UNIVERSITI PUTRA MALAYSIA

EFFICACY OF FEED-BASED MONOVALENT AND MULTIVALENT VACCINES AGAINST BACTERIAL DISEASES IN Epinephelus fuscoguttus (FORSSKAL, 1775) X E. lanceolatus (BLOCH, 1790) (GROUPER) AND Lates calcarifer (BLOCH, 1790) (SEABASS)

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By

MOHAMAD ASLAH BIN MOHAMAD

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

February 2022

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

EFFICACY OF FEED-BASED MONOVALENT AND MULTIVALENT VACCINES AGAINST BACTERIAL DISEASES IN *Epinephelus fuscoguttus* (FORSSKAL, 1775) X *E. lanceolatus* (BLOCH, 1790) (GROUPER) AND *Lates calcarifer* (BLOCH, 1790)

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February 2022

Chair : Assoc. Prof. Ina Salwany Md Yasin, PhD
Institute : Bioscience

Vibriosis, streptococcosis, and MAS are commonly isolated from disease outbreaks in aquaculture farms in Malaysia. The diseases lead to significant economic loss in the country. It is critical to develop highly effective vaccines against these diseases to avoid antibiotics. However, limitations in vaccine development such as injection vaccination, using adjuvants that may stimulate adverse reactions in the host, and using non-cross protective antigens may slow the vaccine development. Therefore, the objectives of the study were (1) to develop a feed-based palm oil adjuvanted multivalent vaccine containing whole cells of inactivated *Vibrio harveyi* strain Vh1, *Streptococcus agalactiae* strain Sa2k, and *Aeromonas hydrophila* strain Ah1sa5, and a feed-based cross-protective monovalent vaccine containing whole cells of inactivated *V. harveyi* strain Vh1, (2) to assess the specific immune response and the relative expression of immune-related genes following vaccination with the feed-based vaccines, (3) to evaluate the protective efficacy of the feed-based vaccines following experimental challenges with multiple *Vibrio spp.* in Asian seabass, *Lates calcarifer* and (4) to evaluate the field efficacy of the feed-based vaccines against vibriosis in farmed hybrid grouper (*Epinephelus fuscoguttus* × *E. lanceolatus*). Briefly, a feed-based multivalent vaccine was developed from formalin-inactivated *V. harveyi* strain Vh1, *S. agalactiae* strain Sa2k, and *A. hydrophila* strain Ah1sa5 incorporated into powdered feed with the addition of palm oil adjuvant. While the monovalent vaccine was developed from formalin-inactivated *V. harveyi* strain Vh1, incorporated into powdered feed with palm oil adjuvant. A commercial feed pellet (Star Feed, Klang, Malaysia) was used as a negative control. The feed-based vaccines and control feed was later assessed for feed quality and safety analysis. The results revealed that the feed-based vaccines contained good nutritional compositions, even after reformulations. Besides, the vaccinated feed was tested to be safe for fish consumption and showed positive effects on fish growth. Further analysis was done on an immune

