Analysis of the efficacy of two molecular adjuvants,flagellin and IFN-γ,on the immune response against Streptococcus agalactiae in Nile tilapia (Oreochromis niloticus)

Preetham elumalai

preetham@cusat.ac.in

Centre for Aquatic Vaccine Development, Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, Kochi, Kerala, India.

Sreeja Lakshmi

King Nandhivarman College of Arts and Science, Thellar, Tamil Nadu, India.

Nandha kumar

Centre for Aquatic Vaccine Development, Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, Kochi, Kerala, India.

Ritam Guha

Centre for Aquatic Vaccine Development, Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, Kochi, Kerala, India.

Alex Wang

Eakapol Wangkahart

Tiehui Wang

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Abstract

Aquaculture industry frequently encounters disease outbreaks, high mortalities as well as emergence of new pathogens due to its intensification. *Streptococcus agalactiae* (Lancefield's group B Streptococcus) is an important pathogen extensively causing infectious diseases in tilapia resulting in huge economic loss and mortality rates. To date, vaccination has been approved to be successful in defending infectious diseases prevailing among farmed fish species. This study aimed to develop an *S. agalactiae* inactivated vaccine (SAIV) using molecular adjuvants- flagellin and tilapia interferon gamma (IFN-γ), and to assess the generated immune response and protective efficacy of the adjuvant incorporated vaccine against the *S. agalactiae* infection in Nile tilapia. The fish were vaccinated with SAIV together with either flagellin or IFN-γ and both together by intraperitoneal injection. The vaccinated fish were challenged with a virulent strain of *S. agalactiae* on day 36 and monitored three weeks for cumulative mortality. The results showed that the vaccine offered significant protection with relative percentage survival (RPS) of 59.37%, 71.87% and 81.25% observed for bacterin vaccine adjuvanted with flagellin, IFN-γ and both, respectively, with an RPS of 15.62% for the unadjuvanted bacterin control group after challenge with *S. agalactiae*. The vaccine induced specific IgM antibodies against *S. agalactiae* in the vaccinated groups and the antibody response was significantly increased following booster vaccination in the fishes administered with vaccine adjuvanted with flagellin, IFN-γ and both. Furthermore, after vaccination MHC-II and IgM gene expression was found significantly upregulated in head kidney and spleen, in line with elevated specific IgM titer. Innate immune parameters including catalase, lysozyme, superoxide dismutase, myeloperoxidase and bactericidal activities were significantly increased in fishes immunized when compared to the unvaccinated controls ($P < 0.05$). Histopathological examinations with tissue sections of head kidney, spleen, liver, kidney, gills and brain were performed from fish vaccinated and non-vaccinated showed mild infiltrations. In conclusion, flagellin and IFN-γ have shown potential for use as molecular adjuvants to enhance the efficacy of fish vaccines against *S. agalactiae* infections.

1. Introduction

Nile tilapia (*Oreochromis niloticus*) is one of the most farmed fish species across the world and its production is expected to reach 7.3 million by 2030 (Kobayashi et al. 2015,WorldBank 2013). This fish species is considered as economically significant fish owing to many factors like its easiness in cultivation, hardiness, sustainability, economical price, and adaptability to various growth conditions (Buentello et al.2007). Intensification of tilapia farming has triggered disease out breaks by infectious pathogens causing mortality and huge economic loss (Aich et al.2022). *S. agalactiae* is a major bacterial pathogen causing mass mortalities in tilapia resulting in huge economic loss worldwide (Wangkahart et al.2022). Streptococcal disease caused by *S. agalactiae* is characterized by exophthalmia, loss of orientation, septicemia, meningoencephalitis, ulcers, lethargy and dead (Klesius et al. 2008).

Vaccination is an alternative method for preventing the infectious diseases in aquaculture, with most vaccines delivered by intraperitoneal (IP) injection (Munang'andu et al. 2016). It is important to realize that to ensure best profit margins by reducing mortality rates of fishes, vaccination along with good

nutrition, sanitary practices and biosecurity maintenance are utmost important as far as aquaculture industry is concerned. Different types of vaccines have been developed against *S. agalactiae* infections in tilapia such as live attenuated vaccines, inactivated vaccines, DNA vaccines, and subunit vaccines (Langemann et al. 2010, Ma et al. 2019, Jorge et al.2017, Su et al. 2018). Recent practice of employing adjuvants to optimize vaccine efficacy as well as to enhance the host immune response is highly studied. Flagellin, the principal structural protein of flagellum in bacteria, is identified as a powerful pathogen-associated molecular patterns (PAMPs) (Zhao et al. 2015). Among the 4 unique globular domains- D0, D1, D2 and D3, D1 domain directly binds with Toll like receptor 5 (TLR5) expressed by antigen-presenting cells and T cells. Following the activation of TLR5, effective immunity is developed by generating chemokines, pro-inflammatory cytokines and co-stimulatory molecules (Hayashi et al. 2001). Previous studies have reported the role of flagellin as an immunostimulant to stimulate the expression of proinflammatory cytokines *in vitro* such as IL-1β, IL-6, TNF-α, IL-8 (Chettri et al.2011, Hynes et al. 2011). Another study has shown that a recombinant flagellin from *Y. ruckeri* rapidly induces the expression of pro-inflammatory cytokines *in vivo* as well as the Th17 cytokine IL-17A/F1 and Th2 cytokine IL-4/13, but not the Th1 cytokine IFN-γ (Wangkahart et al. 2016). It was also observed that some acute phase proteins (APPs) and anti-microbial peptides (AMPs) were upregulated quickly to subsequently impart a potential platform to defend the bacterial infection mediated by flagellin (Wangkahart et al. 2016). Moreover, flagellin in synergy with IFN-γ, upregulated the expression of specific IL-12 family members (Wangkahart et al. 2016). The observations suggest the use of flagellin as adjuvant in stimulating immune response for use in aquaculture. Previous studies have shown the role of IFN-γ in innate as well as adaptive immune responses carrying a pivotal role in elucidating Th1 immune response in all vertebrates. The upregulation of IFN-γ expression was observed in different stimuli like LPS, Poly I: C and bacterial infections. Fish IFNγ can upregulate the expression of adaptive cytokines, eg. IL-12, IL-15 and IL-21, important for vaccine-mediated defense (Liu et al. 2023). Thus, the role of tilapia IFN-γ in immune response against invading pathogens in tilapia is found to be interesting (Velázquez et al. 2017). We hypothesized that a balanced Th1/Th2 and Th17 immune response is important for promoting protective immunity and eliminating tissue injury (pathological inflammation). Inclusion of a flagellin as Th2 and Th17 signals and IFN-γ as a Th1 signal in a vaccine formulation may enhance vaccine efficacy. The current study aimed to investigate the effect of recombinant flagellin and tilapia IFN-γ proteins as molecular adjuvants in enhancing the immune response in tilapia when used along with the SAIV vaccine.

2. Materials and Methods

2.1 Experimental fish and ethics statement

Nile tilapia, weighing approximately 10 g were obtained from a commercial fish farm. Fish were placed in 100 L fiber glass tanks with density 60 fish per tank. Fish were fed with a commercial diet with 32% protein and 4% lipid twice a day for 2 weeks prior to the vaccination trial. Water quality parameters (pH

5.85–6.86, dissolved oxygen 5.1–7.7 mg/L, nitrite 0.012 ± 0.009 mg/L and ammonia 0.04–0.10 mg/L) were checked routinely and water was changed twice per week.

