INCORPORATION OF TRANSGENIC *Nannochloropsis* sp. INTO FISH FEED AS VACCINE DELIVERY SYSTEM AGAINST VIBRIOSIS

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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Chairman : Zetty Norhana Balia Yusof, Ph.D.
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Oral vaccination is one of the potential methods for preventing disease outbreaks, and this method is easier and causes less stress to the fish. However, there is a limitation for oral vaccination which the antigens were easily degraded due to the high acidity of the fish gut. To overcome this limitation, microalgae were used as a vaccine delivery system which it could provide antigen protection from being degraded. Thus, this study aims to evaluate the efficacy of oral vaccines on zebrafish, used as a host model against vibriosis, by incorporating transgenic *Nannochloropsis* sp. into fish feed. In this research, *Nannochloropsis* sp. was transformed with a designated vector (VCP_OmpK_ZA1) harbouring *OmpK* gene fragment, a gene coding for antigenic property protein from *Vibrio harveyi*. 10% of the verified transgenic *Nannochloropsis* sp. were mixed with commercial fish feed (TL+CP). Adult zebrafish were fed with the formulated fish feed for 5 consecutive days in week 1, and the booster given in week 3. For the control groups, zebrafish were fed with 10% wild-type *Nannochloropsis* sp.-formulated feed (WT+CP) and commercial feed (CP). The feeding trials were performed until week 6. The zebrafish then were evaluated through weekly analysis expression of genes via RT-qPCR, and the relative percentage of survival (RPS) and histopathology was analyzed upon bacterial challenge with *V. harveyi* in week 6. Based on the result obtained, the *IgZ* gene was significantly upregulated by 4-fold after 8 days post-first dose of vaccination while *TNFα* and *IL-1β* genes were slightly upregulated after 1 day post-second dose of vaccination by 1.4-fold and 1.6-fold respectively. The RPS of the vaccinated fish (TL+CP) and unvaccinated fish (CP+WT) were 83% and 42%, respectively. For the histopathology analysis, the unvaccinated fish (CP and CP+WT) had severe histopathological changes and vaccinated fish (CP+TL) showed minor histopathological changes. This study has demonstrated that the transgenic microalgae-formulated feed has the potential to stimulate the immune response of the fish.

Keywords: Fish feed**,** *Nannochloropsis* sp., Oral vaccination, Transgenic, Vibriosis

SDG: GOAL 14: Life Below Water

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

PENAMBAHAN *Nannochloropsis* sp. TRANSGENIK KE DALAM MAKANAN IKAN SEBAGAI SISTEM PENYAMPAIAN VAKSIN UNTUK MELAWAN VIBRIOSIS

Oleh

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Pemvaksinan oral adalah salah satu kaedah yang berpotensi untuk mencegah wabak penyakit, dan kaedah ini lebih mudah dan mengurangkan tekanan kepada ikan. Walau bagaimanapun, terdapat had untuk vaksinasi oral yang mana antigennya mudah terdegradasi kerana keasidan yang tinggi di dalam usus ikan. Untuk mengatasi had ini, mikroalga digunakan sebagai sistem penyampaian vaksin yang boleh memberikan perlindungan kepada antigen daripada terdegradasi. Oleh itu, kajian ini bertujuan untuk menilai keberkesanan vaksin oral pada ikan zebra yang digunakan sebagai model haiwan untuk melawan penyakit vibriosis, dengan mencampurkan transgenik *Nannochloropsis* sp. ke dalam makanan ikan. Dalam penyelidikan ini, *Nannochloropsis* sp. telah diubah dengan vektor (VCP_OmpK_ZA1) yang membawa serpihan gen *OmpK*, gen pengekodan protein antigen daripada *Vibrio harveyi*. 10% daripada *Nannochloropsis* sp. transgenik yang disahkan dicampur dengan makanan ikan komersial (TL+CP). Ikan zebra dewasa diberi makan dengan makanan ikan yang diformulasikan ini selama 5 hari berturut-turut pada minggu pertama, dan vaksin penggalak diberikan pada minggu ketiga. Bagi kumpulan kawalan, ikan zebra diberi makan dengan 10% makanan rumusan *Nannochloropsis* sp.- jenis liar (WT+CP) dan makanan ikan komersial (CP). Eksperimen pemberian makanan ini berlangsung sehingga minggu ke-6. Ikan zebra kemudiannya dinilai menerusi ekspresi analisis gen melalui RT-qPCR pada setiap minggu, kemandirian peratus relatif (KPR) dan histopatologi pula dinilai setelah menjalani cabaran bakteria dengan *V. harveyi* pada minggu ke-6. Berdasarkan keputusan yang diperoleh, gen IgZ meningkat dengan ketara sebanyak 4 kali ganda selepas 8 hari selepas dos vaksinasi yang pertama. Bagi gen *TNFα* dan *IL-1β* turut meningkat sebanyak 1.4 kali ganda dan 1.6 kali ganda masing-masing selepas 1 hari diberikan dos penggalak. KPR ikan yang divaksin (TL+CP) dan ikan yang tidak divaksinasi (CP+WT) masing-masing adalah 83% dan 42%. Untuk analisis histopatologi, ikan yang tidak divaksinasi (CP dan CP+WT) mengalami perubahan histopatologi yang teruk dan ikan yang divaksin (CP+TL) menunjukkan perubahan histopatologi yang masih terkawal. Kajian ini telah menunjukkan bahawa

makanan yang dirumuskan oleh mikroalga transgenik berpotensi untuk merangsang tindak balas imun ikan.