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response study with 450 Asian seabass, *Lates calcarifer* juveniles (15.8±2.6g), divided equally into three groups with triplicate. Group 1 was fed with multivalent vaccinated feed, Group 2 with *V. harveyi* monovalent vaccinated feed, and Group 3 with commercial feed. Vaccinations were carried out by feeding the fish with the feed-based vaccine on days 0 and 14 at 5% body weight. Serum and gut samples were collected weekly for IgM antibody, lysozyme activity, and gene expression analysis. A challenged trial was conducted on week 4 post-immunization. The results showed that the specific IgM antibody and non-specific lysozyme activity levels in the serum of the multivalent and monovalent vaccinated groups were more highly stimulated than the control group at most of the sampling period. Besides, the vaccinated fish showed significant upregulation ($P < 0.05$) of the immune-related genes with the highest immune gene expression in the multivalent vaccinated group was seen in the Dendritic Cells gene at week 6 (11.47- fold), followed by the CCL4 gene (7.79-fold) at week 4. While the highest expression in the monovalent vaccinated group was seen in the MHC-I gene at week 3 (5.23-fold), followed by the C3 gene at week 4 (3.67-fold). Bacterial challenge against *V. harveyi*, *S. agalactiae,* and *A. hydrophila* showed that the multivalent vaccine gave an RPS value of 75±7.07%, 85± 7.07% and 70±0.00% against *V. harveyi*, *S. agalactiae* dan *A. hydrophila*, respectively while 70±0.00% against the combined bacteria, while the monovalent vaccine gave 70±0.00% against *V. harveyi* infection. Despite the vast potential applications of the multivalent vaccine against multiple bacterial infections, this current study focuses on the efficacy of the feed-based multivalent vaccine against major *Vibrio* spp in cultured marine fishes in Malaysia, such as *V. harveyi*, *V. alginolyticus,* and *V. parahaemolyticus*. The monovalent *V. harveyi* vaccine was also tested in this study to determine its cross-protective efficacy against multiple *Vibrio* spp. and compared with the multivalent vaccine to determine whether the combined vaccines could induce a more robust immune response without antigen competition. This study revealed significantly higher antibody levels ($P < 0.05$) in the multivalent and monovalent vaccinated fish following the double booster regiment. Post-challenge with specific bacteria at week 10 showed that the IgM antibody levels in serum, body mucus, and gut lavage fluid of the multivalent and monovalent vaccinated group were significantly higher until week 13 than in the control groups. The results were supported by the bacterial challenge study with multiple *Vibrio* spp. The multivalent vaccine an RPS value of 85±7.07%, 80± 0.00% and 75±7.07% against *V. harveyi*, *V. parahaemolyticus,* and *V. alginolyticus*, respectively while 70±0.00% against the combined bacteria, while the monovalent vaccine gave 75±7.07%, 75±7.07% and 70±0.00% against *V. harveyi*, *V. parahaemolyticus,* and *V. alginolyticus*, respectively and 70±0.00% against the combined *Vibrio* spp. infection. Finally, a field trial was conducted on a mariculture farm to study the field efficacy of the multivalent and monovalent vaccines under commercial fish farming conditions. The stimulation of the immune system in the multivalent and monovalent vaccinated group was shown by the increase of the IgM levels after immunization as early as week 2, peaked at week 6, and remained significantly high until week 12. The antibody stimulation indicates that the protection might be possible for a period of 12 weeks (3 months). The vaccine groups resulted in a higher survival rate than the control groups, with 79.75 ±0.07% survival in the multivalent vaccine group and 75.25±4.75% survival in the monovalent vaccine group compared to 65.10±0.14% in the control group after a 16-week field trial. Furthermore, better feed conversion efficiency and

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weight gain were clearly shown in the vaccine groups. Combining the results reveals that the feed-based multivalent and monovalent vaccine effectively induced an immune response against vibriosis, and the multivalent vaccine is potentially effective against streptococcosis and MAS. The monovalent vaccine was found to be as good as the multivalent vaccine against vibriosis, and the combined antigens in the multivalent vaccine do not significantly contribute to higher antibody stimulation and protection against the *Vibrio* spp than the monovalent vaccine. However, the multivalent vaccine could provide wider protection against other diseases than the monovalent vaccine. Both feed-based vaccines are highly potential for commercialization due to their novelty, industrially applicable, and effectiveness in protecting fish. The vaccine can be marketed and applied to the aquaculture and mariculture industries.

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Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KEBERKESANAN VAKSIN MONOVALEN DAN MULTIVALEN BERASASKAN MAKANAN TERHADAP PENYAKIT BAWAAN BAKTERIA PADA *Epinephelus fuscoguttus* (FORSSKAL, 1775) X *E. lanceolatus* (BLOCH, 1790) (KERAPU) DAN *Lates calcarifer* (BLOCH, 1790) (SIAKAP)

