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EVALUATION OF THE EFFICACY OF A BIVALENT INACTIVATED WHOLE-CELL VACCINE AGAINST *Edwardsiella tarda* AND *Vibrio anguillarum* IN TILAPIA (*Oreochromis niloticus*)

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Abstract

Bacterial co-infections constrain tilapia aquaculture. We evaluated the efficacy of an adjuvant-free, formalin-inactivated bivalent whole-cell vaccine combining *Edwardsiella tarda* and *Vibrio anguillarum* in Nile tilapia. Fish received the bivalent formulation, matched monovalent vaccines, or phosphate-buffered saline. Systemic and skin-mucus immunoglobulin M levels were quantified by indirect enzyme-linked immunosorbent assay, and serum and mucus bactericidal activity were assessed at 1, 7, 14, and 21-days post-vaccination. At 21 days post-vaccination, fish were challenged intraperitoneally with *E. tarda* or *V. anguillarum*, or both. By 14–21 days post-vaccination, the bivalent vaccine induced strong systemic and mucosal immunoglobulin M responses and increased bactericidal activity; across readouts, responses were significantly greater than ($p < 0.05$) or equal to ($p > 0.05$) the corresponding monovalent vaccine. After challenge with *E. tarda*, 21-day post-challenge survival was higher in the bivalent (54.17%, Relative Percent Survival = 38.9%) than in the monovalent (29.17%) or control (25.00%) group (both $p < 0.05$). After *V. anguillarum* challenge, survival was similar in the bivalent (87.50%, Relative Percent Survival = 78.6%) and monovalent (75.00%) groups ($p > 0.05$), both of which showed greater survival than the control (41.67%) ($p < 0.05$). After mixed *E. tarda* + *V. anguillarum* challenge, survival was significantly higher (50.00%, Relative Percent Survival = 47.8%) in the bivalent group than in the control group (4.17%) ($p < 0.05$). No acute adverse reactions or behavioral abnormalities were observed. These findings indicate

that an adjuvant-free bivalent vaccine elicits robust mid- to late-phase humoral responses and provides broad protection without evidence of antigenic interference, supporting a practical polyvalent strategy where *E. tarda* and *V. anguillarum* co-circulate.

Keywords: Bactericidal activity, *Edwardsiella tarda*, indirect ELISA, Tilapia, *Vibrio anguillarum*, whole-cell vaccine

Introduction

Bacterial pathogens remain a persistent constraint to tilapia aquaculture, where *Edwardsiella tarda* and *Vibrio anguillarum* are among the most consistently reported agents and may co-circulate under intensive farming stressors (e.g., deteriorating water quality or high stocking density) (Haenen et al., 2023; Frans et al., 2011). *E. tarda* is a Gram-negative, facultative intracellular bacterium that is capable of surviving within phagocytes and evading killing by the complement system (Li et al., 2022), whereas *V. anguillarum* is classically extracellular and rapidly colonizes tissues via adhesins, motility, iron acquisition and secreted toxins (Frans et al., 2011). Outbreaks of either pathogen, and especially their concurrence, may escalate mortality and undermine productivity in warm-water aquaculture systems (Haenen et al., 2023). Although chemotherapeutics have historically been used to treat outbreaks, concerns about antimicrobial resistance and residues have catalyzed a shift toward vaccination as a preventive, pathogen-specific measure (Schar et al., 2020). In practice, fish in intensive systems face multi-pathogen pressure; as a result, polyvalent vaccines are attractive for their broader coverage and reduction in handling events. However, the combination of multiple antigens can also introduce immunological interference (antigenic competition), whereby the immune response to one component is reduced relative to when the antigen is delivered alone, a phenomenon that has been documented in experimental polyvalent fish vaccines (Nikoskelainen et al., 2007). In tilapia and related species, bivalent formulations delivered either orally via feed or by injection have been shown to achieve elevated IgM responses and relatively high percentage survival in experimental and field settings, supporting their operational feasibility (Mohd Ali et al., 2023; Rivas et al., 2023; Shoemaker et al., 2012; Sun et al., 2011). Conceptually, combining antigens from the intracellular pathogen *E. tarda* and the extracellular pathogen *V. anguillarum* may broaden effector engagement across systemic and mucosal compartments and mitigate the risk of antigenic gaps. From a vaccine-development perspective, this potential benefit must be balanced against the risk of antigenic competition in multivalent vaccines, highlighting the importance of evaluating combined formulations alongside matched monovalent vaccines (Kuang et al., 2022) However, the use of adjuvant-free, inactivated whole-cell bivalent approaches targeting these two bacteria in tilapia, and their performance across serological, mucosal, and survival endpoints including mixed-pathogen challenge remain to be explored.

Here, we evaluated a formalin-inactivated, adjuvant-free bivalent vaccine comprising *E. tarda* and *V. anguillarum* antigens. We hypothesized that the bivalent formulation would elicit stronger functional immunity relative to monovalent vaccines without evidence of antigenic competition, thereby offering a practical option for controlling co-circulating *E. tarda* and *V. anguillarum* in tilapia aquaculture. We quantified systemic and mucosal IgM responses alongside serum and mucus bactericidal activity at multiple timepoints, and assessed the protective efficacy of the vaccine against homologous and mixed challenges.

