CHARACTERIZATION AND PROTECTIVE EFFECTS OF FEED-BASED BIVALENT VACCINE AGAINST STREPTOCOCCOSIS AND AEROMONIASIS IN RED HYBRID TILAPIA (*Oreochromis* spp.)

By

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Streptococcosis and aeromoniasis are the major bacterial diseases in tilapia farming. *Streptococcus agalactiae* and *Aeromonas hydrophila* are the dominant causative agents of streptococcosis and aeromoniasis, respectively. Controlling bacterial diseases by vaccination using feed-based bivalent vaccines has been considered an effective method to prevent multiple diseases, which contributes to economic sustainability. Previously, a novel feed-based bivalent vaccine containing formalin-killed whole organisms of *Streptococcus iniae* and *A. hydrophila* for red hybrid tilapia was developed, which provided good immunological responses and high protection against *S. iniae* and *A. hydrophila* infections. However, there is still a lack of vaccine characterization regarding stability and mechanism of the oral delivery route. This study aims to characterize and investigate the immunoprotective efficacy of feed-based bivalent vaccine against the causal agents of streptococcosis and aeromoniasis in red hybrid tilapia. The feed-based bivalent vaccine containing killed whole organisms of *S. agalactiae* and *A. hydrophila* mixed

with 10% palm oil was successfully prepared and characterized regarding stability, nutritional quality, safety and growth performance. The vaccine was delivered orally at 5% of the fish's body weight for three consecutive days on weeks 0, 2 and 6. Lysozyme and enzyme-linked immunosorbent (ELISA) assays were analyzed to evaluate the immunological responses following vaccination. The vaccination efficacy was assessed according to the relative percentage survival (RPS) and histopathological assessment of the vaccinated fish following challenges with pathogenic bacteria. The vaccinated fish's hindgut was subjected to transcript response analyses according to the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and transcriptome for the expression analysis of immune-related genes and pathways following vaccination, respectively. Results showed that in one g of feed-based bivalent vaccine contained 10^9 CFU/g of *S. agalactiae* and *A. hydrophila*, respectively. Acid tolerance analysis of feed-based bivalent vaccine has caused a 10% reduction in the concentration of killed whole organisms without any morphological changes. The concentrations of killed whole organisms in the feed-based bivalent vaccine remained at approximately 90% when stored at room temperature for 60 days. The vaccine did not influence red hybrid tilapia's culture water stability, palatability and growth performances. Moreover, vaccinated fish showed high protective efficacy against *S. agalactiae* (RPS at 80%) and *A. hydrophila* (RPS at 90%) and partial cross-protective efficacy against *S. iniae* (RPS at 63%) and *Aeromonas veronii* (RPS at 60%). The lysozyme activity and IgM antibody level in vaccinated fish's serum, gut lavage and skin mucus were significantly ($p < 0.05$) higher than the control fish. The transcript level of IL-1β (5.2-fold change) was found to be highest in the vaccinated fish at week six after the second

vaccination booster, followed by MHC-II (4.7-fold change) and CD4 (4.6-fold change). Following the higher immune-related gene expression in the vaccinated fish's hindgut at week 6, the hindgut sample was subjected to transcriptomic analysis. The Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis showed numerous (775) differentially expressed genes (DEG) were enriched in 16 immune-related pathways. This study suggested that the feed-based bivalent vaccine is promising for improving the immunological response against the causal agent of streptococcosis and aeromoniasis.

Keywords: Red hybrid tilapia**;** feed-based bivalent vaccine; streptococcosis; aeromoniasis; immune responses

SDG: GOAL 2: Zero Hunger; GOAL 4: Quality Education; GOAL 14: Life Below Water

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

PENCIRIAN DAN KESAN PERLINDUNGAN VAKSIN BIVALEN BERASASKAN MAKANAN TERHADAP STREPTOKOKUSIS DAN AEROMONIASIS PADA HIBRID TILAPIA MERAH (*Oreochromis* spp.)

