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Development of ZnO-Chitosan-Based Nanovaccine with *Chlorella vulgaris* Recombinant Protein against VNN in Hybrid Grouper (*Epinephelus fuscoguttatus* × *lanceolatus*)

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Abstract

Viral Nervous Necrosis (VNN), caused by *Nervous Necrosis Virus* (NNV), is a major constraint in hybrid grouper (*Epinephelus spp*.) aquaculture, with larval and juvenile mortality reaching nearly 100%. Effective prophylactic strategies are urgently required to ensure sustainable production. This study aimed to test the hypothesis that recombinant *Chlorella vulgaris* protein, delivered using ZnO-chitosan nanoparticles, enhances immune response, improves growth, and mitigates VNN-induced tissue damage in hybrid grouper. An *in vivo* challenge experiment was conducted with juvenile fish assigned to 5 groups: Negative control (K−), positive control (K+), and 3 nanovaccine dosages (P1 = 33 µL, P2 = 66 µL and P3 = 112 µL). Vaccination was administered twice via oral sonde, with a 14-day interval between the primary and booster doses prior to viral challenge. The 66 µL dose (P2) yielded the most consistent protective effects, including significant improvements in body length and weight ($p < 0.05$), as well as elevated antioxidant enzyme activities (SOD = 3.997 U/mL; CAT = 109.7 U/mL), indicating enhanced oxidative defense. Histological analyses further confirmed reduced tissue damage in vaccinated groups, with P2 exhibiting attenuated vacuolization in the gills. Interestingly, although P2 was superior in growth and antioxidant responses, the 33 µL dose (P1) demonstrated the lowest tissue damage, suggesting a more favorable safety profile. These findings highlight a dose-dependent trade-off, where P2 maximizes immune and growth benefits, while P1 minimizes histopathological alterations. While ZnO-chitosan nanoparticles are generally considered safe at sub-toxic concentrations reported in fish toxicology studies (< 100 mg/L waterborne exposure), further ecotoxicological and bioaccumulation assessments are required to substantiate their long-term environmental safety. Within the scope of this study, the 33 µL dosage represents the most balanced option between efficacy and safety, supporting its potential as a nanovaccine platform for VNN control in hybrid grouper aquaculture.

Keywords: *Chlorella vulgaris*, Hybrid grouper, Immunostimulant, Nanovaccine, ZnO/chitosan

Introduction

Aquaculture in Indonesia represents a critical and rapidly growing economic sector, driven by increasing demand in both domestic and international markets. According to data from the Ministry of Marine Affairs and Fisheries (MMAF), aquaculture production in Indonesia reached 16,967,518 tons in 2023, reflecting an 11.54% increase compared to the previous year, with substantial contributions from various marine and

freshwater fish commodities [1]. One of the most economically valuable species in marine aquaculture is the hybrid grouper (*Epinephelus fuscoguttatus* × *Epinephelus lanceolatus*), a cross between tiger grouper and giant grouper [2]. This species is favored for its rapid growth rate, high tolerance to environmental fluctuations, and palatable flesh preferred by export markets, particularly in Asia. In 2023, grouper production in Indonesia reached approximately 11.3 thousand tons, with an estimated economic value exceeding 1.2 trillion rupiah [3]. Given this potential, hybrid grouper is considered a priority commodity for strengthening national food security and economic resilience [4,5]. However, the development of hybrid grouper farming faces serious challenges due to viral disease outbreaks, most notably *Viral Nervous Necrosis* (VNN), caused by the *Nervous Necrosis Virus* (NNV). This virus attacks the central nervous system and retina, especially in larval and juvenile stages, with mortality rates reaching up to 100% [6,7]. Conventional vaccines have shown limited effectiveness, primarily due to issues with antigen stability and delivery efficiency. Consequently, nanotechnology-based approaches are being explored to enhance vaccine efficacy and improve disease resistance in cultured fish.

In aquaculture, various nanoparticles have been developed, including silver nanoparticles (Ag-NPs), known for their broad-spectrum antimicrobial activity and applications in disease prevention. Several previous studies have demonstrated that Ag-NPs can inhibit bacterial and viral growth in fish and enhance physiological parameters such as leukocyte counts and antioxidant enzyme activity [8,9]. While effective, Ag-NPs have also been reported to pose toxicity risks, especially to gut microbiota, liver function, and kidney tissues, particularly under high-dose or long-term exposure scenarios [10,11]. Therefore, their application must be carefully regulated and reassessed from a toxicological perspective. As a safer and more environmentally friendly alternative, zinc oxide nanoparticles (ZnO-NPs) have emerged as promising candidates [5]. Zinc oxide nanoparticles (ZnO-NPs) are widely used in aquaculture, not only to control pathogens in ponds but also as dietary supplements to promote growth and boost immunity in farmed species [12,13]. Zinc (Zn), an essential trace element, plays a key role in immune function by supporting white blood cell production and activating antioxidant enzymes that help reduce oxidative stress [14,15]. ZnO is generally considered safe for the environment and is commonly used in pharmaceutical products [16]. ZnO-NPs possess both antimicrobial and immune-boosting properties. They can enhance the body's natural antioxidant defenses by stimulating enzymes such as superoxide dismutase (SOD) and catalase (CAT) [14]. However, high concentrations of these nanoparticles can be harmful, potentially causing blood-related issues and tissue damage especially in sensitive organs like the gills, which are vital for respiration and act as the first line of defense against pathogens [11,17,18]. Sublethal effects such as oxidative tissue damage have been observed in various aquatic species, including common carp (*Cyprinus carpio*) and zebrafish (*Danio rerio*), at concentrations ranging from 0.5 - 10 mg/L [19]. Reported LC_{50} values for ZnO nanoparticles in these fish species under acute exposure conditions generally fall between 1 - 5 mg/L, indicating that the current dosing presents a considerable margin of safety [20,21]. The dose applied in this study is positioned approximately 10^3 to 10^4 times lower than concentrations associated with observable toxicity, suggesting that it remains within a biologically safe range while maximizing the potential immunostimulatory benefits of the formulation [22]. Furthermore, existing literature supports the premise that lower concentrations of ZnO nanoparticles can elicit immunological responses without incurring significant cytotoxicity. Studies have shown that exposure to zinc oxide nanoparticles at sublethal doses can stimulate immune responses in various aquatic organisms, demonstrating favorable outcomes without compromising health [23,24]. Such observations reinforce the conclusion that the ZnO dosage employed in the current study is prudent, optimizing the beneficial aspects of the nanovaccine while mitigating risks associated with toxicity [25].

