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Immunogenicity and cross-protective potential of a sip-derived recombinant vaccine in hybrid red tilapia (*Oreochromis* sp.)

Sabri MY [a,b,*], M Rahmatullah [a], MD Hassan [a], MY Ina-Salwany [a]

[a] Aquatic Animal Health and Therapeutics Laboratory, Institute of Bioscience, Universiti Putra Malaysia, Serdang 43400, Selangor, Malaysia
[b] Department of Veterinary Pathology & Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia, Serdang 43400, Selangor, Malaysia

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ABSTRACT

The recombinant vaccine was prepared from the *Streptococcus iniae* surface immunogenic gene (*Sip*) to determine its potential as a vaccine candidate and its ability to cross-protect against *Streptococcus iniae* and *Streptococcus agalactiae*. The vaccine was administered intraperitoneally (i.p.). Group 1 was vaccinated with the formalin-killed recombinant vaccine (Rvac), while groups 2 (Sivac), 3 (Savac) and 4 (Vvac) were immunised with the formalin-killed whole cells of *S. iniae, S. agalactiae* and *E. coli* (vector only), respectively. Group 5 was an un-vaccinated control group (Cx). A booster dose was administered on day 14 following the first vaccination. Serum, mucus and gut lavage fluids were collected and evaluated using the enzyme-linked immunosorbent assay (ELISA). At challenge week 4, all groups were challenged intraperitoneally with an inoculum containing 1.43×10^9 CFU mL^{-1} live *S. agalactiae* and 1.32×10^9 CFU mL^{-1} live *S. iniae*, respectively. The Rvac group showed a strong immune response in tilapia; however, high antibody levels did not necessarily confer adequate protection, particularly when cross-infected with *S. agalactiae.* Moderate protection (60%) and excellent protection (100%) were observed in the homologous challenge with *S. iniae*. Therefore, further improvement may be required to provide outstanding protection, particularly against *S. agalactiae*.

Introduction

In Malaysia, the Department of Fisheries Malaysia has collaborated with WorldFish to improve tilapia production. InfoFish estimated 50–55 million kg of tilapia, valued at USD135–160 million in production value, in 2024. GIFT tilapia production and genetic improvement of hybrid red tilapia (*Oreochromis* sp.) are among the main projects in sustainable development, aquaculture, and fisheries in Malaysia [1]. With the expansion of aquaculture, particularly tilapia farming, tilapia are vulnerable to severe economic losses from infections caused by *S. agalactiae* and *S. iniae* worldwide, including in Malaysia [2,3].

Due to the outbreaks, farmers attempted to save their fish using antibiotics. However, the treatment is ineffective on moribund fish, leading to environmental pollution. Given the concerns about antibiotic resistance and residues, a vaccine is of paramount importance for controlling streptococcal disease in fish. Several injectable vaccines have been developed to prevent streptococcosis, although many differ in their formulations. In general, vaccination has been established as an effective method of preventing infectious diseases in farmed fish. Currently, most commercial vaccines are inactivated vaccines administered by injection or immersion [4]. Vaccines against streptococcosis in tilapia were previously developed with dubious protection to control the disease. Another available vaccine is MEVAC AQUASTREPT by Kemin, which protects against *S. agalactiae, S. iniae, Lactococcus*, and *Enterococcus* and is administered via immersion or injection. Another product launched in 2023 is AQUAVAC Strep Sa1, and in 2020, MSD Animal Health launched AQUAVAC Strep Sa-Si. The most recent vaccine against streptococcosis and motile aeromonad septicaemia was a feed-based, orally administered bivalent vaccine [3]. This vaccine effectively enhances tilapia's immune response, providing significant protection against bacterial infections.

Surface immunogenic protein (Sip) is a highly conserved cell-surface antigen originally identified in Group B Streptococcus (GBS, *Streptococcus agalactiae*) but is now recognised as a key virulence-associated antigen in *Streptococcus iniae*, a major pathogen of cultured fish. Its high conservation, surface exposure, and immunogenicity have made Sip a strong candidate for a universal vaccine across streptococcal species. A CRISPR interference (CRISPRi) study in *S. agalactiae* revealed that knockdown of the sip gene increases IL-1β secretion by macrophages, suggesting that Sip influences inflammatory signalling

* Corresponding author.
E-mail address: mdsabri@upm.edu.my (S. MY).

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pathways. Moreover, Sip-deficient streptococcal mutants exhibit impaired biofilm formation, reduced adherence to host tissues, and diminished persistence in host infection models, highlighting the importance of Sip in colonisation and virulence regulation. Although these studies were performed in GBS, the strong sequence similarity between Sip from GBS and *S. iniae* suggests that the protein likely performs analogous roles in aquatic hosts (Ye [5]). Recent research on tilapia vaccines has shown that recombinant Sip-based vaccines elicit strong humoral and mucosal immune responses. In particular, nano-carrier formulations (BNC-rSip) significantly enhanced serum antibody titres, stimulated innate immune enzyme activity, and achieved high relative per cent survival (RPS) following challenge with *S. iniae* and *S. agalactiae*. Reported RPS values reached 67.86–78.95%, substantially outperforming non-carrier formulations and confirming Sip's immunoprotective potential in fish vaccinology (Ye [5]).

To date, feed-based recombinant and bivalent vaccines have been tested in hybrid red tilapia with satisfactory results [3,6]. Following the successful development of recombinant and bivalent vaccines, we have developed another novel vaccine using the *S. iniae* surface immunogenic protein (*Sip*) gene, which provides moderate protection against *S. agalactiae* and excellent protection against *S. iniae*.

Materials and methods

Bacterial isolates, culture conditions, PCR and vector assays

For this experiment, the strain *Streptococcus iniae* TSK-2 (accession no. KT722586) was used. The TSK-2 strain was subcultured on tryptic soy agar (TSA, DIFCO) and incubated at 30 °C for 24 h. A single colony was transferred to 5 mL of tryptic soy broth (TSB, DIFCO) and incubated for 24 h at 30 °C with gentle shaking. The GeneJET Genomic DNA Purification Kit (Promega, USA) was used to extract DNA from *the S. iniae* strain TSK-2, following the manufacturer's instructions. To amplify the target gene of interest, primers were designed based on the surface immunogenic gene of *S. iniae* strain TSK-2, using the published *S. agalactiae* sequence (accession number AF151357) as a template. Physical constant and self-complementary analyses of the primers were performed. The forward and reverse primer sequences designed were sip-F (5'-GAC GAC GAC AAG ATG AAA ATG AAT AAA-3') and SIP-R (5'-GAG GAG AAG CCC GGT TAT TTG TTA AA-3'). The primers were designed to generate a product with cohesive vector overhangs (underlined) for efficient cloning into the pET-32 Ek/LIC vector (Novagen, Germany). The extracted DNA from the *S. iniae* strain TSK-2 was then used as a template in this amplification. A Mastercycler (Eppendorf®, Germany) was used to run a polymerase chain reaction in 35 cycles with initial denaturation at 95 °C for 3 min, denaturation at 95 °C for 1 min, annealing at 51 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min.

