

Adjuvant optimization and protective efficacy evaluation of scuticociliate vaccine

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1 Adjuvant optimization and protective efficacy evaluation of scuticociliate vaccine

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Abstract

Scuticociliatosis, caused by *Pseudocohnilembus persalinus*, triggers massive economic losses in turbot (*Scophthalmus maximus*) aquaculture. Our laboratory's existing protective vaccine depends on Freund's adjuvant (FA), which cannot be used for commercial production. We first validated the vaccine assessment system with blank control groups and adopted a stepwise research design for adjuvant screening and antigen optimization to screen alternative adjuvants and assess single-dose vaccines suitable for aquaculture industrialization. In the first trial, turbot were immunized with whole-cell protein (WCP) combined with AA, MF59 or FA respectively; the second trial compared two vaccine formulations containing aluminium adjuvant (AA), namely AA-WCP and AA-membrane protein (MP). All fish received standardized skin abrasion followed by immersion pathogen challenge to detect immune responses and calculate the relative parasite reduction rate. The first trial revealed AA induced higher IgM and better biocompatibility than FA. The second trial showed MP boosted non-specific enzymes (POD, T-SOD, ACH, LZM) and IgM. Challenge assays showed AA-MP reached an 86% parasite reduction rate, higher than AA-WCP (76%). All results were verified by the prevalidated vaccine model. In summary, AA is a biocompatible substitute for FA, and single-dose AA-MP synergistically strengthens protective immunity for commercial turbot farming.

Keywords: *Pseudocohnilembus persalinus*; scuticociliate; fish vaccine; relative parasite reduction rate; aluminium adjuvant; turbot;

1. Introduction

Marine aquaculture constitutes a critical pillar of global food security and economic development, yet its rapid intensification has been increasingly constrained by outbreaks of bacterial, viral, and parasitic diseases (Al-Busaidi et al. 2016; Mol and Coşansu 2022). Among these, scuticociliatosis represents one of the most devastating parasitic threats to high-value marine fish, characterized by rapid onset, high transmission rates, refractory treatment, and substantial economic losses worldwide (Iglesias et al. 2001; Kim et al. 2004; Li et al. 2018; Moustafa et al. 2010). The disease is primarily driven by facultative parasitic scuticociliates such as *P. persalinus*, which readily infect economically important species including turbot (*Scophthalmus maximus*), *Takifugu rubripes*, Japanese flounder (*Paralichthys olivaceus*), and sea bass (*Lateolabrax japonicus*) (Azad 2007; Gao et al. 2018). Pathogens typically gain entry through superficial skin lesions before disseminating to internal organs, triggering systemic infection and mortality (Hewson et al. 2023; Lin et al. 2024). Consequently, eliciting robust mucosal and systemic immune responses are widely recognized as a prerequisite for effective disease interception and long-term prophylaxis (Lord et al. 2014; Mokhtar et al. 2023).

To address this significant challenge, vaccination is widely recognized as the most promising and sustainable strategy for disease prevention, overcoming the limitations of chemical treatments such as drug resistance and environmental pollution (Burridge et al. 2010; Ma et al. 2019). However, vaccine development against scuticociliatosis remains hindered by two persistent bottlenecks: suboptimal adjuvant compatibility and insufficient antigenic protection. Our research group previously developed a scuticociliatosis vaccine with proven protective efficacy under laboratory conditions; however, its formulation relied on Freund's adjuvant (Wang et al. 2024). Freund's adjuvant has long served as a benchmark in experimental fish immunology due to its potent immunostimulatory capacity, making it highly suitable for preliminary laboratory efficacy evaluation (Awate et al. 2013; Sun et al. 2025). Nevertheless, it is fundamentally incompatible with industrial manufacturing. Its commercial translation is increasingly contested owing to concerns over severe tissue residue, chronic inflammatory side effects, and stringent regulatory restrictions in aquaculture (Noia et al. 2014; Tafalla et al. 2014). Therefore, to facilitate industrial-scale production and reduce the costs of clinical application, screening alternative adjuvants to replace Freund's adjuvant is imperative. Antigen formulation presents a parallel dilemma. Whole-cell protein (WCP) extracts offer broad antigenic diversity but frequently contain non-protective intracellular debris that may dilute immune focus or induce immunopathology (Dadar et al. 2017; Kubelka et al. 2004). Conversely, membrane protein (MP) antigens are hypothesized to concentrate surface-exposed protective epitopes, making them attractive subunit candidates for targeted immunity (Sun et al. 2026; Hegde and Keenan 2021). Aluminium adjuvant (AA) has emerged as a safer alternative with proven depot-forming properties. However, the practical protective efficacy and immunological performance of AA–MP vaccine formulations remain largely undetermined, representing a critical knowledge gap hindering the formulation and application of effective and safe vaccines against scuticociliatosis.

A further limitation in current research lies in efficacy assessment methodologies. Conventional vaccine trials predominantly rely on cumulative post-challenge mortality, a delayed endpoint that poorly captures a vaccine's capacity to prevent early mucosal colonization and limit pathogen replication. Instead of relying on general mucus detection reported in previous literature, our research group established a novel standardized evaluation system: fixed-area mechanical abrasion was created on fish body surfaces to simulate natural infection pathways under real mariculture conditions. We quantified parasite loads in wound mucus and calculated the relative parasite reduction rate, which acts as a more sensitive and biologically relevant metric for evaluating the early protective efficacy of vaccines (Wang et al. 2024). Given the distinct immune effects induced by different antigen preparations, comparative studies employing standardized challenge models are essential to dissect antigen-specific immune dynamics and refine vaccine formulation. We adopted a strictly stepwise exploratory design: first conducting adjuvant screening to select the optimal adjuvant, then performing antigen combination optimization and multi-dimensional efficacy verification. To address these challenges, this study implements a sequential experimental framework for scuticociliate vaccine development. An optimal adjuvant was first identified through systematic screening in turbot, and subsequent trials directly compared the protective efficacies of AA mixed with membrane protein (MP) or whole-cell protein (WCP) antigens. Vaccine efficacy was comprehensively validated through multiple quantitative indicators, including wound parasite clearance, relative parasite reduction rate, serum total IgM levels, and innate immune enzyme activities. By integrating dynamic humoral profiling with relative parasite reduction rate-based mucosal assessment, we demonstrate that AA consistently outperforms conventional alternatives across both species, and that MP formulations significantly enhance early protective immunity compared to WCP. These findings establish an optimized adjuvant–antigen framework and validate relative parasite reduction rate as a superior indicator of early vaccine efficacy, providing a scalable, evidence-based foundation for the rational development of marine fish parasite vaccines (Sun et al. 2026; Wang et al. 2024). Specifically, a tiered experimental design was employed, utilizing turbot as the unified experimental animal throughout all trials to establish baseline immune parameters and complete the full optimization of vaccine formulation.

