JIPK. Volume 18 No 1 February 2026
Sinta 1 (Decree No: 158/E/KPT/2021)
e-ISSN:2528-0759; p-ISSN:2085-5842
Available online at https://e-journal.unair.ac.id/JIPK

JIPK
(JURNAL ILMIAH PERIKANAN DAN KELAUTAN)
Scientific Journal of Fisheries and Marine

Research Article

Oil-Adjuvanted Polyvalent Formalin-Killed *Aeromonas hydrophila* Vaccine Enhances Agglutinating Antibodies, Respiratory Burst, and Survival in Giant Gourami

Rozi[1,7], Wiwiek Tyasningsih[2], Jola Rahmahani[2], Eduardus Bimo Aksono[3], Muchammad Yunus[4], Mohammad Anam Al Arif[5], Suryo Kuncorojakti[6], Daruti Dinda Nindarwi[7], Putri Desi Wulan Sari[7], Nina Nurmalia Dewi[7], Woro Hastuti Satyantini[7], Muhammad Browijoyo Santanumurti[7], Dita Wisudyawati[7], Mohammad Noor Amal Azmai[8], Annas Salleh[9], Gazali Salim[10], Suwarno[2]

[1]Doctoral Program of Veterinary Science, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya 60115, Indonesia
[2]Division of Microbiology, Faculty of Veterinary Medicine, Airlangga University, Surabaya 60115, Indonesia
[3]Division of Basic Veterinary Medicine, Faculty of Veterinary Medicine, Airlangga University, Surabaya 60115, Indonesia
[4]Division of Veterinary Parasitology, Faculty of Veterinary Medicine, Airlangga University, Surabaya 60115, Indonesia
[5]Division of Farm, Faculty of Veterinary Medicine, Airlangga University, Surabaya 60115, Indonesia
[6]Division of Veterinary Anatomy, Faculty of Veterinary Medicine, Airlangga University 60115, Surabaya, Indonesia
[7]Department of Aquaculture, Faculty of Fisheries and Marine, Airlangga University, Surabaya 60115, Indonesia
[8]Department of Biology, Faculty of Science, Universiti Putra Malaysia, Serdang, Malaysia
[9]Department of Veterinary Laboratory Diagnosis, Faculty of Veterinary Medicine, Universiti Putra Malaysia, Serdang, Malaysia
[10]Study Program of Aquatic Resources Management, Faculty of Fisheries and Marine Science, Universitas Borneo Tarakan

OPEN ACCESS

ARTICLE INFO

Received: Nov 30, 2025
Accepted: Dec 04, 2025
Published: Dec 05, 2025
Available online: Jan 08, 2026

*) Corresponding author:
E-mail: suwarnofkh2024@gmail.com

Keywords:
Aeromonas hydrophila vaccine
Adjuvanted polyvalent FKC
Osphronemus goramy
Humoral and innate immunity
IL-1β and IFN-γ expression
Life below water

Abstract

Motile *Aeromonas* septicaemia (MAS), predominantly associated with *Aeromonas hydrophila*, remains a major constraint in giant gourami (*Osphronemus goramy*) aquaculture. This study evaluated formalin-inactivated *A. hydrophila* vaccines prepared from MAS-associated field isolates, comparing a monovalent formulation (P2), a non-adjuvanted polyvalent formulation (P3), and an oil-adjuvanted polyvalent formulation (P4) against PBS controls (P1). A total of 240 fish were used (60 per treatment) and assigned to two parallel cohorts (immunology and survival/challenge). Immune endpoints (agglutinating titres, NBT activity, and splenic *il-1β* and *ifn-γ* transcription) were assessed on days 7, 14, 21, 35, and 42 post-vaccination. The survival cohort was challenged intraperitoneally at day 21 with a homologous *A. hydrophila* strain and monitored for 14 days post-challenge. Vaccination was clinically well tolerated and improved survival relative to controls, with P4 showing the highest protection (RPS 81.8%). Agglutinating titres differed by treatment and time; at the peak sampling point (day 35), mean titres in P4 were ~200-fold higher than in P1, and model contrasts indicated significant differences versus controls ($p<0.001$). Splenic *il-1β* and *ifn-γ* transcript levels were higher in vaccinated groups than in controls at later time points. These findings support further evaluation of an oil-adjuvanted polyvalent inactivated *A. hydrophila* vaccine for gourami, including dose optimisation, extended safety assessment, heterologous challenge, and field validation.

Cite this as: Rozi, R., Tyasningsih, W., Rahmahani, J., Aksono, E. B., Yunus, M., Al Arif, M. A., Kuncorojakti, S., Nindarwi, D. D., Sari, P. D. W., Dewi, N. N., Satyantini, W. H., Santanumurti, M. B., Wisudyawati, D., Azmai, M. N. A., Salleh, A., Salim, G., & Suwarno, S. (2026). Oil-Adjuvanted Polyvalent Formalin-Killed *Aeromonas hydrophila* Vaccine Enhances Agglutinating Antibodies, Respiratory Burst, and Survival in Giant Gourami. *Jurnal Ilmiah Perikanan dan Kelautan,* 18(1):17-40. https://doi.org/10.20473/jipk.v18i1.82866

1. Introduction

The bacterial haemorrhagic septicaemia and motile Aeromonas septicaemia (MAS) caused predominantly by *Aeromonas hydrophila* remain major constraints to warm-water freshwater aquaculture, causing substantial mortality, growth loss, and economic damage in Asia and worldwide (Assefa and Abunna, 2018; Fernández-Bravo and Figueras, 2020; Marinho *et al*., 2019). Field reports from South and Southeast Asia indicate that intensive culture of cyprinids and other warm-water species is frequently complicated by *A. hydrophila*-associated septicaemia and dropsy, even under managed husbandry and water-quality conditions (Dash *et al*., 2008; Iqbal, 2016; Podeti and Benarjee, 2017; Sen and Mandal, 2018). In Indonesia and neighbouring countries, expansion of freshwater aquaculture and increasing reliance on high-density systems have magnified the impact of bacterial diseases on livelihoods and food security (Assefa and Abunna, 2018; Lusiastuti *et al*., 2020; Shamsuzzaman *et al*., 2017). Giant gourami (*Osphronemus goramy*) is a culturally and economically important species in this region, yet recurrent MAS outbreaks and co-infections with other bacterial pathogens continue to limit production and often trigger non-selective antimicrobial use (Decostere *et al*., 2004; Gauthier and Rhodes, 2009; Rahmaningsih and Yanuhar, 2014; Rozi *et al*., 2018a, 2018b, 2024; Sularto *et al*., 2026). To link vaccine design to the local epidemiologic context, this study used three previously characterized and archived gourami-derived A. hydrophila isolates (Ah-S1–Ah-S3) as vaccine seed strains, with Ah-S1 serving as the homologous challenge isolate (Rozi *et al*., 2018a, 2018b, 2024).

Vaccination is a cornerstone of bacterial disease control in finfish and has transformed the management of major infections in salmonids and other high-value species (Adams, 2019; Gudding and van Muiswinkel, 2013; Hastein *et al*., 2005; Toranzo *et al*., 2009). Most licensed fish vaccines are inactivated bacterins, frequently formulated with oil-based adjuvants to increase potency and duration of protection (Adams, 2019; Ma *et al*., 2019; Wang *et al*., 2020). Effective vaccines must match pathogen biology and production systems, combining appropriate antigens, adjuvants, and delivery routes to achieve robust yet acceptable safety and performance (Dalmo *et al*., 2016). Oil-adjuvanted emulsions can enhance antibody titers and protection against Gram-negative bacteria, but may also cause adhesions and other side effects, underscoring the need to optimize adjuvant type and dose (Fredriksen *et al*., 2013; Hoare *et al*., 2017; Li *et al*., 2020; Gravningen *et al*., 2008; Jaafar *et al*., 2015; Midtlyng *et al*., 1996; Rømer Villumsen *et al*., 2015).

For *A. hydrophila*, conventional whole-cell approaches using formalin-killed cells (FKC) or heat-killed preparations can provide partial to high protection in several warm-water species, particularly with parenteral delivery and suitable adjuvants (Chandran *et al*., 2002; Dehghani *et al*., 2012; Prasad and Areechon, 2010; Shome and Shome, 1999, 2005; Swain *et al*., 2007). Beyond bacterins, alternative antigen formats and delivery approaches, such as LPS/outer-membrane components, bacterial ghosts, and oral/feed-based strategies, have also been explored for *A. hydrophila* (Alishahi *et al*., 2019; Jiang *et al*., 2016; Liu *et al*., 2011; Thangaviji *et al*., 2012; Yan *et al*., 2018). However, systematic reviews highlight substantial heterogeneity in antigen choice, platform, adjuvant use, and outcome measures, limiting direct cross-study comparison and optimization (Ma *et al*., 2019; Mzula *et al*., 2019; Wang *et al*., 2020). This heterogeneity, together with strain-to-strain variation in field outbreaks, provides a practical rationale for testing polyvalent bacterins that combine multiple local isolates to broaden antigen coverage (Ma *et al*., 2019; Mzula *et al*., 2019; Wang *et al*., 2020).

In Indonesia, mono and bivalent FKC vaccines and alternative delivery regimens have improved survival and basic immune readouts in gourami under experimental or semi-field conditions (Firdaus *et al*., 2013; Ismail *et al*., 2016; Purwaningsih *et al*., 2015; Sugiani *et al*., 2013), and disease investigations reinforce the need for integrated health management in this species (Decostere *et al*., 2004; Lusiastuti *et al*., 2020; Rahmaningsih and Yanuhar, 2014). Nonetheless, most gourami data focus on monovalent or simple bivalently formulated vaccines, with limited head-to-head evidence for polyvalent *A. hydrophila* bacterins based on multiple local strains and limited longitudinal profiling beyond survival and endpoint serology (Monir *et al*., 2020; Purwaningsih *et al*., 2015; Sugiani *et al*., 2013). Moreover, comparative evidence on oil-adjuvanted versus nonadjuvanted polyvalent bacterins remains sparse, and studies only rarely integrate humoral, innate, and cytokine measurements to describe postvaccination immune trajectories (Adams, 2019; Fredriksen *et al*., 2013; Hoare *et al*., 2017; Mutoloki *et al*., 2015; Tafalla *et al*., 2013; Ma *et al*., 2019; Mzula *et al*., 2019; Wang *et al*., 2020).

To address these gaps, we conducted a head-to-head comparison of monovalent versus polyvalent FKC regimens, with and without oil adjuvantation, in giant gourami using a standardized experimental design. We assessed serum agglutinating antibody titers, NBT-reducing activity, and splenic *il-1β/ifn-γ* transcription over time, and quantified protection following a homologous intraperitoneal challenge using sur-

vival and relative percent survival (RPS). The novelty of this work lies in directly isolating the contributions of antigen breadth (monovalent vs. polyvalent) and oil adjuvantation within the same host–pathogen system, using harmonized immunologic endpoints and a homologous challenge model.

2. Materials and Methods

2.1 Materials

2.1.1 The materials

All culture media, biochemical reagents, and buffers were of analytical grade. Tryptic soy broth (TSB) and tryptic soy agar (TSA) were obtained from a single supplier and prepared according to the manufacturer's instructions. Commercial kits for RNA extraction and cDNA synthesis were used according to the protocols described in Section 2.2.17. Primers for il-1β, ifn-γ, and the reference gene were synthesised by a commercial provider based on previously published sequences.

2.1.2 The equipment

Bacterial culture, vaccine preparation and challenge procedures were performed in a Class II biosafety cabinet in a dedicated aquatic microbiology facility. Incubators and shaking incubators were maintained at 28–30°C for *A. hydrophila* growth. Optical density was measured with a benchtop spectrophotometer, and bacterial loads were verified by serial dilution and plating on TSA. RT-qPCR assays were performed on a real-time PCR thermocycler with 96-well format, using optical plates and sealing films recommended by the manufacturer. Detailed model numbers and suppliers for key instruments are provided in the Supplementary Methods to facilitate replication.

2.1.3 Ethical approval

All experimental procedures involving fish were conducted in accordance with national and institutional guidelines for the care and use of animals in research and were approved by the Animal Ethics Committee of Brawijaya University, Indonesia, under approval number 170-Kep-UB-2024**.** Every effort was made to minimise stress, suffering, and the number of animals used.

2.2 Methods

2.2.1 Bacterial strains and origin

Three *A. hydrophila* isolates, designated Ah-S1, Ah-S2, and Ah-S3, were obtained from an existing laboratory collection and not from new field sampling. These isolates were originally recovered from motile *Aeromonas* septicaemia (MAS) outbreaks in giant gourami (*O. goramy*) farms in East Java and have been reported in our previous work on *A. hydrophila* disease in this host, including their isolation, pathogenicity assessment, and molecular characterisation of representative virulent strains (Rozi *et al*., 2018a, 2018b, 2024). No additional diseased fish were collected from farms for bacterial isolation in the present study. Instead, the *A. hydrophila* strains were retrieved from archived cryopreserved isolates in our laboratory collection, whereas the vaccination and challenge experiments were conducted using live giant gourami as described below.

2.2.2 Phenotypic and molecular characterisation (reference to previous work)

The phenotypic and gyrB-based molecular characterisation of the *A. hydrophila* isolates used in this study has been reported in detail in our previous publications (Rozi *et al.,* 2018a, 2018b, 2024). Briefly, isolates recovered from MAS-affected giant gourami showed colony morphology and biochemical profiles consistent with *Aeromonas* spp., and their identity as *A. hydrophila* was confirmed by gyrB sequencing and phylogenetic analysis. In the present experiment, these previously characterised archived isolates (Ah-S1, Ah-S2, and Ah-S3) were revived for vaccine preparation, and Ah-S1 was used as the homologous challenge strain

2.2.3 Revival and maintenance of working cultures

Cryopreserved stocks of *A. hydrophila* isolates Ah-S1, Ah-S2, and Ah-S3 were maintained at −80°C in tryptic soy broth (TSB) supplemented with 10% (v/v) glycerol. For the present study, cryovials were thawed once at room temperature, and a loopful of each suspension was streaked onto tryptic soy agar (TSA) and incubated at 28–30°C for 24–48 h. Single colonies with typical *Aeromonas* morphology were then subcultured on fresh TSA plates to obtain pure, actively growing working cultures. These working cultures were stored on TSA slants at 4°C for short-term use and were periodically reinitiated from the original cryostocks to minimise passage number and reduce the risk of phenotypic drift.