Expression construction, SDS-PAGE and Western blots

The pET-32 Ek/LIC-*Sip* gene-positive recombinant plasmid clones were used to perform recombinant protein expression in the *E. coli* BL21 (DE3) strain. Initially, the recombinant plasmid was extracted using the GeneJET™ Plasmid Miniprep Kit (Thermo Scientific) according to the manufacturer's protocol. Transformation of recombinant plasmids into competent *E. coli* BL21(DE3) was performed. Luria-Bertani (LB) agar cultures of recombinant *E. coli* BL21(DE3) containing the pET-32 Ek/LIC-*Sip* gene were incubated overnight in LB broth supplemented with ampicillin (50 μg mL^{-1}) at 37 °C with shaking until the cells' optical density at 600 nm reached 0.7. For efficient expression, the target DNA was induced to produce protein by 1 mM isopropyl β-D-1-thio-galactopyranoside (IPTG) (Calbiochem, USA). The culture was grown continuously for 10 h at 37 °C, then centrifuged at 10,000 × *g* for 10 min at 4 °C to harvest the cells. Bugbuster protein extraction reagent (Novagen, USA) was added to the harvested pellet to extract the cell lysates containing recombinant *Sip* protein, according to the manufacturer's instructions. To assess the expression of recombinant *Sip* protein, the supernatant obtained from above was subjected to SDS-PAGE and Western blot analysis. The target protein on the nitrocellulose membrane was analysed by Western blot using an anti-His-Tag monoclonal antibody (Merck, Germany) and goat anti-mouse IgG (Calbiochem, USA) as the secondary antibody. Immunoblotting was performed. The membrane was washed with PBST, incubated with anti-His-Tag at a 1:1,000 dilution at 4 °C overnight, and then incubated in goat anti-mouse immunoglobulin G (IgG)-horseradish peroxidase antibody (Calbiochem, USA) at a 1:1,500 dilution at 37 °C for 150 min with gentle shaking. DAB substrate solution (Calbiochem®, USA) was used to visualise the membrane, and finally, the membrane was rinsed with distilled water to stop the reaction.

DNA sequencing and analysis of a recombinant plasmid

The nucleotide sequences were confirmed by sequencing the positive plasmids using the T7 promoter and T7 terminator primers. Sanger sequencing was performed at First Base Laboratories, Malaysia. The nucleotide sequence was translated into an amino acid sequence by the translator tool on the website: http://www.fr33.net/translator.php. Nucleotide and amino acid sequence similarity searches were performed using the BLAST algorithm at the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/blast). The sequence obtained was aligned using the BioEdit sequence alignment editor (version: 7.2.5). Amino acid sequence antigenicity analysis was performed using the Predicted Antigenic Peptide Tool via the website: http://imed.med.ucm.es/Tools/antigenic.pl. The ClustalW programme was used to generate multiple amino acid sequence alignments, and the phylogeny was analysed with MEGA 6©.

Preparation of inactivated recombinant vaccine (IRV)

Stock culture of recombinant *E. coli* BL21(DE3) expressing *S. iniae* surface immunogenic protein (*Sip*) was inoculated into LB broth (Sigma, USA) and incubated at 37 °C for 18 h. An aliquot of 0.5% buffered formalin was added to the broth, and the mixture was incubated overnight at 4 °C to kill the cells before harvesting. The inactivated recombinant cells were washed with sterile phosphate-buffered saline (PBS) and centrifuged at 5,000 × *g* for 10 min at 4 °C. This step was repeated 4 times to ensure the formalin was removed entirely. Finally, the inactivated recombinant cells were resuspended in sterile PBS, and their concentration was measured using the McFarland standard. The cells were then adjusted to 1×10^7 CFU mL^{-1} with sterile PBS. The inactivity of the prepared vaccine was verified by inoculating 0.1 mL of the vaccine onto blood agar and incubating it at 37 °C for 24 h.

Preparation of formalin-killed bacterin (FKB)

As a positive control, three formalin-killed bacterins (FKB) were prepared. The first control bacterin was a formalin-killed whole cell of *S. iniae*. The second and third controls were a formalin-killed whole cell of *S. agalactiae* and *E. coli*, respectively. The protocol was similar to that previously described for IRV preparation.

Experimental design, vaccination, virulence, and challenge

A total of 450 hybrid red tilapia, each weighing 50 ± 5 g, were purchased from SA Agromax Enterprise, Selangor, Malaysia. Fish were divided into five groups of 90 fish each. Fifteen glass aquaria (200 L each) were set up in triplicate for the five groups. Mucus, serum, and gut lavage fluid were sampled from 6 fish per group before the start of the experiment. Fish in Group 1 were immunised with an inactivated recombinant vaccine (Rvac); Group 2 with formalin-killed whole-cell *S. iniae* (Sivac); Group 3 with formalin-killed whole-cell *S. agalactiae*

