UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF FEED-BASED WHOLE-CELLS INACTIVATED BIVALENT VACCINE AND ITS IMMUNOPROTECTIVE ABILITY AGAINST STREPTOCOCCOSIS AND MOTILE AEROMONAD SEPTICEMIA IN RED HYBRID TILAPIA (Oreochromis spp.)

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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Faculty : Agriculture

Streptococcosis and motile aeromonad septicemia (MAS) are well-known bacterial diseases in tilapia culture, which cause mass mortality with significant economic losses to aquaculture globally. As therapy resistance is an increasing problem, development of efficient fish vaccines seems to be an alternative to minimize the streptococcosis and MAS diseases. The development of feed-based monovalent vaccines in controlling these diseases has been attempted; however, the mechanism of immunity of feed-based bivalent vaccine against streptococcosis and MAS infections, and the cross-protective ability of these two diseases are still understudied. To explore the immunological role of the feed-based bivalent vaccine, we compared the immune responses of red hybrid tilapia after immunization with both feed-based bivalent and monovalent vaccines, and compared the relative percentage survival (RPS) and cross-immunization protections of red hybrid tilapia following challenged with *Streptococcus iniae*, *Aeromonas hydrophila*, *S. agalactiae* and *A. veronii*. A total of five groups of fish were vaccinated orally through two different techniques; bivalent vaccine (inactivated *S. iniae* and *A. hydrophila*) sprayed on feed pellets (BS group); bivalent vaccine (inactivated *S. iniae* and *A. hydrophila*) incorporated in fish feed (BI group); monovalent inactivated *S. iniae* and *A. hydrophila* vaccine separately incorporated into feed as monovalent *S. iniae* (MS group) and monovalent *A. hydrophila* (MA group); and control group (without vaccine). The feed based vaccines were delivered orally at 5% of body weight for five consecutive days and also the double booster doses were administered in the same manner on weeks 2 and 6. The haematological results revealed that BI vaccinated group exhibited significantly the highest ($P < 0.05$) number of leucocytes ($45.39 \pm 1.34 \times 10^3/\mu L$) and granulocytes ($7.68 \pm 0.29 \times 10^3/\mu L$) on weeks 3 post-vaccination. The lysozyme activity demonstrated a significant ($P < 0.05$) increase in BI vaccinated group particularly on 8 (313.77 units/mL) and 12 (303.62 units/mL) weeks post-vaccination. The significantly ($P < 0.05$) highest phagocytic activity was also observed in BI group (53.83%), while the lowest was obtained in BS group (37.33%) on weeks 12 post-vaccination. The

enzyme-linked immunosorbent assay (ELISA) analysis showed that BI group developed a strong and significantly ($P < 0.05$) higher systemic and mucosal IgM responses against both *S. iniae* and *A. hydrophila*, and also cross-protective antigen *S. agalactiae* and *A. veronii* as compared to the BS vaccine and unvaccinated groups. On weeks 10 post-vaccination, all fish were challenged through the intraperitoneally (i.p.) route, where relative percentage survival (RPS) in the BI vaccinated group were observed 82.22 ± 3.85% when challenged with *S. iniae*, 77.78 ± 3.85% when challenged with *A. hydrophila* and 77.78 ± 3.85% when co-challenged with both *S. iniae* and *A. hydrophila*, which were significantly higher ($P < 0.05$) compared to the other groups. Simultaneously, the BI vaccinated group also showed significantly ($P < 0.05$) higher partial cross-protections following challenges with *S. agalactiae* (RPS at 60.00 ± 6.67%) and *A. veronii* (RPS at 57.78 ± 7.70%). Quantitative real-time PCR results also showed that the relative expressions of IL-1β, C-type lysozyme, TNF-α, TGF-β, CD4, MHC-I, MHC-II and IgT genes in the BI vaccinated fish spleen, head kidney and hindgut exhibited various significant ($P < 0.05$) rising trends following both the early-phase vaccination and post-infections. Notably, the highest relative expression of IL-1β, MHC-II and IgT genes in BI vaccinated group were observed in the co-infected (*S. iniae* and *A. hydrophila*) fish spleen (9.8 - fold), head kidney (9.6 - fold) and hindgut (24.5 - fold), respectively.