The experiments were performed following the guidelines of the Committee for Purpose of Control and Supervision of Experiments on Animals (CPCSEA) Registration number: 363/GO/Re/S/01/CCSEA/82. This study has been approved by Institutional Animal Ethics Committee of Cochin University of Science and Technology, Kochi, Kerala, India.

2.2 Recombinant flagellin and IFNγ production and purification

Recombinant *Y. ruckeri* flagellin was first produced under denaturing conditions as described previously (Wangkahart et al. 2016). Minor contaminants were further removed by Anion exchange chromatography using HiTrap Q FF column (Cytiva). The buffer system used are Buffer A (50 Tris-HCl, pH7.4, 8 M Urea) and Buffer B (4 parts Buffer A + 1 part 5 M NaCl). The purified protein was refolded in 1X phosphate buffered saline (PBS, pH 7.4) containing 500 mM Arginine and 10% glycerol and changed to storage buffer (1x PBS, pH 7.4 containing 10% glycerol) using a Protein Concentrators PES (30K MWCO Thermo Scientific™ Pierce™). The protein was quantified on SDS-PAGE using BSA as reference and was 1.13 mg/mL.

Nile tilapia IFN-γ was based on NCBI entry (acc. No. NP_001274331) and have a predicted signal peptide (1–22) and a mature peptide (amino acid 23–206). The recombinant IFN-γ has a His tag (MAHHHHHHHHGSGS) at the N-terminal followed by the mature peptide sequence of tilapia IFN-γ. The recombinant protein is 198 amino acids with a molecular weight of 22.70 kDa and a theoretical pI of 10.14. The DNA sequence was codon-optimised according to *E. coli* codon usage using GENEius software (Eurifins Genomics), synthesized and cloned into a pET vector, and purified under denaturing condition as described previously (Costa et al. 2011, Wang et al. 2018). Minor contaminants were further removed as described above. The buffer system used is Buffer A (50mM HEPES, pH8.0, 8 M Urea) and Buffer B (4 parts Buffer A + 1 part 5 M NaCl). The single band purified protein was refolded in PBS, pH 7.4 containing 500 mM Arginine and 10% glycerol and changed to storage buffer (PBS, pH 7.4 containing 10% glycerol) using a Protein Concentrators PES (10K MWCO Thermo Scientific™ Pierce™). The protein was quantified on SDS-PAGE using BSA as reference and was 2.35 mg/ml.

2.3 Bacterial strain and vaccine preparation

The *S. agalactiae* inactivated whole-cell vaccine (SAIV) was prepared in Brain Heart Infusion (BHI) Broth with shaking at 180 rpm at 30°C for 12 hours (h). The bacterial culture was inactivated by adding formalin (0.5%) to the bacterial culture, and then incubated for 24 h at 28°C. The final bacterial density obtained before formalin inactivation was 10^9 colony forming unit (CFU)/mL. The death of bacteria was determined by the absence of growth on BHI agar plates after 48 h of incubation at 30°C. The bacterial culture was centrifuged at 5000 rpm for 30 min to get the pelleted inactive bacteria. The pellets were washed three times with PBS and was resuspended in PBS till 0.67 OD at 600 nm. Initially, the bacterin

was adjusted in PBS at $2x10^9$ CFU/mL and then made different vaccine formulations with 15% bacterin, 70% oil adjuvant plus 15% proteins (protein + PBS).

2.4 Fish immunization and challenge

Fish were randomly divided into 5 groups with triplicates (25 fish per group) as detailed in Table 1. Fish were injected intraperitoneally (i.p.) with each vaccine formulation. Group 1 (G1): fish injected i.p. with 100 µL PBS (control group); Group 2 (G2): fish injected i.p. with 100 µL SAIV vaccine containing Montanide™ ISA 763A VG, Seppic, France); Group 3 (G3); fish injected i.p. with 100 µL SAIV vaccine containing flagellin; Group 4 (G4): fish injected i.p. with 100 µL SAIV vaccine containing IFN-γ; and Group 5 (G5): fish injected i.p. with 100 µL SAIV vaccine containing flagellin and IFN-γ. The SAIV formulation with Montanide™ ISA 763A VG was performed as described previously (Wangkahart et al. 2023, wangkahart et al. 2021). Booster immunization was carried out 21 days post vaccination (dpv) using the same vaccine dose.

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

Group no.	Treatment	Abbreviation
1	Injection with PBS (control group)	G1
2	Injection with SAIV + Montanide™ ISA 763A VG	G2
3	Injection with SAIV + flagellin	G3
4	Injection with SAIV + IFN-g	G4
5	Injection with SAIV + flagellin + IFN-g	G5

For *S. agalactiae* challenge, fish from each experimental group were injected i.p. 36 dpv with a virulent strain (100 µL of 107 CFU/mL) of *S. agalactiae.* The cumulative mortality was recorded for 21 days and relative percent survival (RPS) was calculated.

2.5 Blood and tissue sample collection

After 15 and 36 dpv, fish were fasted for 24 h. Before collecting the sample, the fish were euthanized with a lethal dose of clove oil (250 ppm) and 500 µL blood was collected from the caudal vein. Blood was allowed to clot for 4 h at 4°C. Serum was collected after centrifuging the sample at 3000 rpm for 3 minutes (min) and stored at -20°C. For immune gene expression analysis, the tissues (spleen and head kidney) were collected at fixed time points (days 15 and 36) following immunization. Tissues were collected and stored in sterile tubes having 500 µL RNAlater (Bio-Rad) at − 20 ◦C until use.

2.6 Enzyme linked Immunosorbent assay (ELISA)

96–well ELISA plates (Immulon 4HBX, Thermo Scientific) were pre-treated with 50 µL of 0.05% w/v poly–L–lysine in carbonate–bicarbonate buffer (0.05 M carbonate-bicarbonate pH 9.6 (Sigma-Aldrich,UK). The plates were incubated at room temperature (RT) for 60 min. Plates were washed with PBS 3 times. One-

hundred microliters bacteria at 108 CFU/mL, were added to each well and plates were incubated overnight at 4°C. The bacteria were previously prepared by growing them on TSB at 24°C for 48 h with continuous shaking at 150 rpm and pelleted. The pellet was washed 3 times with 1X PBS, resuspended and adjusted to OD_{600} 1.0. The plates were washed 3 times with 1xPBS and blocked with 2% w/v Bovine serum albumin (BSA) in PBS (300 µL) at RT for 2h. Plates were washed with PBS three times followed by 3 times with PBS containing 0.05% Tween − 20 (PBST). Diluted fish serum (100 µL per well; from 1:128) in PBS were added to the plates and incubated overnight at 4°C. After the overnight incubation, plates were washed 5 times with PBST. Anti–tilapia IgM MAbs (Aquatic Diagnostics Ltd, UK) diluted 1:50 with 0.01% Bovine Serum Albumin (BSA) in PBS was then added to each well (100 µL), and incubated for 90 mins at 37°C. The plates were then washed 3 times with PBS and 3 times with PBST as previously described. Rabbit anti–mouse–horseradish peroxidase (HRP) conjugate (Sigma-Aldrich, UK) diluted 1:4000 with 0.01% BSA in PBS was added to the plates and incubated for 90 mins at 37°C. Chromogen in substrate buffer (prepared by adding 150 µL of chromogen 42 (3, 3', 5, 5' Tetra methyl-benzidine, TMB) to 15 mL of substrate buffer activated by 5 µL H_2O_2 in 6 mL of 50% acetic acid) was then added (100 µL/well) for assay development. The plates were incubated for 10 min at RT and the reaction stopped by adding 50 µL of 2M H_2SO_4. The absorbance was measured at OD_{450} using a 96-well plate spectrophotometer Varioskan LUX Multimode Microplate Reader (Thermo Scientific laboratory, USA). The sensitivity threshold of the assay was determined as 3X the absorbance value of wells containing PBS (background absorbance). Samples above this value were considered positive for specific antibodies.

