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Adjuvants in Microencapsulation-Based Vaccines for Cantang Grouper against Viral Nervous Necrosis: A Review

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Abstract

Viral nervous necrosis (VNN) is one of the most serious diseases attaching Cantang grouper (Epinephelus fuscoguttatus × Epinephelus lanceolatus) with mortality up to 100% in juvenile fish in aquaculture. A promising strategy to control this disease is the development of recombinant vaccines using the microalgae Chlorella vulgaris. In vaccine formulations, the addition of adjuvants is important for enhancing fish immune responses. This study aims to review the microencapsulation techniques using recombinant C.vulgaris protein combined with adjuvants such as Complete Freund's Adjuvant (CFA), Incomplete Freund's Adjuvant (IFA), and chitosan to improve immune responses in Cantang grouper infected with VNN. This review was conducted by synthesizing peer-reviewed journals published over the last decade. The analysis focuses on evaluating the integration of various adjuvants with microencapsulation technologies to enhance vaccine efficacy against VNN in Cantang Grouper. The results indicate that the use of recombinant C.vulgaris-based vaccines combined with encapsulated adjuvants, particularly CFA/IFA and chitosan, can enhance immune responses in Cantang grouper. Microencapsulation technology enhances antigen stability and enables controlled antigen release, thereby improving vaccine delivery efficiency. These findings suggest that adjuvant-assisted microencapsulation systems have significant potential to improve vaccine effectiveness to prevent fish from VNN infection in aquaculture. Technological applications should focus on optimizing encapsulation formulas and large-scale trials to ensure long-term immunity in sustainable grouper farming.

Keywords: *chitosan; Freund's adjuvant (CFA/IFA); microencapsulation; nanovaccine, viral nervous necrosis; aquaculture*

1. Introduction

Farming of Grouper fish is an important sector in marine aquaculture due to the high demand for reef fish from the *Serranidae* family for local and international markets. The commonly cultivated grouper fish is the Cantang grouper (*Epinephelus fuscoguttatus* × *Epinephelus lanceolatus*). Cantang grouper is a hybrid fish with the advantages of rapid growth, high survival rate, and strong environmental adaptability [1]. However, disease outbreaks remain a major challenge in grouper farming, particularly those caused by viruses, bacteria, and parasites under poor environmental conditions. One of the most severe viral diseases is viral nervous necrosis (VNN), which can cause mortality rates of up to 100% in juveniles measuring 2–5 cm. This disease is often associated with environmental stressors, such as poor water quality, that reduce fish immunity [2]. The VNN attacks the central nervous

system of fish, particularly the brain and eye tissue. Damage to this nervous tissue causes behavioral disorders in fish, such as abnormal swimming, loss of balance, and a tendency to stay at the bottom of the water. This disease led to mass mortality in farmed fish populations[3]. Therefore, it needs an effective disease control strategy to mitigate the impact of VNN infection on grouper aquaculture.

One widely developed approach to disease control in fish farming is the use of recombinant vaccines. They have several advantages, including improved safety, efficient antigen production, and the ability to stimulate a specific immune response in the target organism. Furthermore, recombinant vaccine technology allows manipulation of antigen protein synthesis, thereby enhancing the effectiveness of the fish's immune response to specific pathogens [4]. One potential source for recombinant protein production is the microalgae *C.vulgaris*. This microalgae from the Chlorophyceae class are known to possess high nutritional content and various bioactive compounds beneficial to the health of aquatic organisms. These bioactive compounds include carotenoids, sulfated polysaccharides, phenolic compounds, and vitamins, which play a role in enhancing antioxidant activity, cell regulation, and the organism's immune system [5]. Therefore, the use of *C.vulgaris* as a source of recombinant protein has the potential to be developed as a vaccine base for fish farming.