Materials and methods

Fish and maintenance

Tilapia (*Oreochromis niloticus*) weighing approximately 30–50 g were obtained from the Laboratory of Aquaculture Science, Tokyo University of Marine Science and Technology (TUMSAT). Before the experiments, the fish were acclimated for a minimum of 2 weeks in a 60-L freshwater tank maintained at 25°C (±1°C) under a 12-h/12-h light/dark cycle. The fish were fed daily with commercial pellets at 1% of their body weight. One-third of the tank volume was replaced daily to maintain water quality. Dissolved oxygen, pH, and other water parameters were monitored regularly to ensure optimal conditions. All husbandry and experimental procedures involving live fish were conducted in accordance with the Animal Experiment Handling Regulations of TUMSAT. The Edwardsiella strain was maintained and used under its original designation, as reported in previous studies.

Bacterial strains and maintenance

E. tarda FPC498 and *V. anguillarum* were obtained from the Laboratory of Fish Health and Management, TUMSAT. *E. tarda* FPC498 was originally isolated from the ascitic fluid of a naturally infected Japanese flounder (*Paralichthys olivaceus*) (Yamasaki et al., 2015) and previously used by Cao et al. (2023). *V. anguillarum* was isolated from rainbow trout *Oncorhynchus mykiss* (Gallage et al., 2016). Both strains are non-reference field isolates that have been maintained and propagated in the source laboratory as previously described. The identity of each strain was confirmed by standard biochemical profiling and colony characteristics on selective agar; for *V. anguillarum*, species-specific PCR targeting the amiB gene was performed as previously described (Hong et al., 2007).

All bacterial strains were stored at −80 °C until use. For *E. tarda*, frozen stocks were streaked onto tryptic soy agar (TSA); for *V. anguillarum*, the TSA was supplemented with 3% (w/v) NaCl. After incubation at 25–30 °C for 24–48 h, single colonies were inoculated into tryptic soy broth (TSB) for *E. tarda* and TSB containing 3% (w/v) NaCl for *V. anguillarum*. Cultures were incubated at 30 °C with shaking at 150 rpm for 24 h; bacterial cells were harvested by centrifugation, washed three times with phosphate-buffered saline (PBS; pH 7.2), and resuspended in PBS.

Vaccine preparation and experimental groups

Vaccines were prepared as a formalin-inactivated whole-cell suspension (FKC). In brief, log-phase cultures of *E. tarda* and *V. anguillarum* were resuspended in PBS (pH 7.2) to approximately 1 × 10^9 CFU/mL. Formalin (0.3% v/v) was added, and the suspensions were incubated at 4 °C with gentle agitation for 24 h. Complete inactivation was verified by a lack of colonies after plating 100 µL of the treated suspension on TSA (*E. tarda*) or NaCl–TSA (*V. anguillarum*), followed by incubation at 28 °C for 72 h. Cells were washed three times with PBS to remove residual formalin and resuspended to the target concentrations.

Four groups were established and injected intraperitoneally (100 µL/fish) with adjuvant-free suspensions as follows: PBS control (n = 96; PBS only), mono-*E. tarda* (n = 48; 1×10^8 CFU/fish FKC), mono-*V. anguillarum* (n = 48; 1×10^7 CFU/fish FKC), and bivalent (n = 96; both antigens at the same per-fish doses). Sample sizes were chosen to provide adequate numbers for the immunological assays and challenge tests described below. The overall experimental design, including group allocation, dosing, sampling timepoints, and the challenge scheme, is summarized in Figure 1.