2. Materials and Methods

2.1. Experimental animals and maintenance

Juvenile turbot with an average body weight of 50 g for Experiment 1 and juvenile turbot with an average body weight of 200 g for Experiment 2 were purchased from a commercial hatchery in Dalian, Liaoning Province, China. Prior to experimentation, all fish were acclimatized for 7 days in a closed recirculating aquaculture system. Seawater was filtered through a 0.45 µm membrane, and salinity was maintained at 29–32‰. Water temperature was regulated at 16 °C for turbot and 14 °C for *T. rubripes*. During the acclimatization period, fish were considered healthy based on the absence of external clinical signs (e.g., skin lesions, abnormal swimming behavior), active feeding, and zero mortality. To verify the absence of prior pathogen exposure, a serum immobilization assay was performed on randomly netted individuals

with uniform body weight. Results confirmed zero immobilization titers and no detectable agglutination activity, indicating that all experimental fish were pathogen-naïve. Following one-week acclimatization and health verification, each fish was weighed separately, and only individuals with nearly identical body weight were screened to eliminate weight bias. These size-matched fish were gently netted and evenly allocated to different experimental groups one after another; this simplified balanced grouping minimizes weight-related confounding factors and aligns with the core concept of stratified randomization built on uniform fish body size.

For Experiment 1, healthy turbot from the unified weight pool were sequentially netted and evenly split into four treatment groups ($n = 25$ per group): (1) aluminium adjuvant (AA) combined with whole-cell protein (WCP); (2) MF59 combined with WCP; (3) Freund's adjuvant (FA) combined with WCP; and (4) sterile saline control. For Experiment 2, fish with consistent body weight were selected and evenly divided into three treatment groups ($n = 15$ per group): (1) AA combined with WCP; (2) AA combined with membrane protein (MP); and (3) AA combined with PBS control.

2.2. Scuticociliate cultivation

P. persalinus was maintained in axenic culture using standard marine protozoan medium at 16 °C. Cultures were harvested during the logarithmic growth phase for subsequent antigen preparation and challenge experiments (Wang et al. 2024).

2.3. Whole-cell protein (WCP) preparation

Approximately 100 mL of ciliate suspension was collected and filtered to remove particulate debris. Ciliates were inactivated by adding 1% formaldehyde solution (v/v), followed by centrifugation at 10,000 rpm for 2 min. The resulting pellet was washed three times with PBS and resuspended to a final concentration of 2×10^6 cells/mL. Cell lysis was achieved via three freeze–thaw cycles (–80 °C for 10 min, followed by 37 °C for 10 min). The suspension was vortexed immediately after each thawing step to ensure homogeneity and minimize protein degradation. The final lysate was aliquoted into 1.5 mL microcentrifuge tubes (600 µL per tube) and stored at –80 °C until use. This method was adapted from (Wang et al. 2024).

2.4. Membrane protein (MP) preparation

P. persalinus cultures were harvested at the logarithmic growth phase and inactivated with 0.1% formalin (v/v). Membrane proteins were extracted using a commercial membrane protein extraction kit (Biosharp, BL671A, Beijing, China) according to the manufacturer's instructions. Protein concentration was determined using the Bradford assay. Extracted MP was aliquoted and stored at –80 °C. This method was adapted from (Sun et al. 2026).

2.5. Vaccine formulation and administration

Thawed WCP antigen was emulsified with each adjuvant (AA, MF59, FA, or sterile saline) at a 1:1 (v/v)

ratio. Homogenization was achieved by repeatedly passing the mixture between two sterile syringes connected via a silicone tube until a uniform emulsion formed. Each fish received a 0.1 mL intraperitoneal injection. For the MP vaccine, pre-extracted MP was diluted with sterile PBS to 0.6 mg/mL, then mixed with AA at a 1:1 (v/v) ratio to yield a final concentration of 0.3 mg/mL. Each fish received a 0.1 mL intraperitoneal injection of the respective formulation.

2.6. Parasite challenge protocol

Challenge experiments were conducted on day 14 post-immunization for Experiment 1 and day 21 for Experiment 2. Freshly cultured *P. persalinus* suspensions were filtered through a 300-mesh sieve to remove culture medium and debris. Ciliate density was quantified using a hemocytometer and adjusted to 3,000 cells/mL with sterile seawater. Prior to exposure, all scraping instruments were sterilized. Two standardized square wounds (1.5 × 1.5 cm each; total area: 4.5 cm²) were created on the dorsal musculature of each turbot and *T. rubripes* via gentle mechanical abrasion. Mucus was collected from the defined wound area, and all mucus harvested from each wound was transferred into 200 µL broth. After static incubation for 10 min, an aliquot of the suspension was aspirated using a pipette for cell quantification. During immersion challenge, continuous aeration was maintained, and feeding and water exchange were suspended to minimize environmental variables. The calculated volume of seawater was added to challenge tanks, followed by the introduction of the filtered ciliate suspension. At designated time points, five fish were haphazardly sampled by netting from each tank for blood collection.

2.7. Determination of relative parasite reduction rate

Wound mucus was scraped from 10 fish per group at 24 and 48 h post-challenge and diluted to 200 µL using sterile seawater. An aliquot of each suspension was counted under an optical microscope to determine the parasite burden. *Relative parasite reduction rate* = $[(\text{Average number of infected scuticociliates in the control group} - \text{Average number of infected scuticociliates in the experimental group}) / \text{Average number of infected scuticociliates in the control group}] \times 100\%$.

2.8. Blood sampling and serum preparation

In Experiment 1, five fish per group were evenly selected by sequential net capture, euthanized, and systematically dissected. Experiment 2, blood samples were collected on days 1, 3, 7, 14, and 21 post-vaccination. At each sampling time point, fish were differentiated by the healing status of the previous injection puncture sites to exclude individuals bled at earlier time points, ensuring distinct fish were sampled for each time point. Collected blood was transferred to sterile centrifuge tubes and allowed to clot at room temperature for 1 h, followed by overnight incubation at 4 °C. Samples were subsequently centrifuged at 3,000 rpm for 10 min. The supernatant (serum) was carefully transferred to sterile microcentrifuge tubes and stored at -80 °C until analysis.

2.9. Serum immunoglobulin M (IgM) quantification

Serum IgM concentrations were determined using a commercial fish IgM enzyme-linked immunosorbent

assay (ELISA) kit (JW.FI1186; Shanghai, China) following the manufacturer's protocol. The assay utilized a double-antibody sandwich format. Prior to analysis, each serum sample was diluted fivefold (10 μ L serum + 40 μ L sample diluent). Briefly, 50 μ L of serially diluted standards (0–3,200 ng/mL) or diluted samples were added to pre-coated microplate wells, followed by 100 μ L of horseradish peroxidase (HRP)-conjugated detection antibody (blank wells received diluent only). Plates were sealed and incubated at 37 °C for 60 min. After washing five times with wash buffer, 50 μ L each of substrate solutions A and B were added. Plates were incubated at 37 °C in the dark for 15 min. The reaction was terminated by adding 50 μ L of stop solution, and absorbance was measured at 450 nm within 15 min using a microplate reader. IgM concentrations were calculated from a standard curve generated by linear regression, and final values were multiplied by the dilution factor ($\times 5$). All samples were analyzed in duplicate, with intra- and inter-assay coefficients of variation (CV) $< 15\%$ and a standard curve correlation coefficient (R^2) ≥ 0.99 .

