 h, then washed again 3 times with PBST. The sera were diluted at 1:256 in PBST, and added to the wells (50 µL/well) in duplicate and were incubated at 4°C overnight. After washing 3 times with PBST, 50 µL/well of mouse-anti-Nile tilapia IgM (Vertebrate antibodies limited, UK) were added (1:50 dilution in PBST) and incubated at 37°C for 2 h. Subsequently, the ELISA plates were washed with PBST 3 times and 50 µL/well of secondary antibody (anti-mouse IgG labelled with horseradish peroxidase, diluted 1:2,000 in PBST) were added to each well and incubated at 37°C for 1 h. The ELISA plates were then washed 3 times and TMB Liquid Substrate (Sigma) added at 50 uL/well for color development at room temperature (RT) for 20 min. Finally, 50 µL of 0.5 M H_2SO_4 was added to each well to terminate the reaction, and the optical density (OD) at 450 nm was read by an iMark™ Microplate Reader (Bio-Rad).

2.8 Immunohistochemical analysis

Three fish per group were killed at day 21 post-challenge with live *S. agalactiae*. The spleen tissues were sampled and fixed in 10% buffered formalin for 24 h. Afterwards, they were moved to 70% ethanol, dehydrated in 70%, 96%, and 99% ethanol, respectively, and then embedded in paraffin wax before being cut into sections (45 µm) with a Leica RM2245 microtome. The sections were dried for 24 h at 40°C before being placed onto SuperFrost UltraPlus glass slides (Menzel-Glaser, Germany). These slides were utilized for immunohistochemical labeling after being deparaffinized and rehydrated in 99%, 96%, and 70% ethanol and Tris-buffered saline (TBS; Dako, Denmark). To quench endogenous peroxidase activity, slides were pre-treated with 1.5% H_2O_2 for 10 min and heated for 10 min in a microwave for antigen retrieval. Slides were incubated with 2% BSA in TBS for 10 min at RT to prevent non-specific binding. Slides were then incubated

with a specific Nile tilapia anti-IgM monoclonal antibody, which was diluted (1:2,000 in PBST) in 1% BSA, overnight at 4°C. Slides were washed in TBS and incubated for 10 min with the HiDef Detection^{TM} HRP polymer system (Cell Marque, Denmark). Slides were next counterstained with Mayer's hematoxylin (Dako, Denmark), and mounted in a water-soluble mounting media (Aquamount, Merck Millipore, Denmark). Digital images were captured from each slide under a compound microscope (Carl Zeiss) using an EOS-600D Canon camera. Immunohistochemical staining of spleen was presented as the average number of IgM^+ cells [(+) 1–10 positive cells; (++) 11–20 positive cells; (+++) ≥21–50 positive cells] and melanin containing cells (MCCs) counted from 0.5 mm^2 spleen sections.

2.9 Immune gene expression profiling by real-time quantitative PCR (RT-qPCR)

The spleen and liver were randomly collected from 3 fish in each group at 1-, 3-, and 14-days post vaccination (d.p.v.), to assess whether inclusion of Montanide™ ISA 763A VG and ISA 763B VG adjuvants affected the expression of selected cytokine genes (associated with innate and adaptive immunity) after vaccination and relative to fish receiving SAIV alone. The spleen was selected as a major secondary lymphoid tissue in fish, whilst the liver was studied as it is a major contributor to the acute phase response. Total RNA was extracted using Trizol reagent (Invitrogen), cDNA synthesised and gene expression analysis performed by RT-qPCR as described previously [37,38]. The cytokine genes analysed included IL-1β, IL-6, IL-8, IFN-γ and TNF-α. A FX96 Touch Real-Time PCR Detection System (Bio-Rad) was used to investigate the gene expression. The gene-specific primers used for RT-qPCR are listed in Table 2. The expression level of each gene was normalized to that of β-actin and used as an internal control. β-actin is frequently used as internal reference genes for gene expressions in Nile tilapia. The relative quantification of immune genes was presented as a fold change, by calculating the transcription level in the vaccine groups divided by that of the CTRL group.

2.10 Bacterial challenge

For challenge, 45 fish from each experimental group were divided into 3 replicates (15 fish/replicate) and were placed in 500 L aerated fiberglass tanks. Fish were challenged via i.p. injection, with 1.0×10^8 CFU/fish of *S. agalactiae* in 200 μL PBS [22]. All tanks were checked twice daily in order to detect any dead or moribund fish. Both dead and moribund fish were

immediately removed. Fish mortality was recorded daily for 21 days. *S. agalactiae* was re-isolated from spleen and liver (3 fish/group) of dying fish on tryptic soy agar to confirm that morbidity was due to the challenge infection. The relative percent survival (RPS) of each group was calculated as: RPS (%) = 1 – [% mortality rate (vaccinated fish)/% mortality rate (control fish)] × 100 [39].

2.11 Statistical analysis

In this experiment, all data are presented as the mean ± standard error of the mean (SEM) and analyzed using IBM SPSS statistics 22 software (SPSS Inc., Chicago, IL, USA). One-way ANOVA and LSD post hoc tests were used to determine the differences between vaccinated and control groups for each time point for innate immune responses and the level of specific serum antibody (IgM), and considered statistically significant at $P<0.05$.

3. Results

3.1 Vaccine safety assessment

Following injection immunization, there was no mortality in any of the groups, and no abnormalities were seen during the 2 week observation period that followed the injection. Furthermore, throughout the 5 week study period of the main experimental trial, none of the fish died or showed symptoms of acute toxicity or chronic side effects in any of the groups.

3.2 Innate immune response parameter assays

When compared with the CTRL group, the results showed that significantly higher LZM activity was present in sera from the Nile tilapia vaccinated with SAIV+763A from weeks 2-5 w.p.v. and at 1-3 and 5 w.p.v. with sera from the SAIV+763B group (Fig. 1A). In the absence of an adjuvant only at week 3 was a significant difference seen using SAIV alone. The MPO activity of sera from the SAIV+763A and SAIV+763B groups was also significantly increased at 2-4 w.p.v. and 2 and 4 w.p.v., respectively (Fig.1B). Lastly, the bactericidal activities were significantly higher in sera from the SAIV+763A and SAIV+763B groups 2-5 w.p.v. and 1-5 w.p.v., respectively (Fig. 1C), far earlier than in the absence of adjuvant (4 and 5 w.p.v. only).

3.3 Gene expression analysis

Similar expression profiles of the cytokines was observed in spleen and liver in all vaccination groups. Highest induction was typically seen at day 1 post-vaccination, and decreased thereafter (Fig. 2). The exception was IL-6 and TNF-α in the liver of fish given SAIC+763A, which peaked at day 3. At day 1 in spleen, cytokine expression levels in the SAIV+763B fish were significantly higher relative to all the other groups, with the exception of IL-6 and IL-8 where both adjuvanted groups were similarly elevated (Fig. 2). The cytokine transcript levels in fish given SAIV and SAIV+763A were also higher than levels in control (CTRL) fish, with transcript levels in SAIV+763A fish being higher than in SAIV fish in the case of IL-1β and TNF-α. Some increases were still apparent in the vaccinated fish at day 3 but had returned to basal levels by day 14. IFN-γ showed the largest increases overall, with IL-8 the lowest. In the liver, smaller increases were seen typically, with IL-β and IFN-γ significantly higher than the control fish at day 1. These increases were maintained at day 3 for IFN-γ but were decreasing back to basal levels in the case of IL-1β. Curiously, increases in IL-8 and TNF-α expression were only seen at day 3, especially in fish given SAIV+763A. An increase in IL-6

expression was also seen at day 3 in the adjuvanted vaccine groups, and this was maintained to day 14 in the SAIV+763A group.

3.4 Specific antibody response analysis

The levels of specific serum antibody (IgM) are shown in Fig. 3. When compared with the saline only group, the results showed that the levels of antibody against *S. agalactiae* were significantly higher from 4 w.p.v. to 5 w.p.v. in fish receiving the SAIV alone. In the case of the adjuvanted vaccines, the specific antibody levels were significantly higher than in control fish when vaccinated with SAIV+763B at all of the time points tested. Similarly, in sera from fish given SAIV+763A, antibody levels were higher than in control fish at weeks 1 and 3-5. However, no significant differences between sera from the two adjuvanted vaccine groups were found. These findings suggest that the adjuvanted vaccines induce a faster humoral immune response against *S. agalactiae* than in fish given SAIV alone.

3.5 Efficiency of the vaccine against *S. agalactiae* challenge

The protective efficacy of Montanide™ ISA 763A VG and ISA 763B VG was next assessed (Fig. 4). All vaccinated groups were challenged at 5 w.p.v. with virulent *S. agalactiae* at $1x10^8$ CFU/fish. The cumulative mortality in each group were recorded for a period of 21 days, with all of the dead and moribund fish displaying at least one of the known clinical symptoms of streptococcosis, including severe hemorrhaging, corneal opacity, spinning near the water's surface, caudal fin erosion, and exophthalmia. The survival rates of control, SAIV alone, SAIV+763A, SAIV+763B vaccinated group at this time were 31.11%, 73.33%, 84.44%, and 82.22%, respectively. This gave RPS values of 61.29%, 77.42%, and 74.19% for the SAIV, SAIV+763A and SAIV+763B groups, respectively (Fig. 4). No significant differences were found between the two adjuvanted vaccine groups, which both elicited significantly higher protection than use of SAIV alone.

3.6 Analysis of IgM^+ B cell numbers by immunohistochemical analysis

Spleen tissue from all of the vaccinated fish groups was assessed for the presence of IgM^+ B cells by immunohistochemistry (Fig. 5). Noticeable differences in the densities of B cells were apparent after vaccination and after challenge (Table 3). When compared to sections from control fish, spleens from fish given both adjuvanted vaccine groups had more detectable B cells post vaccination but pre-challenge. However, post-challenge all fish showed an increase in detectable B cells. In the case of the control and SAIV groups, the levels increased to levels seen pre-challenge in the adjuvanted vaccine

294 groups, whilst in the latter they increased further with some large clusters seen associated with melanin
295 containing cells (MCC). Indeed, in comparison to unchallenged fish, all challenged fish displayed an
296 increase in MCC incidence (Fig. 3).