2.7 RNA extraction and quantitative real- time PCR (qPCR)

Spleen and head kidney tissues from 3 fish per group were collected at 15 and 36 dpv. The total RNA was extracted by Trizol method (TRIzol™, Sigma Aldrich, France) and converted to cDNA using Bio-Rad iScript™ cDNA synthesis kit (Bio-Rad) according to the manufacturer protocol. The immune related genes, including CD4, IgM, MHC-I, MHC-II, TCRβ, IL-1β, IL-6 and IL-8 were investigated by RT-qPCR method. The primers used in this study were shown in Table 3. The final volume of the RT-qPCR reaction mixture (20 µL) composed of 1 µL cDNA template, 0.5 µL of 10 µM forward primer, 0.5 µL of 10 µM reverse primer, 2 µL enzyme mixture, 4 µLRNAse-free water, and 10 µL 2X master mix. The RT-qPCR analysis was carried out using CFX 96 Touch Real Time PCR detection system (Bio-Rad, USA) and Sso Advanced Universal SYBR green supermix (Bio-Rad, USA). PCR cycling conditions were initial denaturation for 3 min at 95°C followed by 40 cycles of denaturation for 20s at 95°C, annealing for 20s at 64°C, extension for 30s at 72°C and a final extension for 5 min at 72°C. The relative gene expression of each gene was normalized to the reference gene β-actin and presented as fold change.

2.8 Histology

In order to analyse, Head kidney, spleen, liver, kidney, gills and brain were sampled and fixed with the fixative solution (10% Formalin (37–40% stock solution), 4 g/L NaH_2PO_4 and 6.5g/L Na_2HPO_4). Samples tissues were embedded in paraffin, sectioned at 5 µm, and subjected to staining with hematoxylin and eosin (H&E). Light microscopy was used to examine the tissue sections.

2.9 Immunological assays

The superoxide dismutase (SOD) activity in serum was assayed according to the protocol previously described by Paoletti et al. (Paoletti et al. 1990). The catalase (CAT) activity was assessed according to Sinha et al (Sinha et al. 1972) whereas myeloperoxidase (MPO) and lysozyme activity was measured according to the protocol reported in Wangkahart et al. (Wangkahart et al. (2023)). The bactericidal assay was performed in sterile micro-tubes following Takahashi et al. (2013) with modifications (Takahashi et al. 2013). Briefly, 10 µL of 1 × 10^8 CFU of *S. agalactiae* serotype III suspension and 10 µL of fish serum were incubated for 3 h at 37°C in a sterile microcentrifuge tube. The resulting suspension, along with a positive group made up of TSA plates containing bacteria and PBS suspension in place of the fish serum was cultivated on tryptic soy agar (TSA) and incubated overnight at 37°C. After being incubated overnight, the CFU was manually counted. The bactericidal activity was evaluated as: serum bactericidal rate (%) = (1 − the number of viable bacteria after immune serum treatment/the number of viable bacteria after PBS treatment) × 100% (Cao et al. 2018).

2.10 Statistical analysis

The efficacy of vaccines was estimated by calculating the relative percentage survival (RPS). RPS highlights the relationship between mortality in the vaccinated group and the unvaccinated group, and calculated according to Amend (1981) (Amend 1981) using the following equation:

$$\mathrm{RPS} = \left[1 - \left(\frac{\%\ mortality\ of\ vaccinated\ group}{\%\ mortality\ of\ control\ group}\right)\right]\ \mathrm{x}\ 100$$

A Mantel-Cox log-rank test was performed on the survival curves. Statistical analysis was performed using a one-way analysis of variance (ANOVA) and Turkey`s Multiple Range Test using IBM SPSS version 11.5 (SPSS Inc., Chicago, IL, USA) was used to perform the mean comparisons between the treatment groups and considered statistically significant at $P < 0.05$.

3. Results

3.1 Production of recombinant flagellin and IFN-γ

Recombinant protein expression and purification under denaturing condition was described previously (Costa et al. 2011, Wang et al. 2018). Minor contaminants were further removed by ion exchange chromatography. The resulting proteins were refolded and buffer changed to a storage buffer (PBS containing 10% glycerol) and kept at -80°C freezer ready to use (Table S1). Recombinant flagellin and IFN-γ were observed on SDS PAGE with molecular sizes of 45.2 kDa and 22.7 kDa respectively (Fig. S1).

3.2 Kinetics of specific IgM antibody response

The serum samples were collected at 15 and 36 dpv and *S. agalactiae*-specific IgM antibody response were measured by ELISA. There is no difference on IgM titer between groups at 15 dpv, and between

unvaccinated group 1 (G1) and adjuvanted vaccine G2 at 36 dpv (Fig. 1). However, specific IgM antibodies were increased in adjuvanted vaccine G3, G4 and G5 on 36 dpv. In addition, adjuvanted vaccine in G5 showed the highest IgM level amongst the adjuvanted vaccine groups and higher than adjuvanted vaccine of G3, suggesting both flagellin and IFN-γ have contributed to the increased antibody response. The expression of IgM antibodies indicates the enhanced activity of molecular adjuvants in increasing the immunity in tilapia.

3.3 Vaccine efficacy against *S. agalactiae* challenge

The survival rates of G1, G2, G3, G4, and G5, vaccinated group at this time were 60.00%, 28.89%, 20.00%, and 13.33%, respectively. This gave RPS value of 15.62%, 59.37%, 71.87% and 81.25% was observed for G2, G3, G4, and G5, respectively, after challenge with *S. agalactiae* (Fig. 2). The highest protection was observed in fish immunized with *S. agalactiae* adjuvanted with both flagellin and IFN-γ (Group 5).

3.4. Histological Examination

Histopathological examinations of the head kidney, spleen, liver, kidney, gills and brain were performed on fish vaccinated and control groups, the results are presented in Fig. 3 and Table 2. In short, no histological changes were apparently visible in kidney, head kidney, gills, liver and brain tissues of fish administered with SAIV adjuvanted with both flagellin and IFN-γ (Group 5). Decreased melanomacrophage cells with least inflammation was observed in spleen. Unvaccinated control group (G1) was observed with necrosis in most of the tissues. Altogether, no remarkable histological changes were observed in fish administered with SAIV with flagellin (G3) or IFN-γ (G4).

