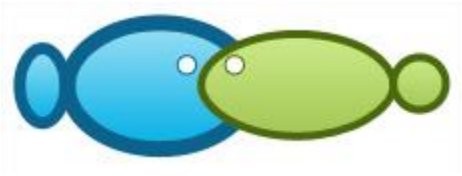

Enteric-coated oral inactivated *Vibrio alginolyticus* vaccine enhances antibody response and protection in *Sciaenops ocellatus* (Linnaeus, 1766)

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Abstract. *Vibrio alginolyticus* is an important bacterial pathogen causing hemorrhagic disease and significant mortality in marine fish, including red drum, *Sciaenops ocellatus* (Linnaeus, 1766). This study evaluated the efficacy of an enteric-coated oral inactivated *V. alginolyticus* vaccine and optimized vaccination strategies to improve protection against vibriosis. A virulent strain (VA15) was selected for challenge experiments, with a median lethal dose (LD_{50}) of 2×10^4 CFU $fish^{-1}$. Juvenile red drum (14-16 g) were orally immunized with vaccine doses ranging from 10^5 to 10^7 CFU equivalent $fish^{-1}$ to determine the optimal antigen concentration. Antibody titres increased significantly following vaccination and reached peak levels at 21 days post-immunization. As no significant difference was detected between the 10^6 and 10^7 CFU equivalent $fish^{-1}$ groups, the 10^6 CFU equivalent $fish^{-1}$ dose was selected for subsequent trials because it provided comparable immunogenicity while requiring less antigen. The protective efficacy of unencapsulated and enteric-coated vaccine formulations was subsequently evaluated using different booster schedules. Fish receiving the unencapsulated vaccine achieved a relative percent survival (RPS) of 44%, whereas enteric coating increased RPS to 54% following a single vaccination. Booster immunization further enhanced protection, with RPS values increasing to 61% and 78% when boosters were administered on days 14 and 21, respectively. These findings demonstrate that enteric coating improves oral vaccine delivery and that a booster vaccination administered 21 days after primary immunization provides the highest level of protection against *V. alginolyticus* infection. Although the study was conducted under laboratory conditions using a single bacterial strain, the results highlight the potential of enteric-coated oral vaccines as a practical disease management strategy for marine aquaculture.
Keywords: antibody titre, booster vaccination, fish immunization, marine aquaculture, vibriosis.

Introduction. Aquaculture is a key economic sector that provides livelihoods for millions of people and contributes significantly to national economic development. Among marine cultured species, red drum, *Sciaenops ocellatus* (Linnaeus, 1766) is a high-value fish characterized by rapid growth and strong environmental adaptability, and has been prioritized for aquaculture development in many coastal regions, particularly in Hue city, Vietnam. This species has also been identified as a target for selective breeding and industrial-scale farming under the national strategy for marine aquaculture development toward 2030, with a vision to 2045 (Prime Minister of Vietnam, 2021).

Despite its economic importance, intensive red drum farming is increasingly threatened by infectious diseases. Among bacterial pathogens, *Vibrio alginolyticus* is one of the most important etiological agents associated with hemorrhagic disease in red drum culture. Field outbreaks in cage-cultured red drum have been reported to cause cumulative mortality of up to 60% under farming conditions in Vietnam (Linh et al., 2022). Disease management has traditionally relied on antibiotic treatment; however, the effectiveness of antibiotics is often limited when infected fish exhibit reduced feed intake. Moreover, excessive antibiotic use contributes to antimicrobial resistance, drug residues in aquatic

products, and environmental contamination (Ayukekbong et al., 2017; Reverter et al., 2020).

Vaccination is widely regarded as a sustainable alternative for disease prevention because it provides specific protection, stimulates active immunity, and reduces dependence on antibiotics (Sommerset et al., 2005). Several studies have also demonstrated the effectiveness of vaccination in controlling bacterial diseases in aquaculture (Ma et al., 2019; Miccoli et al., 2021).

Although injectable vaccines generally provide strong protection, their application is labor-intensive, stressful to fish, and impractical for large-scale aquaculture operations. Consequently, oral vaccination has attracted considerable attention because it is easy to administer, minimizes handling stress, and is suitable for mass immunization under commercial farming conditions (Halimi et al., 2018; Kitiyodom et al., 2019; Bashir et al., 2023).