297 **3.7 Vaccine persistence *in vivo***

298 The persistence of Montanide™ ISA 763A VG and ISA 763B VG *in vivo* was investigated in
299 the adjuvanted vaccine fish groups. Adjuvant residues were found in the peritoneal cavity of
300 SAIV+763A and SAIV+763B injected fish 5 w.p.v. (Fig. 6). The internal organs in the control and
301 SAIV groups had a normal appearance at this time.

302 **4. Discussion**

303 In this report, an inactivated *S. agalactiae* whole cell vaccine (SAIV) was prepared and mixed
304 with one of two adjuvants, Montanide™ ISA 763A VG and ISA 763B VG, to assess the impact of
305 these adjuvants on the immune response and protection in tilapia following vaccination. The results
306 clearly showed the benefits of using these adjuvants in SAIV vaccines, with effects on innate immune
307 parameters, cytokine gene expression, kinetics of antibody production and immunoprotection. Thus,
308 both Montanide™ ISA 763A VG and ISA 763B VG have the potential to be used as adjuvants in fish
309 vaccines to protect against streptococcosis.

310 It is widely recognized that adjuvants have become the most efficient way of giving durational
311 protection and modulating the immunogenicity of fish vaccine components [22,40]. For example, using
312 a range of methods to inactivate *S. agalactiae* (formalin, H_2O_2, pH manipulation), in the absence of
313 adjuvants RPS values of ≤60% are seen post vaccination of Nile tilapia [41]. However, using a number
314 of novel antigenic components of *S. agalactiae* as subunit vaccines, in a prime-boost strategy using
315 FCA as adjuvant followed by FIA, RPS values between 59%-92% were seen, with the latter clearly
316 very promising [42]. Another study of a subunit vaccine for *S. agalactiae* GapA used FCA or
317 Montanide™ ISA 763A VG as adjuvant [43]. Whilst relatively low protection (RPS of 46-63%) was
318 seen with the GapA vaccine, in fact an RPS of 74-77% was achieved using inactivated whole cells with
319 these adjuvants. Freund's adjuvants are unlikely to be used commercially for fish vaccination, but there
320 have been numerous studies using Montanide™ ISA 763A VG as adjuvant in other fish-pathogen
321 systems to explore vaccine effectiveness, and consistently high efficacies have been found [24-32]. A
322 different non-mineral oil-based Montanide adjuvant, Montanide™ ISA 763B VG, was used in a
323 previous study in Nile tilapia with an *S. agalactiae* bacterial ghost vaccine [22], and gave an RPS of

80.8% (vs 73.1% in fish given the unadjuvanted vaccine). However, this adjuvant has yet to be trialed with a more conventional inactivated (formalin-killed) whole cell *S. agalactiae* vaccine. Hence, in the present study ISA 763B VG was used as an adjuvant for vaccination of Nile tilapia with an SAIV. A further group of fish was given an ISA 763A VG adjuvanted SAIV vaccine to allow comparison of the relative performance of these two adjuvants for protection against streptococcosis.

Innate immunity is a critical first line of defense against many invasive diseases [44]. Three innate parameters were studied in the present study post-vaccination. LZM is one of several humoral and cellular factors that plays an important role in natural defense mechanisms in all vertebrates [45]. MPO, an antioxidant enzyme, catalyzes the oxidation of hydrogen peroxide to hypochlorous acid, as well as producing other highly reactive compounds such as tyrosyl radicals and protein cross-links [46]. Following vaccination with the two adjuvanted vaccines, fish showed a significant increase in the activity of these molecules, suggesting that both adjuvanted vaccines enhanced the activation of lysosomal and antioxidant defenses. Additionally, serum antibacterial activity was studied *in vitro* post-vaccination, with considerably higher levels of bactericidal activity found relatively quickly after vaccination with SAIV+763A and SAIV+763B. Bactericidal activity is a key indicator of the overall humoral innate response of fish to pathogen invasion, and may be caused by elevated levels of LZM, complement factors, antimicrobial peptides, and other molecules [47]. Several other studies have shown that adjuvanted vaccines can enhance the non-specific immune response of a variety of fish species, contributing to an early increase in protective immunity [25,27,48-49].

As part of the fish adaptive immune response, the production of specific antibodies plays a crucial role in preventing bacterial infection, although relatively slow to develop [55]. IgM is the main circulating antibody molecule of teleost fish and is an ancient Ig found in all jawed vertebrates [56]. Therefore, specific IgM levels in sera were used to evaluate the humoral immune response induced by injection vaccination of Nile tilapia against *S. agalactiae*. The results showed that specific antibodies were found in all three vaccinated groups (SAIV, SAIV+763A, SAIV+763B) in serum samples collected 1-5 weeks post-vaccination, similar to past studies performed in Nile tilapia and other fish species [41-42, 57-59]. However, the level of specific IgM antibody in the SAIV+763A and SAIV+763B groups appeared earlier and in some cases was significantly higher than in serum from SAIV injected fish. Whilst no differences between the two adjuvanted vaccines were readily apparent, the results show the benefit of adjuvant inclusion to rapidly stimulate humoral immunity in tilapia. This finding was in agreement with the immunohistochemical study, showing higher numbers of detectable

IgM^+ B cells in spleen post-vaccination and post-challenge in these fish in comparison to those given SAIV alone. Interestingly, this was also reflected in the numbers of MMCs detected in the spleen sections from these fish. MCCs, which form characteristic aggregates termed melano-macrophage centers (MMCs), are particularly common in fish lymphoid tissues and have been implicated in a variety of immunological mechanisms, such as antigen trapping [60,61]. In addition, previous reports have shown that MMCs may increase after pathogenic infection [62] or exposure to a potent inflammatory stimulus [63]. Hence, it is possible that these (immune?) cells take part in pathogen eradication during the initial response to *S. agalactiae* infection.

Since the peritoneal cavity is where the vaccine components are first encountered by the fish, the responses measured in the current study in tilapia were concentrated on the expression of specific immune genes in peritoneal cells during the early phase post-vaccination. It is known that these early innate responses have the potential to influence later adaptive responses. Markers of cellular immunity in fish are somewhat limited but are important to study to gain an insight into their relative importance in disease resistance induced by vaccination in addition to analysis of humoral immunity [71]. The spleen and liver are important organs in the teleost fish immune system, contributing to the acute phase response, humoral and cell-mediated immunity [36,37]; the former rich in lymphoid cells containing many immune cell types, such as B cells, T cells, and macrophages that initiate adaptive immune responses via antigen presentation [72]. Therefore, both tissues were used for analysis of cytokine transcript expression, selecting genes involved in the pro-inflammatory response and adaptive immunity (eg IL-1β, IL-6, IL-8, IFN-γ, and TNF-α), that have been used widely as biomarkers of vaccine responsiveness [73]. In the present study, the expression of these genes was evaluated at 1-, 3- and 14-days post-vaccination, with these timings based on our previous work showing many cytokine and antimicrobial genes have highest induction 1 and 3 days after bacterin (ERM) injection [74]. The expression of the cytokine genes in the spleen were consistently modulated at day 1 post-vaccination, with levels of IL-1β, IFN-γ and TNF-α highest in fish given SAIV+763B. IL-1β and TNF-α levels were also significantly higher in fish given SAIV+763A relative to SAIV fish at this time. IFN-γ showed the largest increase in expression and is known to be released from T cells during adaptive immune responses as well as from NK cells as part of innate immunity. In the liver, IL-1β and IFN-γ were also induced at day 1 post-vaccination, but the other genes showed relatively low levels of induction that peaked at day 3. There were no clear differences between expression levels in the two adjuvated-vaccine groups, although IL-6 in liver of SAIV+763A injected fish was still significantly elevated to day 14 (relative to control fish) and this could relate to maintenance of B cells by this

cytokine [75]. These data suggest that Nile tilapia were able to initiate cellular immune responses at early-stages post vaccination, with the increases seen in the adjuvanted-vaccine fish potentially contributing to enhanced downstream activation of adaptive immunity.

Enhanced immune responses post-vaccination hopefully contribute to enhanced protection following disease challenge. In general, the RPS value is usually applied to assess the efficacy of vaccines and an optimal RPS is considered to be ≥60% [50]. In this study, vaccination of Nile tilapia with the three vaccines decreased mortality and the development of streptococcal disease. The RPS of the adjuvanted groups (SAIV+763A and SAIV+763B) was over 70% (77.42% and 74.19%, respectively), higher than in the SAIV group (61.29%), and significantly higher than in the non-vaccinated group (CTRL). Hence, the inclusion of adjuvants improved the response to vaccination, with both adjuvants performing well. Numerous studies in other fish species have shown that using an adjuvant in combination with a vaccine can give good protection against infectious diseases. For instance, turbot immunized with an inactivated vaccine against *Vibrio harveyi* using Montanide™ ISA 763 A VG as adjuvant showed high RPS values (75.86-83.87%) [27]. A high RPS value (97.5%) against *Y. ruckeri* challenge was also seen in rainbow trout given an experimental ERM vaccine formulated with Montanide™ ISA 763A VG [24]. Recently, our previous study in Nile tilapia used a bacterial ghost vaccine against *S. agalactiae* with Montanide™ ISA 763B VG as adjuvant, and again good protection was seen; RPS values of 70.0% vs 60% in fish vaccinated without adjuvant [22]. Despite these promising results, it is well known that resistance to a disease post-vaccination is also connected with other factors, such as the vaccine concentration, durational immunity post vaccination, and the challenge model used (eg strain, dose, route of infection) [51-54]. Thus, more research is needed to evaluate the effectiveness of *S. agalactiae* adjuvanted vaccines when employing higher challenge doses, the effects of various infection routes (eg immersion, cohabitation) and the possibility of cross-protection across isolates from different serotypes. Nevertheless, in this study it was apparent that both Montanide™ adjuvants delivered highly potent vaccines when combined with the inactivated *S. agalactiae* bacterin, and this method may have many more applications for tilapia aquaculture in addition to their use for control of streptococcal disease.