2.10. Non-specific immune enzyme activity assays

Serum enzymatic activities, including peroxidase (POD), total superoxide dismutase (T-SOD), acetylcholinesterase (ACH), and lysozyme (LZM), were quantified using commercial colorimetric kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China; catalog nos. A084-2, A001-1, A105-2, and A050-1, respectively). All procedures, including reagent preparation, incubation conditions, and absorbance measurements, were performed in strict accordance with the manufacturer's guidelines. Enzyme activities were calculated based on standard calibration curves and expressed as units per milliliter (U/mL) of serum. To ensure analytical precision, each sample was analyzed in duplicate.

2.11. Gross anatomical examination of injection site biocompatibility

Upon completion of the immunization, fish from each group were evenly selected by sequential net capture, euthanized, and systematically dissected. Gross anatomical assessment was performed via macroscopic examination of the intraperitoneal injection site and the abdominal cavity. Local tissue biocompatibility was evaluated based on the presence and extent of adjuvant residue, local tissue reactions (e.g., inflammation, hemorrhage), peritoneal adhesion, and the morphological integrity of the surrounding visceral organs.

2.12. Statistical analysis

Statistical analyses were performed using GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA). Prior to analysis, the assumptions of ANOVA (normality and homogeneity of variances) were verified using the Shapiro-Wilk and Levene's tests, respectively. Data meeting these assumptions were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test for multiple comparisons, while non-parametric tests were used for data violating these assumptions. Statistical significance was set at $P < 0.05$.

2.13. Ethics statement

All experimental animal programs in this study were reviewed and approved by the Dalian Ocean University Laboratory Animal Ethics Committee (Approval Letter No. 51, dated 8 June 2023). These programs adhere to internationally accepted animal welfare and ethical guidelines and comply with the relevant laws, regulations, and policies governing experimental animal management in China and Liaoning Province.

3. Results

3.1. Adjuvant screening and protective efficacy

The relative parasite reduction rate and wound-associated parasite loads across adjuvant-vaccinated groups are presented in Table 1 and Figure 1. At 24 h post-challenge (hpc), relative parasite reduction rate values for the aluminium adjuvant (AA), MF59, and Freund's adjuvant (FA) groups were 71%, 67%, and 66%, respectively, with no significant intergroup differences ($p > 0.05$). This uniform early protection indicates that all three formulations successfully primed initial immune defenses, facilitating rapid mucosal and innate responses that effectively limited early parasite colonization. By 48 hpc, protective efficacy diverged significantly among groups. The AA and MF59 groups exhibited enhanced parasite clearance, with relative parasite reduction rate values escalating to 98% and 96%, respectively, while parasite burdens remained at minimal levels. Both groups significantly outperformed the FA group (76% relative parasite reduction rate; $p < 0.05$). In contrast, the AA group not only achieved near-complete parasite elimination at 48 hpc but also demonstrated superior biocompatibility and sustained immunological memory. Collectively, considering the magnitude of immune response, durability of protection, and clinical safety profile, AA was identified as the optimal adjuvant formulation for scuticociliatosis vaccines in turbot.

Table 1. Relative parasite reduction rate of turbot at 24 h and 48 h post-challenge(hpc) across adjuvant treatment groups.

hpc	AA	MF59	FA
24h	71%	67%	66%
48h	98%	96%	76%

Note: Time points presented in this table indicate hours post-challenge. All fish in this table were immunized with whole-cell protein antigen combined with different adjuvants.

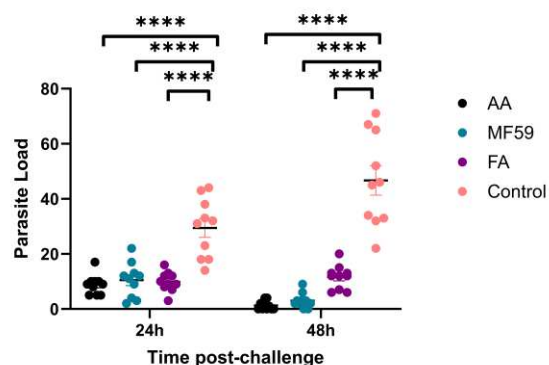


Figure 1. Parasite load in turbot at 24 h and 48 h post-challenge. Fish were immunized with AA, MF59, FA, or sterile saline (control). Parasite counts were quantified from wound mucus. Data are presented as mean $\pm$ standard error of the mean (SEM) ($n = 10$ per group at each time point). Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test (vs. control at the same time point). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns, not significant ($p \geq 0.05$). Abbreviations: AA, aluminium adjuvant; FA, Freund's adjuvant.

3.2. Serum total immunoglobulin M (IgM) levels at 14 days post-immunization

The serum total immunoglobulin M (IgM) levels at 14 days post-immunization are illustrated in Figure 2. At this time point, the AA group demonstrated significantly higher total IgM levels compared to both the MF59 and FA groups ($p < 0.05$). Furthermore, the AA group exhibited the highest total IgM levels among all immunized groups, indicating a superior capacity for inducing systemic humoral immunity. These findings collectively indicate that, compared to the other tested adjuvants, AA elicits a more robust total humoral immune response in *in turbot*, thereby providing a solid immunological foundation for protective efficacy.

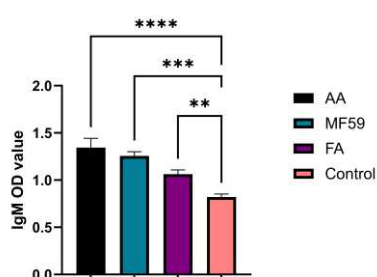


Figure 2. Serum total IgM levels in turbot at 14 days post-intraperitoneal vaccination with different adjuvant. Fish were sampled at 14 days post-immunization. Statistical significance compared to the control group is indicated by asterisks (* $p < 0.05$, ** $p < 0.01$). Abbreviations: AA, aluminium adjuvant; FA, Freund's adjuvant.

3.3. Modulation of non-specific immune enzyme activities

To evaluate the modulatory effects of different adjuvants on the non-specific immune system of turbot, the serum activities of total superoxide dismutase (T-SOD), peroxidase (POD), acetylcholinesterase (ACH), and lysozyme (LZM) were systematically quantified (Figure 3). Compared with the control group, all vaccine-immunized groups exhibited significantly elevated activities of these non-specific immune enzymes ($p < 0.01$). Notably, the AA group demonstrated the most pronounced enhancement, with T-SOD, POD, ACH, and LZM activities surpassing those of the MF59 and FA groups ($**p < 0.001$; $***p < 0.0001$). During the mid-to-late post-immunization phase, the AA group not only sustained peak enzymatic activities but also exhibited the most robust overall activation profile across all measured parameters. These findings indicate that, in addition to eliciting a potent humoral immune response, AA effectively potentiates the innate immune defense network in turbot. By synergistically upregulating antioxidant, antibacterial, and immunomodulatory enzyme activities, AA establishes a more resilient early barrier against pathogen invasion.