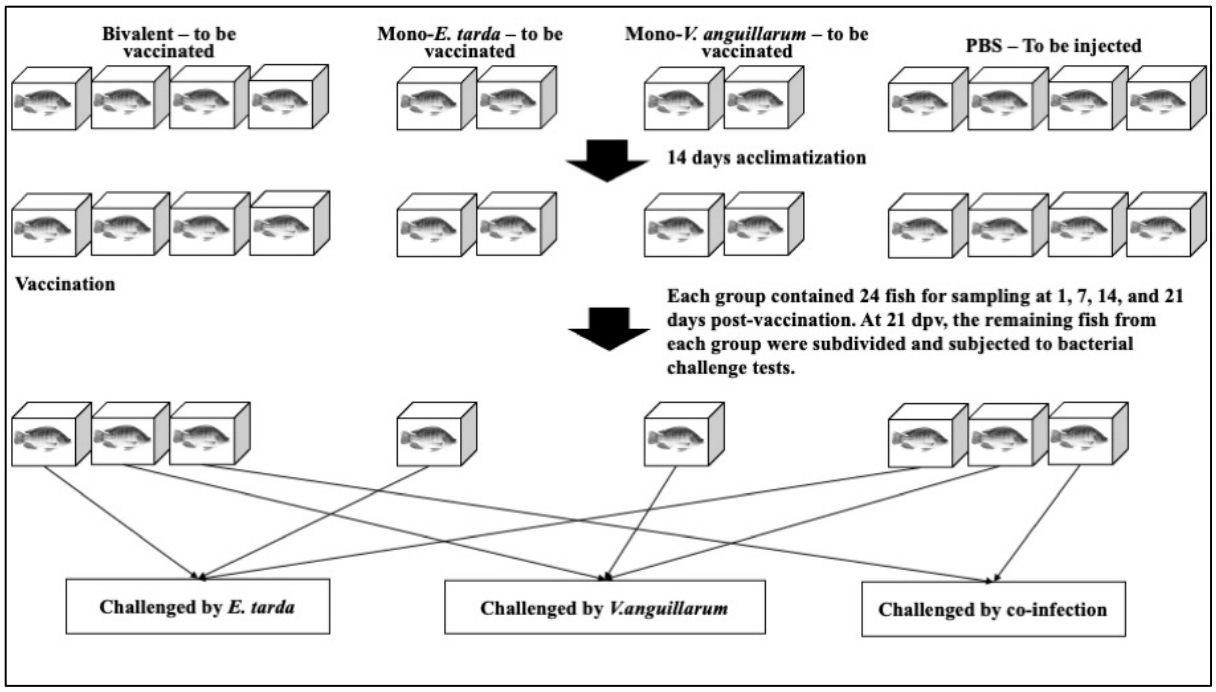

Figure 1. Experimental design. After a 14-day acclimatization, tilapia allocated to the bivalent (n = 96), **Figure 3** monovalent (*E. tarda*, n = 48; *V. anguillarum*, n = 48), or PBS control (n = 96) group were intraperitoneally injected (100 µL/fish) with formalin-killed bacterial suspensions prepared without adjuvant (bivalent, *E. tarda* 1×10^8 + *V. anguillarum* 1×10^7 CFU/fish; monovalent matched) or PBS. Serum and skin-mucus samples were collected at 1, 7, 14, and 21-days post-vaccination (dpv) (n = 6/timepoint/group) for IgM ELISA and serum/mucus bactericidal activity. At 21 dpv, the remaining fish were challenged with *E. tarda* or *V. anguillarum*, or both (3 tanks × 8 fish; n = 24 fish/arm) and observed for 21 days post-challenge (dpc); the co-infection arms comprised only the bivalent and PBS groups.

Sample collection and timepoints

Serum and skin mucus samples were collected at 1, 7, 14, and 21 dpv. At each timepoint, six fish per group were randomly netted and anaesthetised by immersion in 2-phenoxyethanol (300 µL/L; Wako, Japan) until respiratory and opercular movements slowed and the righting reflex was lost. For serum collection, blood was withdrawn from the caudal vein using a plain, non-heparinized 1-mL syringe and transferred into a microtube without anticoagulant. Samples were allowed to clot for 60 min at room temperature before centrifugation at 3,000 × g for 10 min at 4 °C. The clarified serum was aliquoted into single-use vials and stored at –80 °C to avoid repeated freeze–thaw cycles. The sampling timeline is depicted in Figure 1.

While still under anaesthesia, each fish was placed in a sterile polyethylene bag containing 0.5 mL of PBS (pH 7.2) supplemented with EDTA-free protease inhibitor cocktail (1 tablet/10 mL; Roche, Germany). The body surface was gently massaged for approximately 60 s to release mucus into the buffer. The PBS–mucus mixture was transferred to a 1.5-mL microtube and centrifuged at 3,000 × g for 10 min at 4 °C. Supernatants were aliquoted and stored at –80 °C for subsequent immunological assays. All sampling procedures were completed within 3 min per fish to minimize handling stress.

Measurement of specific antibody titers

Serum and mucus levels of IgM specific for *E. tarda* and *V. anguillarum* were measured by indirect ELISA. In brief, Immulon™ Immunoassay plates (Thermo Fisher Scientific, USA)

were coated with 100 µL of FKC of each pathogen (1 × 10^9 CFU/mL) suspended in PBS and incubated overnight at 4 °C. After washing with PBS-Tween, the plates were incubated in 5% skim milk in PBS-Tween to prevent non-specific binding.

Next, 100 µL of appropriately diluted serum or mucus sample was added to each well, and the plates were and incubated for 1 h at room temperature. After a washing step, 100 µL of mouse monoclonal anti-tilapia IgM antibody (Aquatic Diagnostics Ltd., UK; product code F04) was added at a dilution of 1:33 in blocking buffer, according to the manufacturer's recommendation, and the plates were incubated for 1 h. After a second washing step, 100 µL of HRP-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, USA) diluted 1:1000 was added, and the plates were incubated for 1 h. Subsequently, 100 µL of 3,3′,5,5′-tetramethylbenzidine dihydrochloride hydrate substrate (Sigma-Aldrich, USA) was added to each well. After 10 min in the dark, the reaction was stopped by adding 50 µL of 2 M H_2SO_4. Absorbance at 450 nm (OD_{450}) was measured using a Multiskan FC microplate reader (Thermo Fisher Scientific, USA). Antibody levels were expressed as absorbance values and statistically compared among treatment groups and sampling timepoints.