Whilst adjuvants are supportive chemicals that help the fish immune system to respond to vaccination [64], they can also produce a number of minor adverse effects, as seen in various fish species, such as adhesions, granulomas and even autoimmunity [65]. Hence in the present study fish were examined for possible side effects and vaccine persistence. Both SAIV+763A and SAIV+763B

injected fish showed no toxic effects or abnormalities, and hence theses adjuvants appear safe for use in Nile tilapia. However, vaccinated fish were found to have persistent emulsified vaccine droplets in their peritoneal cavity although the internal organs had a normal appearance, as compared to fish given SAIV alone and control fish. Vaccine persistence is considered necessary for long-term protection and augmentation of the fish immune response, and hence is typical of oil adjuvant usage [66]. Indeed, studies in many fish species looking at adverse effects caused by oil adjuvants have shown mild to moderate side-effects, as seen in Atlantic salmon, *Salmo salar* [67-68], turbot [17, 27,69], rainbow trout [70], and Nile tilapia [22]. Modern adjuvant-vaccine formulations strive to decrease such side effects whilst maintaining efficacy.

Conclusion

In conclusion, vaccination of SAIV emulsified with the adjuvants Montanide™ ISA 763A VG or ISA 763B VG induced strong protection against *S. agalactiae* in Nile tilapia. Moreover, selected humoral and cellular elements of innate and adaptive immunity were shown to be enhanced or increase faster in these fish post vaccination relative to fish given SAIV alone. Therefore, the data from this study indicate that Montanide™ ISA 763A VG and ISA 763B VG are safe and effective, and thus have the potential to be used as adjuvants for vaccines being developed to control and prevent streptococcosis in Nile tilapia.

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Author Contributions

Conceptualization, E.W. and R.V.; methodology, E.W., A.T., S.P., P.S., J.P., P.P.; formal analysis, E.W.; investigation, E.W. and R.V.; resources, E.W. and R.V.; data curation, E.W.; writing-original draft preparation, E.W., R.V., T.W., C.J.S.; writing-review and editing, E.W., R.V., T.W., C.J.S.; visualization, E.W., S.P., P.S.; Advice, P.S., T.W. and C.J.S.; project administration, E.W. and R.V.; funding acquisition, E.W. All authors have agreed to the published version of this manuscript.

Ethics statement

The Institute of Animals for Scientific Development (IAD) of Thailand guidelines for the use of animals in research were strictly followed during this study. Mahasarakham University ethics committee authorized the fish handling and experimental techniques used (IACUC-MSU-31/2022).

Conflicts of Interest

Authors declare that there were no known competing financial interests to influence the work reported in this study.

References

[1] C.M. Delannoy, M. Crumlish, M.C. Fontaine, J. Pollock, G. Foster, M.P. Dagleish, J.F. Turnbull, R.N., Zadoks, Human *Streptococcus agalactiae* strains in aquatic mammals and fish, BMC Microbiol., 13(2013), pp.1-9. https://doi.org/10.1186/1471-2180-13-41.

[2] V.N. Raabe, A.L., Shane, Group B streptococcus (*Streptococcus agalactiae*), Microbiol. Spectr., 7(2019), pp.7-2. https://doi.org/10.1128/microbiolspec.GPP3-0007-2018.

[3] E. Wangkahart, B. Bruneel, A. Chantiratikul, M. de Jong, N. Pakdeenarong, P.A. Subramani, Optimum dietary sources and levels of selenium improve growth, antioxidant status, and disease resistance: re-evaluation in a farmed fish species, Nile tilapia (*Oreochromis niloticus*), Fish Shellfish Immunol., 121(2022), pp.172-182. https://doi.org/10.1016/j.fsi.2021.12.003.

[4] C. Li, D.N. Sapugahawatte, Y. Yang, K.T. Wong, N.W.S. Lo, M. Ip, Multidrug-resistant *Streptococcus agalactiae* strains found in human and fish with high penicillin and cefotaxime non-susceptibilities, Microorganisms, 8(2020), p.1055. https://doi.org/10.3390/microorganisms8071055

[5] I. Sommerset, B. Krossøy, E. Biering, P. Frost, Vaccines for fish in aquaculture, Expert Rev. Vaccines, 4(2005), pp.89-101. https://doi.org/10.1586/14760584.4.1.89.

[6] A. Adams, Progress, challenges and opportunities in fish vaccine development, Fish Shellfish Immunol., 90(2019), pp.210-214. https://doi.org/10.1016/j.fsi.2019.04.066.

[7] H.M. Munang'andu, J. Paul, Ø. Evensen, An overview of vaccination strategies and antigen delivery systems for *Streptococcus agalactiae* vaccines in Nile tilapia (*Oreochromis niloticus*), Vaccines, 4(2016), p.48. https://doi.org/10.3390/vaccines4040048.

[8] L.Y. Huang, K.Y. Wang, D. Xiao, D.F. Chen, Y. Geng, J. Wang, Y. He, E.L. Wang, J.L. Huang, G.Y. Xiao, Safety and immunogenicity of an oral DNA vaccine encoding Sip of *Streptococcus agalactiae* from Nile tilapia *Oreochromis niloticus* delivered by live attenuated *Salmonella typhimurium*, Fish Shellfish Immunol., 38(2014), pp.34-41. https://doi.org/10.1016/j.fsi.2014.02.017

[9] G. Liu, J. Zhu, K. Chen, T. Gao, H. Yao, Y. Liu, W. Zhang, C. Lu, Development of *Streptococcus agalactiae* vaccines for tilapia, Dis. Aquat. Org., 122(2016), pp.163-170. https://doi.org/10.3354/dao03084

[10] Q. Wang, X. Wang, X. Wang, R. Feng, Q. Luo, J. Huang, Generation of a novel *Streptococcus agalactiae* ghost vaccine and examination of its immunogenicity against virulent challenge in tilapia, Fish Shellfish Immunol., 81(2018), pp.49-56. https://doi.org/10.1016/j.fsi.2018.06.055.

[11] L.D. Mamede, K.G. de Paula, B. de Oliveira, J.S.C. Dos Santos, L.M. Cunha, M.C. Junior, L.R.C. Jung, A.G. Taranto, D. de Oliveira Lopes, S.Y. Leclercq, Reverse and structural vaccinology approach to design a highly immunogenic multi-epitope subunit vaccine against *Streptococcus pneumoniae* infection, Infect. Genet. Evol., 85(2020), p.104473. https://doi.org/10.1016/j.meegid.2020.104473.

[12] D. Zhang, Y. Gao, Q. Li, X. Ke, Z. Liu, M. Lu, C. Shi, An effective live attenuated vaccine against *Streptococcus agalactiae* infection in farmed Nile tilapia (*Oreochromis niloticus*), Fish Shellfish Immunol., 98(2020), pp.853-859. https://doi.org/10.1016/j.fsi.2019.11.044.

[13] A.A. Laith, M.A. Abdullah, W.W.I. Nurhafizah, H.A. Hussein, J. Aya, A.W.M. Effendy, M. Najiah, Efficacy of live attenuated vaccine derived from the *Streptococcus agalactiae* on the immune responses of *Oreochromis niloticus*, Fish Shellfish Immunol., 90(2019), pp.235-243. https://doi.org/10.1016/j.fsi.2019.04.052.

[14] Z. Zhang, G. Liu, R. Ma, X. Qi, G. Wang, B. Zhu, F. Ling, The immunoprotective effect of whole-cell lysed inactivated vaccine with SWCNT as a carrier against *Aeromonas hydrophila* infection in grass carp, Fish Shellfish Immunol., 97(2020), pp.336-343.

https://doi.org/10.1016/j.fsi.2019.12.069.

[15] J.J. Evans, P.H. Klesius, C.A. Shoemaker, Efficacy of *Streptococcus agalactiae* (group B) vaccine in tilapia (*Oreochromis niloticus*) by intraperitoneal and bath immersion administration, Vaccine, 22(2004), pp.3769-3773. https://doi.org/10.1016/j.vaccine.2004.03.012.

[16] D.A. Austin, P.A.W. Robertson, B. Austin, Recovery of a new biogroup of *Yersinia ruckeri* from diseased rainbow trout (*Oncorhynchus mykiss*, Walbaum), Syst Appl Microbiol., 26(2003), pp.127-131. https://doi.org/10.1078/072320203322337416.

[17] J. Li, L. Tang, S. Li, G. Li, Z. Mo, The efficacy and side-effects of oil-based adjuvants emulsified *Vibrio anguillarum* bivalent inactivated vaccine in turbot (*Scophthalmus maximus*) under production mode, Aquaculture, 524(2020), p.735259. https://doi.org/10.1016/j.aquaculture.2020.735259.

[18] J. Lim, S. Hong, Characterization of *Aeromonas salmonicida* and *A. sobria* isolated from cultured salmonid fish in Korea and development of a vaccine against furunculosis, J. Fish. Dis., 43(2020), pp.609-620. https://doi.org/10.1111/jfd.13158.

[19] R. Hoare, S.J. Jung, T.P. Ngo, K. Bartie, J. Bailey, K.D. Thompson, A. Adams, Efficacy and safety of a non-mineral oil adjuvanted injectable vaccine for the protection of Atlantic salmon (*Salmo salar* L.) against *Flavobacterium psychrophilum*, Fish Shellfish Immunol., 85(2019), pp.44-51. https://doi.org/10.1016/j.fsi.2017.10.005.

