SEED PREPARATION AND FIELD EFFICACY OF PALM OIL ADJUVANTED FEED-BASED STREPTOCOCCOSIS VACCINE

BY

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INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA

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A dissertation submitted in fulfilment of the requirement for the degree of Master of Science

Kulliyyah of Science
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ABSTRACT

Streptococcosis poses a considerable threat to tilapia farming, necessitating the urgent development of an effective vaccine. Previously, the potential usage of palm oil as a vaccine adjuvant in the feed-based streptococcosis vaccine was investigated with promising result following laboratory trials. The present study further investigated the vaccine's efficacy using a similar formulation under field conditions. Prior to the vaccine preparation, the *S. agalactiae* growth curve was developed to understand its growth dynamic and obtain the optimal numbers of bacterial isolates. After that, the proximate composition of the feed-based vaccine was determined to ensure the nutrients in the prepared vaccine is comparable with the commercial feed. The findings indicated that twelve hours incubation of *S. agalactiae* at 30°C reaches the peak of the exponential phase, and the nutrient composition (moisture, ash, crude fiber, crude fat, and crude protein) of the vaccinated pellet was within the recommended nutritional value. Meanwhile, six thousand healthy red hybrid tilapias with an average bodyweight of 40 ± 20g were equitably distributed into six cages at Kenyir Lake, Terengganu. The selected red hybrid tilapias were screened for *S. agalactiae* and ensured to be free from those infections before being transferred to the field trial site. Fish from Group 1 in cages 1 and 2 were not vaccinated, serving as a negative control group. Group 2 in cages 3 and 4 were vaccinated twice on week 0 and week 2 (single booster), while Group 3 in cages 5 and 6 were vaccinated thrice on week 0, week 2, and week 6 (double booster). The palm oil adjuvanted bacterin vaccine was administered orally at an average of 5% body weight. Blood serum and organ samples of the eye, brain, and kidney were collected at two weeks intervals until the conclusion of the 16-week trial period. The collected samples were then subjected to bacterial isolation and immunoglobulin M (IgM), lysozyme, and complement-3 quantification. Following vaccination and the first booster, there was a significant (p<0.05) increase in IgM levels in all vaccinated groups from week 2 that peaked at week 4 before gradually declining. However, an additional booster given at week 6 was significantly (p<0.05) increased the IgM levels and remained highest until the end of the field trial. Similarly, an increasing lysozyme and complement-3 activity were observed following each administration, especially in the double booster group. The average isolation rate of *S. agalactiae* was 19.6 ± 20.2%, 11.5 ± 11.3%, and 8.2 ± 9.4% from unvaccinated, single, and double booster groups, respectively. At the end of the study period, the mortality rate was 23.6 ± 1.8% for unvaccinated, 14.5 ± 5.9% for single booster, and 6.4 ± 0.4% for double booster groups. These results indicate that oral administration of palm oil adjuvanted vaccine stimulates both specific and non-specific immune responses, reduces the incidence of streptococcosis to 11.4%, and improves the survival rate to 93.6%.

خلاصة البحث

تشكل المكورات العقدية تهديدًا كبيرًا لاستزراع البلطي، مما يستلزم التطوير العاجل للقاح الفعالي. في السابق، أظهرنا الاستخدام المحتمل لزيت النخيل كعامل مساعد للقاح في القاح المكورات العقدية المعتمد على التغذية بعد التجارب المعملية. حققت الدراسة الحالية كذلك في فعالية اللقاح باستخدام تركيبة مماثلة في ظل الظروف الميدانية. قبل تحضير اللقاح، تم تطوير منحنى نمو اس. اجالاكتياي (*S. agalactiae*) لفهم ديناميكية نموه للحصول على العدد الأمثل من العزلات البكتيرية. بعد ذلك، تم تحديد التركيب التقريبي للقاح القائم على العلف للتأكد من أن العناصر الغذائية في اللقاح المحضر قابلة للمقارنة مع العلف التجاري. أشارت النتائج إلى أن اثني عشر ساعة من حضانة اس. اجالاكتياي (*S. agalactiae*) عند 30 درجة مئوية تصل إلى ذروة المرحلة الأسية، وأن التركيب الغذائي (الرطوبة، والرماد، والألياف الخام، والدهون الخام، والبروتين الخام) للحبيبات المحصنة كان ضمن القيمة الغذائية الموصى بها. وفي الوقت نفسه، تم توزيع ستة آلاف من البلطي الأحمر المهجن الصحي بمتوسط وزن جسم 40±20 جرام بشكل عادل على ستة أقفاص في بحيرة كينير، تيرينجانو. تم فحص البلطي الأحمر المختار بحثًا عن اس. اجالاكتياي (*S.agalactiae*) والتأكد من خلوه من هذه العدوى قبل نقله إلى موقع التجربة الميدانية. لم يتم تحصين أسماك المجموعة 1 في الأقفاص 1 و 2، حيث تم استخدامها كمجموعة ضابطة سلبية. المجموعة 2 في الأقفاص 3 و 4 تم تطعيمها مرتين في الأسبوع والأسبوع 2 (معزز واحد)، بينما المجموعة 3 في الأقفاص 5 و 6 تم تلقيحها ثلاث مرات في الأسبوع 0 والأسبوع 2 والأسبوع 6 (معزز مزدوج). تم إعطاء لقاح البكتيريا المساعد بزيت النخيل عن طريق الفم بمعدل 5٪ من وزن الجسم. تم جمع عينات من مصل الدم وأعضاء العين والدماغ والكلى على فترات أسبوعين حتى انتهاء فترة التجربة البالغة 16 أسبوعًا. تم بعد

ذلك إخضاع العينات التي تم جمعها للعزل البكتيري والغلوبولين المناعي M (IgM) ، والليزوزيم ، والتقدير الكمي المكمل -3. بعد التطعيم وأول جرعة معززة، كانت هناك زيادة ملحوظة ($p<0.05$) في مستويات IgM في جميع المجموعات التي تم تلقيحها من الأسبوع 2 والتي بلغت ذروتها في الأسبوع 4 قبل أن تنخفض تدريجيًا. ومع ذلك، فإن المعزز الإضافي الذي تم إعطاؤه في الأسبوع 6 أدى بشكل ملحوظ ($p<0.05$) إلى زيادة مستويات IgM وظل أعلى مستوى حتى نهاية التجربة الميدانية. وبالمثل، لوحظ زيادة نشاط الليزوزيم والمكمل 3 بعد كل إدارة، خاصة في مجموعة التعزيز المزدوج. كان متوسط معدل عزل اس. اجالاكتياي (*S.agalactiae*) 19.6 ± 20.2٪، 11.5 ± 11.3٪ و 8.2 ± 9.4٪ من المجموعات المعززة غير المحصنة ، المفردة ، والمزدوجة على التوالي. في نهاية فترة الدراسة، كان معدل الوفيات 23.6 ± 1.8٪ لغير الملقحين، 14.5 ± 5.9٪ للداعم الفردي، و 6.4 ± 0.4٪ للمجموعات المعززة المزدوجة. تشير هذه النتائج إلى أن تناول اللقاح المعزز بزيت النخيل عن طريق الفم يحفز الاستجابات المناعية النوعية وغير النوعية، ويقلل من حدوث المكورات العقدية إلى 11.4٪، ويحسن معدل البقاء على قيد الحياة إلى 93.6٪.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Master of Science.

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DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

Mohd Syafiq Bin Mohammad Ridzuan

Signature ..... Date February 1, 2024

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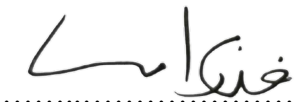

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Signature

February 1, 2024

……..

Date

This dissertation is dedicated to my parents for laying the foundation of what I turned out to be in life.

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Alhamdulillah, and very grateful to Allah s.w.t. blessing, to enable me to complete this dissertation. First and foremost, it is my utmost pleasure to dedicate this work to my dear parents and my family, who granted me the gift of their unwavering belief in my ability to accomplish this goal: thank you for your support and understanding. I would also like to give special thanks to my caring, loving, and supportive wife, Suziyana Binti Shuhadin, for her patience when I was conducting and composing my research: your prayer for me was what sustained me this far.

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CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Fish superiority as an excellent source of protein, vitamin D, and omega-3 fatty acid is one of the factors furnishing to their increasing demand. The United Nations (UN) recently updated their demographic projections, predicting that the world's population would exceed 9.7 billion by 2050 (United Nation, 2019), implying that fish demand will consistently rise in the immediate future. As a result, aquaculture continues to expand faster than other major food-producing industries (FAO, 2018). In recent years, the aquaculture sector has been regarded as essential for increasing fish production and mitigating the burden on capture fisheries. Presently, aquaculture accounts for 49% of total fish production, with Asia contributing more than 91% of worldwide aquaculture production (FAO, 2022). With great potential and ongoing government support, Malaysia's aquaculture sector is flourishing and aiding in meeting the country's rising demand for animal protein to secure the national food security.

Malaysia is blessed with an abundance of rainfall and freshwater resources. Peninsular Malaysia and East Malaysia (Sabah and Sarawak) receive an average of 2,500 and 5,080 mm of precipitation annually (Lung, 2016). The country is drained by 150 river systems stretching a total length exceeding 38,000 km (Chan et al., 2003). In addition, at least 73 man-made lakes have been constructed to serve as reservoir basins for municipal, industrial, agricultural, hydroelectric, and flood mitigation (Sharip & Zakaria, 2008). This abundance of freshwater resources has significantly impacted the expansion of freshwater aquaculture in the country. Keen interest in freshwater aquaculture in Malaysia predated the 1950s with rearing of Mozambique tilapia and common carp in earthen ponds introduced (Department of Fisheries Malaysia, 1955).

More than 70 years later, the significant accomplishment was justified with more than 29 freshwater species being cultured at present in various culture systems, including freshwater ponds, ex-mining pools, freshwater cages, cement tanks, canvas tanks, pen culture, and estate culture (Department of Fisheries Malaysia, 2021). Freshwater aquaculture productivity recently reached 105,904 metric tons, with Perak, Selangor, and Pahang accounting as the top three leading contributors. As of 2021, there were 21,241 fish farmers in with 15,623 (73.5%) of them engaged in freshwater aquaculture (Department of Fisheries Malaysia, 2021).

Tilapia is the one of the country's most important commercial freshwater fish species. The annual production of tilapia in Malaysia has risen from 34,822 metric tons in 2008 to 51,555 metric tons in 2012 (Department of Fisheries Malaysia, 2008, 2012). However, recent production suffered significantly downshift with only 38,543 metric tons in 2021 with a total estimated wholesale value of more than RM 390 million (Department of Fisheries Malaysia, 2021). In general, tilapia's increasing popularity are mainly due to its affordability and tasty, and they are relatively hardy compared to other farmed species. The past decades, however, have demonstrated that tilapia is also vulnerable to a variety of illnesses.

Streptococcosis is regarded as a major freshwater fish disease and is responsible for the high morbidity and mortality in the aquaculture sector. In 1957, the disease initially identified in Japanese rainbow trout *Salmo gairdneri* culture (Hoshina et al., 1958). Since then, there has been a significant increase in streptococcal case reports, posing a threat to freshwater aquaculture worldwide. In Malaysia, streptococcal epidemics have been continuously observed in floating net cages at Pahang River, Kenyir Lake, Pedu Lake, and Pergau Lake, resulting in high tilapia mortality (Amal et al., 2008; Siti-Zahrah et al., 2008). To date, various *Streptococcus* species have been identified as causative agents, including *S. agalactiae*, *S. iniae*, *S. dysagalactiae*, *S. parauberis,* and *S. feacalis* (Soltani et al., 2012). However, the first two species are the

most common threat to tilapia aquaculture worldwide, including Malaysia (Amal, Zamri-Saad, et al., 2013; Doan et al., 2022; Evans et al., 2006).

Currently, streptococcal outbreaks are mitigated by a combination of effective farm management and the use of commercially accessible antibiotics. However, in recent years, antibiotics and other chemotherapeutics have lost their appeal in aquaculture due to environmental concerns, regulatory constraints, cost, and the increasing incidence of resistant microbes (Okeke et al., 2022; Schar et al., 2020). For these reasons, vaccine development is increasingly viewed as a critical area of aquaculture research. The ideal method for mass-vaccinating fish of various sizes or ages is an oral vaccine, which is commonly created by integrating the antigen into feed meals. Oral administration is preferred since it is less time-consuming, reduced stress for the fish, and provide more options when designing a vaccination schedule or regime (Galindo-Villegas et al., 2015).

Unfortunately, vaccines are usually unable to confer protection on their own. Adjuvants are often needed to boost the efficacy of the vaccine. Traditional adjuvants like mineral oil, non-mineral oil, and synthetic oil are commonly used in different bacterial vaccines for fish. However, their use significantly increases vaccine manufacturing cost and adverse side effect (J. Li et al., 2020; Noia et al., 2014). Thus, a preference for a less expensive alternative with no or minimal side effects is beneficial and should be pursued. Previously, the potential usage of palm oil as vaccine adjuvant in the streptococcosis vaccine was investigated producing promising result following laboratory trials (Aminudin et al., 2018). Therefore, in the present study, the vaccine's efficacy was further investigated using a similar formulation under field conditions.

Palm oil is an edible vegetable oil that is distinguish by the high concentration of saturated fatty acid in the 2-position of its triglycerides. Several components in palm

oil have been found to have immune-boosting properties. For example, saponins which are steroid or triterpenoid glycosides, capable of creating foam when a water solution containing these actives is stirred. The pharmaceutical industry has long used saponins as a raw material for producing steroidal drugs and cosmetics. Several studies have shown that palm oil adjuvanted vaccine can stimulate cell-mediated immunity and promote antibody production (Aiyer Harini et al., 2013; Aslah et al., 2021; Monir et al., 2021, 2022; Wanasawaeng et al., 2009). Its use as an adjuvant formulation can therefore be adapted to boost the desired immunological response.

Prior to authority body approval and commercialization, a vaccine must undergo a field-testing to assess its effectiveness, side effects, and protection provided. Field-testing is very crucial since many uncontrolled variables can affect the efficacy of the vaccine outside there. Hence, it's critical to carefully observe and analyze the vaccine efficacy results in both lab and field (OIE, 2018).

1.2 STATEMENT OF THE PROBLEM

Aquaculture has experienced an economic transformation in recent years, but infectious diseases of bacterial, viral, and parasitic origin remain the most critical stumbling blocks to sustainable and intensified aquaculture. *Streptococcus agalactiae* is the main fish bacterial pathogen in many parts of the world, particularly among tilapias. The disease affects tilapia production, causing significant economic losses and jeopardizing the sustainability of aquaculture. In their studies, Shoemaker & Klesius (1997) estimated that global annual losses due to streptococcosis alone totaled USD 150 million and exceeded USD250 million in 2008 (Amal & Zamri-Saad, 2011; Klesius et al., 2008a). Hence, successful streptococcal vaccinations are desperately needed to combat the disease.

Usage of antibiotics in aquaculture can cause substantial environmental effects due to chemical residues in the water and the potential development of multi-resistant bacteria. Antibiotics in fishmeal are costly but may not be compelling enough because streptococcosis infected fish usually lose appetite (Doan et al., 2022; Piamsomboon et al., 2020). Besides causing public health concerns, antibiotic contamination of fish carcasses has also been a barrier to foreign trade (Fidler, 1998; Kümmerer, 2003). Moreover, the infection is usually transmitted from fish to fish, where the bacteria were released from dead and dying fish, which is considered the most important source of infection (Kitao, 1993). As a result, vaccination is widely accepted as the safest, most practical, and cost-effective way of preventing aquatic animal diseases (Sudheesh & Cain, 2017).

Vaccination is the most effective method for managing streptococcosis in fish. The oral route is viewed as the most practical method in mass vaccination compared to other methods such as intraperitoneal injection and immersion. Vaccines alone are not sufficient to provide the immunological protection needed since most of them are poor immunogens requiring conjugation to a large immunogenic carrier. As a result, co-administering with an adjuvant is recommended. Usages of commercially available adjuvants are extremely expensive and associated with adverse side effects. Thus, a search for a cheaper alternative is desirable and should be explored.

1.3 RESEARCH OBJECTIVES

The study aimed to achieve the following objectives:

1- To develop the growth curve of *S. agalactiae* under normal laboratory conditions for seed vaccine preparation.

2- To determine the proximate composition of the experimental feed-based streptococcosis vaccine.

3- To determine the immune responses of cage cultured red hybrid tilapia and the efficacy of palm oil adjuvanted streptococcosis vaccine following oral vaccination regime under field condition.

1.4 RESEARCH QUESTIONS

1. What is the pattern of the *S. agalactiae* growth curve following incubation for seed vaccine preparation?
2. What is the feed-based vaccine's nutritional composition after adding palm oil as a vaccine adjuvant?
3. Does oral vaccination with palm oil adjuvanted streptococcosis vaccine stimulates fish immune response?
4. What is the vaccine's efficacy following an oral vaccination regime against the natural challenge of *S. agalactiae*?

1.5 RESEARCH HYPOTHESES

Meanwhile, the hypotheses of the present study are:

1. In batch culture, bacteria grow in a predictable pattern, resulting in a growth curve composed of four phases of lag, exponential, stationary and death.
2. Formulation used in preparing the vaccine consist of nutritional compositions that are suitable for dietary of farmed red hybrid tilapia.
3. Oral vaccination with palm oil adjuvanted streptococcosis vaccine stimulates fish immune responses and provide adequate protection against streptococcosis in red hybrid tilapia.

1.6 SIGNIFICANCE OF THE STUDY

Tilapia is one of the most important fish species in aquaculture sector in Malaysia and, at present, is threatened by the continuous occurrence of streptococcosis outbreaks. The disease is predominantly caused by a bacterial species named *Streptococcus agalactiae*, resulting in significant mortality of cultured tilapia and economic losses. Currently, there is no locally available aquaculture vaccine to combat the disease; therefore, the production of an effective vaccine is rather urgent. Oral vaccination of fish offers attractive attributes requiring minimal labour without additional infrastructure or specialized equipment. Hence the information gained from this study can be gathered to establish a systematic strategy for producing a low-cost streptococcosis vaccine for tilapia aquaculture.

1.7 SCOPE AND LIMITATIONS OF THE STUDY

The main focus of this research was to demonstrate both specific and non-specific immune responses produced by red hybrid tilapia following oral administration of the vaccine under actual field condition.

However, it is important to note that this study was limited to the definite use of *Streptococcus agalactiae* (SA2BKE) as a seed vaccine and the route of vaccine administration was oral. Besides, the field trial was conducted on a commercial farm for a total of sixteen (16) weeks, where the fish was exposed to the natural challenge of the disease .

CHAPTER TWO

LITERATURE REVIEW

Published in Ridzuan *et al*. (2022) Current status and advances of fish vaccines in Malaysia, *Veterinary World*, 15(2): 465-482. DOI 10.14202/vetworld.2022.465-482.

2.1 INTRODUCTION

Malaysia has recently developed aquaculture facilities for at least 49 marine and freshwater fish species. The most cultivated marine fish species are Asian seabass, snapper, and grouper. In contrast, the most cultured freshwater fish species are catfish, tilapia, and riverine catfish, with cumulative production of 121,553.75 metric tonnes in 2020 (Department of Fisheries Malaysia, 2020). Numerous culture systems, including ponds, cages, ex-mining pools, cement, and canvas tanks, have been adopted and practiced throughout Malaysia's coastal and freshwater locations. The fish farming business is developing with continued government backing and favorable response, resulting in intensification measures.

Unfortunately, the sector has also seen a variety of fish diseases in numerous fish species, resulting in massive economic losses and jeopardizing the company's long-term viability. Current solutions rely on a combination of good farm management and the use of commercially accessible antibiotics. The Malaysian government recently developed a national action plan on antimicrobial resistance (MyAP-AMR) for the period 2017-2021, which discourages the abuse of antibiotics in the food production industry. Furthermore, different efforts have been made to raise national awareness about AMR and antibiotics, expand the information and evidence base through surveillance and research, and optimize antimicrobial drug use (Wan Norhana et al., 2020). Vaccination and alternative medicine are considered as a way out under these

circumstances and have grown in popularity among local researchers, although acceptance among farmers remains questionable.

Several advancements and notable accomplishments have been made locally in the development of aquaculture vaccines against fish diseases. As a result, the purpose of this review is to offer an update on recent aquaculture vaccine development in Malaysia for major fish species and diseases. We genuinely hope that this analysis provides insight into the future potential for aquaculture fish vaccine development.

2.2 GENERAL OVERVIEW OF RECENT AQUACULTURE STATUS IN MALAYSIA

The fisheries sector provides a significant portion of income for more than 106,000 local fishermen and farmers in Malaysia (Department of Fisheries Malaysia, 2020). In addition, fisheries provide up to 12% of agriculture's gross domestic product (GDP) and 0.9% of the country's GDP (Department of Fisheries Malaysia, 2019). While it may seem insignificant, the fisheries sector is vital in supplying food, jobs, nutrition, and a healthy lifestyle and supporting other downstream businesses. Malaysia's capture fisheries and aquaculture production totaled 1.6 million tonnes in 2020, a decrease of 2.36% from 2019. Table 1 (Department of Fisheries Malaysia, 2016, 2017, 2018, 2019, 2020) compares the overall fisheries productivity in Malaysia from 2016 to 2020.

Malaysia's aquaculture sector is thriving with huge potential and ongoing government support to supply the rising demand for animal protein. Aquaculture is critical for boosting fish production, balancing demand on capture fisheries, and preventing wild fish overexploitation. Globally, aquaculture currently accounts for 46% of total fish production (FAO, 2020), with Asia accounting for more than 91% of worldwide aquaculture production (Tacon, 2019). In 2020, Malaysia had a total of

20,262 fish farmers. About 73.5% (7,349 farmers) were engaged in freshwater aquaculture, while 16% (3,349 farmers) were engaged in brackish water aquaculture. In contrast, the remainder were in other sub-sectors classified as ornamental fish, aquatic plants, or seaweed. Malaysian freshwater aquaculture production totaled 97,209.74 tonnes worth RM 766 million in 2020, while brackish aquaculture production totaled 120,739.51 tonnes worth RM 2,289.39 million (Table 1).

On the other hand, seaweed production was estimated to reach 182,061.00 tonnes of wet weight in 2020, accounting for RM 58.87 million of the wholesale value. Malaysia's aquaculture fish production system relies on conventional technologies such as ponds, ex-mining pools, cages, tanks, and pen culture (Table 2). The utilisation of cutting-edge technology like recirculating aquaculture systems (RAS), is currently restricted to government hatcheries.