2.2.4 Experimental fish and husbandry

Sub-adult giant gourami (*Osphronemus goramy*) were obtained from a commercial hatchery in East Java with no recent history of bacterial disease

outbreaks according to farm health records. On arrival at the laboratory, fish were carefully inspected and only clinically normal individuals, without visible external lesions or abnormal behaviour, were included in the experiment. At the start of the trial, a random subsample of fish was weighed and measured to document body weight and total length; these data were used to confirm that size distributions were comparable across treatment groups.

Fish were acclimated for 14 days in indoor fiberglass tanks supplied with aerated, dechlorinated freshwater under a semi-recirculating system. During acclimation and throughout the experiment, fish were held at a density of 10 fish per 80 L tank, with continuous aeration and a natural or near-natural photoperiod (approximately 12 h light : 12 h dark). Water temperature, dissolved oxygen, pH, and total ammonia nitrogen (TAN) were monitored regularly and maintained within ranges suitable for warm-water gourami culture (approximately 27–29°C, dissolved oxygen > 5 mg L^{-1}, pH 7.0–7.5, TAN < 0.02 mg L^{-1}). Fish were fed a commercial pelleted diet formulated for omnivorous freshwater species (floating pellets containing 32–35% crude protein) at a daily ration of approximately 3% body weight, divided into two feedings per day, with uneaten feed and debris removed by siphoning. To minimise the risk of introducing pre-existing Aeromonas infections into the trial, a subset of fish from the acclimation tanks was subjected to baseline health screening. External lesions were assessed visually, and kidney and spleen samples from pooled individuals were streaked onto TSA and Aeromonas-selective media for bacteriological examination. In addition, pooled kidney/spleen tissue was tested by *A. hydrophila*-specific gyrB PCR as described previously (Rozi *et al*., 2018a, 2018b, 2024). No *A. hydrophila* was isolated on culture, and all baseline gyrB PCR assays were negative.

2.2.5 Vaccine preparation and growth of seed cultures

For each isolate (Ah-S1, Ah-S2, and Ah-S3), a single colony from a fresh TSA plate (Section 2.2.3) was inoculated into 10 mL TSB and incubated at 28–30°C for 18–24 h with gentle shaking (120–150 rpm) to obtain an overnight starter culture in logarithmic to early stationary phase. Starter cultures were then transferred to larger TSB volumes (typically 100–250 mL in baffled Erlenmeyer flasks) at a 1:50–1:100 inoculation ratio and incubated under the same conditions until they reached the desired turbidity. Bacterial growth was monitored spectrophotometrically at 600 nm (OD_{600}). An in-house calibration curve relating OD_{600} to colony-forming units (CFU) per mL for *A. hydrophila* Ah-S1 was established in separate preliminary experiments by serial dilution and spread-plating on TSA; this relationship was used to estimate cell densities of Ah-S1, Ah-S2, and Ah-S3 cultures for vaccine preparation and challenge. Immediately before inactivation or dilution, representative samples from each culture were serially diluted and plated on TSA to verify CFU counts by back-titration.

2.2.6 Formalin inactivation and sterility testing

Exponentially growing cultures of *A. hydrophila* (Ah-S1, Ah-S2, and Ah-S3) were adjusted to the desired cell density based on OD_{600} and CFU back-titration (Section 2.2.5), and then inactivated by the addition of sterile neutral-buffered formalin to a final concentration of approximately 0.3% (v/v). Cultures were gently mixed and incubated for 24 h at 4°C with intermittent agitation to ensure uniform exposure of all cells to formalin. After inactivation, bacterial cells were harvested by centrifugation (4,000–5,000 × g for 10–15 min at 4°C), and the supernatant was discarded. Pellets were washed at least three times with sterile phosphate-buffered saline (PBS), repeating centrifugation between washes, to remove residual formalin. The final pellets were resuspended in sterile PBS to the target CFU-equivalent concentration for vaccine formulation (Section 2.2.7). To confirm complete inactivation and sterility of the formalin-killed cell (FKC) preparations, 100 µL aliquots of each final suspension were spread onto TSA plates and incubated at 28–30°C for 48–72 h. Preparations were accepted for use only if no bacterial growth was observed on any of the plates ("no growth = accepted"). FKC suspensions were stored at 4°C and used within a short time frame for vaccine preparation to minimise antigen degradation.

2.2.7 Monovalent and polyvalent FKC vaccine formulations (± oil adjuvanted)

Formalin-killed cell suspensions of *A. hydrophila* Ah-S1, Ah-S2, and Ah-S3 (Section 2.2.6), were adjusted, based on OD_{600}–CFU calibration and back-titration, to 1 × 10^9 CFU-equivalents mL^{-1} in sterile PBS. The monovalent vaccine (P2) contained Ah-S1 only, and fish received 0.1 mL intraperitoneally (i.p.) per fish (1 × 10^8 CFU-equivalents). The non-adjuvanted polyvalent vaccine (P3) was prepared by mixing equal volumes of Ah-S1, Ah-S2, and Ah-S3 FKCs (1:1:1), with each isolate contributing one-third of the final antigen load, and was standardized to 1 × 10^9 CFU-equivalents mL^{-1}; thus, a 0.1 mL i.p. dose delivered 1 × 10^8 CFU-equivalents per fish, equally distributed among isolates. PBS alone served as the control (P1) and was administered i.p. at 0.1 mL per fish. For

the adjuvanted polyvalent formulation (P4), the same P3 antigen suspension (aqueous phase) was emulsified with Montanide ISA 760 VG (Seppic, France) using a two-syringe method at a 30:70 antigen: adjuvant ratio (v/v) to obtain a stable water-in-oil emulsion. The aqueous phase was adjusted so that each 0.1 mL i.p. injection delivered 1 × 10^8 CFU-equivalents per fish, matching the total antigen load of P3. The emulsion was stored at 4°C and gently inverted during vaccination.

2.2.8 Experimental groups, design and randomisation

A total of 240 clinically healthy giant gourami were used in the vaccination trial. Fish were allocated to four treatment groups (P1–P4), each comprising 60 fish per group. For each treatment, fish were divided into two parallel cohorts: an immunology cohort (30 fish per group) and a survival/challenge cohort (30 fish per group). Within each cohort, fish were distributed into three replicate tanks of 10 fish per tank, giving a total of six tanks per treatment (3 tanks for immunological sampling and 3 tanks for challenge). The experiment followed a completely randomised design (CRD) at the tank level, with four vaccination treatments (P1–P4) and three replicate tanks per treatment within each cohort. After acclimation, fish were individually netted and assigned to tanks using a computer-generated randomisation list, stratified by body weight to minimise size differences among groups. Randomisation was conducted at the tank level, and subsequent statistical analyses treated the tank as the experimental unit to avoid pseudo-replication.

2.2.9 Vaccination protocol

On day 0, fish were anaesthetised in aerated freshwater containing buffered tricaine methanesulfonate (MS-222; 100 mg L^{-1}), adjusted to pH 7.0–7.5 with sodium bicarbonate, until loss of equilibrium and reduced response to handling were observed. Anaesthetised fish were gently placed on a wet towel, and each individual received a 0.1 mL intraperitoneal (i.p.) injection of the assigned formulation using a sterile insulin syringe and fine-gauge needle: PBS (P1), monovalent FKC Ah-S1 (P2), non-adjuvanted polyvalent FKC (P3), or oil-adjuvanted polyvalent FKC (P4), as described in Sections 2.2.7. All fish were vaccinated once only (single-dose regimen); no booster injections were administered during the experimental period. Following vaccination, fish were returned to their respective tanks and monitored at least once daily for the remainder of the study. Post-vaccination observations included feed intake, swimming behaviour, external appearance and any visible reactions at the injection site, as well as the occurrence and timing of any mortalities.

2.2.10 Sampling schedule and tissue collection

For the immunology cohort, fish were sampled at day 0 (baseline, immediately before vaccination) and at 7, 14, 21, 35, and 42 days post-vaccination. At each time point, two fish per tank were randomly selected from each of the three replicate tanks per treatment (n = 6 fish per treatment per time point).After blood collection, fish were euthanised by MS-222 overdose (300 mg L^{-1}; buffered to pH 7.0–7.5 with sodium bicarbonate) prior to aseptic spleen collectionas described in Section 2.2.9. Blood was collected from the caudal vein using sterile syringes without an anticoagulant. Blood samples were allowed to clot at room temperature and then centrifuged (3,000 × g for 10–15 min) to separate serum. Serum was aliquoted into sterile microtubes and stored at −80°C until analysis of agglutinating antibody titres and NBT-reducing activity (Sections 2.2.15–2.2.16).

After blood collection, fish were euthanised by MS-222 overdose, and the spleen was aseptically excised, blotted to remove excess blood, and snap-frozen or placed in appropriate tubes for RNA preservation. Spleen samples were stored at −80°C until RNA extraction and RT-qPCR analysis of il-1β and ifn-γ transcription (Section 2.2.17). Fish in the survival/challenge cohort were not subjected to blood or tissue sampling during the pre-challenge period; they were only monitored clinically (behaviour, appetite, external signs of disease) until the challenge trial was conducted (Section 2.2.12).

2.2.11 Challenge test, LD_{50} reference, and bacteriological confirmation

The primary challenge strain used in this study was Ah-S1 (corresponding to isolate AH3 in our earlier work). Ah-S1 was selected as the homologous challenge strain because it originated from a local MAS outbreak in giant gourami and its LD_{50} had been previously established, enabling a standardized and reproducible challenge modelTo maintain comparability across regimens, P2 used Ah-S1 as the monovalent seed strain. Accordingly, the protection outcomes in this study reflect homologous protection against Ah-S1, and the breadth of protection against Ah-S2/Ah-S3 was not assessed and warrants future heterologous challenge testing. In our earlier study, intraperitoneal injection of graded doses of *A. hydrophila* AH3 (10^4–10^8 CFU/fish) followed by Dragstedt–Behrens analysis yielded an LD_{50} of 4.53 × 10^6 CFU/fish (Rozi *et al.*, 2018b). In the present vaccine–challenge experiment,

we did not re-estimate LD_{50}. Instead, the challenge dose was chosen a priori based on this published value to produce reproducible, high but submaximal mortality in unvaccinated controls. Specifically, fish were challenged intraperitoneally with 1.0×10^7 CFU/fish (≈2× LD_{50}; Section 2.2.12), consistent with standard practice in fish vaccine efficacy trials.

2.2.12 Challenge procedure

To standardize challenge pressure and enable a controlled head-to-head comparison of formulation effects (monovalent vs. polyvalent and ± oil adjuvant), a single homologous challenge model was used. Accordingly, protection outcomes are interpreted as homologous protection, and cross-strain protection against Ah-S2/Ah-S3 was not assessed and should be evaluated in future heterologous challenge studies. Fish were challenged at 21 days post-vaccination (dpv) to target a biologically relevant window when adaptive responses to inactivated bacterins become established; in our longitudinal measurements, agglutinating titers increased markedly by day 21. No pilot study was performed to optimize the challenge timing.

The vaccine efficacy trial was conducted using the survival/challenge cohort (Section 2.2.8), which was challenged intraperitoneally with *A. hydrophila* Ah-S1. For the challenge inoculum, Ah-S1 was revived from cryostock and grown in TSB at 28–30°C with shaking as described in Section 2.2.5. An overnight culture was adjusted spectrophotometrically to the OD_{600} corresponding to the target density and then washed twice by centrifugation (4,000–5,000 × g, 10 min, 4°C) and resuspended in sterile PBS to remove residual medium components. The final suspension was adjusted to deliver a nominal dose of 1.0×10^7 CFU in 0.1 mL PBS per fish, based on the previously established OD_{600}–CFU calibration for Ah-S1. Immediately before use, the actual bacterial concentration was verified by serial dilution and spread-plating on TSA (back-titration).

On challenge day, fish were anaesthetised with buffered MS-222 (Section 2.2.9). Each fish then received an intraperitoneal injection of 0.1 mL of the Ah-S1 suspension at the designated CFU dose, using sterile insulin syringes. After injection, fish were returned to their original tanks and observed until complete recovery from anaesthesia. Post-challenge, fish were monitored for 14 days, with twice-daily inspections for changes in behaviour, appetite, external lesions, and mortality. Dead and moribund fish (reaching predefined humane endpoints) were removed promptly, recorded (tank, treatment, time post-challenge), and processed for bacteriological examination as described in Section 2.2.13. No therapeutic treatments were administered during the observation period.

2.2.13 Re-isolation and confirmation of A. hydrophila

To verify that post-challenge mortality was attributable to *A. hydrophila* infection, a subset of moribund or freshly dead fish from each treatment group was examined bacteriologically. Following euthanasia (for moribund fish) by MS-222 overdose, the body surface was disinfected with 70% ethanol, and aseptic necropsy was performed. Kidney swabs (and, when present, samples from haemorrhagic internal lesions) were streaked onto TSA and Aeromonas-selective agar and incubated at 28–30°C for 24–48 h. Predominant colonies displaying typical Aeromonas morphology were subcultured and identified based on standard phenotypic criteria (Gram-negative, oxidase-positive rods with the expected biochemical profile), consistent with the previously characterised *A. hydrophila* isolates used in this study (Rozi *et al.*, 2018a, 2018b, 2024). Because the challenge strain Ah-S1 had already been taxonomically and molecularly confirmed in earlier work, routine PCR was not repeated for each reisolate. The primary purpose of these examinations was to confirm that mortality following challenge was associated with systemic *A. hydrophila* infection, rather than unrelated opportunistic bacteria.

2.2.14 Serum handling and immunological assays

Blood samples collected from the caudal vein (Section 2.2.10) were transferred into plain microtubes and allowed to clot at room temperature for approximately 1 h, then held at 4°C until processing. Samples were centrifuged (3,000 × g, 10–15 min), and the supernatant serum was carefully aspirated, avoiding disturbance of the clot. Serum from each fish was aliquoted into sterile, labelled microtubes to avoid repeated freeze–thaw cycles and stored at −80°C until analysis. For each assay, serum aliquots were thawed once on ice, mixed gently, and used immediately. Baseline sera collected prior to vaccination (day 0) showed undetectable/near-baseline agglutinating activity in all groups.