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Streptokokusis dan aeromoniasis adalah penyakit bakteria utama dalam penternakan tilapia. *Streptococcus agalactiae* dan *Aeromonas hydrophila* masing-masingnya adalah penyebab utama bagi streptokokusis dan aeromoniasis. Pengawalan penyakit melalui vaksinasi menggunakan vaksin bivalen berasaskan makanan telah dianggap sebagai kaedah pencegahan yang berkesan dan menyumbang kepada kelestarian ekonomi. Sebelum ini, vaksin bivalen berasaskan makanan yang mengandungi *killed whole organisms of Streptococcus agalactiae* dan *Aeromonas hydrophila* bagi kegunaan hibrid tilapia merah telah dibangunkan dan memberikan tindak balas imunologi yang baik dan perlindungan yang tinggi terhadap *S. iniae* dan *A. hydrophila.* Namun, masih terdapat kekurangan pencirian vaksin mengenai kestabilan dan mekanisma laluan oral. Kajian ini bertujuan untuk mencirikan dan menyiasat keberkesanan imunoprotektif vaksin bivalen berasaskan makanan yang baharu terhadap agen penyebab streptokokusis dan aeromoniasis pada hibrid tilapia merah. Vaksin bivalen berasaskan makanan

yang mengandungi *killed whole organisms* of *S. agalactiae* dan *A. hydrophila* dengan campuran 10% minyak sawit telah berjaya dihasilkan dan dicirikan berdasarkan kestabilan, kualiti nutrisi, keselamatan dan prestasi pertumbuhan. Vaksin telah diberi secara oral pada 5% daripada berat badan ikan selama tiga hari berturut-turut pada minggu ke-0, diikuti oleh dos penggalak pada minggu ke-2 dan -6. Ujian lisozim dan imunojerapan berpaut enzim (ELISA) telah dijalankan bagi menilai tindakbalas imunologikal selepas vaksinasi. Keberkesanan vaksinasi pada ikan setelah dicabar dengan bakteria-bakteria patogen telah dinilai melalui kelangsungan peratusan relatif (RPS) dan ujian histopatologi. Gut belakang ikan yang divaksin selanjutnya tertakluk kepada kuantitatif tindak balas berantai polimerase masa nyata (RT-qPCR) dan transkriptom untuk menganalisis ekspresi gen-gen dan laluan berkaitan imun. Satu gram vaksin bivalen berasaskan makanan masing-masingnya mengandungi 10^9 CFU/g *S. agalactiae* dan *A. hydrophila*. Analisis daya tahan asid terhadap vaksin bivalen berasaskan makanan telah mengurangkan 10% jumlah *killed whole organisms* tanpa menyebabkan perubahan morfologi. Jumlah *killed whole organisms* dalam vaksin bivalen berasaskan makanan didapati pada 90% (10^9 CFU/g) apabila vaksin disimpan pada suhu bilik selama 60 hari. Vaksin didapati tidak mempengaruhi kestabilan air, keperisaan dan prestasi pertumbuhan hibrid tilapia merah. Malah, ikan yang divaksin menunjukkan keberkesanan perlindungan yang tinggi terhadap *S. agalactiae* (RPS, 80%) dan *A. hydrophila* (RPS, 90%) serta keberkesanan perlindungan silang separa terhadap *S. iniae* (RPS, 63%) dan *Aeromonas veronii* (RPS, 60%). Aktiviti lisozim dan paras antibodi IgM dalam serum, lavaj usus dan mukus kulit pada ikan yang divaksin adalah lebih tinggi ($p < 0.05$) berbanding ikan kawalan. Tahap transkrip IL-1β (perubahan 5.2 kali ganda) didapati

tertinggi pada ikan yang divaksin pada minggu ke-6 selepas dos penggalak kedua, diikuti oleh MHC-II (perubahan 4.7 kali ganda) dan CD4 (perubahan 4.6 kali ganda). Berikutan ekspresi gen-gen berkaitan imun yang tinggi di dalam gut belakang ikan yang divaksin pada minggu ke-6, sampel gut belakang telah digunakan dalam analisis transkriptomik. Analisis KEGG telah menunjukkan banyak (775) gen-gen pembezaan terekspres (DEG) terlibat dalam 16 laluan berkaitan imun. Kajian ini mencadangkan bahawa vaksin bivalen berasaskan makanan adalah vaksin berasaskan makanan yang mampu meningkatkan tindak balas imunologi terhadap agen penyebab streptokokusis dan aeromoniasis.