Table 1 Fisheries and aquaculture production between 2016 – 2020 in Malaysia (Department of Fisheries Malaysia, 2016, 2017, 2018, 2019, 2020)

Year	**2016**	**2017**	**2018**	**2019**	**2020**
Production					
Capture					
Inland (Tonnes)	5,847.97	5,177.19	6,089.08	5,568.70	5,625.14
Marine (Tonnes)	1,574,447	1,465,113	1,452,862	1,455,446	1,383,299
Total capture (Tonnes)	1,580,295.0	1,470,290.2	1,458,951.1	1,461,014.7	1,388,924.1
Total value of capture fisheries (RM Million)	10,265.78	10,912.16	11,437.19	11,426.49	10,181.43
Aquaculture[1]					
Freshwater (Tonnes)	103,348.21	102,596.83	101,269.88	104,601.56	97,209.74
Total value of freshwater aquaculture (RM Million)	789.11	728.18	711.09	780.50	766.47
Marine (Tonnes)	98,049.9	121,453.02	116,112.08	119,069.47	120,739.51
Total value of marine aquaculture (RM Million)	1,891.63	2,268.09	2,293.68	2,458.27	2,289.39
Total aquaculture	201,398.11	224,049.85	217,381.96	223,671.03	217,949.25
Total fisheries and aquaculture[2]	1,781,693.1	1,694,340.0	1,676,333.0	1,684,685.7	1,606,873.4

[1]Exclude production of seaweed. [2]Total may not match due to rounding.

Table 2 Fractions of aquaculture production systems and area adopted in Malaysia (2020) (Department of Fisheries Malaysia, 2020)

Production system / Area	**Pond (Ha)**	**Ex-mining pool (Ha)**	**Cages (m^2 x10^3)**	**Tank[1] (m^2 x10^3)**	**Pen culture (Ha)**	**Molluscs culture[2] (Ha)**	**Seaweed culture (Ha)**
Freshwater	3,725.29	3,033.00	550.00	417.99	8.00	-	-
Marine	7,511.00	-	2,304.47	239.00	392,368.00	9,714.00	9,828.00
Total	11,236.29	3,033.00	2,854.47	656.99	392,376.00	9,714.00	9,828.00
Total value (RM Million)	1,849.23	100.39	921.27	4.71	10.73	126.71	58.87

[1]Include cement and canvas tank. [2]Include cockle (bottom culture), mussel and oyster (raft culture).

2.3 INFECTIOUS DISEASES OF FISH IN MALAYSIA

Infectious diseases are any abnormalities caused by a wide range of microorganism infections. Bacteria and viruses are the main culprits in Malaysia's outbreaks of infectious illnesses in the aquaculture industry (Sayuthi, 1993). The most common bacterial diseases that affect cultured fish are *Streptococcus agalactiae*, *Streptococcus iniae, Aeromonas hydrophila* and *Vibrio alginolyticus* (Aisyhah et al., 2015; Siti Hawa et al., 2020; Siti-Zahrah, 1992). Meanwhile, betanodavirus and iridovirus frequently affect cultured marine fish species, and recently the outbreaks of emerging diseases of tilapia lake virus (TiLV) were reported to affect the wild (Abdullah et al., 2018) and cultured tilapia (Amal et al., 2018). These diseases have contributed substantial economic losses to the country, and actions must reduce the impact. Vaccination was considered the best control measure to protect the fish from infectious diseases by developing herd immunity (Miccoli et al., 2021). A recent study by Standish et al. (2016) proved the existence of herd immunity in aquatic animals, which played a crucial role in increasing the survival rate as well as disease resistance.

2.4 OVERVIEW OF FISH VACCINES LICENSING IN MALAYSIA

The approval of any veterinary vaccine registration in Malaysia, including fish vaccines, is governed by the Department of Veterinary Services (DVS), Ministry of Agriculture and Food Industry, and is governed by the Animal Act 1953. As stated in Section 30(1) of the Animal Act 1953 (revised in 2006), the Director-General of DVS has the authority to provide a license authorizing the holder to possess live cultures or veterinary vaccinations and to administer them to animals or birds. In addition, Section 84(1) of the Animal Act 1953 (Amendment 2013) stated that no person should knowingly import or possess any living noxious insect, pest, or living germ, virus, or bacterial culture, that is harmful or dangerous to animals or birds without the Director-prior General's written permission. Therefore, the DVS as a regulatory agency controls the production, importation, distribution, sale, and use of veterinary vaccines for

diagnosis, treatment, research, and disease prevention. The Technical Committee of Veterinary Product (TCVP) and National Veterinary Product Control Committee (NVPCC) under DVS act as a licensing authority to register animal vaccines and other biologics. The licensure of new animal vaccines is subjected to a well-defined regulatory process. A general framework for the regulatory approval of veterinary vaccines is shown in Figure 1 (Department of Veterinary Services Malaysia, 2019).

At present, Malaysia has approved only three aquatic vaccines, which are shown in Table 3 (Department of Veterinary Services Malaysia, 2020). These vaccines are inactivated and are meant to protect against streptococcosis, vibriosis, and iridoviral disease. Notably, other vaccine forms, such as recombinant, DNA, or live-attenuated vaccinations, have not been licensed for aquaculture use in Malaysia.

Table 3 List of approved aquatic vaccines in Malaysia (Department of Veterinary Services Malaysia, 2020)

Trade name	**Causative agent**	**Manufacturer**	**Nature of vaccine**	**Recommended species**	**Delivery methods**[1]	**Dose and recommended fish size**	**Further information**
AquaVac Strep SI	*Streptococcus iniae*	Intervet, Holland	Killed	Tilapia; Asian seabass; Other susceptible fish species	IP; IMM	Injection: 0.1 ml/fish; for fish 20 g or above. Immersion: 1 L of vaccine and 9 L of seawater; for fish 3 g and above.	www.aquavac-vaccines.com/
AquaVac IridoV	Iridovirus	Intervet, Holland	Killed	Asian seabass; Grouper; Pompano; Japanese yelowtail	IP	0.05 ml/fish; for fish 5 g or above.	www.aquavac-vaccines.com/
Vibri-Fishvax	*Vibrio anguillarum*	Fatro S.p.A, Italy	Killed	Trout; Salmon; Seabass; Seabream	IP; IMM	Injection: 0.1 ml/fish; for fish 50 g or above. Immersion: dip 7.5-10 kg of fish per 1 L of vaccine (1:10 ratio); fish between 1-8 g	www.fatro.it

[1]IP: intraperitoneal injection; IMM: immersion

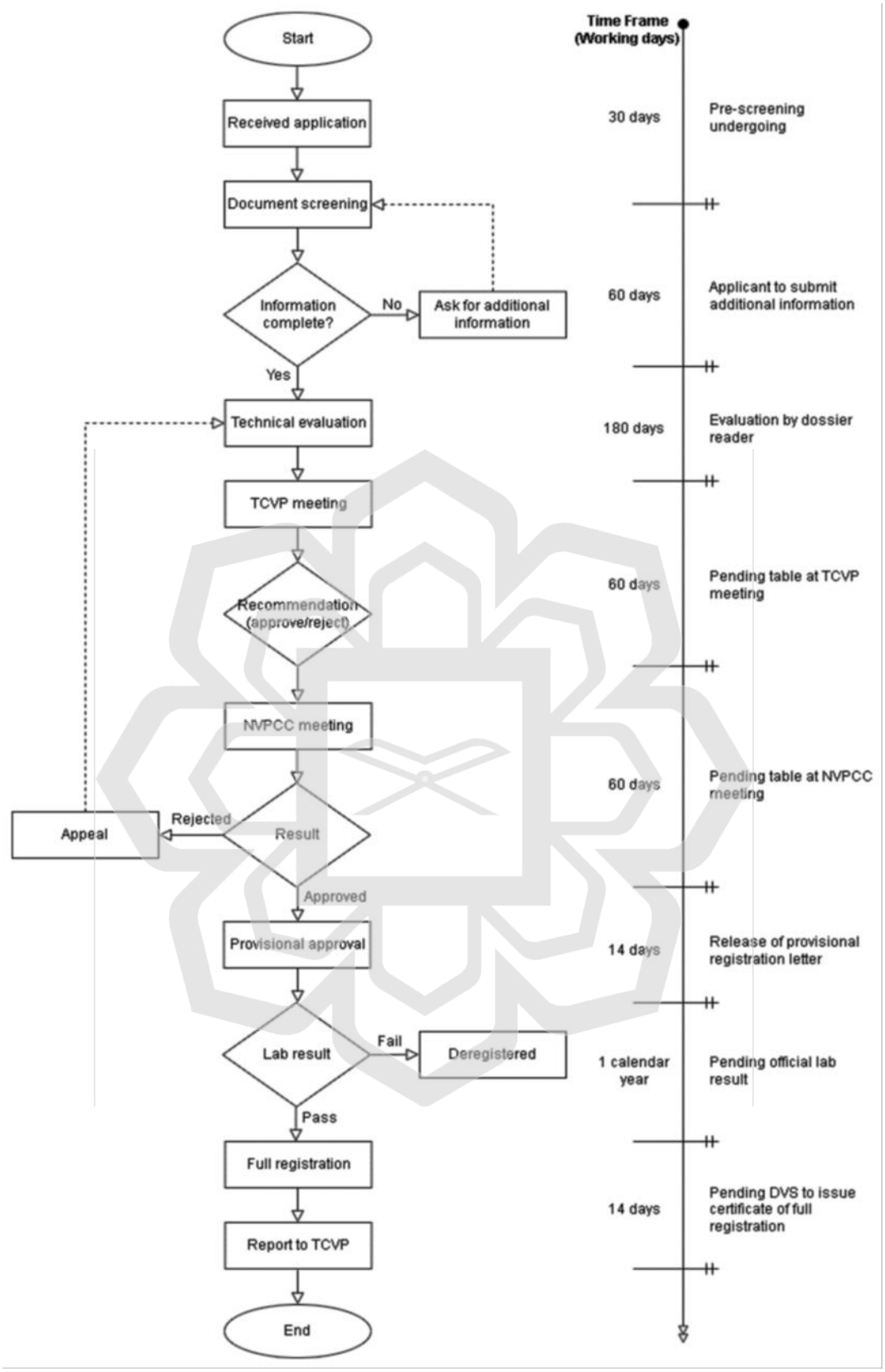

Figure 1 Flow chart and time frame of veterinary vaccines registration in Malaysia (Department of Veterinary Services Malaysia, 2019)

2.5 OVERVIEW OF RECENT RESEARCH AND DEVELOPMENT (R&D) OF THE FISH VACCINE IN MALAYSIA

Malaysian research on fish vaccines predates 1979 and has lagged much behind other countries. Initially, this project was sparked by the rapid expansion of marine fish mariculture, including sea bass (*Lates calcarifer*), grouper (*Epinephelus coioides*), and golden snapper (*Lutjanus johni*), which began in 1973 and accelerated in the 1980s. Fingerlings or juveniles were brought in large quantities from neighboring countries such as Chinese Taipei, Indonesia, and Thailand to deal with vast demand and concentration (Seng, 1997). Due to the increased intensity of aquaculture, an episode of disease outbreak is frequently experienced during the initial stocking and grow-out stage. Throughout a one-year survey conducted by (Chuah, 2001), it was discovered that groupers were primarily linked to viral diseases (50%), with bacterial infections accounting for 47% and cryptocaryoniasis accounting for the remaining 3%. Based on the culturists' descriptions of the discoloration (blackening) of the fish body without other external symptoms, it was suspected that the virus-associated disease was iridoviral infection, whereas the bacterial-associated disease of *Vibrio* spp. and *Flexibacter* spp caused ulceration or rotting of the groupers' fins and tail.

One of the earliest studies on the fish vaccine in Malaysia was recorded in the late seventies. Wong et al. (1979) explored the root cause of red boil disease in estuary grouper (*Epinephelus salmoides*) with the possibility of vaccination control. Their study found that *Vibrio parahaemolyticus* is the cause of disease based on colony morphology on selective Thiosulfate-citrate-bile salts-sucrose media and pathogenicity test. Unfortunately, they were unable to develop effective vaccines against *V. parahaemolyticus* utilizing several means of inactivation, including heat-killed and formalin-killed, with and without adjuvant. When challenged 1 week after immunization, all the vaccines failed to protect the fish, with 60-70% mortality rates. However, the absence of protection was most likely caused by the bacterium being challenged before the immunity developed.

Another mass vaccination trial was conducted in Malaysia in the late 1990s, with special permission granted to a government research institute, the National Fish Health Research Centre (NaFisH), Fisheries Research Institute (FRI) Batu Maung, Penang, to conduct a field vaccination trial using Alpharma vaccine on *Lutjanus argentimaculatus* against vibriosis (Siti-Zahrah et al., 2003). A total of 19,000 fish were vaccinated in-house at the hatchery before being sent to Langkawi Island for cage culture. Unfortunately, between 24 and 48 h after immunization aberrant mortality (100%) was seen, along with bulging of the ocular membrane on the dead fish. As a result, a replicated study on a laboratory scale was done to ascertain any predisposing conditions that may have contributed to the situation. Initially, it was suspected that the vaccine or its storage conditions were to blame. However, the vaccine had no detrimental effects, and no abnormalities were observed during the laboratory testing. In addition, the physical and behavioral alterations observed within 24-48 h post-vaccination were missing after 2 weeks of monitoring, as described in the field trial. As a result, they hypothesized that prior catastrophic incidents could have been caused by handling stress and a high stocking density in the holding net prior to injection. Since then, local researchers have made enormous strides, as seen by an increase in the volume of study on epidemiology, pathogenicity, vaccine development, and other relevant topics. The following section will discuss the state of fish vaccine research and development in Malaysia for specific fish species and diseases.

2.6 STREPTOCOCCOSIS VACCINE

Streptococcosis is one of the most extensive vaccines studied in Malaysia due to the aggressive series of outbreaks affecting tilapia culture in Malaysia, dating back to 1997 (Siti-Zahrah et al., 2004, 2008). The disease is a bacterial infection caused by *Streptococcus* species. *S. agalactiae* and *S. iniae* are the major species affecting tilapia aquaculture production worldwide, including Malaysia (Mishra, Nam, et al., 2018; Pei-Chih et al., 2020; Siti Hawa et al., 2020). The clinical symptoms and signs of streptococcosis infected fish include exophthalmia or pop-eye, corneal opacity, hemorrhage at the gill and body surface, inflammation and soft brain, ascites, and

display erratic swimming behaviour (Mishra, Nam, et al., 2018; Zamri-Saad et al., 2010). Histological examination of hybrid tilapia naturally infected by *S. agalactiae* revealed marked congestion and infiltration of inflammatory cells in the eye, brain, kidney, liver, and spleen (Laith et al., 2017).

The first reported case of streptococcosis in Malaysia was documented in the late 1990s at Pahang River, Pahang affecting tilapia weighing between 300 to 400 g and resulting in 60% mortality of cultured red hybrid tilapia. In 2000, a series of disease outbreaks were recorded in Kenyir Lake, Terengganu, and Pergau Lake, Kelantan, resulting in approximately 50% mortality of cultured tilapia (Amal et al., 2008; Siti-Zahrah et al., 2004, 2008). Repeated cases were encountered in Aquaculture Industrial Zone at Kenyir Lake (Najiah et al., 2012) and Temerloh, Pahang (Laith et al., 2017), causing mass mortality of cage-cultured red hybrid tilapia. The outbreaks commonly occur during hot and dry months and are correlated with high water temperature (≥29°C) (Amal et al., 2010). A similar finding by Pei-Chih et al. (2020) reported that water temperature above 27°C was predisposed tilapia to streptococcosis infection in Taiwan. Furthermore, in an experimental trial, Rodkhum et al. (2011) showed Nile tilapia that were exposed to a high water temperature of 30°C and 33°C, after immersion challenged with *S. agalactiae*, resulted in high cumulated mortality compared to the tilapia maintained at 25°C where most of the fish survived without any clinical signs.

More recently, Siti Hawa et al. (2020) reported that *S. iniae* was responsible for some of the cases in cultured red tilapia since 2006. Currently, reported disease cases are widespread, covering all over Peninsular Malaysia. An extensive review of the status of the disease in Malaysia was previously described by Zamri-Saad et al. (2014). Although the evidence of *S. agalactiae* serotype Ia was recently discovered (Syuhada et al., 2020), local isolates predominantly corresponded to serotype III and Biotype 1 (Suphia-Amiera, 2019). In Malaysia, infection by *S. agalactiae* has also been reported in golden pompano, *Trachinotus blochii* (Amal, Zamri-Saad, et al., 2013).

An epidemic of streptococcosis outbreaks was also documented worldwide. For instance, in his recent article, Pei-Chih et al. (2020) reported that the clinical cases of *Oreochromis* spp. in Taiwan were predominated by bacterial infection, where streptococcosis accounted for 53.7% of the cases, and the infection rate was recorded at 29.5%. Meanwhile, in China, sporadic cases of streptococcosis involving *S. agalactiae* have been reported in several provinces of Guangdong, Guangxi, Hainan, and Fujian, which led to massive cumulative mortality and economic losses (J. Sun et al., 2016). It is worth noting that these four central tilapia-producing provinces accounted for 40 percent of global tilapia production. In addition, between 2016 and 2017, six disease outbreaks were reported in Brazil, affecting Nile tilapia with total death rates of 25 – 35% (Leal et al., 2019). Further investigation revealed that the primary etiological agent was *S. agalactiae* serotype III. Recently, the disease was reported to have caused enormous losses in Thailand's freshwater farmed seabass, with a cumulative mortality rate of 35-65% (Piamsomboon et al., 2020). Interestingly, Al-Harbi (2016) previously reported evidence of non-hemolytic group B *S. agalactiae* infection resulting in typical streptococcal clinical symptoms, culminating in acute or chronic infections of cultured tilapia in Saudi Arabia. Streptococci-related global losses were previously estimated at USD250 million in 2008 (Amal & Zamri-Saad, 2011; Klesius et al., 2008b).

Hence, it is now realized that these bacteria pose a considerable threat to tilapia farming, so producing an effective vaccine is rather urgent. In Malaysia, several types of vaccines, such as inactivated, recombinant, and live attenuated vaccines, and different routes of vaccination, either oral, injectable and immersion, were extensively explored by local researchers against both *S. agalactiae* and *S. iniae* (Table 4).

2.6.1 Development of Vaccine Against *Streptococcus agalactiae*

Rather than developing an injectable or immersion vaccine against streptococcosis, significant resources were expended in developing an oral vaccine. The oral route

becomes the most preferred route because 80% of the local tilapia culturists are small-scale operators. Utilizing injectable or immersion courses necessitated additional infrastructure and labor, a massive endeavor that added to the load on these farmers. Oral vaccines, which are normally prepared by infusing the antigen into feed, are suited for mass vaccination of fish of all sizes and ages. Oral administration is the most appealing way since it is straightforward, less stressful for fish, and provides a more flexible approach to vaccination regime formulation (Mutoloki et al., 2015).

Despite offering attractive features, it is essential to note that oral vaccination also has its limitations and usually provides a shorter duration of protection when compared to injection immunization. Furthermore, oral administration makes it impossible to determine the exact dose of antigen each fish takes and occasionally results in inconsistent responses because of the fish's harsh acidic stomach contact. As a result, the antigen is susceptible to being destroyed prior to rousing the gut-associated lymphoid tissues (GALTs) and priming immune cells (Somamoto & Nakanishi, 2020). Therefore, conjugation with a large immunogenic carrier called an adjuvant is crucial to permit a prolonged antigen release (Sharma, 1999). Different types of adjuvants have been tested for fish vaccines, such as alginate microspheres encapsulated (de las Heras et al., 2010), nanoparticle (W. Zhang et al., 2021), Freund's incomplete adjuvant (FIA) (Ramos-Espinoza et al., 2020), and polysaccharides (B. Sun et al., 2018), with promising results.

The effect of oral vaccination of killed whole-cell *S. agalactiae* vaccine on stimulating GALTs in tilapia was previously investigated by Firdaus Nawi et al. (2011). The study discovered that once-weekly vaccination exposure was adequate to stimulate the GALT, but repeated doses elicited stronger responses. Moreover, the size of GALT and the number of aggregated lymphoid cells in tilapia's gastrointestinal tract were much greater than in unvaccinated fish. Their finding provides an early indication that oral exposures to the killed antigen incorporated in feed could be used in a vaccination procedure to protect tilapia from *S. agalactiae* infection.

The protective capacity of locally produced oil adjuvanted oral vaccine against streptococcosis in tilapia was subsequently reported (Firdaus-Nawi et al., 2013). They investigated the effect of immunological response between fish fed with adjuvanted feed-based vaccine, feed-based vaccine (without adjuvant), and unvaccinated groups, following a vaccination regime in a laboratory vaccination trial. They found that following the vaccination regime, the antibody level (IgM) in serum, mucus, and gut lavage fluid of both vaccinated groups was significantly higher ($p<0.05$) as early as 1-week post-immunization, and the increasing pattern was constantly observed till the end of the experiment of week 7. Moreover, the antibody level produced in fish fed with the adjuvanted feed-based vaccine was also significantly higher ($p<0.05$) than fish provided with the feed-based vaccine (without adjuvant). Intraperitoneal challenge against high concentration of live virulent *S. agalactiae* resulted in 100% survival in fish fed with adjuvanted vaccine group, while 50 percent and 12.5 percent survival of fish fed with the feed-based vaccine (without adjuvant) and control group, respectively. Another laboratory trial of a feed-based vaccine against *S. agalactiae* in red tilapia showed that a double booster vaccination regime (week 0, 2, and 6) at 4% of body weight successfully provided a consistently high level of serum IgM antibody for 4 months (Md. S. Ismail et al., 2016). Following the excellent results in the laboratory setting, a field trial was undertaken and tested in an endemic farm (M. S. Ismail et al., 2017). A similar vaccination regime was employed in the field study and resulted in 75% survival of the vaccinated group compared to 45% survival in the unvaccinated group. Interestingly, the author and coworkers demonstrated a comparable serum antibody (IgM) pattern between laboratory and field trials.