Recent advances in vaccine delivery systems have improved the potential of oral vaccination. The micropolymer Eudragit E100 can protect antigens from degradation and facilitate antigen release under acidic conditions in the stomach, thereby enhancing antigen stability and immune responses. Using this approach, Bashir et al., (2023) reported that an oral inactivated *Streptococcus agalactiae* vaccine encapsulated with Eudragit E100 achieved a relative percent survival (RPS) of up to 75% in tilapia. More recent studies have further highlighted the growing potential of encapsulation technologies for improving oral vaccine performance in aquaculture (Radhakrishnan et al., 2023; Nazari & Jeddi-Tehrani, 2026).

However, vaccine efficacy depends strongly on antigenic compatibility between vaccine strains and circulating pathogens. Therefore, developing an inactivated vaccine based on a locally isolated *V. alginolyticus* strain and incorporating Eudragit E100 encapsulation may provide a practical and effective solution for disease prevention in red drum culture. The present study evaluated the immunogenicity and protective efficacy of an enteric-coated oral inactivated *V. alginolyticus* vaccine and investigated the effects of different booster vaccination schedules.

Material and Method

Experimental fish and husbandry. A total 1150 of juvenile *S. ocellatus* with a total length of 10-12 cm and an average body weight of 14-16 g were obtained from a commercial hatchery in Hue City, Vietnam. Prior to the experiment, fish were certified free of *Vibrio* spp. by the Hue veterinary authority.

Fish were acclimated for 14 days in 2 m^3 composite tanks supplied with aerated seawater at a salinity of 20‰. During the acclimation period, fish were fed a commercial diet (NRD 2/3, INVE, Thailand) twice daily (08:00 and 15:00) at 2.5% of body weight.

To verify the absence of *Vibrio* infection, five fish were randomly sampled at the end of the acclimation period. Kidney tissues were aseptically collected, streaked onto thiosulfate citrate bile salts sucrose (TCBS, Merck, Germany) agar, and incubated at 28°C for 24 h. No presumptive *Vibrio* colonies were detected.

Bacterial strain and preparation. Ten virulent *V. alginolyticus* strains carrying the *flaA* virulence gene were cultured in tryptic soy broth (TSB, Merck, Germany) supplemented with 2% NaCl at 28°C for 24 h with shaking at 150 rpm. Bacterial density was estimated spectrophotometrically at 600 nm (U2900, Hitachi, Japan) and adjusted to an optical density of OD_{600} = 1.0, corresponding to approximately 1×10^9 CFU mL^{-1}.

Selection of virulent strains and LD_{50} determination. Ten virulent *V. alginolyticus* isolates carrying the *flaA* gene were screened for pathogenicity in *S. ocellatus*. Ten fish were intraperitoneally injected with 0.1 mL of bacterial suspension containing 1×10^9 CFU mL^{-1}, while control fish received 0.1 mL sterile saline solution. Mortality was monitored four times daily for three days. Among the tested isolates, strain VA15 caused 100% mortality within three days and was therefore selected for vaccine production and challenge experiments.

The median lethal dose (LD_{50}) of strain VA15 was determined using serial bacterial dilutions ranging from 10^3 to 10^8 CFU mL^{-1}. Fish were injected intramuscularly with 0.1 mL of bacterial suspension at the base of the pectoral fin. Each treatment consisted of three replicate tanks containing 20 fish per tank. Mortality and clinical signs were recorded daily for 14 days. Dead and moribund fish were subjected to bacterial re-isolation from the liver, kidney, and spleen. The LD_{50} value was calculated according to the method of Reed & Muench (1938).

Preparation of formalin-inactivated vaccine. The selected *V. alginolyticus* strain VA15 was cultured as described above. Bacterial suspensions were inactivated by adding formalin (37%) to a final concentration of 2% and incubating at 28°C for 24 h. Residual formalin was removed by washing the bacterial pellet with phosphate-buffered saline (PBS).

Complete inactivation was confirmed by plating treated suspensions onto tryptic soy agar (TSA) supplemented with 2% NaCl and incubating at 28°C for seven days. The treatment condition showing no bacterial regrowth was used for vaccine preparation.

Encapsulation with Eudragit E100. The inactivated bacterial suspension was mixed with Eudragit E100 polymer solution and subjected to ultrasonication for 1 h according to the method described by Bashir et al., (2023).