In the development of recombinant protein-based vaccines, microencapsulation technology can be used to improve antigen stability and the efficiency of vaccine delivery systems. Microencapsulation is a technique for processing micro-/nano-sized particles coated with a polymer-based coating. The technology aims to protect antigens from degradation during storage and after administration to target organisms, and to control antigen release to enhance the effectiveness of the immune response [6]. Vaccine formulations are supplemented with adjuvants to increase the immunogenicity of the antigen [7]. Adjuvants enhance the organism's immune response, reduce the amount of antigen required, and increase the vaccine effectiveness [8]. Some types of adjuvants used in vaccine delivery systems are Complete Freund's Adjuvant (CFA), Incomplete Freund's Adjuvant (IFA) [9], and chitosan [10]. However, information regarding the effectiveness of each adjuvant in enhancing the immune response in Cantang grouper against VNN infection is still limited. Therefore, this literature review aims to analyze the role of CFA, IFA, and chitosan adjuvants in the microencapsulation system of *C.vulgaris* recombinant protein and the immune response of Cantang grouper against VNN infection.

2. Materials and Methods

The study applied a Systematic Literature Review (SLR) approach to identify, analyze, and synthesize relevant studies related to the application of recombinant proteins derived from *C.vulgaris*, microencapsulation technology, and the use of adjuvants in improving immune responses in grouper infected with VNN. The literature search was conducted using several scientific databases in range of from 2016 to 2026, including Google Scholar, ScienceDirect, and PubMed, to obtain relevant peer-reviewed articles, books, and research reports with combinations of keywords such as "*Chlorella vulgaris*," "recombinant vaccine," "microencapsulation," "adjuvant," "chitosan," "Freund's adjuvant," "viral nervous necrosis," and "grouper immune response."

The selection of literature was carried out in several stages. The first stage was collecting all publications related to the keywords. The second was the screening and reviewing of titles and abstracts to determine the relevance of the studies. The third was a full-text evaluation of selected articles to ensure their relevance to the study objectives. The final stage was analyzing and comparing to identify the effects of different adjuvants and microencapsulation techniques on the immune response of grouper against VNN infection.

The inclusion criteria for the selected literature were:

1. Scientific publications discussing *C.vulgaris*, recombinant proteins, or microalgae-based vaccines;
2. Studies related to microencapsulation technology or vaccine delivery systems;
3. Research examining immune responses in fish, particularly grouper species infected with VNN; and
4. Articles published in peer-reviewed journals.

Meanwhile, publications not directly related to fish immunology, microalgae-based vaccines, or VNN infection were excluded from the analysis. All selected studies were then analyzed descriptively and comparatively to evaluate the effectiveness of adjuvants such as CFA, IFA, and chitosan in enhancing immune responses, particularly through indicators such as NF-κB and IFN-γ expression.

3. Results and Discussion

3.1. Cantang Grouper

Cantang grouper is a hybrid species widely cultivated in marine aquaculture. They were produced through crossbreeding between male giant grouper (*Epinephelus lanceolatus*) and female tiger grouper (*Epinephelus fuscoguttatus*)[11]. The hybridization was first successfully conducted in 2010 at the Brackish Water Aquaculture Fisheries Center (BPBAP), Situbondo, Indonesia. The name “Cantang” is derived from the combination of the names of its parent species, namely “macan” (tiger grouper) and “kertang” (giant grouper).

Cantang grouper has a morphology of a laterally compressed and relatively rounded body shape. The mouth is superior, with the lower lip slightly longer than the upper lip, while the scales are classified as ctenoid scales similar to those of its parent species. As a carnivorous fish, Cantang grouper possesses sharp canine-like teeth used for capturing prey. The body coloration is generally dark brown to blackish with several transverse dark stripes, while the fins resemble those of the giant grouper, with a pale-yellow base color and scattered dark spots[12].

Cantang grouper typically inhabits tropical and subtropical coastal waters. In the larval stage, groupers generally occupy shallow waters with depths ranging from 0.5 to 3 m [13]. These ecological characteristics make the species suitable for aquaculture development in tropical marine environments. However, intensive aquaculture systems may increase the risk of disease outbreaks, particularly when biosecurity and water quality management are inadequate. One of the most serious viral diseases affecting grouper aquaculture is VNN, which can cause mass mortality, especially during larval and juvenile stages. The rapid spread of viral infections is often associated with poor environmental conditions and weakened immune systems in fish [14].

