BIOENCAPSULATION OF TRANSGENIC *Nannochloropsis* sp. WITH *Artemia* AS A VACCINE DELIVERY SYSTEM TO ZEBRAFISH AS A FISH MODEL AGAINST VIBRIOSIS

By

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December 2023

Chair : Zetty Norhana Balia Yusof, PhD
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Aquaculture production is a significant food source, but it has been declining due to aquatic diseases, such as vibriosis caused by *Vibrio* spp. For fish larvae, given their diminutive size and great susceptibility to the disease, oral vaccination was deemed superior to injection or immersion methods. Therefore, a bioencapsulation technique was utilized, where transgenic *Nannochloropsis* sp. expressing the OmpK gene was incorporated into live *Artemia*, a natural diet for fish at their early stage. This study utilized *Nannochloropsis* sp. transformed with a vector VCP_OMPK_ZA1 harboring OmpK gene fragment originating from *Vibrio* sp. which acts as an antigen. The cuticle of *Artemia* offers an additional layer of protection to the antigen, protecting it against degradation in the gastrointestinal tract. Transgenic *Nannochloropsis* sp. was incubated with *Artemia* for 2 hours and the presence of the transgene was validated using PCR analysis. The transgenic microalgae-incorporated *Artemia* was then fed to zebrafish, an excellent model for studying infection and immune responses because of its compact size, swift growth, and suitability for genetic manipulation. The objective was to investigate immune response activation via gene expression analysis, with a specific focus on assessing the IgZ, TNF-α, and IL-1β genes. The levels of expression for IgZ and IL-1β were found to be elevated by factors of 0.8, 1.2, 1.9, and 2.6-fold respectively on Day 7 and Day 14, whereas TNF-α significantly upregulated by 2.9-fold in zebrafish fed with the transgenic microalgae-incorporated *Artemia* in comparison to zebrafish given with wild-type *Artemia*. IL-1β (4.0-fold) gene transcripts increased significantly in groups fed with wild-type bioencapsulated *Artemia* after being infected with bacteria while IgZ levels did not increase significantly for both groups. The effectiveness of the transgenic microalgae was evaluated by subjecting them to a bacterial challenge of *Vibrio* sp. and measuring the relative percent survival (RPS). The outcomes of the bacterial challenge experiment revealed that the vaccinated groups displayed complete immunity against pathogenic *Vibrio* spp., with RPS of 100%, and demonstrated significantly increased survival rates when compared to those that were fed with wild-type *Nannochloropsis* sp. bioencapsulated *Artemia* (RPS = 40%). The histopathological analysis revealed that the fish that were not vaccinated had severe changes such as hemorrhages and necrosis, whereas those that were vaccinated showed only mild changes such as tubular degeneration and muscle regeneration following the bacterial challenge trial. Overall, the results obtained proved that the incorporation of transgenic microalgae harboring an antigenic peptide administered through the live feed may enhance the fish's immune system to combat vibriosis and be effectively applicable for vaccinating fishes during the early larval stage.

Keywords: Bioencapsulation, Live feed, *Nannochloropsis* sp., Oral vaccination, Transgenic microalgae, Vibriosis

SGD: GOAL 14: Life Below Water

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Master Sains

BIOENKAPSULASI *Nannochloropsis* sp. TRANSGENIK DENGAN *Artemia* SEBAGAI SISTEM PENGHANTAR VAKSIN KEPADA IKAN ZEBRA SEBAGAI MODEL IKAN UNTUK MENENTANG PENYAKIT VIBRIOSIS