[20] X. Wu, J. Xing, X. Tang, X. Sheng, H. Chi, W. Zhan, Protective cellular and humoral immune responses to *Edwardsiella tarda* in flounder (*Paralichthys olivaceus*) immunized by an inactivated vaccine, Mol. Immunol., 149(2022), pp.77-86. https://doi.org/10.1016/j.molimm.2022.06.008

[21] F.C. Ramos-Espinoza, V.A. Cueva-Quiroz, J. Yunis-Aguinaga, N.C. Alvarez-Rubio, N.P. de Mello, J.R.E. de Moraes, Efficacy of two adjuvants administrated with a novel hydrogen peroxide-inactivated vaccine against *Streptococcus agalactiae* in Nile tilapia fingerlings, Fish Shellfish Immunol., 105(2020), pp.350-358. https://doi.org/10.1016/j.fsi.2020.07.051.

[22] E. Wangkahart, S. Deville, B. Wang, P. Srisapoome, T. Wang, C.J. Secombes, Immune response and protective efficacy of two new adjuvants, Montanide™ ISA 763B VG and Montanide™

GEL02, administered with a *Streptococcus agalactiae* ghost vaccine in Nile tilapia (*Oreochromis niloticus*), Fish Shellfish Immunol., 116(2021), pp.19-29. https://doi.org/10.1016/j.fsi.2021.06.014

[23] SEPPIC. MontanideTM adjuvant ranges. https://www.seppic.com/en/montanide-gel/ (2022), Accessed 4th October 2022

[24] R.M. Jaafar, J.K. Chettri, I. Dalsgaard, A. Al-Jubury, P.W. Kania, J. Skov, K. Buchmann, Effects of adjuvant Montanide™ ISA 763 A VG in rainbow trout injection vaccinated against *Yersinia ruckeri*, Fish Shellfish Immunol., 47(2015), pp.797-806. https://doi.org/10.1016/j.fsi.2015.10.023

[25] M. Soltani, A. Mokhtari, S.S. Mirzargar, A. Taherimirghaed, A. Zargar, S. Shafiei, S.P. Hosseini-Shekarabi, Efficacy and immune response of intraperitoneal vaccination of rainbow trout (*Oncorhynchus mykiss*) with a *Yersinia ruckeri* bacterin formulated with Montanide™ ISA 763 AVG adjuvant, Bull. Eur. Assoc. Fish Pathol., 36(2016), pp.225-236.

[26] K.A. Veenstra, T. Wang, K.S. Russell, L. Tubbs, J.B. Arous, C.J., Secombes, Montanide™ ISA 763A VG and ISA 761 VG induce different immune pathway responses in rainbow trout (*Oncorhynchus mykiss*) when used as adjuvant for an *Aeromonas salmonicida* bacterin, Fish Shellfish Immunol., 114(2021), pp.171-183. https://doi.org/10.1016/j.fsi.2021.04.024.

[27] W. Xu, C. Jiao, P. Bao, Q. Liu, P. Wang, R. Zhang, X. Liu, Y. Zhang, Efficacy of Montanide™ ISA 763 A VG as aquatic adjuvant administrated with an inactivated *Vibrio harveyi* vaccine in turbot (*Scophthalmus maximus* L.), Fish Shellfish Immunol., 84(2019), pp.56-61. https://doi.org/10.1016/j.fsi.2018.09.024.

[28] J.G. Ramírez-Paredes, M.A. Mendoza-Roldan, B. Lopez-Jimena, K. Shahin, M. Metselaar, K.D. Thompson, D.J. Penman, R.H. Richards, A. Adams, Whole cell inactivated autogenous vaccine effectively protects red Nile tilapia (*Oreochromis niloticus*) against francisellosis via intraperitoneal injection, J. Fish Dis., 42(2019), pp.1191-1200. https://doi.org/10.1111/jfd.13041

[29] J. Galindo-Villegas, A. García-Alcazar, J. Meseguer, V. Mulero, Aluminum adjuvant potentiates gilthead seabream immune responses but induces toxicity in splenic melanomacrophage centers, Fish Shellfish Immunol., 85(2019), pp.31-43. https://doi.org/10.1016/j.fsi.2018.02.047.

[30] C.F. Lin, H.K. Jiang, N.C. Chen, T.Y. Wang, T.Y. Chen, Novel subunit vaccine with linear array epitope protect giant grouper against nervous necrosis virus infection, Fish Shellfish Immunol., 74(2018), pp.551-558. https://doi.org/10.1016/j.fsi.2018.01.029.

[31] H.H. Hoang, P.C. Wang, S.C. Chen, Recombinant resuscitation-promoting factor protein of *Nocardia seriolae*, a promissing vaccine candidate for largemouth bass (*Micropterus salmoides*), Fish Shellfish Immunol., 111(2021), pp.127-139. https://doi.org/10.1016/j.fsi.2021.01.015.

[32] X. Liu, H. Gong, D. Zhang, C. Shi, H. Pan, O. Chang, Q. Wang, Y. Ren, Antigenicity and immunity of recombinant OmpAII from *Aeromonas veronii* and protection against virulent *Aeromonas* infections in *Carassius auratus gibelio*, Aquaculture, 552(2022), p.737979. https://doi.org/10.1016/j.aquaculture.2022.737979.

[33] E. Wangkahart, P. Kersanté, P.T. Lee, O. Sanbut, S. Nontasan, A. Chantiratikul, Effect of Kera-Stim® 50, a feed additive containing free amino acid mix on growth, antioxidant and immune responses, digestive enzymes, and fatty acid composition in Nile tilapia (*Oreochromis niloticus*), Aquaculture, 551(2022), p.737874. https://doi.org/10.1016/j.aquaculture.2021.737874.

[34] E. Wangkahart, S. Wachiraamonloed, P.T. Lee, P.A. Subramani, Z. Qi, B. Wang, Impacts of *Aegle marmelos* fruit extract as a medicinal herb on growth performance, antioxidant and immune responses, digestive enzymes, and disease resistance against *Streptococcus agalactiae* in Nile tilapia (*Oreochromis niloticus*), Fish Shellfish Immunol., 120)2022), pp.402-410. https://doi.org/10.1016/j.fsi.2021.11.015.

[35] S. Mutoloki, S. Alexandersen, Ø. Evensen, Sequential study of antigen persistence and concomitant inflammatory reactions relative to side-effects and growth of Atlantic salmon (*Salmo salar* L.) following intraperitoneal injection with oil-adjuvanted vaccines, Fish Shellfish Immunol., 16(2004), pp.633-644. https://doi.org/10.1016/j.fsi.2003.10.002.

[36] E. Wangkahart, C.J. Secombes, T. Wang, Studies on the use of flagellin as an immunostimulant and vaccine adjuvant in fish aquaculture, Front. Immunol., 9(2019), p.3054. https://doi.org/10.3389/fimmu.2018.03054.

[37] E. Wangkahart, S. Jumpalueang, S. Ardprachan, J. Phudkliang, P. Sunthamala, S. Pholchamat, Z. Qi, Molecular characterization and expression analysis of novel interleukin-1 family member

(nIL-1F) gene in Nile tilapia (*Oreochromis niloticus*), J. Mar. Sci. Eng, 10(2022), p.1272. https://doi.org/10.3390/jmse10091272.

[38] B. Wang, E. Wangkahart, C.J. Secombes, T. Wang, Insights into the evolution of the suppressors of cytokine signaling (SOCS) gene family in vertebrates, Mol. Biol. Evol., 36(2019), pp.393-411. https://doi.org/10.1093/molbev/msy230.

[39] D.F. Amend, Potency testing of fish vaccines. Fish biologics: serodiagnostics and vaccines, 1981, pp.447-454.

[40] K.P. Plant, S.E. LaPatra, Advances in fish vaccine delivery, Dev. Comp. Immunol., 35(2011), pp.1256-1262. https://doi.org/10.1016/j.dci.2011.03.007.

[43] Z. Zhang, A. Yu, J. Lan, H. Zhang, M. Hu, J. Cheng, L. Zhao, L. Lin, S. Wei, GapA, a potential vaccine candidate antigen against *Streptococcus agalactiae* in Nile tilapia (*Oreochromis niloticus*), Fish Shellfish Immunol., 63(2017), pp.255-260. https://doi.org/10.1016/j.fsi.2017.02.019.

[42] Y. Ma, L. Hao, Z. Liang, J. Ma, H. Ke, H. Kang, H. Yang, J. Wu, G. Feng, Z. Liu, Characterization of novel antigenic vaccine candidates for nile tilapia (*Oreochromis niloticus*) against *Streptococcus agalactiae* infection, Fish Shellfish Immunol., 105(2020), pp.405-414. https://doi.org/10.1016/j.fsi.2020.07.024.

[41] F.C. Ramos-Espinoza, V.A. Cueva-Quiroz, J. Yunis-Aguinaga, J.R.E. de Moraes, A comparison of novel inactivation methods for production of a vaccine against *Streptococcus agalactiae* in Nile tilapia *Oreochromis niloticus*, Aquaculture, 528(2020), p.735484. https://doi.org/10.1016/j.aquaculture.2020.735484

[44] C.J. Secombes, T. Wang, The innate and adaptive immune system of fish. In Infectious disease in aquaculture 2012, (pp. 3-68). Woodhead Publishing. https://doi.org/10.1533/9780857095732.1.3.

[45] L. Li, J.C. Cardoso, R.C. Félix, A.P. Mateus, A.V. Canário, D.M. Power, Fish lysozyme gene family evolution and divergent function in early development, Dev. Comp. Immunol., 114(2021), p.103772. https://doi.org/10.1016/j.dci.2020.103772.

[46] B. Pulli, M. Ali, R. Forghani, S. Schob, K.L. Hsieh, G. Wojtkiewicz, J.J. Linnoila, J.W. Chen, Measuring myeloperoxidase activity in biological samples, PLoS One, 8(2013), p.e67976. https://doi.org/10.1371/journal.pone.0067976.

[47] A.E. Ellis, Immunity to bacteria in fish, Fish Shellfish Immunol., 9(1999), pp.291-308. https://doi.org/10.1006/fsim.1998.0192.