2.2.15 Microagglutination assay

Serum agglutinating antibody titres against *A. hydrophila* were measured using a microagglutination assay with formalin-killed Ah-S1 as antigen. Before testing, serum samples were heat-inactivated at 45°C for 30 min to inactivate complement. Heat-inactivated sera were subjected to twofold serial dilutions in sterile PBS in 96-well U-bottom microplates, typically starting at a 1:2 or 1:4 dilution. For each well, 50 µL of

diluted serum was mixed with 50 µL of the FKC Ah-S1 suspension, previously adjusted to a concentration of approximately 1×10^8 CFU-equivalents mL^{-1}, giving a final reaction volume of 100 µL per well. Plates were gently tapped to mix, covered, and incubated at room temperature (or 25–28°C) for a defined period (18–24 h) without disturbance. After incubation, wells were examined visually for agglutination. A positive reaction was defined as a diffuse mat or veil of bacteria covering the bottom of the well, whereas a negative reaction appeared as a compact button of settled bacteria in the centre of the well. Each plate included negative control wells containing antigen plus PBS without serum and, where available, a reference positive control serum. Agglutination titres were expressed as the reciprocal of the highest serum dilution showing a clear positive agglutination reaction.

2.2.16 Respiratory burst (NBT) assay

Respiratory burst activity of circulating phagocytes was assessed in whole blood using a nitroblue tetrazolium (NBT) reduction assay. Freshly collected blood (Section 2.2.10) was processed immediately after sampling; samples showing visible clotting were discarded. To standardize input across fish, a fixed whole-blood volume (100 µL) was used for each reaction. Whole blood was mixed with 100 µL of 0.2% (w/v) NBT solution prepared in PBS (final reaction volume 200 µL; NBT: blood ratio 1:1, v/v), gently mixed, and incubated at room temperature (25–28°C) for 30 min. The reaction was stopped by adding 100 µL of ice-cold 100% methanol, followed by centrifugation to pellet cells/formazan. The supernatant was discarded, and the pellet was washed twice with 70% methanol to remove unreacted NBT and then air-dried. Intracellular formazan was solubilized by adding 120 µL of 2 M KOH followed by 140 µL DMSO, with gentle mixing until complete dissolution. Absorbance was measured at 620 nm using a microplate reader, with wells containing NBT but no blood as blanks. NBT-reducing activity was expressed as blank-subtracted OD_{620} and interpreted as a standardized whole-blood index of respiratory burst activity. Because the assay was performed on whole blood using a fixed input volume, results were not normalized to leukocyte counts.

2.2.17 RNA extraction, cDNA synthesis, and RT-qPCR for il-1β and ifn-γ

For gene expression analysis, spleen samples collected as described in Section 2.2.9 were processed individually. Approximately 20–30 mg of splenic tissue from each fish was excised aseptically, blotted to remove excess blood, placed into pre-labelled tubes, and snap-frozen in liquid nitrogen or transferred to RNA preservation medium as appropriate, then stored at −80°C until RNA extraction. Total RNA was extracted using a commercial column-based kit (Total RNA Mini Kit, Geneaid Biotech, Taiwan) according to the manufacturer's instructions, including an on-column DNase I treatment to remove residual genomic DNA. RNA concentration and purity were assessed spectrophotometrically (NanoDrop, Thermo Fisher Scientific) by measuring absorbance at 260 and 280 nm, and RNA integrity was checked by agarose gel electrophoresis to confirm the presence of sharp 28S and 18S rRNA bands without degradation. Only samples with A_{260}/A_{280} ratios within the acceptable range and intact rRNA profiles were used for downstream analyses. For cDNA synthesis, 1 µg of total RNA from each sample was reverse transcribed in a 20 µL reaction volume using a reverse transcription kit (iScript™ cDNA Synthesis Kit, Bio-Rad, USA) with random hexamer and/or oligo(dT) primers, following the supplier's protocol. The resulting cDNA was diluted (1:5–1:10 in nuclease-free water) and stored at −20°C until use in quantitative PCR. Relative mRNA expression of pro-inflammatory (il-1β) and Th1-like (ifn-γ) cytokines was quantified by real-time quantitative PCR (RT-qPCR) using a real-time PCR system (StepOne™ Real-Time PCR System, Applied Biosystems, USA) and a SYBR/EvaGreen-based master mix in 20 µL reactions, typically containing 1–2 µL diluted cDNA, 10 µL 2× SYBR/EvaGreen master mix, and 0.2–0.5 µM of each forward and reverse primer.

Primer sequences for il-1β, ifn-γ, and the reference genes β-actin and ef-1α are listed in Table 1. The thermal cycling programme consisted of an initial denaturation step (95°C for 2–3 min), followed by 40 cycles of denaturation (95°C for 10–15 s), annealing/extension at the gene-specific annealing temperature (typically 58–60°C for 20–30 s), and a final melt-curve analysis to verify the specificity of amplification. Melting curve profiles were inspected to confirm a single peak for each amplicon. Primer efficiency for each target and reference gene was evaluated using standard curves generated from serial dilutions of pooled cDNA and was found to be within an acceptable range (approximately 90–110%). For selected representative amplicons, PCR products were purified and sequenced to confirm identity with the expected *O. goramy* cytokine or housekeeping gene fragments. Relative gene expression was calculated using the 2^−ΔΔCt method, with β-actin as the primary reference gene. The stability of β-actin and ef-1α expression across treatments and sampling times was evaluated, and β-actin was retained as the main normaliser, with ef-1α serving as a secondary check. For each sample, ΔCt values were obtained by subtracting the Ct of β-actin from the Ct of the target gene, and ΔΔCt values were calculated relative to the mean ΔCt of the PBS control group at day 0. Fold-change expression values (2^−ΔΔCt) were used for statistical analysis of il-1β and ifn-γ transcription.

2.3 Statistical Analysis

All analyses treated the tank as the experimental unit to avoid pseudo-replication: individual fish measurements were first averaged at the tank level (means per tank per time point) before modelling. Unless otherwise stated, data are presented as tank-level means ± standard error of the mean (SEM). Agglutination titres were $\log_2$-transformed before analysis. NBT optical density values and 2^−ΔCt or 2^−ΔΔCt cytokine expression values were inspected for skewness and, where necessary, $\log_{10}$- or square-root–transformed to better meet model assumptions; the transformation used for each endpoint is reported with the corresponding results. No formal prospective power calculation was performed; the number of tanks and fish per tank was chosen pragmatically based on prior experience with similar vaccine trials and logistical constraints, and is reported to allow readers to judge precision and power ex post.

Among the immunological variables, splenic ifn-γ transcription was pre-specified before data collection as the primary mechanistic endpoint, with splenic il-1β, serum agglutinating antibody titres, and NBT-reducing activity treated as secondary endpoints. For each endpoint, pre-planned contrasts focused on the oil-adjuvanted polyvalent FKC (P4): P4 vs P1 (PBS control; primary contrast), P4 vs P2 (monovalent FKC; secondary contrast), and P4 vs P3 (non-adjuvanted polyvalent FKC; secondary contrast). Exploratory contrasts not defined a priori are reported descriptively without claims of confirmatory inference.

For time-course immunological endpoints, the primary analyses used linear mixed-effects models of the form:

Response ~ Treatment × Day + (1 | Tank),

with Treatment (P1–P4) and Day (0, 7, 14, 21, 35, 42 days post-vaccination) as fixed effects and Tank as a random intercept. When useful for interpretation, simpler one-way models (Treatment only) were also fitted separately at each sampling day. Model assumptions (normality and homoscedasticity of residuals, and influential observations) were evaluated by inspection of residual and Q–Q plots and standard influence diagnostics. For each mixed model, marginal and conditional R^2 and the intraclass correlation coefficient (ICC) for Tank were reported to quantify the proportion of variance attributable to treatment and to clustering.

Table 1. qPCR primers for immune-related genes

Gene	Primer (5′–3′)	Product (bp)	GenBank
β-actin	F:AACCATGGATGATGAAATCGCCGCA R:TGATGCCTGGGGCGACCGACGATGG	126	AB604946
IL-1β	F:TAACACTGAGAGGACAACTG R:GAAGAGAAACCGCACCAT	90	KT884611.1
IFN-γ	F:TAGGCTGTTGCAGCACTATAAA R:AACACCACCCATGAAGATCAA	105	KF294754.1
EF-1α	F:CAGGGCATCCATCAACAAGA R:GCATAAGCCAGTCCTTGAGTATAG	121	NM001279647.1

Vaccine performance was also summarised as relative percent survival (RPS) for each vaccinated group compared with the PBS control, calculated according to Amend (1981):

$$\mathrm{SR}(\%) = \left(\frac{\text{Number of surviving fish at day 14}}{\text{Number of fish challenged}}\right) \times 100.$$

$$\mathrm{RPS}(\%) = \left[1 - \left(\frac{\text{Mortality in vaccinated group (\%)}}{\text{Mortality in control group (\%)}}\right)\right] \times 100.$$

Estimated marginal means (EMMs) and 95% confidence intervals (CIs) for each Treatment × Day combination, as well as contrast estimates and adjusted p-values, were obtained using emmeans. Tukey–Kramer adjustments were used for all pairwise comparisons within each endpoint. To control multiplicity across the pre-specified contrasts, Holm-adjusted p-values are additionally reported as a sensitivity analysis. Where relevant, standardised effect sizes (Cohen's *d* for pre-planned contrasts and partial η^2 for omnibus tests) were calculated and are presented alongside the corresponding estimates in the results tables to aid interpretation of the magnitude and biological relevance of treatment effects; they were not used to define additional significance thresholds. All tests were two-sided, and p-values < 0.05 were interpreted in the context of effect sizes and CIs rather than as strict decision boundaries.

Survival over the 14-day post-challenge period was first visualised by Kaplan–Meier curves and compared among treatments by log-rank tests. To account for tank-level clustering and obtain hazard-based effect estimates, Cox proportional-hazards mixed-effects models were fitted with Treatment as a fixed effect and Tank as a frailty term, yielding hazard ratios (HRs) and 95% CIs relative to P1. The proportional hazards assumption was evaluated using Schoenfeld residuals. Relative percent survival (RPS) at day 14 was calculated for each vaccinated group according to Amend (1981). All analyses were conducted using the jamovi statistical platform (jamovi project), interfacing with the underlying R engine and relevant packages, including lme4 for mixed-effects models, emmeans for EMMs and contrasts, and survival/coxme for survival and Cox regression analyses.

3. Results and Discussion

3.1 Results

3.1.1 Molecular identification and Phylogenetic analysis of A. hydrophila isolates

The three vaccine/challenge isolates (Ah-S1, Ah-S2, and Ah-S3) were previously characterised phenotypically and confirmed as *A. hydrophila* (Rozi *et al.*, 2018a, 2018b, 2024). In the present study, gyrB PCR produced a single amplicon of the expected size in all isolates (Figure 1A). BLASTn of the gyrB fragment (Ah-S1 shown as a representative in Table 2) returned top matches annotated as *A. hydrophila* with 97.47% identity (98% query cover) and 97.10% identity (92% query cover), whereas a third *A. hydrophila* match showed lower identity (93.22%; 98% query cover). Given that this lower identity may reflect greater sequence divergence, BLAST similarity is interpreted together with phylogenetic placement. In the gyrB phylogeny, Ah-S1, Ah-S2 and Ah-S3 clustered tightly (bootstrap = 99) within the broader *A. hydrophila* group and were separated from closely related *Aeromonas* taxa and outgroups (Figure 1B). Taken together, gyrB PCR, BLAST results, and phylogenetic evidence support the classification of these isolates as *A. hydrophila.*

3.1.2 Agglutination titres

Serum agglutinating antibody titres against *A. hydrophila* varied by sampling time and treatment (Table 3). In PBS controls (P1), titres remained low throughout the study (mean 9.14 ± 0.06 to 16.00 ± 0.28 over days 7–42). Vaccinated groups showed higher titres than P1, and mean titres were generally higher in P4 than in P2 and P3 across the sampling period (Table 3). At day 7, mean titres were 100.57 ± 4.00 in P4 and 10.29 ± 0.28 in P1. Group differences were more pronounced at later time points (e.g., day 21: 2,413.71 ± 5.68 in P4 vs 9.14 ± 0.06 in P1; day 35: 3,218.29 ± 19.13 in P4 vs 16.00 ± 0.28 in P1; Table 3). Titres in P2 and P3 peaked at days 21–35 (512.57–1,316.57) and were higher than P1 at these later time points, while remaining lower than P4 (Table 3). By day 42, titres declined in vaccinated groups but remained higher in P4 (512.00 ± 19.13) than in P1 (10.85 ± 0.06). Linear mixed-effects modelling of $\log_2$-transformed titres identified significant effects of Treatment ($F = 38.4$, df = 3,8, $p < 0.001$, partial $\eta^2 = 0.935$), Day ($F = 78.3$, df = 4,32, $p < 0.001$, partial $\eta^2 = 0.907$) and their interaction ($F = 12.4$, df = 12,32, $p < 0.001$, partial $\eta^2 = 0.823$) (Table 5). Tukey-adjusted contrasts showed P4 differed from P1 at all sampling days ($p < 0.001$; Table 6), and model-derived estimated marginal means for P4 were higher than P2 and P3 at most time points (Table 6). Overall, these analyses indicate that vaccination was associated with higher agglutinating titres than PBS controls, with the largest treatment differences observed in P4 during days 21–35 (Tables 3, 5–6).