(Savac); Group 4 with formalin-killed *E. coli* (Vvac); and Group 5 served as the unvaccinated control. Vaccination was administered intraperitoneally at 250 μL containing 10^7 CFU per fish. 14 days after the first vaccination, a booster dose was administered to elicit a stronger immune response. Two fish from each replicate (6 fish per group) were sacrificed once a week for four weeks following vaccination. Mucus, serum, and gut lavage fluid were sampled from the sacrificed fish. These samples were analysed by ELISA to detect antibodies. Prior to challenge week, 10 hybrid red tilapia were injected (i.p.) with 250 μL of 1.43×10^7 CFU/mL of live *S. iniae*, TSK-2, and live *S. agalactia*e, Millud II, respectively, in two separate aquariums. After 48 h of exposure, the bacteria were re-isolated from the infected fish and cultured on blood agar. The isolates were then subcultured into brain heart infusion broth (BHIB) and incubated at 37 °C for 18 h in a shaker incubator. After incubation, the inoculum concentration was estimated using the spread plate method and expressed as CFU/mL (Jeffrey C [7]). The dose for the challenge trial was determined as 250 μL/fish with a concentration of 1.32×10^9 CFU/mL of live *S. iniae* and live *S. agalactiae*. The inocula were used immediately in the challenge week. At this point (week 4), the remaining 200 fish were challenged separately with virulent strains of *S. agalactiae* (Set-A) and *S. iniae* (Set-B) at a concentration of 1.32×10^9 CFU/mL, with each challenge duplicated. Ten tanks (200 L each, Set-A) were allocated for the challenge study with *S. agalactiae*, and another 10 tanks (same size, Set-B) were set up for the challenge with *S. iniae*. Fish in Group 1 were divided into two subgroups, Group 1A and Group 1B. Fish in Group 1A were challenged with live *S. agalactiae*, whereas fish in Group 1B were challenged with live *S. iniae*. Similarly, the remaining fish in the other 4 groups were subdivided into two groups and challenged with live *S. agalactiae* and *S. iniae*, respectively. 10 fish were allocated to each individual tank (replicate). After the challenge trial, clinical signs and mortality were observed for all fish from week 4 until week 6.

Sampling of mucus, serum and gut-lavage fluid

Approximately 500 μL of blood from fish samples was collected through the caudal peduncle vein of the fish into a centrifuge tube. The blood samples were stored at 4 °C for several hours to allow clotting, which separated the serum from the blood cells. The next day, the tubes containing fish blood were centrifuged at 3,000 × *g* for 3 min at 4 °C. The serum was collected using a pipette and stored at −20 °C until use. To collect the mucus, a swab was applied to the surface of the fish's body by swabbing 10 times with the sterile cotton swab from head to tail on one side of the fish. The cotton swab was then immersed in 1 mL of sterile PBS with 0.02% (w/v) sodium azide in a tube and kept at 4 °C overnight. After that, the mixture was centrifuged at 3,000 × *g* for 3 min at 4 °C, and 500 μL of the supernatant was collected from each tube and stored at −20 °C. The gut-lavage fluid was collected from the hindgut. Approximately 10 cm of the hindgut was selected before the gut was infused with 1 mL of sterile PBS + 0.02% (w/v) sodium azide using a 0.5-inch 27-gauge needle in 3 cc syringes. Fluids were collected directly and filled into a microtube by gentle palpation. The collected fluid was then refrigerated at 4 °C before centrifugation at 3,000 × *g* for 3 min to remove debris. Finally, ~500 μL of supernatant was collected and stored at −20 °C until further use.

Histology preparation

Histology was performed to investigate the development of gut-associated lymphoid tissue (GALT) in tilapia from pre-vaccination to post-vaccination and post-challenge stages. The guts of surviving and dead fish were collected every week from day 0 to week 6 for histological examination. About 5 cm of hindgut was fixed in 10% buffered formalin for at least 24 h before being trimmed and dehydrated for 2 h in a series of alcohol solutions (50%, 70%, 90%, and 100% alcohol) followed by xylene wash. Then, each tissue was soaked in paraffin at 56–58 °C. Tissue samples were embedded in melted paraffin and sectioned at 4 μm for slide preparation. The tissue sections were transferred to glass slides and left to dry overnight at 40 °C. All sections were stained with haematoxylin and eosin (H&E) and then observed on the FIVE Image Analyser (Olympus®, USA).

Serology

Pre- and post-vaccination sera, body mucus, and gut-lavage fluid samples were subjected to an indirect enzyme-linked immunosorbent assay (ELISA) following the method described by Nur-Nazifah et al. [6] to determine IgM antibody levels against *S. agalactiae* and *S. iniae*, respectively. ELISA plates were coated with 100 μL of pre-prepared *coating antigens for S. agalactiae and S. iniae* and incubated overnight at 4 °C. The next day, the plates were washed 3 times with wash buffer (PBS + 0.05% Tween-20), then 200 μL of blocking buffer (PBS + 0.05% Tween-20 + BSA) was added to each well, and the plates were incubated for 1 h at 37 °C. The plates were washed twice with wash buffer, then 100 μL of diluted sample (serum, mucus, or gut lavage fluid) was added to each well. The plates were then incubated at 37 °C for 1 h. The plates were again washed three times with the wash buffer. After washing, 100 μL of goat anti-tilapia hyperimmune serum (in-house production) diluted 1:5,000 was added to each well and incubated at 37 °C for 1 h. Following incubation, the plates were washed three times, and then 100 μL of rabbit anti-goat IgM HRP (Nordic, The Netherlands) diluted 1:5, 000 was added to each well and incubated at 37 °C for 1 h. The plates were washed three times with wash buffer, then 100 μL of TMB One solution conjugate (Promega, USA) was added to each well, and the plates were incubated at 37 °C for 30 min. The reaction was stopped by adding 50 μL of stop solution (2.5 M sulphuric acid), and the optical density was measured at 450 nm.

Bacterial isolation

The brain, eyes, and kidneys were collected at weeks 5–6 for bacterial isolation. The swab was plated onto blood agar and incubated at 30 °C for 24 to 48 h. To characterise *S. agalactiae* and *S. iniae*, gramme staining was performed and followed by PCR analysis using specific primers for *S. iniae* [8] and *S. agalactiae* [9], respectively.

Statistical analysis

MedCalc ver. 23.3.7 (Mariakerke©, Belgium) was used to analyse the data obtained from the *in vivo* study. All data (survival, antibody responses, and GALT size between groups) were analysed using one-way ANOVA, and significant differences were determined at $p < 0.05$ using pairwise comparisons with the Student-Newman-Keuls test. The clustered multiple-comparison graph shows the SEM for each treatment group.

Results

PCR amplification of the surface immunogenic gene of Streptococcus iniae

The TSK-2 strain of *S. iniae* produced a single product, as determined by agarose gel electrophoresis with a 1-kb DNA ladder (Thermo Scientific; Fig. 1). PCR amplification with designed primers confirmed the presence of the *S. iniae* surface immunogenic gene (*Sip*), which is approximately 1305 bp in length.

DNA sequence analysis of the recombinant plasmid

The DNA sequencing of the pET32-Ek/LIC recombinant plasmid revealed a 1305 bp nucleotide sequence that is 99% similar to the *S. agalactiae* surface immunogenic gene (*Sip*) by BLASTn analysis (Table 1). Analysis with Bioedit software revealed that the start codon

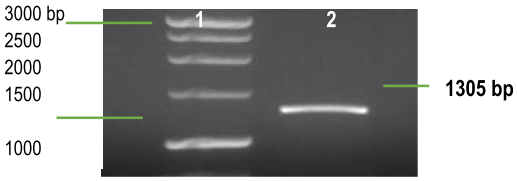

Fig. 1. Agarose gel electrophoresis of PCR amplification of the surface immunogenic gene of *S. iniae*. Lane 1: 1 kb DNA ladder; Lane 2: PCR product of *S. iniae* isolate, TSK-2.