[48] R. Hoare, S.J. Jung, T.P. Ngo, K.L. Bartie, K.D. Thompson, A. Adams, Efficacy of a polyvalent injectable vaccine against *Flavobacterium psychrophilum* administered to rainbow trout (*Oncorhynchus mykiss* L.), J. Fish. Dis., 42(2019), pp.229-236. https://doi.org/10.1111/jfd.12919

[49] X. Sun, P. Jin, Q. Liu, Q. Wang, Y. Zhang, X. Liu, A CpG-riched plasmid as vaccine adjuvant reduce antigen dose of an inactivated *Vibrio anguillarum* vaccine in turbot (*Scophthalmus maximus* L.), Fish Shellfish Immunol., 98(2020), pp.312-317. https://doi.org/10.1016/j.fsi.2020.01.031

[50] P.J. Midtlyng, Methods for measuring efficacy, safety and potency of fish vaccines. In Fish vaccines 2016, pp. 119-141, Springer, Basel. https://doi.org/10.1007/978-3-0348-0980-1_6.

[51] M. Karlsen, T. Tingbø, I.T. Solbakk, Ø. Evensen, A. Furevik, A. Aas-Eng, Efficacy and safety of an inactivated vaccine against Salmonid alphavirus (family Togaviridae), Vaccine, 30(2012), pp.5688-5694. https://doi.org/10.1016/j.vaccine.2012.05.069.

[52] X.M. Dan, T.W. Zhang, Y.W. Li, A.X. Li, Immune responses and immune-related gene expression profile in orange-spotted grouper after immunization with *Cryptocaryon irritans* vaccine, Fish Shellfish Immunol., 34(2013), pp.885-891. https://doi.org/10.1016/j.fsi.2012.12.011.

[53] L.P. Li, R. Wang, W.W. Liang, T. Huang, Y. Huang, F.G. Luo, A.Y. Lei, M. Chen, X. Gan, Development of live attenuated *Streptococcus agalactiae* vaccine for tilapia via continuous passage *in vitro*, Fish Shellfish Immunol., 45(2015), pp.955-963. https://doi.org/10.1016/j.fsi.2015.06.014

[54] D.H. Xu, D. Zhang, C. Shoemaker, B. Beck, Dose effects of a DNA vaccine encoding immobilization antigen on immune response of channel catfish against *Ichthyophthirius multifiliis*, Fish Shellfish Immunol., 106(2020), pp.1031-1041.

https://doi.org/10.1016/j.fsi.2020.07.063

[55] T. Wang, C.J. Secombes, The cytokine networks of adaptive immunity in fish, Fish Shellfish Immunol., 35(2013), pp.1703-1718. https://doi.org/10.1016/j.fsi.2013.08.030.

[56] J. Ye, I.M. Kaattari, C. Ma, S. Kaattari, The teleost humoral immune response, Fish Shellfish Immunol., 35(2013), pp.1719-1728. https://doi.org/10.1016/j.fsi.2013.10.015.

[57] L. Zhu, Q. Yang, L. Huang, K. Wang, X. Wang, D. Chen, Y. Geng, X. Huang, P. Ouyang, W. Lai, Effectivity of oral recombinant DNA vaccine against *Streptococcus agalactiae* in Nile tilapia, Dev. Comp. Immunol., 77(2017), pp.77-87. https://doi.org/10.1016/j.dci.2017.07.024.

[58] Q. Wang, T. Fu, X. Li, Q. Luo, J. Huang, Y. Sun, X. Wang, Cross-immunity in Nile tilapia vaccinated with *Streptococcus agalactiae* and *Streptococcus iniae* vaccines, Fish Shellfish Immunol., 97(2020), pp.382-389. https://doi.org/10.1016/j.fsi.2019.12.021.

[59] N.T. Yu, W.W. Zeng, Z. Xiong, Z.X. Liu, A high efficacy DNA vaccine against Tilapia lake virus in Nile tilapia (*Oreochromis niloticus*), Aquac. Rep., 24(2022), p.101166. https://doi.org/10.1016/j.aqrep.2022.101166.

[60] F.A. Vigliano, R. Bermúdez, M.I. Quiroga, J.M. Nieto, Evidence for melano-macrophage centres of teleost as evolutionary precursors of germinal centres of higher vertebrates: an immunohistochemical study, Fish Shellfish Immunol., 21(2006), pp.467-471.

https://doi.org/10.1016/j.fsi.2005.12.012

[61] N.C. Steinel, D.I. Bolnick, Melanomacrophage centers as a histological indicator of immune function in fish and other poikilotherms, Front. Immunol., 8(2017), p.827.

https://doi.org/10.3389/fimmu.2017.00827.

[62] K.A. Veenstra, A. Alnabulsi, L. Tubbs, J.B. Arous, C.J. Secombes, Immunohistochemical examination of immune cells in adipose tissue of rainbow trout (*Oncorhynchus mykiss*) following intraperitoneal vaccination, Fish Shellfish Immunol., 87(2019), pp.559-564.

https://doi.org/10.1016/j.fsi.2019.02.001.

[63] W.G. Manrique, G. da Silva Claudiano, T.R. Petrillo, M.P. De Castro, M.A. Pereira Figueiredo, M.A. de Andrade Belo, J.E. De Moraes, F.R. De Moraes, Response of splenic melanomacrophage centers of *Oreochromis niloticus* (Linnaeus, 1758) to inflammatory stimuli by BCG and foreign bodies, J. Appl. Ichthyol., 30(2014), pp.1001-1006. https://doi.org/10.1111/jai.12445.

[64] C. Tafalla, J. Bøgwald, R.A. Dalmo, Adjuvants and immunostimulants in fish vaccines: current knowledge and future perspectives, Fish Shellfish Immunol., 35(2013), pp.1740-1750. https://doi.org/10.1016/j.fsi.2013.02.029.

[65] A.B. Romstad, L.J. Reitan, P. Midtlyng, K. Gravningen, Ø. Evensen, Antibody responses correlate with antigen dose and *in vivo* protection for oil-adjuvanted, experimental furunculosis (*Aeromonas salmonicida* subsp. *salmonicida*) vaccines in Atlantic salmon (*Salmo salar* L.) and can be used for batch potency testing of vaccines, Vaccine, 31(2013), pp.791-796. https://doi.org/10.1016/j.vaccine.2012.11.069.

[66] C. Tafalla, J. Bøgwald, R.A. Dalmo, H.M. Munang'andu, Ø. Evensen, Adjuvants in fish vaccines. Fish vaccination, 2014, pp.68-84. https://doi.org/10.1002/9781118806913.ch7.

[67] P.J. Midtlyng, L.J. Reitan, L. Speilberg, Experimental studies on the efficacy and side-effects of intraperitoneal vaccination of Atlantic salmon (*Salmo salar* L.) against furunculosis, Fish Shellfish Immunol., 6(1996), pp.335-350. https://doi.org/10.1006/fsim.1996.0034.

[68] P.J. Midtlyng, L.J. Reitan, A. Lillehaug, A. Ramstad, Protection, immune responses and side effects in Atlantic salmon (*Salmo salar* L.) vaccinated against furunculosis by different procedures, Fish Shellfish Immunol., 6(1996), pp.599-613. https://doi.org/10.1006/fsim.1996.0055.

[69] M. Noia, B. Domínguez, J. Leiro, J. Blanco-Méndez, A. Luzardo-Álvarez, J. Lamas, Inflammatory responses and side effects generated by several adjuvant-containing vaccines in turbot, Fish Shellfish Immunol., 38(2014), pp.244-254. https://doi.org/10.1016/j.fsi.2014.03.020

[70] K.A. Veenstra, T. Wang, A. Alnabulsi, A. Douglas, K.S. Russell, L. Tubbs, J.B. Arous, C.J. Secombes, Analysis of adipose tissue immune gene expression after vaccination of rainbow trout

with adjuvanted bacterins reveals an association with side effects, Mol. Immunol., 88(2017), pp.89-98. https://doi.org/10.1016/j.molimm.2017.05.026.

[71] C.J. Secombes, Fish immunity: the potential impact on vaccine development and performance, Aquac. Res., 42(2011), pp.90-92. https://doi.org/10.1111/j.1365-2109.2010.02673.x.

[72] C.J. Secombes, R. Belmonte, Overview of the fish adaptive immune system. In Fish vaccines 2016, pp. 35-52, Springer, Basel. https://doi.org/10.1007/978-3-0348-0980-1_2.

[73] C.J. Secombes, T. Wang, S. Bird, The interleukins of fish, Dev. Comp. Immunol., 35(2011), pp.1336-1345. https://doi.org/10.1016/j.dci.2011.05.001.

[74] E. Wangkahart, C.J. Secombes, T. Wang, Dissecting the immune pathways stimulated following injection vaccination of rainbow trout (*Oncorhynchus mykiss*) against enteric redmouth disease (ERM), Fish Shellfish Immunol., 85(2019), pp.18-30. https://doi.org/10.1016/j.fsi.2017.07.056.

[75] A. Attaya, Y. Jiang, C.J. Secombes, T. Wang, Distinct response of immune gene expression in peripheral blood leucocytes modulated by bacterin vaccine candidates in rainbow trout *Oncorhynchus mykiss*: A potential *in vitro* screening and batch testing system for vaccine development in aquaculture, Fish Shellfish Immunol., 93(2019), pp.631-640. https://doi.org/10.1016/j.fsi.2019.08.002.

Figure Legends and Tables

Fig. 1. Innate immune parameters of vaccinated fish. Fish were immunized with the SAIV vaccine alone (SAIV), or SAIC adjuvanted with ISA 763A VG (SAIV+763A) and ISA 763B VG (SAIV+763B), or PBS as control (CTRL). Sera were collected at weeks 1, 2, 3, 4 and 5. Eight biological replicates were used per group, and data expressed as the mean + SEM. (A) LZM activity, (B) MPO activity, and (C) bactericidal activity. Bars with different letters indicates a significant difference ($P<0.05$) between the different groups at each time point.