The immune defense system of the grouper, similar to that of other vertebrates, consists of both innate (non-specific) and adaptive (specific) immune responses to protect the fish from pathogenic infections, including viruses, bacteria, parasites, and fungi. Therefore, enhancing the immune response of grouper through vaccination strategies, including recombinant vaccines derived from microalgae such as *C.vulgaris* [15], has become an important approach for preventing VNN infection in aquaculture [16].

3.2. Viral Nervous Necrosis

VNN is one of the most important viral diseases affecting marine fish species, including groupers, seabass, and other economically important fish [17]. VNN includes the genus Betanodavirus in the family Nodaviridae. Transmission of VNN can occur through both

vertical and horizontal pathways. Vertical transmission occurs when infected broodstock transmit the virus to their offspring through reproductive processes, while horizontal transmission occurs through water, contaminated equipment, or direct contact between infected and healthy fish. In Indonesia, the first outbreak of VNN was reported in seabass hatcheries in East Java in 1997, causing significant economic losses in marine aquaculture [18].

The prevalence of virus outbreaks is majorly influenced by the worst environmental conditions. Bad environmental conditions cause stress in fish. Stress reduces the immune system of the fish, making it easier for viruses to attack the fish. The other factors contributing to the infection are fish age, species susceptibility, viral dose, water quality, and environmental stability. Relatively high water temperatures and unstable environmental conditions may also accelerate viral replication and transmission [19]. Larval and juvenile stages are the most susceptible to VNN infection, with mortality ranging 80–100% [20].

The infection process starts with the virus adhering to host cells through interactions between viral adhesins and specific receptors on the cell surface. Structural components of the virus include a protein capsid and nucleic acids. The capsid protein acts as a viral entry that facilitates the virus's binding to host cell receptors and allows the virus to enter host cells. After adhesion, the virion penetrates, uncoats, and undergoes subsequent transcription [21]. In the inner host, the VNN primarily targets the central nervous system, especially the brain and retina. Infection of nerve cells leads to pathological changes such as necrosis, vacuolization, and hypertrophy, which are identified as histopathological features of VNN infection [22]. As a result, internal organs such as the spleen and kidneys may swell as a systemic response to infection [23].

Clinically, infected fish exhibit abnormal swimming behavior such as whirling or spiral swimming, loss of equilibrium, lethargy, and darkened body. In severe cases, infected fish may remain near the water surface or sink to the bottom before eventually dying. These neurological symptoms reflect the severe damage to the nervous system caused by viral replication in neural tissues. Following infection, fish initiate an immune response involving both innate (non-specific) and adaptive (specific) immune mechanisms. The innate immune response represents the first line of defense and includes inflammatory responses and the activation of immune signaling pathways. One important pathway involved in antiviral responses is the activation of nuclear factor kappa B (NF-κB) and interferon-related signaling pathways such as interferon gamma (IFN-γ), which play key roles in regulating antiviral defense mechanisms [2]. Experimental studies indicate that healthy fish may become infected within approximately 1-2 days after exposure to viral suspensions [24]. VNN may also be detected in fish that do not exhibit visible clinical symptoms, suggesting the presence of asymptomatic carriers that can act as reservoirs for viral transmission within aquaculture systems [25].

Given the high mortality rate and rapid transmission of VNN, effective preventive strategies are essential for sustainable aquaculture production. One promising approach involves the development of recombinant vaccines and immunostimulants to enhance fish immune responses. In this context, bioactive compounds derived from microalgae such as *C.vulgaris* have attracted considerable attention due to their potential to stimulate immune responses and improve disease resistance in fish.

3.3. Chlorella vulgaris

C.vulgaris is a unicellular photosynthetic microalga belonging to the family Chlorellaceae, with advantages such as a rapid growth rate, high biomass productivity, and adaptability to various environmental conditions [26]. It makes *C. vulgaris* a promising biological resource for applications such as nutraceuticals, pharmaceuticals, and aquaculture feed additives.

As shown in Fig. 1, *C.vulgaris* shows morphology such as a smooth cell wall and a cup-shaped chloroplast containing a single pyrenoid. The cell diameter ranges from 2.5 to 3.5 μm.