Table 1
BLASTn analysis of the sequence pET-*sip* gene of *S. iniae* TSK-2.

Organism	Accession No	Query Cover (%)	Similarity (%)
S. agalactiae strain GB00059 Sip (sip) gene, sip-4 allele, complete cds	DQ914270.1	100	99
S. agalactiae strain COH1 group B streptococcal surface immunogenic protein (sip) gene, complete cds	AF151358.1	100	99
S. agalactiae Sip (sip) gene, complete cds	HQ878436.1	99	99
Streptococcus iniae strain TSK-2 Sip gene, complete cds	KT898957	99	99
Streptococcus dysgalactiae Sd-Sip gene for surface immunogenic protein, complete cds	AB769377.1	25	70
Streptococcus pyogenes MGAS8232	AE009949.1	25	70

(ATG) of the *S. iniae* surface immunogenic gene was in frame with the LIC site insertion (Fig. 2). The full-length sequence of the *S. iniae Sip* gene was the first to be deposited in GenBank, under accession number KT898957.

Expression of pET-32 Ek/LIC-surface immunogenic protein

Successful expression of the pET-32 Ek/LIC-*Sip* gene was confirmed using anti-His-Tag monoclonal antibody and goat anti-mouse immunoglobulin G (IgG)-horseradish peroxidase as a secondary antibody. SDS-PAGE analysis confirmed the presence of ~70 kDa fusion protein (Fig. 3), and Western immunoblot reconfirmed that the ~70 kDa protein band corresponded to the recombinant fusion protein derived from 17.8 kDa tagged protein and ~52 kDa of recombinant protein (Fig. 4).

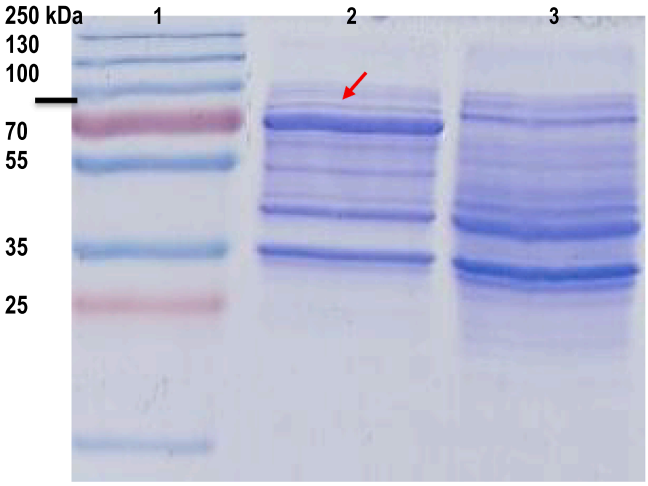

Fig. 3. SDS-PAGE of pET32 Ek/LIC-Sip expression in *Escherichia coli* BL21 (DE3). Lane 1: PageRuler Plus Prestained Protein Ladder (Thermo Fisher Scientific); Lane 2: Soluble cell protein from *E. coli* BL21(DE3) expressing pET-32 Ek/LIC-Sip; Lane 3: *E. coli* BL21(DE3) without the insert. Note: The arrow indicates the expressed protein around 70 kDa.

GACGACGACAAGATGAAAATGAATAAAAGGTACTATTGACATCGACAATGGCAGCTTCGCTATTATCAGTC
GCAAGTGTTCAAGCACAAGAAACAGATACGACGTGGACAGCACGTACTGTTTCAGAGGTAAAGGCTGATTT
GGTAAAGCAAGACAATAAATCATCATATACTGTGAAATATGGTGATACACTAAGCGTTATTTTCAGAAGCA
ATGTCAATTGATATGAATGTCTTAGCAAAAATTAATAACATTGCAGATATCAATCTTATTTATCCTGAGA
CAACACTGACAGTAACTTACGATCAGAAGAGTCATACTGCCACTTCAATGAAAATAGAAACACCAGCAAC
AAATGCTGCTGGTCAAACAACAGCTACTGTCGATTTGAAAACCAATCAAGTTTCTGTTGCAGACCAAAAA
GTTTCTCTCAATACAATTTTCGGAAGGTATGACACCAGAAGCAGCAACAACGATTGTTTCGCCAATGAAG
ACATATTCTTCTGCGCCAGCTTTGAAATCAAAAGAAGTATTAGCACAAGGGCAAGCTGTTAGTCAAGCAG
CAGCTAATGAACAGGTATCACCAGCTCCTGTGAAGTCGATTACTTCAGAAGTTCCAGCAGCTAAAGAGGA
AGTTAAACCAACTCAGACGTCAGTCAGTCAGTCAACAACAGTATCACCAGCTTCTGTTGCCGCTGAAACA
CCAGCTCCAGTAGCTAAAGTAGCACCGGTAAGAACTGTAGCAGCCCCTAGAGTGGCAAGTGTTAAAGTAG
TCACTCCTAAAGTAGAAACTGGTGCATCACCAGAGCATGTATCAGCTCCAGCAGTTCCTGTGACTACGAC
TTCAACAGCTACCGACAGTAAGTTACAAGCGACTGAAGTTAAGAGCGTTCCGGTAGCAAAAAAGCTCCAA
CAGCAACACCGGTAGCACAACCAGCTTCAACAACAAATGCAGTAGCTGCCCATCCTGAAAATGCAAGGC
TCCAACCTCATGTTGCAGCTTATAAAGAAAAAGTAGCGTCAACTTATGGAGTTAATGAATTCAGTACATA
CCGTGCGGGAGATCCAGGTGATCATGGTAAAGGTTTAGCAGTTGACTTTATTGTAGGTAAAACCAAGCAC
TTGGTAATGAAGTTGCACAGTACTCTACACAAAATATGGCAGCAAATAACATTTTCATATGTTATCTGGC
AACAAAAGTTTTACTCAAATACAAATAGTATTTATGGACCTGCTAATACTTGGAATGCAATGCCAGATCG
TGGTGGCGTTACTGCCAACCACTATGACCACGTTCACGTATCCTTTAACAAATAAGAGGAGAAGCCCGG

Fig. 2. Complete nucleotide sequence of the surface immunogenic protein (*sip*) gene of *Streptococcus iniae* strain TSK-2 (GenBank accession no. KT898957). Ligation-independent cloning (LIC) sites introduced by the amplification primers for cloning into the pET-32 Ek/LIC expression vector are **underlined**. The *sip* start codon (ATG) is located immediately downstream of the 5′ LIC site.