Fig. 2. Cytokine gene expression of vaccinated fish, as assessed by RT-qPCR analysis. Fish were immunized with the SAIV vaccine alone, SAIV+763A, SAIV+763B, or PBS as the control (CTRL). The spleen and liver were sampled at day 1, 3, and 14 after immunization. Three biological replicates

were conducted and expressed as the mean + SEM. Bars with different letters denote significant differences (P<0.05) between the different groups at each time point.

Fig. 3. The specific IgM response of Nile tilapia after vaccination against *S. agalactiae*, as determined by ELISA. Fish were immunized with the SAIV vaccine alone (SAIV), SAIV+763A, SAIV+763B or PBS as the control (CTRL). Sera were collected at weeks 1, 2, 3, 4 and 5. Eight biological replicates were used per group and data expressed as the mean + SEM. Bars with different letters indicate a significant (P<0.05) difference between the different groups at each time point.

Fig. 4. Survival rate of vaccinated and control Nile tilapia, following injection (i.p.) challenge with *S. agalactiae* (at a concentration of 1×10^8 CFU/fish) 5 weeks after vaccination. Fish were prior injected with PBS as control (CTRL), SAIV alone (SAIV), SAIV+763A or SAIV+763B, respectively. Survival was analysed for 21 days post-challenge, when RPS values were calculated.

Fig. 5. Immunohistochemical staining of spleen tissue from fish vaccinated with different types of *S. agalactiae* vaccines at day 21 post challenge, showing the presence of IgM^+ B cells and melanin containing cells (MMCs) at 40X magnification. 1-3A) Control group (CTRL); 1-3B) SAIV alone; 1-3C) SAIV+763A; 1-4D) SAIV+763B. The white arrowheads show IgM^+ B cells and the yellow arrowheads show MCCs. N=3 fish per group.

Fig. 6. Adjuvant persistence in vaccinated Nile tilapia at 5 w.p.v. (A), saline injected fish (CTRL); (B), SAIV injected fish; (C) SAIV+763A injected fish; and (D) SAIV+763B injected fish. Arrowheads indicate persistence of Montanide™ ISA 763A VG and Montanide™ ISA 763B VG in the peritoneal cavity of fish that received the adjuvanted vaccines.

Table 1. Treatment groups and details of experimental vaccines in the trial.

Table 2. Primers used in this study.

Table 3. Relative IgM^+ B cell and melanin containing cell densities in spleen tissue of fish injected with PBS (CTRL) or different types of SAIV vaccine, as assessed by immunohistochemical staining. Analysis was performed at day 21 post-challenge with live *S. agalactiae*. N=3 fish per group.

Table 3. (**Note):** Average number of IgM^+ B cells [(+) 1–10 positive cells, (++) 11–20 positive cells and (+++) ≥21 positive cells], and melanin containing cells (MCCs) [(+) 1–50 MCCs, (++) 51–100 MCCs and (+++) >100 MCCs] in the spleen.

Table 1. Treatment groups and details of experimental vaccines in the trial.

Group no.	Treatment	Abbreviation
1	Injection with PBS (control group)	CTRL
2	Injection with *S. agalactiae* inactivated vaccine alone	SAIV
3	Injection with *S. agalactiae* inactivated vaccine and Montanide^{TM} ISA 763A VG	SAIV+763A
4	Injection with *S. agalactiae* inactivated vaccine and Montanide^{TM} ISA 763B VG	SAIV+763B

Table 2. Primers used in this study.

Gene	Accession no.	Primer	Nucleotide sequence (5' → 3')	Annealing Temp °C
β-actin	XM003443127	Fw	ACAGGATGCAGAAGGAGATCACAG	60
		Rv	GTACTCCTGCTTGCTGATCCACAT	
IFN-γ	NM_001287402	Fw	GAAACTTCTGCAGGGATTGG	60
		Rv	CTCTGGATCTTGATTTCGGG	
IL-1β	FF280564	Fw	AAGATGAATTGTGGAGCTGTGTT	60
		Rv	AAAAGCATCGACAGTATGTGAAAT	
IL-6	XM_019350387	Fw	ACAGAGGAGGCGGAGATG	60
		Rv	GCAGTGCTTCGGGATAGA	
IL-8	NM001279704	Fw	GCACTGCCGCTGCATTAAG	59
		Rv	GCAGTGGGAGTTGGGAAGAA	
TNF-α	NM001279533	Fw	AGGGTGATCTGCGGGAATACT	60
		Rv	GCCCAGGTAAATGGCGTTGT	

Abbreviations: β-actin: beta actin; IFN-γ: interferon gamma; IL-1β: interleukin 1 beta; IL-6: interleukin 6; IL-8: interleukin 8; TNFα: tumor necrosis factor alpha; Fw: forward; Rv: reverse; Temp: temperature

Table 3. Relative IgM^+ B cell and melanin containing cell densities in spleen tissue of fish injected with PBS (CTRL) or different types of SAIV vaccine, as assessed by immunohistochemical staining. Analysis was performed at day 21 post-challenge with live *S. agalactiae*. N=3 fish per group.

Cell-type/time point	CTRL	SAIV	SAIV+763A	SAIV+763B
IgM^+ cells				
Pre-challenge	+	+	++	++
Post-challenge	+	++	+++	+++
Melanin containing cells (MCCs)				
Pre-challenge	+	+	++	++
Post-challenge	++	++	+++	+++

Note: Average number of IgM^+ B cells [(+) 1–10 positive cells, (++) 11–20 positive cells and (+++) ≥21 positive cells], and melanin containing cells (MCCs) [(+) 1–50 MCCs, (++) 51–100 MCCs and (+++) >100 MCCs] in the spleen.