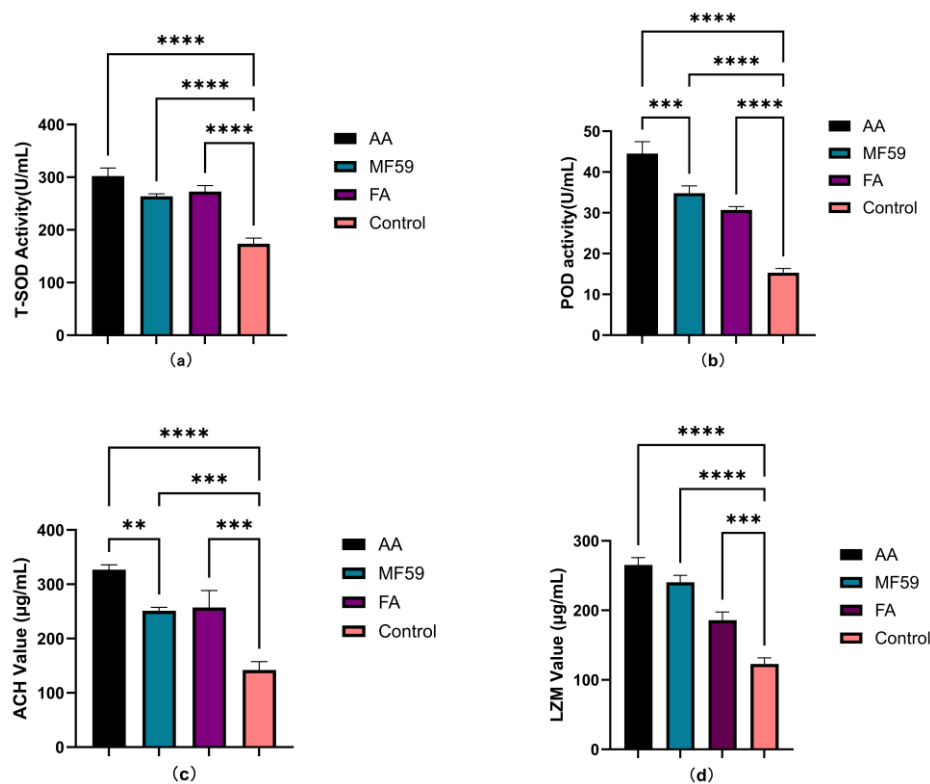


Figure 3. Effects of different adjuvants on serum non-specific immune enzyme activities in turbot. Fish were intraperitoneally injected with vaccines emulsified with AA, MF59, FA, or saline (control). Serum samples were collected to assess immune parameters. (a) Total superoxide dismutase (T-SOD) activity; (b) Peroxidase (POD) activity; (c) Acetylcholinesterase (ACH) value; (d) Lysozyme (LZM) value. Statistical significance compared to the control group is indicated by asterisks ($**p < 0.01$, $***p < 0.001$, $****p < 0.0001$). Abbreviations: AA, aluminium adjuvant; FA, Freund's adjuvant.

3.4. Gross anatomical assessment and local tissue biocompatibility

The biocompatibility and tissue safety of adjuvants are critical criteria for evaluating their potential application in aquaculture. Upon completion of the immunization cycle, turbot were systematically dissected to macroscopically assess adjuvant residue and local tissue reactions at the intraperitoneal injection site, thereby comprehensively evaluating the *in vivo* absorption profiles and biosafety of the different formulations. Macroscopic examination revealed distinct differences in intraperitoneal residue among the groups. The FA group exhibited evident adjuvant residue, indicating slow absorption of the water-in-oil formulation, with localized peritoneal adhesion observed in some individuals. In contrast, no obvious residue was detected in the abdominal cavities of the AA and MF59 groups. The injection sites in these groups showed excellent tissue recovery, suggesting rapid *in vivo* clearance and minimal local tissue irritation. The control group displayed normal visceral morphology without any pathological lesions. Collectively, AA and MF59 demonstrated superior biocompatibility, aligning better with the safety requirements for aquaculture vaccine adjuvants (Figure 4).

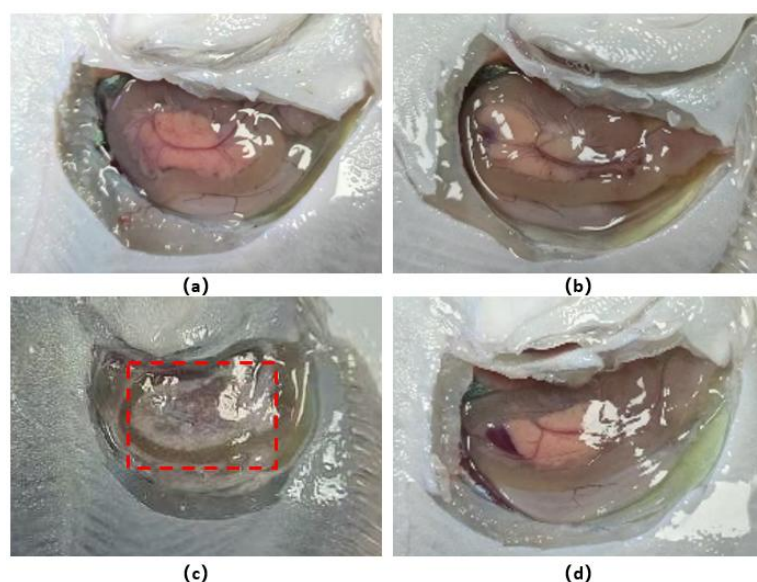


Figure 4. Macroscopic assessment of abdominal cavity conditions following intraperitoneal vaccine administration in turbot. (a) AA adjuvant group showing no visible residue and healthy tissue morphology. (b) MF59 adjuvant group exhibiting complete absorption with normal visceral appearance. (c) FA group displaying evident adjuvant residue and localized peritoneal adhesion. (d) Control group with normal abdominal cavity and no pathological changes. Abbreviations: AA, aluminium adjuvant; FA, Freund's adjuvant.

3.5. Antigen comparison and protective efficacy

To further optimize the vaccine formulation, the protective efficacy of the membrane protein (MP) antigen was evaluated against the whole-cell protein (WCP) antigen, both adjuvanted with the optimal aluminium adjuvant (AA), in a *T. rubripes* challenge model. Parasite load quantification and relative parasite reduction rate analysis revealed distinct advantages of the MP formulation. As illustrated in Figure 5, the MP vaccine significantly accelerated parasite clearance compared to the WCP formulation.

At 24 h post-challenge (hpc), both vaccinated groups exhibited reduced parasite burdens relative to the AA-phosphate-buffered saline (PBS) control; however, the AA-MP group showed a lower mean parasite load than the AA-WCP group. By 48 hpc, the disparity became statistically significant. The AA-MP group demonstrated near-complete parasite elimination, with burdens dropping to minimal levels (significantly lower than the control, **** $p < 0.0001$), whereas the AA-WCP group retained a moderate parasite load comparable to early-stage infection levels. This enhanced pathogen clearance directly correlated with improved protective outcomes, as summarized in Table 2. While both formulations conferred protection at 24 h, the AA-MP group achieved a higher relative parasite reduction rate of 68% compared to 57% in the AA-WCP group. At the 48h endpoint, the protective efficacy of the MP vaccine peaked at 86% relative parasite reduction rate, significantly outperforming the WCP vaccine (76% relative parasite reduction rate; $p < 0.05$). These results indicate that the MP fraction, when combined with AA, elicits a more potent and rapid immune response capable of effectively neutralizing *P. persalinus* invasion in *T. rubripes*.