A more advanced approach in antigen preparation of *S. agalactiae* involving recombinant DNA technology such as insertion of genetic materials that encode antigen into an expressing vector was also explored by local researchers. As such, Nur-Nazifah et al. (2014) previously reported that an adequate protective capacity of an inactivated recombinant vaccine expresses the cell wall surface anchor family protein of *S. agalactiae* in tilapia, following high dose challenge in a heat-stress environment with a record of 70% survival. Again, an elevated antibody level in blood serum, body mucus,

and gut lavage fluid was significantly higher (p<0.05) and correlated with protection rate. Thus, they believed that the application of appropriate adjuvant could tremendously enhance vaccine efficacy. This finding has led to the vaccination field trials carried out in Kenyir Lake, Terengganu, East Coast of Malaysia (Nadirah, 2015). The same recombinant vaccine that expresses the cell wall surface anchor family protein of *S. agalactiae* in the presence of FIA was used and administered orally during both rainy and dry seasons. In both seasons, the vaccinated group showed elevated levels of serum, mucus, and gut lavage fluid antibody (IgM) (p<0.05) as early as 2nd month post-vaccination and continued to increase after booster (given at month-2) until the end of the trial at 4th month. Stimulation of the GALT was also observed after the 1st month of antigen exposure in the intestine as individual cells or small aggregations in both the epithelium and lamina propria. Better stimulation of GALT was found at the 4th month correlated with repeated exposure of antigen of a booster dose.

More recently, Aminudin et al. (2018) reported the utilization of palm oil as an adjuvant substitute in *S. agalactiae* oral vaccine with promising results. Palm oil is unique among vegetable oils because of its high saturated fatty acid content at the 2-position of its triglycerides and is an excellent source of cheaper adjuvant alternatives due to the vitamin E and saponin content. Both have previously been shown to enhance cell-mediated immunity and antibody production (Aiyer Harini et al., 2013; Aminudin et al., 2018). In addition, another work by Mufti et al. (2017) showed that a mixture of palm and coconut oil adjuvants in a duck pasteurellosis nanovaccine was physiochemically stable following a 6-month pretreatment period at 4, 25, and 40 ˚C.

Another route of administration of vaccine against the disease was also extensively explored. For example, Noraini et al. (2013) reported the potential use of spray administration of the formalin-killed *S. agalactiae* vaccine. Antigen administration through spray could theoretically transfer antigens to mucosal tissues, such as the skin, gill, and nasal, where they can activate innate and adaptive immune responses in the fish (Q. Wang et al., 2020). The authors evaluated the frequency effect

of spray vaccination on fish that received a single spray, fish that received a daily spray for 5 consecutive days, and fish that were not vaccinated. In the 2nd and 4th weeks, each group received two boosts. Serological analysis revealed that both serum and mucus IgM levels of vaccinated fish were significantly higher (p<0.05) after the 1st week of post-vaccination and after each booster compared to control. The challenge trial against *S. agalactiae* at a 10^9 CFU ml^{-1} concentration using both immersion and intraperitoneal methods resulted in 80% and 70% survival rates, respectively. These findings indicated that spray administration effectively generated protective IgM antibodies providing moderate to high protection.

Another study by Kahieshesfandiari et al. (2019) investigated the potential use of feed-based heat-inactivated biofilm vaccine of *S. agalactiae*. Chitin flakes were used to encapsulate the biofilm cells, and they compared the efficacy of the biofilm vaccine against the conventional heat-killed whole-cell vaccine in red hybrid tilapia. The vaccination regime was standardized between both types of vaccines. The vaccines were administered on week 0 and a booster on week 2 before the fish were subjected to the i.p challenge of a high concentration of *S. agalactiae* (10^9 CFU ml^{-1}). The results showed that tilapia vaccinated with biofilm vaccine exhibited significantly robust immune response (p<0.05) and high protection with the relative percentage survival (RPS) of 87% as compared to fish vaccinated with the whole-cell vaccine (57%). Besides that, they said that fish immunized with biofilm vaccine had significantly higher serum, mucus, and gut lavage antibody levels (p<0.05) than fish inoculated with the whole-cell vaccine or the control group. Futhermore, they discussed evidence for a high level of GALT activation in the form of lymphoid cell aggregations within the lamina propria.

Furthermore, continuous effort to develop an efficacious vaccine against streptococcosis has led the local researcher to investigate the potential application of a live-attenuated vaccine, another modern type of vaccine whereby the bacteria hold weakened or less virulent forms. Laith et al. (2019) previously demonstrated that

repeated 187 laboratory passage and chemical attenuation using acriflavine dye could weaken *S. agalactiae*. Primary oral administration of live-attenuated vaccine and booster at week four resulted in relative percent survival (RPS) of 82% after i.p challenge with a median lethal dose (LD_{50}) of *S. agalactiae*. In addition, significant enhancements on the immune responses of tilapia were described, including elevated serum antibody (IgM) level and lysozyme activity.

2.6.2 Development of Vaccine Against *Streptococcus iniae*

R&D of an oral vaccine against *S. iniae* were also undertaken by local researchers. Evidence of antigen localization in the gut of oral vaccinated and non-vaccinated red hybrid tilapia post-challenge with *S. iniae* was recently described by Hayat et al. (2020). A low number of antigens at 72 h post-challenge in the group receiving the routine oral vaccine (5 consecutive days and booster on days 14 and 21) displayed the simulation and irregular projection of M-cells with variable morphology and antigen distribution pattern. Further, they reported that fish immunized with biofilm vaccine had significantly higher antibody levels in their serum, mucus, and gut lavage ($p<0.05$) than fish immunized with whole-cell vaccine or the control group (Hayat et al., 2021). Moreover, they discussed evidence indicating a high level of GALT stimulation in the lamina propria in the form of lymphoid cell aggregations.

2.7 MOTILE AEROMONAS SEPTICEMIA (MAS) VACCINE

Infection with aeromonad bacteria causes MAS, which is most usually associated with *Aeromonas hydrophila* (Fowoyo & Achimugu, 2019; Yardimci & Turgay, 2021) but other species such as *A. sobria* (Majtán et al., 2012; Yardimci & Turgay, 2021), *A. veronii* (T. Li et al., 2020; Shameena et al., 2020), and *A. caviae* (Roy et al., 2018) have been documented to have a similar etiology in the past. The *Aeromonas* genus consists of many species. They are all motile, except for *A.*

salmonicida, and most of them are ubiquitous inhabitants of different aquatic ecosystems (Stratev & Odeyemi, 2017). The foremost, *A. hydrophila*, is predominantly responsible for most cases of MAS in freshwater fish worldwide. For example, in Malaysia, cases involving *A. hydrophila* have been reported in *Clarias* sp. (Q. Wang et al., 2020), *Pangasius* sp. (Yardimci & Turgay, 2021), and tilapia (Fowoyo & Achimugu, 2019).

Perhaps this disease is sometimes overlooked since the infection's nature is typically associated with external stressors such as poor water quality or infection by another pathogen. Although the bacterial is often regarded as an opportunistic pathogen, it is easily transmitted from fish to fish, with hemorrhagic and ulcerated skin indicating the severity of infection. Depending on the strain of bacteria, the portal of entry, and mechanism of infection, fish infected with MAS can produce a diverse pattern of skin lesions (Baumgartner et al., 2017), which indirectly affects the aesthetic quality of fish and market value, particularly in trading live fish. The previous research shows that the combination of various virulence factors might influence the pathogenicity of *Aeromonas* spp. in fish. Some of these virulence factors include exotoxins (Roges et al., 2020), exoenzymes (Ji et al., 2015), outer membrane protein (Omp) (Yadav et al., 2016), and quorum sensing-controlled virulence factor (T. Li et al., 2020).

The disease has resulted in severe economic losses to aquaculture. In 2009, an outbreak of *A. hydrophila* was reported in Brazil, affecting farmed hybrid surubim amounting to 20 tonnes in mortality and approximately USD 160,000 losses (da Silva et al., 2012). More recently, Peterman & Posadas (2019) reported an estimated farm-gate loss of USD 2.6 million due to the disease within catfish aquaculture in east Mississippi during 2016. Another alarming concern on *Aeromonas* spp. is their increased resistance pattern against multiple antibiotics, especially those currently used in aquaculture (Saengsitthisak et al., 2020; Stratev & Odeyemi, 2016).

Several countries have actively worked to address this issue, and vaccination is viewed as a viable option for preventing the disease. India, for example, has conducted substantial research on different types of vaccines, including inactivated vaccines, live attenuated vaccines, and biofilm vaccines, all of which are based on various components of *A. hydrophila*. Previously, extracellular protein, S-layer protein, lipopolysaccharide (LPS), and Omp were identified as viable candidates (Nayak, 2020). Although local researchers in Malaysia are not interested in developing a vaccine against *A. hydrophila*, this is likely due to its complicated serotype and variable in virulence gene expression (Mzula et al., 2019; Q. Wang et al., 2020); many efforts have been conducted in recent years and are summarized in Table 4.

Table 4 Different experimental approaches and trials in the development of fish vaccine in Malaysia

Name of pathogen(s)	Type of vaccine	Vaccination route	Stage of trial	Fish species	Finding	References
S. agalactiae	Inactivated	Oral	Lab trial	Red tilapia	• Adjuvanted vaccine group resulted in the highest survival rate (100%), followed by non-adjuvanted group (50%) and control (12.5%) • Adjuvanted vaccine group resulted in significant higher IgM level, increase in size of gut-associated lymphoid tissue, and number of lymphocytes	(Firdaus-Nawi et al., 2013)
S. agalactiae	Inactivated	Oral	Lab trial	Red tilapia	• Double booster group resulted in highest survival (70%), followed by single booster group (45%) and control (0%) • Following first administration and booster dose, both vaccinated groups showed significant higher serum IgM level and reached peak at week-3 before declining. Following second booster in week 6, the antibody level significantly increased again and remain high until week-12	(Md. S. Ismail et al., 2016)
S. agalactiae	Inactivated	Oral	Field trial	Red tilapia	• The survival rate was 75% for double booster group, 65% for single booster and 45% for unvaccinated. • Both vaccinated groups showed a significant increase in the antibody level that reached peak at week-4 but gradually declined thereafter. However, following another booster at week-6 (double booster group), the antibody level showed a significant increase and remained high until end of study period	(M. S. Ismail et al., 2017)
S. agalactiae	Recombinant	Oral	Lab trial	Red tilapia	• The survival rate was 70% for recombinant vaccine. • Feed-based recombinant vaccine developed a strong and significantly higher IgM antibody response in serum, mucus, and gut lavage fluid	(Nur-Nazifah et al., 2014)

(Contd…)

Table 4 *(Continued)*.

Name of pathogen(s)	Type of vaccine	Vaccination route	Stage of trial	Fish species	Finding	References
S. agalactiae	Recombinant	Oral	Field trial	Red tilapia	• No outbreak of streptococcosis was recorded during the study period • The IgM antibody level in serum, mucus, and gut lavage was significantly higher in vaccinated groups. Aggregation of lymphocytes and development of GALT was observed in vaccinated fish	(Nadirah, 2015)
S. agalactiae	Inactivated	Oral	Lab trial	Red tilapia	• The highest survival rate (70%) was recorded in vaccinated group using 10% palm oil, followed by 7% Freund's incomplete adjuvant (45%) • Both vaccinated groups displayed higher antibody response with similar pattern	(Aminudin et al., 2018)
S. agalactiae	Inactivated	Spray immersion	Lab trial	Red tilapia	• A higher percentage of survival was noted for fish challenged through immersion (80%) compared with intraperitoneal injection (70%) • Serum and mucus antibody correspond to each booster. Both antibody levels remained high after the last booster until the end of the 8-week study period	(Noraini et al., 2013)
S. agalactiae	Inactivated and biofilm	Oral	Lab trial	Red tilapia	• Fish vaccinated with biofilm vaccine showed the highest survival percentage (87%), followed fish fed with whole-cell vaccine (57%) • The serum, mucus, and gut lavage antibody level of fish vaccinated with biofilm vaccine was significantly higher than other groups	(Kahieshesfandiari et al., 2019)

(Contd…)

Table 4 *(Continued)*.

Name of pathogen(s)	Type of vaccine	Vaccination route	Stage of trial	Fish species	Finding	References
S. agalactiae	Live attenuated	Oral	Lab trial	Red tilapia	• The RPS of fish vaccinated with live attenuated vaccine was 82% compare to 2.5% in control • After first and booster dose, the serum IgM levels increased significantly and reached the peak on the week-5	(Laith et al., 2019)
S. iniae	Inactivated	Oral	Lab trial	Red tilapia	• No efficacy data provided • Group of fish vaccinated for three and five consecutive days with booster on day-14 and day-21 recorded the lowest antigen positivity • Group of fish vaccinated for five consecutive days with booster on day-14 and day-21 showed the stimulation and irregular projection of M-cells with variable morphology and antigen distribution pattern	(Hayat et al., 2020)
S. iniae	Inactivated	Oral	Lab trial	Red tilapia	• Groups of fish vaccinated continuously for 9 days with booster on day-14 and day-21 recorded the highest survival of 70% • All vaccinated groups showed a significant increase in IgM levels in serum, mucus, and gut lavage	(Hayat et al., 2021)
A. hydrophila	Recombinant	Intra-peritoneal	Lab trial	African catfish	• The RPS of all vaccinated groups was significantly higher (100 %) compared to placebo (29.42 %)	(Matusin, 2015)

(Contd…)

Table 4 *(Continued)*.

Name of pathogen(s)	Type of vaccine	Vaccination route	Stage of trial	Fish species	Finding	References
S. iniae; *A. hydrophila*	Inactivated; bivalent	Oral	Lab trial	Red tilapia	• Group of fish vaccinated by bivalent vaccine incorporated in feed achieved the highest RPS of 80%, 77%, and 77% following challenged against *S. iniae*, *A. hydrophila*, and co-infection of both bacteria, respectively • Lysozyme, phagocytic activity and serum antibody was significantly higher against *S. iniae* and *A. hydrophila* in vaccinated groups in the pre and post-challenged	(Monir et al., 2020)
V. harveyi; *S. agalactiae*; *A. hydrophila*	Inactivated; Polyvalent	Oral	Lab trial	Asian seabass	• The vaccine provided a RPS of 75%, 80%, and 80% after challenge with *V. harveyi*, *A. hydrophila*, and *S. agalactiae*, respectively • The serum antibody and lysozyme were remained significantly higher at the end of the 6 weeks trial. The immune-related gene, dendritic cells, Chemokine ligand 4, and MHC I were highly expression after the fish were vaccinated	(Aslah et al., 2021)
V. harveyi	Live attenuated	Intra-peritoneal	Lab trial	Tiger grouper	• The RPS of vaccinated group was calculated at 52%. • Vaccinated fish displayed upregulation of autophagosome pathway, the coagulation and complement cascade pathways, and antigen processing and presentation pathways	(Mohd-Aris et al., 2019)
V. harveyi	Live attenuated	Bath immersion	Lab trial	Asian seabass	• Fish vaccinated with live attenuated vaccine resulted in a significantly high rate of survival (68%) after challenged with the wild type strain • Increase expressions of the Chemokine ligand 4 and major histocompatibility complex I genes in the skin and liver of the vaccinated fish	(Chin et al., 2020)

(Contd…)

Table 4 *(Continued)*.

Name of pathogen(s)	Type of vaccine	Vaccination route	Stage of trial	Fish species	Finding	References
V. harveyi	Inactivated	Intra-peritoneal	Lab trial	Marine red tilapia	• Vaccinated group resulted in a significantly higher rate of survival (87%) • The IgM antibody titer and lysozyme of vaccinated fish were significantly higher throughout the experiment	(Mohd-Aris et al., 2019)
V. alginolyticus	Recombinant	Intra-peritoneal	Lab trial	Hybrid grouper	• The RPS for the rOmpK vaccinated group was 100%, followed by 63% for the rOmpW group. • Both rOmpK and rOmpW vaccinated groups displayed an increasing pattern of serum IgM following vaccinations • The IgM against rOmpK showed significantly higher values than rOmpW at challenge on day 28 post vaccination	(Nehlah et al., 2016, 2017)
V. alginolyticus; *V. harveyi*	Recombinant; bivalent	Intra-peritoneal	Lab trial	Asian seabass	• Fish vaccinated with rOmpK had a 90% survival rate against *V. harveyi* and 100% against *V. alginolyticus* and *V. parahaemolyticus*. • The blood and gut lavage antibody of fish vaccinated with rOmpK were increase significantly and TLR2, MyD88, and MHC I genes were upregulated in the kidney and intestinal tissues of rOmpK vaccinated fish.	(Silvaraj et al., 2020)

S. iniae=Streptococcus iniae, A. hydrophila=Aeromonas hydrophila, V. harveyi=Vibrio harveyi, A. hydrophila=Aeromonas hydrophila, S. agalactiae=Streptococcus agalactiae, V. alginolyticus=Vibrio alginolyticus, RPS=Relative percent survival

2.7.1 Development of Vaccine Against *Aeromonas hydrophila*

Matusin (2015) reported her successful attempt in developing a recombinant vaccine expressing the immunogenic genes of Omp (OmpTs and OmpW) of *A. hydrophila*. The genes were positively cloned into the pET102/D-TOPO vector, and trials were conducted in African catfish (*Clarias gariephinus*). Two vaccines were administered by injection at week-0 followed by a booster at week-2 before being challenged with live virulent *A. hydrophila*. The RPS of vaccinated groups was recorded at 100% compared to the placebo group (29.42%).

In contrast, Monir et al. (2020) previously reported the effectiveness of a newly developed feed-based bivalent vaccine against *S. iniae* and *A. hydrophila* in combating both pathogens in tilapia. They discovered that following double booster vaccination regime (given on 0, 14, 42 days), the number of leucocytes, monocytes, granulocytes, lysozyme, and phagocytic activity, and serum antibody (IgM) levels were significantly higher ($p<0.05$) in the vaccinated group as early as 21 days post-vaccination. In addition, the challenge test against *S. iniae*, *A. hydrophila*, and co-infection of these bacteria showed the RPS of 80%, 76.67%, and 76.67%, respectively.

More recently, another study by Aslah et al. (2021) reported the effectiveness of a newly developed feed-based polyvalent vaccine against *Vibrio harveyi*, *S. agalactiae*, and *A. hydrophila* in Asian seabass. This appealing feature effectively controls several fish diseases by a single vaccination. They found that oral vaccination at 5% body weight has successfully stimulated high serum antibody (IgM) production ($p<0.05$) against *V. harveyi*, *A. hydrophila*, and *S. agalactiae*, and serum lysozyme level. Gene expression analysis from vaccinated fish gut samples revealed significantly higher expression ($p<0.05$) of dendritic cells, Complement-3, Chemokine ligand 4, and major MHC I compared to the unvaccinated group. The experimental challenge resulted in

RPS of 75%, 80%, and 80% after i.p injection with 10^7 CFU ml^{-1} of *V. harveyi*, *A. hydrophila*, and *S. agalactiae,* respectively.

2.8 VIBRIOSIS VACCINE

Vibriosis has been an issue in Malaysian marine aquaculture since 1973 (Seng, 1997), when the culture of marine finfish was established. However, it was not until the 1990s that sea bass, grouper, and snapper were frequently reported to be infected with the disease. Throughout the hatchery and grow-out phases, the fish are susceptible. Several *Vibrio* spp. have been discriminated against as etiological agents of the disease, including *V. alginolyticus*, *V. vulnificus*, and *V. harveyi*. In 1979, (Wong et al., 1979) reported red boil disease affecting cultured estuary grouper (*E. salmoides*) in floating cages in the Straits of Penang, Malaysia. Based on colony morphology on selective media and pathogenicity, they concluded that *V. parahaemolyticus* is the causative agent. The diseased fish were found darker in color with hemorrhagic abscesses. In addition, they reported that most cases were asymptomatic due to an acute course of infection. Another case involving greasy grouper (*E. malabaricus*), silver seabass (*L. calcarifer*), and golden snapper (*L. johni*) were formerly reported by Wong & Leong (1990) affecting floating cages area in Penang and Perak, the northern region of Peninsular Malaysia. They found that Group I (consist of *V. parahaemolyticus*, *V. alginolyticus*, *V. harveyi*, and *V. vulnificus*) with 3 fish species, greasy grouper, silver seabass, and golden snapper predominately with 42%, 56%, and 62% prevalence, respectively.

Moreover, cases of vibriosis outbreak are not limited to Peninsular Malaysia but rather spread across in Borneo, East Malaysia. For example, Ransangan & Mustafa (2009) reported a vibriosis outbreak of Asian seabass cultured in net cages in Sabah, Malaysia, in February 2008. Their work revealed that *V. harveyi* is the causative pathogen. The fish were characterized by deep skin and fin ulceration, dark

pigmentation, lack of appetite, ascites in the body cavity, and enlarged liver and spleen. Further analysis discovered that the isolated *V. harveyi* displayed high virulence with a median lethal dose (LD50) of 1.4×10^4 CFU g^{-1} in Asian seabass and 8.33×10^3 CFU g^{-1} in humpback grouper (Ransangan et al., 2012).

Recently, in September 2016, a concurrent infection by *V. harveyi* and *V. alginolyticus* was reported affecting cultured hybrid groupers reared in marine floating cage-culture of Selangor, Malaysia (Nurliyana et al., 2019). Within ten days, 29% of the farm's juvenile groupers died. Clinical symptoms and signs such as lethargy, increased mucus secretion, rotting fins, liver and kidney congestion, and spleen enlargement were all seen in the diseased groupers, whereas generalized congestion of the brains and internal organs were also reported. Interestingly, other cultured fish species produced in the exact location, such as Asian seabass, snapper, and golden pompano, were not harmed. More recently, according to Amalina et al. (2019), the distribution of *Vibrio* spp. isolated from cultured groupers in Peninsular Malaysia was predominated by *V. vulnificus*, *V. alginolyticus*, and *V. parahaemolyticus* with 33%, 24%, and 22%, respectively. Thus, vibriosis has remained a severe problem in Malaysian cultured marine fish since the 90s, resulting in significant economic losses. Total losses among Malaysia's three main grouper, snapper, and seabass culture species were USD 7.4 million in 1990 (Amalina et al., 2019).

According to recent assessment on the management costs of Asian seabass cage culture in Malaysia, the average loss due to vibriosis was calculated to be USD0.24/tail, which can be broken down into mortality of 79.2%, treatment of 20.8%, and diagnosis of 1.25% (Norhariani et al., 2019). As a result, the development of an effective vaccination approach to prevent disease epidemics is desirable.