Fig.1

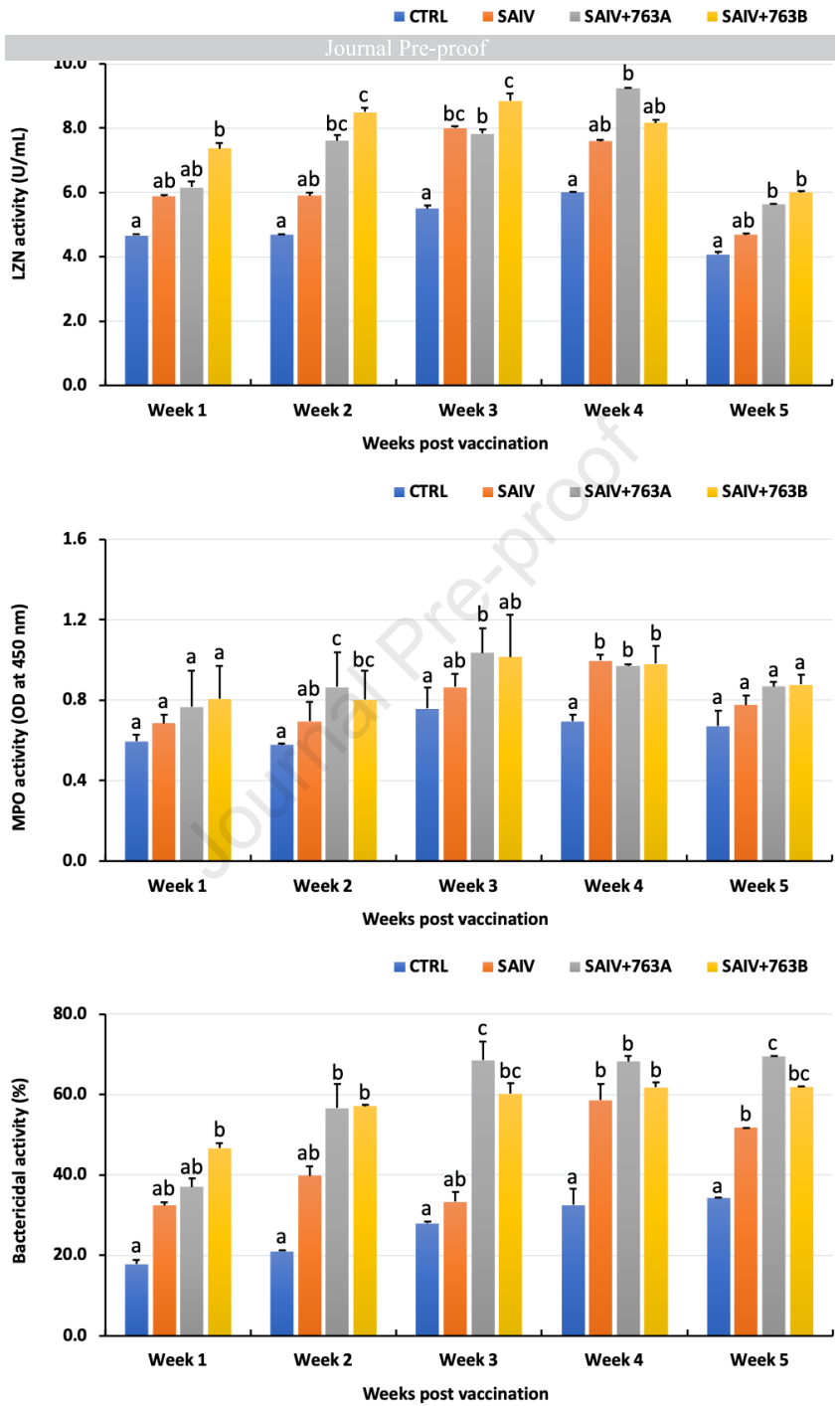


Fig.2

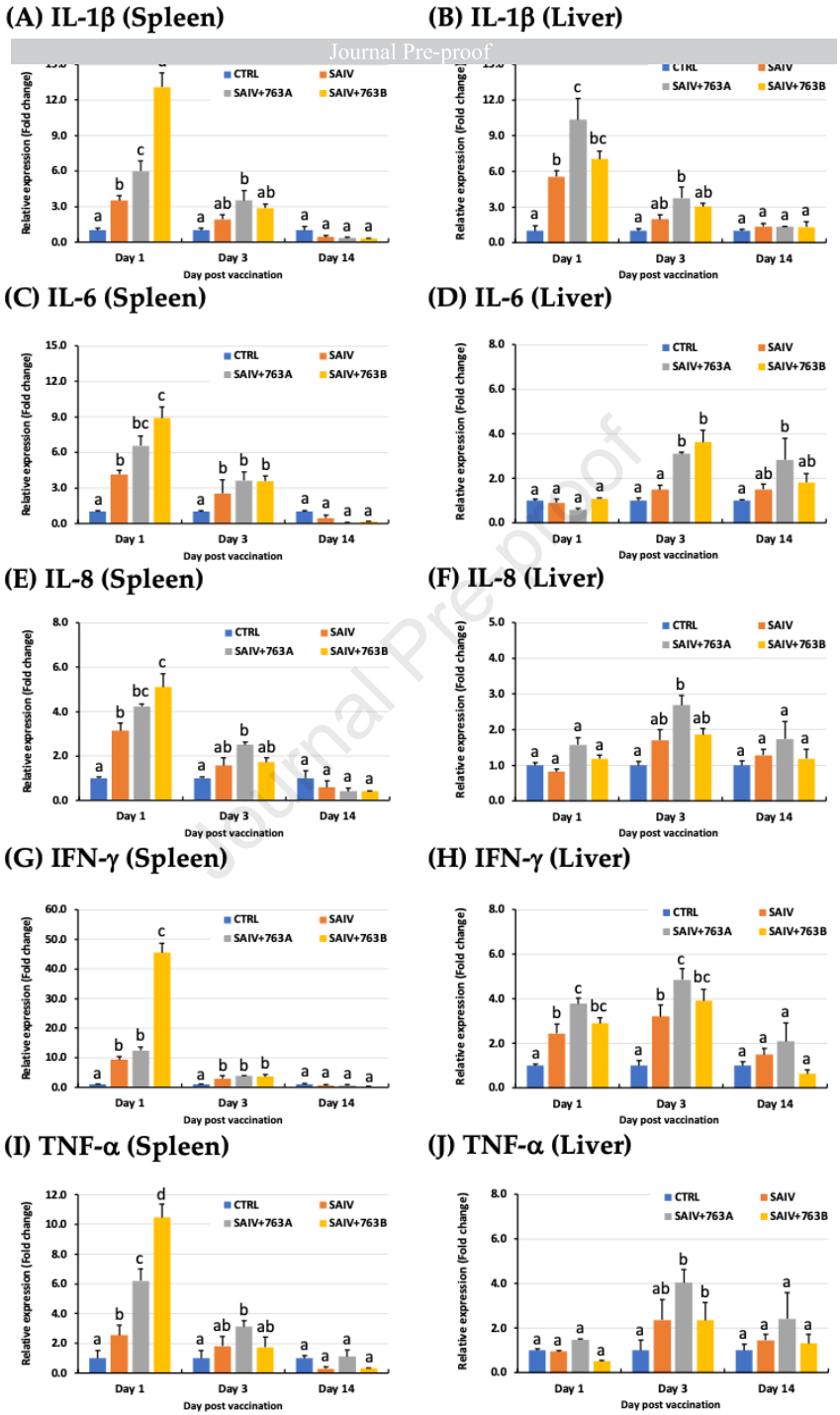


Fig.3

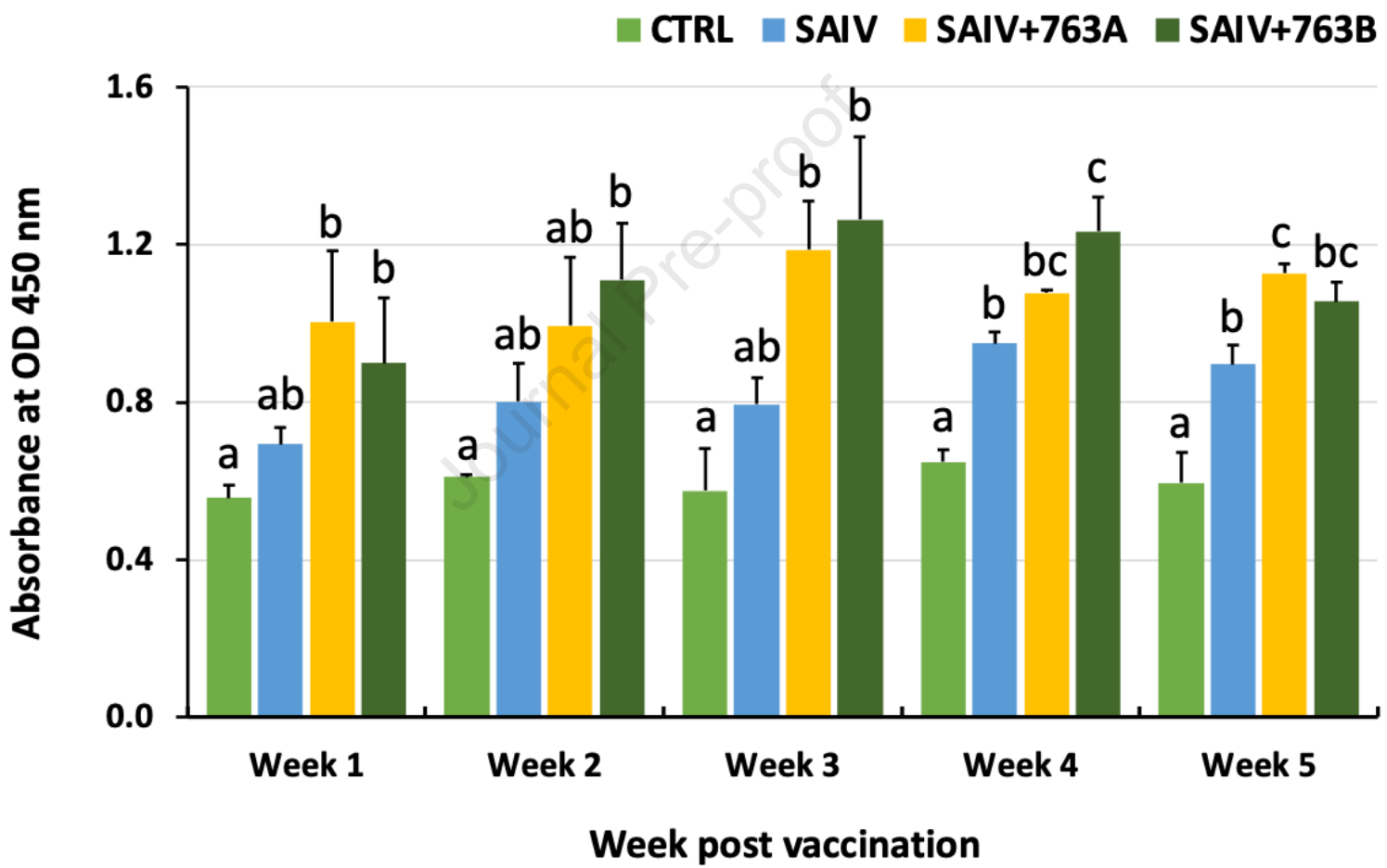
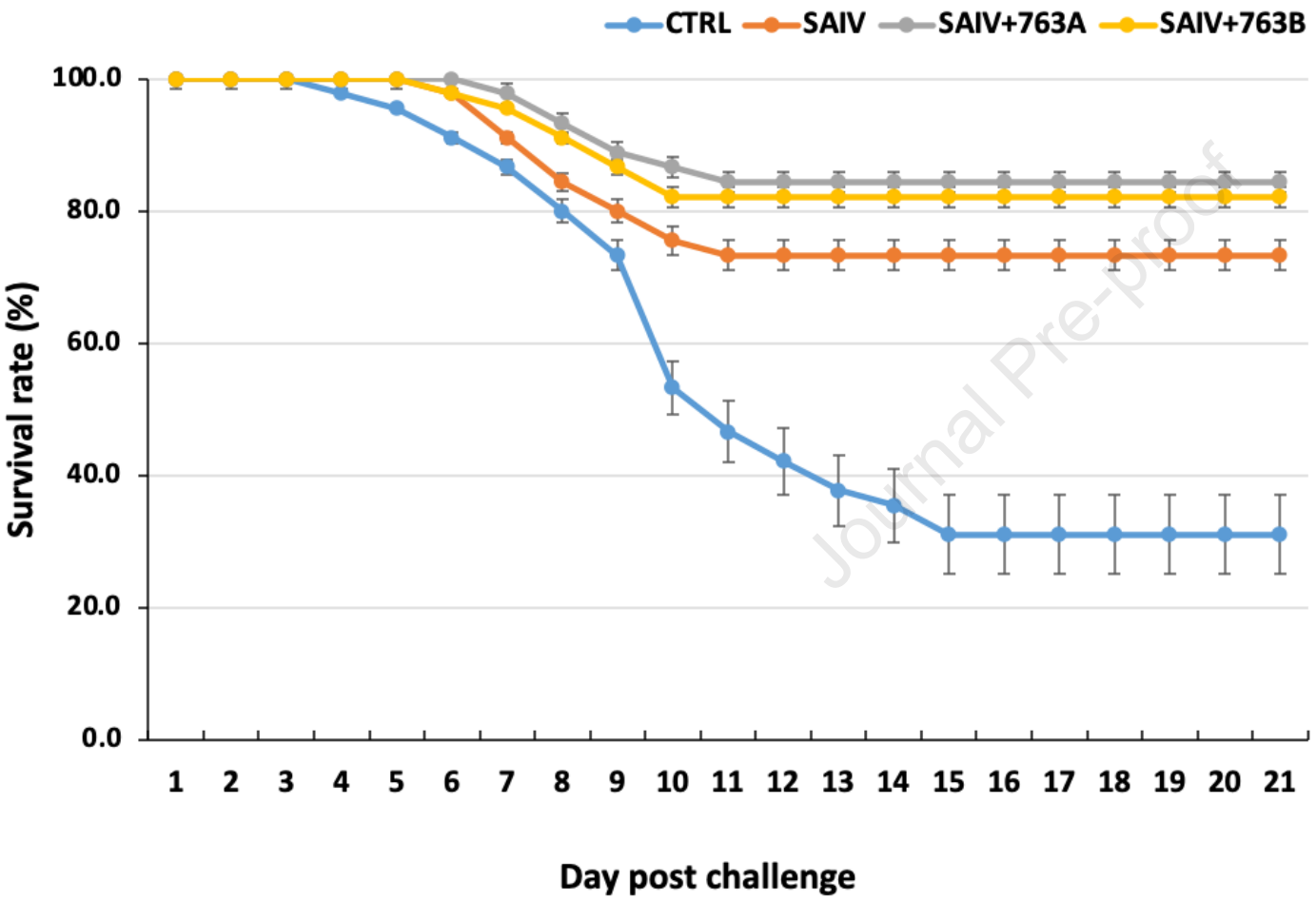