Table 2. Relative parasite reduction rate of experimental fish at 24 h and 48 h post-challenge across antigen treatment groups.

hpc	AA-WCP	AA-MP
24h	57%	68%
48h	76%	86%

Note: Time points presented in this table indicate hours post-challenge. Group AA-WCP represents the whole-cell protein vaccine-immunized group, whereas group AA-MP denotes the membrane protein vaccine-immunized group.

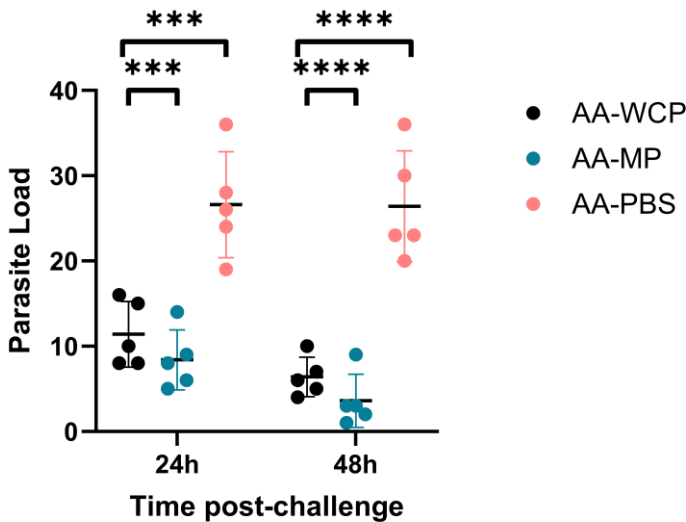


Figure 5. Parasite load of experimental fish at 24 h and 48 h post-challenge. Fish were immunized with AA combined with either WCP (AA-WCP) or MP (AA-MP), or treated with AA-PBS as a control. Statistical significance compared to the control group (AA-PBS) is indicated by asterisks (* $p < 0.05$,

****p < 0.01, ***p < 0.001, ****p < 0.0001. ns, not significant (p ≥ 0.05). Abbreviations: AA, aluminium adjuvant; FA, Freund's adjuvant.**

3.6. Dynamics of serum total immunoglobulin M (IgM) responses

The temporal kinetics of serum total immunoglobulin M (IgM) levels following immunization are illustrated in Figure 6. Both vaccine formulations elicited a progressive humoral response, with IgM levels rising steadily and peaking at 14 days post-immunization before plateauing. Notably, the AA–MP group consistently maintained higher IgM optical density (OD) values than the AA–WCP group across all monitoring time points, with the divergence being most pronounced during the peak response phase. This superior total IgM production aligns with the enhanced innate immune activation previously observed in the MP group, indicating a coordinated engagement of humoral defenses. The robust and sustained IgM response elicited by the MP formulation suggests that surface-exposed membrane epitopes efficiently drive B-cell activation and IgM secretion in *T. rubripes*.

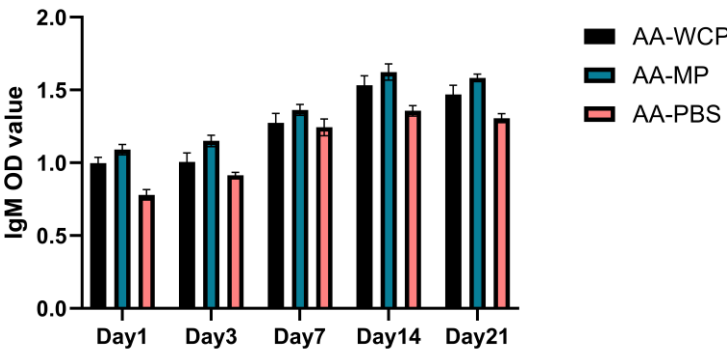


Figure 6. Temporal profile of serum total IgM levels in immunized experimental fish. Serum samples were collected at 1, 3, 7, 14, and 21 days post-immunization to assess total IgM responses. Groups included AA–WCP, AA–MP, and AA–PBS (control). Data are presented as mean OD values ± SD. The MP formulation elicited a consistently higher and more sustained IgM response compared to WCP, with peak levels observed at day 14. Abbreviations: AA, aluminium adjuvant; FA, Freund's adjuvant.

3.7. Modulation of non-specific immune enzyme activities

To evaluate the modulatory effects of the antigen formulations on the non-specific immune system of *T. rubripes*, serum activities of peroxidase (POD), total superoxide dismutase (T-SOD), acetylcholinesterase (ACH), and lysozyme (LZM) were systematically quantified. Vaccination with the AA–MP formulation elevated the activities of all measured enzymes compared to both the AA–WCP and AA–PBS control groups. The AA–MP group demonstrated the most pronounced enhancement, with POD, T-SOD, ACH, and LZM activities surpassing those of the AA–WCP group (**p < 0.001; ***p < 0.0001). During the mid-to-late post-immunization phase, the AA–MP group sustained peak enzymatic activities, indicating robust innate immune priming. These findings confirm that MP vaccination effectively potentiates the non-specific defense network in *T. rubripes*, synergizing with the specific

humoral response to establish a comprehensive immunological barrier (Figure 7).

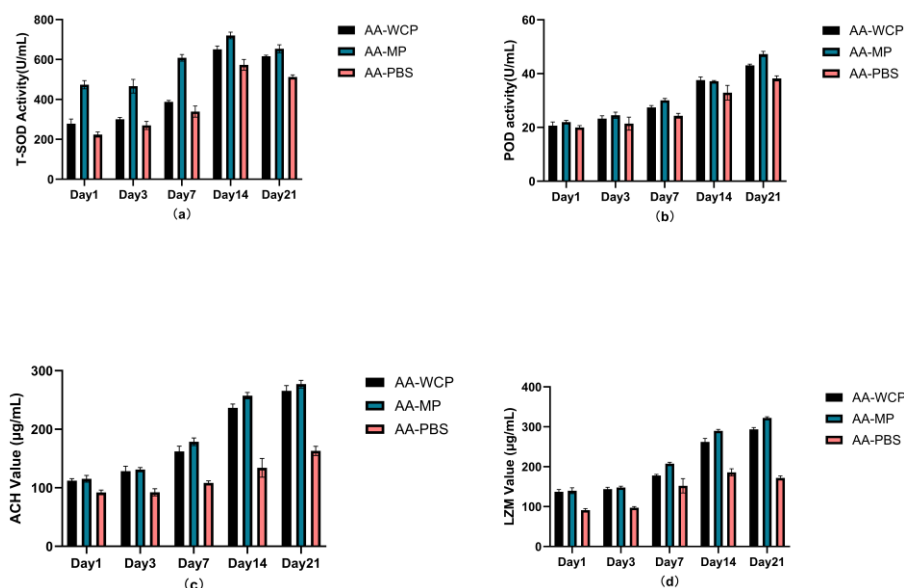


Figure 7. Temporal dynamics of serum non-specific immune enzyme activities in immunized experimental fish following intraperitoneal vaccination. Fish were immunized with aluminium adjuvant combined with whole-cell protein (AA–WCP), membrane protein (AA–MP), or AA–phosphate-buffered saline (AA–PBS) as a control. Serum samples were collected at 1, 3, 7, 14, and 21 days post-immunization. (a) Total superoxide dismutase (T-SOD) activity; (b) Peroxidase (POD) activity; (c) Acetylcholinesterase (ACH) activity; (d) Lysozyme (LZM) activity. Statistical significance compared to the AA–PBS control group at corresponding time points was determined by one-way analysis of variance followed by Tukey’s multiple comparisons test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Abbreviations: AA, aluminium adjuvant; FA, Freund’s adjuvant.