Kata Kunci: Hibrid tilapia merah, vaksin bivalen berasaskan makanan, streptokokusis**;** aeromoniasis; tindak balas imun

SDG: MATLAMAT 2: Kelaparan sifar; MATLAMAT 4: Pendidikan berkualiti; MATLAMAT 14: Kehidupan di dalam air

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α	Alpha
β	Beta
°C	Degree celsius
%	Percentage
16S rRNA	16S ribosomal ribonucleic acid
µM	Micromolar
µm	Mirometer
BLAST	Basic local alignment search tool
bp	Base pair
BP	Biological processes
CC	Cellular components
cfb	CAMP-factor
CFU	Colony forming unit
DEG	Differentially expressed genes
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
DPI	Day post infection
ELISA	Enzyme-linked immunosorbent
FOA	Food and agriculture organization
FPKM	Fragments per kilobase of transcript sequence per millions mapped reads
g	Gram
GALT	Gut-associated lymphoid tissues
GBS	Group B *Streptococcus*

GO	Gene ontology
gyrB	DNA gyrase subunit B
Ig	Immunoglobulin
IL-1*β*	Interleukin-1 beta
IP	Intraperitoneal
KEGG	Kyoto encyclopedia of genes and genomes
Kg	Kilogram
L	Litre
MF	Molecular functions
mg	Miligram
MHC	Major histocompatibility complex
mL	Mililitre
mRNA	Messenger RNA
NaCl	Sodium chloride
NADP	Nicotinamide adenine dinucleotide phosphate
ng	Nanogram
nm	nanometer
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
PPAR	Peroxisome proliferator-activated receptors
qPCR	Quantitative PCR
RNA	Ribonucleic acid
rpm	Revolutions per minute

RPS	Relative percent survival
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
SEM	Scanning electron microscopy
sp.	A single unnamed species
spp.	More than one unnamed species
TEM	Transmission electron microscopy
TGF-*β*	Transforming growth factor beta
TLR2	Toll-like receptor 2
TNF-*α*	Tumor necrosis factor alpha
U/mg	Unit per miligram
v/v	Volume over volume
w/v	Weight over volume

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CHAPTER 1

INTRODUCTION

1.1 Background of the study

Tilapia is an important freshwater fish that is intensively cultured worldwide. Global production for tilapia is forecast to grow by 3.7% in 2022, breaking the 6 million metric tons barrier (FAO, 2022). Tilapia, including the Mozambique tilapia (*Oreochromis mossambicus*), the Nile tilapia (*O. niloticus*), the blue tilapia (*O. aureus*) and different hybrids of the red hybrid tilapia (*Oreochromis* sp.) are commonly cultured worldwide (Zeng et al., 2021). In Malaysia, red hybrid tilapia has become the dominant aquaculture species (> 90%) due to high consumer demand (Lim et al., 2016; Mohamad, S.N. et al., 2021). Although tilapia culture is expanding worldwide, it also faces enormous microbial diseases caused by bacteria, fungi, viruses, and parasite infections that have led to severe economic losses (FAO, 2022).

Among the bacterial diseases, streptococcosis and aeromoniasis are the primary barriers to sustainable tilapia production (Abdel-Latif et al., 2020). *Streptococcus agalactiae* and *Streptococcus iniae* have been recognized as the primary causal agents for streptococcosis disease (Suwanbumrung et al., 2023; Wangkahart et al., 2023; Vela-Avitúa., 2023). Meanwhile, *Aeromonas hydrophila* and *Aeromonas veronii* have been reported as the primary causal agents for aeromoniasis disease (Aly et al., 2023; Mursalim et al., 2022). Nevertheless, the mortality of cultured hybrid tilapia is not only caused by a

single infection but can also be caused by the co-infection of different bacterial genera. Several cases of co-infections include *Streptococcus* sp. and *Aeromonas* sp. (Puneeth et al., 2022; Basri et al., 2020), *Streptococcus* sp. and *Francisella* sp. (Assis et al., 2017) and *Aeromonas* sp. and *Flavobacterium* sp. (Dong et al., 2015) have been reported.

So far, the main strategy to control bacterial diseases in tilapia culture worldwide includes chemical control by applying antibiotics (Assane et al., 2019). However, this strategy is expensive and inefficient in the development of antibiotic resistance within the pathogenic strains (Haenen et al., 2023; Akter et al., 2023). Moreover, severe complications arise from accumulations of antibiotic residue in the food chain that could harm humans and the environment (Arsène et al., 2022). Interestingly, vaccination offers a potential alternative strategy for controlling bacterial diseases in tilapia culture (Wang et al., 2023; Thompson et al., 2023). In aquaculture, vaccination can be administered through injection, immersion and oral methods (Bedekar & Kole, 2022; Plant & LaPatra, 2011). Controversially, a complex method of vaccine administration has raised another concern, particularly involving large-scale fish culture in large farming systems. It is important to develop an easy and practical vaccine administration that applies to all sizes and stages of fish and can stimulate lasting immune responses that protect the fish against multiple infections (Kuang et al., 2022).