To reduce these risks, researchers have developed hybrid ZnO/chitosan nanoparticles. Chitosan is a natural, biodegradable polymer known for its compatibility with biological systems. It helps stabilize the nanoparticles and improves their ability to deliver antigens effectively [26]. In addition to using nanoparticle-based adjuvants, recombinant proteins from *Chlorella vulgaris* are also being explored as

promising antigens for vaccine development. This microalga is rich in proteins and bioactive pigments that have shown antiviral and immune-enhancing properties [27]. Exposure to *C. vulgaris* derived proteins has been shown to enhance lymphocyte proliferation and activate interferon related antiviral signaling, as well as increase specific antibody titers in species such as tilapia and carp [28,29]. When encapsulated within nanoparticle systems, these proteins exhibit improved bioavailability and antigen presentation, which significantly enhances the activation of both humoral and cellular immune responses [30]. Furthermore, the incorporation of chitosan in the nanoparticle formulation can facilitate the sustained release and delivery of these antigens, further underpinning the potential of *C. vulgaris* recombinant proteins as effective immunogens when combined with nanoparticle adjuvants for aquaculture vaccine applications [30,31]. When these proteins are incorporated into nanoparticles, they can be absorbed more efficiently and stimulate a stronger immune response [32,33]. This study aims to assess the effectiveness and safety of a nanovaccine that combines *C. vulgaris* recombinant protein with ZnO/chitosan nanoparticles as an adjuvant, tested in hybrid grouper exposed to viral nervous necrosis (VNN). The underlying hypothesis is that the combination of these elements will produce synergistic effects at both pharmacokinetic and immunological levels. Chitosan, due to its cationic nature and mucoadhesive properties, significantly enhances antigen uptake through mucosal surfaces while ensuring efficient delivery to antigen-presenting cells. It has been observed that chitosan can enhance endosomal escape, thus increasing the likelihood of recombinant proteins being effectively processed and presented by major histocompatibility complex (MHC) molecules to T lymphocytes, culminating in stronger adaptive immune responses [34,35]. Meanwhile, ZnO nanoparticles not only provide structural stability to the antigen but also release Zn^{2+} ions, which are important in the activation of immune-related enzymes such as superoxide dismutase (SOD) and in regulating transcription factors involved in cytokine signaling [36,37]. The release of Zn^{2+} may enhance antioxidant defenses and stimulate innate immune pathways, creating a dual mode of protection against viral threats encountered by the fish [37,38]. The evaluation in hybrid grouper focuses on growth performance, antioxidant enzyme activity (including SOD and catalase), and gill histology; this is particularly relevant because gills play a critical role in respiration and serve as key immune and physiological barriers [39]. Findings from this work are expected to provide mechanistic insights and practical evidence supporting the development of safe, effective, and sustainable nanotechnology-based vaccines for aquaculture.

Materials and methods

Ethical approval

The Research Ethics Committee of Universitas Brawijaya reviewed the experimental design and approved the study under protocol number 184-KEP-UB-2024.

Materials

Zinc oxide nanoparticles (ZnO-NPs) used in this study were purchased from Hongwu Materials, China, with an average particle size of 20 - 40 nm. Chitosan nanoparticles (50 nm) were obtained from CV. ChiMultiguna, Cirebon, Indonesia. Recombinant *Chlorella vulgaris* protein was produced via an *Escherichia coli* expression system and purified using affinity chromatography. Hybrid grouper (*Epinephelus fuscoguttatus*×*Epinephelus lanceolatus*), with an average weight of 15 ± 2 g and total length of 11 ± 1 cm, were sourced from a certified disease-free local hatchery in Situbondo, East Java, Indonesia.

Experimental design

The study was conducted using a completely randomized design (CRD) with 5 experimental groups: Negative control (K−, unvaccinated and unchallenged), positive control (K+, unvaccinated and challenged with VNN), and 3 vaccinated groups receiving different nanovaccine doses (P1 = 33 µL, P2 = 66 µL and P3 = 112 µL per fish). Each treatment was replicated 3 times, with 20 hybrid grouper (*Epinephelus fuscoguttatus*×*E. lanceolatus*) juveniles allocated per replicate, resulting in a total of 300 fish. Allocation of fish into tanks was performed randomly to minimize allocation bias, and tanks were maintained under uniform environmental conditions (temperature, salinity, aeration and feeding regime). The experimental timeline consisted of 3 main phases: (i) acclimatization (7 days), (ii) vaccination and booster administration (days 0 and 14), and (iii) viral

challenge followed by 14 days of post-challenge monitoring (days 28 - 56). All outcome assessments, including growth performance, survival, behavioral observations, antioxidant enzyme activity, and histopathological scoring, were performed in a blinded manner to ensure objectivity. This design enabled both within-group and between-group comparisons, thereby increasing the statistical power and reproducibility of the findings.

Acclimatization

Fish were acclimated for 7 days in fiberglass tanks filled with clean, aerated seawater at a temperature of 28 ± 1 °C and a salinity of 30 ppt. Commercial pellets were administered twice daily at 3% of body weight. Feeding was halted 24 h prior to vaccination [40].