Immunization and antibody response. To determine the optimal vaccine dose, fish were randomly assigned to four treatment groups and orally immunized with feed containing 10^5, 10^6, or 10^7 CFU equivalent $fish^{-1}$ of formalin-inactivated *V. alginolyticus* vaccine (NT1, NT2, and NT3, respectively). A non-vaccinated group served as the control.

Each treatment consisted of three replicate tanks containing 20 fish per tank. Vaccine-coated feed was prepared by surface coating and stored at 4°C until use. Fish received a single oral vaccination on day 0, with the vaccine-coated feed formulated to provide the designated antigen dose (10^5, 10^6, or 10^7 CFU equivalent $fish^{-1}$) in a single feeding.

Blood samples were collected from three fish per treatment (one fish randomly sampled from each replicate tank) on days 1, 7, 14, 21, and 28 post-immunization. Serum was separated and stored at -20°C until analysis.

Antibody titres were determined using a microagglutination assay following Roberson (1990). Briefly, 50 µL of two-fold serially diluted serum was mixed with 50 µL of formalin-killed *V. alginolyticus* antigen suspension in U-bottom 96-well microplates. The plates were incubated at room temperature (25-28°C) for 2 h and examined visually for agglutination. The antibody titre was defined as the reciprocal of the highest serum dilution showing visible agglutination and was expressed as log_2 values.

Booster immunization and challenge test. Based on the antibody response experiment, the vaccine dose of 10^6 CFU equivalent $fish^{-1}$ was selected for subsequent vaccination trials.

Fish were randomly allocated to five experimental groups: NT1, single oral vaccination with unencapsulated vaccine; NT2, single oral vaccination with encapsulated vaccine; NT3, encapsulated vaccine with a booster administered on day 14; NT4, encapsulated vaccine with a booster administered on day 21; and NT5, non-vaccinated control. Each treatment consisted of three replicate tanks containing 20 fish per tank. Fish were orally administered a single primary vaccination dose of 10^6 CFU equivalent $fish^{-1}$ on day 0. Booster vaccinations were subsequently administered on day 14 (NT3) or day 21 (NT4) according to the experimental design.

To evaluate vaccine efficacy, fish were challenged on day 29 by intramuscular injection with strain VA15 at the previously determined LD_{50} dose. Mortality and clinical signs were recorded daily for 14 days post-challenge. Vaccine efficacy was assessed as RPS according to Ellis (1999).

Statistical analysis. Data are presented as mean ± SD. Differences among treatments were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test in SPSS version 20.0. Survival data were analyzed using Kaplan-Meier survival curves and compared using the log-rank test. Differences were considered statistically significant at $p < 0.05$.

Results

Virulence of Vibrio alginolyticus strain VA15 and LD_{50} determination. The virulence of *V. alginolyticus* strain VA15 was evaluated in *S. ocellatus* by intramuscular challenge with bacterial concentrations ranging from 10^3 to 10^8 CFU mL^{-1} (Figure 1). No mortality was observed in fish challenged with 10^3 CFU mL^{-1}. Mortality increased progressively with increasing bacterial concentration, reaching 30%, 50%, 70%, 90%, and 100% in fish challenged with 10^4, 10^5, 10^6, 10^7, and 10^8 CFU mL^{-1}, respectively. Fish receiving the highest bacterial dose exhibited rapid disease progression and complete mortality within 7 days post-challenge.

Based on the method described by Reed and Muench (1938), the LD_{50} of strain VA15 was estimated at approximately 2×10^4 CFU $fish^{-1}$. These results confirmed the high virulence of strain VA15 and justified its selection for vaccine efficacy evaluation.