The implementation of feed-based vaccines to control bacterial-based diseases has received great attention because it is less stressful, applicable for mass immunization of many fish, reduces the labour cost and provides a procedure

for oral boosting during grow-out periods in ponds and cages (Radhakrishnan et al., 2023; Ram et al., 2019). Many studies have reported the potential of feed-based vaccines containing one type of bacterial antigen (monovalent) to improve fish immunological responses such as oral cationic-based nanoemulsion vaccine against *S. agalactiae* (Suwanbumrung et al., 2023), oral vaccine GC5-Sip spores against *S. agalactiae* (Yao et al., 2019) and coated formalized killed *A. hydrophila* vaccine against *A. hydrophila* (Ayoub, 2018). However, it has been reported that monovalent vaccines can only protect against a single infection (Aly et al., 2021). Consequently, to vaccinate against various bacterial diseases, vaccination must be carried out several times, resulting in excessive handling stress and vaccination costs. Therefore, a better vaccination strategy is needed by implementing feed-based bivalent vaccines with cross-protective ability to prevent multiple diseases and contribute to economic sustainability.

Previously, in 2020, a novel feed-based bivalent vaccine pellet containing formalin killed whole organisms of *S. iniae* and *A. hydrophila* mixed with 10% palm oil for red hybrid tilapia was successfully developed (Monir et al., 2020) (MyIPO Malaysia, patent number: PI20222001807). The vaccine was effective and has successfully improved the immunological responses in the vaccinated fish (Monir et al., 2022). Moreover, the vaccine provided high protective efficacies against *S. iniae* (RPS, 82.2%) and *A. hydrophila* (RPS, 77.8%) and partial cross-protective efficacies against *S. agalactiae* (RPS, 60.0%) and *A. veronii* (RPS, 57.8%) (Monir et al., 2021). However, there is a lack of characterization studies on the developed feed-based bivalent vaccine in terms of stability and mechanism of the oral delivery route. Noteworthy, Syuhada et

al., (2020) also stated that the *S. agalactiae* serotype III was the dominant serotype infecting tilapia farming in Malaysia. Thus, improvements in the previously developed feed-based bivalent vaccine regarding the type of killed whole organisms used and detailed characterization must be conducted to fulfil the current understanding of the potential of the feed-based bivalent vaccine in controlling streptococcosis and aeromoniasis. Consequently, further investigation on the potential of feed-based bivalent vaccine containing formalin killed whole organisms of *S. agalactiae* serotype III and *A. hydrophila* mixed with 10% palm oil in protecting red hybrid tilapia merits scientific investigations.

1.2 Problem statement

Streptococcosis and aeromoniasis are significant problems affecting tilapia aquaculture industries worldwide. Vaccination is one of the most important tools for managing infectious diseases in fish culture because of the ability of vaccines to stimulate protective immunity and produce a memory response in vaccinated fish (Wang et al., 2023; Ma et al., 2019). Many monovalent vaccines have been developed worldwide to suppress the infection against steptococcosis and aeromoniasis (Suwanbumrung et al., 2023).

Some effective commercial streptococcosis vaccines are available worldwide, such as injectable AquaVac® Strep *Sa* (Merck Animal Health Company, USA) vaccine against *S. agalactiae*, injectable and immersion AquaVac® Strep *Si* (Merck Animal Health Company, USA) vaccine against *S. iniae* and injectable NORVAX® Strep *Si* (Merck Animal Health Company, USA) vaccine against *S. iniae*. However, these developed monovalent vaccines only have high protective

efficacy against a single target of bacteria and are delivered only through injection or immersion immunization. Owing to the issue, developing a feed-based bivalent vaccine to control multiple bacterial diseases in a single immunization is highly desirable due to the cost-effectiveness and saving time of fish vaccination (Abu-Elala et al., 2019; Guo et al., 2020). The effort to develop a bivalent or polyvalent oral vaccine is novel and ongoing.