Nanovaccine formulation and administration

The nanovaccine was formulated by incorporating recombinant *C. vulgaris* protein into a ZnO/chitosan nanoparticle suspension. The recombinant protein stock solution was prepared at a concentration of 2.436 µg/µL. The ZnO-NPs were included in the formulation at a final concentration of 0.005 ppm (5 µg/L). The nanoparticle suspension was prepared with differences ratio of ZnO:chitosan:protein adjusted according to the treatment dose. Three dosage levels were prepared: 33 µL (P1), 66 µL (P2), and 112 µL (P3) per fish was guided by preliminary toxicological studies indicating that the LC_{50} of ZnO-NPs in fish is approximately 0.005 ppm, with both doses being 10^3 - 10^4 times lower than reported sublethal levels. This ensured safety while maintaining a standardized antigen exposure of 8 µg recombinant protein per fish, corresponding to approximately 0.533 µg/g body weight for an average fish weight of 15 ± 2 g. This ensured consistency in protein exposure across treatments. Statistical analysis confirmed that weight variation among fish was not significant ($p > 0.05$), so dosing was standardized per fish rather than per gram of body weight. Vaccination was performed twice using an oral sonde, with a 14-day interval between the first and booster administration.

Virus challenge

Fourteen days after the second vaccination, all fish (except the K-group) were challenged with *Nervous Necrosis Virus* (VNN). The viral strain used was RGNNV, originally obtained from Humpback grouper in Situbondo Brackish Water Aquaculture Centers, Indonesia and tested positive for VNN using Polymerase Chain Reaction (PCR) analysis following standard diagnostic protocols. The VNN-positive fish tissues were subsequently preserved, homogenized, and finely minced to prepare challenge feed for hybrid grouper, following the method described by Yanuhar *et al.* [41]. This approach ensured the presence of infectious viral particles in the challenge material, enabling reliable simulation of natural oral exposure routes in experimental fish. The experiment consisted of 5 treatment groups: K−, K+, P1, P2, and P3, each with 3 replicates. For 14 days post-challenge, daily observations were conducted for mortality, swimming activity, and feeding behavior.

Growth measurement and gill sampling

Fish length and weight were measured on day 0 and day 56. Specific growth rate (SGR) was calculated using a standard formula. At the end of the experiment, gill tissues from 3 fish per treatment group were collected for enzymatic and histopathological analysis. Prior to sampling, fish were anesthetized using 100 ppm clove oil.

Antioxidant enzyme analysis (SOD and CAT)

Gill tissues were homogenized in phosphate buffer solution (pH 7.4) and centrifuged at 10,000 rpm for 10 min at 4 °C. The resulting supernatant was analyzed using a spectrophotometric assay kit (Elabscience, China) to determine Superoxide Dismutase (SOD) and Catalase (CAT) activities. Results were expressed in U/mg protein.

Gill histopathology

Gill tissues were fixed in 10% neutral buffered formalin (NBF) for 24 h at room temperature, followed by routine histological processing. Dehydration was carried out through a graded ethanol series (70%, 80%, 90%, 95% and absolute ethanol), each for 1 h at room temperature, and subsequently cleared in xylene for 2×30 min. Samples were then embedded in paraffin at 56 - 58 °C, and sectioned at 5 µm thickness using a rotary microtome (Leica RM2235, Germany). The sections were mounted on glass slides, deparaffinized, and stained with hematoxylin for 5 min and eosin for 2

min. Histological alterations were examined under a light microscope (Olympus BX51, Japan) at magnifications of 100× and 400×. Tissue changes, including epithelial lifting, edema, hyperplasia, lamellar fusion, and necrosis, were semi-quantitatively scored based on lesion severity (0 = none, 1 = mild, 2 = moderate and 3 = severe) following established criteria [42]

Data analysis

Data were analyzed using SPSS software version 25.0. One-way analysis of variance (ANOVA) was applied to assess significant differences among groups ($p < 0.05$), followed by the Honestly Significant Difference (HSD) post-hoc test to identify pairwise differences. Results are presented as mean ± standard deviation (SD).

Results and discussion

Growth performance of hybrid grouper

Body length and weight are important indicators used to assess how effective different treatments are in aquaculture. In this study, we evaluated the impact of a *Chlorella vulgaris* based nanovaccine formulated with a ZnO nanoparticle adjuvant on the growth performance of hybrid grouper over 2 distinct periods: From the day of vaccination to day 28 (post-vaccination), and from day 28 to day 56 following exposure to viral nervous necrosis (post-challenge). Statistical analysis showed that the nanovaccine had a significant positive effect on both body length and weight in both phases ($p < 0.05$). The growth trends are presented in **Figures 1** and **2**.

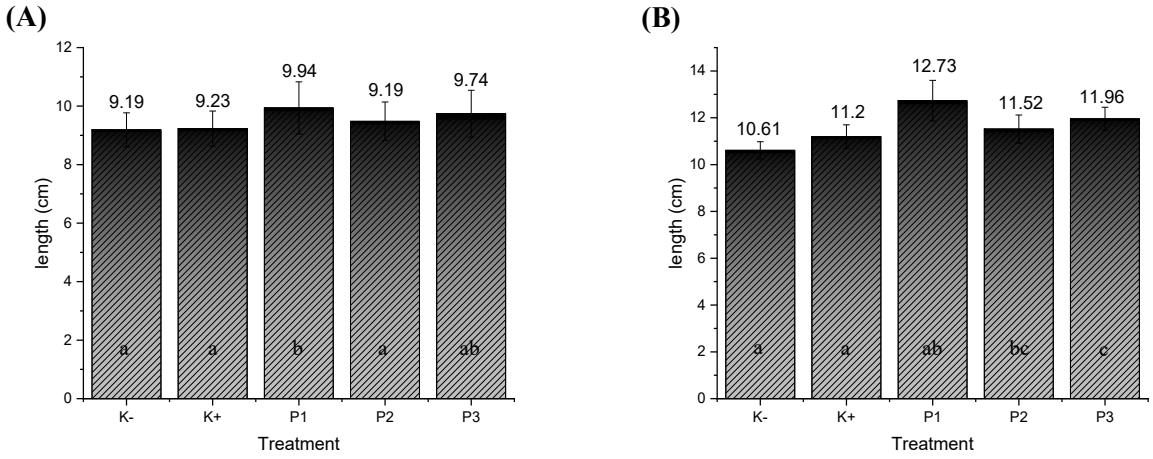

Figure 1 Body length growth of hybrid grouper ($p < 0.05$): A) Post-nanovaccination; B) Post-VNN challenge.