Table 2
Histopathological observations of head kidney, spleen, liver, kidney, gills and brain of fish groups G1, G2, G3, G4, G5

	G1	G2	G3	G4	G5
Head kidney	necrotic centers with hyaline degeneration	macrophages with hemorrhage	Necrotic tubular epithelial Fibrin with bacterial accumulation	No histological changes with mild infiltration.	No histological changes with mild infiltration.
Spleen	Severe multifocal necrotic splenitis with splenic necrosis	Vascular sinuses congestion	Increased inflammation observed	Increased Melano macrophage cells	Decreased Melano macrophage cells with decreased inflammation.
Liver	Necrosis and vacuolar degeneration	Liver congestion	Hepatocytes swelling and pancreatic vascular congestion with accumulation	No histological changes seen.	No histological changes seen.
Kidney	Granulomas containing necrotic centers, macrophages and fibroblast with leucocytes infiltration	necrosis in tubular epithelial cells	Fibrin precipitation with bacterial accumulation	swelling of renal tubular cells with fibroblast	No histological changes with mild infiltration.
Gills	lamellar sinusoids with congestion and multifocal hemorrhages	enlarging and congesting central venous sinus	in gill lamellar epithelial cells with mild hyperplasia	Hyperplasia in the gill lamella	No histological changes in gill and lamella.
Brain	Thickening and congestion of the meninges with inflammatory cells infiltration (arrow) with micro venous thrombosis	Hyperplasia of microglial cells show strong positive signa	Capillary swelling shows weakly positive signal	Few inflammatory cells infiltration in meninges	No histological changes

Table 3
Primers used in this study for qRT-PCR

Genes	Oligo sequence (5'-3')	Product size (base pair)	Reference
IgM	F: GGATGACGAGGAAGCAGACT R: CATCATCCCTTTGCCACTGG	122	KJ676389
CD4	F: GCTCCAGTGTGACGTGAAA R: TACAGGTTTGAGTTGAGCTG	106	XM_025911776
MHC-I	F: TTCTCACCAACAATGACGGG R: AGGGATGATCAGGGAGAAGG	132	XM026157132
MHC-II	F: GAGGAACAAGCTCGCCATCG R: AGTCGTGCTCTGACCTCGAG	106	JN967618
TCR β	F: GGACCTTCAGAACATGAGTGCAGA R: TCTTCACGCGCAGCTTCATCTGTT	113	HM162889
IL-6	F: ACAGAGGAGGCGGAGATG R: GCAGTGCTTCGGGATAGA	161	XM_019350387
IL-8	F: GCACTGCCGCTGCATTAAG R: GCAGTGGGAGTTGGGAAGAA	135	NM001279704
IL-1β	F: AAGATGAATTGTGGAGCTGTGTT R: AAAAGCATCGACAGTATGTGAAAT	175	FF280564
β-actin	F: ACAGGATGCAGAAGGAGATCACAG R: GTACTCCTGCTTGCTGATCCACAT	155	XM003443127

3.5. Immune response parameter analysis

The antioxidant defense parameters of the vaccine groups are described in Fig. 4. The lysozyme (LZM) and catalase (CAT) activity (Fig. 4A and 4B) in serum were significantly increased in vaccinated groups at the two sampling points. We found that G5 showed the significantly higher in both the time points. The enzyme increased at 36 dpv indicated the efficacy of the booster vaccine increasing the innate defense mechanism in fish. The highest bactericidal activity was also seen in G5 in both the time points examined compared to the G1 group (Fig. 4C). Similarly, the G5 showed the significant upregulation in the SOD and MPO activities at 15 and 36 dpv (Fig. 4D and 4E).

3.6. Immune-related gene expression analysis by RT-qPCR

The RT-qPCR was performed to analyze the effect of flagellin and IFN-γ on the SAIV vaccine to enhance the cell-mediated immunity in terms of gene expression analysis in the spleen and head kidney, are shown in Fig. 5. There was no significant increased expression of the pro-inflammatory cytokine genes IL-1β, IL-6 and IL-8 in spleen and head kidney at 15 and 36 dpv, with the exception of IL-6 in head kidney where it was up-regulated in G4. No induction of expression was also observed for CD4 and TCRb. However, IgM expression was significantly increased at 36 dpv in the spleen of all vaccinated groups (G2-G5) and in the head kidney of G4 and G5 containing IFN-γ in the vaccine formulation. Increased IgM expression was also observed at 15 dpv in the head kidney of G4 and G5. A small increase of MHC-I expression was observed at 36 dpv in the spleen of G2 and G5. Remarkably, MHC-II expression was highly increased in the spleen at 36 dpv of all molecular adjuvanted groups (G3, G4 and G5) and on 15 dpv of G3. Its expression was also increased in head kidney at 36 dpv of G4 and G5.

4. Discussion

Potential role of adjuvants is moving on with success stories in controlling bacterial diseases in fishes. Montanide™ ISA 763A VG and Montanide™ ISA 763B VG- developed by SEPPIC, commercial adjuvants used for i.p. injection in fish, are proved to be excellent in animal vaccines. The efficiency of these adjuvants in protection and in improving immune response (Wangkahart et al. 2023) including aluminum hydroxide, flagellin, chitosan oligosaccharide, CpG oligonucleotides, liposome, poly I:C, MARCOL 52, Freund's complete and incomplete adjuvants, are a range of effective adjuvants tried in vaccine studies (Jiao et al. 2010, Zheng et al. 2012, Dalmo et al. 2016).

Nile tilapia aquaculture is affected by Streptococcosis caused by *S. agalactiae* (Dangwetngam et al.2016, Barato et al. 2015). In this study, we have analysed the effects of flagellin and IFN-γ mixed with SAIV vaccine in providing protection and enhanced immune response in Nile tilapia. Recent studies have shown up with the role of flagellin as promising vaccine adjuvant and immunostimulant for fish aquaculture. It has been observed that flagellin from *Y. ruckeri*, potently activate the expression of inflammatory cytokines, antimicrobial peptides and acute phase proteins *in vitro* and *in vivo* (Wangkahart et al. 2019). *In vivo* studies showed that there is a remarkable induction in the expression of IL-12 family members and cytokines in Th2 and Th17 pathways but have little effects in Th1 pathway following flagellin stimulation (Wangkahart et al. 2019).

This study emphasizes the role of flagellin and IFN-γ to boost the immune response and protective efficacy in Nile tilapia infected with *S. agalactiae.* The immune parameters are analysed post vaccination. We administered vaccine through injection mode and fishes were injected with a booster at 21 dpv. Fish serum samples were collected at two time points (15 dpv and 36 dpv), before booster and after booster respectively. It was observed that the SOD and CAT activity in serum was remarkably enhanced in the fish administered with SAIV adjuvanted with flagellin and IFN-γ. The same effect was observed in the activities of LZM and MPO. The enhancement in the innate immune parameters was observed after booster vaccination at 36 dpv. LZM activity is essential in non-specific immune response in the fish and enhancement in immune response and is crucial to fight the invading bacteria (Ashfaq et

al.2019). We also tested serum bactericidal activity post vaccination and found that higher levels of its activity in fish treated with SAIV adjuvanted with flagellin and IFN-γ. This finding further supports the related outcome from the previous studies where adjuvanted vaccines enhancing non-specific immune response in the fishes with their potential bactericidal activity accompanied by elevated LZM activity, complement factors and other associated molecules (Ellis 1999, Hoare et al. 2019).