4. Discussion

Marine aquaculture has become a cornerstone of global protein supply, yet its rapid intensification has been severely challenged by the emergence of infectious diseases. Among these, scuticociliatosis, caused by opportunistic ciliates such as *P. persalinus*, stands out as one of the most destructive parasitic threats to high-value marine fish. The pathogen exploits superficial skin injuries to penetrate host tissues, rapidly disseminating to internal organs and triggering mass mortality within days. Traditional control measures, including chemical treatments and antibiotic applications, are increasingly constrained by drug resistance, environmental contamination, and strict food safety regulations (Miller and Harbottle 2018). Consequently, prophylactic vaccination has emerged as the most sustainable and economically viable strategy for long-term disease management. However, the protective efficacy of fish vaccines is not solely determined by the antigen itself but relies heavily on the precise pairing of adjuvants and immunogenic components (Kayser and Ramzan 2021). While several preliminary studies have explored vaccine candidates against scuticociliatosis, these efforts often lack a systematic, stepwise optimization

process. Many rely on empirical formulations or evaluate protection using delayed mortality endpoints, which fail to capture the critical early stages of mucosal defense and limit the reproducibility of results across different farming systems (Moustafa et al. 2010). Addressing these critical gaps, our study demonstrates that a systematic, stepwise optimization process is essential to effectively capture the early stages of mucosal defense and ensure reproducible protective outcomes.

We adopted a strictly empirical two-stage optimization strategy to gradually establish and validate a reliable anti-scudicociliatosis vaccine formulation. In Experiment 1, multiple adjuvants were systematically screened and compared to identify the optimal and biocompatible adjuvant candidate. In Experiment 2, the selected superior adjuvant was further combined with different antigens to achieve enhanced protective efficacy. The final vaccine effectiveness was fully verified through multiple independent quantitative indicators, including wound parasite abundance, relative parasite reduction rate, serum IgM levels, and key innate immune enzyme activities, providing solid and multi-dimensional evidence for adjuvant optimization and vaccine validation.

We constructed a standardized challenge model that simulates natural marine infection. Mechanical skin abrasion combined with parasite immersion was adopted to reproduce the natural invasion pathway of *P. persalinus* under real mariculture conditions. We screened and replaced traditional adjuvants with a safer aluminium adjuvant, and further combined this optimal adjuvant with membrane protein antigen. This integrated experimental system allows precise quantification of the early mucosal protective potency induced by our optimized vaccine formulation.

Historically, Freund's complete adjuvant (FA) has served as a reference standard in experimental fish immunology due to its strong immune-activating properties. Nevertheless, its application in commercial aquaculture faces substantial practical and regulatory barriers (Stills 2005). The oil-based nature of FA frequently leads to persistent injection-site residues, chronic granulomatous inflammation, and tissue necrosis, which not only compromise fish welfare and growth performance but also conflict with stringent food safety standards (Jiao et al. 2010). Our macroscopic findings in the present study strongly corroborate these limitations, confirming the unsuitability of FA for routine aquaculture use. These limitations have driven the search for safer, commercially viable alternatives. Aluminium-based adjuvants (AA) have gained widespread acceptance in human and veterinary medicine owing to their proven safety record, minimal tissue retention, and favorable biocompatibility. AA functions by forming a localized antigen depot that ensures sustained release, promotes efficient uptake by phagocytic cells, and stimulates protective immune signaling without inducing severe inflammatory damage (Exley et al. 2019; Xiong et al. 2015). Although AA has been tested in various fish species, its systematic evaluation in combination with specific parasitic antigens for scudicociliatosis control remains insufficiently refined. By establishing a structured comparison in this study, we provide direct empirical evidence confirming the true practical value and superior biocompatibility of AA over conventional oil-based adjuvants in marine vaccine development (Lindblad 2004).

To address this need, the present study implemented a sequential, two-phase experimental design to

systematically identify the optimal vaccine configuration against *P. persalinus*. The first phase was dedicated to adjuvant screening rather than vaccine validation, in which three adjuvants (AA, FA, MF59) were comprehensively compared using whole-cell protein antigens. Only after confirming AA as the optimal adjuvant with superior immunity and biosafety did we proceed to the second-phase antigen optimization. In the second experiment, we further improved vaccine efficacy by matching the screened optimal AA with membrane protein antigen, achieving significantly enhanced protection compared with the WCP group. The advantages of this tiered experimental design are fully reflected in our experimental results.

The initial screening in turbot demonstrated that AA induces a stronger and more total IgM response than both FA and MF59, while maintaining excellent tissue compatibility. Although FA generated a measurable immune response, it was consistently accompanied by localized tissue damage and prolonged inflammation, confirming its unsuitability for routine aquaculture use. MF59, despite its established safety in mammals, produced a comparatively weaker response in this parasitic model. The superior performance of AA likely results from its balanced mechanism of action: it retains antigens at the injection site long enough to ensure repeated stimulation of immune cells, yet it avoids the aggressive inflammatory cascade triggered by oil-based emulsions (Danielsson and Eriksson 2021). The high total IgM levels observed in AA-vaccinated fish, combined with the complete absence of injection-site pathology, strongly support its role as a reliable and scalable carrier for marine fish vaccines.

Building on these findings, the second phase revealed that the choice of antigen fundamentally shapes the protective outcome. The AA-MP formulation achieved an 86% relative parasite reduction rate at 48 hours post-challenge, significantly outperforming the AA-WCP group (76%). This advantage stems from the targeted nature of membrane proteins (Nakano et al. 1992), which concentrate surface-exposed epitopes directly involved in parasite attachment and host cell invasion. In contrast, WCP contains abundant intracellular materials that may dilute the immune focus or generate non-protective antibodies. Importantly, the MP-vaccinated group exhibited pronounced upregulation of key innate immune enzymes, each playing a distinct role in early pathogen clearance. Peroxidase (POD) catalyzes the production of reactive oxygen species and hypohalous acids that directly damage parasite membranes; its elevation indicates enhanced oxidative killing capacity at the infection site (Nakano et al. 1992). Total superoxide dismutase (T-SOD) rapidly neutralizes superoxide radicals, preventing oxidative stress from damaging host tissues and preserving the functional integrity of immune cells during acute infection (Vutukuru et al. 2006). Lysozyme (LZM) degrades structural components of microbial and parasitic cell walls, exerting direct lytic activity while simultaneously recruiting phagocytes to clear invading organisms (Subbotkin and Subbotkina 2018). Acetylcholinesterase (ACH), although traditionally associated with neural signaling, modulates the cholinergic anti-inflammatory pathway in fish, helping to balance immune activation and prevent excessive tissue damage (Soreq and Seidman 2001). The coordinated increase in these enzymes demonstrates that the MP antigen effectively primes a multi-layered innate defense, which works synergistically with the subsequent IgM surge to neutralize parasites

before they can establish systemic infection (Keyt et al. 2020).