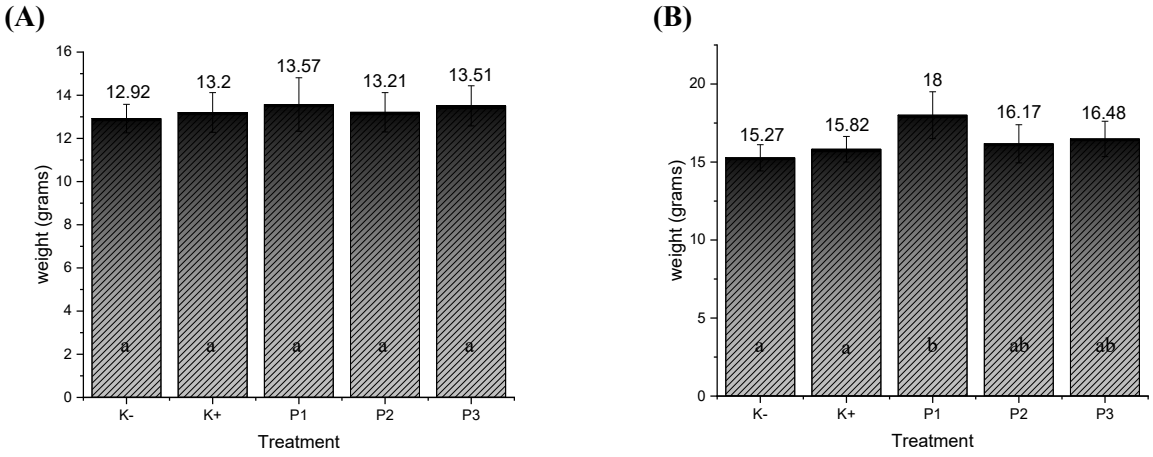

Figure 2 Body weight growth of hybrid grouper ($p < 0.05$): A) Post-nanovaccination; B) Post-VNN challenge.
Note: Different letters indicate significant differences among treatments (ANOVA, HSD test, $p < 0.05$).

Between days 0 and 28 (the post-vaccination phase), all vaccinated groups (P1, P2 and P3) showed higher growth rates in both body length and weight compared to the negative (K−) and positive (K+) control groups. Among them, the P1 group demonstrated the most significant improvement, with an average length increase of 2.79 cm (28.1%) and a weight gain of 4.43 g (32.6%). In comparison, the K+ group showed more modest growth, gaining just 1.97 cm in length (21.3%) and 2.62 g in weight (19.8%). Even after being challenged with VNN (days 28 to 56), the P1 group continued to outperform all others in terms of growth, suggesting that the nanovaccine formulation consisting of *Chlorella vulgaris* recombinant protein combined with ZnO-chitosan nanoparticles at a dose of 33 µL was effective not only in promoting early growth but also in supporting the fish's recovery and physiological stability post-infection.

These findings are consistent with previous studies. For instance, Tang *et al.* [15] found that ZnO nanoparticles enhance digestive enzyme activity and feed utilization, leading to better growth outcomes under stress. Likewise, Grasso *et al.* [43] highlighted the nutritional value of *C. vulgaris* proteins, which are rich in essential amino acids and bioactive compounds that aid tissue repair and accelerate growth [43]. Overall, the optimal vaccine dose used in the P1 group consistently delivered strong results in promoting growth and improving resilience against viral infection. The superior growth outcomes at P1 compared to P2 can be explained by an energy trade-off: Lower antigen/adjuvant stimulation (P1) allows more metabolic energy to be allocated for somatic growth, whereas higher stimulation (P2) redirects resources towards antioxidant defense and immune activation, resulting in enhanced SOD/CAT activity but relatively reduced growth performance. This trade-off is common in fish physiology during immune challenges. Detailed patterns of body length and weight changes are shown in **Figures 3** and **4**.

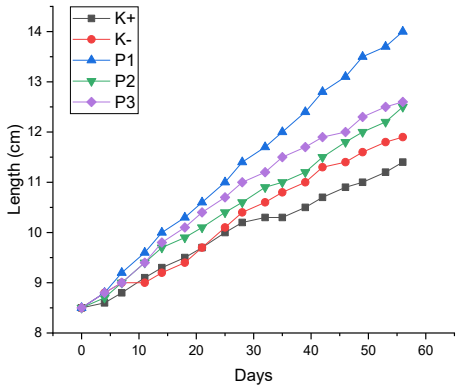

Figure 3 Growth pattern chart of hybrid grouper length.

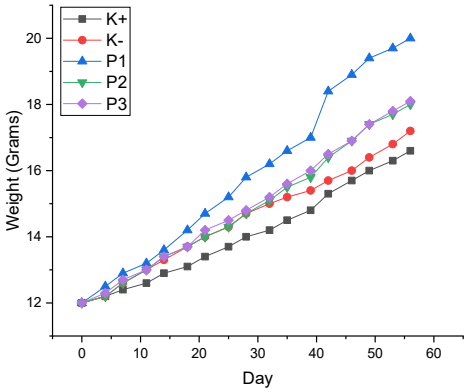

Figure 4 Growth pattern chart of hybrid grouper weight.