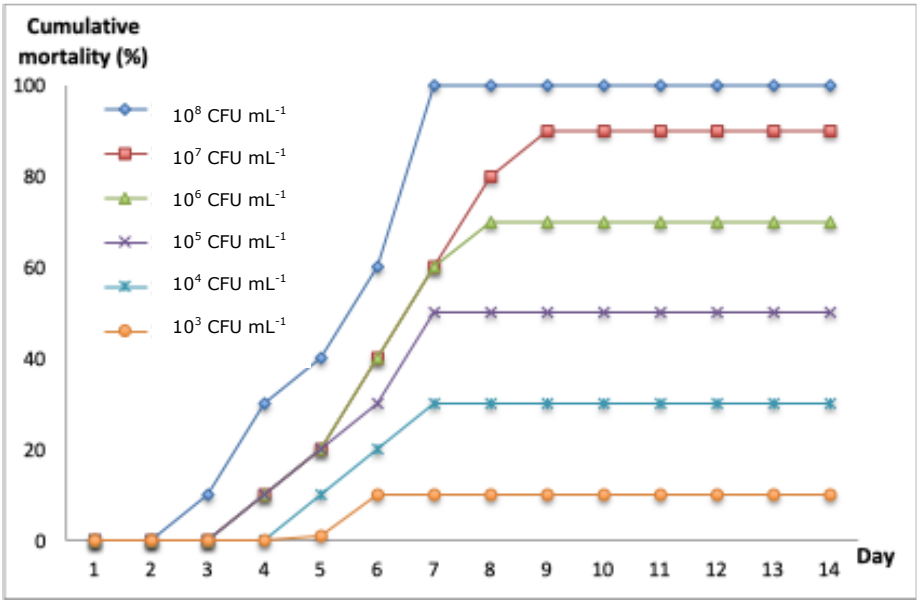

Figure 1. Cumulative mortality of *Sciaenops ocellatus* following intramuscular challenge with *Vibrio alginolyticus* strain VA15 at different bacterial concentrations (10^3-10^8 CFU mL^{-1}).

Antibody response. Oral vaccination induced a marked increase in antibody titres compared with the non-vaccinated control (Table 1). Antibody levels increased from day 7 post-immunization and generally reached their highest values on day 21. Fish vaccinated with 10^6 CFU equivalent $fish^{-1}$ (NT2) and 10^7 CFU equivalent $fish^{-1}$ (NT3) exhibited stronger antibody responses than those receiving 10^5 CFU equivalent $fish^{-1}$ (NT1). Although antibody titres peaked at day 21 in both NT2 and NT3, the response in NT2 remained stable through day 28, whereas a slight decline was observed in NT3. In contrast, NT1 showed a lower but gradual increase throughout the experimental period.

No significant differences were detected between NT2 and NT3 at later sampling points ($p > 0.05$). Therefore, the vaccine dose of 10^6 CFU equivalent $fish^{-1}$ was selected for subsequent experiments because it achieved comparable immunogenicity while requiring less antigen. The sustained antibody level observed in NT2 at day 28 also suggests that increasing the vaccine dose beyond 10^6 CFU equivalent $fish^{-1}$ did not provide additional immunological benefits.

Table 1
Antibody titres (log_2) in *Sciaenops ocellatus* following oral vaccination at different sampling times

Treatment	*Day 0*	*Day 7*	*Day 14*	*Day 21*	*Day 28*
NT1	6.3±0.3	7.3±0.7	6.7±0,3	7.0±0.7	7.3±0.3
NT2	6.0±0.3	7.7±0.7	8.7±0.7	9.7±0.7	9.7±0.3
NT3	5.7±0.7	7.3±0.3	8.3±0.3	9.7±0.3	9.3±0.3
Control	6.3±0.3	5.7±0.7	6.3±0.3	6.0±0.3	5.7±0.7

Note: NT1 = fish vaccinated with 10^5 CFU equivalent $fish^{-1}$; NT2 = fish vaccinated with 10^6 CFU equivalent $fish^{-1}$; NT3 = fish vaccinated with 10^7 CFU equivalent $fish^{-1}$; Control = non-vaccinated fish.

Vaccine efficacy. The protective efficacy of the oral vaccine was assessed using RPS following challenge with *V. alginolyticus* strain VA15 (Figure 2). All vaccinated groups showed higher survival than the non-vaccinated control group.

Encapsulation improved vaccine performance, increasing RPS from 44% in fish receiving the unencapsulated vaccine (NT1) to 54% in fish receiving a single dose of the encapsulated vaccine (NT2). Booster vaccination further enhanced protection. Fish receiving a booster on day 14 (NT3) achieved an RPS of 61%, whereas the highest protection was observed in fish receiving a booster on day 21 (NT4), with an RPS of 79%.