A critical component of this optimization strategy is the use of relative parasite reduction rate measured at 48 hours post-infection as the primary efficacy indicator. Traditional parasitic vaccine trials in aquaculture typically depend on cumulative mortality recorded over 14 to 21 days. By quantifying the reduction in skin mucus parasite load shortly after challenge, the relative parasite reduction rate model captures the decisive window of early immune interception. This approach not only shortens the evaluation timeline but also provides a more accurate reflection of functional protection, closely matching the rapid infection kinetics of *P. persalinus*. The consistent protection levels observed at both 14-day and 21-day challenge intervals further confirm the reliability and durability of the AA-MP formulation (Sun et al. 2026).

The skin abrasion and immersion challenge method used here effectively mimics natural infection routes, validating the experimental approach (Sun et al. 2026; Wang et al. 2024). While these results provide a clear path forward, further studies are needed to optimize vaccine application under diverse commercial farming conditions. Additionally, this study focused on systemic immune markers and did not deeply investigate local mucosal IgM responses or local immune cell responses at the skin surface. Future research should aim to identify the exact protein fragments within the MP mixture that trigger the strongest protection, test the vaccine under real farming conditions to monitor long-term immunity, and evaluate how factors like storage temperature, shelf life, and practical administration methods affect its overall performance. Furthermore, while this study demonstrates robust short-term protection, the long-term duration of immunity, the impact of fluctuating environmental challenge pressures, and the direct extrapolation of efficacy to other teleost species require further investigation. Before commercial deployment, critical steps including large-scale field trials, GMP-compliant mass production, and comprehensive ecological safety assessments must be completed.

In summary, through a structured, step-by-step experimental approach, this study identifies the combination of aluminium adjuvant and membrane protein as the most effective and safe vaccine formulation against scuticociliatosis. By replacing residue-prone adjuvants with biocompatible AA and shifting from broad whole-cell extracts to targeted membrane antigens, the developed strategy delivers rapid, high-level protection without compromising fish health. All conclusions were empirically derived from stepwise adjuvant screening and antigen optimization, rather than pre-assumed vaccine effectiveness. Multiple quantitative readouts including wound parasite burden, parasite reduction rate, serum total IgM levels, and innate enzyme activities collectively validate the efficacy of the optimized AA-MP vaccine. This optimized framework offers a practical and reproducible model for advancing parasitic vaccine development in commercial marine aquaculture.

5. Conclusions

This study successfully developed a safe and highly effective single-dose vaccine against *Pseudocohnilembus persalinus*-induced scuticociliatosis in marine fish. This work adopted a two-step

empirical optimization strategy: adjuvant screening in turbot, followed by independent antigen optimization trials. By replacing Freund's adjuvant with aluminum adjuvant and utilizing membrane proteins as the optimal antigen, the AA-MP formulation delivered robust protection without injection-site lesions or tissue residues. The vaccine efficacy was fully validated by multiple independent indexes, including reduced wound parasite abundance, elevated parasite reduction rate, significantly enhanced innate immune enzymes (POD, T-SOD, LZM), and increased serum total IgM levels, indicating synergistic activation of both innate and adaptive immunity. Additionally, adopting a 48-hour skin mucus relative parasite reduction rate enabled a more sensitive and rapid efficacy assessment than traditional mortality endpoints. Overall, this work establishes a scalable, biocompatible vaccine platform, providing a reproducible framework for advancing parasitic disease control in sustainable aquaculture.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/doi/s1>, Figure S1: title; Table S1: title; Video S1: title.

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References

1. Al-Busaidi MA, Jukes DJ, Bose S (2016) Seafood safety and quality: an analysis of the supply chain in the Sultanate of Oman. *Food Control* 59:651-662. <https://doi.org/10.1016/j.foodcont.2015.06.023>

- 541 2. Mol S, Coşansu S (2022) Seafood safety, potential hazards and future perspective. Turk J Fish
542 Aquat Sci 22:TRJFAS20533. <https://doi.org/10.4194/TRJFAS20533>
- 543 3. Iglesias R, Paramá A, Alvarez MF et al (2001) *Philasterides dicentrarchi* (Ciliophora,
544 Scuticociliatida) as the causative agent of scuticociliatosis in farmed turbot *Scophthalmus*
545 *maximus* in Galicia (NW Spain). Dis Aquat Org 46:47-55. <https://doi.org/10.3354/dao046047>
- 546 4. Kim, S.M.; Cho, J.B.; Kim, S.K.; et al. Occurrence of scuticociliatosis in olive flounder
547 *Paralichthys olivaceus* by *Philasterides dicentrarchi* (Ciliophora: Scuticociliatida). Dis Aquat
548 Org 2004, 62, 233–238.
- 549 5. Li R, Gao Y, Hou Y et al. (2018) Mitochondrial genome sequencing and analysis of
550 scuticociliates (*Uronema marinum*) isolated from *Takifugu rubripes*. Mitochondrial DNA B
551 3:736-737. <https://doi.org/10.1080/23802359.2018.1483757>
- 552 6. Moustafa EM, Tange N, Shimada A, Morita T (2010) Experimental scuticociliatosis in Japanese
553 flounder (*Paralichthys olivaceus*) infected with *Miamiensis avidus*: pathological study on the
554 possible neural routes of invasion and dissemination of the scuticociliate inside the fish body. J
555 Vet Med Sci 72:1557-1563. <https://doi.org/10.1292/jvms.10-0123>
- 556 7. Azad IS (2007) Scuticociliatosis-associated mortalities and histopathology of natural infection
557 in cultured silver pomfret (*Pampus argenteus* Euphrasen) in Kuwait. Aquaculture 264:311-
558 317. <https://doi.org/10.1016/j.aquaculture.2006.10.033>
- 559 8. Gao Y, Jin S, Dang H et al (2018) Mitochondrial genome sequencing of notorious scuticociliates
560 (*Pseudocohnilembus persalinus*) isolated from turbot (*Scophthalmus maximus* L.).
561 Mitochondrial DNA Part B 3:1077-1078. <https://doi.org/10.1080/23802359.2018.1508388>
- 562 9. Hewson I, Ritchie IT, Evans JS et al (2023) A scuticociliate causes mass mortality of *Diadema*
563 *antillarum* in the Caribbean Sea. Sci Adv 9: eadg3200. <https://doi.org/10.1126/sciadv.adg3200>
- 564 10. Lin N, Pan Y, Zhan Z et al (2024) *Miamiensis avidus*, a novel scuticociliate pathogen isolated
565 and identified from cultured large yellow croaker (*Larimichthys crocea*). Pathogens 13:618.
566 <https://doi.org/10.3390/pathogens13080618>
- 567 11. Lord JM, Midwinter MJ, Chen YF et al (2014) The systemic immune response to trauma: an
568 overview of pathophysiology and treatment. Lancet 384:1455-1465.
569 [https://doi.org/10.1016/S0140-6736\(14\)60687-5](https://doi.org/10.1016/S0140-6736(14)60687-5)
- 570 12. Mokhtar DM, Zacccone G, Alesci A et al (2023) Main components of fish immunity: an overview
571 of the fish immune system. Fishes 8:93. <https://doi.org/10.3390/fishes8020093>
- 572 13. Burrridge T, Weis JS, Cabello F et al. (2010) Chemical use in salmon aquaculture: A review of
573 current practices and possible environmental effects. Aquaculture 306:7-