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Di Malaysia, penyakit bawaan bakteria seperti vibriosis, streptokokusis, dan aeromonad septisemia motil (MAS) kerap dijumpai menjangkiti ternakan akuakultur di Malaysia. Penyakit ini membawa kepada kerugian ekonomi yang sangat besar kepada negara. Bagi mengelakkan penggunaan antibiotik, penghasilan vaksin yang berkesan adalah penting bagi mengawal jangkitan penyakit-penyakit ini. Walau bagaimanapun, limitasi seperti vaksinasi melalui suntikan, penggunaan adjuvan yang boleh memberi kesan buruk terhadap hos, dan antigen pelindung bukan silang, boleh melambatkan pembangunan vaksin. Oleh itu, objektif kajian ini adalah (1) untuk membangunkan vaksin multivalen berasaskan makanan yang mengandungi sel-sel utuk tidak aktif *Vibrio harveyi* strain Vh1, *Streptococcos agalactiae* strain Sa2k dan *Aeromonas hydrophila* strain Ah1sa5 dengan tambahan minyak sawit sebagai adjuvan, serta vaksin monovalen pelindung silang berasaskan makanan yang juga mengandungi sel utuk tidak aktif *V. harveyi* strain Vh1 dengan tambahan adjuvan minyak sawit, (2) untuk menilai tindak balas imun spesifik dan ekspresi relatif gen berkaitan imun selepas vaksinasi dengan vaksin berasaskan makanan tersebut, (3) untuk menilai keberkesanan perlindungan vaksin berikutan infeksi dengan beberapa *Vibrio* spp. pada ikan siakap, *Lates calcarifer* dan (4) untuk menilai keberkesanan lapangan vaksin-vaksin tersebut terhadap vibriosis dalam kerapu hibrid ternakan (*Epinephelus fuscoguttus* × *E. lanceolatus*). Bagi mencapai objektif-objektif tersebut, vaksin multivalen berasaskan suapan telah dibangunkan dengan menggabungkan vaksin *V. harveyi* strain Vh1 dengan vaksin *S. agalactiae* Sa2k, dan vaksin *A. hydrophila* Ah1sa5 dan dicampur dalam pelet makanan ikan yang telah dihancurkan dengan tambahan minyak sawit sebagai adjuvan. Manakala vaksin monovalen dibangunkan daripada vaksin *V. harveyi* strain Vh1, dicampur ke dalam pellet ikan yang telah dihancurkan dengan tambahan adjuvan minyak sawit. Pelet makanan komersial (Star Feed, Klang, Malaysia) digunakan sebagai kawalan negatif. Vaksin-vaksin berasaskan

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makanan serta pelet komersial (kawalan) kemudiannya dinilai untuk analisis kualiti dan keselamatan makanan terhadap ikan ternakan. Keputusan menunjukkan bahawa vaksin berasaskan makanan mengandungi komposisi pemakanan yang baik, walaupun selepas perumusan semula. Selain itu, makanan yang telah divaksin telah diuji selamat untuk penggunaan ikan dan menunjukkan kesan positif terhadap pertumbuhan ikan. Selanjutnya, kajian tindak balas imun selepas vaksinasi diuji dengan 450 ikan siakap, *L. calcarifer* juvenil (15.8±2.6g), dibahagikan sama rata kepada tiga kumpulan dengan tiga gandaan. Kumpulan 1 diberi makan vaksin multivalen, Kumpulan 2 dengan vaksin monovalen *V. harveyi*, dan Kumpulan 3 dengan makanan komersial. Vaksinasi dilakukan dengan memberi makan ikan pada hari 0 dan 14 dengan kadar 5% daripada berat badan. Sampel serum dan usus diambil setiap minggu bagi analisa antibodi IgM, aktiviti lisozim, dan analisis ekspresi gen berkaitan imun. Cabaran infeksi dengan bakteria virulen telah dijalankan pada minggu ke-4 selepas imunisasi. Keputusan menunjukkan bahawa antibodi IgM spesifik dan tahap aktiviti lisozim tidak spesifik dalam serum kumpulan vaksin multivalen dan monovalen lebih banyak dihasilkan daripada kumpulan kawalan sepanjang tempoh vaksinasi. Selain itu, ikan yang divaksin menunjukkan peningkatan gen yang berkaitan dengan imun yang ketara ($P < 0.05$) dengan ekspresi gen imun tertinggi dalam kumpulan vaksin multivalen dilihat dalam gen sel dendritik pada minggu ke-6 (11.47 kali ganda), diikuti oleh gen CCL4. (7.79 kali ganda) pada minggu ke 4. Manakala ekspresi tertinggi dalam kumpulan vaksin monovalen dilihat pada gen MHC-I pada minggu ke 3 (5.23 kali ganda), diikuti oleh gen C3 pada minggu ke 4 (3.67 kali ganda). Cabaran bakteria terhadap *V. harveyi, S. agalactiae*, dan *A. hydrophila* menunjukkan bahawa vaksin multivalen memberikan nilai RPS 75±7.07%, 85± 7.07% dan 70±0.00% masing-masing terhadap jangkitan bakteria *V. harveyi*, *S. agalactiae* dan *A. hydrophila* manakala 70±0.00% terhadap jangkitan gabungan bakteria tersebut, manakala vaksin monovalen memberikan 70±0.00% kemandirian terhadap jangkitan *V. harveyi*. Walaupun aplikasi vaksin multivalen mempunyai potensi yang meluas terhadap pelbagai jangkitan bakteria, kajian semasa ini memfokuskan pada keberkesanan vaksin multivalen berasaskan makanan terhadap jangkitan vibriosis dalam ternakan ikan marin di Malaysia. Vaksin monovalen *V. harveyi* juga turut diuji dalam kajian ini untuk menentukan keberkesanan perlindungan silang terhadap jangkitan pelbagai *Vibrio* spp. Vaksin ini turut dibandingkan dengan vaksin multivalen untuk menentukan sama ada vaksin gabungan boleh mendorong tindak balas imun yang lebih baik tanpa persaingan antigen. Keputusan kajian menunjukkan paras antibodi IgM yang lebih tinggi ($P < 0.05$) dalam ikan yang divaksin dengan vaksin multivalen dan monovalen selepas dos penggalak. Pasca-cabaran infeksi dengan bakteria pada minggu ke-10 menunjukkan bahawa paras antibodi IgM dalam serum, lendir di bahagian badan dan cecair lavaj usus bagi kumpulan yang divaksin multivalen dan monovalen adalah meningkat lebih tinggi berbanding sebelum cabaran dan kumpulan kawalan sehingga minggu ke-13. Keputusan ini disokong oleh kajian cabaran bakteria dengan pelbagai *Vibrio* spp., terhadapt vaksin multivalent yang memberikan nilai RPS yang tinggi iaitu 85±7.07%, 80± 0.00% dan 75±7.07%, masing-masing selepas dicabar dengan *V. harveyi*, *V. parahaemolyticus,* dan *V. alginolyticus*, manakala 70±0.00% selepas dicabar dengan campuran bakteria tersebut, dalam masa yang sama, vaksin monovalent juga memberikan nilai RPS yang tinggi iaitu 75±7.07%, 75±7.07% dan 70±0.00% masing-masing selepas dicabar dengan *V. harveyi*, *V. parahaemolyticus,* dan *V. alginolyticus*, serta 70±0.00%