Combining our results demonstrate that the BI vaccine could elicit significant immunological responses and this vaccine is highly effective to control *S. iniae* and *A. hydrophila* virulence in red hybrid tilapia, but have moderate efficacy when challenged with *S. agalactiae* and *A. veronii*. Nevertheless, this newly developed feed-based bivalent incorporated (BI) vaccine can effectively protect tilapia against streptococcosis and MAS infections, and also could offer a promising strategy for mass fish vaccination in aquaculture industry.

Keywords: Feed-based; Bivalent vaccine; IgM responses; Cross-protection; Gene expression; Red hybrid tilapia

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PENGHASILAN VAKSIN SEL UTUK TIDAK AFTIF SECARA BIVALEN BERASASKAN MAKANAN SERTA KEUPAYAAN IMUNOPROTEKTIFNYA DALAM MENENTANG JANGKITAN STREPTOKOKUSIS DAN AEROMONAD SEPTISEMIA MOTIL PADA TILAPIA MERAH HIBRID *(Oreochromis* spp.)

Oleh

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Jun 2021

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Penyakit bakteria, streptococcus and aeromonad septicemia motile (MAS) cukup sinonim dalam kultur ikan tilapia. Penyakit bakteria ini menyebabkan kematian ikan yang sangat besar dengan kerugian yang signifikan dari sudut ekonomi bagi industri akuakultur di peringkat global. Oleh kerana rintangan terhadap terapi semakin meningkat, penghasilan vaksin ikan yang efisien dilihat sebagai alternatif bagi meminimumkan jangkitan penyakit seperti streptokokus dan MAS. Penghasilan vaksin monovalen berasaskan makanan dalam mengawal penyakit-penyakit ini telah dijalankan; namun, mekanisme imuniti disebalik vaksin bivalen berasaskan makanan terhadap streptokokus dan jangkitan MAS, dan kemampuan perlindungan silang kedua penyakit ini masih kurang difahami. Bagi meneroka peranan imunologi vaksin bivalen berasaskan makanan, kami membandingkan tindak balas imun dari tilapia merah hibrid selepas imunisasi dengan vaksin bivalen dan monovalen berasaskan makanan, dan membandingkan kelangsungan peratusan relatif (RPS) dan perlindungan silang-imunisasi tilapia merah hibrid selepas dicabar dengan bakteria *Streptococcus iniae*, *Aeromonas hydrophila*, *S. agalactiae* dan *A. veronii*. Sejumlah lima kumpulan ikan divaksinasi berasaskan makanan melalui dua teknik yang berbeza; vaksin bivalen (*S. iniae* dan *A. hydrophila* yang tidak aktif) disembur pada pelet makanan (kumpulan BS); vaksin bivalen *(S. iniae* dan *A. hydrophila* yang tidak aktif) yang dimasukkan dalam makanan (kumpulan BI); vaksin monovalent *S. iniae* dan *A. hydrophila* yang tidak aktif secara berasingan dimasukkan ke dalam makanan sebagai monovalen *S. iniae* (kumpulan MS) dan monovalen *A. hydrophila* (kumpulan MA); dan kumpulan kawalan (tanpa vaksin). Vaksin berasaskan makanan tersebut diberikan secara oral pada kadar 5% dari berat badan selama lima hari berturut-turut dan juga dos pengganda diberikan dengan cara yang sama pada minggu ke-2 dan 6. Hasil hematologi pula menunjukkan kumpulan vaksin BI memberikan jumlah leukosit tertinggi ($P < 0.05$) ($45.39 \pm 1.34 \times 10^3$/µL) dan granulosit ($7.68 \pm 0.29 \times 10^3$/µL) pada minggu ke-3 selepas vaksinasi. Aktiviti lisozim