Fig.4



Group	Mortality	% Mortality	RPS
CTRL	31	68.89	–
SAIV	12	26.67	61.29 [b]
SAIV+763A	7	15.56	77.42 [a]
SAIV+763B	8	17.78	74.19 [a]

Group	SAIV	SAIV+763A	SAIV+763B
CTRL	P < 0.0001	P < 0.0001	P < 0.0001
SAIV		P < 0.0001	P < 0.0001
SAIV+763A			P = 0.076

Fig.5

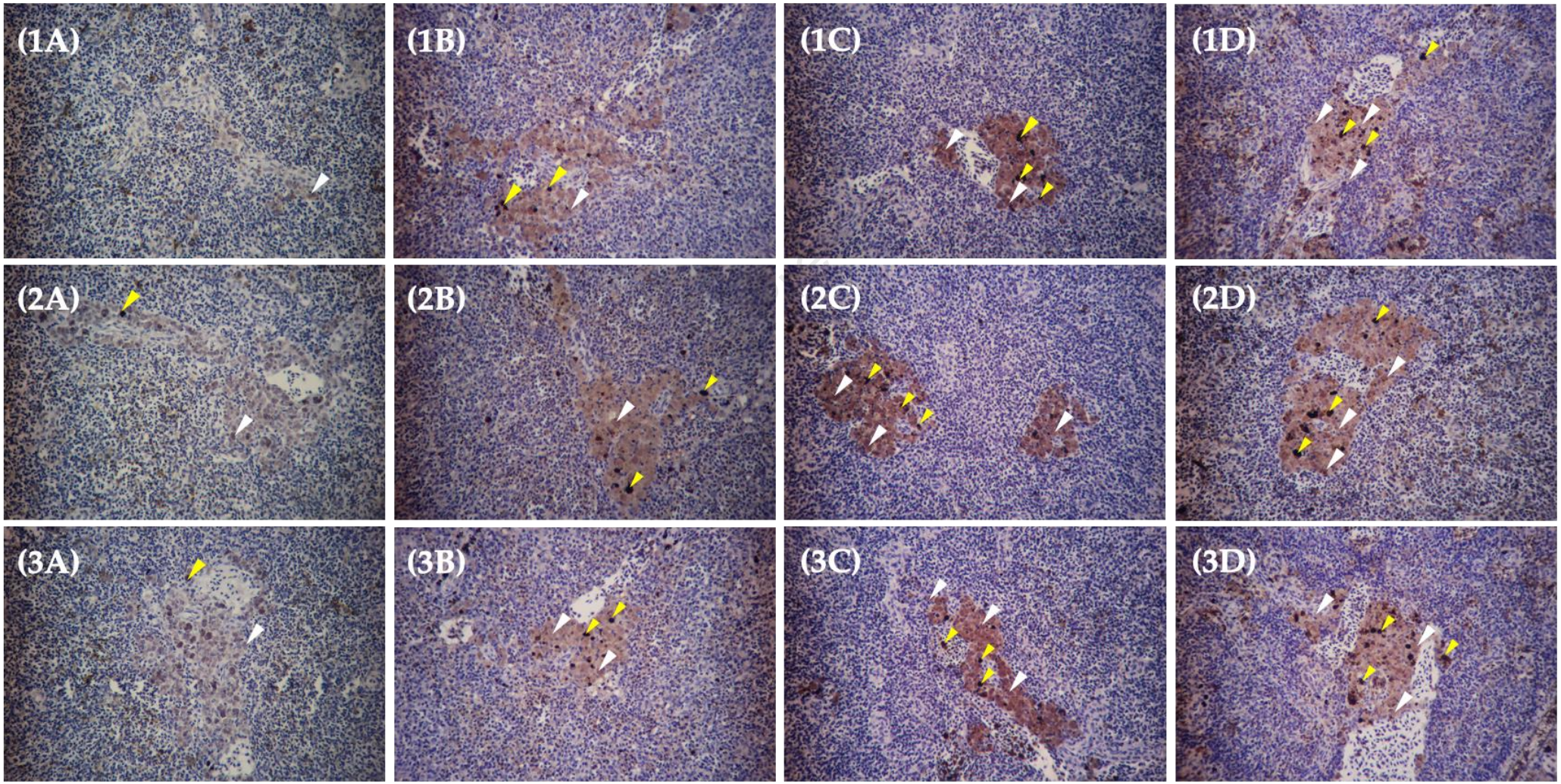
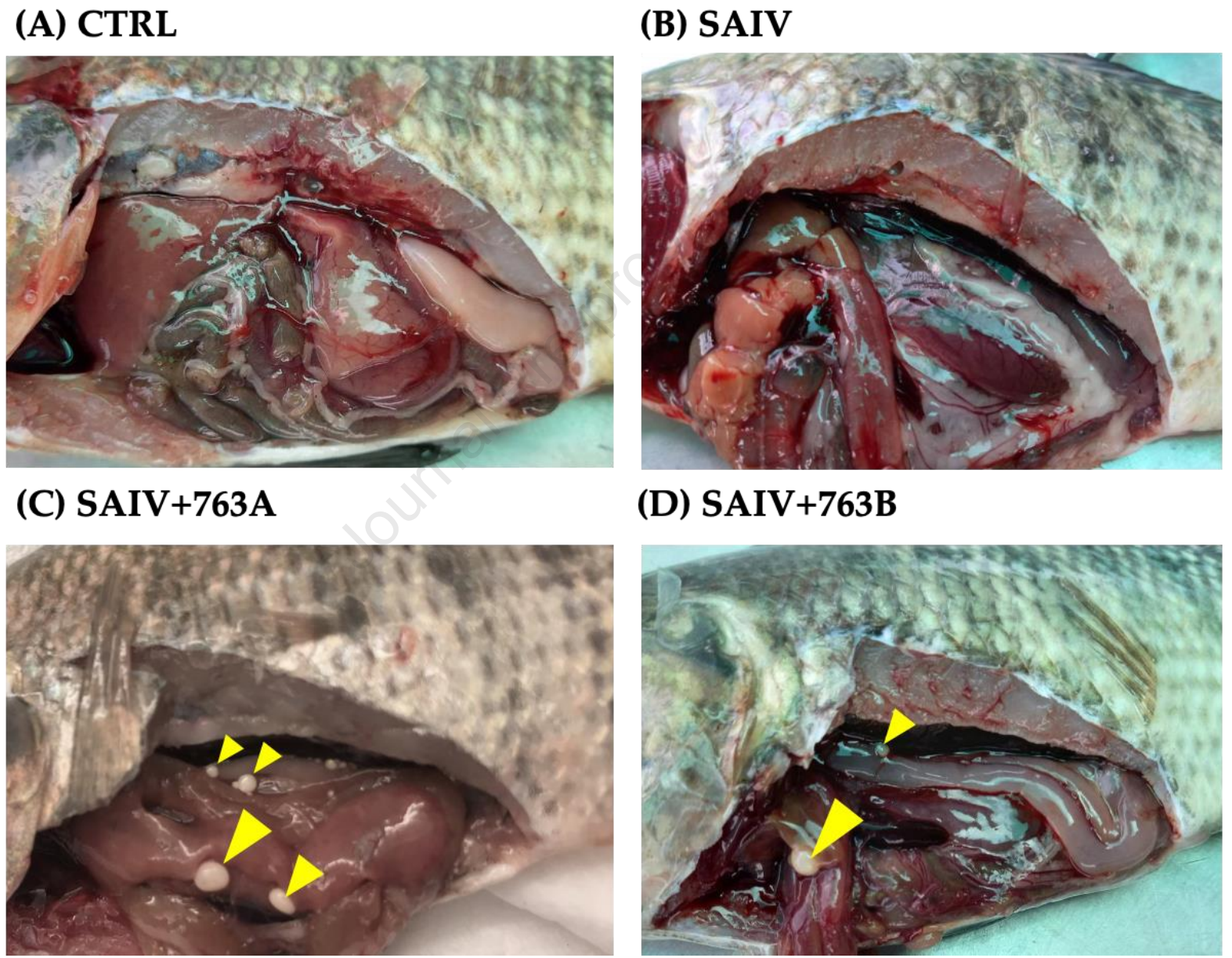


Fig.6



Highlights

- An *Streptococcus agalactiae* inactivated vaccine (SAIV) was made to vaccinate Nile tilapia
- Tilapia were injected with SAIV plus Montanide™ ISA 763A VG and Montanide™ ISA 763B VG
- SAIV with Montanide™ ISA 763A VG or Montanide™ ISA 763B VG increased innate immunity
- SAIV with Montanide™ ISA 763A VG or Montanide™ ISA 763A VG induced specific IgM and pro-inflammatory cytokine expression
- SAIV with Montanide™ ISA 763A VG or Montanide™ ISA 763B VG gave excellent protection to *S. agalactiae*