Reproduction occurs through asexual cell division by producing autospores, allowing rapid population growth under favorable environmental conditions [27]. This microalga has high tolerance to environmental fluctuations, so it can grow efficiently in various aquatic environments exposed to sunlight, including lakes, rivers, springs, and marine waters [28].

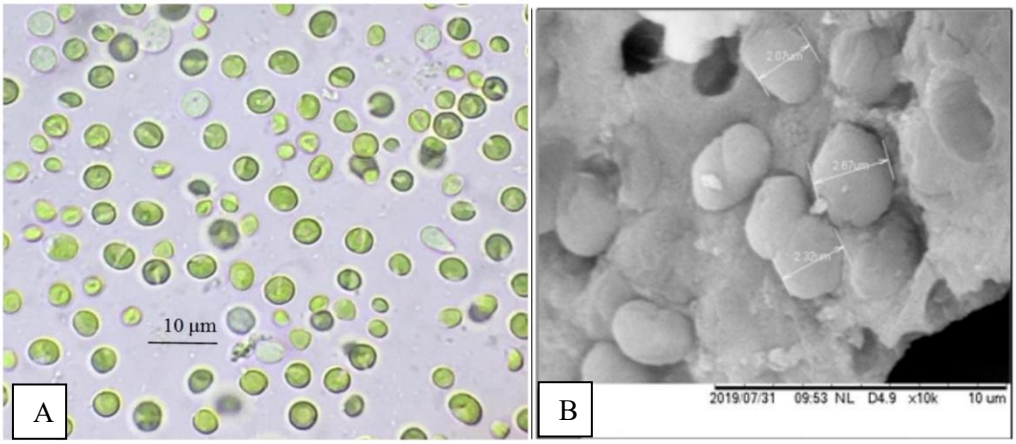

Fig. 1. *Chlorella vulgaris* [29], [30]

C.vulgaris has high protein content and constitutes approximately 50–60% of dry biomass. So, they make a valuable source of nutritional and functional proteins [26]. The molecular weight of proteins found in ranges from 12 to 120 kDa, with most proteins concentrated between 39 and 75 kDa [31]. These proteins are distributed throughout different cellular compartments, including the cell wall, cytoplasm, and chloroplast. The nutritional quality of these proteins is influenced by their composition of essential and non-essential amino acids. The other constituents of *C.vulgaris* are bioactive compounds such as pigments, polysaccharides, vitamins, and antioxidants. Major pigments identified in this microalga include chlorophyll a, chlorophyll b, canthaxanthin, lutein, β-carotene, astaxanthin, pheophytin, and violaxanthin [31]. These pigments (as shown in Table 1) act as antioxidants and may provide protective effects against oxidative stress.

Table 1. Pigment composition of *C.vulgaris* [32]

Pigment	µg/g (dw)
Chlorophyll-a	250–9,630
Chlorophyll-b	72–5,770
Canthaxanthin	362,000
Lutein	52–3,830
β-Carotene	7–12,000
Astaxanthin	550,000
Violaxanthin	10–37
Pheophytin-a	2,310–5,640
Pheophytin-b	N/A

Bioactive compounds present in *C.vulgaris* have been reported to exhibit antiviral, anti-inflammatory, and immunomodulatory activities. According to Masitha et al. [33], bioactive substances in *C.vulgaris* extract can inhibit pathogen adhesion to host cells. The antiviral

activity occurs when bioactive compounds interact with viral glycoproteins, preventing viral attachment to host cell receptors and thereby blocking viral adsorption and infection processes. Furthermore, these bioactive compounds may also act as anti-inflammatory agents in fish infected with VNN [34]. This effect has been associated with the regulation of cellular stress responses, including the expression of heat shock proteins (HSPs) that help maintain cellular stability during viral infection. In the context of vaccination, antigenic proteins derived from VNN capsid proteins (CP) are known to stimulate immune responses in fish. The activation of the immune system leads to the production of specific antibodies capable of neutralizing viral particles [35]. As a result, vaccinated fish exhibit greater resistance to viral infection compared to non-vaccinated fish.