menunjukkan peningkatan yang signifikan ($P < 0.05$) dalam kumpulan vaksin BI terutamanya pada minggu ke-8 (313.77 units/mL) dan minggu ke-12 (303.62 units/mL) selepas vaksinasi. Aktiviti fagositik tertinggi ($P < 0.05$) yang tinggi juga diperhatikan pada kumpulan BI (53.83%), sementara yang terendah diperoleh pada kumpulan BS (37.33%) pada minggu ke-12 selepas vaksinasi. Analisis imunosorben berkait enzim (ELISA) menunjukkan bahawa pemberian dos pertama vaksin berasaskan makanan telah merangsang tahap antibodi IgM yang berlanjutan sehingga minggu ke-4, sementara penggalak kedua memastikan tahap IgM tetap tinggi selama 16 minggu pada Kumpulan vaksin BI dan MS. Di samping itu, kumpulan BI telah membentuk tindak balas IgM sistemik (serum) dan mukosa (lendir badan dan cecair lavaj usus) yang kuat dan lebih tinggi ($P < 0.05$) secara signifikan terhadap *S. iniae* dan *A. hydrophila* dan juga memberikan perlindungan silang terhadapa antigen *S. agalactae* dan *A. veronii* berbanding dengan kumpulan BS dan kawalan. Pada minggu ke-10 selepas vaksinasi, semua ikan dicabar melalui intraperitoneum (i.p.), di mana peratusan survival relatif (RPS) dalam kumpulan vaksin BI dilihat pada kadar 82.22 ± 3.85% ketika dicabar dengan *S. iniae*, 77.78 ± 3.85% ketika dicabar dengan *A. hydrophila* dan 77.78 ± 3.85% ketika dicabar bersama dengan *S. iniae dan A. hydrophila*, yang jauh lebih tinggi ($P < 0.05$) berbanding kumpulan lain. Pada masa yang sama, kumpulan yang diberi vaksin BI juga menunjukkan perlindungan silang separa yang lebih tinggi ($P < 0.05$) berikutan cabaran dengan *S. agalactiae* (RPS pada 60.00 ± 6.67%) dan *A. veronii* (RPS pada 57.78 ± 7.70%). Hasil PCR kuantitatif masa nyata juga menunjukkan bahawa gen IL-1β, lisozim jenis C, TNF-α, TGF-β, CD4, MHC-I, MHC-II dan IgT dalam limpa, ginjal bahagian atas dan usus bahagian belakang ikan yang divaksinasi BI menunjukkan corak peningkatan ekspresi yang pelbagai secara signifikan ($P < 0.05$) selepas vaksinasi fasa awal dan selepas jangkitan. Ekspresi relatif tertinggi gen IL-1β, MHC-II dan IgT dalam kumpulan vaksin BI yang dilihat tertinggi masing-masing pada limpa ikan (9.8 kali ganda), ginjal bahagian atas (9.6 kali ganda) dan usus bahagian belakang (24.5 kali ganda) yang dicabar secara rentas dengan *S. iniae* and *A. hydrophila*.

Gabungan hasil kajian kami menunjukkan bahawa vaksin BI dapat menimbulkan tindak balas imunologi yang signifikan dan vaksin ini sangat berkesan untuk mengawal jangkitan *S. iniae* dan *A. hydrophila* pada ikan tilapia merah hibrid, namun mempunyai keberkesanan sederhana ketika dicabar dengan *S. agalactiae dan A. veronii*. Walaupun begitu, vaksin bivalen (BI) berasaskan makanan yang baru dihasilkan ini dapat melindungi ikan tilapia merah hibrid dengan berkesan terhadap streptokokus dan jangkitan MAS, dan juga dapat menawarkan strategi vaksinasi ikan yang berkesan dan efektif dalam industri akuakultur.