3.1.3 Respiratory burst (NBT-reducing) activity

Nitroblue tetrazolium (NBT)-reducing activity in whole blood varied with time and vaccination status (Table 4). In PBS controls (P1), mean OD_{620} values remained low to moderate and relatively stable, ranging from 0.29 ± 0.11 to 0.48 ± 0.01 over the 42-day period. In vaccinated fish, patterns were more variable, and the magnitude of change was smaller than for agglutinating antibody titres. At days 7 and 14, P4 showed higher NBT activity (0.63 ± 0.07 and 0.61 ± 0.27, respectively) than P1 (0.48 ± 0.01 and 0.31 ± 0.08). At day 21, mean NBT activity in P4 (0.22 ± 0.11) was lower than in P1 (0.42 ± 0.10), where as at day 35, values were again higher in P4 (0.42 ±

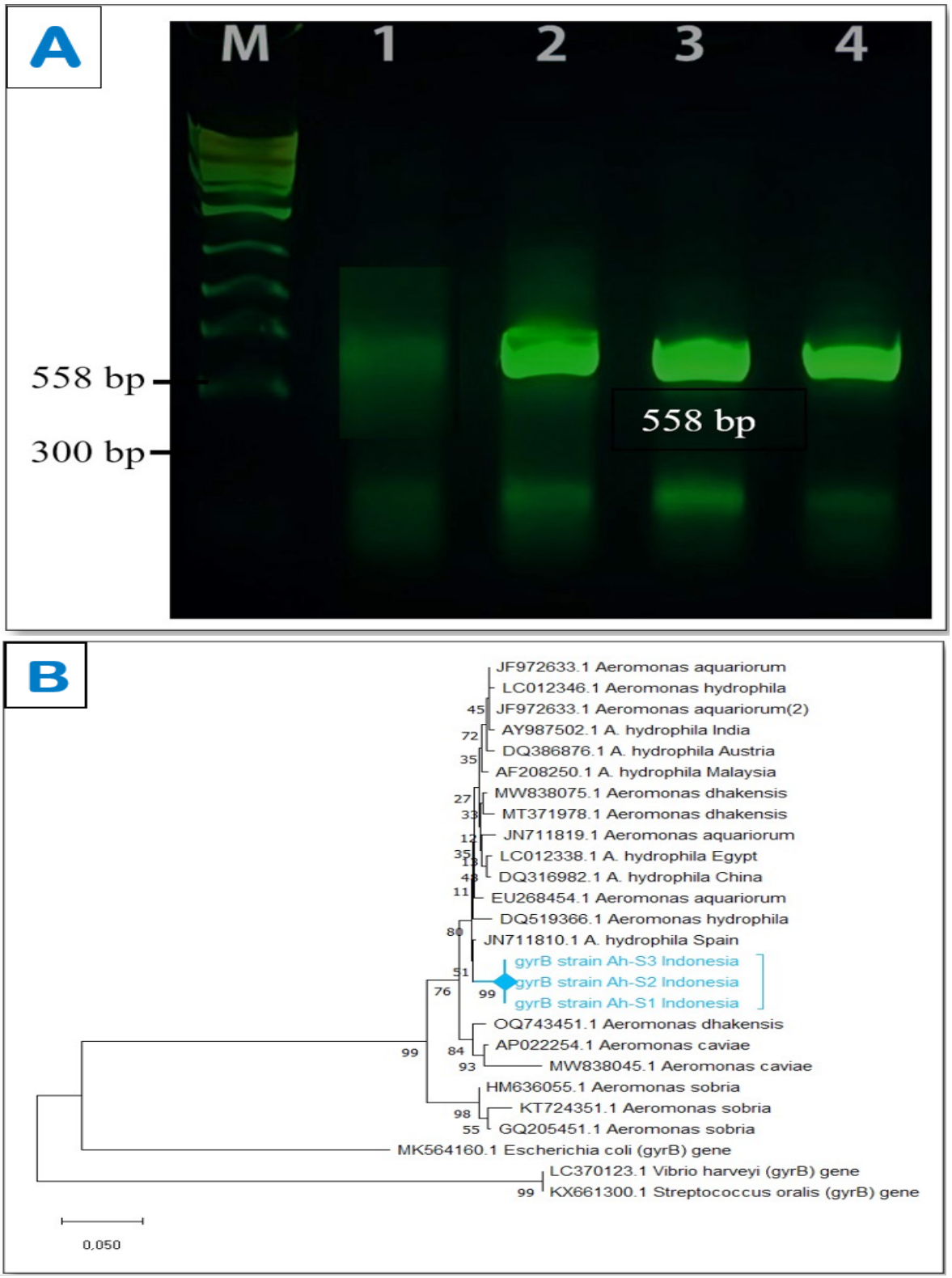

Figure 1. PCR amplification of the *gyrB* gene from *A. hydrophila* isolates (1A). Phylogenetic placement of Surabaya *A. hydrophila* isolates based on gyrB sequences. Maximum parsimony tree inferred from partial *gyrB* sequences of Aeromonas isolates. The Surabaya isolates Ah-S1, Ah-S2, and Ah-S3 (bold) cluster within the *A. hydrophila* complex together with reference *A. hydrophila* strains from China, Egypt, Spain, Austria, Malaysia, and India, and are clearly separated from *A. dhakensis, A. aquariorum*, *A. caviae*, and *A. sobria*. *Escherichia coli*, *Vibrio harveyi*, and *Streptococcus oralis* gyrB sequences were used as outgroups. Node labels indicate bootstrap support values from 1,000 replicates (only values ≥50% are shown). Branch lengths are proportional to the number of inferred character changes.

Table 2. Top BLASTn matches of the partial *gyrB* fragment from the Surabaya *A. hydrophila* isolate

No.	*A. hydrophila* isolates used in this study	Description	Query-cover	Percent Indentity	Number of Access
1.	Ah-S1	*Aeromonas hydrophila* strain AE-57 GyrB (*gyrB*) gene, partial cds	98%	97.47%	JN711810.1
2.	Ah-S1	*Aeromonas hydrophila* isolate 295 GyrB (gyrB) gene, partial cds	92%	97.1%	DQ519366.1
3.	Ah-S1	*Aeromonas hydrophila* strain D14 DNA gyrase subunit B (gyrB) gene, partial cds	98%	93.22%	MT967985.1

Table 3. Serum agglutinating antibody titres in *O. goramy* vaccinated with monovalent and polyvalent FKC vaccines over a 42-day follow-up

Observation time	PBS control (P1)	Monovalent FKC (P2)	Non-adjuvanted polyvalent FKC (P3)	Adjuvanted polyvalent FKC (P4)
D7	10.29 ± 0.28^{cd}	8.00 ± 0.28^{a}	9.14 ± 5.17^{b}	100.57 ± 4.00^{i}
D14	9.71 ± 0.20^{bc}	68.57 ± 3.13^{f}	82.28 ± 3.29^{h}	667.42 ± 020^{l}
D21	9.14 ± 0.06^{b}	512.57 ± 5.68^{j}	548.85 ± 5.42^{k}	2413.71 ± 5.68^{n}
D35	16.00 ± 0.28^{e}	1316.57 ± 8.82^{m}	1316.57 ± 8.76^{h}	3218.29 ± 19.13^{o}
D42	10.85 ± 0.06^{d}	68.57 ± 8.82^{f}	77.71 ± 8.76^{g}	512.00 ± 19.13^{j}

Serum agglutinating antibody titres against *A. hydrophila* in giant gourami (*O. goramy*) at 7, 14, 21, 35, and 42 days post-vaccination. P1 = phosphate-buffered saline (PBS) control; P2 = monovalent formalin-killed cell (FKC) vaccine; P3 = non-adjuvanted polyvalent FKC vaccine; P4 = oil-adjuvanted polyvalent FKC vaccine. Values are presented as mean ± standard error (SE) of tank means. Different superscript letters within a column indicate significant differences among sampling days for the same treatment (linear mixed-effects model followed by Tukey's test, $p < 0.05$).

Table 4. Nitroblue tetrazolium (NBT)-reducing activity in *O. goramy* following vaccination with monovalent and polyvalent FKC vaccines

Observation time	PBS control (P1)	Monovalent FKC (P2)	Non-adjuvanted polyvalent FKC (P3)	Adjuvanted polyvalent FKC (P4)
D7	0.48 ± 0.01^{h}	0.50 ± 0.01^{h}	0.47 ± 0.07^{gh}	0.63 ± 0.07^{i}
D14	0.31 ± 0.08^{f}	0.49 ± 0.08^{gh}	0.23 ± 0.27^{bcd}	0.61 ± 0.27^{i}
D21	0.42 ± 0.10^{gh}	0.27 ± 0.10^{cdef}	0.39 ± 0.11^{g}	0.22 ± 0.11^{bcd}
D35	0.29 ± 0.11^{def}	0.12 ± 0.11^{a}	0.20 ± 0.11^{bcd}	0.42 ± 0.11^{gh}
D42	0.31 ± 0.12^{ef}	0.49 ± 0.12^{h}	0.17 ± 0.03^{ab}	1.18 ± 0.03^{j}

Nitroblue tetrazolium (NBT)-reducing activity (respiratory burst) in whole blood of giant gourami (*Osphronemus goramy*) at 7, 14, 21, 35, and 42 days post-vaccination. P1 = phosphate-buffered saline (PBS) control; P2 = monovalent formalin-killed cell (FKC) vaccine; P3 = non-adjuvanted polyvalent FKC vaccine; P4 = oil-adjuvanted polyvalent FKC vaccine. Values are presented as mean absorbance at 620 nm ± standard error (SE) of tank means. Different superscript letters within a column indicate significant differences among sampling days for the same treatment (linear mixed-effects model followed by Tukey's test, $p < 0.05$).

Table 5. Omnibus mixed-model tests for immunological markers. Summary of linear mixed-model omnibus tests for Treatment (B), Day (C), and their interaction (B×C) for each immunological marker. Fill F, df, p, and partial η^2 directly from the jamovi output

Marker	Effect	F	df1	df2	p	partial η^2
IFN-γ	Treatment (B)	359.7	3	40	<0.001	0.964
IFN-γ	Day (C)	159.2	4	40	<0.001	0.941
IFN-γ	B×C	28.0	12	40	<0.001	0.894
IL-1β	Treatment (B)	1527.0	3	8	<0.001	0.998
IL-1β	Day (C)	1312.0	4	32	<0.001	0.994
IL-1β	B×C	198.0	12	32	<0.001	0.987
Agglutination titre	Treatment (B)	38.4	3	8	<0.001	0.935
Agglutination titre	Day (C)	78.3	4	32	<0.001	0.907
Agglutination titre	B×C	12.4	12	32	<0.001	0.823
Respiratory burst (NBT)	Treatment (B)	617.0	3	8	<0.001	0.996
Respiratory burst (NBT)	Day (C)	350.0	4	32	<0.001	0.978
Respiratory burst (NBT)	B×C	233.0	12	32	<0.001	0.989

0.11) than in P1 (0.29 ± 0.11). The largest difference occurred at day 42, when mean OD_{620} reached 1.18 ± 0.03 in P4 versus 0.31 ± 0.12 in P1. P2 and P3 generally showed intermediate values between P1 and P4, with no consistent ranking across all time points.

Analysis of log-transformed NBT data demonstrated strong omnibus effects of Treatment ($F = 617.0$, $df = 3,8$, $p < 0.001$, partial $\eta^2 = 0.996$), Day ($F = 350.0$, $df = 4,32$, $p < 0.001$, partial $\eta^2 = 0.978$) and their interaction ($F = 233.0$, $df = 12,32$, $p < 0.001$, partial $\eta^2 = 0.989$) (Table 5). Planned contrasts for P4 vs P1 yielded statistically significant differences at all sampling days (Tukey-adjusted $p < 0.001$; Table 6), with particularly large effect sizes at days 14 and 42. Given the more modest absolute changes and some fluctuations over time, these NBT data are interpreted as supportive evidence of vaccine-associated modulation of innate immune activity, rather than as a primary outcome in this study.

3.1.4 Splenic il-1β mRNA expression

Relative splenic il-1β mRNA expression (2^−ΔΔCt relative to PBS at day 0) varied by sampling time and treatment (Table 5; Figure 2). At day 7, fold-changes were close to baseline across treatments. From days 21–42, mean fold-changes were higher in P2, P3 and P4 than in P1; values in P4 were generally higher (approximately ~3–5) over this period, whereas P1 remained near 1-fold (Table 5; Figure 2). Mixed-effects analysis of log-transformed values showed significant effects of Treatment, Day, and their interaction (Table 5). Within-day ANOVA followed by Tukey's test indicated il-1β expression in P4 differed from P1 at all post-vaccination sampling times ($p < 0.05$), with P3 and P2 also differing from P1 at several later time points (Table 5). Across time points, il-1β transcript levels were higher in vaccinated groups than in PBS controls at later sampling points, with the largest group differences observed for P4 during days 21–42 (Table 5; Figure 2).

3.1.5 IFN-γ expression

Splenic ifn-γ transcription varied by treatment and sampling time (Table 6; Figure 3). In PBS controls (P1), estimated marginal means remained low (0.16–0.31) across days 7–42. In vaccinated groups, ifn-γ expression was higher than in P1 at multiple post-vaccination time points, and mean values were generally higher in P4 than in P3 and P2 (Table 6). Mean values in P4 were 1.09 at day 7, 3.11 at day 14, 2.87 at day 21, 3.79 at day 35, and 5.03 at day 42, compared with 0.16–0.31 in P1 across the same time points (Table 6). Planned contrasts for P4 vs P1 were significant at all days (Tukey-adjusted $p < 0.001$; Table 6), with large effect sizes across sampling times. The mixed-effects model for ifn-γ (log-transformed 2^−ΔCt) showed significant effects of Treatment ($F = 359.7$, $df = 3,40$, $p < 0.001$, partial $\eta^2 = 0.964$), Day ($F = 159.2$, $df = 4,40$, $p < 0.001$, partial $\eta^2 = 0.941$) and their interaction ($F = 28.0$, $df = 12,40$, $p < 0.001$, partial $\eta^2 = 0.894$) (Table 5). Within-day ANOVA with Tukey's test indicated P4 differed from P1 at all sampling times ($p < 0.05$), with P3 and P2 also differing from P1 at several later time points (Tables 5–6). Considering il-1β and ifn-γ together, mean transcript levels at later sampling points were higher in vaccinated groups than in P1, with larger group differences observed for P4 (Tables 5–6; Figures 2–3). In summary, vaccination was associated with higher ifn-γ transcript levels than PBS controls at later time points, particularly in P4 (Tables 5–6; Figure 3).