3.4. Microencapsulation

Microencapsulation is a technique used to encapsulate or coat active substances, such as antigenic proteins, with protective materials to form microparticles. In vaccine development, this technique aims to protect antigens from environmental degradation, enhance protein stability, and enable controlled release of the antigen within the host organism. In addition to encapsulating materials, vaccine formulations generally require additional components such as adjuvants, which function to enhance the immune response to the administered antigen. Adjuvants are substances added to vaccine formulations to enhance the adaptive immune response against antigens [36]. The use of adjuvants can stimulate both humoral and cellular immune responses, thereby improving vaccine efficacy in preventing infections and reducing mortality rates in fish exposed to pathogens. Comparison of some adjuvants is presented in Table 2.

Complete Freund's Adjuvant (CFA)

CFA is one of the most widely used adjuvants in immunological studies [37]. CFA is a water-in-oil (W/O) emulsion composed of mineral oil and heat-killed Mycobacterium cells. The mycobacterial components act as a strong immune stimulator, enabling CFA to induce both humoral and cellular immune responses effectively. Due to its strong immunostimulatory properties, CFA is considered highly effective in enhancing immune responses to antigens. However, its application is often limited because it may cause severe local inflammatory reactions and other side effects.

Incomplete Freund's Adjuvant (IFA)

IFA is similar to CFA but does not contain mycobacterial components. It is also formulated as a W/O emulsion using mineral oil as the continuous phase. IFA has been shown to enhance antibody production and stimulate immune responses, including both humoral and mucosal immunity [38]. In aquaculture applications, adjuvants have been used as vaccines against VNN administered through feed in grouper, resulting in improved immune responses in fish [39].

Chitosan

Chitosan is a natural polymer obtained from the partial deacetylation of chitin, which is the second most abundant polysaccharide in nature after cellulose. Chitosan possesses several advantageous properties, including biocompatibility, biodegradability, non-toxicity, and antimicrobial activity, making it widely used in various biomedical and biotechnological applications, including drug and vaccine delivery systems [40].

In microencapsulation systems, chitosan acts as a coating material to protect antigens from degradation while improving protein stability during storage and administration. Besides, chitosan exhibits excellent mucoadhesive properties that allow it to adhere to mucosal surfaces and enhance antigen absorption through mucosal tissues. It makes chitosan highly suitable for

oral vaccine delivery systems in fish [41]. Chitosan-based microcapsules can provide controlled antigen release, enabling prolonged immune stimulation compared with direct antigen administration. However, several limitations were detected for chitosan, such as low solubility at pH values above 6 and variability in physicochemical properties that may affect reproducibility during the encapsulation process [42].

Several studies demonstrate that encapsulation materials, such as oil-based adjuvants and natural polymers, have been known to enhance antigen stability and improve immune responses in fish. The comparison of adjuvants and encapsulating materials in Table 2 shows that oil-based systems such as CFA and IFA are effective in enhancing immune responses in stimulating antibody production [36,43]. But their application is limited by toxicity and potential side effects, including inflammation and tissue damage. Natural polymers such as chitosan offer a safer alternative due to their biocompatibility, biodegradability, and ability to provide controlled antigen release [10,42]. The studies summarized in Table 3 demonstrate that microencapsulation-based vaccine systems improve antigen stability and immune responses across various fish species. Oil-based adjuvants have been shown to increase protection levels and antibody responses, as reported by Rodríguez et al. [44] and Rizkiantino et al. [45]. Chitosan-based systems enhance antigen delivery efficiency and support both systemic and mucosal immunity, as demonstrated in several studies [40-42]. Chitosan in micro- and nanoparticle forms shows strong potential for oral vaccine applications, offering a more practical and less invasive approach compared to injection-based methods [40]. However, current studies are still limited in directly comparing the effectiveness of different encapsulation materials under similar experimental conditions. Therefore, further research is needed to optimize encapsulation systems by balancing immunogenic effectiveness, safety, and delivery efficiency, particularly for practical applications in aquaculture.