Kata kunci: Vaksin berasaskan makanan; Vaksin bivalen; Tindak balas IgM; Perlindungan rentas; Ekspresi gen; Tilapia merah hibrid

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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s	percentage
µL	microlitre
µM	micromolar
®	registeredp
™	trademark
°C	degree celcius
A. hydrophila	*Aeromonas hydrophila*
APC	antigen-presenting cell
A. veronii	*Aeromonas veronii*
BSA	bovine serum albumin
BHIA	brain heart infusion agar
BHIB	brain heart infusion broth
BLAST	basic local alignment search tool
bp	base pair
$CaCl_2$	calcium chloride
CFU	colony forming units
CD4	cluster of differentiation 4
dATP	deoxyadenosine triphosphate
dNTP	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
ECPs	extracellular products
g	gram
GALT	gut-associated lymphoid tissue

H & E	haematoxylin and eosin
hpi	hour post infection
Ig	immunoglobulin
IL-1β	Interleukins -1 beta
IP	intraperitoneal
kb	kilobase pair
LD_{50}	median lethal dosage
LPS	lipopolysaccharide
M	molar
MAS	motile aeromonad septicemia
MHC	major histo-compatibility complex
mg	milligram
$MgCl_2$	magnesium chloride
mM	millimolar
MS-222	tricaine methanesulfonate solution
NaCl	sodium chloride
OMPs	outer membrane proteins
PAMPs	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RNA	ribonucleic acid
RPM	revolutions per minute
RPS	relative percentage survival
rRNA	ribosomal ribonucleic acid

S. agalactiae	*Streptococcus agalactiae*
S. iniae	*Streptococcus iniae*
sp	species
TGF-β	transforming growth factor-beta
TNF-α	tumor necrosis factor-alpha
TSA	tryptic soy agar
TSB	tryptic soy broth
V	voltage
v/v	volume per volume
wpv	week post vaccination
w/v	weight per volume

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Tilapia (*Oreochromis* spp.) is one of the most important freshwater fish species that is cultured intensively all over the world. The global production of cultured tilapia represent 6.93 million tons in 2020, around 40 percent of the combined production of the most important commercial whitefish species (FAO, 2021). Tilapia is very popular due to rapid growth, suitability for aquaculture, low production cost, high acceptability in the market (Gu et al., 2017; Dawood et al., 2019), and it is easy to adapt in tropical and sub-tropical regions of the world (Shelton, 2002). Subsequently, tilapia is increasingly demanded in global markets ranging from the poorest segments in developing countries to highly developed western markets (Aminudin, 2017). However, the production of tilapia is increasing day by day from the particular ecosystem, but this sector in globally has been facing several health risks for affecting with emerging and re-emerging bacterial diseases, which causes the severe economic losses in this industry (Adikesavalu et al., 2017; Vásquez-Machado et al., 2019).

Amongst infectious bacterial diseases, the occurrence of streptococcosis and motile aeromonad septicemia (MAS) have been characterized as one of the major obstacle to the sustainable establishment of tilapia sector particularly in Asian countries (Sumiati et al., 2015; Verner-Jeffreys et al., 2017; Pasaribu et al., 2018; Abdelkhalek et al., 2020; Han et al., 2020). Streptococcosis is a septicemic infection mainly by Gram-positive bacteria *Streptococcus agalactiae* and *S. iniae*, and major bacterial pathogens of tilapia that can cause severe symptoms, and result in high morbidities and mortalities rates of up to 60 - 80% (Noraini et al., 2013; Abdelkhalek et al., 2020; Wang et al., 2020a). In Malaysia, the outbreak of streptococcosis is frequently reported in red hybrid tilapia (*Oreochromis* spp.), particularly in dry and hot (April - June) seasons and causes economic losses every year in tilapia industry (Zamri-Saad et al., 2014; Ali et al., 2020; Syuhada et al., 2020). The annual global economic loss due to streptococcosis has been estimated at around USD 10 billion (Xu et al., 2007; Cui et al., 2019). In another bacterial disease of motile aeromonad septicemia (MAS) mainly by Gram-negative bacteria of *Aeromonas hydrophila*, recognized to have a devastating effect on tilapia production (Aboyadak et al., 2015; Pasaribu et al., 2018; Mahrous et al., 2020), and is also responsible for huge economic losses in the aquaculture sector globally (AlYahya et al., 2018; Han et al., 2020). The motile aeromonads of *A. veronii* and *A. sobria* also observed to cause adverse effects in tilapia farming (Dong et al., 2017; Amal et al., 2018; Chirapongsatonkul et al., 2019).