Table 6. Primary contrast P4 vs P1 for each marker and sampling day. Model-derived estimated marginal means (EMMs) and planned contrasts comparing the adjuvanted polyvalent FKC group (P4) with the PBS control (P1) at each sampling day (D7–D42). Fill in the cells using jamovi "Estimated Marginal Means" and Tukey post-hoc output

Marker	Day	EMM P1	EMM P4	ΔEMM (P4–P1)	95% CI for Δ	p (Tukey)	Cohen's d	Interpretation
IFN-γ	D7	0.16	1,087	0.927	[2.719, 3.147]	<0.001	3.65	very strong
IFN-γ	D14	0.22	3,113	2,893	[2.719, 3.147]	<0.001	11.39	very strong
IFN-γ	D21	0.237	2,870	2,633	[2.719, 3.147]	<0.001	10.37	very strong
IFN-γ	D35	0.293	3,793	3,500	[2.719, 3.147]	<0.001	13.78	very strong
IFN-γ	D42	0.313	5,027	4,713	[2.719, 3.147]	<0.001	18.56	very strong
IL-1β	D7	0.19	0.897	0.707	[2.829, 3.035]	<0.001	6.47	very strong
IL-1β	D14	0.25	1,310	1,060	[2.829, 3.035]	<0.001	9.71	very strong
IL-1β	D21	0.173	3,557	3,383	[2.829, 3.035]	<0.001	30.98	very strong
IL-1β	D35	0.20	4,403	4,203	[2.829, 3.035]	<0.001	38.49	very strong
IL-1β	D42	0.23	5,537	5,307	[2.829, 3.035]	<0.001	48.60	very strong
Agglutination titre	D7	9,907	94,473	84,567	[965.988, 1505.592]	<0.001	0.33	small
Agglutination titre	D14	9,903	615,803	605,900	[965.988, 1505.592]	<0.001	2.40	very strong
Agglutination titre	D21	9,523	2,047,997	2,038,473	[965.988, 1505.592]	<0.001	8.07	very strong
Agglutination titre	D35	14,097	2,950,097	2,936,000	[965.988, 1505.592]	<0.001	11.63	very strong
Agglutination titre	D42	10,280	524,283	514,003	[965.988, 1505.592]	<0.001	2.04	very strong
Respiratory burst (NBT)	D7	0.47	0.647	0.177	[0.234, 0.272]	<0.001	7.93	very strong
Respiratory burst (NBT)	D14	0.327	0.617	0.290	[0.234, 0.272]	<0.001	13.01	very strong
Respiratory burst (NBT)	D21	0.433	0.227	-0.207	[0.234, 0.272]	<0.001	-9.27	very strong
Respiratory burst (NBT)	D35	0.280	0.440	0.160	[0.234, 0.272]	<0.001	7.18	very strong
Respiratory burst (NBT)	D42	0.330	1,177	0.847	[0.234, 0.272]	<0.001	37.99	very strong

3.1.6 Survival after homologous challenge and relative percent survival

Following 21 days post-vaccination, fish in the survival cohort were challenged intraperitoneally with 1 × 10^7 CFU/fish of virulent *A. hydrophila* Ah-S1, and survival was monitored for 14 days (Figure 4; Table 7). PBS controls (P1) experienced rapid and extensive mortality: deaths began around days 3–4 post-challenge and accumulated steadily thereafter, so that by day 14 survival had declined to 8.3%, corresponding to 55 deaths. Dead and moribund fish commonly showed skin and fin haemorrhages and visceral congestion, and *A. hydrophila* was consistently re-isolated from kidney tissue, confirming that mortality was attributable to the challenge strain. All vaccinated groups exhibited higher survival than the PBS controls. Day-14 survival proportions were 61.7% in the monovalent FKC group (P2), 75.0% in the non-adjuvanted polyvalent FKC group (P3), and 83.3% in the adjuvanted polyvalent group (P4) (Table 7; Figure 4).

Kaplan–Meier analysis showed significant differences among survival curves (log-rank test, $p < 0.001$; Figure 4). Cox proportional hazards models with treatment as a fixed effect and tank as a frailty term yielded hazard ratios (HRs) of 0.33 (95% CI 0.20–0.54) for P2, 0.21 (0.12–0.37) for P3, and 0.13 (0.07–0.26) for P4, each versus P1 (Table 7). Relative percent survival (RPS) at day 14 further summarised these differences (Figure 5), with values of 58.2% for P2, 72.7% for P3, and 81.8% for P4. Taken together, these survival outcomes show that all FKC vaccines conferred considerable protection against homologous A. hydrophila challenge under the present experimental conditions, with numerically highest survival, lowest hazard, and highest RPS in the oil-adjuvanted polyvalent group (P4).

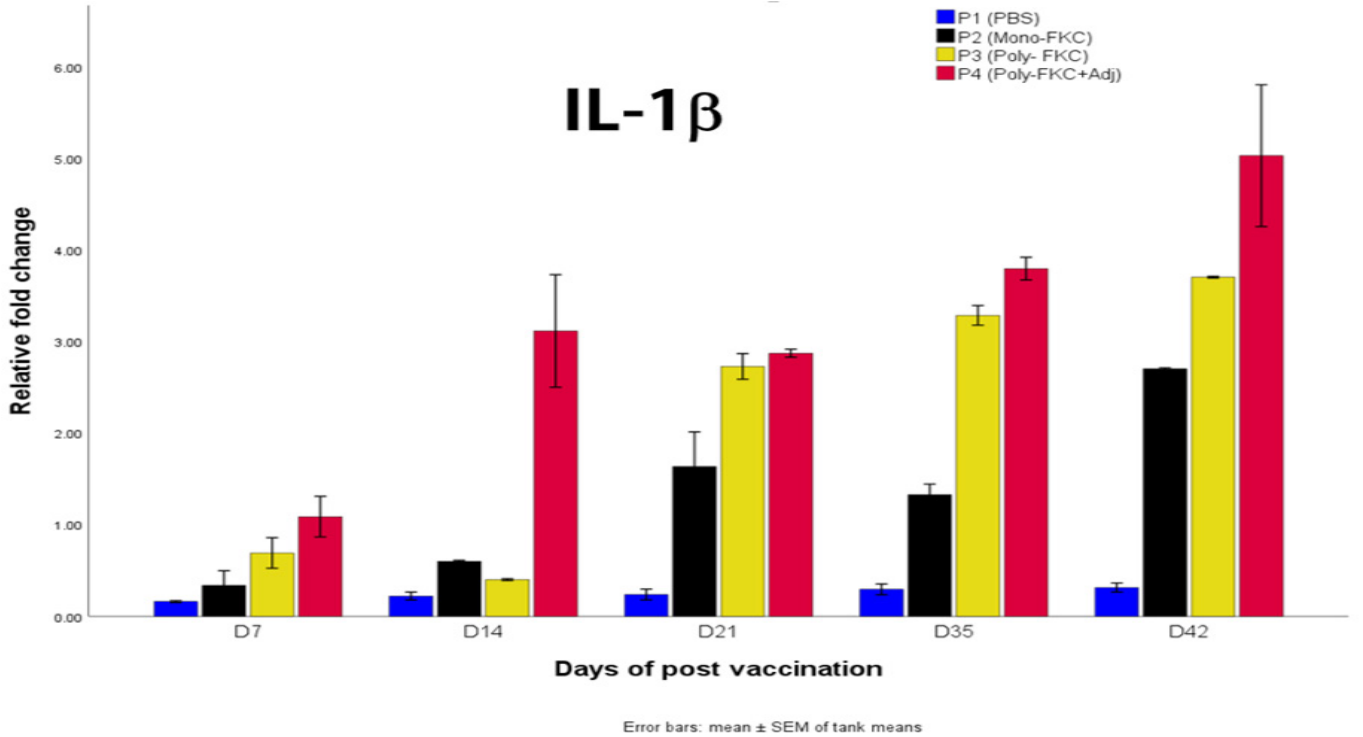

Figure 2. Temporal profile of splenic il-1β expression following vaccination in *O. goramy*. Relative fold change of splenic il-1β mRNA expression in giant gourami (*O. goramy*) at days 7, 14, 21, 35, and 42 post-vaccination. Treatment groups were PBS (P1), monovalent FKC (P2), non-adjuvanted polyvalent FKC (P3), and oil-adjuvanted polyvalent FKC (P4). Expression levels were determined by RT-qPCR and expressed as 2^−ΔΔCt relative to the PBS control after normalisation to β-actin. Bars indicate mean ± SEM of tank means (n = 3 tanks per treatment). Where present, different lowercase letters above bars denote significant differences among treatments within each sampling day (one-way ANOVA followed by Tukey's test, $p < 0.05$).

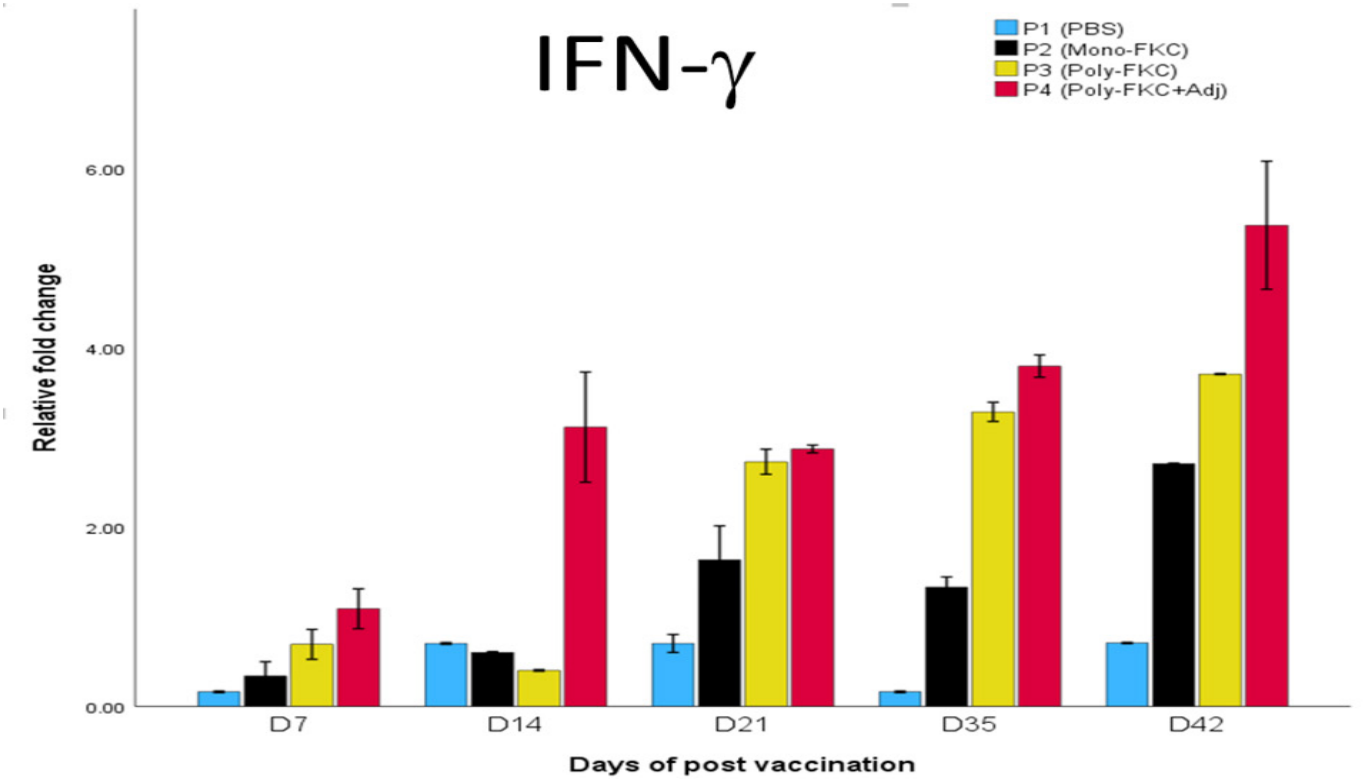

Figure 3. Temporal profile of splenic ifn-γ expression following vaccination in *Osphronemus goramy*. Relative fold change of splenic ifn-γ mRNA expression in giant gourami (*Osphronemus goramy*) at days 7, 14, 21, 35, and 42 post-vaccination. Fish were injected intraperitoneally with PBS (P1), monovalent formalin-killed cell vaccine (mono-FKC; P2), non-adjuvanted polyvalent FKC (poly-FKC; P3), or oil-adjuvanted polyvalent FKC (poly-FKC + adjuvant; P4). Gene expression was quantified by RT-qPCR and calculated as relative fold change using the 2^−ΔΔCt method, normalised to β-actin and referenced to the PBS control group. Bars represent mean ± SEM of tank means (n = 3 tanks per treatment).

Table 7. Survival outcomes and approximate hazard ratios after *A. hydrophila* challenge

Treatment	Deaths (n)	SR day 14 (%)	HR vs P1	95% CI for HR
P1 (PBS)	55	8.3	1.00 (reference)	–
P2 (Mono-FKC)	23	61.7	0.33	0.20–0.54
P3 (Poly-FKC)	15	75.0	0.21	0.12–0.37
P4 (Poly-FKC + adjuvant	10	83.3	0.13	0.07–0.26