2.8.1 Development of Vaccine Against *Vibrio harveyi*

One of the earliest attempts by local researchers was reported by Mohd-Aris et al. (2016), who discovered virulence-associated genes, serine protease (VHS), and Omp from pathogenic *V. harveyi*. Using computational prediction, further characterization of these genes revealed that both VHS and Omp are composed of 62 and 36 antigenic sites, potentially developing an effective live-attenuated vaccine candidate. Following success in the characterization of *V. harveyi* vaccine candidates, a live-attenuated vaccine was developed by selective gene deletion of virulence-associated protease and subjected to vaccination trial in tiger grouper (Mohd-Aris et al., 2019). In week 4, a single dose vaccination regime was employed before the fish was challenged with *V. harveyi*. Their work achieved moderate satisfactory RPS of 52%. At the same time, transcriptomic profiling revealed the upregulation of the autophagosome pathway, coagulation, and complement cascade pathways, and antigen processing and presentation pathways. Another trial of the same vaccine was reported by Chin *et al*. (Chin et al., 2020), embarking on different fish species of Asian seabass, different vaccine routes of bath immersion, and double dose regime, resulting in improved efficacy. The vaccine was administered on day 0 and 14 before the fish were challenged by immersion at 10^7 CFU ml^{-1} of *V. harveyi*. RPS was recorded at 68%, and higher expression ($p<0.05$) of the chemokine ligand-4 and MHC I genes in the skin and liver of vaccinated fish was noted.

Classical preparation of the killed vaccine has also proved effective in combating *V. harveyi* infection. Abu Nor et al. (2020) recently reported that double dose injection of formalin-killed whole-cell vaccine of *V. harveyi* was successfully reduced mortality in marine red hybrid tilapia to 16% after the fish were intraperitoneally challenged with 10^8 CFU ml^{-1} of *V. harveyi* at week 4. A significant increment ($p<0.05$) in IgM serum, mucus, and gut lavage antibody titer and lysozyme activities was achieved as early as 1-week post-vaccination.

2.8.2 Development of Vaccine Against *Vibrio alginolyticus*

Omp has long been recognized as a potential vaccine candidate in most Gram-negative bacteria, owning to its highly conserved region across serotypes and virulence attributes (Maiti et al., 2020). Besides, scholars reported the antigenic sites of OmpK and OmpW of *Vibrio alginolyticus* consisting of 34 and 27 antigenic sites respectively and deemed suitable for vaccine candidates (Nehlah et al., 2016). Interestingly, in an experimental trial, juvenile hybrid groupers were injected with inactivated *Escherichia coli* expressing the OmpK and OmpW resulted in the RPS of 100% and 63%, respectively, after i.p challenged with a high dose (10^9 CFU ml^{-1}) of a virulent strain of *V. alginolyticus* (Silvaraj et al., 2020). Besides, the IgM level against OmpK was significantly higher ($p<0.05$) than OmpW suggesting the superiority of the OmpK recombinant vaccine against *V. alginolyticus* infection.

More recently, recombinant OmpK vaccine versatility in providing cross-protection against different *Vibrio* species of *V. harveyi*, *V. alginolyticus*, and *V. parahaemolyticus* in Asian seabass has been reported (Silvaraj et al., 2020). Furthermore, in an experimental trial, a single i.p injection of recombinant OmpK vaccine resulted in 90–100% survival after being challenged with a high concentration of *V. harveyi*, *V. alginolyticus*, and *V. parahaemolyticus*.

2.9 CHALLENGES AND FUTURE OF FISH VACCINE IN MALAYSIA

2.9.1 Commercialization of Local Vaccine

Increase frequencies of emerging and re-emerging diseases among aquaculture fish species as witnessed in the past few years, resulting in considerable economic losses. This trend is expected to drive demand for commercial aquaculture vaccines locally and

globally. Unfortunately, although local researchers have achieved a lot and massive investment in research grants, there is still no locally produced fish vaccine available in the country. In fact, other veterinary medical treatments for use in farmed aquatic animals are in severely short supply. This scenario is a severe impediment to disease prevention and response, resulting in welfare issues and stifling the growth of Malaysian aquaculture.

The commercialization of vaccines or any research product is a complex process, and there is no one-size-fits-all formula that will ensure its success (N. Ismail et al., 2015). It usually entails a lengthy process and requires a hefty investment; both are frequently a source of difficulty for researchers. Aside from these two crucial factors, several scholars have identified several issues that local researchers may confront when commercializing their research output. Lack of entrepreneurial knowledge and skill, the inefficiency of the Technology Transfer Center (TTC), the availability of potential licensee, linkage gaps between industry and researchers, limited workforce, and poor marketing strategies are additional challenges in bringing research outputs into the viable market (Heng et al., 2012; N. Ismail et al., 2017; Khademi et al., 2015; Latif et al., 2016). A dynamic framework for effective commercialization of research products was proposed by N. Ismail et al. (2015), consisting of eight elements: The researchers' knowledge, skills, and personal traits, product idea creation, development, packaging, and promotion, commercialization paths, gaining a competitive advantage in the market, selecting business partners, fostering a positive working relationship with business partners, and providing facilities and support.

Another hurdle in commercializing aquaculture vaccines in Malaysia is the limited availability of animal vaccine manufacturing plants. Like any other biological product, vaccines for commercial use should comply with good manufacturing guidelines. However, unlike conventional pharmaceutical goods, vaccine production involves biological processes such as microbe cultivation and extraction from living cells, which sometimes display intrinsic variability (WHO, 2016). Therefore, stringent

protocol at all production stages is applied, which can only be accomplished in a high-tech manufacturing facility. Because of the cost and complexity of the operation, the vast majority of aquaculture vaccine supply currently comes from a handful of developed countries. Despite the fact that Southeast Asian countries produce some of the world's best fish and fisheries goods (SEAFDEC, 2017), Southeast Asian countries venturing into fish vaccine manufacturing is unheard of. Although the alliance is mostly composed of developing nations, establishing a regional aquaculture vaccine supply chain makes sense in terms of collective food security and supply, socioeconomic growth, and the sector's sustainability. A medium-sized central manufacturing facility and open-system bioreactors will ensure a low-resource environment and simplify the production of aquaculture vaccines against regionally significant fish diseases. Several factors, however, should be considered before committing to this capacity.

2.9.2 Limited Studies on Vaccination Field Trial

Field trials are required to accurately assess a vaccine's efficacy, safety, and overall performance in real-world settings. In contrast to controlled laboratory circumstances, the dynamic interaction between host, pathogen, and environment in a production setting can result in variances in immune response and vaccine effectiveness (Mitchell, 1997). This event is significant for a fish vaccine for at least two reasons: first, site-specific water physiochemical profiles can affect fish immunity and disease occurrence, and second, physical rearing conditions can vary greatly depending on the rearing technology used (Midtlyng, 2016). As a result, field testing is a mandatory requirement for innovative vaccines intended for commercial use, and regulatory agencies require it prior to granting a full authorization. To achieve successful testing, meticulous planning, full cooperation from farm operators, and massive investment are frequently required. Unfortunately, vaccine field trials in Malaysia are insufficient and should be expanded to expedite the commercialization process. The following sections describe some critical aspects of the fish vaccinology field trial.

2.9.2.1 Establishing the Vaccination Protocol

Researchers, farm managers, and operators should collaboratively develop a suitable field vaccination protocol. This step is crucial to ensure all parties involved in the study are aware of their roles and responsibilities and the importance of adhering to the protocol. This is because any deviation that occurs throughout the implementation might affect the trial's outcome. Therefore, investigators should carefully justify any unforeseen scenarios and provide immediate responses to minimize the study's outcome's impact. Besides, the vaccination regime adopted in the field study must be supported by laboratory trial findings, and other practices should be resembling regular activities on the farm. A set of guidelines from the EU commission (EMA/CVMP/IWP/314550/2010) entitled "Guideline on the design of studies to evaluate the safety and efficacy of fish vaccines" is an excellent reference for this purpose.

2.9.2.2 Site Selection, Epidemiology, and Immune Response Monitoring

The fish will be subjected to the disease's natural challenge in the field trial. Therefore, when selecting a trial site, researchers should examine the scale of the disease's spread and the number of new cases. There is a considerable probability of failure in the field vaccination trial due to the absence of the pathogen. The lack of disease outbreaks in the vaccinated fish population will result in insufficient evidence of vaccine efficacy because the challenge pressure remains unknown. Therefore, investigators should conduct important on-farm epidemiological surveillance to ascertain the critical period of infection and disease prevalence before conducting the vaccine trial.

Furthermore, the quality of the raw data collected during the trial is critical. Farm operators should be educated on the value of accurate record-keeping, which reflects the quality of the final product following the trial period. In addition, the use of

mortality records between the vaccinated and control groups is frequently insufficient. It should be complemented by routine response monitoring, such as antibody titer, pathogen isolation, side effect score, and assessment of water quality. These parameters must be explicitly established in the study protocol and justified considering the vaccine's indications. If no outbreak occurs during the trial, this data can be used to provide evidence of the vaccine's immunological response. This correlate of protection can be used as an alternative outcome for vaccine evaluation (Knight-Jones et al., 2014).

2.9.2.3 Control Groups

In most vaccination studies, a group of vaccinated fish is compared to an equivalent group of unvaccinated or placebo fish. The investigator should choose the controls to provide evidence of infection and represent a group of fish to which the vaccinated group can be compared legitimately. Two approaches are most commonly chosen in comparing these groups: (1) Comparison of vaccinated and control groups in the same captivity and (2) Comparison between vaccinated and control groups kept in different but identical captivity within a similar farm. The first approach is customarily preferred since both fish groups will be subjected to equivalent conditions and challenges. Individual identification, such as pit tagging or specialized dye, can be used to accomplish this. This approach, however, is only possible for a vaccine that can be administered on an individual fish basis and is unsuitable for oral and immersion treatment. Besides, researchers should also consider the likelihood of the herd immunity effect, which occurs when a population of vaccinated fish becomes immune, making disease transmission to an unvaccinated group improbable.

2.9.3 Lack of Studies on Viral Vaccine

For decades, R&D on fish vaccines in Malaysia has been concentrated based on bacterial etiology. Although virus disease has been implicated in the local aquaculture industry since the 1990s, it is not the subject of interest by local researchers. This is most likely owing to the country's inadequate capacity and capability in this subject. Nowadays, only a few laboratories are prepared to maintain fish cell cultures and virus purification. In addition, producing a fish virus vaccine is indeed very challenging and time-consuming process. The fish virus has many subtypes or serotypes, and vaccines derived from one subtype are typically less effective against other subtypes (Crane & Hyatt, 2011). In addition, changes or mutations can occur when the virus reproduces rapidly in the infected fish (Stepien et al., 2015), especially with the RNA virus. The mutation process is often disadvantageous for the viruses, but not always since certain viruses become stronger due to the mutation. Thus, in some cases, the previously developed vaccines are less effective or not effective at all against the newly mutated virus (Kennedy & Read, 2018). This phenomenon has caused vaccine development to be a never-ending story that consumes a great deal of expense.

2.9.4 Future of Fish Vaccines in Malaysia

Malaysia's aquaculture industry will continue to proliferate while preserving a competitive edge as market demand for fish increases. This tendency is highly correlated with population growth, rapid urbanization, increase in household income, and international trade expansion, all of which contributed to increased fish consumption per capita (Naylor et al., 2021). Besides, the industry has generated numerous employment and income generation to the locals and ensures national food security and advancing the sustainable fisheries agenda. Continuous industrial growth will reduce dependence on imported fisheries products while easing pressure on wild fish stocks. Regrettably, the aquaculture industry's rapid rise has been stymied by recurrent infectious disease outbreaks. Vaccination is considered a viable solution and

might offer a brighter future to the industry. At present, fish vaccinations in Malaysia are still limited to a few farms and hatcheries. These operators engage in large-scale farming, hence have the financial means to invest in vaccinations.

The active role played by various organizations, including the Department of Fisheries, local fisheries associations, and universities in fish vaccines gaining wider local attention. Other than that, an increment in the public call for healthy (antibiotics-free) food is probably molding Malaysia's future of fish vaccinations. The use of vaccinations in aquaculture is being considered as a substitute for antimicrobial medications and chemotherapy. In response, the MyAP-AMR for 2017-2021 opposed the irresponsible and uncontrol use of antibiotics in the food production industry. Indiscriminate use of antibiotics in aquaculture would greatly influence the environment due to drug residues, affecting water's natural microbiota, and accelerating the development of antibiotic-resistant bacteria.

Furthermore, water contaminated by these untreated aquaculture effluents is recycled into agricultural land, resulting in elevated antibiotic residue levels in groundwater, hence further increasing the risk of antibiotic-resistant microbes. Alarmingly, evidence of multiple resistant bacteria and resistance genes has been previously reported in Malaysian aquaculture and its environs (Atyah et al., 2010; Fauzi et al., 2021; Lihan et al., 2020). Vaccines, unlike antibiotics, are widely accepted as the safest, practical, and cost-effective way of preventing aquatic animal diseases. Vaccination has been shown to be critical to the success of Norwegian Atlantic salmon farming (Gudding & Van Muiswinkel, 2013). To summarise, salmon farming in Norway suffered significant losses in the 1980s because of salmonid *Vibrio* infections and furunculosis, and farmers relied heavily on antibiotics to battle the diseases. In response, a salmonid vibriosis immersion vaccine and an injectable *Aeromonas salmonicida* vaccine were developed and applied in the farms (Eggset et al., 1997; Kashulin et al., 2017; Midtlyng, 1996). The vaccines' high effectiveness has successfully controlled the disease and immediately resulted in a decline of antibiotic

dependence (Midtlyng et al., 2011). Therefore, we believe that the mass application of vaccines in the local aquaculture industry is beneficial by increasing the industry's resilience.

CHAPTER THREE

DEVELOPMENT OF *Streptococcus agalactiae* GROWTH CURVE FOR SEED VACCINE PREPARATION

3.1 INTRODUCTION

Bacterial growth or replication is a coordinated process involving the formation of two identical daughter cells, and its path is determined by an organism's ability to recognise and adapt to its surrounding (Murray et al., 2021). Bacterial population increase occurs through asexual reproduction via binary fission. However, sufficient metabolites must be present to permit the synthesis of bacterial components, particularly the nucleotides required for DNA synthesis and growth. Besides, initiating a replication cycle will also trigger a series of protein and RNA synthesis regulatory processes. This replication of chromosomes begins at the cell membrane, where each daughter's chromosomes are assembled and proceed in opposite directions (O'Donnell et al., 2013). Simultaneously, bacteria membrane synthesis and peptidoglycan synthesis will initiate the process of cell division, resulting in the segregation of daughter chromosomes as the bacterial membrane expands.

Growth curve is a graphical representation of the growth rate of microorganisms, such as bacteria, over a specific period. It illustrates the population size of bacteria over time under controlled conditions (L. Wang et al., 2015). Typically, a bacterial vaccine is produced in a closed system or batch culture, where the bacteria growth curve displays a classical pattern encompasses four distinct phases: the lag phase, the exponential (log) phase, the stationary phase, and the death phase. Each phase offers unique insights into bacterial physiology and metabolism during cultivation. Bacterial seed vaccines demand precise and consistent inoculum sizes to produce reliable and reproducible results during large-scale production (Okonkowski et al.,

2005). Developing *S. agalactiae* growth curves help determine numbers of the bacterial colonies and the exact stage of bacterial growth to harvest the optimal inoculum size, leading to a consistent vaccine production process. The increase in cell mass can be measured as a function of time under pure culture conditions, where the nutrients and environment are regulated for vaccine preparation. Understanding the growth curve of the *S. agalactiae* seed allows us to identify the optimal culture conditions required for its propagation. A well-defined growth curve aids in pinpointing the conditions that yield the highest bacterial count and viability.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial Strain and Growth Condition

The clinical isolates of *Streptococcus agalactiae* (SA2BKE) used as a seed vaccine in the present study were acquired from strain collection of the National Fish Health Research Centre (NaFisH), Fisheries Research Institute (FRI) Batu Maung. The isolates were initially isolated from a local outbreak in 2007 at Pergau Lake, Kelantan, Malaysia (Siti-Zahrah et al., 2008). The isolates were previously identified using the API®20 STREP identification system (bioMérieux, France) and confirmed using the polymerase chain reaction (PCR) technique (16's rRNA gene) (Nur-Nazifah et al., 2014). The strains were maintained in 1 ml aliquots at -80°C in Brain Heart Infusion (BHI) broth containing 10% glycerol. All microbiological procedure was performed under strict Biosafety Level 2 (BSL-2).

3.2.2 Examination of the Purity of the Isolates

The cultures were sub-cultured overnight on Blood Agar (BA) at 30 ± 2°C. The purity of the bacteria culture was examined based on specific colonies' morphology on the differential agar media. Being β-hemolytic, *S. agalactiae* will appear as grey-white

colonies surrounded by a narrow colourless zone on the agar medium, indicating the red blood cell have undergone complete lysis (Edwards & Baker, 2018). The pure cultures were then subjected to biochemical assay using the API®20 STREP identification system (bioMérieux, France) as per manufacturer instruction and further confirmed using PCR utilizing the 16s rRNA region.

3.2.3 Gram staining

A single colony from an overnight culture of *S. agalactiae* was inoculated onto a clean microscope glass slide containing a drop of distilled water to facilitate colony transfer. The culture was then spread with an inoculation loop to achieve an even thin film and air-dried. Crystal violet stain (BD, Switzerland) was flooded over the fixed culture for 60 seconds, poured off, and the excess stain was raised with tap water. After that, iodine solution (BD, Switzerland) was added to cover the smear for another 60 seconds and poured off. Subsequently, a few drops of decolorizer (BD, Switzerland) were added to the slide and raised with tap water in 5 seconds. Finally, the smear was counterstained with safranin stain (BD, Switzerland) for 60 seconds, washed off with tap water, and excess water was blotted with filter paper.

3.2.4 Oxidase and Catalase Test

The bacterial colony was aseptically spread over the surface of oxidase strip (MilliporeSigma, USA) and observed after 1 minutes. The development of a violet colour indicated a positive oxidase reaction. Meanwhile, a loopful of freshly grown colonies from a BA was first suspended onto a clean microscope slide. Then a drop of 3% hydrogen peroxide was placed onto the smear and observed for immediate bubble formation which indicated a positive catalase reaction.

3.2.5 Serotyping of *S. agalactiae*

The serotyping of *S. agalactiae* was carried out using ImmuLex™ *Streptococcus* group B antisera (SSI Diagnostica, Denmark) utilising a rapid agglutination principle. Approximately, one drop (10 µL) of ImmuLex™ antisera was applied on a reaction card for each reaction. Subsequently, a loopful of freshly grown colonies from a BA was suspended in 100 µL saline, and a drop of this suspension was added next to the ImmuLex™ antisera. The two drops were combined with a mixing stick, equally distributed, and observed for agglutination within 5 to 10 seconds. Visible agglutination within 10 seconds suggested a positive reaction.

3.2.6 Development of *S. agalactiae* Growth Curve

A single colony from an overnight culture of *S. agalactiae* was inoculated into 10 ml of BHI broth and incubated overnight at 30 ± 2°C, 150 rpm. Then, 1 ml of bacterial suspension was inoculated onto a 1 L conical flask containing 600 ml of BHI broth. Immediately after inoculation, 3 ml of the mixture was pipetted and subjected to optical density (OD) measurement at 600 nm wavelength. At the same time, another 1 ml of the suspension was serially diluted in 9 ml of sterile PBS (pH 7.4) and plated on BA in duplicate. After overnight incubation at 30 ± 2°C, the colonies were counted and expressed as colony-forming units (CFU ml^{-1}). The bacterial suspension was further incubated at 30 ± 2°C, 150 rpm and similarly repeated after 15 min, 30 min, 1 hr, 2 hr, 4 hr, 8 hr, 12 hr, 16 hr, 20 hr, and 24 hr post-incubation. The assay was conducted at least two times independently, and the standard growth curve was plotted using SlideWrite™ (Advance Graphics Software Inc., USA) software.

3.3 RESULT

3.3.1 Biochemical Profiles and Serotypes of *S. agalactiae* (SA2BKE)

The phenotypic characterization of *S. agalactiae* (SA2BKE), used as a seed vaccine is presented in Table 5. The isolates first underwent gram staining, oxidase, and catalase test. The isolates that indicated gram-positive cocci, oxidase, and catalase-negative were further characterized based on their biochemical profiles using the API®20 STREP identification system (bioMérieux, France). The kit is a standardized combination of 20 biochemical assays that permits the identification of streptococci, enterococci, and the most frequently related organisms by group or species. The isolates exhibited identical phenotypic traits, as shown in Table 5, with a 99.9% likelihood level (Figure 2). The isolates tested positive for the Voges-Proskaeur test, producing hippurate hydrolase, alkaline phosphatase, leucine aminopeptidase, and arginine dihydrolase enzymes, and were able to ferment ribose and inulin. In addition, serotyping assay revealed that the isolates belong to serotypes III (Figure 3).

Table 5 Phenotypic characterization of *Streptococcus agalactiae* (SA2BKE) by using conventional method and API®20 STREP identification system (bioMérieux, France)

Tests/Reaction	**Phenotypic characteristics**	
	Conventional	**API®20 STREP**
Gram's staining	+	
Cell morphology	Cocci (in chain)	
Haemolysis	β	
Oxidase	−	
Catalase	−	
Voges-Proskauer		+
Hydrolysis of Hippurate		+
Esculin		−
Pyrrolidonyl arylamidase		−
α-galactosidase		−
β-glucuronidase		−
β-galactosidase		−
Alkaline phosphatase		+
Leucine aminopeptidase		+
Arginine dihydrolase		+
D-ribose		+
L-arabinose		−
D-mannitol		−
D-sorbitol		−
D-lactose		−
D-trehalose		+
Inulin		−
D-raffinose		−
Starch		−
Glycogen		−

Figure 2 API20 STREP strip displaying an excellent identification (99.9%) of *S. agalactiae*

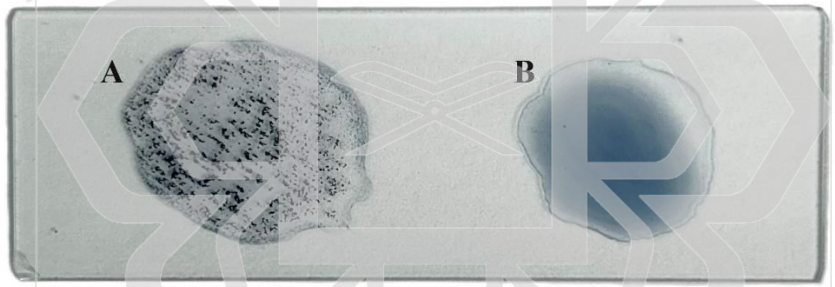

Figure 3 Serotyping of *S. agalactiae* (SA2BKE) using rapid latex agglutination test revealed serotype III identity. A positive reaction (A) has visible agglutination and clearing, whilst a negative reaction (B) is smooth and cloudy

3.3.2 Growth Curve of *S. agalactiae*

The growth curve of *S. agalactiae* (SA2BKE) following incubation at 30℃ in BHI broth is presented in Figure 2. Over the first two hours, the bacteria do not actively replicate, displaying a flat line; hence this stage could be designated as the lag phase. After two hours post-incubation, the rapid growth of the bacteria was observed, marked by predictable doublings of the population, and peaked at 12 hours post-incubation. Hence this stage of bacteria growth could be designated as the log or exponential phase. Subsequently, between 12 to 16 hours post-incubation, the growth rate of the cells appeared stagnant, resulting in a smooth horizontal linear line marking the stationary phase's duration. Prolonged incubation caused the bacteria's growth to follow a steeply declining trajectory between 16 to 24 hours post-incubation, indicating the onset of the death phase.