Interestingly, a previous study reported that the novel feed-based bivalent vaccine mixed with 10% palm oil has improved immunological response and gives high protection against streptococcosis and aeromoniasis in red hybrid tilapia without affecting the fish's growth performance (Monir et al., 2022; Monir et al., 2021). However, more studies are needed to characterize the stability and the mechanism of the oral delivery route following vaccination with feed-based bivalent vaccine mixed with palm oil.

Similar to 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 (Muktaar et al., 2016). Several studies reported palm oil as an adjuvant for the 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).

Meanwhile, Aminudin et al., (2018) observed a high protection level in orally immunized tilapia with feed-based vaccine containing killed whole organism of *S. agalactiae* with addition of palm oil against *S. agalactiae*. Recently, Monir et

al. (2021) reported feed-based bivalent vaccine mixed with 10% palm oil has improved immunological response and gives high protection against *S. iniae* ansd *A. hydrophila* infections. However, the effect of mixing palm oil with the whole killed organisms' stability in vaccine preparation remains unclear.

1.3 Significance of the study

Streptococcosis and aeromoniasis are two major bacterial diseases infecting tilapia farming that must be controlled. In Malaysia, there is no commercialized vaccine to combat these diseases. Developing a bivalent vaccine to protect against multiple diseases in a single immunization, especially by oral administration, is highly beneficial to farmers. Previously, a novel feed-based bivalent vaccine containing formalin killed whole organisms *S. iniae* and *A. hydrophila* mixed with 10% palm oil for red hybrid tilapia was successfully developed, with the ability to improve fish immunity and give high protection against *S. iniae* and *A. hydrophila* (Monir et al., 2020; Monir et al., 2021).

However, *S. agalactiae* serotype III is currently reported as the dominant serotype infecting tilapia farming in Southeast Asia, including Malaysia, Indonesia and Thailand (Syuhada et al., 2020). Furthermore, there is a lack of characterization on the previously developed novel feed-based bivalent vaccine (Monir et al., 2020) in terms of the killed whole organism's stability, mechanism of the oral delivery route and effect of mixing palm oil with the whole killed organisms' stability in vaccine preparation.

In addition, preparing the feed-based bivalent vaccine involves many steps, including culturing vaccine seed, inactivation and pelleting process before storage. Thus, detailed characterization is required to ensure that the vaccine preparation process and storage are not affecting the outcome of vaccination.

This study aims to characterize the feed-based bivalent vaccine containing killed whole organisms of *S. agalactiae* serotype III and *A. hydrophila* mixed with 10% palm oil for red hybrid tilapia. The vaccine potential in improving immunological responses in red hybrid tilapia against the causal agent of streptococcosis and aeromoniasis diseases is also highlighted in this study. The effort to use the local feed-based vaccine in Malaysia is limited and ongoing.

Thus, a concerted effort to investigate the immunoprotective potential of a newly developed feed-based bivalent vaccine containing killed whole organisms of *S. agalactiae* serotype III and *A. hydrophila* mixed with palm oil for red hybrid tilapia is necessary and merits scientific investigation. The study investigating newly developed feed-based bivalent vaccines may provide an alternative for sustainable tilapia production and bacterial diseases management.

1.4 Research objectives

The research framework of this study is elaborated in Figure 1.1. The objectives of this study are:

i. to characterize the physical stability, nutritional quality, safety and growth performance of a newly developed feed-based bivalent vaccine containing killed whole organisms of *S. agalactiae* and *A. hydrophila* mixed with 10%

palm oil in red hybrid tilapia.

ii. to determine the immunological response and its protective efficacy in vaccinated red hybrid tilapia against experimental streptococcosis and aeromoniasis infections.

iii. to determine the expression of immunological response in red hybrid tilapia following vaccination with the feed-based bivalent vaccine through transcript response analyses.