Bactericidal activity assay

E. tarda and *V. anguillarum* were cultured to the logarithmic growth phase, harvested, washed twice with PBS, and resuspended to approximately 1 × 10^5 CFU/mL (for *V. anguillarum*, PBS and NaCl–TSA were used as appropriate). For each assay, 50 µL of serum or mucus was combined with 10 µL of bacterial suspension in a tissue culture treated 96-well test plate (TPP, Switzerland) and incubated at 30 °C for 2 h. The mixtures were then serially diluted in PBS and plated onto NaCl–TSA. Plates were incubated at 30 °C for 24 h, and colonies were enumerated as CFU.

Bactericidal activity was determined by the method of Gallage et al. (2016) and expressed as the percentage of growth inhibition relative to the PBS control:

$$\text{Inhibition (\%)} = \left(1 - \frac{CFU_{treated}}{CFU_{PBS\ control}}\right) \times 100$$

where $CFU_{treated}$ denotes the colony count from serum- or mucus-treated wells, and $CFU_{PBS\ control}$ the count from bacteria incubated with PBS alone.

Challenge tests

At 21 dpv, three separate challenge models were conducted: *E. tarda*, *V. anguillarum*, and co-infection (see Figure 1). All challenges were performed by intraperitoneal injection using a final volume of 100 µL per fish. The fish remaining in each vaccination group were allocated to challenge arms in three replicate tanks (8 fish/tank; n = 24 fish/arm). Fish were observed twice daily for 21 dpc; moribund or dead individuals were recorded and promptly removed. The challenge organism was confirmed by re-isolation on TSA for *E. tarda* and NaCl–TSA for *V. anguillarum*.

For the *E. tarda* model, the mono-*E. tarda*, bivalent, and PBS control groups were injected with 100 µL of *E. tarda* suspension at 1×10^8 CFU/fish. For the *V. anguillarum* model, the mono-*V. anguillarum*, bivalent, and PBS control groups were injected with 100 µL of *V. anguillarum* suspension at 1×10^7 CFU/ fish. For the co-infection model, the bivalent and PBS control groups received a mixed inoculum prepared immediately before use: 50 µL of *E. tarda* (1×10^8 CFU/fish) plus 50 µL of *V. anguillarum* (1×10^7 CFU/fish). The co-infection challenge in the

present study did not include monovalent comparator groups. Accordingly, this model was not intended to determine whether the bivalent formulation outperforms monovalent vaccines under mixed-pathogen exposure, but rather to confirm that protection is maintained when both pathogens are encountered simultaneously. Future studies incorporating matched monovalent vaccine groups in co-infection models will be necessary to directly compare relative efficacy and to determine whether bivalent vaccination confers additive or synergistic protection.

Cumulative survival was summarized at 21 dpc for each arm. Relative percent survival (RPS) was calculated as:

$$\text{RPS} = [1 - \frac{Mortalit\_vaccinated}{Mortalit\_PBS\ control}] \times 100,$$

where higher values indicate greater vaccine-induced protection.

Statistical analysis

All data are presented as mean ± standard deviation (SD). Antibody titers, bactericidal activities, and survival rates at 21 dpc were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test to determine statistical significance among groups. The significance level was set at $p < 0.05$. All statistical analyses were performed using IBM SPSS Statistics for macOS, version 30.0 (IBM Corp., Armonk, NY, USA).

Results

IgM antibody responses in serum and mucus

First, we measured specific IgM antibody responses in fish serum and mucus for 21 dpv. From 7 to 21 dpv, vaccination elicited antigen-specific IgM responses in both serum and skin mucus, with vaccinated groups generally exceeding the PBS control group.

For *E. tarda*-specific responses (Figure 2A–B), in serum at 21 dpv the bivalent group (OD_{450} = 0.22) was numerically higher than the monovalent group (OD_{450} = 0.20) but not significantly different; both vaccinated groups were significantly higher than the PBS group (OD_{450} = 0.08; $p < 0.05$). In mucus, at 21 dpv the bivalent group (OD_{450} = 0.43) did not differ significantly from the monovalent group (OD_{450} = 0.38), while both groups were significantly higher than the PBS group (OD_{450} = 0.09; $p < 0.05$).

For *V. anguillarum*-specific responses (Figure 2C–D), in serum at 14 dpv the bivalent group (OD_{450} = 0.27) was not significantly different from the monovalent group (OD_{450} = 0.18), but was significantly higher than the PBS control (OD_{450} = 0.08; $p < 0.05$). At 21 dpv, the bivalent group was significantly higher than both the monovalent and PBS groups; and the monovalent group was significantly higher than the PBS group ($p < 0.05$). In mucus, at 14–21 dpv the bivalent group (1.38–1.44) was significantly higher than the monovalent (0.35–0.34) and PBS (0.08–0.09) groups at corresponding timepoints ($p < 0.05$).