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Akuakultur merupakan sektor pengeluaran sumber makanan yang penting namun ia mengalami kemerosotan akibat penyakit akuatik seperti vibriosis yang disebabkan oleh *Vibrio* spp. Vaksinasi secara oral dianggap lebih baik bagi larva ikan berbanding kaedah suntikan atau rendaman kerana saiznya yang kecil. Oleh itu, teknik bioenkapsulasi telah digunakan di mana *Nannochloropsis* sp. transgenik yang menghasilkan gen OmpK dimasukkan ke dalam *Artemia*, iaitu makanan semulajadi untuk ikan pada peringkat larva. Kajian ini menggunakan mikroalga *Nannochloropsis* sp. yang telah diubahsuai dengan menggunakan vektor VCP_OMPK_ZA1 yang membawa serpihan gen OmpK yang berasal dari *Vibrio* sp. yang bertindak sebagai antigen. Membran *Artemia* bertindak sebagai lapisan perlindungan tambahan kepada antigen daripada pencernaan di dalam system gastrointestinal ikan. Di dalam kajian ini, *Nannochloropsis* sp. telah berjaya ditransformasi dengan vektor VCP_OMPK _ZA1 yang membawa fragmen gen OmpK dan diverifikasi melalui analisis PCR. *Nannochloropsis* sp. transgenik kemudian diinkubasi dengan *Artemia* selama 2 jam dan kehadiran transgen di dalam *Artemia* diverifikasi dengan menggunakan analisis PCR. *Artemia* yang mengandungi *Nannochloropsis* sp. transgenik kemudian diberi kepada ikan zebra, model yang sangat baik untuk mengkaji tindak balas sistem imun ikan kerana saiznya yang kecil, mudah dikultur dan dimanipulasi genetiknya. Tujuannya adalah untuk mengkaji pengaktifan tindak balas sistem imun ikan melalui analisis ekspresi gen, dengan tumpuan kepada gen IgZ, TNF-α, dan IL-1β. Tahap ekspresi bagi IgZ dan IL-1β didapati meningkat sebanyak 0.8 kali ganda dan 1.2 kali ganda, 1.9 kali ganda dan 2.6

kali ganda masing-masing pada Hari ke-7 dan Hari ke-14, sementara TNF-α mengalami peningkatan yang signifikan sebanyak 2.9 kali ganda pada ikan zebra yang diberi makan dengan *Artemia* bioenkapsulasi *Nannochloropsis* sp. transgenik berbanding dengan ikan zebra yang diberi Artemia sahaja. Gen IL-1β (4.0 kali ganda) meningkat secara signifikan bagi kumpulan ikan yang diberi makan *Artemia* bioenkapsulasi mikroalga jenis liar setelah diuji dengan bakteria, sementara tahap IgZ tidak meningkat secara signifikan bagi kedua-dua kumpulan. Keberkesanan mikroalga transgenik telah dinilai melalui cabaran bakteria *Vibrio* sp. dan diukur peratusan kelangsungan hidup relatif (RPS). Hasil eksperimen cabaran bakteria mendapati bahawa kumpulan yang divaksinasi menunjukkan kekebalan sepenuhnya terhadap *Vibrio* spp. yang patogenik, dengan RPS sebanyak 100%, dan mempamerkan kadar kelangsungan hidup yang meningkat dengan ketara berbanding dengan kumpulan ikan yang diberi makan dengan *Artemia* bioenkapsulasi *Nannochloropsis* sp. jenis liar (RPS = 40%). Analisis histopatologi menunjukkan bahawa ikan yang tidak divaksinasi mengalami perubahan histopatologi yang teruk seperti pendarahan dan nekrosis, manakala ikan yang divaksinasi hanya mengalami perubahan yang ringan seperti degenerasi tubular dan regenerasi otot selepas ujian cabaran bakteria. Keseluruhan hasil kajian membuktikan bahawa penggunaan mikroalga transgenik yang mengandungi peptida antigenik yang diberikan melalui makanan hidup dapat meningkatkan sistem imun ikan untuk melawan vibriosis dan boleh digunakan untuk memberi vaksin kepada ikan di peringkat larva.

Kata kunci: Bioenkapsulasi, Makanan hidup, *Nannochloropsis* sp., Vaksinasi oral, Mikroalga transgenik, Vibriosis

SDG: MATLAMAT 14: Hidup di Bawah Air

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LIST OF ABBREVIATIONS

%	Percentage
°C	Degree Celsius
A	Absorbance
Bp	Base pair
cDNA	Complementary DNA
Cells/mL	Cells per milliliter
Chl	Chlorophyll
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
et al.,	And friends
EtBr	Ethidium bromide
F, R	Forward, Reverse
FAO	Food and Agriculture Organization
g	Gram
h	Hours
hpf	Hour post-fertilization
L	Liter
min	Minutes
mL	Milliliter
NaCl	Sodium chloride
PCR	Polymerase chain reaction
qPCR	Quantitative PCR