Specific antibody production is a measure of preventing bacterial infection and we examined the specific IgM antibody levels since it is the main antibody in the teleost fish (Ye et al. 2013). ELISA is a widely used sensitive method for the detecting and quantifying specific humoral antibody response to fish pathogens (Ristow et al. 1993). Vaccinated fishes are examined for humoral immune response by detecting and quantifying specific IgM antibodies in the sera post vaccination on 15 dpv and 36 dpv. We observed that specific IgM antibody level were enhanced in fish sera vaccinated with SAIV adjuvanted with flagellin, IFN-γ and its combination (G3, G4 and G5 respectively) when compared to negative control (G1) and positive control (G2) groups. Enhanced IgM antibody levels were significantly elevated in the fish sera at 36 dpv. The findings clearly indicate the potential role of molecular adjuvants in stimulating the humoral immunity in fish which again promisingly support the previous data where adjuvants enhance the immune response in tilapia infected with *S. agalactiae* (Wangkahart et al. 2023, Wangkahart et al. 2021). It was observed that specific IgM antibodies level were increased in SAIV vaccine adjuvanted with Montanide 763A VG and 763B VG. The IgM antibody level in these groups were also increased at an early stage (Wangkahart et al. 2023).

The role flagellin and IFN-γ in enhancing the efficacy of SAIV vaccine in conferring protection was measured. Many studies have proved a consistent correlation between the high expression of specific antibodies and protective efficacy of vaccine (Wangkahart et al. 2021). The RPS value of 59.37%, 71.87% and 81.25% was observed for SAIV vaccine adjuvanted with flagellin, IFN-γ and its combination, respectively, while the RPS of the positive control group was 15.62%. The RPS of fish injected with vaccine adjuvanted with both flagellin and IFN-γ showed enhanced protection. The protective efficacy of flagellin was already studied (Wangkahart et al. 2019) and the present finding is found to be in accordance with the earlier observation. An enhanced RPS was observed in fish injected with SAIV adjuvanted with Montanide 763A VG (77.42%) and Montanide 763B VG (74.19%) when compared to the unadjuvanted vaccine control group (61.29%) (Wangkahart et al. 2023). In another study, survival rate of fish administered with *S. agalactiae* ghost vaccine (SAGV) adjuvanted with Montanide 763B VG (80.0%), SAGV with Montanide GEL02 (84.4%) was found to be improved when compared to control group which was only 33.3% (Wangkahart et al. 2021). Our findings are supporting the previous studies with the analogous observations.

The histopathological examinations of head kidney, spleen, liver, kidney, gills and brain were performed in vaccinated and non-vaccinated fish. No histological changes were observed significantly in the head kidney, kidney, gills, liver and brain tissues of the fish vaccinated with SAIV adjuvanted with both flagellin and IFN-γ and challenged with *S. agalactiae* whereas decreased melanomacrophage cells was observed in the spleen tissues.

Immune related gene expression analysis remains well linked with the fish vaccination studies giving insights to the disease resistance offered by the respective vaccine administered. In this study, the expression levels of eight adaptive immune-related genes in spleen and head kidney of fish following immunization, such as CD4, IgM, MHC-I, MHC-II, TCRb, and innate immune response, such as IL-1β, IL-6 and IL-8 were analyzed to see whether the molecular adjuvants flagellin and IFN-γ affected their expression in vaccinated fish. There were no upregulation of the expression of major proinflammatory cytokines at 15 dpv and 36 dpv., suggesting that the fish at these time points retained to resting state without heightened inflammation or due to trafficking of inflammatory cells from tissues to the vaccination site. Interestingly, the expression of both MHC-II and IgM was coordinately increased at 36 dpv in the spleen of adjuvanted vaccines fish and in the head kidney in IFN-γ containing vaccine groups. The heightened transcript expression of IgM is in agreement of the increased specific IgM antibody titer in molecular adjuvanted vaccine groups found in this study. MHC-II molecules are constitutively expressed on the surface of professional antigen-presenting cells (APCs), including dendritic cells, B cells and macrophages, and can be upregulated by IFN-γ leading to antigen presentation to $CD4^+$ T cells and immune activation (Wijdeven et al. 2018). It is noteworthy that MHC-II expression in head kidney was only heighten in IFN-γ containing vaccine groups and these groups also showed higher antibody titer and protection against live bacterial infection, implying different mechanisms initiated by flagellin and IFN-γ. Our study suggests that MHC-II upregulated by IFN-γ present processed peptide to $CD4^+$ T cells leading to activation and differentiation into T helper cell subsets and specific antibody response and protection.

Conclusion

This study suggests that both flagellin or IFN-γ alone or in combination can improve the efficacy of SAIV vaccine against *S. agalactiae* infection in Nile tilapia. The combination of flagellin and IFN-γ had better effects by providing different signals to activate the vaccine immune response where IFN-γ upregulated the expression of MHC-II that present processed peptide to $CD4^+$ T cells leading to activation and differentiation into T helper cell subsets and specific antibody response and protection. The findings emphasize safe potential approach for vaccination strategies employing novel vaccines incorporating molecular adjuvants, providing protection to fish and inducing protective immunity against infectious bacterial diseases.

Declarations

Conflicts of Interest

The authors declare no conflict of interest.

Author Contribution

Sreeja Lakshmi: Conceptualization, Methodology, Formal analysis, Writing-original draft, Writing-review and editing, funding acquisition. Nandhakumar: Methodology, Formal analysis. Ritam Guha: Methodology, Formal analysis. Alex Wang: Methodology, Formal analysis. EakapolWangkaghart: Conceptualization, Methodology, Data curation, Writing- review and editing, supervision, funding acquisition. Tiehui Wang: Conceptualization, Methodology, Data curation, Writing- review and editing, supervision, funding acquisition. Preetham Elumalai: Conceptualization, Methodology, Data curation, Writing- review and editing, supervision, project administration, funding acquisition.All authors have read and agreed to the current version of the manuscript.

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Data availability

The data that has been used is confidential.