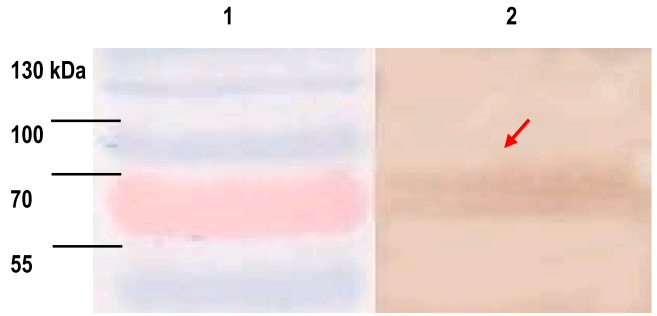

Fig. 4. Immunoblot showing the banding pattern of the soluble fusion protein of the recombinant surface immunogenic protein of *S. iniae*. Lane 1: PageRuler Plus Prestained Protein Ladder (Thermo Fisher Scientific); Lane 2: Soluble cell surface protein of recombinant-Sip gene.

Serum antibody response against S. iniae

Before vaccination, the serum antibody was comparatively low. However, serum antibody levels increased after vaccination, particularly in the Rvac, Sivac, and Savac groups from week 0 onward, and remained higher than those in the Vvac and Cx groups through week 6 (Fig. 5). Nonetheless, a decreasing pattern was observed at week 1, but this trend reversed at week 2 following the booster dose. The IgM levels in the immunised groups were significantly ($p < 0.05$) higher than those in the Vvac and Cx groups. However, no significant difference ($p > 0.05$) was observed between Rvac and Sivac; however, Rvac showed a substantial increase compared to the rest of the group.

Serum antibody response against S. agalactiae

ELISA with *S. agalactiae* coating antigen revealed a significant increase ($p < 0.05$) in serum antibody levels in the Rvac groups compared with the other groups. The Sivac and Savac groups also showed the same increase pattern as the Rvac group, but with significantly lower values ($p < 0.05$) than the Rvac group and higher values than the other groups. However, no significant difference was observed between the Vvac and Cx groups ($p > 0.05$). After the challenge, antibody levels in the Vvac and Cx groups could not be measured because all fish in these groups died (Fig. 6).

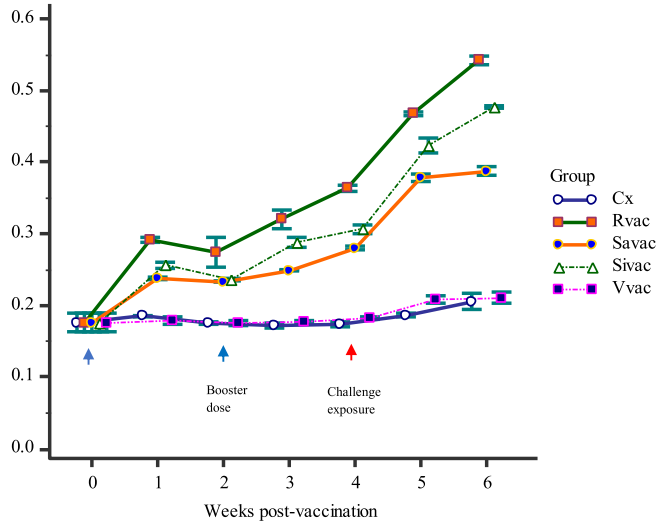

Fig. 5. Antibody levels of serum IgM against *S. iniae* following vaccination with an injectable recombinant vaccine (Rvac), an injectable whole-cell vaccine of *S. iniae* (Sivac), *S. agalactiae* (Savac), *E. coli* (Vvac), and an unvaccinated control group (Cx).

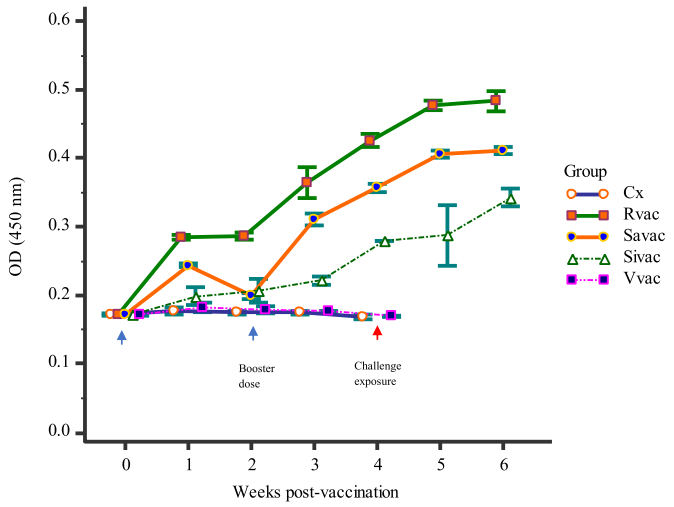

Fig. 6. Serum IgM antibody levels against *S. agalactiae* following vaccination with an injectable recombinant vaccine (Rvac), an injectable whole-cell vaccine of *S. iniae* (Sivac), *S. agalactiae* (Savac), *E. coli* (Vvac), and an unvaccinated control group (Cx).

Mucus antibody responses against S. iniae

Following vaccination, mucus antibody levels in the Rvac, Sivac, and Savac groups increased significantly ($p < 0.05$) from week 1 to week 6. Meanwhile, the mucus IgM levels of the Vvac and Cx groups did not differ significantly ($p < 0.05$) until week 6 (Fig. 7). However, the IgM level in the Rvac group remained the highest throughout the experiment.

Mucus antibody responses against S. agalactiae

Like serum and gut-lavage fluid antibodies, mucus IgM levels were significantly higher ($p < 0.05$) in the vaccinated groups than in the Vvac and Cx groups. However, no significant difference was found between groups Rvac and Sivac ($p > 0.05$), and a similar pattern of antibody responses was observed in both groups. Antibody levels began to increase from week 0 to week 6 (Fig. 8).