Fish growth patterns are often described usings the relationship between body length and weight, typically modeled by the allometric regression equation: $W = aL^b$. In this study, all treatment groups showed a negative allometric growth pattern ($b < 3$), meaning the fish grew more in length than in weight. This physiological phenomenon is often observed in fish recovering from stress or infection, where energy resources are prioritized for maintaining vital organ function, tissue repair, and essential metabolic processes [44]. The observation that vaccinated groups maintained stable length increments suggests that the nanovaccine formulation helped preserve growth capacity amidst viral challenges by mitigating oxidative stress and preventing severe tissue pathology, particularly in the gills [45]. These findings are significant in the context of aquaculture; despite a slower rate of weight gain, consistent length growth under disease pressure implies that vaccinated fish are more resilient, demonstrating sustained metabolic efficiency and the ability to retain marketable size trajectories, even in the face of viral infections [46]. Such results underscore the dual function of the vaccine in not only providing immunological protection but also supporting growth performance during stressful production conditions [47,48].

This type of growth is often seen during periods of physiological stress or recovery such as after a viral infection which aligns with observations reported by Ramírez-Coronel *et al.* [49]. Their research also noted that fish tend to prioritize length growth overweight gain when recovering from health challenges. Post-vaccination, immunological responses and physiological recovery processes may redirect energy allocation from somatic growth to the maintenance of vital functions and tissue repair. Ahmad *et al.* [50] also emphasized that the regression coefficient (b) is highly sensitive to environmental conditions, diet composition, and health status, suggesting that it reflects the physiological impact of external interventions. Although the growth pattern did not fully conform to isometric growth, the nanovaccine-treated groups maintained stable length development during the recovery phase, suggesting a protective role of the nanovaccine formulation against the long-term physiological consequences of VNN infection. As a nano-adjuvant agent, *Chlorella vulgaris* offers numerous health benefits. Agwa and Abu [51] reported significant improvements in growth and health parameters in various fish species following *C. vulgaris* supplementation. Similarly, Ramírez-Coronel *et al.* [49] demonstrated that dietary supplementation with *C. vulgaris* enhanced both growth performance and immune responses. Furthermore, Ahmad *et al*. [50] stated that the application of *Chlorella vulgaris* can help mitigate the effects of environmental stress and disease infections, making it a promising component in sustainable aquaculture strategies.

The observed enhancement in fish growth performance among nanovaccine-treated groups indicates that the vaccine functions not only as an immunostimulant but also supports nutrient metabolism and absorption. Zinc (Zn) is an essential micronutrient involved in protein synthesis, metabolic enzyme activity, and cell proliferation key processes for tissue formation and regeneration [52,53]. Köse *et al.* [54] found that dietary Zn supplementation improved fish growth, stress resilience, and pathogen resistance attributes crucial for intensive aquaculture operations. The recombinant protein derived from *C. vulgaris* contains essential amino acids and bioactive compounds that enhance digestive efficiency and tissue repair. Chen *et al.* [55] showed that diets enriched with Zn and other micronutrients promote immune responses and long-term health in fish. Ibrahim [56] also noted that nutritional composition, particularly amino acids and trace elements, significantly influences fish growth, especially in plant-based diets. Therefore, the Zn/*C. vulgaris*-based nanovaccine not only provides immune protection but also offers nutritional benefits that support fish growth and recovery. ZnO used in the nanovaccine formulation further enhances bioactivity by improving Zn absorption and demonstrating antibacterial properties, thereby reducing pathogen load and promoting overall health [13]. Studies by Kumar [57]; Rahimnejad *et al.* [58] highlighted that dietary ZnO supplementation enhances vaccine efficacy, immune system performance, and nutrient utilization efficiency. Thus, incorporating Zn and *Chlorella vulgaris* into nanovaccine formulations presents an innovative and effective approach to boosting fish

growth and disease resilience, especially under high-stress environmental conditions.

Antioxidant enzyme activity (SOD and CAT)

Oxidative stress is a physiological response that commonly arises during viral infections and, if unmanaged, can lead to cellular damage. Antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) play crucial roles in mitigating oxidative damage. SOD catalyzes the dismutation of superoxide radicals into hydrogen peroxide and oxygen, while CAT decomposes hydrogen peroxide into water and oxygen, thereby protecting tissues from oxidative injury [59]. In this study, nanovaccine administration significantly enhanced the activity of both SOD and CAT in gill tissues ($p < 0.05$). Average enzyme activity levels for each treatment group are presented in **Figures 5** and **6**.

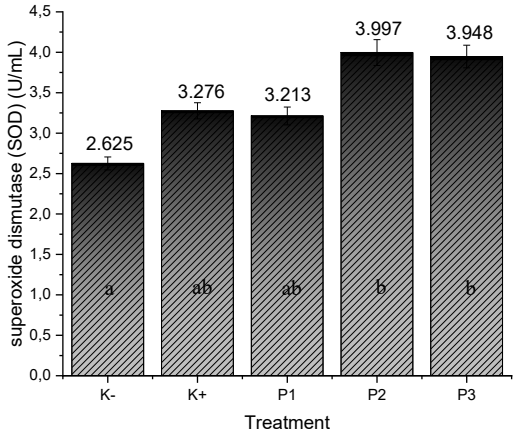

Figure 5 Mean SOD enzyme activity across treatment groups.

Note: Bars with different letters indicate significant differences among treatments based on ANOVA followed by BNJ test ($p < 0.05$). Identical letters denote no difference, while combined letters (e.g., ab) indicate overlapping groups that are not significantly different from either subset.