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Figures

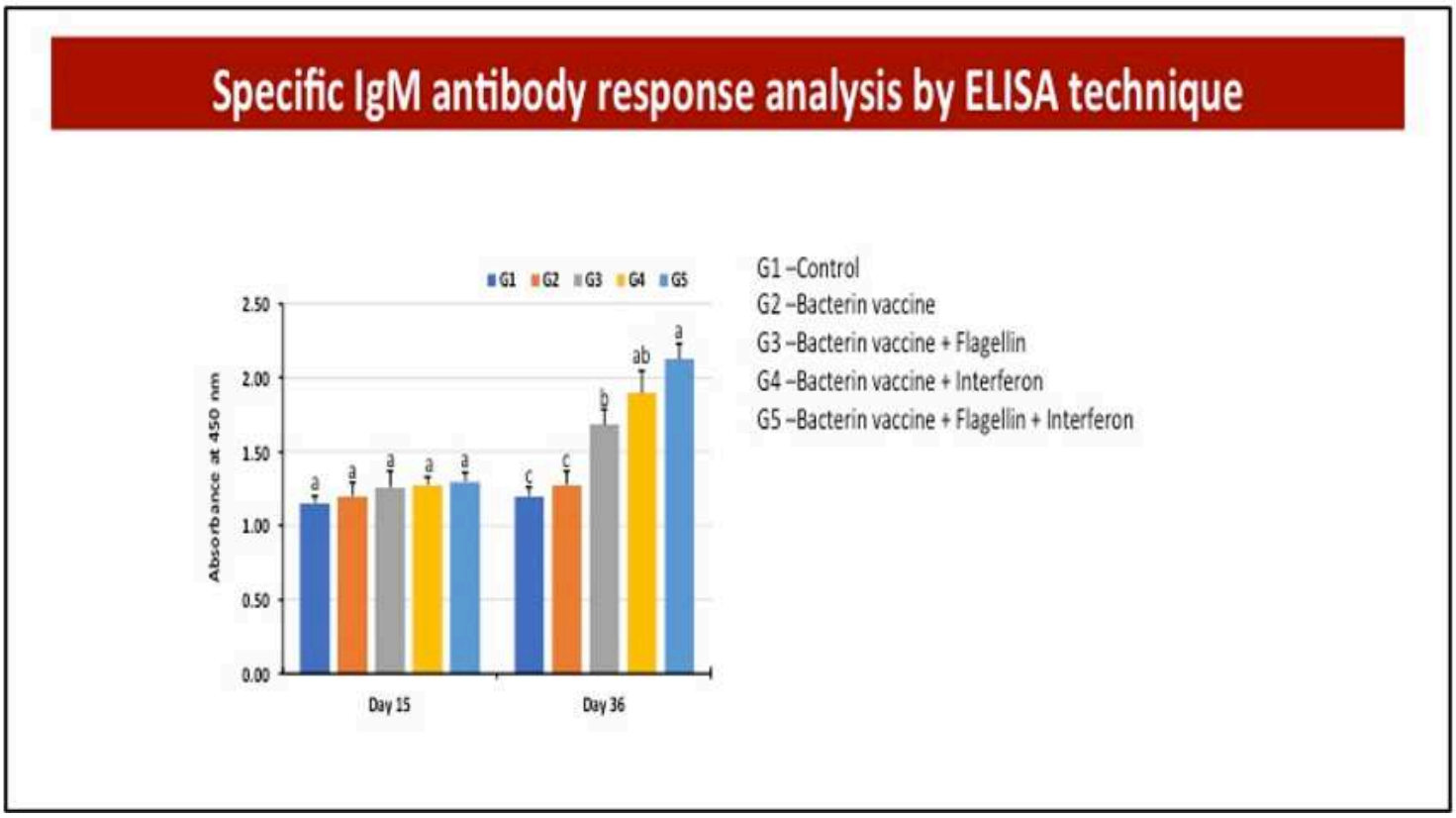
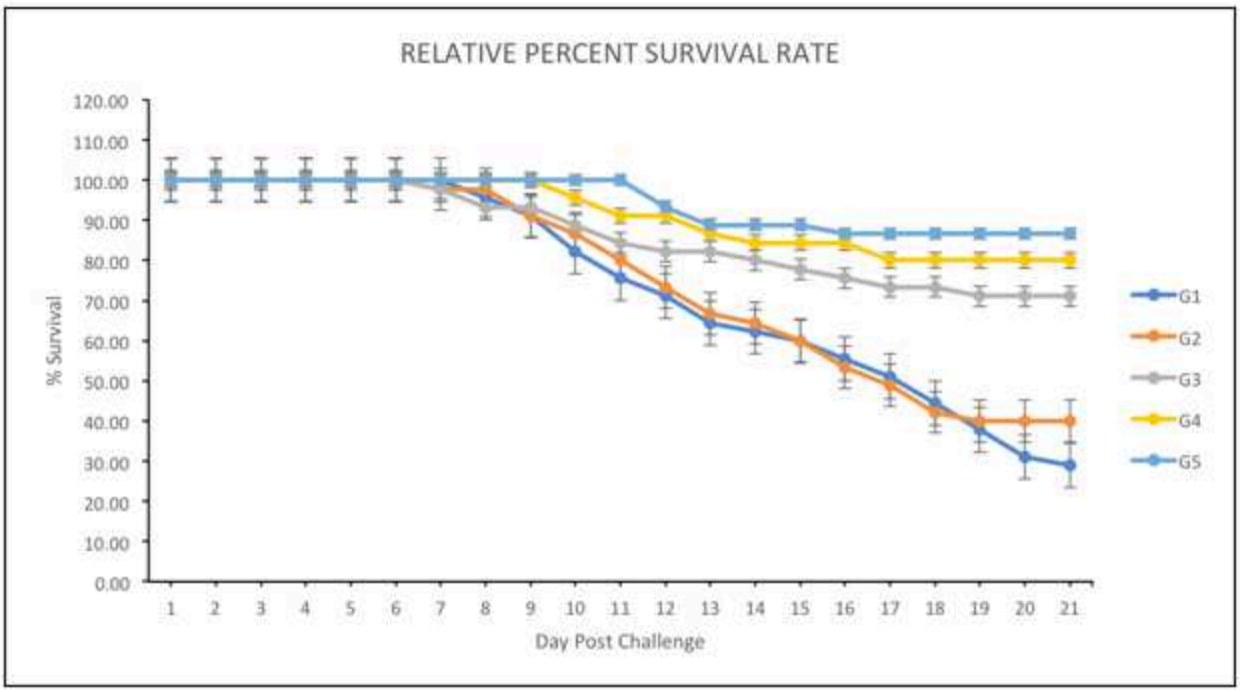


Figure 1

Specific IgM antibody response in the serum (diluted 1:128) of Nile tilapia on days 15 and 36 of the injection vaccination trials determined by ELISA. The optical density (OD) was measured at 450 nm. Serum samples from eight fish were measured in each group, and data expressed as the mean + SEM (n=8). Bars with letters indicate a statistical significance ($P < 0.05$) between different groups at the mentioned time points.



Groups	Mortality	Cumulative motality (%)	RPS
G1	32	71.11	–
G2	27	60.00	15.62
G3	13	28.89	59.37
G4	9	20.00	71.87
G5	6	13.33	81.25

Figure 2

Analysis of vaccine efficacy based on survival of infected fish. Fish were

vaccinated with formalin killed S. *agalactiae* adjuvanted with flagellin, IFN-γ and with both via intraperitoneal injection. Fish were then challenged with virulent strain

of *S. agalactiae* at 1x107 CFU/fish through injection and monitored for 21 days. G1 = Injection with PBS (control group); G2 = Injection with SAIV + Montanide^{TM} ISA 763A VG; G3 = Injection with SAIV + flagellin; G4 = Injection with SAIV + IFN- γ; G5 = Injection with SAIV + flagellin + IFN- γ