Rpm	Revolution per minute
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse-transcriptase PCR
s	Second
sp.	Species
UPM	Universiti Putra Malaysia
UV-Vis	Ultraviolet-visible
V	Volt
µg	Microgram
µl	Microliter

CHAPTER 1

INTRODUCTION

1.1 Background of the study

The aquaculture industry is estimated to be worth over RM14.88 billion in 2021, a rise of 7.5% from 2020 which is from RM13.84 billion (DOF, 2022). Despite the growth, Chiew et al. (2019) reported that it has been noted that numerous viral and microbial diseases still exist on farms in Malaysia. The occurrence of these diseases has led to substantial financial setbacks (Chiew et al., 2019). In this context, diseases caused by bacteria resulted in mass mortalities in fish farms with *Aeromonas*, *Pseudomonas,* and *Vibrio* being the most common pathogen (Austin et al., 2012). *Vibrio harveyi* is frequently identified as a primary causative agent of Vibriosis, with outbreaks commonly occurring in hatcheries, where fish at early stages are particularly susceptible to the disease (Kalatzis et al., 2018; Ina-Salwany et al., 2018). Antibiotics have been consistently employed for preventing bacterial disease outbreaks but constant antibiotics used resulted in the emergence of bacteria pathogens. Thus, vaccination which is economically and environmentally sustainable is currently being used to treat bacterial, and parasitic infections and prevent viral infections (Assefa and Abunna, 2018). However, ensuring that the organisms receive a vaccination dose in an effective manner also poses a challenge. Vaccination is actively employed to enhance disease resistance; however, challenges such as high labor and stress during injection, costliness of vaccine quantity in immersion, and the low efficacy and instability of orally delivered vaccines, underscore the need for a robust delivery system to ensure effective protection against diseases (Flores-Kossack et al., 2020; Bøgwald and Dalmo, 2021; Kwong et al., 2023). This study utilized unicellular microalgae *Nannochloropsis* sp. which is known for its high nutritive value, thus it is actively used in hatcheries for feeding rotifers and fish. *Nannochloropsis* sp. was transformed through electroporation using VCP_OMPK_ZA1 which harbor outer membrane protein kinase (OmpK) fragments originating from *Vibrio* sp. pathogenic bacteria and can act as antigen properties against vibriosis disease. The transgenic *Nannochloropsis* sp. was bioencapsulated with *Artemia*, offering dual protection to antigens. This environmentally friendly method ensures the effective delivery of antigens to small fish at an early age, addressing the inherent low efficacy drawback associated with oral vaccination. The bioencapsulated live feed was administered to zebrafish, a versatile and valuable animal model commonly used to comprehend the mechanisms of infection and immune responses associated with diseases affecting fish.

1.2 Problem statements

While injection-based vaccination delivery reduces usage and guarantees efficient administration, the process of capturing the animal, carefully handling it, administering anesthesia, and then giving injections to the fish is both resource-intensive and expensive, with the potential to inflict harm upon the fish (Noorhisham and Yusof, 2022). Moreover, it is impractical for small fish or crustaceans and poses a challenge when multiple doses of vaccine are required. Additionally, before they are mature enough to be immunized and before their systems of immunity have completely grown, some fish species may suffer from major diseases in their juvenile stages (Muktar et al., 2016). Another approach entails immersing the fish in a vaccine suspension; however, this results in stress for the fish and proves costly due to the substantial amount of vaccine needed. The most straightforward and suitable approach is to provide the vaccine orally, which is accomplished by adding it to aquatic animals' food.

Vaccination via the mucosal pathway, especially through oral administration, is deemed the best method concerning animal well-being and handling procedures. Administering vaccines orally is a highly attractive option for the aquaculture industry due to its various benefits, which include the simplicity of the delivery method, reduced stress on the fish compared to other forms of administration, suitability for fish of all sizes, and the possibility of performing oral boost vaccinations during their growth phases in cultivation ponds (Radhakrishnan et al., 2023). Despite the advantages of oral vaccination, there is a limited number of approved oral vaccines applicable to the aquaculture industry.