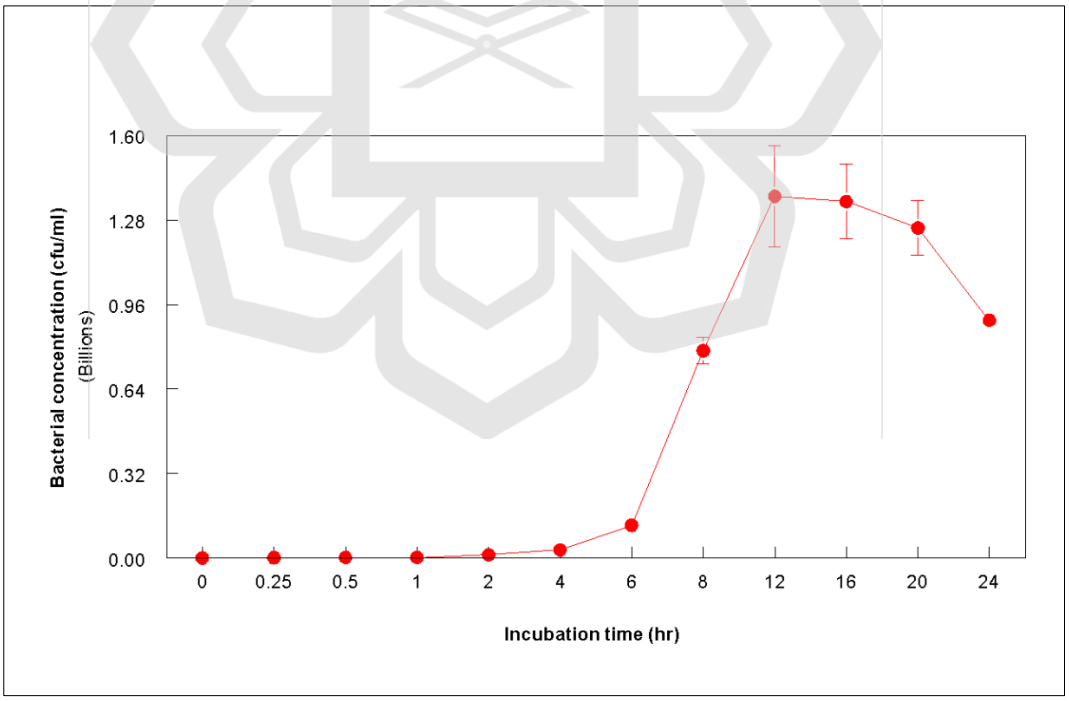

Figure 4 Growth curve of *S. agalactiae* (SA2BKE) following incubation at 30 ± 2℃, 150 rpm in BHI Broth for 24 hours

3.4 DISCUSSION

The term growth describes an increase in cell mass and size (Guertin & Sabatini, 2015). Both nutritional and physical elements influence bacterial growth. Some examples of physical variables are the pH, temperature, osmotic pressure, and moisture content of the medium in which the bacteria are cultured. In contrast, nutritional parameters include carbon, nitrogen, sulphur, phosphorus, and other trace elements. Under optimum conditions, a bacterium proliferates in a process known as binary fission, where a single cell splits into two equally sized daughter cells. The process begins with cell elongation, which requires careful cell membrane and cell wall enlargement (Hobot, 2015). The cell replicates its DNA to have two copies of its chromosome, one for each daughter cell. After nucleoids are segregated to each end of the elongated cell, septum formation is completed, dividing the elongated cell into two equally sized daughter cells. The period of the entire cell cycle process varies for different bacteria species (Gibson et al., 2018).

In the present study, the growth curve of *S. agalactiae* was developed in order to determine the total incubation time required for seed vaccine preparation. *S. agalactiae* was cultured in nutritious culture media, Brain-Heart Infusion (BHI) broth, a modification of the Rosenow (1919) and Haden (1923) original formula, and the incubation condition was controlled at 30 ± 2°C and 150 rpm. It is important to note that different culture mediums, incubation conditions, and initial bacteria load may result in different growth curves (Davis et al., 2005). The development of a growth curve for *S. agalactiae* in a closed system for various applications has been reported elsewhere. For instance, Sitkiewicz & Musser (2009) previously categorized different logarithmic and stationary phases, including mid-logarithmic (ML), late-logarithmic (LL), and early stationary (ES) phases of *S. agalactiae* cultivated as static cultures in Todd Hewitt medium supplemented with 0.5% yeast extract. In addition, Yildirim-Aksoy et al. (2019) previously reported *S. agalactiae* growth kinetics in M9 minimal medium containing various concentrations of chitosan and chitin in order to determine the bacteria's ability to degrade and utilize these polymers as carbon source. The present

study identified four distinct phases of microbial growth: the lag, log, stationary and death phases.

The bacteria utilised multiple mechanisms to regulate gene expression and respond to a dynamic environment. The lag phase, represented by a flat line during the initial stage after incubation (the first two hours), indicated that *S. agalactiae* was adapting to the new environment and preparing for cell division. In this stage, the bacteria are metabolite active, producing essential material for activating signalling pathways and transcriptional modifications. These processes result in upregulated respiration, lipopolysaccharide biosynthesis, protein synthesis, nucleotide metabolism, and other processes essential for differentiation and multiplication (Hamill et al., 2020). Together, these processes result in cell division and can promote exponential development. A few variables that could affect the length of the lag phase include the inoculum size, the physiochemical environment of the new and old growth mediums, and the physiological history of the inoculated culture (Bertrand, 2019).

The bacterial cells enter the log or exponential phase shortly after the lag phase. This stage is distinguished by bacterial cells growing at a logarithmic rate and lasts until all of the nutrients and energy sources in the setup have depleted. In the present study, the exponential phase of *S. agalactiae* reaches peaked at 12 hours post-incubation. A previous study by Liu et al. (2014) characterized the growth characteristics of a batch culture of *S. agalactiae* at different incubation temperatures of 37℃ and 28℃ and reported a peak of logarithmic phase after 6 and 10 hours, respectively. More recently, S. Li et al. (2023), who studied the growth characteristics of a wild strain of *S. agalactiae* isolated from infected tilapia in a BHI medium, achieved a faster rate of reaching the logarithmic growth phase at 8 hours post-incubation. Several factors, such as inoculum size and culture conditions (growth media, incubation temperature and agitation rate), may account for the glaring differences in growth rate observed in the present study. Nevertheless, the cells are the healthiest and preferred for seed vaccine preparation at this stage.

As the nutrients in the medium diminish, metabolic waste accumulates, and in the presence of stress factors such as changes in pH, the cells enter the stationary phase. This phase is characterized by a plateau in which the apparent increase in biomass ceases. Interestingly in this stage, the bacterial cells remain metabolically active, where genes essential for DNA repair, glycogen production, thermotolerance, and osmotolerance are highly expressed (Jaishankar & Srivastava, 2017). A previous study by Sitkiewicz & Musser (2009) indicates that in the stationary phase, transcription of *S. agalactiae* genes implicated in the stress response, such as *hrcA*, *grpE*, *dnaK*, *clpE*, and *clpL*, as well as a cluster of genes involved in arginine/ornithine transport and metabolism, was upregulated. The latter allows bacteria to utilise arginine as an energy source once carbohydrate is depleted from the environment. Prolonged incubation following the stationary phase led to death, where poisonous by-products and a lack of nutrients caused the surviving cells to discontinue metabolic functions and begin the death process. As a result, the rate of growth becomes negative.

3.5 CONCLUSION

The current study concluded that 12 hours of incubation of *S. agalactiae* at 30℃ in BHI broth reaches the peak of the exponential phase and recorded the highest cell mass and, therefore, is suitable for seed vaccine preparation.

CHAPTER FOUR

DETERMINATION OF PROXIMATE COMPOSITION OF PALM OIL ADJUVANTED FEED-BASED STREPTOCOCCOSIS VACCINE

4.1 INTRODUCTION

Protein, fat, carbohydrates, moisture, ash, fiber, and nitrogen-free extract (NFE) are some primary components in fish feeds. The first three components are commonly recognized as energy-yielding nutrients, which the body metabolizes to provide the energy required for various physiological processes and physical activities. The ability of various fish species to utilize energy-producing nutrients differs substantially. This variability is influenced by their natural feeding habits, which might be herbivorous, omnivorous, or carnivorous. Tilapia is an omnivore fish that consumes a wide range of foods, such as phytoplankton, zooplankton, suspended detritus, macrophytes, and insect larvae (L. Zhang et al., 2022).

In the context of aquaculture, nutrition plays a critical role because it influences operation costs, fish growth, and general health. Therefore, understanding the fish's nutritional requirements and implementing appropriate feeding practices are vital in formulating a nutritious and cost-effective diet. Most notably, protein and fat are essential for the production and utilization of feed. Digestible fat and protein are essential in providing fish with the necessary energy for grow and development. The fat in the feed is a vital source of energy and for the growth of adipose tissue, whereas protein is primarily necessary to build muscle tissues (Weil et al., 2013).

Fish feed accounts for more than half of overall production costs depending on the cultured fish species and scale of aquaculture practices. An unbalanced diet or ineffective feeding practices results in food waste and economic losses and can potentially deteriorate water quality in the aquaculture system (L. Wang et al., 2021). Inadequate energy can result in protein waste, as a higher fraction of dietary protein is utilized for energy increases, leading to unnecessary ammonia production. On the other hand, excessive energy in meals might result in increased fat deposition and reduced growth due to a lack of essential nutrients (Kader et al., 2005).

Proximate analysis is a laboratory technique used to determine the nutritional composition of a sample. In the context of feed-based fish oral vaccines, the analysis assists scientists to understand the feed-based vaccine's essential components (Naqtahnain Hamid et al., 2015). The approximate composition of moisture, protein, ash, fat, and fiber can be measured using an established laboratory procedure commonly known as the Weende System, and the NFE was calculated as the differences between the total quantity of feed and these five components. This system provides access to feed quality evaluation and enables valid comparisons of diets based on specific nutrients. The present study used palm oil as an adjuvant substitute, and the vaccine was formulated in fish feed. Therefore, information about nutritional composition, particularly for protein, fat, and energy provided by the feed-based vaccine provides valuable insights into the formulation to ensure a balanced diet is provided as well as effectiveness of the vaccine.

4.2 MATERIALS AND METHOD

4.2.1 Preparation of Formalin-killed Bacteria

The formalin-killed bacteria used as a seed vaccine was prepared according to the method of Firdaus-Nawi et al. (2013). Briefly, the bacteria were cultured in Brain Heart

Infusion (BHI) broth at 30 ± 2°C, 150 rpm for 12 hr. Following incubation, the bacterial concentration was determined using a previously developed standard growth curve and standard plate count technique. Subsequently, the cells were harvested through centrifugation at 5,000 rpm for 15 min at 4°C and washed thrice with sterile PBS (pH 7.4). The harvested cells were suspended in 0.5% formalin to kill the bacteria and kept overnight at 4°C. The formalin residue was then removed through centrifugation and repetitive washing in PBS. The formalin-killed cell was re-suspended in PBS to obtain the final bacteria concentration of 10^9 CFU ml^{-1}. Lastly, the suspension was streaked onto BA to ensure the entire bacterial cell was killed and stored at 4°C until further used.

4.2.2 Preparation of Palm Oil Adjuvanted Feed-Based Vaccine (FBV)

The formalin-killed bacteria (10^9 CFU ml^{-1}) was first diluted with an equal volume of PBS (pH 7.4) and absolute ethanol (1:1 ratio). After that, palm oil was added to a final concentration of 10%. The suspension was then thoroughly mixed, sprayed onto the feed formulation and subjected to a pressed pelleting machine. The final concentration of bacteria cells is 10^6 cells/kg of feed. The feed formulation is summarized in Table 6

Table 6 Feed formulation of the palm oil adjuvanted vaccine

Ingredients	**Percentage (%)**
Danish fishmeal	15.00
Soybean meal	23.43
Rice bran	39.77
Maize / Starch	15.00
Squid meal	5.00
Di-calcium phosphate (DCP)	1.00
CMC (Binder)	0.10
Mineral premix	0.40
Vitamin premix	0.30

4.2.3 Proximate Analysis

4.2.3.1 Sample Preparation

Approximately 300 g of the FBV, feed formulation (FF) pellet (without vaccine), and commercial pellet (CP) were ground into a fine powder using a laboratory mill grinder for 8 minutes. The resulting powder was transferred into plastic containers and stored in a borosilicate glass desiccator until further used.

4.2.3.2 Moisture Content

The empty porcelain crucibles were cleaned, dried in an oven at 105°C for 3 hours, and transferred into a desiccator to cool down for at least 1 hour. The empty crucibles were weighted and assigned as W0. Then, 1.000 g of the sample was weighted into the crucible and assigned as W1 in triplicates. The crucibles containing the sample were then dried in an oven at 105°C for 5 hours before being transferred into a desiccator. After 1 hour, each crucible containing the dried sample was weighted, assigned as W2, and the moisture composition was measured using the formula:

$$Moisture\ (\%) = \frac{W1 - (W2 - W0)}{W1} \times 100\%$$

4.2.3.3 Ash Content

Initially, the cleaned crucibles were combusted in a muffle furnace at 550°C for 5 hours before being transferred into a desiccator to cool down for at least 1 hour. The empty crucibles were weighted and assigned as W0. Then, 1.000 g of the sample was weighted into the crucible and assigned as W1 in triplicates. The crucibles containing the sample

were combusted in the furnace at 550℃ for 16 hours before being transferred into a desiccator. After 1 hour, each crucible containing combusted samples was weighted, assigned as W2, and the ash composition was measured using the formula:

$$Ash\ (\%) \quad = \frac{W2 - \mathrm{W0}}{W1} \times 100\%$$

4.2.3.4 Crude Fat

The aluminium thimbles were first cleaned in an oven at 105℃ for 3 hours and transferred into a desiccator to cool down for at least 1 hour. The empty thimbles were weighted and assigned as W0. Then, 1.000 g of the sample was weighted on filter paper, placed into a cellulose thimble and assigned as W1 in triplicates. The 4-step extraction protocol of boiling (135℃, 20 min), rinsing (135℃, 35 min), recovery (135℃, 10 min), and pre-drying was performed in a FOSS Soxtec Avanti 2055 machine (Foss Analytical, Denmark) using petroleum ether as solvent. The extracted fat was transferred into an oven at 105℃ for 1 hour to ensure all petroleum ether vapor was fully evaporated. After drying, the thimbles were placed in a desiccator to cool down for at least 1 hour before the thimbles were weighed and assigned as W2. The proximate composition of extracted crude fat was determined using the formula:

$$Crude\ fat\ (\%) \quad = \frac{W2 - \mathrm{W0}}{W1} \times 100\%$$

4.2.3.5 Crude Fiber

1.000 g of samples were initially weighted into a filter crucible in triplicate, assigned as W1, before adding 1.000 g of acid-washed celite powder. In the presence of 1.25% of sulphuric acid and sodium hydroxide, the hydrolysis and extraction of crude fiber were

carried out in Fibertec 2010 hot extraction unit (Foss Analytical, Denmark). Then, the filter crucible containing extracted fiber was transferred into an oven at 105℃ for 2 hours before being cooled in a desiccator. Following that, the filter crucibles were weighted again and assigned as W2. The sample was combusted in the furnace at 525℃ for another 8 hours before being weighed and assigned as W3. The composition of extracted crude fiber was determined using the formula:

$$Crude\ fiber\ (\%) = \frac{W2 - \mathrm{W3}}{W1} \times 100\%$$

4.2.3.6 Crude Protein

The crude protein was quantified using the Kjeldahl method, which involves a 3-step extraction protocol of digestion, distillation and titration. At first, 1.000 g of samples were weighed on triplicate filter paper, assigned as W1, and placed into a digestion flask. 7 g of Kjedahl catalyst and 3-5 glass beads were added to each digestion flask before 16.5 ml of 96 % of sulphuric acid and 5 ml of prechilled hydrogen peroxide were added. The digestion process was done using FOSS digestor DT208 (Foss Analytical, Denmark) at 420°C for 90 minutes. After digestion, 60 ml of distilled water was dispensed into each tube. Then, 25 ml of 4 % boric acid (Chem Lab, Belgium) and 1 ml of methyl red indicator were added into a 250 ml conical flask. Prior to distillation in Kjeltec 2100 unit (Foss Analytical, Denmark), 60 ml of 40% sodium hydroxide (Fisher scientific, USA) was dispensed into a digestion tube. The distillation constituent was then titrated with 0.5 mol/L of hydrochloric acid using an 876 manual titrator plus (Metrohm, Switzerland), and the titrate volume was assigned as W1. The composition of extracted crude protein was determined using the formula:

$$Crude\ protein\ (\%) = \frac{W2 - W\ (blank) \times (normality\ acid\ \times 14 \times 100)}{W1}$$

4.2.4 Data Analysis

Statistical analysis was calculated using one-way analysis of variance (ANOVA) coupled with Tukey HSD in Statistix version 10 software (Analytical Software, USA). The results were considered significant at $p<0.01$.

4.3 RESULT

Table 7 summarizes the mean ± SD proximate composition of feed-based streptococcosis vaccine (FBV), feed formulation (FF), and commercial pellet (CP).

Table 7 Mean ± SD proximate composition of feed-based streptococcosis vaccine (FBV), feed formulation (FF), and commercial pellet (CP)

Samples	Moisture (%)	Ash (%)	Crude fat (%)	Crude fiber (%)	Crude protein (%)
FBV	6.8378 ± 0.3493[a]	8.5829 ± 0.3474[b]	7.7033 ± 0.9735[a]	6.3110 ± 1.8526[a]	30.220 ± 0.8907[a]
FF	5.2977 ± 1.2214[b]	8.8727 ± 0.1493[b]	6.7356 ± 1.3329[a]	5.6397 ± 2.6170[a]	32.496 ± 2.5750[a]
CP	7.8438 ± 0.0529[c]	9.6728 ± 0.1012[a]	2.5625 ± 0.0410[b]	6.1979 ± 0.1216[a]	31.561 ± 0.3438[a]

[a,b,c]Different superscript letters represent a significantly different ($p<0.01$) between the same row

4.3.1 Moisture

The moisture content was found to be 6.8378 ± 0.3493, 5.2977 ± 1.2214, and 7.8438 ± 0.0529% in FBV, FF, and CP, respectively. A significant (p<0.01) difference was observed among all feed samples tested. A variation in moisture content between FBV and FF is most likely due to the seed vaccine mixture's inclusion prior to pelleting. Although the moisture was significantly higher than the control pellet (FF), it remained within an acceptable range of less than 10% (de Koning, 2002). Moisture content in animal feed should be regulated to avoid mold growth and overcome a decomposition response, allowing for safe storage and improving feed shelf life.

4.3.2 Ash

Ash is an indicator of the total amount of minerals present in a feed sample, following the dehydration process and combustion in a muffle furnace. This study found the highest ash content in CP with 9.6728 ± 0.1012%, followed by FF and FBV with 8.8727 ± 0.1493 and 8.5829 ± 0.3474%, respectively. There was a significant (p<0.01) increase in the ash content of CP between FF and FBV. Meanwhile, a comparison between FF and FBV revealed insignificant (p>0.01) different. The ash content of all the feed samples was found within the recommended ash content in the tilapia diet of 7-12% (Villarino, 2020).

4.3.3 Crude Fat

The feed samples analysed contain various amounts of crude fat. The crude lipid content of FBV and FF was 7.7033 ± 0.9735, and 6.7356 ± 1.3329%, respectively. The addition of palm oil as a vaccine adjuvant caused a slight increase (p>0.01) in crude fat content in FBV compared to FF. Surprisingly, crude fat in CP was severely low (2.5625 ±

0.0410%) and deviated from the recommended value for farmed tilapia of 5-12% (Lim et al., 2011). Dietary lipids are essential sources of highly digestible energy and provide essential fatty acids fish need for normal growth and development. Besides, lipids are primarily included in the formulated diet to maximize their protein-sparing effect by increasing their digestible energy value (Hasan, 2001).

4.3.4 Crude Fiber

The crude fiber was found to be 6.3110 ± 1.8526, 5.6397 ± 2.6170, and 6.1979 ± 0.1216% in FBV, FF, and CP, respectively. No significant (p>0.01) difference was observed in the crude fiber of these feed samples, and its composition was within the suggested values for the tilapia diet of 4-8% (Ng & Romano, 2013).

4.3.5 Crude Protein

The highest crude protein content was found in FF with 32.496 ± 2.5750%, followed by CP and FBV with 31.561 ± 0.3438 and 30.220 ± 0.8907%, respectively. Although a variation was observed, there was no significant difference (p>0.01) in the crude protein composition of all feed samples, which fell within the recommended values for farmed tilapia of 27-37% (Khattab et al., 2000).

4.3.6 Carbohydrate, Energy, and Nitrogen-Free Extract (NFE)

The calculated percentage of carbohydrates, energy, and NFE is summarized in Table 8. The approximate composition of carbohydrates and NFE between FBV, FF, and CP showed no significant (p>0.01) different. Carbohydrates constituted the highest

proportion of all proximate components in all feed samples. CP had the highest carbohydrate content (48.360 ± 0.3606%), whereas FF had the lowest (46.598 ± 2.8362). On the other hand, NFE, which is represented as soluble carbohydrates and other digestible such as starch and sugar, is calculated by the difference between the original sample and the percentage of other proximate (moisture, fat, protein, fiber, and ash). The NFE was 42.771 ± 1.7814, 43.052 ± 3.1556, and 42.162 ± 0.3556% in FBV, FF, and CP, respectively. Eventually, the highest energy yield was computed at 389.45 ± 8.3017% in FBV. It was significantly (p<0.01) higher than FF and CP and associated with increased crude fat content due to the inclusion of palm oil as a vaccine adjuvant.