The mortality of cultured fish mostly in intensive farming system is not only caused by single bacteria, but most of the cases due to multiple co-infection with different bacteria (Dong et al., 2015). Tilapia mortalities due to bacterial co-infections has also been observed, such as co-infections of *Streptococcus* sp. and *Aeromonas* sp. (Sugiani et al.,

2012; Sumiati et al., 2015), *S. agalactiae* and *Francisella noatunensis* (Assis et al., 2017), *A. veronii* and *Flavobacterium columnare* (Dong et al., 2015), and *F. noatunensis* and *Shewanella putrefaciens* (Pradeep et al., 2016). In Malaysia, a recent report revealed that mass mortality due to co-infection of tilapia lake virus (TiLV) and *A. veronii* was observed in cultured red hybrid tilapia (Amal et al., 2018).

The most common practice in treating fish bacterial diseases of streptococcosis and MAS involves the application of different antibiotics (Darwish et al., 2002; Zamri-Saad et al., 2014; Assane et al., 2019). Unfortunately, serious complications raised through the spread of antibiotics resistance bacteria and the accumulation of antibiotics residues in the environment and food product, creating serious problem to human and environmental health (Defoirdt et al., 2011; Cabello et al., 2016; Stratev & Odeyemi, 2016a; Watts et al., 2017). Moreover, antibiotics are presently ineffective for controlling streptococcosis and MAS infections (Klesius et al., 2000; Musa et al., 2009; Aisyhah et al., 2014; Mahrous et al., 2020). Owing to the issues, the application of antibiotic is no longer encouraged. Therefore, vaccination is now considered as the best approach and feasible way to prevent bacterial diseases outbreaks.

To date, three widely vaccine delivery systems have been applied in aquaculture with variable success, depending on the nature of each vaccine and production stages of fish; injection, bath immersion and oral (Adams, 2019; Abu-Elala et al. 2019). In general, Southeast Asian countries particularly Indonesia, Malaysia, Thailand and Vietnam, most fish farmers operate on small-scale basis with little technical supports (Aminudin, 2017; Kayansamruaj et al., 2020). Thus, the costs of manpower and technical supports, and facilities required to carry out vaccination using injection and immersion routes are unaffordable (Aminudin, 2017; Ismail et al., 2017). However, the oral route is considered to be the ideal approach as it is less stressful, more practical, applicable to all sized fish, mass immunization of large scale fish, reduces the cost of labour and provide a good technique for boosting several times during culture period in ponds and cages (Firdaus-Nawi et al., 2014; Laith et al., 2019; Jun et al., 2020; Sotomayor-Gerding et al., 2020). Moreover, vaccines administered via oral route have the ability to elicit both mucosal and systemic immunities; thereby protecting the fish at the portal of entry of the pathogen and preventing the spread of infections systemically (Munang'andu et al., 2015; Somamoto & Nakanishi, 2020). As for now, Chile, Scotland and Norway are the only few countries that apply feed-based vaccines for fish (Laith et al., 2019). The limited application of this feed-based vaccine could be due to little information and research documentation on the assessment of immunogenicity and protective efficacy of feed-based vaccination in different cultured fish other than injection and immersion vaccination methods.

Inactivated vaccines prepared in formalin solution have been widely applied in aquaculture in recent decades and reached a satisfactory level of protections against pathogens (Nguyen et al., 2017; Wei et al., 2020). Meanwhile, some previous studies have observed that inactivated vaccine combined with adjuvant could provide more effective protections to prevent bacterial diseases compared to the use of alone vaccine (Nguyen et al., 2017; Xu et al., 2019a). Thus, commercially available different fish vaccines are currently developed from different mineral oil as an adjuvant, whereas these

oils can cause damage to fish, with consequent retardation of growth and negative effects on the welfare of the fish (Mutoloki et al., 2006; Heegaard et al., 2011; Xu et al., 2019a; Li et al., 2020). On the other hand, Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FIA) are very effective in fish vaccination with low toxicity levels (Tafalla et al., 2013; Jiang et al., 2015; Wang et al., 2016), however, it extremely expensive, particularly for the commercial fish vaccine development (Aminudin, 2017). Some studies showed palm oil as an adjuvant for newcastle disease virus (NDV) vaccine in chicken and caseous lymphadenitis vaccine in rat model could effectively enhance immune protection without any side effects (Wanasawaeng et al., 2009; Roslindawani et al., 2016). Therefore, palm oil can be a potential adjuvant for fish vaccine that could stimulate good immunities and subsequent protections at a cheaper rate (Roslindawani et al., 2016; Aminudin et al., 2018).