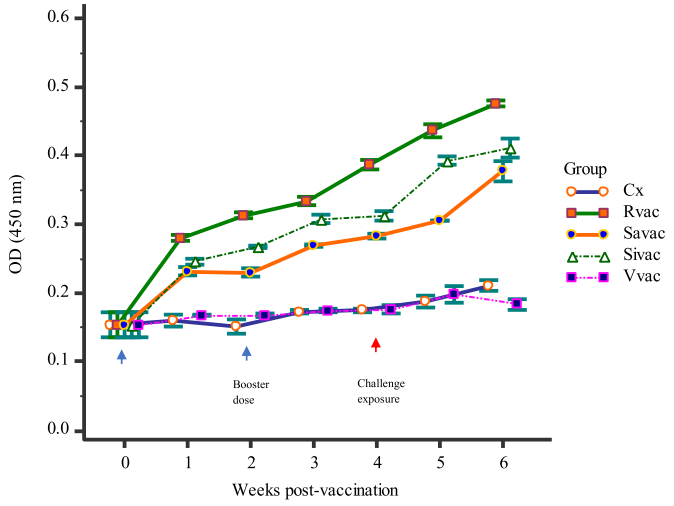

Fig. 7. Antibody levels of mucus IgM against *S. iniae* following vaccination with an injectable recombinant vaccine (Rvac), an injectable whole-cell *S. iniae* vaccine (Sivac), *S. agalactiae* vaccine (Savac), *E. coli* vaccine (Vvac), and an unvaccinated control group (Cx).

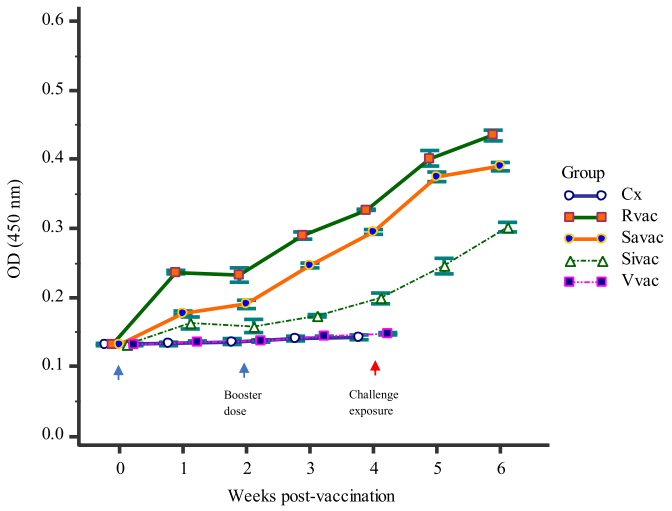

Fig. 8. Antibody levels of mucus IgM against *S. agalactiae* after vaccination with an injectable recombinant vaccine (Rvac), an injectable whole-cell vaccine of *S. iniae* (Sivac), *S. agalactiae* (Savac), *E. coli* (Vvac), and an unvaccinated control group (Cx).

Gut-lavage fluid antibody responses against S. iniae

After vaccination, antibody levels in gut lavage fluid from fish in the Rvac, Sivac, and Savac groups increased significantly as early as week 1 ($p < 0.05$). In contrast, no significant change was observed in the Vvac and Cx groups throughout the experiment (Fig. 9). At the following challenge in week 4, IgM levels remained higher in the Rvac to Savac group, and a significant difference ($p < 0.05$) was observed between the Rvac and Savac groups. However, the Rvac and Sivac groups were found to be statistically unchanged. In addition, antibody levels peaked at week 6 (Fig. 9).

Gut-lavage fluid antibody responses against S. agalactiae

Following vaccination, antibody levels in the gut-lavage fluid of fish from groups Rvac, Sivac, and Savac began to increase significantly ($p < 0.05$) as early as week 1. However, the Vvac and Cx groups showed no statistically significant differences throughout the study. As observed in serum and mucus, IgM levels in the gut lavage also decreased slightly at week 2 (Fig. 10) but slowly recovered after the booster dose. After the challenge in week 4, antibody levels remained high, peaking at week 6. The observed IgM levels in the Rvac group were significantly higher ($p < 0.05$) than those in the other groups, except the Savac group. After the challenge, IgM levels in groups Vvac and Cx could not be determined because all fish in these groups died.

Histological analysis

Gut-associated lymphoid tissue (GALT) was detected by histological analysis of the hybrid red tilapia gut. Vaccination with Rvac, Sivac and Savac vaccines revealed aggregation of lymphoid cells within the lamina propria and scattered lymphocytes in the epithelium as early as week 1 post-vaccination (Fig. 11). However, after the specified vaccination, the gut of tilapia in the Vvac and Cx groups (Fig. 12) showed no GALT development.

Relative Percentage Survival (RPS)

Following the challenge trial, mortality data for all groups were recorded from week 4 until week 6. At the end of the experiment, RPS was calculated following the formula described by Amend [10].

$$\text{RPS} = 1 - \left(\frac{\text{Mortality of vaccinated fish}}{\text{Mortality of unvaccinated control fish}}\right) \text{x } 100\%$$

Challenge with S. agalactiae

During the challenge phase, fish showed mortalities with the clinical signs of erratic swimming, lethargy and anorexia within 24 h post-challenge (hpc), and some fish died acutely with no clinical signs. The highest mortality occurred on day 1 in most groups (Table 2). However, *S. agalactiae* caused 100% mortality in the Vvac and Cx groups within 24–48 hpc. Mortality in the Rvac group reached up to 40% within 14 days post-challenge (dpc), resulting in a significant difference in mortality ($p < 0.05$) between the Rvac group and the other groups. However, no significant differences were observed between the Sivac and Savac groups or between the Vvac and Cx groups ($p > 0.05$).

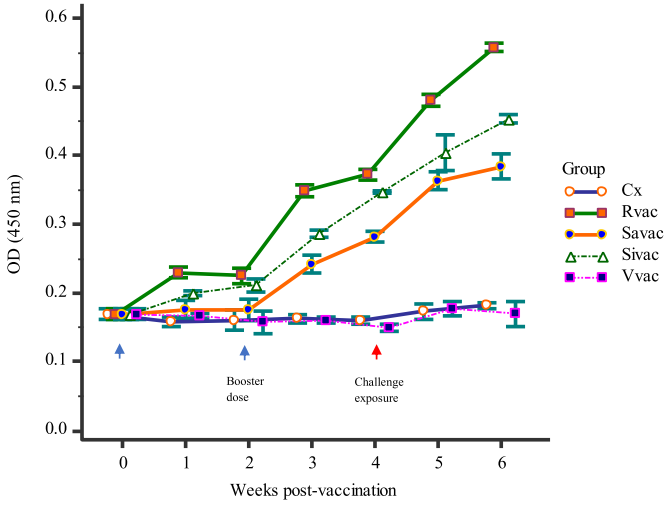

Fig. 9. Antibody levels of gut-lavage IgM against *S. iniae* following vaccination with an injectable recombinant vaccine (Rvac), an injectable whole-cell vaccine of *S. iniae* (Sivac), *S. agalactiae* (Savac), *E. coli* (Vvac), and an unvaccinated control group (Cx).