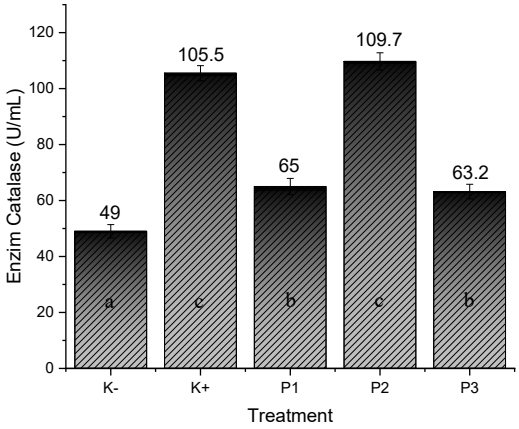

Figure 6 Mean CAT enzyme activity across treatment groups.

Note: Bars with different letters indicate significant differences among treatments based on ANOVA followed by BNJ test ($p < 0.05$). Identical letters denote no difference, while combined letters (e.g., ab) indicate overlapping groups that are not significantly different from either subset.

The negative control group (K−) exhibited the lowest SOD (2.625 ± 0.08 U/mL) and CAT (49.0 ± 2.4 U/mL) activities, consistent with normal physiological conditions without stress or infection. In contrast, the positive control group (K+), exposed to VNN without nanovaccine treatment, showed a marked increase in antioxidant enzyme activity (SOD: 3.276 ± 0.10 U/mL; CAT: 105.5 ± 2.7 U/mL), reflecting severe oxidative stress induced by viral infection. Among the nanovaccine-treated groups, SOD activity increased significantly compared to K−, but remained more regulated than in the K+ group. The P2 group (66 µL dose) recorded the highest SOD (3.997 ± 0.16 U/mL) and CAT (109.7 ± 3.1 U/mL) activities, indicating that this intermediate dose most effectively activated the antioxidant defense system under VNN-induced stress. Interestingly, although the P3 group showed high SOD activity (3.948 ± 0.14 U/mL), its CAT activity decreased substantially (63.2 ± 2.6 U/mL). This discrepancy suggests a potential enzymatic imbalance possibly linked to dose-related toxicity resulting from the higher concentrations of ZnO nanoparticles. Excess Zn^{2+} ions can generate reactive oxygen species (ROS) that may overwhelm the antioxidant defense system. Under such conditions, the elevated SOD activity could be interpreted as a compensatory response to the increased levels of superoxide radicals, while the decline in CAT activity may result from enzyme inactivation or substrate overload. This pattern is consistent with reports indicating that supra-optimal doses of ZnO can disrupt redox homeostasis and compromise physiological functions in aquatic species [60]. Meanwhile, the P1 group (33 µL dose) exhibited moderate SOD (3.213 ± 0.11 U/mL) and CAT (65.0 ± 2.9 U/mL) activity. This finding signifies an effective yet controlled response to oxidative stress, implying that lower nanoparticle doses may optimize the synergistic relationship between Zn^{2+} cofactor activity and bioactive compounds from Chlorella vulgaris, thereby circumventing adverse effects associated with excessive immune activation. From an immunological standpoint, the results indicate that while higher dosages may initially elicit stronger antioxidant responses, they also risk inciting oxidative dysregulation and metabolic strain that could ultimately compromise fish health [61]. Thus, the optimal efficacy of the nanovaccine appears to hinge on attaining a delicate balance between stimulating protective enzyme activity and preventing toxicity-related consequences [62].

The results demonstrate that administration of a nanovaccine based on *Chlorella vulgaris* recombinant protein with a ZnO-chitosan adjuvant significantly enhanced antioxidant enzyme activity, particularly in the P2 treatment group. Increased levels of SOD and CAT indicate an upregulated cellular defense mechanism against oxidative stress caused by VNN infection. The medium dose (P2) was found to be optimal in stimulating the immune system, as it synergistically enhanced the activities of both antioxidant enzymes. A linear correlation analysis between nanovaccine dose and enzyme activity revealed a strong relationship for SOD (R^2 = 0.773; **Figure 5**), while the correlation for CAT was minimal (R^2 = 0.001; **Figure 6**), suggesting that CAT activity may be influenced by other factors such as physiological condition, environmental variables, or infection severity. This variability implies that while the nanovaccine effectively enhances SOD activity, the CAT response may be modulated by more complex regulatory mechanisms.

The increased antioxidant activity observed in this study suggests that the nanovaccine helps strengthen the hybrid grouper's oxidative defense system an essential factor in combating the oxidative stress caused by VNN infection [63]. Zinc ions (Zn^{2+}) in the ZnO-chitosan formulation serve as important cofactors for antioxidant enzymes like superoxide dismutase (SOD), which play a vital role in protecting cells from oxidative damage [59]. Additionally, *Chlorella vulgaris* a high quality plant based protein source contains bioactive compounds such as chlorophyll and phycocyanin, both known for their strong antioxidant properties. These compounds help maintain redox balance and support overall cellular health [64]. The combined effects of Zn^{2+} and bioactive compounds from Chlorella vulgaris have been shown to enhance the antioxidant response in fish. This enhancement is thought to be facilitated by the ZnO-chitosan complex, which increases the activities of superoxide dismutase (SOD) and catalase (CAT) through 2 primary mechanisms. First, Zn^{2+} ions can act as cofactors for antioxidant enzymes, helping to scavenge reactive oxygen species (ROS) and maintain

redox balance. This mechanism is substantiated by studies that highlight Zn's role in regulating antioxidant systems via upregulation of antioxidant enzymes during oxidative stress [65,66]. Second, Zn^{2+} has been implicated in stimulating antioxidant gene expression pathways, particularly through its regulatory influence on the Nrf2 transcription factor, which plays a significant role in oxidative stress responses [67,68]. This not only reduces oxidative tissue damage but also supports faster recovery and improved growth after infection [63]. The ability of the nanovaccine to regulate antioxidant activity is a key mechanism in providing long-term protection against the physiological stress induced by viral infections. Overall, using nanotechnology-based vaccines in aquaculture represents a promising and innovative strategy not only for reducing oxidative stress but also for building stronger systemic immune defenses [57,69,70].