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selepas dicabar dengan gabungan jangkitan *Vibrio* spp. tersebut. Akhirnya, percubaan lapangan telah dijalankan di ladang marikultur untuk mengkaji keberkesanan lapangan vaksin multivalen dan monovalen terhadap ternakan ikan secara komersial. Rangsangan sistem imun dalam kumpulan vaksin multivalen dan monovalen ditunjukkan oleh peningkatan tahap IgM selepas imunisasi seawal minggu ke-2, memuncak pada minggu ke-6, dan kekal tinggi dengan ketara sehingga minggu ke-12. Rangsangan antibodi menunjukkan bahawa perlindungan mungkin boleh dicapai untuk tempoh 12 minggu (3 bulan). Kumpulan vaksin menghasilkan kadar kelangsungan hidup yang lebih tinggi daripada kumpulan kawalan, dengan kadar 79.75 ±0.07% dalam kumpulan vaksin multivalent dan 75.25±4.75% dalam kumpulan vaksin monovalen.berbanding 65.10±0.14% bagi kumpulan kawalan selepas percubaan vaksinasi di lapangan selama 16 minggu. Tambahan pula, kecekapan penukaran makanan yang lebih baik dan pertambahan berat telah ditunjukkan dengan jelas dalam kumpulan vaksin. Keputusan-keputusan yang diperoleh daripada kajian ini menunjukkan bahawa vaksin multivalen dan monovalen berasaskan makanan amat berkesan bagi mendorong tindak balas imun di dalam ikan yang divaksin. Vaksin monovalen didapati sama baiknya dengan vaksin multivalen terhadap vibriosis, dan gabungan antigen dalam vaksin multivalen tidak menyumbang secara signifikan kepada rangsangan dan perlindungan antibodi yang lebih tinggi terhadap *Vibrio* spp. Walau bagaimanapun, vaksin multivalen berpotensi memberikan perlindungan yang lebih luas terhadap penyakit lain seperti streptokokusis dan MAS. Kedua-dua vaksin berasaskan makanan sangat berpotensi untuk dibangunkan secara komersial kerana aplikasinya yang sangat releven dengan ternakan berskala besar serta terbukti mampu memberikan perlindungan yang efektif terhadap ikan ternakan.