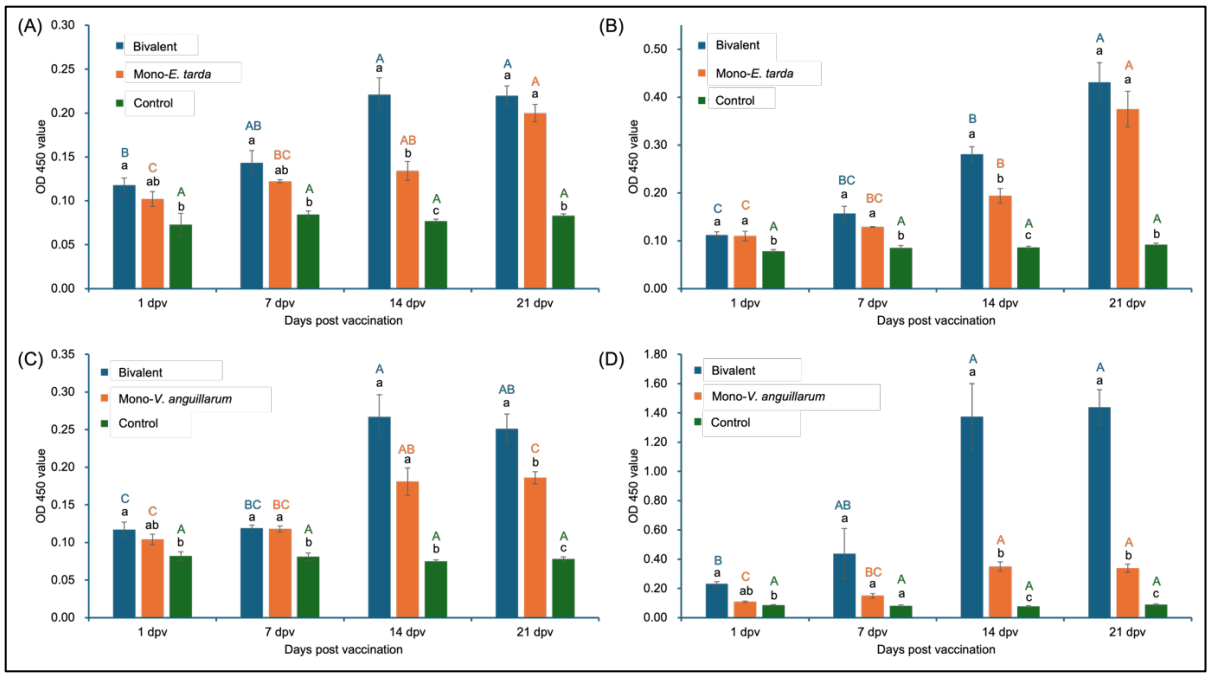

Figure 2. Serum and mucosal IgM antibody responses against *E. tarda* and *V. anguillarum* in tilapia immunized with monovalent vaccines, a bivalent vaccine, or PBS control. (A) Serum IgM responses against *E. tarda*. (B) Mucosal IgM responses against *E. tarda*. (C) Serum IgM responses against *V. anguillarum*. (D) Mucosal IgM responses against *V. anguillarum*. All responses were measured at 1, 7, 14, and 21 dpv. Data are mean ± SD (n = 6 fish/group), and the error bars represent standard deviation. At each timepoint, different lowercase letters denote significant differences among groups (ANOVA with Tukey's test, $p < 0.05$), and uppercase letters indicate significant differences over time within the same group.

Collectively, mid- to late-phase IgM responses were robust across systemic and mucosal compartments, with the bivalent vaccine performing better than or equal to the monovalent vaccine in most comparisons and showing a consistent advantage in mucosal IgM and *V. anguillarum* serum IgM at 14 dpv.

Bactericidal activity in serum and mucus

We also measured the bactericidal activity of serum and mucus collected post-vaccination. Overall, vaccination increased bactericidal activity across compartments compared with PBS controls from 7 to 21 dpv. For *E. tarda*-specific bactericidal activity (Figure 3A–B), the bivalent group showed significantly higher serum activity than the monovalent group at 7 and 21 dpv ($p < 0.05$), while the difference at 14 dpv was not significant ($p > 0.05$). Serum activity in the PBS group remained <7% at each timepoint, and values within the bivalent group did not differ significantly between 14 and 21 dpv ($p > 0.05$). In mucus, all pairwise comparisons among the three groups were significant at 14 dpv ($p < 0.05$). At 21 dpv, activity in the bivalent group (40.53%) was significantly higher than that in the PBS group (1.40%; $p < 0.05$), but not significantly different from that in the monovalent group (23.10%; $p > 0.05$).

For *V. anguillarum*-specific bactericidal activity (Figure 3C–D), serum activity in the bivalent group reached 76.00% and 80.12% at 14 and 21 dpv, respectively; these values were significantly higher than those in the monovalent group (60.50% and 66.70%) and PBS controls (<7%; $p < 0.05$). In mucus, activity at 21 dpv was 48.60% in the bivalent group and 44.80% in

the monovalent group; although these values did not differ significantly ($p > 0.05$), both were significantly higher than the inhibition observed in the PBS group (1.50%; $p < 0.05$).