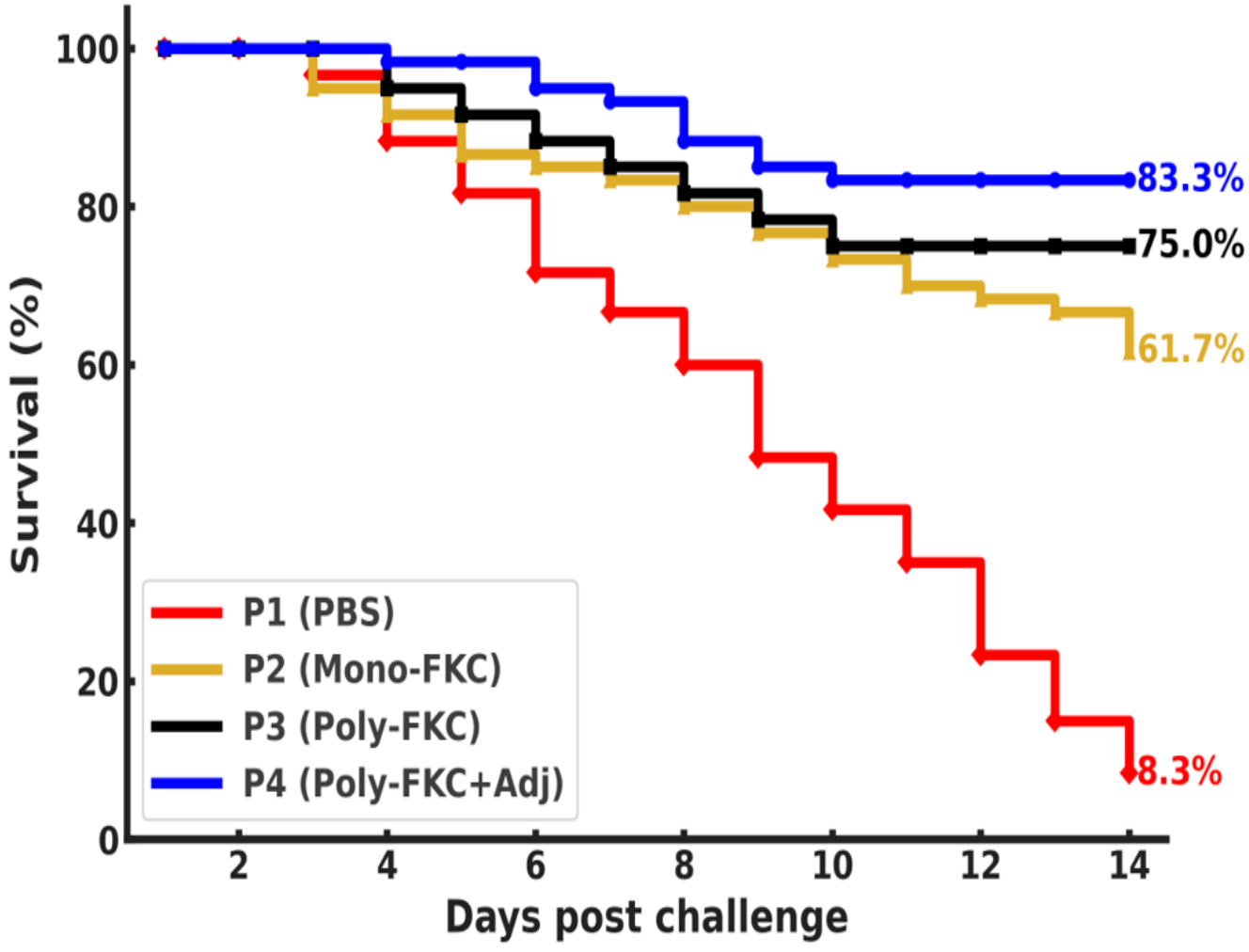

Figure 4. Kaplan–Meier survival curves of vaccinated giant gourami following *A. hydrophila* challenge. Kaplan–Meier plots showing the cumulative survival of giant gourami (*O. goramy*) over 14 days following intraperitoneal challenge with virulent *A. hydrophila* (1×10^7 CFU fish^{-1}) at 21 days post-vaccination. Fish were injected with PBS (P1, blue), a monovalent formalin-killed cell vaccine (P2, black), a polyvalent FKC vaccine (P3, yellow), or an oil-adjuvanted polyvalent FKC vaccine (P4, red). Each curve represents the pooled survival of 120 fish per treatment. Final survival on day 14 was 8.3% (P1), 61.7% (P2), 75.0% (P3), and 83.3% (P4). Percentage survival at day 14 is indicated at the right end of each curve.

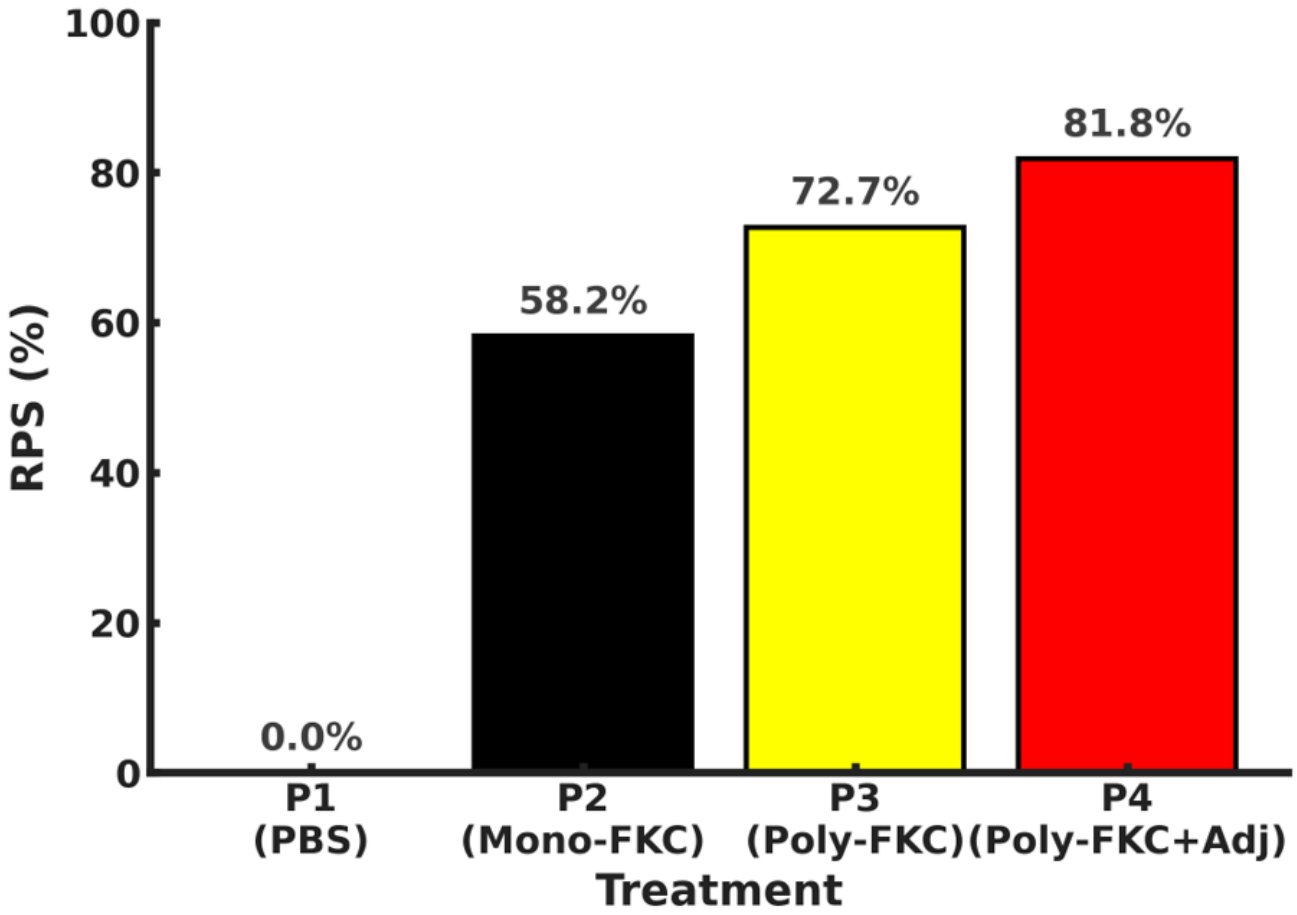

Figure 5. Relative percent survival of vaccinated giant gourami at 14 days post-challenge. Relative percent survival (RPS) of giant gourami vaccinated with different formalin-killed cell (FKC) formulations following intraperitoneal challenge with virulent *A. hydrophila* at 21 days post-vaccination. Treatments were PBS control (P1, blue), monovalent FKC (P2, black), polyvalent FKC (P3, yellow), and oil-adjuvanted polyvalent FKC (P4, red). RPS was calculated at day 14 post-challenge as RPS = (1−MvMc)×100%, where Mc and Mv are the cumulative mortalities in the control and vaccinated groups, respectively. Final RPS values were 0.0% (P1 by definition), 58.2% (P2), 72.7% (P3), and 81.8% (P4). Bars are annotated with the corresponding RPS (%) for ease of comparison.

3.2 Discussion

3.2.1 Overall vaccine performance and novelty in O. goramy

This study demonstrates that formalin-killed *A. hydrophila* vaccines based on field isolates from MAS outbreaks in *O. goramy* can reduce mortality under a controlled *homologous* intraperitoneal challenge, with the largest effects observed for a water-in-oil–adjuvanted polyvalent formulation. Within the broader context of bacterial disease in warm-water aquaculture, these findings are consistent with the established role of inactivated bacterins as the main workhorse of fish vaccination (Hastein *et al*., 2005; Gudding and van Muiswinkel, 2013; Adams, 2019; Ma *et al*., 2019; Wang *et al*., 2020). To our knowledge, however, comparative evaluation of monovalent versus polyvalent inactivated vaccines, with and without oil adjuvantation and with multiple immunological endpoints assessed alongside survival, has not previously been reported for giant gourami. The present work, therefore, refines the existing picture by directly benchmarking three formalin-killed vaccine regimens in a single, standardised experiment using locally prevalent *A. hydrophila* strains.

At the level of clinical outcome, all three FKC regimens conferred clear protection against intraperitoneal challenge with a virulent *A. hydrophila* strain, as reflected by higher Kaplan–Meier survival and hazard ratios well below 1.0 compared with PBS controls (Kaplan and Meier, 1958; Peto *et al*., 1977). The resulting RPS values (58.2% for the monovalent FKC, 72.7% for the non-adjuvanted polyvalent vaccine, and 81.8% for the adjuvanted polyvalent formulation) fall within the range typically considered

biologically meaningful in experimental fish vaccine trials (Amend, 1981; Chandran *et al*., 2002; Shome and Shome, 1999, 2005; Dehghani *et al*., 2012; Prasad and Areechon, 2010; Sugiani *et al*., 2013). The stepwise pattern, with P4 showing the highest level of protection, followed by P3 and P2, and with P1 clearly lower than all vaccinated groups, is broadly compatible with earlier evidence that combining antigens from multiple strains and adding an oil adjuvant can enhance protection against bacterial pathogens in salmonids and other species (Hoel *et al*., 1997; Toranzo *et al*., 1997, 2009; Fredriksen *et al*., 2013; Jaafar *et al*., 2015; Monir *et al*., 2020; Yan *et al*., 2018). In addition, the concept that antigenic relatedness and shared epitopes can influence cross-reactive immune recognition across bacterial taxa has been demonstrated experimentally in fish and mammalian models (Hoel *et al*., 1998), supporting the rationale for broadening antigenic input when field strain diversity is expected. At the same time, because efficacy was evaluated using a single-strain, intraperitoneal challenge at a relatively high dose and at a single challenge time point, these outcomes should be interpreted as homologous protection and should not be extrapolated to natural exposure conditions or heterologous Aeromonas genotypes without additional data.

3.2.2 Humoral responses and antibody-mediated protection

The humoral responses observed here are consistent with the central role of specific antibody in teleost immunity to extracellular bacteria (Ellis, 1999; Anderson and Siwicki, 1993; Swain *et al*., 2007). The marked increase in serum agglutination titres, particularly in the adjuvanted polyvalent group, mirrors findings from FKC or biofilm-derived *A. hydrophila* vaccines in carp and tilapia, where higher antibody titres have generally been associated with improved survival after challenge (Chandran *et al*., 2002; Dehghani *et al*., 2012; Thangaviji *et al*., 2012; Prasad and Areechon, 2010; Sugiani *et al*., 2013; Monir *et al*., 2020). Agglutination assays are functional readouts reflecting the ability of serum antibodies to bind and aggregate whole bacteria, and related approaches have long been used in mammalian and veterinary diagnostics (Purcell *et al*., 1969; Rahman *et al*., 2003; Corona-Vargas *et al*., 2016). In teleosts, such antibodies can promote opsonisation and complement activation (Ellis, 1999; Carroll, 1998; Wang and Zhang, 2010). However, agglutination does not resolve Ig isotypes or strain-specific reactivity to individual vaccine isolates (Ah-S1–Ah-S3), and therefore the present titres should be interpreted as an overall whole-cell humoral readout rather than isolate-specific immunity. Although the present study did not measure complement activity or isotype-specific responses, the concordant ranking of higher agglutination titres and higher survival across regimens is consistent with (but does not prove) a contribution of antibody-associated mechanisms to protection under this homologous intraperitoneal challenge model. The challenge at 21 dpv coincided with a period when agglutination titres had risen substantially relative to baseline, which is compatible with expected early protection windows in warm-water fish following bacterin vaccination; nonetheless, the duration of protection beyond this time point was not evaluated here. More broadly, experimental work in mammalian infection models has shown that antibody-mediated protection can depend critically on intact complement function (Han *et al*., 2001), which is conceptually consistent with the In future, antigenic cross-reactivity assessments can also be framed in light of experimental evidence that immunological cross-reactions may occur across related bacterial pathogens (Hoel *et al*., 1998), strengthening the rationale for formal cross-reactivity testing among local Aeromonas variants. View that higher functional antibody readouts (e.g., agglutination/opsonisation potential) may translate into improved bacterial clearance when complement pathways are engaged.

3.2.3 Innate responses and cytokine profiles

The innate and cytokine endpoints measured here complement the serological data by providing a limited view of early and intermediate events in the response to vaccination. Nitroblue tetrazolium reduction in whole blood, while less dramatic than the changes in antibody titre, indicated a vaccine- and time-dependent modulation of phagocyte respiratory burst, with the most pronounced late increase in the adjuvanted polyvalent group. Similar use of NBT and related functional assays has been reported in carps and catfish to monitor innate responses after vaccination or immunostimulant administration (Lygren *et al*., 1999; Verho *et al*., 2005; Sirimanapong *et al*., 2014; Yin *et al*., 2009). Given assay variability and the modest absolute effect sizes for some time points, NBT is best interpreted here as supportive evidence that the most intensive regimen (P4) engaged phagocyte functions more strongly by day 42, rather than as mechanistic proof that innate responses are the dominant driver of protection.