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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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LIST OF ABBREVIATIONS

%	percentage
microlitre	ml
µM	micromolar
®	registered
™	trademark
ºC	degree Celcius
APC	antigen-presenting cell
BSA	bovine serum albumin
BHIA	brain heart infusion agar
BLAST	basic local alignment search tool
bp	base pair
CaCl2	calcium chloride
CFU	colony forming units
DCs	Dendritic cells
dNTP	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
g	gram
H&E	haematoxylin and eosin
Ig	immunoglobulin
IP	intraperitoneal
IM	intramuscular
kb	kilobase pair
LD50	median lethal dosage

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LPS	lipopolysaccharide
M	molar
MAS	motile aeromonad septicemia
MHC	major histo-compatibility complex
mg	milligram
MgCl2	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
sp	species
TGF	transforming growth factor
TSA	tryptic soy agar
TSB	tryptic soy broth
V	voltage
v/v	volume per volume
wpv	week post-vaccination

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CHAPTER 1

INTRODUCTION

1.1 Background of the study

Globally, intensive aquaculture has been implemented for years to increase productivity. Despite the advantages of intensive aquaculture techniques, the industry has some possible challenges Intensive farming systems usually increase the stress level in fish due to high stocking density and high feeding levels, resulting in decreased water quality (Hastuti et al., 2019). The stressors increase susceptibility to infectious diseases causing substantial losses to fish farming. Thus, the need to protect the highly intensive culture system from infectious disease has become a priority among the aquaculturists community, as the intensive culture of aquatic animals brings along more severe diseases that cause economic and welfare issues (Kole et al., 2018).

In Malaysia, *Vibrio* spp., *Streptococcus agalactiae,* and *Aeromonas hydrophila* are the primary bacterial pathogens affecting fresh and marine water aquaculture industries (Ismail et al., 2017; Pauzi et al., 2020; Mohd Yazid et al., 2021). They can infect and cause diseases in fish, namely vibriosis, streptococcosis, and motile aeromonad septicemia (MAS), respectively, which the outbreak can pose a severe economic loss to the fish farming operations.

Vibriosis is one of the most prevalent bacterial diseases affecting diverse marine fishes and shellfishes (Mougin et al., 2021). Approximately 66.7% of diseases reported in groupers (*Epinephelus* spp.) are due to vibriosis, affecting all stages of growth and leading to 50% of mortality (Amalina et al., 2019). Important *Vibrio* spp. known to cause a substantial economic impact on aquaculture are *Vibrio anguillarum*, *V. ordalii*, *V. salmonicida*, *V. vulnificus*, *V. alginolyticus*, *V. harveyi,* and *V. parahaemolyticus* (Haenen et al., 2014: Amalina et al., 2019). There was a reported outbreak caused by *V. harveyi* among cultured Asian seabass (*Lates calcarifer*) in Sabah, Malaysia, leading to high mortalities among the farmed fish (Albert and Ransangan, 2013).

On the other hand, streptococcosis is one of the important tilapia diseases with high morbidity and mortality worldwide, including in Malaysia (Ridzuan et al., 2022). Examples of pathogens that can cause streptococcosis are *S. agalactiae* and *S. iniae* (Ismail et al., 2017). Aquaculture outbreaks of streptococcosis can result in up to 80% mortalities in fish, while some can be potentially zoonotic, which can be transferred from fish to humans and cause foodborne outbreaks (FAO, 2021). This infection's significant characteristics in fish include infarctions in the liver and spleen, along with meningoencephalitis (Zamri-Saad et al., 2014).

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In comparison, the genus *Aeromonas* can cause aeromoniasis, which can manifest in terms of motile aeromonad septicemia (MAS) and epizootic ulcerative syndrome (EUS) (Rahman et al., 2002). *Aeromonas dhakensis, A. hydrophila, A. veronii, A. jandaei,* and *A. caviae* are among the *Aeromonas* that cause a significant impact on aquaculture in Malaysia (Azzam-Sayuti et al., 2021). *Aeromonas* spp. has been previously reported in various freshwater fish, including channel catfish (*Ictalurus punctatus*), Siberian sturgeon (*Acipenser baerii*), Russian sturgeon (*Acipenser gueldenstaedtii*), banded knifefish (*Gymnotus omarorum*), and Nile tilapia (*Oreochromis niloticus*) (Zhang et al., 2016; Pauzi et al., 2020).