1.2 Problem statement

A number of monovalent vaccines have been developed to combat *S. iniae*, *S. agalactiae* (Klesius et al., 2000; Chen et al., 2012; Diab et al., 2019) and *A. hydrophila* (Aly et al., 2015; Sukenda et al., 2017a). Moreover, the effective commercial streptococcosis vaccines such as AQUAVAC® Strep Sa (Merck Animal Health Company, USA) and NORVAX® STREP Si (Merck Animal Health Company, USA), are administered via intraperitoneal (i.p.) and immersion routes, respectively. These developed monovalent vaccines have high protective properties against single target bacteria through injection or immersion immunization, but they are unable to protect multiple bacteria through a single vaccination. However, considering the cost and timing of fish vaccination, developing feed-based bivalent or cross-protective vaccines to prevent these diseases in a single immunization is desirable (Bastardo et al., 2012; Shoemaker et al., 2012; Abu-Elala et al., 2019; Guo et al., 2020).

The commercial adjuvants of FCA, FIA and montanide have been currently used for developing fish vaccines against *S. iniae* (Wang et al., 2016), *S. agalactiae* and (Abu-Elala et al. 2019) and *A. hydrophila* (Heloisa et al., 2020), respectively and showed 70 - 75% relative percent survival (RPS) with long-term protection, but the cost of using these commercial adjuvants in fish vaccines is too expensive. Thus, we require to find a lower ratio of adjuvant to minimize the cost of vaccine production and give comparable protection to the fish. Due to the increasing awareness of animal welfare and environmental issues, a safer and natural ingredient of palm oil was selected as the best candidate to substitute the present expensive commercial adjuvants. However, this research provides a better understanding of the use of feed-based palm oil combined bivalent vaccine that is capable of providing adequate immuno-protective against streptococcosis and MAS infections.

1.3 Justification of the research

To date from our knowledge, there is no report that has been claimed to develop feed-based bivalent vaccine, which can confer protection against *S. iniae*, *A. hydrophila* and cross-protection ability of *S. agalactiae* and *A. veronii* in red hybrid tilapia. However,

preliminary investigations performed by Wang et al. (2020b) suggest sufficient antigenic similarity exists between *S. iniae* and *S. agalactiae*, and cross-protection was observed, as the *S. iniae* monovalent vaccine protected fish from *S. agalactiae* infection (Diab et al. 2019; Rahmatullah, 2018). On the other hand, cross-protection of *A. hydrophila* vaccine against *A. veronii* in tilapia has not been reported. Given the increased incidence and prevalence of *S. agalactiae* and *A. veronii* also associated with red hybrid tilapia production in Malaysia, it would be fortuitous if the newly developed feed-based bivalent vaccine based on whole-cells inactivated *S. iniae* and *A. hydrophila* would provide adequate cross-protection against *S. agalactiae* and *A. veronii*.

Moreover, as described by Behera & Swain (2014), Firdaus-Nawi et al. (2014), Dubey et al. (2016) and Wang et al. (2018a), developed feed-based adjuvant added monovalent vaccine or antigen encapsulated oral vaccines those stimulated immune responses with high protection against target bacterial strains in fish. In general however, the manufacture of these non-cross-protective encapsulated or commercial adjuvant added oral vaccine formulations is elaborate and expensive, and could protect only one pathogen with a single vaccination. In order to fill in the research gap, the main purpose of this study is to develop a feed-based bivalent or cross-protective vaccine formulated from two prominent pathogenic bacteria, *S. iniae* and *A. hydrophila*, which can be a novel vaccine strategy for the prevention of both streptococcosis and MAS infections in the aquaculture industry.