Table 8 Estimation of carbohydrate, energy, and nitrogen-free extract (NFE)

Parameters	**Samples**	**Formula**	**Results**
Carbohydrate (%)	FBV	100% - (% moisture + % protein + % fat + % ash)	46.655 ± 0.3997[a]
	FF		46.598 ± 2.8362[a]
	CP		48.360 ± 0.3606[a]
Energy (%)	FBV	(% fiber × 2) + (% protein × 4) + (% fat × 9) + (% carbohydrate × 4)	389.45 ± 8.3017[a]
	FF		353.44 ± 22.032[b]
	CP		355.14 ± 0.0322[b]
Nitrogen-free extract (%)	FBV	100% - (% moisture + % protein + % fat + % ash + % fiber)	42.771 ± 1.7814[a]
	FF		43.052 ± 3.1556[a]
	CP		42.162 ± 0.3556[a]

[a,b]Different superscript letters represent a significantly different (p<0.01) between the same row

4.4 DISCUSSION

Fish growth, health, and reproduction largely depend on an adequate supply of nutrients, both in quality and quantity, regardless of the culture system in which they are

maintained. Proximate analysis of feed-based vaccines is a crucial step in understanding and optimizing their formulation. As a result, it is essential for aquafeed to effectively match the nutrient and energy requirements of the fish while simultaneously meeting the production goals. Proximate analysis based on the Weende system was founded by Henneberg and Stohmann in the mid-eighteenth century, which permits the quantitative analysis of the various macronutrients in animal feed, including moisture, ash, protein, fat, and fiber (McCleary, 2003). Following the discovery, extensive research has been directed to hone the system to its current state, allowing feeds to be compared based on specific nutrients.

Moisture content in the feed-based vaccine formulation is essential for its stability and shelf life. Proximate analysis ensures that the moisture levels are within acceptable limits, preventing degradation and maintaining vaccine integrity. A high-moisture pellet can hasten decomposition and promote the growth of spoilage and pathogenic microbes. As a result, analysing moisture content throughout the feed processing and manufacturing process is critical in feed mills to ensure a high-quality end product. The moisture level of all feed samples found in the study was within the recommended maximum range of less than 10% (de Koning, 2002). However, the inclusion of the seed vaccine mixture in the feed formulation (FBV) resulted in a noticeable increase in moisture level. Previous research by Oehme et al. (2014) indicated that the physical quality of the dry pellet had no effect on the growth and feed uptake of Atlantic salmon (*Salmo salar*). More recently, Lee et al. (2016) revealed that a feeding regime of an extruded pellet containing an average moisture content of 7.28% led to significantly higher weight gain, specific growth rate, and feed efficiency without deteriorating water quality compared to a highly moist pellet (65%) following 6-week feeding trial in olive flounder.

The second proximate composition analysed in the present study is ash content. Ash of animal feeds indicates the total quantity of minerals present. In this study, a dry ashing procedure was performed by heating the sample in an open crucible in a muffle

furnace to ensure the destruction of organic matter, converting minerals into carbonate or oxide forms (Hoenig, 2005). However, this approach has limitations because it does not retain certain elements, such as iron and selenium, which may be partially volatile throughout the process. It is important to note that several minerals, including calcium, phosphorus, magnesium, zinc, iron, copper, manganese, iodine, and selenium, have been reported in fish and are essentially required for normal growth and development (Lall & Kaushik, 2021). Even though its specific metabolism in fish remains inadequately understood, it is believed that an adequate level of this mineral can promote survival and growth, induces non-specific immunity, and increase disease resistance (Muralisankar et al., 2022). Certain disorders, including cataracts, anaemia, and bone deformities, have been associated with mineral deficiencies in the diet (Lall & Kaushik, 2021). The ash content in CP from this study resulted in the highest percentage, followed by FF and FBV. This difference provides an insight into the mineral content in each feed sample and can be attributed to several factor, including (1) differences in the basic raw ingredients for protein sources, such as the use of white fish meal, brown fish meal or soybean meal; (2) variations caused by incorporation of macro or trace mineral premixes; and (3) present of contaminants in conventional and unconventional feed constituents (Tacon & De Silva, 1983). Nonetheless, the composition in each feed sample fell within the recommended range of 7-12% for the tilapia diet (Villarino, 2020).

The fat present in fish feed commonly exists in triglycerides, which provide a concentrated energy source and essential fatty acids that fish cannot produce. Fat is the primary component of cell membranes in the body and serves as a precursor for steroid hormones and other signalling molecules such as prostaglandins, prostacyclins, and thromboxanes, which are responsible for blood clotting, immunological and inflammatory responses, renal and neural function (Araujo et al., 2021). Due to the mobilization of essential fatty acids from endogenous tissue lipids, a low-fat diet can reduce the weight of cultured fish. However, a previous study by He et al. (2015) found that dietary lipid content of 1% (low fat), 7% (medium fat), and 13% (high fat) in juvenile Nile tilapia did not significantly affect final body weight following ten weeks

of trial, indicating that the fish have the ability to adapt to low-fat diet by synthesizing lipid and maintaining lipid homeostasis. Excess dietary fat, conversely, can harm fish's normal growth, feed utilization and efficiency, liver and immune function, and antioxidant capacity, ultimately affecting the quality of cultured fish (Naiel et al., 2022). In the present study, the addition of palm oil as a vaccine adjuvant to the feed-based vaccine resulted in a negligible increase in fat content, which remained within the recommended range of 5-12% for farmed tilapia (Lim et al., 2011). Interestingly, proximate analysis of a commercial pellet (as a comparison) revealed that the fat content was severely low and deviated from the recommended value. This event might be due to the quality of raw material and the type of processing used since the composition of the final product highly depends on both.

Proteins is the most abundant and critical component in feed-based fish oral vaccines. It is necessary for the growth of fish and also serves as the main antigenic agent, stimulating the fish's immune response and promoting the production of specific antibodies (Aoki et al., 2015; Jauralde et al., 2021). Proteins are made up of numerous amino acids, and the unique combination of these amino acids determines the features of each protein. Similar to other animals, fish produce body proteins from amino acids in their diet and other sources. Several essential amino acids vital for fish growth and development include arginine, leucine, isoleucine, lysine, phenylalanine, histidine, tryptophan, threonine, methionine and valine (Furuya et al., 2023). A shortage of essential amino acids may hinder protein synthesis, impairing healthy growth. Several studies have shown that weight gain and specific growth rate increase with increasing dietary protein levels (Kim et al., 2012; Rahimnejad et al., 2021; Sankian et al., 2017). Excess protein, on the other hand, is both economically and environmentally unsound because it is the most expensive dietary component and can produce nitrogenous waste build-up. A previous study by Khattab et al. (2000) recommended a range of 27 – 37% of crude protein in farmed tilapia diet depending on fish age, culture system, and environmental conditions. In the present study, all of the samples analysed showed that the protein composition is within the recommended value for commercial tilapia farming. Although insignificant compositional differences were observed, they may be

attributed to the distinct formulation (or protein source raw ingredient) used in commercial pellets as well as the homogeneity of the sample during analysis. This coincides with the findings of Torrecillas et al. (2021), who showed that experimental diets containing different formulations of fishmeal (7.5 – 15%) and krill meal (0 – 7.5%) resulted in variations of crude protein proximate composition, which were not statistically significant. In addition, the Kjeldahl method use in the analysis determines the nitrogen content of the sample, which can be converted to crude protein content using nitrogen-to-protein conversion factors (Nielsen, 2006); therefore, it is reasonable to anticipate minor variations.

Another critical nutrient usually included in fish feed is fiber. Crude fiber represents an indigestible proportion of fish diet which aids in waste excretion from the gastrointestinal system by binding with water molecules and thus producing firmer stools. In addition, a certain amount of crude fiber in the feed is advantageous for nutrient assimilation and promotes the production of beneficial microorganisms in the digestive system (Kamarudin et al., 2018). Previous work by Sun et al. (2019) found that a rich crude fiber diet in Taiwanese loaches had no significant effect on specific growth rates. However, it did influence the feeding rate, feed conversion rate, and protein efficiency ratio. More recently, Zhong et al. (2020) reported that supplementation with high dietary fiber (8.5%) decreased the largemouth bass's weight gain and protein efficiency ratio, decreased hepatic fat deposition, and enhanced antioxidant capacity. Consequently, a balanced composition of crude fiber in feed formulation is essential for optimizing its impact on fish growth performance and health. Tilapia, as omnivorous, preferred a higher range of fiber (4-8%) in their diet (Ng & Romano, 2013). In the present study, the crude fiber content of all tested feed samples fell within the optimal range, with FBV having the highest crude fiber proximate composition, followed by CP and FF. The crude fiber composition in FBV from the present study coincides with the findings of Aslah et al. (2021), who formulizes a feed-based vaccine containing whole-cell polyvalent vaccine against vibriosis, streptococcosis, and motile aeromonad septicemia. Although the formulation of the

author's vaccinated pellets was not disclosed, it is probable both used a similar plant-based protein source (soybean meal).

4.5 CONCLUSION

The current study concluded that the nutrient composition of the vaccinated pellet (FBV) was within the recommended nutritional value, therefore, is suitable for farmed red hybrid tilapia diet.

CHAPTER FIVE

DETERMINATION OF IMMUNE RESPONSES OF CAGE CULTURED RED HYBRID TILAPIA AND THE EFFICACY OF PALM OIL ADJUVANTED STREPTOCOCCOSIS VACCINE FOLLOWING ORAL VACCINATION REGIME UNDER FIELD CONDITION

5.1 INTRODUCTION

In recent years, the world has witnessed a significant increase in the demand for effective vaccines to combat various infectious diseases in fish. The rapid development of vaccines has been vital in protecting fish from diseases and preventing outbreaks. Vaccines work by stimulating the immune system to recognize and mount a response against specific pathogens, such as viruses and bacteria (Marshall et al., 2018). To achieve this, vaccines often contain antigens derived from these pathogens. However, some antigens may not be potent enough to elicit a robust immune response on their own and adjuvants are needed. Adjuvants are added to vaccines to enhance the body's immune response to the antigens (Facciolà et al., 2022). They achieve this by activating and prolonging the immune system's reaction, leading to the production of more antibodies and memory cells (Awate et al., 2013). Consequently, the fish's immune system becomes better equipped to recognize and neutralize the pathogen if encountered in the future. One such promising adjuvant candidate is palm oil, a versatile and sustainable resource that holds great potential in revolutionizing fish vaccine development and distribution.

The fish immune system comprises two immune mechanisms, innate (non-specific) and adaptive (specific), both of which are subdivided into cellular and humoral responses. Upon initial infection, the innate immune system is the first to respond. The innate immune system of fish encompasses physical barriers such as fish skin, gill, and stomach, as well as non-specific cellular components such as toll-like receptors (TLRs), macrophages, granulocytes (neutrophil and eosinophil), and non-specific cytotoxic cells. Additionally, it includes humoral components such as the complement system, lysozyme, interferon, C-reactive protein, transferrin, lectin, and alkaline phosphatase (Firdaus-Nawi & Zamri-Saad, 2016; Mokhtar et al., 2023; Smith et al., 2019). Although innate immunity responses are nonspecific and provide only temporary and limited protection, it is crucial to prevent the severity of the infection and act as a buffer, allowing adaptive immunity sufficient time to take over.

On the other hand, the adaptive immune system comprises highly specialized cells against specific antigens, offering long-term immunity and memory. The adaptive immune system's principal components are cytotoxic T-lymphocytes cells, immunoglobulins, and major histocompatibility complex (MHC) (Mokhtar et al., 2023). The interrelationship between innate and adaptive immune systems is facilitated by the functioning of dendritic cells and macrophages (also known as antigen-presenting cells), which process the invader antigenic protein into peptides. Subsequently, these peptides are displayed on the cell surface of the major histocompatibility complex (MHC). This display indicates the reactivity of T-lymphocytes and initiates the acquired response of cell-mediated (Biller-Takahashi & Urbinati, 2014; Mokhtar et al., 2023). As a result, specific cytokines are released, which induce the maturation of B-lymphocytes into memory and plasma cells, ensuing secretion of specific immunoglobulin or antibodies and resulting in complete antigen destruction (Firdaus-Nawi & Zamri-Saad, 2016).

In the present investigation, red hybrid tilapia's immune responses were determined by continuous monitoring and quantifying the serum immunoglobin M

(IgM), lysozyme, and complement-3 using an enzyme-linked immunosorbent assay (ELISA) technique following a vaccination regimen. The assay combines a biochemical and serological technique that employs enzymatic reactions to detect and quantify a specific protein in the samples (S. Zhang & Vrient, 2020). In general, ELISA involves the successive addition and washing of antibodies, conjugates, and substrates, which results in the generation of a colorimetric signal for spectrophotometric analysis.

5.2 MATERIALS AND METHOD

5.2.1 Animal Ethics

The experimental procedure was conducted in this field trial in accordance with the outlined by The Institutional Animal Care and Use Committee (IACUC), International Islamic University Malaysia, for animal utilization for scientific purposes, as indicated by permission number IACUC-2020-015.

5.2.2 Study Site

The field trial was conducted on a private farm at Kenyir Lake, an artificial lake located in the province of Terengganu, Malaysia. The main fish species culture on site is red hybrid tilapia. Other fish species, such as catfish and Hoven's carp, were also cultured but in low density. The selected farm had a history of endemic streptococcal problems, which led to a cumulatively high mortality rate of cultured red hybrid tilapia during the hot and dry season of Mac to June.

5.2.3 Experimental Design and Vaccination Protocol

The field vaccination trial was conducted according to Ismail et al. (2017) with minor modifications during the annual outbreak season (Mac to June). Briefly, a total of 6,000 red hybrid tilapias with an average bodyweight of 40 ± 20 g were acquired from a local hatchery and evenly distributed into six 4 m length x 4 m width x 4 m depth floating net cages. Each cage was represented as a single booster (SB), double booster (DB), and unvaccinated control group in duplicate. Prior to the experiment, ten fish from each group were randomly screened and their health status was ascertained by routine diagnostic procedures for parasite, bacteria, and tilapia lake virus (TiLV) detection.

A total of 16 weeks were devoted to the vaccination trial. The fish was fasted a day prior to each vaccine administration to ensure maximum vaccine uptake. The SB group was fed the vaccine on weeks 0 and 2, whereas the DB group was on weeks 0, 2, and 6. Meanwhile, regular commercial feed was given as a replacement for the control group. The feed-containing vaccine was given at 5% of the fish's average weight twice; at 1 pm and 6 pm. It is important to note that other husbandry practices adopted on the farm were not intervened.

5.2.4 Field Sampling

Post-mortem was performed *in-situ* from 30 fish per group at two weeks intervals for 16 weeks. Brain, eye, and kidney samples were collected and subjected to bacterial isolation and identification. Blood was also withdrawn from the caudal vein and subjected to serum antibody (IgM), lysozyme, and complement-3 determination. Any clinical signs observed and mortality was recorded throughout the study period.

5.2.5 Bacterial Identification

Swab samples of the brain, eye, and kidneys were aseptically streaked on blood agar (BA) and incubated at 30 ± 2℃ for 48 hours. The clinical isolates were subcultured multiple times to ensure their purity before performing the gram staining, oxidase, and catalase test. Following manufacture instructions, the isolates were further characterized based on their biochemical profiles using the API®20 STREP identification system (bioMérieux, France).

5.2.6 Water Quality Measurement

During each sampling period, water quality parameters were collected at three different locations within the farm and measured on-site between 2:00 and 3:00 pm (local time in Malaysia). Using a hand-held YSI meter (YSI Inc., USA), the following parameters were measured: water temperature, pH, and dissolved oxygen. Meanwhile, nitrogen ammonia, sulfide, and nitrate concentration were measured using salicylate, methylene blue, and cadmium reduction method (HACH company, USA). Nitrite and iron were measured using USEPA Diazotization and the FerroVer method (HACH company, USA). On the other hand, reactive phosphorus or orthophosphate was carried out using the Molybdovanadate method (HACH company, USA).

5.2.7 Determination of Serum Antibody (IgM) Titer by ELISA

The blood serum was subjected to indirect ELISA to determine the antibody (IgM) levels following the method described by Firdaus-Nawi et al. (2013). Five colonies of *S. agalactiae* from the BA were briefly sub-cultured into 100 ml of the BHI broth and incubated at 30 ± 2℃, 150 rpm for 24 hr. Following incubation, the bacterial concentration was determined using the standard plate count technique. Subsequently,

the cells were harvested through centrifugation at 5000 rpm for 15 min at 4°C and washed thrice in 40 ml of sterile PBS (pH 7.4). The resulting pellet was then suspended in Carbonate Bicarbonate coating buffer (pH 9.6) to a final concentration of 10^5 CFU ml^{-1} and boiled in a water bath at 97°C for 20 minutes. The suspension was stored at -20°C prior to use as coating antigen.

The flat-bottomed microtitre plates were coated with 100 µL coating antigen and left overnight at 4°C. The plates were washed twice with PBS containing 0.05% of Tween-20 (PBS-T) to remove unbound antigens. The coating antigens were then blocked with 200 µL of 1 % bovine serum albumin (BSA) and washed twice. At 37°C for 1 hr, 100 µL of each diluted serum sample (1:1000) was added and incubated. After that, 100 µL of goat anti-tilapia immunoglobulin (GAT) serum, diluted at 1:10,000, was added to each well and incubated for 1 hr. Next, 100 µL of conjugated rabbit anti-goat (RAG) IgM-horseradish peroxidase (Nordic Immunology, Netherlands) (1:10,000) was added and incubated for another 1 hr. The bound conjugates were captured by adding 100 µL of TMB One Substrate solution (Promega, USA) before the reaction was terminated by adding 50 µL of 2.5 M Sulphuric Acid. The absorbance was determined at 450 nm wavelength. Using serum from juvenile and disease-free red hybrid tilapia, the cut-off value was determined and set at mean plus three times the standard deviation value (Crowther, 2000).

5.2.8 Determination of Lysozyme Activity

Determination of serum lysozyme level was carried out by using a fish lysozyme (renal amyloidosis) ELISA kit (Cusabio, China), which employs the competitive inhibition enzyme immunoassay technique with a detection range between 3.12 – 200 ng ml^{-1} lysozyme. Briefly, reagents, samples, and standards were prepared per manufacturer instruction, and 50 µl of standards and sample was loaded per well. Then, 50 µl of HRP-conjugate (1x) was added to each well, excluding the designated blank well with gentle

agitation for 60 seconds, followed by incubation for 40 minutes at 37°C. The plate underwent five washes to eliminate unbound conjugate before adding 90 µl of TMB substrate to each well. The plate was incubated for 20 minutes at 37°C before the reaction was terminated by adding 50 µl of stop solution. The optical density was measured by employing a microplate reader at 450 nm. The lysozyme values are expressed as µg ml^{-1}.

5.2.9 Determination of Complement-3 Activity

Determination of serum complement-3 activity was carried out by using a fish Complement-3 (C3) ELISA kit (Cusabio, China), which employs a similar competitive inhibition enzyme immunoassay technique with a wider detection range of 12.5 – 500 µg ml^{-1} of Complement-3. Briefly, reagents and samples were prepared per manufacturer instruction, and 50 µl of standards or samples were loaded per well. Then, 50 µl of HRP-conjugate mixed solution was added to each well except the designated blank well with gentle agitation and incubated for 1 hour at 37°C. Before adding 50 µl of Substrate A and Substrate B to each well, the plate was washed thrice to remove unbound conjugate. The plate was incubated for 15 minutes at 37°C before the reaction was terminated by adding 50 µl of stop solution. The optical density was measured using a microplate reader at 450 nm. The lysozyme values are expressed as mg ml^{-1}.

5.2.10 Data Analysis

The statistical analysis was performed using one-way analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) in Statistix version 10 software (Analytical Software, USA). The results were considered significant at $p<0.05$.

5.3 RESULT

5.3.1 Isolation of *S. agalactiae*

The average of *S. agalactiae* isolation rate in red hybrid tilapia regardless of the experimental groups was summarized in Figure 5. Initially, during health screening and first sampling (week 0), the bacteria species was not isolated from any experimental groups, indicating that the fish acquired from the local hatchery was free from *S. agalactiae*. However, from week 2 onward, *S. agalactiae* were successfully isolated throughout the study period, with the highest isolation rate recorded at week 10 (30%), followed by week 4 and week 14 (both with 21%). In the remaining sampling weeks, a lower prevalence of *S. agalactiae* ranged between 2 – 11%. As shown in Figure 6, *S. agalactiae*-infected tilapia ranged in weight from 13 to 165 g. However, the highest incidence of positive detection was recorded in tilapia weighing between 51 and 107 g (Figure 7).