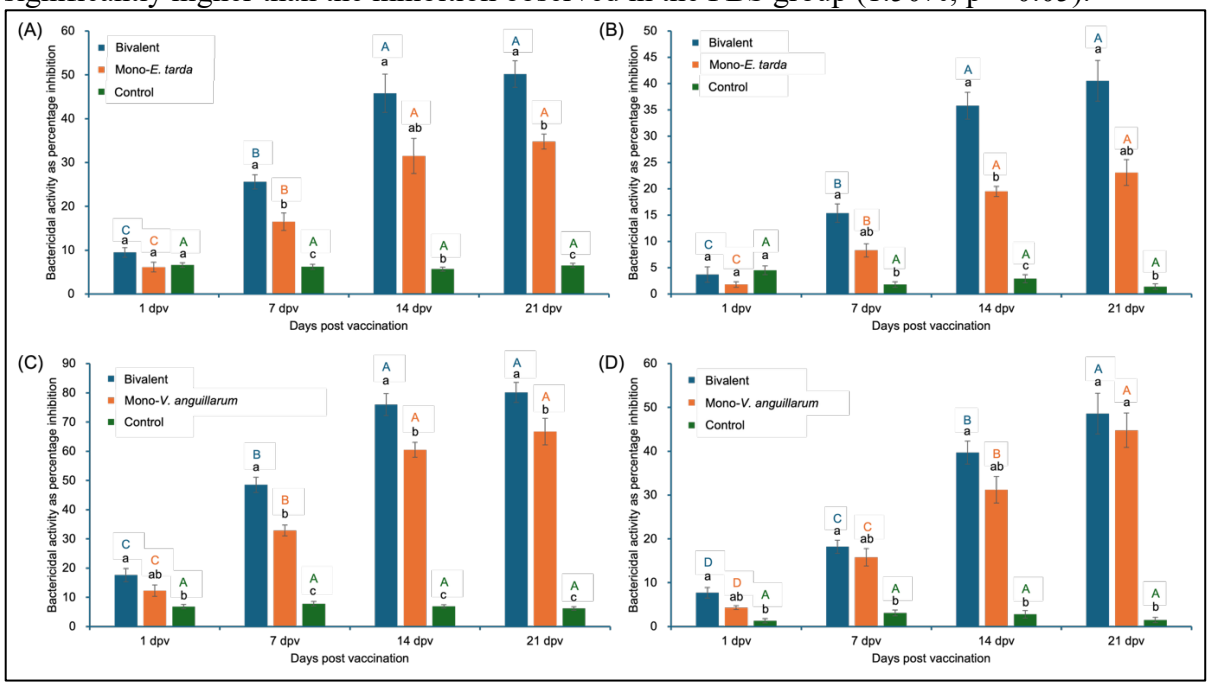

Figure 3. Serum and mucosal bactericidal activity against *E. tarda* and *V. anguillarum* in tilapia immunized with monovalent vaccines, a bivalent vaccine, or PBS control. (A) Serum bactericidal activity against *E. tarda*. (B) Mucosal bactericidal activity against *E. tarda*. (C) Serum bactericidal activity against *V. anguillarum*. (D) Mucosal bactericidal activity against *V. anguillarum*. All responses were measured at 1, 7, 14, and 21 dpv. Data are mean ± SD (n = 6 fish/group), and the error bars represent standard deviation. At each timepoint, different lowercase letters denote significant differences among groups (ANOVA with Tukey's test, $p < 0.05$), and uppercase letters indicate significant differences over time within the same group.

Overall, the observed bactericidal responses aligned with the IgM kinetics. The bivalent vaccine showed clear advantages over the monvalent vaccine in serum bactericidal activity and comparable activity to the monovalent vaccine against *V. anguillarum* at later timepoints.

Protective efficacy against single and co-infections

Lastly, we monitored survival of the fish for 21 dpc. After *E. tarda* challenge (Figure 4A), survival was significantly higher in the bivalent group (54.17%) than in the monovalent (29.17%) or PBS (25.00%) group ($p < 0.05$). After *V. anguillarum* challenge (Figure 4B), survival was 87.50% in the bivalent group and 75.00% in the monovalent group; the difference between the vaccinated groups was not significant ($p > 0.05$), but survival in both groups was significantly higher than that in the PBS group (41.67%; $p < 0.05$).

Under mixed (*E. tarda* + *V. anguillarum*) challenge (Figure 4C), survival in the bivalent group (50.00%) was significantly higher than that in the PBS group (4.17%; $p < 0.05$).

Taken altogether, these results demonstrate that the bivalent vaccine was superior or comparable to monovalent formulations in single-pathogen models and conferred a clear benefit under co-infection, consistent with the humoral and bactericidal data.