Kata Kunci: Makanan Ikan, *Nannochloropsis* sp., Transgenik, Vibriosis, Vaksinasi oral

SDG: GOL 14: Hidupan bawah air

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Date: 16 May 2024

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LIST OF ABBREVIATIONS

%	Percentage
°C	Degree Celsius
A	Absorbance
Bp	Base pair
cDNA	Complementary DNA
Cells/ml	Cells per millilitre
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
L	Litre
min	Minutes
ml	Milliliter
NaCl	Sodium chloride
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
Rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid

RT-PCR	Reverse-transcriptase PCR
s	Seconds
sp.	Species
UPM	Universiti Putra Malaysia
UV-Vis	Ultraviolet-visible
V	Volt
μg	Microgram
μl	Microlitre

CHAPTER 1

INTRODUCTION

1.1 Background Study

In Malaysia, the total aquaculture production in 2021 was 1.75 million tonnes which comprises 1.33 million tonnes of fish captured and 417 187 million of farmed fish (Department of Fisheries Malaysia [DOF], 2022). Other than that, in 2021, 24 million production of aquatic plants and 242 million ornamental fish were recorded. The total sales recorded for all of the aquaculture productions were approximately RM 14.8 billion (DOF, 2022). This shows that aquaculture production in Malaysia is significant and profitable and this sector needs to be sustained throughout the year, especially for food production.

In the fish farm, there is a higher possibility for infectious diseases to occur as the fish are more susceptible to bacterial pathogens due to the higher density of the farmed fish and the limitation of using clean water (Watts *et al*., 2017). The common bacterial pathogens that cause fish disease are *Vibrio*, *Edwardsiella, Aeromonas,* and *Streptococccus* (Sudheesh *et al*., 2012; Wamala *et al*., 2018). *Vibrio* spp. is a significant pathogenic microbe that can cause severe impacts on the health of both animals and humans. The outbreak of vibriosis disease raises concerns, especially when it affects a large number of fish, particularly in fish farms, as it can cause mass mortality, leading to huge losses in the aquatic industry.

Antibiotics have been widely introduced for controlling diseases; however, it is difficult to determine the suitable amount to completely diminish the pathogens. Furthermore, extensive use of antibiotics will result in the development of antibiotic-resistant bacteria which can cause harm to humans, as well as the environment. In Peninsular Malaysia, tetracycline is one of the common antibiotics used to treat and prevent disease outbreaks in aquatic animals. A study by Thiang *et al.* (2022), detected low concentrations of tetracycline residues in five farms in the region, suggesting its usage in the area. Additionally, the study also reported that out of 93 different bacteria types, 48 were found to carry the *tet* gene, including *Enterobacter, Vibrio, Photobacterium,* and *Pseudoalteromona*. The presence of tetracycline residues and the emergence of bacteria carrying the *tet* gene pose risks to aquatic plants, such as algae. These findings suggest that the use of antibiotics may have contributed to the development of antibiotic-resistant pathogens. Therefore, the utilization of antibiotics for treating or preventing disease outbreaks in aquatic animals needs to be carefully regulated and monitored.

Vaccination is another strategy for treating bacterial and viral diseases. Vaccination works by stimulating the immune system of the fish to produce antibodies against the pathogen which will protect the fish from the pathogen as well as prevent the disease from recurring (Mondal and Thomas, 2022). Most commercial vaccines are made of inactivated bacterial pathogens, and they are introduced into the fish through injection (Adam *et al.* 2021, Du *et al.* 2022) However, this practice is costly and reduces the vaccine efficacy as it introduces stress to the fish.

Recently, an alternative was developed by introducing genetically modifying microalgae *Nannochloropsis* sp. into a vaccine carrier to vaccinate the fish orally (Abidin *et al*., 2020b; Abidin *et al*., 2021a). This invention is known to be a promising technology that could become one of the practical methods for vaccinating a larger amount of fish or small-sized fish, helping to reduce the use of antibiotics in aquatic animals. Other than that, the features of the microalgae itself are potential for vaccine candidates as they have rigid cell walls which can protect the antigen from easily degraded and improve the efficiency of the vaccine effects on fish (Kwon et al., 2013; Specht & Mayfield, 2014). Also, *Nannochloropsis* sp. plays a significant role in nutrition enrichment for aquatic animals as they have high lipid content and high levels of eicosapentaenoic fatty acid (Guimarães et al., 2021). Thus, by utilizing transgenic *Nannochloropsis* sp. as an oral vaccine for the fish, they will provide protection as well as nutrition to the fish. However, studies on using the microalgae such as *Nannochloropsis* sp. as a vaccine delivery system for vaccinating the fish via oral route are still lacking.

1.2 Problem Statements

The problem statements in this research study were:

1. Outbreak of the diseases will cause a huge loss of aquaculture production.

2. A long-term treatment with an antibiotic will lead to the development of antibiotic- resistance bacteria.

3. Oral vaccination has low efficiency as the oral vaccine tends to degrade in the fish gut due to high acidity.

4. Lack of studies related to the utilization of the microalgae as a vaccine delivery system for fish

1.3 Objective

The research on the development of transgenic *Nannochloropsis* sp. by Abidin *et al*. in 2021a had not been tested on fish. Therefore, this study aimed to continue exploring the effectiveness of the newly developed vaccine. The primary objective was to evaluate the efficacy of oral vaccines on zebrafish, used as a host model against vibriosis, by incorporating transgenic *Nannochloropsis* sp. into their feed.
The specific objectives for this research study were:

1. To transform *Nannochloropsis* sp. with vector VCP_OMPK_ZA1 harbouring OmpK gene fragment.

2. To formulate a feed-based oral vaccine and evaluate the efficiency of the transgenic *Nannochloropsis* sp. – formulated feed in inducing an immune response in zebrafish through gene expression analysis (Rt-qPCR), relative percentage survival (RPS) evaluation and histopathological changes.

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