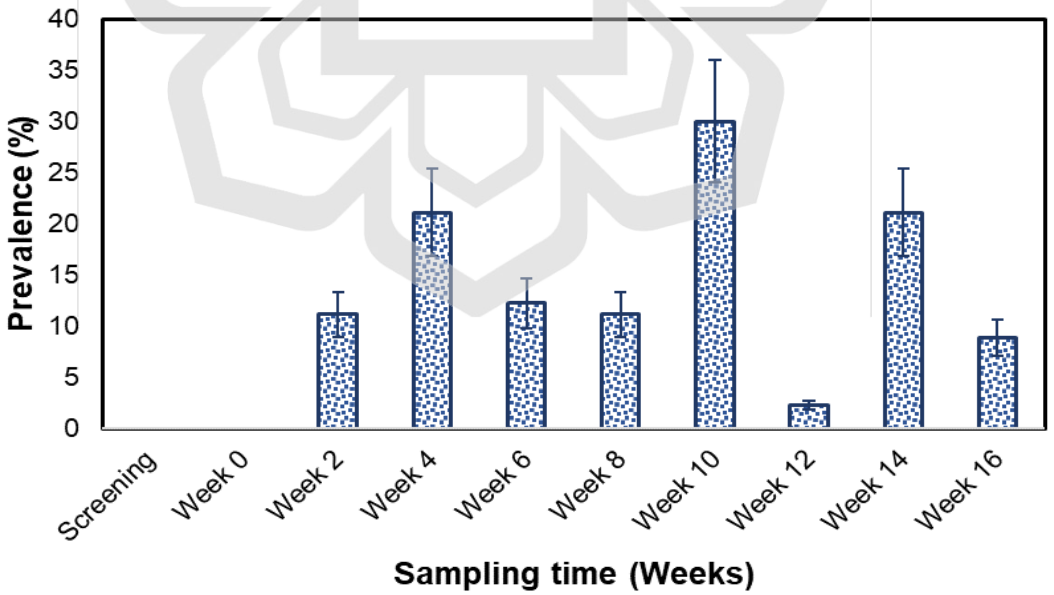

Figure 5 The average rate of *Streptococcus agalactiae* isolation from red hybrid tilapia samples

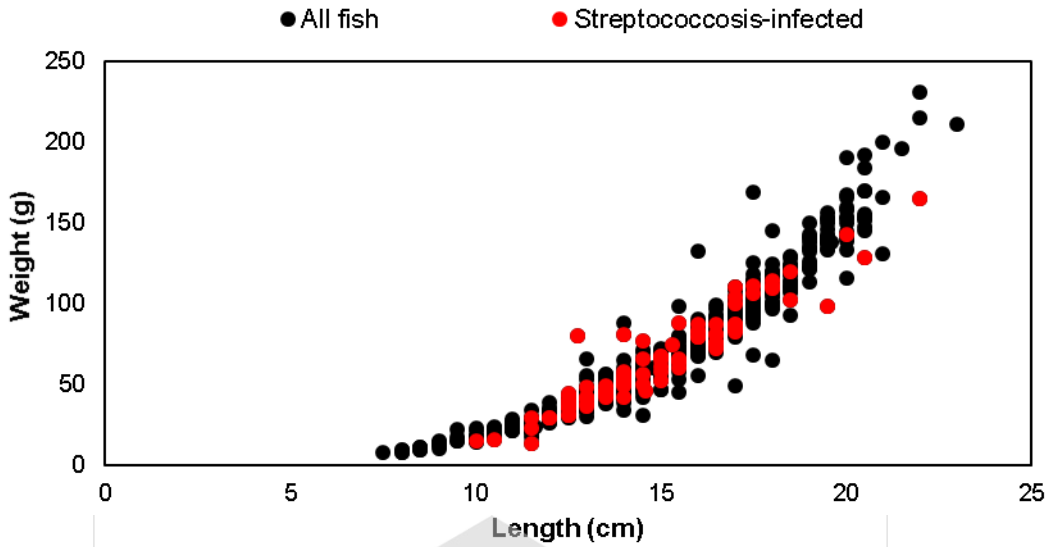

Figure 6 Range of weight and length of *Streptococcus agalactiae*-infected red hybrid tilapia

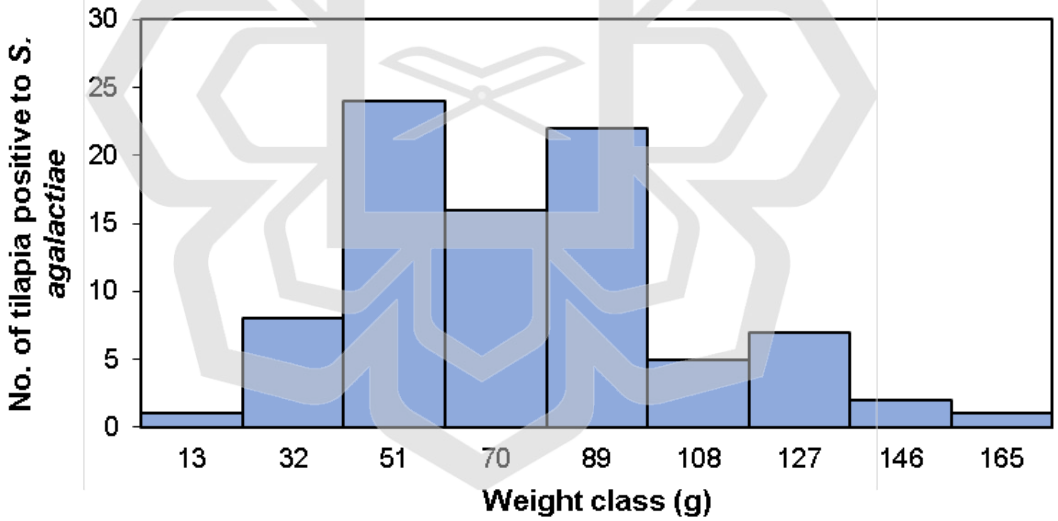

Figure 7 Infected red hybrid tilapia weight class

Meanwhile, Table 9 summarizes the isolation rate of *S. agalactiae* from each experimental group. In the unvaccinated control group, *S. agalactiae* was successfully isolated from week 4 and throughout the sampling period, except in week 12. However, in the single booster group, the bacteria species were isolated almost every sampling

week starting from week 2, except in week 6. Similarly, in the double booster group, *S. agalactiae* was isolated throughout the sampling period with a lower prevalence, except for week 2, week 6, and week 12. The cumulative incidence of *S. agalactiae* during the duration of the study was 19.6 %, 11.5 %, and 8.2 % for unvaccinated control, SB, and DB groups, respectively (Table 9).

Table 9 Rate of *Streptococcus agalactiae* isolated from red hybrid tilapia samples (n=30)

Week	**Rate of isolation (%)**			**Mean ± SD**
	Unvaccinated	**Single booster**	**Double booster**	
0	0	0	0	0
2	0	33.3	0	11.1 ± 19.2
4	20	26.7	16.7	21.1 ± 5.1
6	36.7	0	0	12.2 ± 21.2
8	20	6.7	6.7	11.1 ± 7.7
10	60	10	20	30.0 ± 26.5
12	0	6.7	0	2.2 ± 3.9
14	30	10	23.3	21.1 ± 10.2
16	10	10	6.7	8.9 ± 1.9
Mean ± SD	19.6 ± 20.2	11. 5 ± 11.3	8.2 ± 9.4	

5.3.2 Clinical Signs and Symptoms of Infected Fish

Several clinical signs and gross lesions related to streptococcosis in red hybrid tilapia were observed throughout the entire study period, which included bilateral exophthalmia (pop-eye), soft and watery brain, enlarged spleen, and erratic swimming behavior (Figure 8).

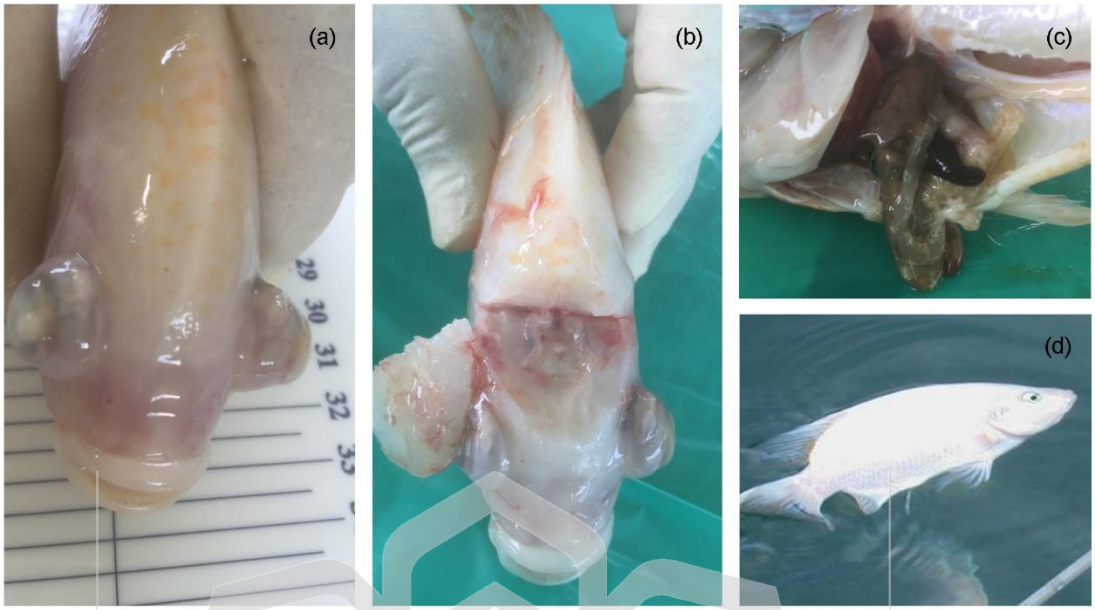

Figure 8 Several clinical signs and gross lesions were observed during routine sampling that is associated with streptococcosis (a) bilateral exophthalmia, (b) soft and watery brain, (c) enlarged spleen, (d) erratic swimming

5.3.3 Water Quality

The water quality parameters measured throughout the study period are summarized in Table 10. Most parameters were within the permissible range for farmed tilapia except the water temperature (30.73 ± 1.08°C), which might predispose fish to infections.

Table 10 Water quality parameters were measured during the study period. The values are mean ± SD

Parameter	Mean ± SD
Temperature (°C)	30.73 ± 1.08*
pH (1-14)	7.06 ± 0.17
Dissolved oxygen (mg L^{-1})	5.56 ± 0.84
Iron (mg L^{-1})	0.03 ± 0.01
Ammonia (mg L^{-1})	0.02 ± 0.02
Nitrate (mg L^{-1})	1.87 ± 1.22
Nitrite (mg L^{-1})	0.01 ± 0.00
Sulfide (µg L^{-1})	2.74 ± 1.23
Phosphate (mg L^{-1})	0.13 ± 0.14

* Indicates not within the normal parameter.

5.3.4 Serum Antibody (IgM) Response Against *S. agalactiae* Following Vaccination Regime

Figure 9 depicts the progression of serum antibody (IgM) response and *S. agalactiae* isolation rate in each experimental group following vaccination. Prior to the vaccination, serum antibody levels against *S. agalactiae* did not exhibit significant ($p>0.05$) differences among groups and were marginally below the cut-off value. However different phenomenon was observed after vaccination where both vaccinated groups demonstrated a significant ($p<0.05$) increase in the serum antibody level, reaching their peak at week 4 and subsequently declining to the non-protective cut-off value by week 12 in the SB group. Nonetheless, following a second booster at week 6, the serum antibody level in the DB group increased significantly ($p<0.05$) at week 8 before gradually reducing. However, it remained above the cut-off value until the end of the experimental period at week 16. Meanwhile, the serum antibody level in the unvaccinated control group rose between weeks 6 and 10 before sharply reducing, approaching the cut-off value at week 16. This event was probably due to the high

infection rate of *S. agalactiae*, which started from week 4 to week 10 in the unvaccinated control group.

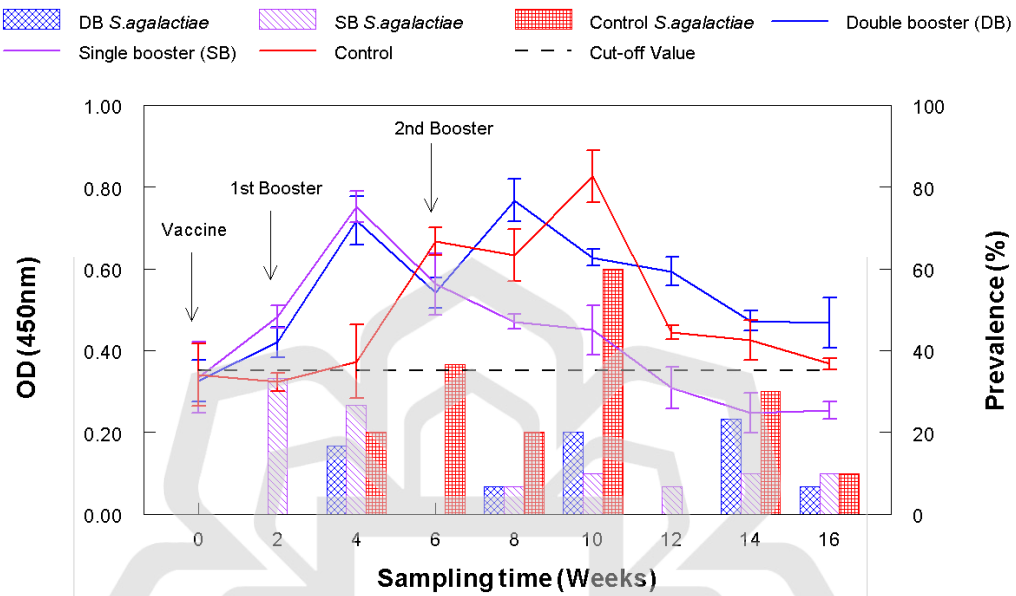

Figure 9 Serum antibody (IgM) response of red hybrid tilapia and isolation rate of *Streptococcus agalactiae* following vaccination regime (n=30). The values are mean ± SD

5.3.5 Lysozyme Level

The serum lysozyme concentration of red hybrid tilapia sampled during the study is presented in Figure 10. Prior to vaccine administration, the serum lysozyme level in unvaccinated (control) and vaccinated fish (SB and DB) showed an insignificant ($p>0.05$) difference. Following initial vaccine administration and first booster, the lysozyme activity in all vaccinated fish samples at weeks 2 and 4 increased significantly ($p<0.05$) compared to the unvaccinated fish. However, in week 6, the lysozyme level in unvaccinated fish was significantly ($p<0.05$) higher compared to both vaccinated groups. It remained high until week 10, likely correlating with the high infection rate of

S. agalactiae, which was started from week 4 to week 10 in the unvaccinated control group. Moreover, following a second booster at week 6, the serum lysozyme levels of fish in the DB group were significantly (p<0.05) higher than those of fish in the SB group at week 8 before gradually declining thereafter. Besides, the serum lysozyme level in unvaccinated (control) and vaccinated fish did not differ significantly (p>0.05) from week 12 through week 16 of the experiment.

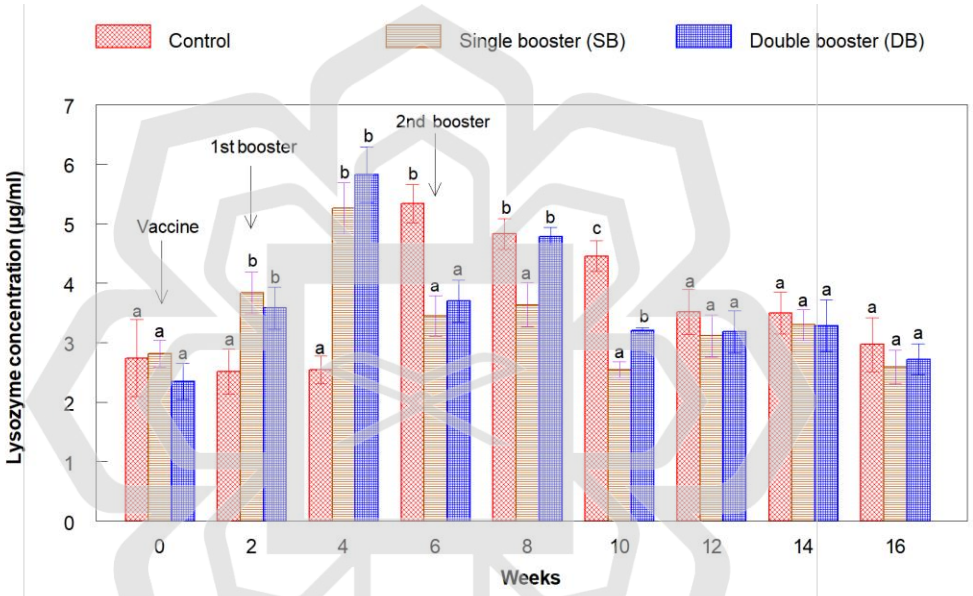

Figure 10 The serum lysozyme activity of red hybrid tilapia following the vaccination regime (n=10). The values are mean ± SD. Mean values with different superscripts differ significantly at p<0.05

5.3.6 Complement-3 Level

The serum complement-3 concentration of red hybrid tilapia sampled during the study is presented in Figure 11. Prior to vaccine administration, the serum complement-3 level in unvaccinated (control) and vaccinated fish showed an insignificant (p>0.05) difference. Following initial vaccine administration and first booster, the complement-

3 activity in all samples of the vaccinated fish (SB and DB) was significantly (p<0.05) higher than the unvaccinated fish from week 2 until week 6. Besides, following another booster on week 6, the serum complement-3 level of fish in the DB group was significantly (p<0.05) more than in the SB group at week 8 before gradually declining thereafter. Nevertheless, the complement-3 level in unvaccinated control fish resulted in a significant (p<0.05) higher compared to SB groups in week 8 and remained higher (p<0.05) than both vaccinated groups (SB and DB) at week 10. This sudden increase in complement-3 activity of fish in the unvaccinated control group might be due to the high infection rate of *S. agalactiae*, which peaked at week 10. Eventually, the serum complement-3 level in unvaccinated (control) and vaccinated fish showed an insignificant (p>0.05) difference starting from week 12 until the end of the experiment in week 16.

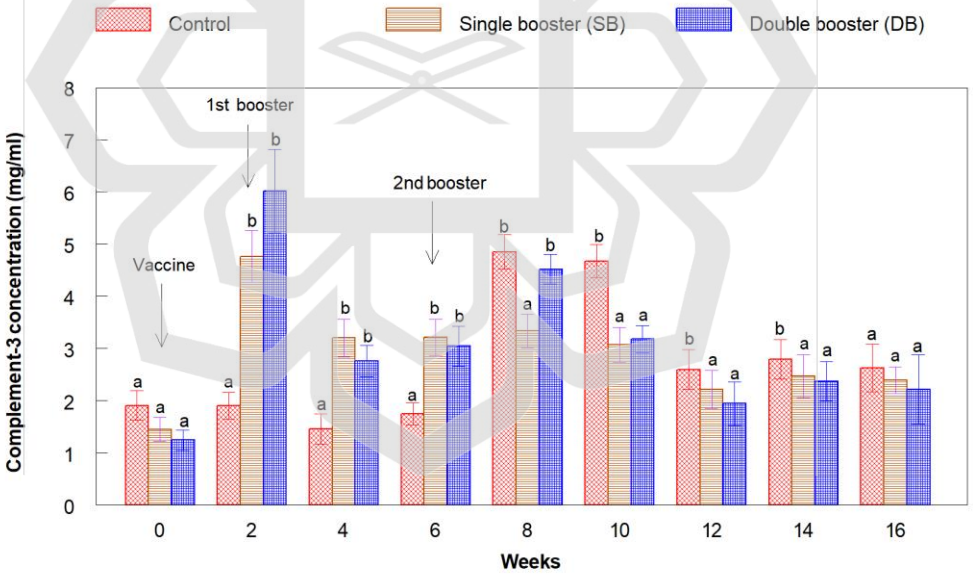

Figure 11 The serum complement-3 variations of red hybrid tilapia following the vaccination regime (n=10). The values are Mean ± SD. Mean values with different superscripts differ significantly at p<0.05

5.3.7 Efficacy of the Vaccine

Table 11 summarizes fish mortality records within each of the experimental groups. The unvaccinated control group recorded the highest mortality with 23.55 ± 1.77 %, followed by SB and DB groups with 14.45 ± 5.87 % and 6.40 ± 0.42 %, respectively. The average mortality rate between the unvaccinated control group and the DB group showed significant (p<0.05) different. It is important to note that slightly lower mortality in the unvaccinated group is expected since the incidence rate of *S. agalactiae* was relatively low.

Table 11 Mortalities of red hybrid tilapia in experimental trial groups. Values are the Mean ± SD. Different superscript letters indicate a significant difference at p<0.05

Experimental groups	**Cage no**	**Dead/Total**	**Mortality (%)**	**Average mortality (%)**
Unvaccinated control	1	248/1000	24.8	23.55 ± 1.77^{a}
	2	223/1000	22.3	
Single booster (SB)	3	186/1000	18.6	14.45 ± 5.87^{ab}
	4	103/1000	10.3	
Double booster (DB)	5	61/1000	6.1	6.40 ± 0.42^{b}
	6	67/1000	6.7	

5.4 DISCUSSION

Streptococcosis is documented to recur globally, especially in major tilapia-producing countries, and causes significant economic losses to tilapia aquaculture. As such, in 2011, China, the world's leading producer of cultured tilapia, suffered an estimated $40 million loss due to the disease, causing approximately 80% livestock mortality during outbreaks (Zhang, 2021). Besides, a previous report by Jantrakajorn et al. (2014)

revealed that *S. agalactiae* emerged as predominant disease-causing species in tilapia culture areas across Thailand. In southern Taiwan, a series of streptococcosis outbreaks were observed in tilapia farming during the period of 2016 to 2018, and further phenotypic characterization revealed the presence of β-hemolytic and γ-hemolytic strains of *S. agalactiae* (Sudpraseart et al., 2021). Similarly, Suhermanto et al. (2019) formerly reported that both β-hemolytic and non-hemolytic were discovered in tilapia culture and distributed from various regions in Indonesia, including Papua, Jambi, South Borneo, Java, and Gorontalo. In January 2020, a recent case of streptococcosis was documented in Malaysia at a tilapia farm in Selangor. This incident resulted in high mortality rate of 70% among adult red hybrid tilapia, and further investigation revealed co-infection of *S. agalactiae*, *A. hydrophila*, and Tilapia Lake Virus (TiLV) (Basri et al., 2020). Thus, developing and applying effective vaccines as preventive and control measures in the tilapia farming industry is rather urgent.

The oral vaccination of fish has attracted attention due to its lack of dependence on additional manpower or specialized facilities. In Malaysia, oral vaccines are favored because 80% of local tilapia farmers are small-scale producers (Ridzuan et al., 2022). In addition, it is simple to administer (mimics natural feeding behavior), permits minimal physical contact between fish and operator, and allows flexibility in vaccine regimen formulation depending on the culture system, species, and size of fish in captivity. Despite these enticing benefits, oral immunization typically results in a shorter duration of protection than other routes and can cause inconsistent responses due to the hostile gastrointestinal environment of fish. Conjugation with an adjuvant and application of repetitive doses as boosters are essential to ensure sustained antigen release. Previously, we reported the promising results and potential use of palm oil as a fish vaccine adjuvant to replace commercially available Freund's adjuvant (Aminudin et al., 2018). Following an experimental challenge, it was determined that 10% palm oil adjuvant effectively stimulated systemic immune response and resulted in the highest survival rate of red hybrid tilapia. Palm oil derived from the fruit of the oil palm tree (*Elaeis guineensis*), has gained widespread use in various industries due to its versatile properties. It is an abundant and economically viable natural resource, making it an

attractive candidate for numerous applications (Pande et al., 2012). Palm oil contains bioactive compounds, such as tocopherols, carotenoids, and phenolics, which possess immunostimulatory properties. These compounds can modulate the immune system, enhancing the response to vaccine antigens (Gonzalez-Diaz & García-Núñez, 2021; Maheshwari et al., 2022). Therefore, the present study investigated the efficacy of palm oil adjuvanted feed-based streptococcosis vaccine under field conditions. In an actual production setting, the dynamic interaction between the host, pathogen, and environment can result in variations in immune response and vaccine efficacy.