Following the ban on antibiotics in aquaculture, vaccination has been advocated as one of the options for reducing disease-related mortality and morbidity (Sudheesh and Cain, 2016; Zhang et al., 2018). Vaccines are more effective and less harmful to individuals and the environment than antibiotics (Jun et al., 2020). It stimulates the fish's immune system to produce antibodies that protect them against disease compared to antibiotics, which kill or halt infections. Vaccines have been shown to be more effective and lower mortality significantly (Tafalla et al., 2014). Vaccination often produces memory cells, which can trigger a fast-anamnestic response upon infection by the targeted pathogen, making it the most effective technique for controlling infectious illnesses in animals with adaptive immunity (Yamaguchi et al., 2019).

Apparently, vaccines against vibriosis, streptococcosis, and MAS have been developed to target single infection of the respective pathogens (Firdaus-Nawi et al., 2013; Gudding and Van Muiswinkel, 2013; Wali and Balkhi, 2016). Compared to a single vaccination, developing a multivalent vaccine that can cure various illnesses caused by *Vibrio* spp., *S. agalactiae*, and *A. hydrophila* improves practical use while reducing effort. Controlling many illnesses with a single vaccination is more cost-effective than employing numerous different immunizations. Besides, some studies reported that a combined two or more vaccines could induce a more robust immune response without antigen competition (Bao et al., 2019; R. Hoare et al., 2019). Instead, a synergistic effect can be observed, triggering an innate immune response and stimulating adaptive immunity (Bao et al., 2019).

Three types of vaccine delivery systems have been applied in aquaculture with variable success; immersion, injection (e.g., intra-peritoneal and intra-muscular), and oral routes (Reyes et al., 2017; Feng et al., 2018). Overall, antigens directly incorporated in or adhered to the feed are preferable routes for many farmers since they are needle-free, have no size limitation, lower cost, and are more convenient for farmer operation (Tang et al., 2017; Wang et al., 2018). Small-scale private farmers in Southeast Asia, such as Vietnam, Malaysia, Indonesia, and Thailand, are unlikely to afford labor-intensive vaccine protocols requiring special equipment; thus, an oral vaccine is highly preferable (Ismail et al., 2016). Moreover, vaccines administered via the oral route can elicit both mucosal and systemic immunities, thereby protecting the fish at the portal of entry and preventing infections systemically (Munang'andu et al., 2015; Somamoto and

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Nakanishi, 2020). However, due to a poor understanding of the requirements for proper immune induction following oral administration, only a few countries, such as Chile, Scotland, and Norway, use oral vaccines for fish (Laith et al., 2019).

Inactivated vaccines have been used regularly in aquaculture and have achieved good protection against pathogens (Nguyen et al., 2017; Wei et al., 2020). Meanwhile, several research studies have shown that inactivated vaccines incorporated with adjuvants can better protect against bacterial infection (Shahin et al., 2019; Li et al., 2020). As a result, commercially accessible fish vaccines are being created by incorporating various mineral oil as an adjuvant (Noia et al., 2014; Labarca et al., 2015). Vaccines incorporated with Freund's Complete or Incomplete Adjuvants (FCA or FIA), for example, were highly efficient in low-toxicity fish immunization (Liu et al., 2016; Sanchez et al., 2018; Wang et al., 2020). Nevertheless, this commercial adjuvant is extremely expensive, especially for the commercial preparation of the vaccine. Thus, an alternative adjuvant that could provide good stimulation of immunity and subsequent protection at a lower price should be considered (Aminudin et al., 2018). According to some studies, using cheap oilseed such as palm oil as an adjuvant for Newcastle disease virus (NDV) vaccination in chickens and caseous lymphadenitis vaccination in rats had successfully boosted immune protection while causing no adverse effects (Wanasawaeng et al., 2009; Roslindawani et al., 2016).