1.4 Objectives

The general objective of this study is to develop a feed-based bivalent vaccine combined palm oil as an adjuvant, which can protect against two major bacterial diseases of streptococcosis and motile aeromonad septicemia (MAS) in red hybrid tilapia (*Oreochromis* spp.).

There are four specific objectives of this study, which are stated as below:

1) to develop a feed-based palm oil combined bivalent vaccine containing whole-cells inactivated *Streptococcus iniae* and *Aeromonas hydrophila.*
2) to assess the systemic and mucosal immune responses by red hybrid tilapia (*Oreochromis* spp.) following oral vaccination using the newly developed feed-based bivalent vaccine.
3) to evaluate the effectiveness of the newly developed feed-based bivalent vaccine in protecting red hybrid tilapia (*Oreochromis* spp.) against *S. iniae, A. hydrophila, S. agalactiae* and *A. veronii* infections.
4) to analyze the relative expression of immune-related genes at early phase-immunization and post-infections by quantitative real-time PCR in feed-based bivalent vaccinated red hybrid tilapia (*Oreochromis* spp.).

1.5 Research hypotheses

Hypothesis 1:

H_0: The newly developed feed-based bivalent vaccines against streptococcosis and motile aeromonad septicemia (MAS) are not safe and negative impact on growth performances in red hybrid tilapia (*Oreochromis* spp.).

H_a: The newly developed feed-based bivalent vaccines against streptococcosis and motile aeromonad septicemia (MAS) are safe and no negative impact on growth performances in red hybrid tilapia (*Oreochromis* spp.).

Hypothesis 2:

H_0: Vaccination of red hybrid tilapia (*Oreochromis* spp.) with newly developed feed-based bivalent vaccine could not induce certain level of systemic and mucosal immune responses against *Streptococcus* sp. and *Aeromonas* sp.

H_a: Vaccination of red hybrid tilapia (*Oreochromis* spp.) with newly developed feed-based bivalent vaccine could induce certain level of systemic and mucosal immune responses against *Streptococcus* sp. and *Aeromonas* sp.

Hypothesis 3:

H_0: Vaccination with the newly developed feed-based bivalent vaccine could not confer better protection against *S. iniae* and *A. hydrophila* as well as cross-protection against *S. agalactiae* and *A. veronii* in red hybrid tilapia (*Oreochromis* spp.).

H_a: Vaccination with the newly developed feed-based bivalent vaccine could confer better protection against *S. iniae* and *A. hydrophila* as well as cross-protection against *S. agalactiae* and *A. veronii* in red hybrid tilapia (*Oreochromis* spp.).

Hypothesis 4:

H_0: The newly developed feed-based bivalent vaccine could not induce the expression of immune-related genes at early phase-immunization and post-infections in vaccinated red hybrid tilapia (*Oreochromis* spp.).

H_a: The newly developed feed-based bivalent vaccine could induce the expression of immune-related genes at early phase-immunization and post-infections in vaccinated red hybrid tilapia (*Oreochromis* spp.).

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Md. Shirajum Monir was born on November 3, 1985 in Thakurgaon, Bangladesh. He completed his primary, secondary and higher secondary education in the same district. Then, he pursued his study at Bangladesh Agricultural University, Bangladesh for First degree in Bachelor of Science (Fisheries) (Hons) and Master of Science degree in Aquaculture at same university. Previously, he has experienced working at Department of Fisheries (DoF), Bangladesh for almost 1.5 years as Extension Officer. Thereafter, he appointed to hold the position as Senior Scientific Officer in Bangladesh Fisheries Research Institute (BFRI) from May 2010 until recent years.

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Patent

Composition and method for preparing and using oral vaccine for vibriosis in marine fish. Inventors: Ina-Salwany, M.Y., Zamri-Saad, M., Amal, M. N. A. Al-sarri, N. B., Mohamd, M. A. & Monir, M. S. Patent No:PA-2010/024, University Putra Malaysia, Malaysia and Date of Patent: Jan. 08, 2021.

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