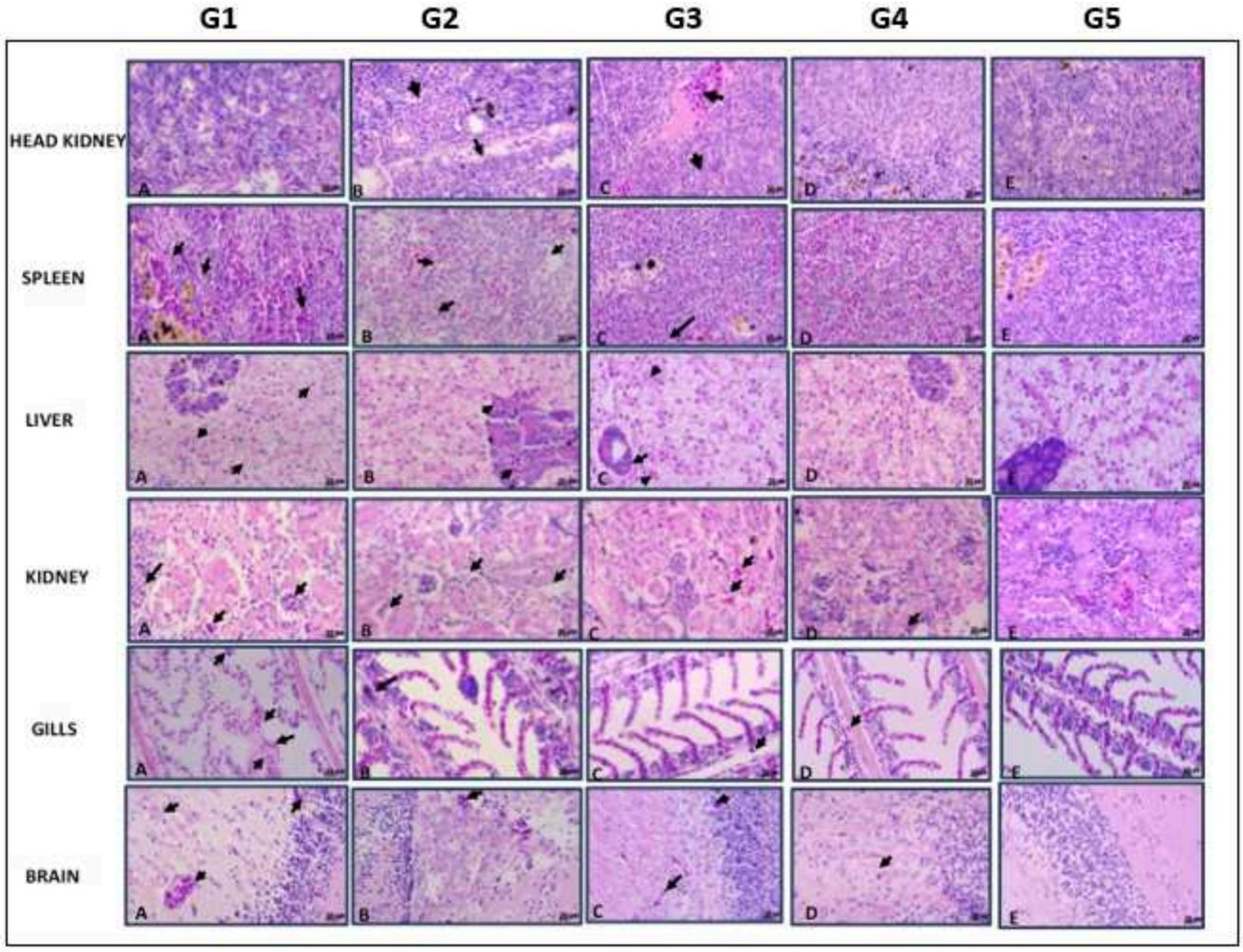


Figure 3

Histological sections of Head kidney, Spleen, Liver, kidney, gills and brain from fishes of treated groups. The tissue sections were collected from 58 days post-vaccination, from vaccinated and control groups. No histological changes were apparently visible in kidney, head kidney, gills, liver and brain tissues of fish administered with SAIV adjuvanted with both flagellin and IFN-γ (Group 5). Decreased melanomacrophage cells with least inflammation was observed in spleen. Unvaccinated control group (G1) was observed with necrosis in most of the tissues. No remarkable histological changes were observed in fish administered with SAIV with flagellin (G3) or IFN-γ (G4). G1=Injection with PBS (control group); G2 = Injection with SAIV + Montanide^{TM} ISA 763A VG; G3 = Injection with SAIV + flagellin; G4 = Injection with SAIV + IFN- γ; G5 = Injection with SAIV + flagellin + IFN- γ.

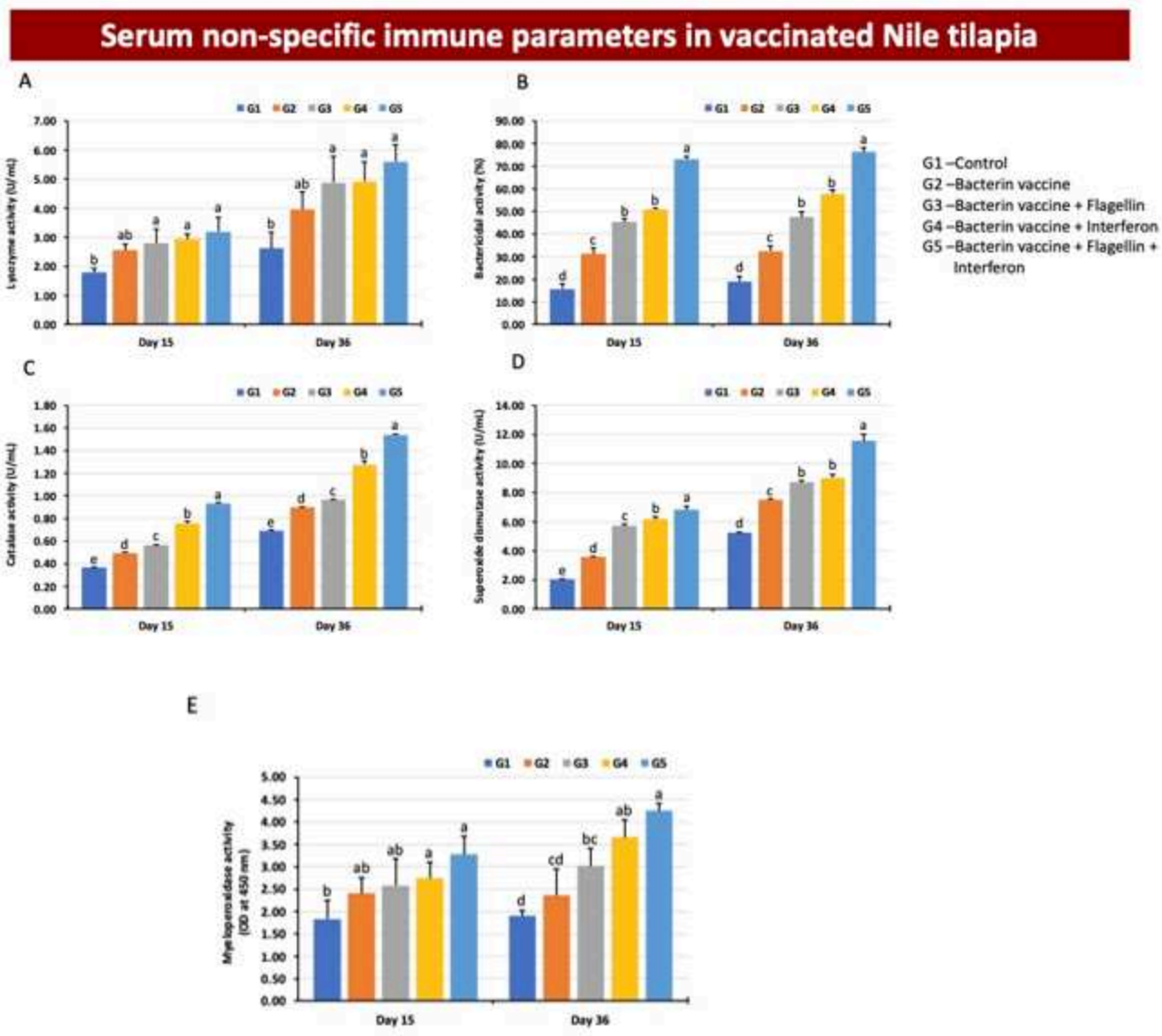
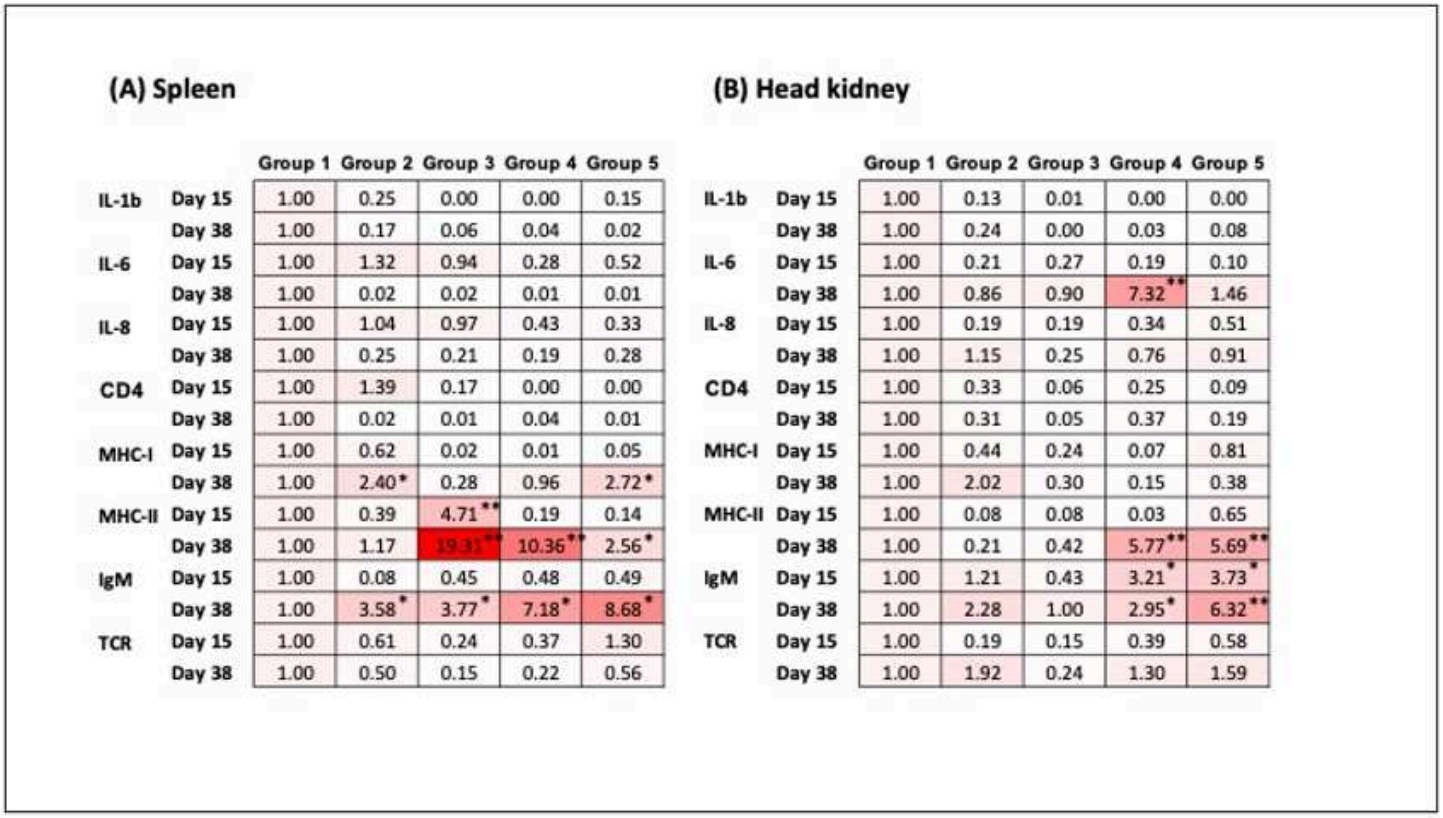