1.2 Problem statement

The application of chemicals and drugs has become the main strategy in fish culture for effective farming and high production (Hamdan et al., 2016; Hutchings et al., 2020; Novriadi, 2016). In Malaysia, Mohd Nor et al. (2019) reported that 50% of farmers from the total respondents in their study might use antibiotics to treat sick fish (for any reason). However, using different antimicrobial agents increased the existence of antimicrobial-resistant bacteria and drug residues in fish, which became one of the biggest problems (Chen et al., 2019; Deng et al., 2020). Antibiotic resistance can be transmitted to wild and human pathogenic bacteria, creating health risks and environmental issues, as has been proved in various nations (Manage, 2018; Zhou et al., 2021). Thus, an alternative to the usage of chemicals and drugs on farms should be considered. Vaccination is important for preventing aquaculture infectious diseases (Guo et al., 2019; Jiang et al., 2014). Several studies have described the effectiveness of vaccines in marine and freshwater fishes, such as inactivated vaccines (Hwang et al., 2017; Kai et al., 2014; Wei et al., 2020), live attenuated vaccines (Choi et al., 2016; Lampe et al., 2017; Li et al., 2015), and subunit vaccines (Guo et al., 2018; Ho et al., 2018; Tian et al., 2013) for the control of vibriosis, streptococcosis, motile aeromonad septicemia (MAS) and other fish diseases. Besides, effective commercial vibriosis and streptococcosis vaccines are available such as AQUAVAC® VIBRIO and AQUAVAC® Strep Sa, respectively, from Merck Animal Health Company, USA. When administered by injection or immersion, these monovalent vaccines provide excellent protection against a single target bacterium, but they are unable to protect multiple bacteria with a single

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vaccination and may cause stress on the fish, increase labor costs, and others (Gong et al., 2015; Wali and Balkhi, 2016).

Given the cost and timing of fish vaccination, feed-based multivalent or cross-protective vaccines that prevent multiple diseases in a single immunization are desirable (Li et al., 2016a; Peng et al., 2018; Ji et al., 2020). However, the issues regarding antigen degradation and competition might slow down their development.

Freund's Complete Adjuvants, Freund's Incomplete Adjuvants, and Montanide are commercial adjuvants that have been frequently used to develop fish vaccines against *Vibrio* spp., *S. agalactiae,* and *A. hydrophila* (Gjessing et al., 2012; Firdaus-Nawi et al., 2013; Rauta and Nayak, 2015; Abdelhamed et al., 2017; Yang et al., 2018), and have shown 70-100% relative percent survival (RPS) with long-term protection. However, the cost of using these commercial adjuvants in fish vaccines is too high (Aminudin et al., 2018). As a result, an alternate adjuvant that might stimulate immunity and give future protection at a lower cost should be considered. Several studies have reported using palm oil as a vaccine adjuvant (Abdul Hafid et al., 2010; Aminudin et al., 2018; Roslindawani et al., 2016; Wanasawaeng et al., 2009) as well as an immunostimulant (Larbi Ayisi et al., 2018). Like mineral oil in commercial adjuvant, palm oil can enhance and prolong the vaccine's antigenic properties due to the protection of the antigen by the oily coating that serves as an antigen depot (Coscelli et al., 2015; Muktar et al., 2016). Besides, the attraction of the antigen by the oil that may take part in antibody formation and an accompanying slow, continuous release of antigen could stimulate antibody production in suitable cells (Bastardo et al., 2012; Coscelli et al., 2015; Steinberg and Norman, 1972). Nevertheless, the applications of palm oil in fish vaccine adjuvant are still limited.

1.3 Significance of the study

The effort to develop a multivalent vaccine for oral administration is novel and is ongoing. Currently, there is no report on developing a feed-based multivalent vaccine that can confer protection against *Vibrio* spp., *S. agalactiae,* and *A. hydrophila* in farmed fish, especially in Malaysia. However, some monovalent vaccines from locally isolated pathogens of *Vibrio* spp., *S. agalactiae,* and *A. hydrophila* have been developed by Chin et al. (2020a), Ismail et al. (2017), and Matusin (2015), respectively. Countering multiple infections with multivalent feed-based vaccinations was proven to induce a stronger immune response, provide ease of application and be cost-effective (Ismail et al., 2017; Hoare et al., 2019). Furthermore, there is a lack of studies using palm oil as a potential fish vaccine adjuvant that could stimulate good immunities and provide protection at a cheaper rate (Aminudin et al., 2018).