These results demonstrate that both enteric coating and booster immunization contributed to improved protection against *V. alginolyticus* infection. Among the vaccination strategies evaluated, administration of a booster dose on day 21 provided the greatest protective efficacy.

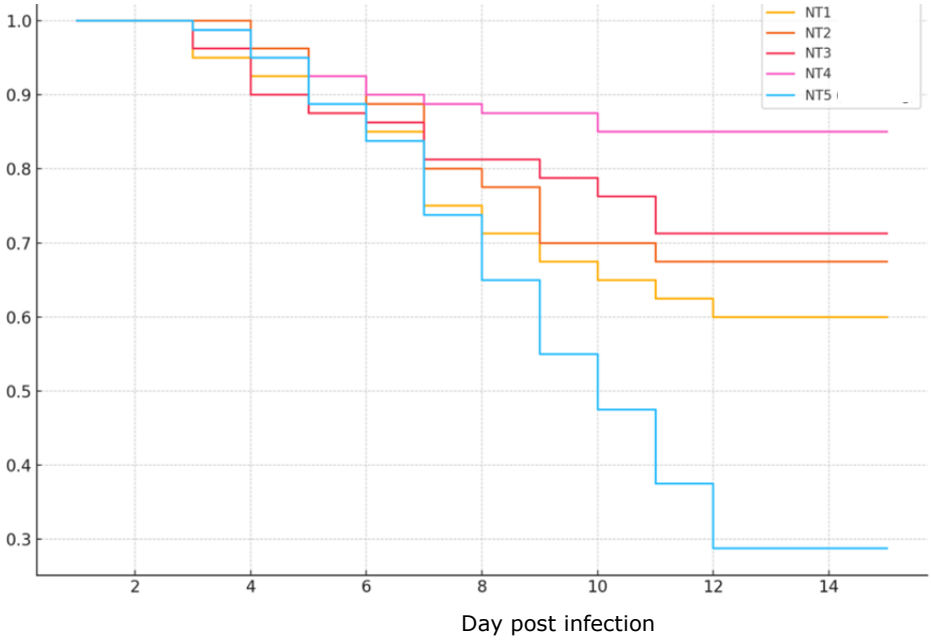

Figure 2. Survival of *Sciaenops ocellatus* following oral vaccination and experimental challenge with *Vibrio alginolyticus* strain VA15. Survival curves of *Sciaenops ocellatus* were analyzed using the Kaplan-Meier method, and differences among treatments were compared using the log-rank test.

Discussion

Virulence of Vibrio alginolyticus strain VA15. The present study demonstrated that *V. alginolyticus* strain VA15 was highly pathogenic to *S. ocellatus*, with an LD_{50} of 2×10^4 CFU $fish^{-1}$. This level of virulence is comparable to that reported for pathogenic *V. alginolyticus* isolates infecting marine fish under experimental conditions (Liang et al., 2011). The relatively low LD_{50} indicates a strong capacity of the strain to establish infection and cause disease in *S. ocellatus*. Therefore, VA15 provided a robust and reproducible challenge model for evaluating vaccine efficacy.

The use of a highly virulent challenge strain is important for vaccine studies because it allows protective effects to be evaluated under stringent conditions. In addition, the successful re-isolation of *V. alginolyticus* from challenged fish confirmed that mortality was directly associated with bacterial infection, supporting the validity of the challenge model.

Antibody response following oral vaccination. Oral vaccination stimulated a clear humoral immune response in *S. ocellatus*, as reflected by the progressive increase in antibody titres following immunization. Peak antibody levels were observed approximately three weeks after vaccination, which agrees with previous reports describing the kinetics of adaptive immune responses in teleost fish (Sommerset et al., 2005; Brudeseth et al., 2013). An important finding of the present study was that the 10^6 CFU equivalent $fish^{-1}$ dose induced an antibody response comparable to that produced by the higher 10^7 CFU equivalent $fish^{-1}$ dose. This suggests that increasing antigen concentration beyond a certain threshold does not necessarily enhance immune stimulation. Similar observations have been reported in vaccine studies involving fish bacterial pathogens, where excessive antigen loading provided little additional immunological benefit (Ma et al., 2019; Miccoli et al., 2021).