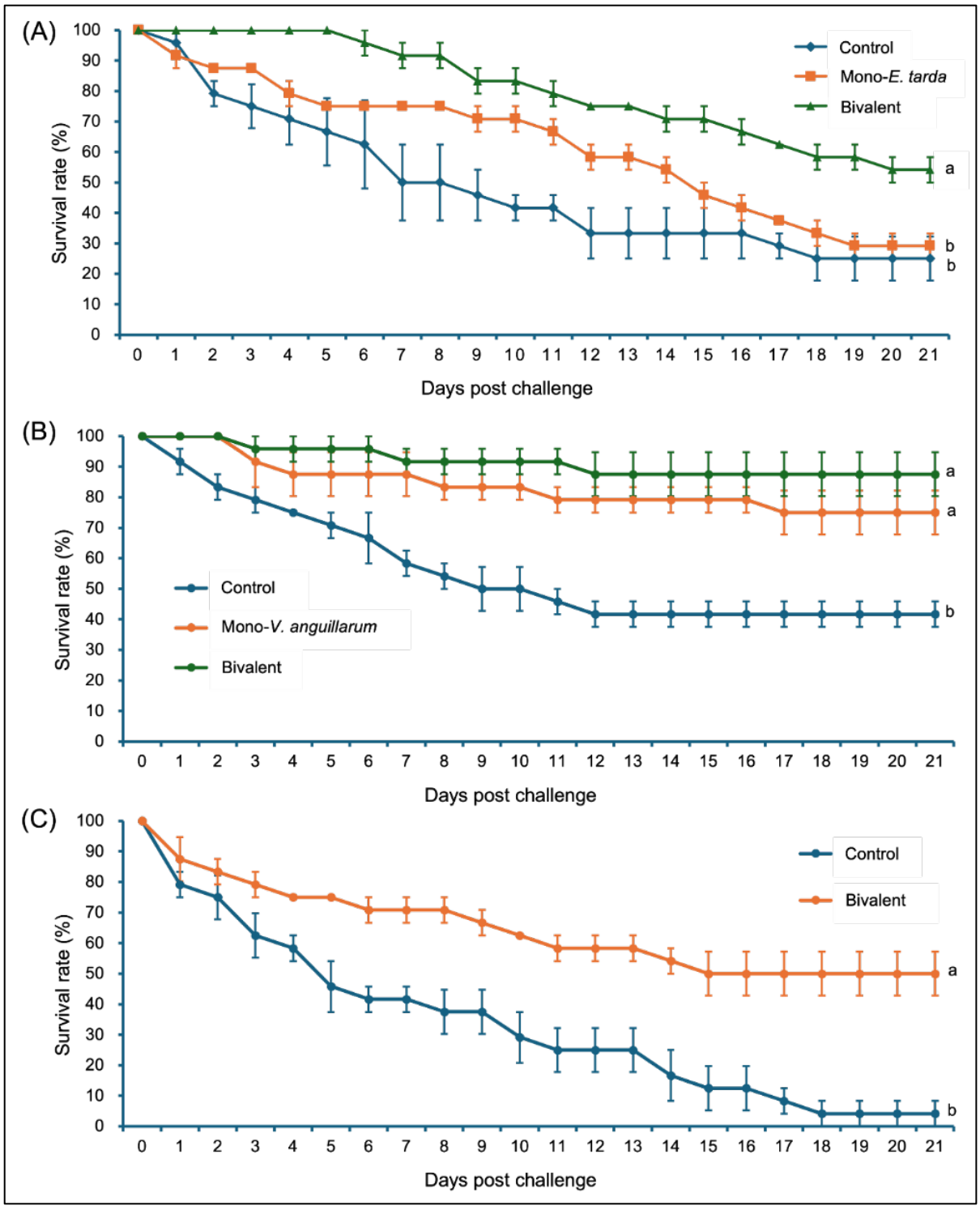

Figure 4. Cumulative survival of tilapia following experimental challenge with *E. tarda*, *V. anguillarum*, or a combination of both after immunization with monovalent vaccines, a bivalent vaccine, or PBS control. (A) Cumulative survival after *E. tarda* challenge. (B) Cumulative survival after *V. anguillarum* challenge. (C) Cumulative survival after mixed (*E. tarda* + *V. anguillarum*) challenge. Data are the cumulative survival rate (%) over 21 dpc. Each treatment had three replicate tanks (8 fish/tank; n = 24 fish/group). Different letters indicate significant differences among groups at 21 dpc (ANOVA with Tukey's test, $p < 0.05$).

Discussion

This study has shown that a formalin-inactivated, adjuvant-free bivalent vaccine targeting *E. tarda* and *V. anguillarum* elicits robust systemic and mucosal IgM responses, together with higher serum/mucus bactericidal activity, which translate into improved survival under homologous and mixed-pathogen challenges (Figures 2–4). Two features of our findings stand out: (i) there was no evidence of antigenic interference within the tested dose ratio and time window, because the bivalent-induced responses were equal to or better than the monovalent-induced responses against each pathogen; and (ii) there was functional concordance between serology and killing. Collectively, our study highlights the potential practical value of an adjuvant-sparing polyvalent approach for farms facing concurrent bacterial risks. From an operational and economic standpoint, delivering protection against both pathogens in a single vaccination event can reduce handling and injection frequency compared with administering two separate monovalent vaccines, thereby lowering labor demands and minimizing handling-associated stress and potential losses. Future field-scale cost–benefit analyses will be valuable to quantify these practical savings under commercial conditions.

Mechanistically, the observed activity and responses align with known teleost humoral biology. Vaccine-induced IgM promotes agglutination/opsonization and classical-pathway complement activation, providing a plausible bridge to the observed increases in serum and mucus bactericidal activity. The rise in mucosal IgM despite intraperitoneal immunization is consistent with systemic–mucosal crosstalk and polymeric immunoglobulin receptor-mediated translocation to epithelial surfaces, reinforcing frontline defenses (Rombout et al., 2014; Salinas, 2015; Sunyer, 2013; Xu et al., 2013). We infer that this framework may underlie the similar kinetics of IgM and bactericidal activity at mid–late timepoints, although direct mechanistic validation (e.g., complement inhibition) will be needed to verify this.