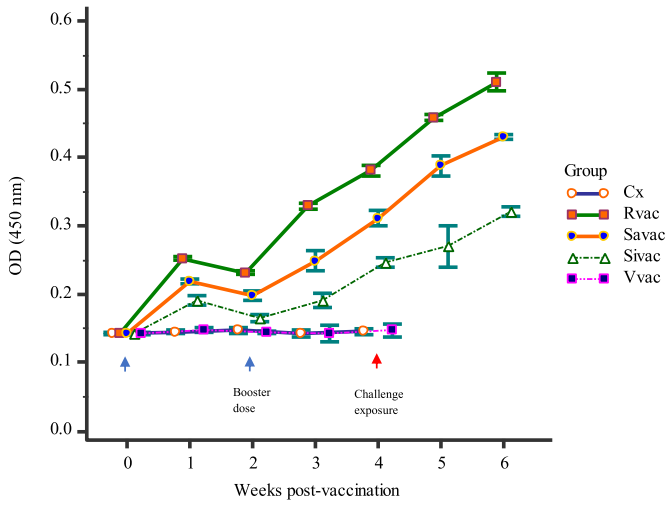

Fig. 10. Antibody levels of gut-lavage IgM against *S. agalactiae* following vaccination with an injectable recombinant vaccine (Rvac), an injectable whole-cell vaccine of *S. iniae* (Sivac), *S. agalactiae* (Savac), *E. coli* (Vvac), and an unvaccinated control group (Cx).

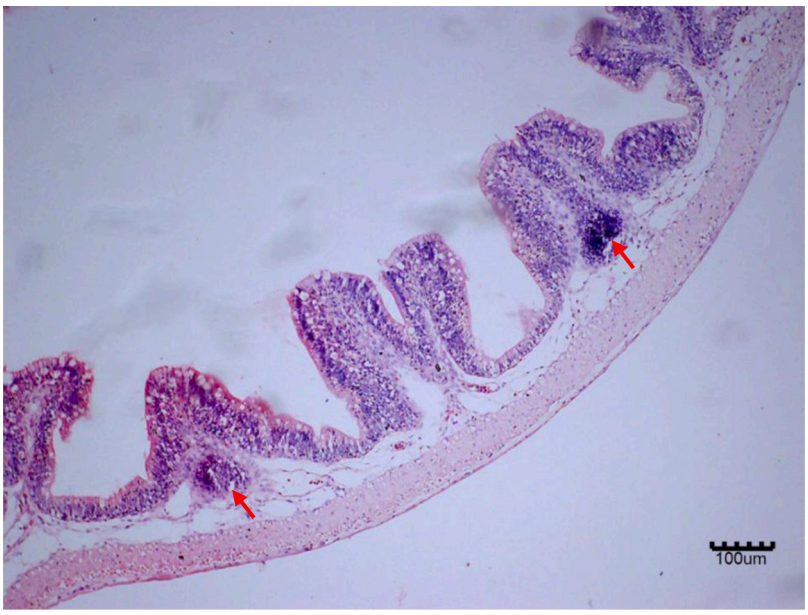

Fig. 11. Cross-section of the gut of hybrid red tilapia vaccinated with an injectable recombinant-Sip (Rvac) and challenged by *S. iniae* (H&E, × 100). The red arrow marks the gut-associated lymphoid tissues.

Fig. 12. Cross-section of the gut of hybrid red tilapia without any vaccination (Cx) (H&E, × 100).

Table 2
Total cumulative mortality and relative percentage of survival (RPS) recorded for juvenile hybrid red tilapia after challenge with 1.32 × 10^9 CFU/ml live *S. agalactiae* by i.p. injection.

	No. of dead fish (hpi)							
Group	24	48	72	96	336	Total no. dead	Mortality (%)	RPS (%)
1	3	3	1	1	0	8/20	40	60
2	5	4	2	2	0	13/20	65	35
3	4	5	1	1	0	11/20	55	45
4	11	7	2	0	0	20/20	100	0
5	14	6	0	0	0	20/20	100	0

Note: Group 1: Rvac, Group 2: Sivac, Group 3: Savac, Group 4: Vvac, Group 5: Cx.

Challenge with S. iniae

During the challenge phase, the fish in the Rvac group did not exhibit any behavioural changes or clinical signs. However, the highest mortality occurred in most groups on day 2 compared to the *S. agalactiae*-challenged groups. The fish in the Rvac group (recombinant vaccine) remained active and showed no mortality or clinical signs. In contrast, fish from groups Vvac and Cx exhibited mortality and clinical signs at 24 hpc and persisted through 120 hpc. Meanwhile, the whole-cell-vaccinated groups (Sivac and Savac) showed only abnormal swimming and mortality at 48 hpc and later. Corneal opacity and exophthalmia were observed in some fish at 3 days post-challenge (dpc) in both bacterium challenges. Furthermore, mortality or clinical signs were observed from 120 h post-challenge (hpc) to the end of the trial at 336 hpc across all groups, except in Group 1 (Rvac) for the *S. iniae* challenge

Table 3
Total cumulative mortality and relative percentage of survival (RPS) recorded for juvenile hybrid red tilapia after challenge with 1.32×10^9 CFU/ml live *S. iniae* by i.p. injection.

No. of dead fish (hpi)								
Group	24	48	72	96	336	Total no. dead	Mortality (%)	RPS (%)
1	0	0	0	0	0	0/20	0	100
2	0	2	1	1	0	4/20	20	64
3	0	3	2	1	0	6/20	30	45
4	2	5	2	1	0	10/20	50	9
5	2	6	1	1	1	11/20	55	0

Note: Group 1: Rvac, Group 2: Sivac, Group 3: Savac, Group 4: Vvac, Group 5: Cx.

(Table 3).