In the spleen, *il-1β* and *ifn-γ* transcription provided a simple window into a pro-inflammatory and Th1-like axis. Both cytokines were up-regulated in vaccinated fish, with the largest and most sustained changes again observed in the oil-adjuvanted polyva-

lent group. Up-regulation of *il-1β* after vaccination or pathogen exposure has been documented in multiple fish species and is generally taken as a marker of inflammasome-linked or early inflammatory activation (Dan *et al*., 2013; Sirimanapong *et al*., 2014; Song *et al*., 2014). Increased *ifn-γ* expression has been associated with cellular responses and macrophage activation, particularly in the context of intracellular pathogens and more "Th1-like" profiles (Dan *et al*., 2013; Yin *et al*., 2009; Ma *et al*., 2019). Our data align with this literature in suggesting that oil adjuvantation amplifies pro-inflammatory and interferon-like signalling in the spleen of *O. goramy*, but they do not allow causal attribution between the observed cytokine changes and survival. The design targeted a small set of cytokines at discrete time points; more detailed pathway analyses, including additional cytokines and cell-type–resolved readouts (Inoue *et al*., 2002; Mutoloki *et al*., 2015), would be needed to define mechanistic links between early gene expression patterns and protection. Overall, when considered together with agglutination and NBT readouts, the cytokine results support a descriptive interpretation that regimens associated with higher immune activation across these endpoints also showed higher survival; however, no formal correlates-of-protection analysis was performed, and the panel does not permit mechanistic conclusions. Taken together, the data indicate that P4 generally showed higher mean humoral, innate, and cytokine readouts than P3/P2, which paralleled the observed ranking in survival under the homologous challenge conditions used here

3.2.4 Role of oil adjuvant and safety considerations

The superiority of the oil-adjuvanted polyvalent FKC in this experiment is consistent with the established adjuvant effects of water-in-oil emulsions in fish, which can enhance antigen persistence, promote depot formation, and skew responses toward higher and longer-lasting antibody titres (Hastein *et al*., 2005; Dalmo *et al*., 2016; Tafalla *et al*., 2013; Adams, 2019). Montanide™ ISA 763 A VG and related formulations have previously improved efficacy against *Yersinia ruckeri* and *Flavobacterium psychrophilum* in salmonids and rainbow trout, with acceptable safety profiles under controlled conditions (Gravningen *et al*., 2008; Fredriksen *et al*., 2013; Jaafar *et al*., 2015; Hoare *et al*., 2017). Conversely, some oil adjuvants have been associated with adhesions, chronic inflammation, or reduced welfare when formulations or dosing are suboptimal (Midtlyng *et al*., 1996; Rømer Villumsen *et al*., 2015; Li *et al*., 2020). In the present study, the antigen: adjuvant ratio followed manufacturer guidance and published practice, but dose/ratio optimisation was not performed specifically for giant gourami. In the present study, we did not observe gross injection-site pathology or excess background mortality in the adjuvanted group during the 42-day pre-challenge period, but our assessments were macroscopic and limited in duration. Accordingly, more detailed safety evaluation (adhesion scoring, histopathology, and longer-term growth/performance monitoring) will be essential before recommending widespread use in commercial gourami farming.

3.2.5 Polyvalent formulation and implications for vaccine development

The use of multiple *A. hydrophila* isolates from MAS outbreaks in *O. goramy* to construct the polyvalent vaccine connects this work to earlier studies showing that antigenic breadth can increase cross-protection against strain diversity in bacterial fish pathogens (Hoel *et al*., 1997; Toranzo *et al*., 1997, 2009; Shome and Shome, 2005; Jiang *et al*., 2016; Mzula *et al*., 2019). Our earlier characterisation of these gourami isolates, including virulence profiling and haemolysin/aerolysin gene analysis (Rozi *et al*., 2018a, 2018b), provided a rationale for their inclusion as vaccine seeds. However, antigenic differences among Ah-S1–Ah-S3 were not quantified (e.g., by cross-agglutination, ELISA against each isolate, or antigen profiling), and therefore the polyvalent formulation should be viewed as field-informed rather than constructed from measured antigenic distances. Because the challenge was performed using Ah-S1 only, the present experiment does not directly test whether including Ah-S2/Ah-S3 broadens protection; instead, it demonstrates that polyvalent formulations can perform well under a homologous Ah-S1 challenge, while breadth remains to be established. From this perspective, the present FKC data are best viewed as defining a pragmatic baseline regimen derived from locally prevalent strains, against which alternative platforms and heterologous challenge scenarios can be benchmarked in future work (Liu *et al*., 2011; Yan *et al*., 2018; Jiang *et al*., 2016; Thangaviji *et al*., 2012). In future, antigenic cross-reactivity assessments can also be framed in light of experimental evidence that immunological cross-reactions may occur across related bacterial pathogens (Hoel *et al*., 1998), strengthening the rationale for formal cross-reactivity testing among local Aeromonas variants.

3.2.6 Limitations, future directions, and practical implications

This study has several limitations that should be considered when interpreting the findings. First, the experiment was conducted in a single host species us-

ing one *A. hydrophila* isolate delivered intraperitoneally at a fixed dose, which may not fully reflect natural exposure routes, mixed infections, or the broader strain heterogeneity encountered in farms (Amend, 1981; Dash *et al*., 2008; Podeti and Benarjee, 2017; Marinho-Neto *et al*., 2019; Fernández-Bravo and Figueras, 2020). This limitation is fundamental: intraperitoneal single-strain challenges standardise exposure but may overestimate performance relative to natural infection conditions (immersion exposure, variable doses, co-infections, and environmental stress). Second, the number of tanks per treatment was modest; although mixed-effects models accounted for tank clustering, precision around some estimates, particularly differences among vaccinated groups, remains limited. Third, immunological readouts were restricted to serum agglutination, NBT activity, and splenic *il-1β* and *ifn-γ* mRNA, and other relevant components such as complement, lysozyme, mucosal and cellular responses, and additional cytokines were not measured (Carroll, 1998; Díaz de Stahl *et al*., 2003; Ellis, 1999; Wang and Zhang, 2010; Ma *et al*., 2019). Finally, the work was performed under controlled laboratory conditions without field trials or economic analyses, so any implications for antimicrobial-use reduction or farm-level resilience remain speculative (Assefa and Abunna, 2018; Lusiastuti *et al*., 2020; Shamsuzzaman *et al*., 2017). These limitations point to clear priorities for future work. In the short term, dose–response and duration-of-immunity studies, alternative challenge scenarios (including immersion or cohabitation and heterologous strains), and extended safety assessments with histopathology would help refine the risk–benefit profile of the oil-adjuvanted polyvalent FKC. Such histopathological follow-up is also relevant because *A. hydrophila* infection is known to produce characteristic tissue lesions and immunopathological changes in carp and other cyprinids (Ahmed *et al.,* 1995), and comparable assessments would strengthen welfare and safety conclusions in gourami. In parallel, route optimisation including immersion strategies and booster concepts could be evaluated, building on evidence that booster immersion vaccination can confer meaningful protection against bacterial disease in salmonids (Chettri *et al.,* 2015). In the longer term, complementary studies on subunit and multi-epitope vaccines, adjuvant and route optimisation (oral or immersion delivery), and well-designed on-farm trials will be needed to determine how, and under what conditions, laboratory efficacy can translate into practical gains in survival and reduced antibiotic reliance in *O. goramy* culture.

Within these boundaries, the present work provides experimental evidence that a polyvalent FKC based on gourami MAS isolates, particularly when formulated with an oil adjuvant, can enhance humoral, innate, and cytokine responses and reduce mortality in *O. goramy* under controlled challenge. This supports continued development of inactivated vaccines as one component of integrated fish health management for gourami, while also motivating complementary efforts on subunit and multi-epitope vaccines, route optimisation, and field evaluation to determine how laboratory efficacy translates into practical benefits in diverse farming systems. However, claims regarding breadth of protection should remain cautious because cross-strain efficacy was not directly evaluated. Within this framework, the present data support the oil-adjuvanted polyvalent FKC (P4) as a pragmatic lead candidate in this experimental model; however, this designation should be regarded as hypothesis-generating and confirmed through optimisation and field-validation studies before large-scale deployment is recommended.

4. Conclusion

Formalin-killed *A. hydrophila* vaccines derived from MAS-associated field isolates improved survival of giant gourami (*O. goramy*) in a homologous intraperitoneal challenge model. Protective performance differed among formulations: the oil-adjuvanted polyvalent vaccine (P4) produced the highest protection, followed by the non-adjuvanted polyvalent (P3) and monovalent (P2) regimens, and all vaccinated groups showed higher survival than PBS controls (P1). Immune readouts assessed here (agglutinating titres, NBT activity, and splenic il-1β and ifn-γ transcripts) broadly tracked the survival pattern. These findings provide a basis for further development of vaccination as a disease-control option in gourami aquaculture, but require dose optimisation, extended safety assessment, and field validation under farm-relevant exposure conditions before wider implementation can be recommended.

Acknowledgement

The authors would like to express their sincere gratitude to the organizing committee of the 8th International Conference on Fisheries and Marine Sciences (INCOFIMS) & 6th International Conference on Biotechnology & Fisheries Product (INCOBIFS) 2025, Faculty of Fisheries and Marine, Universitas Airlangga, for the opportunity to present and discuss this work and for the valuable scientific forum they provided.

Authors' Contributions

All authors contributed to data interpretation,

reviewed the manuscript, and approved the final version. Rozi collected the data and drafted the initial version of the manuscript. Eduardus Bimo Aksono and Jola Rahmahani designed and prepared the figures. Rozi and Suwarno conceived the study, developed the main conceptual framework, and critically revised the manuscript.

Conflict of Interest

The authors declare that they have no competing interests.

Declaration of Artificial Intelegence (AI)

During the preparation of this work, the authors used ChatGPT (OpenAI) and Grammarly solely to improve English readability (grammar, flow, and terminology consistency). No scientific content, data analysis, results, figures, or artwork were generated or altered by AI. The authors reviewed and edited all text and take full responsibility for the content of this publication.

Funding Information

This work was supported by the RKAT of the Faculty of Fisheries and Marine, Universitas Airlangga, under Grant Number 771/UN3.1.12/LT/2019

References

Adams, A. (2019). Progress, challenges and opportunities in fish vaccine development. *Fish & Shellfish Immunology*, 90(7):210-214.

Ahmed, S. A., Marzouk, M. S., Megid, R. M. A., Moustafa, M., & Gado, M. (1995). Histopathological and immunological studies on *A. hydrophila* infection in common carp. *Veterinary Medical Journal* (*Giza*), 43(4):389-396.

Alishahi, M., Tollabi, M., & Ghorbanpour, M. (2019). Comparison of the adjuvant effect of propolis and Freund on the efficacy of *A. hydrophila* vaccine in common carp (*Cyprinus carpio*). *Iranian Journal of Fisheries Sciences*, 18(3):428-444.

Amend, D. F. (1981). Potency testing of fish vaccines. *Developments in Biological Standardization*, 49:447–454. (International Symposium on Fish Biologics: Serodiagnostics and Vaccines)

Anderson, D. P., & Siwicki, A. K. (1993, October). Basic hematology and serology for fish health programs. In Proceedings of the Second Symposium on Diseases in Asian Aquaculture: Aquatic Animal Health and the Environment, Phuket, Thailand, pp. 185–202. Fish Health Section, Asian Fisheries Society, Manila.

Assefa, A., & Abunna, F. (2018). Maintenance of fish health in aquaculture: Review of epidemiological approaches for prevention and control of infectious disease of fish. *Veterinary Medicine International*, 2018(1):1-10.

Awate, S., Babiuk, L. A., & Mutwiri, G. (2013). Mechanisms of action of adjuvants. *Frontiers in Immunology*, 4(1):1-10.

Carroll, M. C. (1998). The role of complement and complement receptors in induction and regulation of immunity. *Annual Review of Immunology*, 16(1):545-568.

Chandran, M. R., Aruna, B. V., Logambal, S. M., & Michael, R. D. (2002). Immunisation of Indian major carps against *A. hydrophila* by intraperitoneal injection. *Fish & Shellfish Immunology*, 13(1):1-9.

Chettri, J. K., Jaafar, R. M., Skov, J., Kania, P. W., Dalsgaard, I., & Buchmann, K. (2015). Booster immersion vaccination using diluted *Yersinia ruckeri* bacterin confers protection against ERM in rainbow trout. *Aquaculture*, 440(6):1-5.

Corona-Vargas, J. L., Vicencio-Mallén, M. A., Salmerón-Sosa, F., Carrillo-Casas, E. M., Trigo-Tavera, F. J., & Miranda-Morales, R. E. (2016). Detection of antibodies against *Mycoplasma mycoides* subsp. capri in goats with the complement fixation test. *Advances in Microbiology*, 6(13):959-964.

Dalmo, R., Bøgwald, J., & Tafalla, C. (2016). Adjuvants and delivery methods: Current and novel. In Adams, A. (Ed.), Fish Vaccines, pp. 75-103. Springer, Cham.

Dan, X. M., Zhang, T. W., Li, Y. W., & Li, A. (2013). Immune responses and immune-related gene expression profile in orange-spotted grouper after immunization with *Cryptocaryon irritans* vaccine. *Fish & Shellfish Immunology*, 34(1):885-891.

Dash, S., Swain, P., Swain, M. M., Nayak, S. K., Behura, A., Nanda, P. K., & Mishra, B. K.

(2008). Investigation on infectious dropsy of Indian major carps. *Asian Fisheries Science*, 21(4):377-384.

Decostere, A., Hermans, K., & Haesebrouck, F. (2004). Piscine mycobacteriosis: A literature review covering the agent and the disease it causes in fish and humans. *Veterinary Microbiology*, 99(3):159-166.

Dehghani, S., Akhlaghi, M., & Dehghani, M. (2012). Efficacy of formalin-killed, heat-killed and lipopolysaccharide vaccines against motile aeromonads infection in rainbow trout (*Oncorhynchus mykiss*). *Global Veterinaria*, 9(4):409-415.

Díaz de Stahl, T., Dahlström, J., Carroll, M. C., & Heyman, B. (2003). A role for complement in feedback enhancement of antibody responses by IgG3. *Journal of Experimental Medicine*, 197(1):1183-1190.

Ellis, A. E. (1999). Immunity to bacteria in fish. *Fish & Shellfish Immunology*, 9(4):291-308.

Fernández-Bravo, A., & Figueras, M. J. (2020). An update on the genus *Aeromonas*: Taxonomy, epidemiology, and pathogenicity. *Microorganisms*, 8(1):1-39.

Firdaus-Nawi, M., Sabri, M. Y., Hanan, Y., Siti-Zahrah, A., & Zamri-Saad, M. (2013). Efficacy of feed-based adjuvant vaccine against *Streptococcus agalactiae* in *Oreochromis* spp. *Aquaculture Research*, 45(1):87-96.

Fredriksen, B. N., Olsen, R. H., Furevik, A., Souhoka, R. A., Gauthier, D., & Brudeseth, B. (2013). Efficacy of a divalent and a multivalent water-in-oil formulated vaccine against a highly virulent strain of *Flavobacterium psychrophilum* after intramuscular challenge of rainbow trout. *Vaccine*, 31(15):1994-1998.

Gauthier, D. T., & Rhodes, M. (2009). Mycobacteriosis in fishes: A review. *The Veterinary Journal*, 180(1):33-47.

Gravningen, K., Sakai, M., Mishiba, T., & Fujimoto, T. (2008). The efficacy and safety of an oil-based vaccine against *Photobacterium damsela* subsp. piscicida in yellowtail: A field study. *Fish & Shellfish Immunology*, 24(5):523-529.