Figure 4

Innate immune parameters of Nile tilapia vaccinated with molecular adjuvants. (A) Lysozyme activity (LYZ), (B) Bactericidal activity, (C)Catalase activity (CAT) (D) Superoxide dismutase (SOD) (E) myeloperoxidase activity (MPO), of Nile tilapia was vaccinated with different formulations with molecular adjuvants. Sera were collected at day 15 and day 36 post vaccination. Values represent the mean ± SEM (n = 6) at a significant level of $P < 0.05$. G1 = Injection with PBS (control group); G2 = Injection with SAIV + Montanide^{TM} ISA 763A VG; G3 = Injection with SAIV + flagellin; G4 = Injection with SAIV + IFN- γ; G5 = Injection with SAIV + flagellin + IFN- γ



(A) Spleen

		Group 1	Group 2	Group 3	Group 4	Group 5
IL-1b	Day 15	1.00	0.25	0.00	0.00	0.15
	Day 38	1.00	0.17	0.06	0.04	0.02
IL-6	Day 15	1.00	1.32	0.94	0.28	0.52
	Day 38	1.00	0.02	0.02	0.01	0.01
IL-8	Day 15	1.00	1.04	0.97	0.43	0.33
	Day 38	1.00	0.25	0.21	0.19	0.28
CD4	Day 15	1.00	1.39	0.17	0.00	0.00
	Day 38	1.00	0.02	0.01	0.04	0.01
MHC-I	Day 15	1.00	0.62	0.02	0.01	0.05
	Day 38	1.00	2.40*	0.28	0.96	2.72*
MHC-II	Day 15	1.00	0.39	4.71**	0.19	0.14
	Day 38	1.00	1.17	19.31**	10.36**	2.56*
IgM	Day 15	1.00	0.08	0.45	0.48	0.49
	Day 38	1.00	3.58*	3.77*	7.18*	8.68*
TCR	Day 15	1.00	0.61	0.24	0.37	1.30
	Day 38	1.00	0.50	0.15	0.22	0.56

(B) Head kidney

		Group 1	Group 2	Group 3	Group 4	Group 5
IL-1b	Day 15	1.00	0.13	0.01	0.00	0.00
	Day 38	1.00	0.24	0.00	0.03	0.08
IL-6	Day 15	1.00	0.21	0.27	0.19	0.10
	Day 38	1.00	0.86	0.90	7.32**	1.46
IL-8	Day 15	1.00	0.19	0.19	0.34	0.51
	Day 38	1.00	1.15	0.25	0.76	0.91
CD4	Day 15	1.00	0.33	0.06	0.25	0.09
	Day 38	1.00	0.31	0.05	0.37	0.19
MHC-I	Day 15	1.00	0.44	0.24	0.07	0.81
	Day 38	1.00	2.02	0.30	0.15	0.38
MHC-II	Day 15	1.00	0.08	0.08	0.03	0.65
	Day 38	1.00	0.21	0.42	5.77**	5.69**
IgM	Day 15	1.00	1.21	0.43	3.21*	3.73*
	Day 38	1.00	2.28	1.00	2.95*	6.32**
TCR	Day 15	1.00	0.19	0.15	0.39	0.58
	Day 38	1.00	1.92	0.24	1.30	1.59

Figure 5

Transcript expression analysis of innate and adaptive immune-related genes with heatmap depiction of qPCR data. The spleen (A) and head kidney (B) of tilapia were sampled at 15 and 36 dpv, and gene expression assessed by RT-qPCR analysis. G1 = Injection with PBS (control group); G2 = Injection with SAIV + Montanide^{TM} ISA 763A VG; G3 = Injection with SAIV + flagellin; G4 = Injection with SAIV + IFN- γ; G5 = Injection with SAIV + flagellin + IFN- γ. Three biological replicates were used per group. The asterisks indicated significant differences between the vaccinated and the control groups (* = $P < 0.05$, ** = $P < 0.01$) at each time point.

Supplementary Files

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