Bacterial isolation and PCR

Both *S. agalactiae* and *S. iniae* were successfully cultured from the brains and kidneys of dead fish in both challenges. To our surprise, we failed to isolate pathogens from organs of live fish in any group except the Vvac and Cx groups on day 5 post-challenge. Isolates were confirmed by PCR assay using F1/IMOD primers for *S. agalactiae,* which generated a 220 bp band and for *S. iniae* using Sin1/Sin2 primers, which generated a 300 bp band (data not provided).

Discussion

In vaccination, various methods or techniques of vaccine delivery, such as injection, immersion (including ultrasound, attenuated bacterial delivery, and novel immersion methods), oral administration (including microalgae, nano- and micro-particles, alginate microparticles, chitosan, poly(lactide-co-glycolide: PLGA), biofilms, attenuated bacterial delivery, artemia, plant-expressed vaccines and feed-based vaccines) are extensively discussed in terms of either advantages or disadvantages [11,6,12,13]. Despite these techniques, injection vaccination remains far ahead and remains popular. However, the limitations of the injectable vaccine prompted further investigation into alternative methods, such as oral vaccines.

In this study, the injectable vaccine demonstrated efficacy against post-challenge with *S. agalactiae* and *S. iniae*, resulting in 60% and 100% reduction in RPS, respectively. The basis for this difference is not readily elucidated; however, differences in antigenicity, including variations in gene expression, have been suggested between these two isolates [14,15]. Tilapia vaccinated i.p. with the recombinant-Sip vaccine showed significantly improved protection compared to the Cx group following challenge with *S. iniae* and *S. agalactiae*. Vaccination significantly reduced abnormal behaviour and mortality ($p < 0.05$). Thus, the ability to prevent streptococcal disease by administering the recombinant-*Sip* vaccine intraperitoneally (i.p.) significantly increased ($p < 0.05$) the survival of hybrid red tilapia. However, no significant differences ($p > 0.05$) were observed between the Sivac and Savac groups and the Vvac and Cx groups.

In another study, i.p. injection of *S. iniae* vaccine reduced mortality by 91.3% in tilapia weighing 25 g [16]. The difference in potency between 55 g and 25 g tilapia against challenge with the homologous *S. iniae* isolate may indicate that younger tilapia are less effective at generating the same level of protective immunity against both *S. iniae* and *S. agalactiae* after immunisation with a single isolate vaccine. This may be due to underdeveloped immunity in young fish. Muzquiz et al. [17] demonstrated that the pathogenicity of streptococcal disease in rainbow trout is age dependent. Our data suggest that the efficacy of homologous vaccines may also be age dependent. However, studies on recombinant streptococcal vaccines, particularly those targeting *S. iniae* in tilapia or other farmed fish species, remain limited for comparison.

Antibody titres in serum, mucus, and gut lavage fluid following i.p. injection of the recombinant vaccine were significantly higher ($p < 0.05$) than in the control group. Nevertheless, IgM levels against *S. iniae* indicated that the whole-cell Si vaccine had higher titres than the whole-cell Sa vaccine in serum, mucus, and gut lavage. However, they did not differ significantly ($p > 0.05$). In contrast, IgM levels against *S. agalactiae* showed that the whole-cell Sa vaccine was significantly higher ($p < 0.05$) than the whole-cell Si vaccine, either in serum, mucus, or gut-lavage fluids. The vector vaccine administered i.p. elicited significantly lower titres ($p < 0.05$) than those elicited by all other vaccines in this experiment. The previous study showed that protective immunity against *S. iniae* depended on antibodies against *S. iniae*.

In this study, the developed recombinant Sip vaccine elicited high levels of both systemic and mucosal immunity, resulting in 100% protection against infection with a virulent *S. iniae* strain. However, the potency was insufficient, providing only 60% protection against a virulent strain of *S. agalactiae*. However, the efficacy of the developed recombinant vaccine is acceptable when we compare it with the positive control group (whole cell-killed vaccine), the unvaccinated group (Cx group) and the negative Vvac group (*E. coli* without vector insert), which showed a significant difference ($p < 0.05$) between the recombinant-*Sip* vaccine and the rest of the groups. At 72 h post-challenge, no fish survived in the Vvac and Cx groups, indicating that the unvaccinated fish had no protection whatsoever. A similar study by Nur-Nazifah et al. [6] showed 70% protection in hybrid red tilapia vaccinated with a recombinant cell wall surface anchor family protein against *S. agalactiae*. The efficacy of the recombinant *Sip* vaccine can be enhanced by using an oil adjuvant. Firdaus et al. [11] demonstrated that adjuvants are immune enhancers that can be incorporated into vaccines to augment the immune response, thereby enhancing protective immunity against the target pathogen.

In addition, the injectable Rvac vaccine not only induces high antibody levels and protection in tilapia but also elevates mucosal antibody levels. There is no doubt that injectable vaccination has many advantages, including effectiveness, long-lasting immunity, a known, precise amount of vaccine antigen that can be administered to fish, and a small volume required for delivery [13]. Based on our experience, we found that an injectable vaccine delivered into the peritoneal cavity does not significantly stimulate gut-associated lymphoid tissues (GALTs) compared to oral administration. GALT is a site in the lamina propria of the intestine to manufacture a local cellular immune response to combat the pathogen entering through the fish gastrointestinal tract. Again, although injection vaccination has been widely used in humans and animals since the vaccine's introduction, it has some well-known limitations, notably impracticality for large numbers of fish and its incompatibility with small fish under 20 g [18].

To summarise the results, we concluded that the *S. iniae* surface immunogenic protein (*Sip*) is a far superior antigen, contributing to protective immunity and controlling streptococcosis in hybrid red tilapia. The developed vaccine conferred high levels of systemic and mucosal immunity on hybrid red tilapia. However, the level of protection was insufficient to achieve 100% survival in hybrid red tilapia when challenged with *S. agalactiae.* Therefore, it is proposed to enhance the vaccine's efficacy by mixing it with an oil adjuvant to increase its protective capacity in fish, aiming to have the Rvac vaccine perform similarly to that observed in the previous study [11]. In addressing the challenges posed by various *Streptococcus* spp., it is therefore imperative to explore additional alternatives in the manufacture and combination of recombinant vaccines to develop vaccines that exhibit high antigenicity and immunogenicity against the diseases to be protected against.

Ethical approval

The authors followed all applicable institutional guidelines for the care and use of animals.

CRediT authorship contribution statement

Sabri MY: Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **M Rahmatullah:** Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **MD Hassan:** Writing – review & editing, Supervision, Investigation, Data curation, Conceptualization. **MY Ina-Salwany:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Data availability

Data will be made available on request.

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