Interestingly, antibody titres remained stable in fish receiving 10^6 CFU equivalent $fish^{-1}$, whereas a slight decline was observed in the 10^7 CFU equivalent $fish^{-1}$ group at the final sampling point. Although the difference was not statistically significant, this trend indicates that higher antigen doses may not improve the persistence of the humoral response. Consequently, the lower dose was selected for subsequent experiments because it provided comparable immunogenicity while reducing vaccine production requirements.

Vaccine efficacy and the role of encapsulation. Vaccinated fish exhibited higher survival than non-vaccinated controls following challenge with *V. alginolyticus*, demonstrating that oral administration of the inactivated vaccine induced protective immunity. Protection was further enhanced when the vaccine was encapsulated with Eudragit E100. The improved efficacy of the encapsulated formulation is likely related to protection of the antigen from degradation during passage through the digestive tract, allowing greater antigen availability for uptake by mucosal immune tissues.

These findings are consistent with previous studies demonstrating that polymer-based delivery systems improve oral vaccine performance in fish (Halimi et al., 2018; Kitiyodom et al., 2019; Bashir et al., 2023). Similar benefits of encapsulation have been reported for oral *Streptococcus agalactiae* vaccines in tilapia, where enhanced antigen stability resulted in stronger immune responses and increased protection after bacterial challenge (Bashir et al., 2023).

Booster vaccination also contributed substantially to vaccine efficacy. Fish receiving a booster on day 21 achieved the highest RPS, exceeding that observed in fish boosted on day 14. This result suggests that a longer interval between primary and secondary immunization may allow more effective maturation of adaptive immune responses before re-exposure to the antigen. Comparable effects of booster vaccination have been described in several fish vaccine studies, where secondary antigen exposure promoted stronger and more durable protection (Brudeseth et al., 2013; Miccoli et al., 2021).

Practical implications for aquaculture. The combination of oral delivery, enteric coating, and booster vaccination represents a practical approach for controlling vibriosis in *S. ocellatus*, culture. Compared with injectable vaccines, oral vaccination is easier to administer, reduces handling stress, and is more suitable for large-scale farming operations. The use of an encapsulated oral vaccine may therefore provide an effective strategy for improving fish health while reducing reliance on antibiotics.

Given increasing concerns regarding antimicrobial resistance in aquaculture, vaccination-based disease prevention is becoming increasingly important (Ayukekbong et al., 2017; Reverter et al., 2020). Although the present study was conducted under controlled laboratory conditions using a single challenge strain, the results provide a strong foundation for future field evaluations and broader assessments of vaccine performance against diverse *V. alginolyticus* isolates.

Conclusions. The present study demonstrated that an oral formalin-inactivated vaccine against *V. alginolyticus* successfully induced a humoral immune response and enhanced protection against experimental vibriosis in juvenile *S. ocellatus*. Among the antigen doses evaluated, 10^6 CFU equivalent $fish^{-1}$ provided an antibody response comparable to the higher dose while reducing antigen requirements, making it the most suitable formulation for oral immunization. Encapsulation with Eudragit E100 further improved vaccine efficacy, and administration of a booster dose 21 days after primary vaccination produced the highest level of protection following bacterial challenge. Overall, these findings indicate that combining oral delivery, Eudragit E100 encapsulation, and an appropriate booster schedule can substantially enhance the protective efficacy of an inactivated vaccine against *V. alginolyticus***.** Although the study was conducted under controlled laboratory conditions using a single challenge strain, the results provide a strong foundation for future field validation and support the development of practical oral vaccination strategies for the prevention of vibriosis and the reduction of antibiotic use in marine aquaculture.

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Authors Contributions. Conceptualization: PNN; Methodology: ADQN, PNN; Formal analysis: ADQN, PNN, HTXN, QNN, LTHN, NNT; Investigation: ADQN, HTXN, QNN, LTHN, NNT; Data curation: NNT, LTHN; Writing - original draft: ADQN, PNN; Writing - review & editing: ADQN, HTXN, LTHN, PNN; Supervision: PNN.

Conflict of Interest. The authors declare that there is no conflict of interest.

Data Availability. The datasets generated and analyzed during the current study are stored by the corresponding author and are available upon reasonable request.

Ethical Approval. All experimental procedures involving animals were conducted in accordance with the institutional guidelines for the care and use of laboratory animals of Hue University. The study was approved by the Hue University Animal Ethics Committee under certificate number HUVN0054 (issued on December 10, 2024).

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