Table 2. Comparison of commonly used adjuvants and encapsulating materials in vaccines

Adjuvant	General Description	Advantages	Limitations	Ref.
CFA	Water-in-oil (W/O) emulsion composed of mineral oil mixed with heat-killed *Mycobacterium*.	Induces a strong immune response, both humoral and cellular.	Highly toxic and may cause severe inflammatory reactions.	[36] [43]
IFA	W/O emulsion using mineral oil without heat-killed *Mycobacterium*.	Produces fewer side effects compared with CFA and enhances antibody production.	1. Weaker immune-modulatory effect compared with CFA. 2. Local irritation that may lead to granuloma or cyst formation.	[36] [43]
Chitosan	A natural polymer obtained from the partial deacetylation of chitin, one of the most abundant polysaccharides in nature after cellulose.	1. Non-toxic, biocompatible, biodegradable, and non-allergenic. 2. Suitable for mucosal delivery systems. 3. Enables controlled antigen release. 4. Mucosal administration can induce antibody and T-cell responses.	1. Poor reproducibility in some formulations. 2. Limited solubility at pH above 6.	[42] [10]

Table 3. Previous studies on microencapsulation-based vaccines in aquaculture

Author	Target Fish Species	Disease/Pathogen	Encapsulation Material	Main Findings
Rodríguez et al. [44]	Atlantic salmon	Copepodids	Oil-based adjuvant systems	A higher degree of protection coincided with higher circulating antibody levels against homologous antigens.
Rizkiantino et al [45]	Red tilapia (*Oreochromis* sp.)	*Streptococcus*	IFA	Improved antibody production and enhanced resistance against bacterial infection.
Angulo et al. [41]	Various fish species	Multiple pathogens	Chitosan-based delivery system	Improved antigen stability and enhanced mucosal immune responses in fish.
Sanina et al. [42]	Aquatic organisms	Viral and bacterial infections	Chitosan nanoparticles	Demonstrated effective antigen delivery and controlled release properties.
Lei and Langrish [40]	Aquaculture species	Vaccine delivery systems	Chitosan microparticles	High potential for oral vaccine delivery due to biocompatibility and biodegradability.

3.5. Mechanism of Microencapsulation in Fish Vaccine Delivery

Microencapsulation technology has important role in improving vaccine delivery systems in fish. They provide a protective barrier for the antigen from degradation. Conventional vaccination methods often show antigen degradation caused by digestive enzymes, pH fluctuations, and instability in aquatic environments [46], and inefficient antigen uptake by fish tissues. Antigenic proteins are enclosed within protective polymer matrices, forming stable microparticles. These microcapsules protect the antigen against such environmental factors and allow controlled and sustained antigen release, which can prolong immune system stimulation.

In aquaculture applications, microencapsulation technology offers significant potential for developing more effective, stable, and easily administered vaccines. They facilitate oral vaccination through feed, which is more practical and less stressful for fish compared with injection methods, especially in large-scale aquaculture operations. After ingestion, the microcapsules pass through the digestive tract and interact with intestinal mucosal tissues, where antigen-presenting cells recognize the antigen and initiate immune responses with 86% survival in pathogen-challenged fish [47]. Encapsulation materials such as chitosan further enhance vaccine effectiveness due to their mucoadhesive properties [48], which increases the retention time of antigen particles on mucosal surfaces. This prolonged contact improves antigen uptake and stimulates stronger immune responses, thereby enhancing fish resistance to pathogens, including viral infections such as VNN. This approach contributes to sustainable disease control strategies in fish farming. The mechanism of the microencapsulated *C.vulgaris* vaccine and its interaction with the immune system of grouper infected with VNN is illustrated in Fig. 2.

The recombinant antigen derived from *C.vulgaris* is encapsulated using adjuvants such as chitosan and CFA/IFA. The microencapsulation system protects the antigen and enables controlled release during administration. This delivery mechanism stimulates the fish immune system, leading to the activation of immune responses including IgM production, NF-κB

signaling, and IFN-γ expression. As a result, viral replication is suppressed, and the survival rate of grouper infected with VNN can be improved.