1.3 Significance of the study

It is crucial to administer effective vaccines at the initial larval stage before any infection. The innate immune system is pivotal in regulating swift immune responses and safeguarding the recipient against adverse circumstances during the initial stages of fish developmental growth, encompassing expanding embryos and the following larval phases (Chin et al., 2019). Oral vaccination is a preferable method of immunization for fish larvae, given their small size and increased vulnerability to stress, compared to injection or immersion methods. To date, numerous oral fish vaccines have been created, and among them, the encapsulation of vaccines within live *Artemia* seems to hold great promise (Ma et al., 2020). A key benefit of this oral vaccination approach is that the antigen is housed within a live feed, *Artemia*, which guarantees the intake of the antigen for the larvae (Lin et al., 2007). Bioencapsulation, which involves integrating the drug within live feed such as rotifers and *Artemia*, is regarded as an eco-friendly approach to delivering antigens to marine fish larvae (Noorhisham et al., 2022).

Certain authors have asserted that individual Artemia may not fulfil the nutritional needs for larval development, as they could be deficient in specific essential n3 and n6 polyunsaturated fatty acids (PUFAs), notably arachidonic acid (AA, 20:4n6), docosahexaenoic acid (DHA, 22:6n3), and eicosapentaenoic acid (EPA, 20:5n3) (Aya et al., 2021). Some microalgae are known to be rich in essential PUFAs. Various studies have shown that fatty acids can be transferred from prey to predator, suggesting that enriching *Artemia* with microalgae might address this nutritional gap (Méndez-Martínez et al., 2018).

Microalgae are essential food sources capable of improving the quality of aquaculture organisms. It has shown resistance to bacteria while some possess biomolecules that act as immunostimulants (Shah et al., 2018). Since microalgae are easily available in nature, it has emerged as a significant contributor to the food chain for several aquatic organisms together with mollusks, shrimp, and rotifers (Kaparapu, 2018). Furthermore, microalgae have been employed to enhance the quality of live feed because they can inhibit bacterial pathogens such as *Vibrio* sp. and preventing transmission to smaller fish (El-Sadoony et al., 2022). Recent breakthroughs in the field of genetic modifications of microalgal species are being developed for oral delivery of antimicrobial agents, and recombinant molecules, including growth promoters (Doron et al., 2016). However, up to the present moment, advancements in the utilization of genetically modified microalgae as carriers for the oral administration of bioactive compounds for aquaculture disease management have been constrained because most oral vaccines available are not applicable for smaller fish.

Due to its small size and brief life cycle, zebrafish are a particularly good model for scientists to investigate infectious mechanisms and the immune responses of hosts to pathogens. It is also invaluable for evaluating the safety and efficacy of new substances aimed at preventing and treating diseases in animals, permitting in vivo and real-time observation of substance effects (Rasheed et al., 2021). Therefore, within this research, the process of bioencapsulation of transgenic *Nannochloropsis* sp. with *Artemia* to feed zebrafish *(Danio rerio)* was presented. We evaluated the antigen's stability against digestion, its delivery to the fish gut, and the protective immune response of the fish in this study.

1.4 Hypotheses of the study

The hypotheses of this study are outlined as follows:

Hypothesis 1:
H0: Transgenic *Nannochloropsis* sp. transformed using vectors VCP_OMPK_ZA1 does not carry OmpK gene fragment to deliver to the fish
H1: Transgenic *Nannochloropsis* sp. transformed using vectors VCP_OMPK_ZA1 harbored OmpK gene fragment to deliver to the fish

Hypothesis 2:
H0: Zebrafish vaccinated with transgenic *Nannochloropsis* sp. bioencapsulated *Artemia* have a low survival rate when challenged with pathogenic *Vibrio harveyi* and do not induce the immune response of the fish against vibriosis
H1: Zebrafish vaccinated with transgenic *Nannochloropsis* sp. bioencapsulated *Artemia* have a high survival rate when challenged with pathogenic *Vibrio harveyi* and are able to trigger an immune reaction of the fish against vibriosis

1.5 Objectives of study

The objective of this study is to bioencapsulate transgenic *Nannochloropsis* sp. harboring the OmpK gene fragment originated from *Vibrio* sp. with *Artemia* for oral vaccine delivery to fish.

The specific objectives of this project were:

1. To transform *Nannochloropsis* sp., with vectors VCP_OMPK_ZA1 harboring OmpK gene fragment
2. To bioencapsulate transgenic *Nannochloropsis* sp. with *Artemia*
3. To evaluate the effects of transgenic microalgae-encapsulated *Artemia* in inducing zebrafish immune system

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