The results indicated that fish vaccinated with palm oil adjuvanted feed-based streptococcal vaccine produced higher and sustained antibody levels in blood serum, particularly in the DB group, compared to those unvaccinated control. Interestingly, the elevation pattern of IgM level induced by the vaccinated fish is comparable to a prior vaccination trial conducted in 2017, where Freund's incomplete adjuvant (FIA) was utilized (M. S. Ismail et al., 2017). Promising effect of palm oil as a candidate for fish vaccine adjuvant was supported by other studies (Amir-Danial et al., 2023; Aslah et al., 2021; Mohd Ali et al., 2023; Monir et al., 2021, 2022), suggesting its ability to stimulate gradual antigen release and extend immune response. The comparison of IgM levels between the SB and DB groups demonstrated that repeated immunization booster doses are required to provide protection for a minimum of 16 weeks. A high degree of antibody fluctuation following the initial booster in the SB group indicated a briefer duration of protection provided by the vaccine, necessitating another immunization (second booster), as observed in the DB group. This is in accordance with previous findings that recommended booster immunization doses under field conditions (Abou-Okada et al., 2021; Chettri et al., 2015; Mondal & Thomas, 2022). In contrast, an abrupt increase in IgM level in the unvaccinated control group, observed between week 6 and week 10, correlates with the high infection rate of *S. agalactiae*, which began between week 4 to week 10. In response, the fish's natural immune system is stimulated, producing antibodies that could be detected in the analysis.

Lysozyme and complement-3 have been used in the present study as an indicator for non-specific immune responses induced following field vaccination trials. Both of these specialized proteins are essential defense molecules of the innate immune system and are routinely employed to indicate fish duress or disease (Abdollahi et al., 2016; Biller et al., 2021). During an invasion, the pathogen is initially recognized by a pattern recognition receptor (PRR) that initiates an innate immune response and subsequent host immunity via multiple signaling pathways, which increase lysozyme production and complement system activation, thereby contributing to the pathogen's eradication (Firdaus-Nawi & Zamri-Saad, 2016). Lysozyme disrupts the peptidoglycan layer of the bacterial cell wall, whereas the complement system engages in multiple defense modes, such as the formation of membrane attack complex, opsonization, and antibody production (Abdollahi et al., 2016). Each vaccine administration from this study significantly increased lysozyme and complement-3 activity in the vaccinated groups. The findings were in conformity with previous research by Raju et al. (2023) and Dong et al. (2022) that demonstrated orally vaccinated fish exhibit elevated levels of lysozyme and complement-3 activity in gut mucus and serum samples. Moreover, a sudden rise in lysozyme activity in the unvaccinated control during week 6 and complement-3 level during week 8 is anticipated, as this corresponds to a period in which the fish were exposed to a high infection of *S. agalactiae*.

S. agalactiae was successfully isolated and identified from fish organ samples as early as week 2 and persisted until the study's conclusion at week 16, confirming that the selected farm had an endemic streptococcal problem. Positive detection of *S. agalactiae* was observed across a broader range of red hybrid tilapia weight, with the highest frequency between 51 – 107 g. Previous research has demonstrated that streptococcosis can be a problem in tilapia culture once the fish reach 20 g and persists until they reach a harvestable size of more than 500 g (Amal et al., 2015). On the basis of these findings, we proposed a vaccination regime to be implemented before the fish attained 50 g in weight to boost the fish's natural immune system before the onset of the disease. Besides, the overall isolation rate of *S. agalactiae* in the vaccinated group of DB showed a significant reduction than the unvaccinated control group throughout

the 16-week trial period. This was consistent with prior studies, which found a lower incidence of pathogen isolation in vaccinated fish compared to those unvaccinated (Amir-Danial et al., 2023; M. S. Ismail et al., 2017). The streptococcosis-infected red hybrid tilapia manifested typical clinical signs and symptoms as reported elsewhere (Md. S. Ismail et al., 2016; Mishra, Gyu-Hwi, et al., 2018; Zamri-Saad et al., 2010). Most notably, exophthalmia, soft and watery brain, enlarged spleen, lethargy, loss of appetite, isolated from schooling, and displayed erratic swimming behavior.

5.5 CONCLUSION

Oral vaccination by double booster regimens with palm oil adjuvanted streptococcosis induced a protective immune response in both specific and non-specific immunes system translating into a lower incidence of streptococcosis and a higher survival rate of red hybrid tilapia.

CHAPTER SIX

GENERAL DISCUSSION

The streptococcal disease has posed a concern for the global tilapia aquaculture industry. Its severity is worsened by intensified cage-culture systems, especially in large waterbodies such as lakes or rivers. This disease corresponds with increasing water temperature that induced stress factors in fish under captivity. Past research has shown that an increase in temperature induces pathogenicity of *S. agalactiae* due to overexpression of β-hemolysin, pore-forming toxin (CAMP factor), and Pilus 2b backbone protein (PI-2B) (Kayansamruaj et al., 2014). The transcriptome and proteome analyses revealed the differential upregulation of genes and proteins implicated in cellular metabolic pathways, virulence factors, and bacterial adaptation (Tavares et al., 2018). Once infected, fish exhibited a significant upregulation of inflammatory-related genes, including cyclooxygenase-2, interleukin-1 beta (IL-1β), and tumor necrosis factor-alpha (TNF-α), which may result in acute mortality (Kayansamruaj et al., 2014). Due to the year-round moderate climate in Malaysia, periodic outbreaks of streptococcosis with abrupt high mortality of cultured tilapia were associated with the onset of the dry and hot season from March until June (Amal et al., 2015; Siti-Zahrah et al., 2008). Consequently, these four critical months are crucial for disease intervention, especially thru vaccination.

Vaccination is one of the most significant science advancements in history. It has saved countless lives and eradicated or controlled several deadly infectious diseases in the world. Among the different types of vaccines, the whole-cell killed bacterin vaccine holds a prominent position with a long-standing history of successful disease prevention (Amanna & Slifka, 2020). One of the key advantages of whole-cell killed bacterin vaccines is that they can elicit a broad immune response. Unlike subunit vaccines that contain only specific components of the pathogen, whole-cell killed bacterin vaccines present a diverse range of bacterial antigens to the immune system.

This stimulates the production of a wider array of antibodies and T-cells, contributing to a more robust and comprehensive defense against the targeted pathogen (Pollard & Bijker, 2021). Several similar types of fish vaccines against streptococcosis are available abroad, including Aquavac® Strep Sa, Aquavac® Strep Sa1, Aquavac® Strep Si (Intervet, MSD Animal Health), and Alpha Ject® micro 1 Tila (Pharmaq). All of these vaccines require an injection route for delivery, and currently, only Aquavac® Strep Si (intended against *S. iniae* infection) is approved for use in Malaysia. Unfortunately, most local farmers prefer not to use injection vaccine for aquaculture. This predicament might be because 80% of local tilapia farmers are small-scale operators and do not have the financial means to invest in extra labor, specialized equipment, and facilities necessary to apply injectable or immersion vaccines (Ridzuan et al., 2022). In contrast, oral fish vaccination allows for mass vaccination on the farm without unnecessary stress associated with fish handling, which can occasionally lead to mortality (Mutoloki et al., 2015; Stevens et al., 2017). In addition, oral immunization offers ease of operation and permits flexibility in formulating a vaccination regimen. Therefore, considerable effort was devoted to developing an oral vaccine.

Typically, whole-cell bacterin vaccine production entails a series of fundamental procedures culminating in the end product. The first step involves cultivating bacteria in an optimal culture medium and condition to maximize antigen yield while preserving the cell's integrity (Gomez & Robinson, 2018). Bioreactors are commonly employed for this purpose. Huser et al. (1983) previously recommended fermenter cultivation of *S. agalactiae* in Todd-Hewitt broth containing 2% (w/v) glucose, pH 6.2, with a constant flow of CO_2 for mass production. In contrast, the BHI medium was chosen in this study due to its superior sensitivity, specificity, and accuracy while reducing material and supply costs (Huu et al., 2019). The subsequent step is bacterial harvesting, which can be easily achieved by centrifugation to release the antigen from the growth substrate, followed by the inactivation procedure. Inactivating the whole-cell bacterial vaccine is crucial to destroy disease-producing capacity and is typically accomplished through exposure to heat, radiation, or chemically induced. The latter is the most widely used in human and animal vaccines, such as formaldehyde and

β-propiolactone, due to its high inactivation potency without alteration of antigenic properties (Habib et al., 2006; Nurulaini et al., 2020). The toxicity mechanisms of formaldehyde and β-propiolactone toxicity have been described in detail by Delrue et al. (2012). These chemicals are alkylating agents that act through the alkylation of carboxyl and hydroxyl group of DNAs, resulting in monohydroxymethylation of amino acids and other carboxyethyl derivatives, which blocks genome reading. In addition, formaldehyde and β-propiolactone also induce crosslinking of proteins, preventing normal transcriptional processes as a consensus mechanism and causing cell death.

In an attempt to produce a vaccine for *S. agalactiae* seed in bulk quantities, the bacterial growth curve was established following the predetermined culture conditions. After 12 hours of incubation in a nutrient-rich medium of BHI broth, the bacterial growth reached the apex of the exponential phase. At this stage, the cells are the healthiest and metabolically active and are preferred for seed vaccine preparation. In addition, a recent study disclosed higher expression levels of *S. agalactiae* virulence genes, including major nuclease, genetic pilus island (PI-2a), other virulence-associated genes involved in translation, ribosome structure and biogenesis occurred during the exponential growth phase (Silvestre et al., 2022). Elevated virulence expression is advantageous for seed vaccine candidates as it may induce a more robust immune response from the host (Arya, 2021). Therefore, incubation periods exceeding 12 hours must be avoided.

In the current investigation, the fish were not fed the day before each administration and the vaccine were evenly distributed to ensure maximum vaccine uptake by all fish. However, it is essential to note that it is impossible to determine the exact dose of antigen each fish consumes, potentially leading to inconsistent responses. In the present study, the oral vaccine was prepared by incorporating the seed vaccine in the feed formulation before being subjected to a pressed pelleting machine. The nutritional compositions of the prepared feed-based vaccine were determined in proximate analysis, and it was revealed that the moisture, ash, crude fat, crude fiber,

and crude protein of the vaccinated pellet were within the recommended nutritional value for the red hybrid tilapia diet. These dietary components in a fish's diet are essential in influencing growth, feed conversion, physiological effects, disease susceptibility, and overall health (Blazer, 1992). Besides, adding palm oil as an adjuvant did not significantly alter the vaccine's proximate composition, which is consistent with prior research by Aslah et al. (2021). The key advantages of using palm oil as a fish vaccine adjuvant is its biocompatibility and safety. Being a naturally derived product, palm oil is well-tolerated by fish and does not induce adverse reactions. It is non-toxic and does not accumulate in fish tissues, ensuring the safety of both the vaccinated fish and consumers (Ayisi et al., 2019; Ng et al., 2007). This aspect is especially important as the aquaculture industry aims to prioritize sustainability and eco-friendly practices. Other than that, palm oil is abundantly produced in Malaysia, making it readily available and cost-effective as a fish vaccine adjuvant. Compared to some synthetic adjuvants, palm oil offers a more affordable option for aquaculture producers and insignificantly increasing production costs. This affordability also makes it more accessible to small scale farmers, which can benefit from improved disease prevention measures.

Following the vaccination trial in an endemic farm, the specific antibody (IgM), non-specific immune response activity (lysozyme and complement-3), bacterial isolation, and mortality record were thoroughly monitored within four months of the culture period. The serum IgM antibody revealed a significant difference between the vaccinated and unvaccinated groups. It exhibited an ascending pattern after the initial administration and first booster, reaching a peak at week 4. The IgM level of fish that received a second booster dose displayed further elevation above the cut-off value throughout the trial period. The findings suggest that oral immunization of fish can induce a systemic response, as previously demonstrated by Firdaus-Nawi et al. (2013) and Md. S. Ismail et al. (2016). Furthermore, using a subsequent dose of vaccine as a booster to the prime dose is critical for increasing the amplitude of the fish's immune response and providing extended protection (Abou-Okada et al., 2021).

Both lysozyme and complement-3 were evaluated in the present study as indicators of the innate immune component. Following vaccine administration, the innate humoral system serves as the initial responder and significant concentrations of these compounds are typically generated in response to antigen entry (Magnadóttir, 2006). The serum lysozyme and complement-3 levels revealed a significant difference between the vaccinated and unvaccinated groups following prime and booster doses. The sudden increase in lysozyme and complement-3 in unvaccinated fish during week-6 and week-8 coincides with a high infection rate of *S. agalactiae*, reflecting the host's ongoing response against invading pathogens. An earlier study by Desvignes et al. (2002) demonstrated that an experimental infection of Atlantic salmon with salmon pancreas disease virus (SPDV) provoked increased lysozyme and complement excretion at 9- and 16-days post-infection. More recently, Charlie-Silva et al. (2019) reported that tilapia inoculated with *Aeromonas hydrophila* showed a significant increase in complement-3 levels 6 and 24 hours after inoculation compared to control animals.

Throughout the field trial, *S. agalactiae* was consistently recovered from various organs of the sampled red hybrid tilapia. Most notably, the presence of *S. agalactiae* was detected as early as week-2, with the highest isolation rate recorded at week-10, reaching a prevalence of 30%. A significant reduction in *S. agalactiae* isolation rate was observed between the vaccinated and unvaccinated groups suggesting that the application of vaccine in the fish farm can lessen the incidence rate of the pathogen, as shown in the study done by M. S. Ismail et al. (2017) and Raju et al. (2023). At the end of the trial, vaccinated groups of single and double boosters recorded the highest survival rate with 85.5 and 93.6%, respectively, compared to the unvaccinated group of 76.4%. Although the study's incidence rate of *S. agalactiae* is considerably low, cumulative mortality caused a significant profit reduction and jeopardized sustainable aquaculture practices. Therefore, the application of aquaculture vaccines plays a vital role as a preventive measure and should be included as part of fish health management on fish farms.

CHAPTER SEVEN

CONCLUSION

The persistent recurrence of streptococcosis outbreaks negatively impacts tilapia aquaculture, and this situation has been further exacerbated by the rapid expansion of tilapia farming around the world. Under these circumstances, the aquaculture vaccine is viewed as a way out.

This study shows that the administration of palm oil adjuvanted feed-based streptococcal vaccine as a tool to control and prevent streptococcosis in tilapia farms is a viable option as it can effectively induce both specific and non-specific immune responses against *S. agalactiae*. Numerous advantages of palm oil as a fish vaccine adjuvant presents a promising solution for enhancing disease prevention in aquaculture. From its ability to enhance immunogenicity and provide long-lasting immunity to its cost-effectiveness and biocompatibility, the use of palm oil can significantly benefit the aquaculture industry. A double booster regimen of oral immunization is recommended since it produced significantly high lysozyme and complement-3 levels and resulted in a sustained IgM level for at least 16 weeks. The field efficacy of the vaccine reduces the incidence of streptococcosis to 11.4% and improves the survival rate to 93.6%.

Developing efficacious oral vaccines against other economically important fish diseases, most notably vibriosis, aeromoniasis, and edwardsiellosis, is proposed. On the other hand, a thorough analysis of the prospective market and sales of local fish vaccines is highly recommended and should be pursued. Then, with a better grasp of the industry's needs, competent authorities and local experts can continue their R&D of fish vaccines.

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APPENDIX A

Appendix A1: Blood Agar (BD Difco, Cat. no.: 211037)

1. Suspend 40 g of Blood Agar Base in 1 liter of distilled water.
2. Heat with frequent agitation and boil to dissolve homogenously.
3. Autoclave at 121°C for 15 minutes.
4. Cool the base agar media to 45 - 50°C, before aseptically add 5% defibrinated blood.
5. Mix gently (avoid bubble formation) and pour into clean sterile petri dish.
6. Store the solidified agar at 4°C.

Appendix A2: Brain Heart Infusion Broth (Merck, Cat. no.: 110493)

1. Suspend 52 g of the powder in 1 liter of distilled water.
2. Heat with frequent agitation and boil to dissolve homogenously.
3. Sterile by autoclaving at 121°C for 15 minutes.
4. Pour into clean sterile petri dish.
5. Store the solidified agar at 4°C.

Appendix A3: Phosphate Buffered Saline (Calbiochem, Cat. no.: 524650-1EA)

1. Reconstitute 1 tablet into 1 liter of distilled water.
2. Gently agitate to ensure complete dissolve.
3. Sterile by autoclaving at 121°C for 15 minutes.
4. Store at 4°C.

Appendix A4: Normal Saline Solution, 0.85%

1. Dissolve 8.5 g NaCI (Merck, Cat. no.: 106404) in 1 liter of distilled water.
2. Gently agitate to ensure complete dissolve.
3. Sterile by autoclaving at 121°C for 15 minutes.
4. Allow to cool at room temperature.

APPENDIX B

Appendix B1: 0.5% (v/v) Formalin Solution

1. Pipette 5 ml of Formaldehyde Solution 37% (QReC, Cat. no.: F5023-1-250) into 995 ml of distilled water.
2. Label the bottle properly and store at room temperature.

APPENDIX C

Appendix C1: 1.25% (m/v) Sulphuric Acid

1. Carefully pipette 13 ml of Sulphuric Acid 96% (Fisher scientific, Cat. no.: AC133610025) into 987 ml of distilled water.
2. Label the bottle properly and store at room temperature.

Appendix C2: 1.25% (m/v) Sodium Hydroxide

1. Weight 12.5 g of Sodium Hydroxide pellet (Merck, Cat. no.: 106462) and pour into volumetric flask containing 1 L of distilled water.
2. Shake well to ensure complete dissolved.
3. Label the bottle properly and store at room temperature.

APPENDIX D

Appendix D1: Measurement of Nitrogen Ammonia (NH_3-N) using Salicylate Method (Hach, Method 8155)

1. Prepare the blank by filing a sample cell with 10 ml of deionized water.
2. Prepare the sample of the same volume in another sample cell.
3. Add the content of one Ammonia Salicylate powder pillow to each sample cell.
4. Shake to dissolve the reagent and start a 3-minute reaction time.
5. After time expires, add the content of one Ammonia Cyanurate powder pillow to each sample cell.
6. Shake to dissolve the reagent and start a 15-minute reaction time.
7. Zero the spectrophotometer using blank sample.
8. Read the prepared sample at 655 nm

Appendix D2: Measurement of Sulfide (S^{2-}) using USEPA Methylene Blue Method (Hach, Method 8131)

1. Prepare the blank by filing a sample cell with 10 ml of deionized water.
2. Prepare the sample of the same volume in another sample cell.
3. Add 0.5 ml of Sulfide 1 Reagent and swirl to mix.
4. Add 0.5 ml of Sulfide 2 Reagent and swirl to mix.
5. Start a 5-minute reaction time.
6. After time expires, zero the spectrophotometer using blank sample.
7. Read the prepared sample at 690 nm.

Appendix D3: Measurement of Nitrate (NO_3-N) using Cadmium Reduction Method (Hach, Method 8039)

1. Fill a sample cell with 10 ml of sample.
2. Add the content of one NitraVer 5 Nitrate Reagent powder pillow.
3. Start 1-minute reaction time. Simultaneously, put a stopper on the sample cell and shake vigorously until the timer expires.
4. Start a 5-minute reaction time.
5. After time expires, prepare the blank by filling a second sample cell with 10 ml of sample.
6. Zero the spectrophotometer using blank sample.
7. Read the prepared sample at 500 nm.

Appendix D4: Measurement of Nitrite (NO_2^--N) using USEPA Diazotization Method (Hach, Method 8507)

1. Fill a sample cell with 10 ml of sample.
2. Add the content of one NitriVer 3 Reagent powder pillow and swirl to mix.
3. Start a 20-minute reaction time.
4. When the timer expires, fill a second sample cell with 10 ml of sample as blank.
5. Zero the spectrophotometer using blank sample.
6. Read the prepared sample at 507 nm.

Appendix D5: Measurement of Total Iron (Fe) using USEPA FerroVer Method (Hach, Method 8008)

1. Fill a sample cell with 10 ml of sample.
2. Add the content of one FerroVer Iron Reagent powder pillow and swirl to mix.
3. Start a 3-minute reaction time.
4. When the timer expires, fill a second sample cell with 10 ml of sample as blank.
5. Zero the spectrophotometer using blank sample.
6. Read the prepared sample at 510 nm.

Appendix D6: Measurement of Reactive Phosphorus (Orthophosphate) (PO_4^{3-}) using USEPA PhosVer 3 (Ascorbic Acid) Method (Hach, Method 8048)

1. Fill a sample cell with 10 ml of sample.
2. Add the content of one PhosVer 3 Phosphate Reagent powder pillow and immediately close the sample cell and shake vigorously for 30 seconds.
3. Start a 2-minute reaction time.
4. When the timer expires, fill a second sample cell with 10 ml of sample as blank.
5. Zero the spectrophotometer using blank sample.
6. Read the prepared sample at 880 nm.

APPENDIX E

Appendix E1: Preparation of 0.05 M Carbonate Bicarbonate Coating Buffer (Sigma, Cat. no.: C3041-100CAP)

1. Reconstitute one capsule into 100 ml of sterile distilled water.
2. Shake well to ensure complete dissolve.
3. Store at 4°C.

Appendix E2: Preparation of Phosphate-Buffered Saline with 0.05% Tween-20 Detergent (PBST)

1. Add 0.5 ml of Tween-20 (Promega, Cat. no.: H5151) into 1 liter of sterile PBS.
2. Gently mix and store at room temperature.

Appendix E3: Preparation of Blocking Solution (2% Bovine Serum Albumin)

1. Weight 0.8 g of lyophilized Bovine Serum Albumin (Calbiochem, Cat. no.: 126593-100GM) and transfer into a conical centrifuge tube containing 40 ml of sterile PBS.
2. Agitate using vortex to ensure complete dissolve and use immediately.

Appendix E4: Preparation of Stop Solution (0.2 M Sulphuric Acid)

1. Carefully transfer 111 ml of Sulphuric Acid 96.3% (Sigma, Cat. no.: 258105) into clean glass bottle containing 889 ml of distilled water.
2. Label the bottle properly and store at room temperature.