The increase in activities of key antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) is important; however, these increases alone do not fully capture the broader non-specific immune status in aquaculture species. It is critical to explore molecular immunological markers such as IL-1β, TNF-α, and IFN-γ, as their expression provides deeper insights into cytokine-mediated immune responses within aquatic organisms [71-73]. A comprehensive approach that incorporates these immunological parameters, alongside enzymatic data, will yield a holistic view of immune efficacy. The effectiveness of these nanotechnology-based vaccine formulations depends significantly on their ability to influence redox signaling pathways, which is essential for enhancing the activity of these crucial antioxidant enzymes [74,75]. Such modulation is vital for supporting fish under stressful conditions, particularly during viral challenges, thus reinforcing the need for integrated immune assessments in aquaculture research.

Gill histopathology

The gills are essential for respiration in fish and are particularly sensitive to environmental stressors, including viral infections like Nervous Necrosis Virus (VNN) and exposure to harmful substances. In this study, we examined the gill health of hybrid grouper (*Epinephelus fuscoguttatus*×*E. lanceolatus*) after vaccination with a nanovaccine containing *Chlorella vulgaris* recombinant protein and a ZnO-chitosan adjuvant. VNN infection is known to trigger inflammation and oxidative stress, which can lead to significant damage in gill tissues [59,76]. To evaluate this, histological analysis was performed using light microscopy, focusing on 3 common types of gill lesions: Edema, secondary lamellar hyperplasia, and lamellar fusion. The severity of these lesions was assessed by estimating the percentage of tissue affected and classified into 3 categories: Mild (less than 15%), moderate (15% - 70%), and severe (more than 70%) [77]. Histopathological observations of the gill tissue revealed clear differences among treatment groups. The positive control group (K+) exhibited marked structural damage, as illustrated in **Figure 7**.

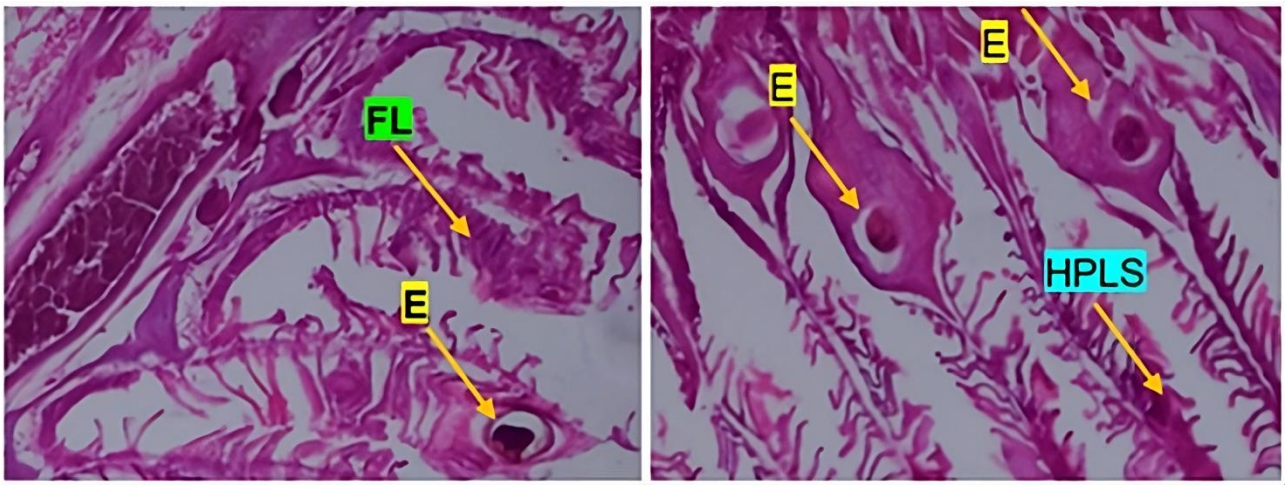

Figure 7 Histological observation of gill tissue lesions: E = Edema; HPLS = Hyperplasia; FL = Lamellar fusion.

Table 1 Scoring of gill tissue lesions in hybrid grouper.

	Replicates			Mean	Percentage (%)	Score	Severity
	1	2	3				
Edema							
K−	0	0	0	0	0	0	None
K+	103	104	101	102.53	36.62	3	Severe
P1	32	35	35	34.00	12.14	1	Mild
P2	45	46	47	46.13	16.48	2	Moderate
P3	55	56	52	54.27	19.38	2	Moderate
Hyperplasia							
K−	0	0	0	0	0	0	None
K+	106	104	105	104.80	37.43	3	Severe
P1	39	41	42	40.60	14.50	1	Mild
P2	55	56	53	54.27	19.38	2	Moderate
P3	63	65	64	63.93	22.83	2	Moderate
Lamellar Fusion							
K−	0	0	0	0	0	0	None
K+	108	109	107	107.80	38.50	3	Severe
P1	42	43	41	41.67	14.88	1	Mild
P2	51	54	54	53.07	18.95	2	Moderate
P3	73	74	71	72.93	26.05	2	Moderate

Each lesion type was assessed by scoring microscopic fields based on a defined formula, with results summarized in **Table 1**.

Results from histopathological scoring revealed that the positive control group (K+) experienced the most severe gill damage across all lesion types, with scores of 3 and lesion coverage exceeding 36%. In contrast, the P1 treatment group (low-dose nanovaccine) exhibited mild damage (score = 1) with mean lesion percentages around 12% - 15%. Groups P2 and P3 (medium and high doses) showed moderate damage (score = 2), with higher lesion percentages than P1 but substantially lower than K+. These findings indicate that nanovaccine administration mitigated gill tissue damage caused by VNN infection, with the strongest protective effect observed in the P1 group, which demonstrated the least histopathological disruption aside from the negative control. The presence of edema, hyperplasia, and lamellar fusion in the K+ group confirms the detrimental impact of VNN-induced oxidative stress and inflammation on gill morphology.