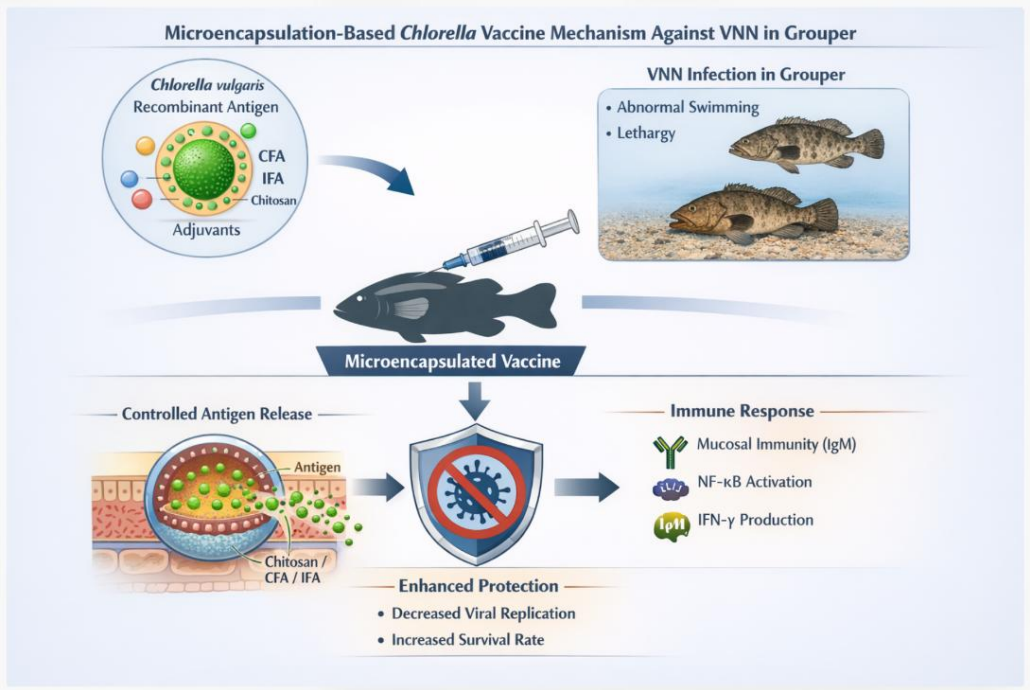

Fig. 2. Mechanism of microencapsulation-based recombinant *C.vulgaris* vaccine against VNN in Cantang grouper.

The use of adjuvants in vaccine formulations plays an important role in enhancing immune responses in fish against viral infections. According to Huda et al [39], the administration of a recombinant *C.vulgaris*-based vaccine supplemented with adjuvants such as CFA or IFA in Cantang grouper (*Epinephelus fuscoguttatus–lanceolatus*) infected with VNN showed promising results. The addition of CFA/IFA significantly improved immune responses, which was indicated by the highest antibody titers detected in IgM-labeled antifish antibody cells.

4. Conclusions

A review of the potential of adjuvant-assisted microencapsulation systems to enhance vaccine effectiveness against viral nervous necrosis (VNN) in Cantang grouper has been conducted. Recombinant proteins derived from *Chlorella vulgaris*, combined with adjuvants such as Complete Freund's Adjuvant (CFA), Incomplete Freund's Adjuvant (IFA), and chitosan, can improve immune responses in fish. Microencapsulation technology enhances antigen stability and enables controlled antigen release, thereby improving vaccine delivery efficiency. Increased immune responses indicated by markers such as IgM, NF-κB, and IFN-γ demonstrate the effectiveness of this approach in strengthening fish immunity against VNN infection.

With their stability and suitability for oral delivery systems, microencapsulation-based vaccines offer promising potential for practical application in aquaculture. Future studies should focus on optimizing encapsulation formulations, evaluating long-term immune protection, and conducting large-scale trials to support the development of effective vaccination strategies for sustainable grouper aquaculture.

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Author Declaration

Data availability statement: Data will be made available on request.

CRediT authorship contribution statement: DMF: Visualization, Writing – Original Draft, Writing – Review & Editing. UY: Conceptualization, Writing – Original Draft, Writing – Review & Editing. ASA: Formal Analysis, Software, Visualization. RR: Formal Analysis, Writing – Review & Editing.

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Artificial Intelligence Statement: The authors declare that generative AI tools were used solely to assist with language editing and grammar improvement.

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