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This study tried to develop a feed-based multivalent vaccine against several common bacterial fish diseases in Malaysia, particularly vibriosis, streptococcosis, and MAS in a different host. Vibriosis, one of the most prevalent bacterial diseases affecting diverse marine fish, is highlighted in this study. At the same time, a cross-protective feed-based monovalent *Vibrio harveyi* vaccine against multiple *Vibrio* spp. was also developed and tested its efficacy along with the multivalent vaccine against vibriosis. The multivalent and monovalent *V. harveyi* vaccine, which contains a local isolate of *V. harveyi* strain VH1, has been demonstrated to stimulate strong antigenic responses on its homologous OMPs antigen and cross-reacted against heterologous OMPs antigens of *V. parahaemolyticus* strain VPK1, *V. alginolyticus* strain VA2, and *Photobacterium damselae* strain PDS1 at a molecular weight of 32 kDa (Mursidi, 2018) (Appendix A). However, the efficacy of the multivalent and monovalent *V. harveyi* vaccine incorporated inside feed pellet against multiple *Vibrio* spp. in marine fish is still understudied. For streptococcosis and MAS, a similar study was done by Lee et al. (unpublished data, Appendix B) to study the efficacy of the multivalent vaccine against streptococcosis and MAS in freshwater fish, red hybrid tilapia (*Oreochromis niloticus* × *O. mossambicus*) with promising results.

1.4 Objectives

The general objective of this study is to develop a feed-based multivalent vaccine and cross-protective monovalent *V. harveyi* vaccine with palm oil as an adjuvant that can provide protection against multiple bacterial diseases in fish

There are four objectives of this study:

i. To develop a feed-based palm oil adjuvanted multivalent vaccine containing whole cells of inactivated *V. harveyi* strain Vh1, *S. agalactiae* strain Sa2k and *A. hydrophila* strain Ah1sa5 and a feed-based palm oil adjuvanted monovalent *V. harveyi* vaccine containing whole cells of inactivated *V. harveyi* strain Vh1

ii. To assess the specific immune response and the relative expression of immune-related genes following vaccination of Asian seabass (*L. calcarifer*) with the newly developed feed-based vaccines

iii. To evaluate the protective efficacy of the newly developed feed-based vaccine in Asian seabass (*L. calcarifer*) following experimental challenges with *V. harveyi*, *V. alginolyticus,* and *V. parahaemolyticus*

iv. To evaluate the field efficacy of the newly developed feed-based vaccines against vibriosis in farmed hybrid grouper (*Epinephelus fuscoguttus* × *E. lanceolatus*)

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1.5 Hypotheses of the study

i. Objective 1:

H_0: The multivalent vaccine against vibriosis, streptococcosis, and aeromoniasis and the cross-protective monovalent vaccine against multiple *Vibrio* spp. in fish cannot be developed.
H_1: The multivalent vaccine against vibriosis, streptococcosis, and aeromoniasis and the cross-protective monovalent vaccine against multiple *Vibrio* spp. in fish can be developed.

ii. Objective 2:

H_0: Vaccination with the newly developed feed-based vaccines could not induce immune responses and relative expression of immune-related genes against bacterial infections in Asian seabass (*L. calcarifer*).
H_1: Vaccination with the newly developed feed-based vaccines could induce immune responses and relative expression of immune-related genes against bacterial infections in Asian seabass (*L. calcarifer*).

iii. Objective 3:

H_0: Vaccination with the newly developed feed-based vaccines could not confer protection against *V. harveyi*, *V. alginolyticus,* and *V. parahaemolyticus* in Asian seabass (*L. calcarifer*).
H_1: Vaccination with the newly developed feed-based vaccines could confer protection against *V. harveyi*, *V. alginolyticus,* and *V. parahaemolyticus* in Asian seabass (*L. calcarifer*).

iv. Objective 4:

H_0: The newly developed feed-based vaccines could not confer protection against vibriosis in farmed hybrid grouper (*Epinephelus fuscoguttus* × *E. lanceolatus*).
H_1: The newly developed feed-based vaccines could confer protection against vibriosis in farmed hybrid grouper (*Epinephelus fuscoguttus* × *E. lanceolatus*).

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