The bivalent pairing may benefit from biological complementarity. *E. tarda* is a facultative intracellular pathogen that persists within phagocytes, whereas *V. anguillarum* is classically extracellular; combining their antigens will broaden effector recruitment across both phagocyte-mediated pathways and complement-dependent killing. We further speculate that distinct pathogen-associated molecular patterns engage different pattern-recognition receptors (e.g., TLR5 and TLR9/21) on teleost antigen-presenting cells, favoring additive cytokine programs that support B-cell and macrophage functions (Palti, 2011; Yeh et al., 2013; Sui et al., 2017; Zhang et al., 2016). This molecular logic is concordant with our finding that bivalent immunity was at least as strong as monovalent against each target and clearly advantageous during co-infection (Figure 4A–C).

Within the 21-dpv observation period and the tested formulation ratio, no antigenic interference was detected: most readouts in the bivalent group were not lower and often higher than those in the corresponding monovalent groups, although certain endpoints (e.g., 21 dpv mucus specific bactericidal activity) showed no significant difference. The co-infection challenge in the present study did not include monovalent comparator groups. Accordingly, this model was not intended to determine whether the bivalent formulation outperforms monovalent vaccines under mixed-pathogen exposure, but rather to confirm that protection is maintained when both pathogens are encountered simultaneously. Future studies incorporating matched monovalent vaccine groups in co-infection models will be necessary to directly compare relative efficacy and to determine whether bivalent vaccination confers additive or synergistic protection.

Our data also align with previous studies in tilapia and related systems, where feed-based or oral bivalent vaccines showed elevated IgM and improved protection, and injectable

formulations achieved laboratory and field efficacy (Shoemaker et al., 2012; Mohd Ali et al., 2023; Rivas et al., 2023; Sun et al., 2011), further supporting the operational feasibility of our approach. Notably, we observed broad immunogenicity without exogenous adjuvants, consistent with the idea that antigen valency and stoichiometry may functionally "adjuvantize" responses; it will be valuable in future work to optimize antigen ratios because ratio effects on protection have been demonstrated for other bivalent vaccines in fish (Kuang et al., 2022).

In the bivalent group, IgM and serum/mucus specific bactericidal activity rose in parallel, showing consistent mid-to-late-phase trends rather than proven causal linkage. In tilapia and salmonids, passive transfer and field serology have linked antibody levels with protection and informed booster decisions, suggesting that IgM and specific bactericidal activity may serve as practical surrogate endpoints to guide on-farm schedules (Pasnik et al., 2005; Pasnik et al., 2006; Holten-Andersen et al., 2012). Similarly, in Atlantic cod, IgM-mediated protection has been demonstrated despite the evolutionary loss of MHC class II and CD4 (Jonsson et al., 2025). Together with the survival advantage under mixed-pathogen exposure, these surrogates indicate a potential advantage of the bivalent approach under co-circulating pathogen pressure, although whether protection will exceed a simple additive effect remains to be determined.

We found that short-term tolerability was favorable, observing no acute adverse reactions. Whereas oil-adjuvanted injections may elicit adhesions or granulomatous changes in finfish, our adjuvant-free formulation reduces, but does not eliminate, the risk of adjuvant-related reactions; histopathological assessment was not conducted and should be included in future evaluations (Tammas et al., 2024; Tziouvas & Varvarigos, 2021).

Because challenges were conducted via intraperitoneal injection using homologous strains, and survival was analyzed as endpoint proportions (three tanks × eight fish each), our findings primarily reflect controlled laboratory efficacy. Future work should incorporate Kaplan–Meier/log-rank or Cox modeling, durability and boosting schedules, ratio optimization, and heterologous-strain challenges across environmental conditions. Inclusion of cellular readouts (e.g., memory B-cell markers) would further resolve the contribution of long-lived humoral immunity.

Conclusion

This study has demonstrated that a formalin-inactivated, adjuvant-free bivalent vaccine combining *E. tarda* and *V. anguillarum* antigens elicits robust mid- to late-phase systemic and mucosal IgM responses, enhances serum and mucus bactericidal activity, and is associated with improved survival in Nile tilapia under the conditions tested. In single-pathogen challenges, bivalent performance was greater than or comparable to matched monovalent formulations depending on the pathogen, with no evidence of antigenic interference. Monovalent comparators were not included in the co-infection arm; therefore, we refrain from inferring superiority or more-than-additive effects. No acute adverse reactions or behavioral abnormalities were observed during the study period. Collectively, these findings support an adjuvant-sparing polyvalent strategy for farms facing co-circulating *E. tarda* and *V. anguillarum* and highlight IgM and serum/mucus bactericidal activity as practical surrogate readouts to inform booster timing, while field-scale and long-term validation remain necessary.

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Ethical approval
The experimental procedures involving animals were reviewed and approved by the Animal Ethics Committee of Tokyo University of Marine Science and Technology (Japan).

Informed consent
Not applicable.

Data availability statement
The authors declare that data can be provided by corresponding author upon reasonable request.

Conflicts of interest
There is no conflict of interests for publishing this study.

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Contribution of authors
Zheyu Liu: Investigation, methodology, formal analysis, writing – original draft preparation
Masato Endo: Resources, validation, review and editing.
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