Gudding, R. & van Muiswinkel, W. B. V. (2013). A history of fish vaccination: Science-based disease prevention in aquaculture. *Fish & Shellfish Immunology*, 35(6):1683-1688.

Han, Y., Kozel, T. R., Zhang, M. X., MacGill, R. S., Carroll, M. C., & Cutler, J. E. (2001). Complement is essential for protection by an IgM and an IgG3 monoclonal antibody against experimental, hematogenously disseminated candidiasis. *Journal of Immunology*, 167(3):1550-1559.

Hastein, T., Gudding, R., & Evensen, O. (2005). Bacterial vaccines for fish, an update of the current situation worldwide. *Developments in Biologicals*, 121(1):55-74.

Hoare, R., Jung, S. J., Ngo, T. P. H., Bartie, K., Bailey, J., Thompson, K. D., & Adams, A. (2017). Efficacy and safety of a non-mineral oil adjuvanted injectable vaccine for the protection of Atlantic salmon against *Flavobacterium psychrophilum*. *Fish & Shellfish Immunology*, 67(8):346-357.

Hoel, K., Reitan, L. J., & Lillehaug, A. (1998). Immunological cross reactions between *Aeromonas salmonicida* and *Vibrio salmonicida* in Atlantic salmon and rabbit. *Fish & Shellfish Immunology*, 8(3):171-182.

Hoel, K., Salonius, K., & Lillehaug, A. (1997). *Vibrio* antigens of polyvalent vaccines enhance the humoral immune response to *Aeromonas salmonicida* antigens in Atlantic salmon (*Salmo salar* L.). *Fish & Shellfish Immunology*, 7(2):71-80.

Inoue, T., Moritomo, T., Tamura, Y., Mamiya, S., Fujino, H., & Nakanishi, T. (2002). A new method for fish leucocyte counting and partial differentiation by flow cytometry. *Fish & Shellfish Immunology*, 13(5):379-390.

Iqbal, Z. (2016). An overview of diseases in commercial fishes in Punjab, Pakistan. *Fish Pathology*, 51(1):S30-S35.

Ismail, M. S., Siti-Zahrah, A., Syafiq, M. R. M., Amal, M. N. A., Firdaus-Nawi, M., & Zamri-Saad, M. (2016). Feed-based vaccination regime against streptococcosis in red tilapia (*Oreochromis niloticus* × *Oreochromis mossambicus*). *BMC Veterinary Research*, 12(1):1-6.

Jaafar, R. M., Chettri, J. K., Dalsgaard, I., Al-Jubury,

A., Kania, P. W., Skov, J., & Buchmann, K. (2015). Effects of adjuvant Montanide™ ISA 763 A VG in rainbow trout injection vaccinated against *Yersinia ruckeri*. *Fish & Shellfish Immunology*, 47(2):797-806.

Jiang, X., Zhang, C., Zhao, Y., Kong, X., Pei, C., Li, L., Nie, G., & Li, X. (2016). Immune effects of the vaccine of live attenuated *A. hydrophila* screened by rifampicin on common carp (*Cyprinus carpio* L.). *Vaccine*, 34(27):3087-3092.

Kaplan, E. L., & Meier, P. (1958). Nonparametric estimation from incomplete observations. *Journal of the American Statistical Association*, 53(282):457-481.

Li, J., Tang, L., Li, S., Li, G., & Mo, Z. (2020). The efficacy and side-effects of oil-based adjuvants emulsified *Vibrio anguillarum* bivalent inactivated vaccine in turbot (*Scophthalmus maximus*) under production mode. *Aquaculture*, 524(11):1-7.

Liu, M., Clemons, K. V., Bigos, M., Medovarska, I., Brummer, E., & Stevens, D. A. (2011). Immune responses induced by heat-killed *Saccharomyces cerevisiae*: A vaccine against fungal infection. *Vaccine*, 29(9):1745-1753.

Lusiastuti, A. M., Taukhid, Maskur, Murwantoko, Prayitno, S. B., Sugiani, D., & Caruso, D. (2020). Building and improving the capacity of fish and environmental health management strategy in Indonesia. *IOP Conference Series: Earth and Environmental Science*, 521(1):1-11.

Lygren, B., Sveier, H., Hjeltnes, B., & Waagbo, R. (1999). Examination of the immunomodulatory properties and the effect on disease resistance of dietary bovine lactoferrin and vitamin C fed to Atlantic salmon (*Salmo salar*) for a short-term period. *Fish & Shellfish Immunology*, 9(2):95-107.

Ma, J., Bruce, T. J., Jones, E. M., & Cain, K. D. (2019). A review of fish vaccine development strategies: Conventional methods and modern biotechnological approaches. *Microorganisms*, 7(11):1-18.

Marinho-Neto, F. A., Claudiano, G. S., Yunis-Aguinaga, J., Cueva-Quiroz, V. A., Kobashigawa, K. K., Cruz, N. R. N., Moraes, F. R., Moraes, J. R. E. (2019). Morphological, microbiological and ultrastructural aspects of sepsis by *A. hydrophila* in *Piaractus mesopotamicus*. *PLoS ONE*, 14(9):1-20.

Midtlyng, P. J., Reitan, L. J., Lillehaug, A., & Ramstad, A. (1996). Protection, immune responses and side effects in Atlantic salmon vaccinated against furunculosis by different procedures. *Fish & Shellfish Immunology*, 6(8):599-613.

Monir, M. S., Yusoff, M. S., Zulperi, Z. M., Hassim, H. A., Muhamad, A., Ngoo, M. S., & Salwany, M. Y. I. S. (2020). Haematoimmunological responses and effectiveness of feed-based bivalent vaccine against *Streptococcus iniae* and *A. hydrophila* infection in hybrid red tilapia (*Oreochromis mossambicus* × *O. niloticus*). *BMC Veterinary Research*, 16(1):1-14.

Mutoloki, S., Munang'andu, H. M., & Evensen, O. (2015). Oral vaccination of fish – Antigen preparations, uptake, and immune induction. *Frontiers in Immunology*, 6(1):1-10.

Mzula, A., Wambura, P. N., Mdegela, R. H., & Shirima, G. M. (2019). Current state of modern biotechnological-based *A. hydrophila* vaccines for aquaculture: A systematic review. *BioMed Research International*, 2019(1):1-11.

Peto, R., Pike, M. C., Armitage, P., Breslow, N. E., Cox, D. R., Howard, S. V., Mantel, N., McPherson, K., Peto, J., & Smith, P. G. (1977). Design and analysis of randomized clinical trials requiring prolonged observation of each patient. II. Analysis and examples. *British Journal of Cancer*, 35(1):1-39.

Podeti, K. R., & Benarjee, G. (2017). Haematological changes in South Indian freshwater murrel, *Channa punctatus* having both EUS and *A. hydrophila* infection. *Journal of Parasitic Diseases*, 41(2):329-335.

Prasad, S., & Areechon, N. (2010). Efficacy of formalin-killed *A. hydrophila* and *Streptococcus* sp. vaccine in red tilapia. *Our Nature*, 8(1):231-240.

Purcell, R. H., Holland, P. V., Walsh, J. H., Wong, D. C., Morrow, A. G., & Chanock, R. M. (1969). A complement-fixation test for measuring Australia antigen and antibody. *Journal of Infectious Diseases*, 120(3):383-386.

Purwaningsih, U., Taukhid, Lusiastuti, A. M., Sugiani, D., & Sumiati, T. (2015). A vaccine preparation of *Mycobacterium fortuitum*, a local isolate,

that is effective for preventing *mycobacteriosis* in giant gourami (*Osphronemus gourami*). *Jurnal Riset Akuakultur*, 10(3):423-433.

Rahman, S., Siddique, M. J., Hussain, I., Khushi, M. Z., & Rasool, M. H. (2003). Standardization of indirect haemagglutination test for monitoring *Mycoplasma mycoides* subspecies *capri* antibodies raised in rabbits and goats. *International Journal of Agriculture and Biology*, 5(1):295-297.

Rahmaningsih, S., & Yanuhar, U. (2014). Zoonotic bacterium *Mycobacterium tuberculosis* that infects gourami fish (*Osphronemus goramy* Lac.). *Jurnal Harpodon Borneo,* 7(1):1-5.

Rømer Villumsen, K., Koppang, E. O., & Raida, M. K. (2015). Adverse and long-term protective effects following oil-adjuvanted vaccination against *Aeromonas salmonicida* in rainbow trout. *Fish & Shellfish Immunology*, 42(1):193-203.

Rozi, R., Rahayu, K., & Daruti, D. N. (2018b). Detection and analysis of hemolysin genes in *A. hydrophila* isolated from gourami (*Osphronemus gouramy*) by polymerase chain reaction (PCR). *IOP Conference Series: Earth and Environmental Science,* 137(012001):1-8.

Rozi, R., Rahayu, K., Daruti, D. N., & Stella, M. S. P. (2018a). Study on characterization, pathogenicity and histopathology of disease caused by *A. hydrophila* in gourami (*Osphronemus gouramy*). *IOP Conference Series*: *Earth and Environmental Science,* 137(1):1-10.

Rozi, R., Tyasningsih, W., Rahmahani, J., Aksono, E. B., Yunus, M., Al Arif, M. A., Kuncorojati, S., Kusdarwati, R., Sari, P. D. W., Azmai, M. N. A., Salleh, A., Khanand, N., & Suwarno. (2024). Designing a novel aerolysin-based multi-epitope vaccine against *A. hydrophila* isolated from *Osphronemus goramy* using reverse vaccinology: An in silico approach. *Jurnal Ilmiah Perikanan dan Kelautan,* 16(2):298-321.

Sen, K., & Mandal, R. (2018). Fresh-water fish diseases in West Bengal, India. *International Journal of Fisheries and Aquatic Studies*, 6(5):356-362.

Shamsuzzaman, M. M., Islam, M. M., Tania, N. J., Al Mamun, M. A., Barman, P. P., & Xu, X. (2017). Fisheries resources of Bangladesh: Present status and future direction. *Aquaculture and Fisheries*, 2(4):145-156.

Shome, R., & Shome, B. (1999). Evaluation of killed *A. hydrophila* whole cell vaccine against acute infectious abdominal dropsy in Indian major carps. *Indian Journal of Fisheries*, 46(3):313-317.

Shome, R., & Shome, B. (2005). Evaluation of three types of *A. hydrophila* vaccines against acute infectious dropsy disease in Indian major carps. *Indian Journal of Fisheries*, 52(1):405-412.

Sirimanapong, W., Thompson, K. D., Kledmanee, K., Thaijongrak, P., Collet, B., Ooi, E. L., & Adams, A. (2014). Optimisation and standardisation of functional immune assays for striped catfish to compare their immune response to live and heat-killed *Aeromonas hydrophila. Fish & Shellfish Immunology*, 40(2):374-383.

Song, X., Zhao, J., Bo, Y., Liu, Z., Wu, K., & Gong, C. (2014). *A. hydrophila* induces intestinal inflammation in grass carp: An experimental model. *Aquaculture*, 434(12):171-178.

Sugiani, D., Sukenda, Haris, E., & Lusiastuti, A. M. (2013). Vaccination of tilapia fish (*Oreochromis niloticus*) using monovalent and bivalent vaccines for the prevention of Motile Aeromonas Septicemia and Streptococcosis. *Jurnal Riset Akuakultur*, 8(2):230-239.

Sularto, Listiyowati, N., & Nurdiansyah. (2026). The status of domestication and aquaculture system on the giant gourami *Osphronemus goramy* in Indonesia. In: Nugroho, E., Imron, Gunadi, B., Rosmiati, Syahputra, K. (eds) Aquaculture Development in Archipelago. Springer, Singapore.

Swain, P., Behura, A., Dash, S., & Nayak, S. (2007). Serum antibody response of *Labeo rohita* to three species of pathogenic bacteria: *Aeromonas hydrophila*, *Edwardsiella tarda* and *Pseudomonas fluorescens*. *Veterinary Immunology and Immunopathology*, 117(1-2):137-141.

Tafalla, C., Bøgwald, J., & Dalmo, R. A. (2013). Adjuvants and immunostimulants in fish vaccines: Current knowledge and future perspectives. *Fish & Shellfish Immunology*, 35(6):1740-1750.

Thangaviji, V., Michaelbabu, M., Anand, S. B., Gunasekaran, P., & Citarasu, T. (2012). Immunization with the Aeromonas OMP provides

protection against *A. hydrophila* in goldfish (*Carassius auratus*). *Journal of Microbial & Biochemical Technology*, 4(2):45-49.

Toranzo, A. E., Romalde, J. L., Magariños, B., & Barja, J. L. (2009). Present and future of aquaculture vaccines against fish bacterial diseases. In Rogers, C., & Basurco, B. (Eds.), *The use of veterinary drugs and vaccines in Mediterranean aquaculture. Options Méditerranéennes: Série A. Séminaires Méditerranéens*, 155-176.

Toranzo, A. E., Santos, Y., & Barja, J. L. (1997). Immunization with bacterial antigens: *Vibrio* infections. *Developments in Biological Standardization*, 90(1):93-105.

Verho, S., Järvinen, S., Nikoskelainen, S., & Lilius, E. M. (2005). Biological effect of vaccination can be assessed directly from diluted whole blood of rainbow trout using homologous blood phagocytes as immunosensors. *Fish & Shellfish Immunology*, 19(2):175-183.

Wang, Q., Ji, W., & Xu, Z. (2020). Current use and development of fish vaccines in China. *Fish & Shellfish Immunology*, 96(1):223-234.

Wang, Z., & Zhang, S. C. (2010). The role of lysozyme and complement in the antibacterial activity of zebrafish (*Danio rerio*) egg cytosol. *Fish & Shellfish Immunology*, 29(5):773-777.

Yan, M., Liu, J., Li, Y., Wang, X., Jiang, H., Fang, H., Guo, Z., & Sun, Y. (2018). Different concentrations of *Edwardsiella tarda* ghost vaccine induces immune responses *in vivo* and protects *Sparus macrocephalus* against a homologous challenge. *Fish & Shellfish Immunology*, 80(9):467-472.

Yin, G., Ardo, L., Thompson, K. D., Adams, A., Jeney, Z., & Jeney, G. (2009). Chinese herbs *(As tragalus radix* and *Ganoderma lucidum*) enhance immune response of carp, *Cyprinus carpio* and protection against *Aeromonas hydrophila*. *Fish & Shellfish Immunology*, 26(1):140-145.