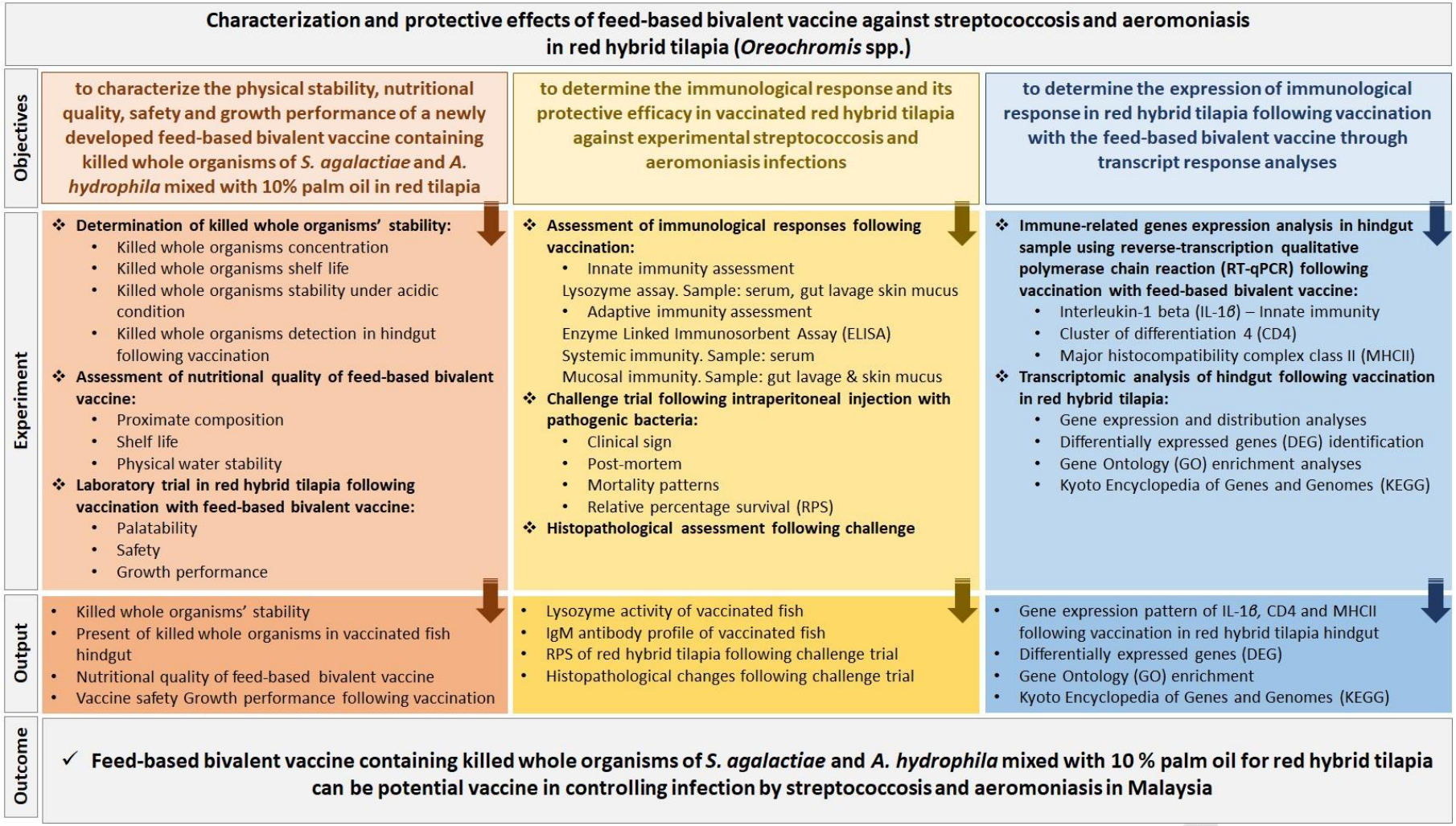

Figure 1.1: Research framework of this study.

1.5 Hypotheses of the study

Objective 1:

H0: The feed-based bivalent vaccine pellet containing killed whole organisms of *S. agalactiae* and *A. hydrophila* mixed with 10% palm oil in red hybrid tilapia cannot be characterized.

H1: The feed-based bivalent vaccine pellet containing killed whole organisms of *S. agalactiae* and *A. hydrophila* mixed with 10% palm oil in red hybrid tilapia can be characterized.

Objective 2:

H0: The feed-based bivalent vaccine pellet could not improve immunoprotective efficacy against the causal agent of streptococcosis and aeromoniasis diseases in red hybrid tilapia.

H1: The feed-based bivalent vaccine pellet could improve immunoprotective efficacy against the causal agent of streptococcosis and aeromoniasis diseases in red hybrid tilapia.

Objective 3:

H0: The transcript responses in red hybrid tilapia following vaccination with the feed-based bivalent vaccine pellet could not be elucidated.

H1: The transcript responses in red hybrid tilapia following vaccination with the feed-based bivalent vaccine pellet could be elucidated.

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