Conversely, the vaccinated groups showed reduced lesion scores, indicating the protective effect of *C. vulgaris* and ZnO-chitosan in modulating immune responses and reducing tissue damage. These results are consistent with Fu *et al.* [63]; Wulandari *et al.* [77], who reported that adjuvanted vaccines could significantly reduce tissue lesions caused by pathogenic infections [33]. In the P2 and P3 groups, moderate lamellar fusion was still observed (score = 2), suggesting that while vaccination did not completely prevent structural damage, it significantly reduced its severity. Lamellar fusion may represent a chronic adaptive response to irritation, as reported by Kaur et al. in their study on pesticide exposure and gill morphology [78]. Notably, the P1 group exhibited improved tissue integrity with relatively normal lamellar structures and minimal damage. In particular, P2 showed well-organized secondary lamellae without signs of fusion or degeneration, suggesting that the nanovaccine successfully suppressed viral replication and inflammation. The presence of edema, hyperplasia, and

lamellar fusion in the gills of fish from the control groups highlights the damaging effects of VNN infection and the resulting oxidative stress on gill tissue [79,80]. In contrast, fish that received the nanovaccine showed clear signs of protection, with significantly fewer and less severe tissue lesions. These results support the effectiveness of the *Chlorella vulgaris*-based nanovaccine, formulated with a ZnO-chitosan adjuvant, in safeguarding gill structure and reducing the impact of virus-induced damage [69].

The comparative analysis of gill tissue histology among different treatment groups demonstrates that the P1 group shows significant recovery, with lesions reduced to approximately 12% - 15%, whereas the P2 group exhibits partial recovery characterized by moderate lesions of 16% - 19%. This observation indicates that both treatment dosages are effective in promoting tissue healing, with P1 facilitating a restoration of gill morphology that is closer to baseline conditions. The dual functionality of ZnO-chitosan nanoparticles extends beyond their role as adjuvants; they also provide a protective effect on tissues. Chitosan's mucoadhesive properties create a physical barrier that attenuates pathogen adhesion at mucosal surfaces, while the Zn^{2+} ions enhance epithelial integrity and accelerate tissue repair processes, which likely contributed to the lesser gill lesions noted in the vaccinated groups [81,82].

Furthermore, when compared to conventional vaccines, such as inactivated or DNA vaccines, the evaluated nanovaccine demonstrates comparable or superior outcomes in promoting growth performance and enhancing antioxidant activity [83]. However, it is critical to note that while this presentation of results is promising, the absence of measurements regarding direct viral load reduction signals that further optimization is necessary. Consequently, field-scale comparative trials against existing standard vaccines are essential for validating the full potential of this nanovaccine formulation. In operational terms, the oral sonde injection method utilized within this study may lack practicality for large-scale farming applications, leading future research efforts to focus on the development of oral or immersion delivery systems that are more practical for extensive aquaculture practices [84,85].

Conclusions

The nanovaccine formulated with recombinant *Chlorella vulgaris* protein and ZnO/chitosan hybrid nanoparticles demonstrated promising protective effects in hybrid grouper (*Epinephelus fuscoguttatus* × *E. lanceolatus*) challenged with Viral Nervous Necrosis (VNN). Instead of directly measuring viral replication, the protective response was reflected through improved physiological performance, antioxidant status, and tissue integrity. Among the different doses tested, the 33 µL group (P1) achieved the best growth results, with an average final length of 12.73 cm and weight of 18.00 g both significantly higher than the control groups ($p < 0.05$). Meanwhile, the 66 µL group (P2) showed the highest levels of antioxidant enzyme activity, with superoxide dismutase (SOD) reaching 3.997 ± 0.16 U/mL and catalase (CAT) at 109.7 ± 3.1 U/mL. These elevated enzyme levels point to a strengthened cellular defense system, helping the fish counteract the oxidative stress caused by viral infection. Histopathological analysis of the gills showed substantially reduced tissue damage in vaccinated fish compared to the positive control. For instance, the P1 group exhibited only mild edema (12.14%), while the K+ group showed severe damage (score 3), with lesion coverage exceeding 36% of the tissue. Instead of directly measuring viral replication, the protective response was reflected through improved physiological performance, antioxidant status, and tissue integrity. The 33 µL dose (P1) appears optimal, offering maximal protection without observable toxicity. This study did not include direct viral quantification or long-term field validation, which should be addressed in future research to reinforce the antiviral claim. Importantly, by reducing the severity of VNN-related pathology and enhancing host resilience, this nanovaccine has the potential to decrease reliance on antibiotics and chemical therapeutics in grouper farming, thereby supporting sustainable aquaculture practices and aligning with antimicrobial resistance (AMR) mitigation goals.

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Generative AI tools (specifically ChatGPT by OpenAI) were used solely to assist in improving the readability, grammar, and language clarity of this manuscript. All content was developed, reviewed, and approved by the authors with full human oversight. The AI tool was not involved in the generation of scientific content, analysis, interpretation, or conclusions presented in this article. No AI tools were listed or considered as authors or co-authors. The authors remain fully responsible for the integrity and accuracy of the content.

CRediT author statement

Nezya Pramudya Wardani: Conceptualization, Investigation, Data processing, Writing - Original Draft; **Uun Yanuhar**: Supervision, Methodology, Validation, Funding acquisition, Writing - Review & Editing; **Muhammad Musa**: Formal analysis, Data curation, Visualization; **Heru Suryanto**: Resources, Software, Methodology; **Nico Rahman Caesar**: Project administration, Investigation, Validation, Data curation; **Defa Rifqi Machfuda**: Experimental design, Visualization, Writing - Review & Editing; **Nafal Naqi Ramadhan**: Sample collection, Laboratory work.

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