- 574 23. <https://doi.org/10.1016/j.aquaculture.2010.05.020>
- 575 14. Ma J, Bruce TJ, Jones J et al. (2019) A review of fish vaccine development strategies:
576 Conventional methods and modern biotechnological approaches. *Microorganisms*
577 7:569. <https://doi.org/10.3390/microorganisms7110569>
- 578 15. Wang X, Gao Y, Ni X et al (2024) Transcriptomic analysis of gene expression in immune
579 pathways in the spleen of *Takifugu rubripes* after immunization with scuticociliate vaccine.
580 *Aquaculture* 581:740380. <https://doi.org/10.1016/j.aquaculture.2023.740380>
- 581 16. Awate S, Babiuk LA, Mutwiri G (2013) Mechanisms of action of adjuvants. *Front Immunol*
582 4:114. <https://doi.org/10.3389/fimmu.2013.00114>
- 583 17. Sun Q, Gao Y, Wang X et al (2025) Transcriptomic Analysis of Gene Expression in Immune
584 Pathways in the Spleen of *Takifugu rubripes* After Infection with Scuticociliate *Uronema*
585 *marinum*. *Acta Parasitologica* 70:140. <https://doi.org/10.1007/s11686-025-01076-1>
- 586 18. Noia M, Domínguez B, Leiro J et al (2014) Inflammatory responses and side effects generated
587 by several adjuvant-containing vaccines in turbot. *Fish Shellfish Immunol* 38:244-254.
588 <https://doi.org/10.1016/j.fsi.2014.03.018>
- 589 19. Tafalla C, Bøgvold J, Dalmo RA et al (2014) Adjuvants in fish vaccines. In: Gudding R,
590 Lillehaug A, Evensen Ø (eds) *Fish vaccination*. Wiley, Chichester, pp 81-106.
591 <https://doi.org/10.1002/9781118806913.ch7>
- 592 20. Dadar M, Dhama K, Vakharia VN et al (2017) Advances in aquaculture vaccines against fish
593 pathogens: global status and current trends. *Rev Fish Sci Aquac* 25:250-283.
594 <https://doi.org/10.1080/23308249.2016.1261277>
- 595 21. Kubelka J, Hofrichter J, Eaton WA (2004) The protein folding ‘speed limit’. *Curr Opin Struct*
596 *Biol* 14:76-88. <https://doi.org/10.1016/j.sbi.2004.01.013>
- 597 22. Sun Q, Wu B, Ao Y et al (2026) Development and evaluation of immunoprotective efficacy of
598 membrane protein vaccine against scuticociliatosis. *Vaccines* 14:142.
599 <https://doi.org/10.3390/vaccines14020142>
- 600 23. Hegde RS, Keenan RJ (2021) The mechanisms of integral membrane protein biogenesis. *Nat*
601 *Rev Mol Cell Biol* 23:107-124. <https://doi.org/10.1038/s41580-021-00413-2>
- 602 24. Miller RA, Harbottle H (2018) Antimicrobial drug resistance in fish pathogens. *Microbiol*
603 *Spectr* 6: ARBA-0017-2017. <https://doi.org/10.1128/microbiolspec.ARBA-0017-2017>
- 604 25. Kayser V, Ramzan I (2021) Vaccines and vaccination: history and emerging issues. *Hum Vaccin*
605 *Immunother* 17:5255–5268. <https://doi.org/10.1080/21645515.2021.1935488>
- 606 26. Moustafa EMM, Naota M, Morita T et al (2010) Pathological study on the scuticociliatosis

607 affecting farmed Japanese flounder (*Paralichthys olivaceus*) in Japan. *J Vet Med Sci* 72:1359–
608 1362. <https://doi.org/10.1292/jvms.10-0096>

609 27. Stills HF Jr. (2005) Adjuvants and antibody production: dispelling the myths associated with
610 Freund's complete and other adjuvants. *ILAR J* 46:280–
611 293. <https://doi.org/10.1093/ilar.46.3.280>

612 28. Jiao X-d, Cheng S, Hu Y-h, Sun L (2010) Comparative study of the effects of aluminum
613 adjuvants and Freund's incomplete adjuvant on the immune response to an *Edwardsiella tarda*
614 major antigen. *Vaccine* 28:1832-1837. <https://doi.org/10.1016/j.vaccine.2009.12.036>

615 29. Exley C, Siesjö P, Eriksson H (2019) The immunobiology of aluminium adjuvants: how do they
616 really work? *Trends Immunol* 40:477-479. <https://doi.org/10.1016/j.it.2019.04.001>

617 30. Xiong J, Wang G, Cheng J et al (2015) Genome of the facultative scuticociliatosis pathogen
618 *Pseudocohnilembus persalinus* provides insight into its virulence through horizontal gene
619 transfer. *Sci Rep* 5:14174. <https://doi.org/10.1038/srep14174>

620 31. Lindblad EB (2004) Aluminium adjuvants—in retrospect and prospect. *Vaccine* 22:3658-
621 3668. <https://doi.org/10.1016/j.vaccine.2004.03.014>

622 32. Danielsson R, Eriksson H (2021) Aluminium adjuvants in vaccines – A way to modulate the
623 immune response. *Semin Cell Dev Biol* 115:3-9. <https://doi.org/10.1016/j.semedb.2020.12.008>

624 33. Vinothkumar KR, Henderson R (2010) Structures of membrane proteins. *Quarterly Reviews of*
625 *Biophysics* 43:65–158. <https://doi.org/10.1017/S0033583510000041>

626 34. Nakano T, Sato M, Takeuchi M (1992) Glutathione Peroxidase of Fish. *Journal of Food Science*
627 57:1116–1119. <https://doi.org/10.1111/j.1365-2621.1992.tb11276.x>

628 35. Vutukuru SS, Chintada S, Madhavi KR et al (2006) Acute effects of copper on superoxide
629 dismutase, catalase and lipid peroxidation in the freshwater teleost fish, *Esomus danricus*. *Fish*
630 *Physiology and Biochemistry* 32:221–229. <https://doi.org/10.1007/s10695-006-9003-3>

631 36. Subbotkin MF, Subbotkina TA (2018) Effects of Environment and Physiological State of an
632 Organism on the Activity and Content of Lysozyme in Fishes of the Family Cyprinidae: A
633 Review. *Inland Water Biology* 11:184–194. <https://doi.org/10.1134/S1995082918020037>

634 37. Soreq H, Seidman S (2001) Acetylcholinesterase – new roles for an old actor. *Nature Reviews*
635 *Neuroscience* 2:294–302. <https://doi.org/10.1038/35067583>

636 38. Keyt BA, Baliga R, Sinclair AM et al (2020) Structure, Function, and Therapeutic Use of IgM
637 Antibodies. *Antibodies* 9:53. <https://doi.org/10.3390/antib9040053>

638