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THE EFFECT OF FEED-BASED VACCINATION AGAINST VIBRIOSIS ON STIMULATION OF THE GUT-ASSOCIATED LYMPHOID TISSUE (GALT) IN ASIAN SEABASS (*LATES CALCARIFER*)

DWAYNE NORMAN ROMANUS

THE EFFECT OF FEED-BASED VACCINATION AGAINST VIBRIOSIS ON STIMULATION OF THE GUT-ASSOCIATED LYMPHOID TISSUE (GALT) IN ASIAN SEABASS (*LATES CALCARIFER*)

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CERTIFICATIONS

I hereby certify that I have read this project paper entitled "The Effect of Feed-Based Vaccination against Vibriosis on Stimulation of the Gut-Associated Lymphoid Tissue (GALT) in Asian Seabass (*Lates Calcarifer*)" by Dwayne Norman Romanus and in my opinion, it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirements for the course VPD 4999 – Project.

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CONTENTS

LIST OF ABBREVIATIONS

AHPND	Acute Hepatopancreatic Necrosis Disease
FAO	Food and Agriculture Organization
GALT	Gut-Associated Lymphoid Tissue
GIALT	Gill-Associated Lymphoid Tissue
H&E	Hematoxylin and Eosin
Ig	Immunoglobulin
MALT	Mucosa-Associated Lymphoid Tissue
SALT	Skin-Associated Lymphoid Tissue
SEA	Southeast Asia
WHO	World Health Organization

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ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 – Projek.

KESAN VAKSIN BERASASKAN MAKANAN KE ATAS JANGKITAN VIBRIOSIS TERHADAP PEMBENTUKAN TISU LIMFOID BERKAIT USUS (GALT) PADA IKAN SIAKAP PUTIH (*LATES CALCARIFER*)

Oleh

Dwayne Norman Romanus

2022

Penyelia: Professor Madya Dr. Annas Salleh

Vibriosis merupakan salah satu jangkitan penting di dalam industri penternakan ikan sangkar di seluruh dunia, termasuklah Malaysia. Pada masa kini, vaksinasi merupakan pilihan utama dalam usaha untuk mengawal jangkitan ini. Disebabkan pemberian vaksin secara suntikan memerlukan tenaga buruh yang tinggi, satu vaksin berasaskan makanan daripada *formalin-killed Vibrio harveyi* telah direka untuk meringankan beban kerja. Kajian ini dijalankan untuk mengenal pasti kesan pemberian vaksin berasaskan makanan terhadap tisu limfoid berkait usus (GALT) pada ikan siakap putih. Kajian ini menggunakan arkib blok sampel usus yang disimpan dalam minyak parafin daripada kajian lepas yang dilakukan di Pulau Ketam, Selangor. Sampel tersebut terdiri daripada dua kumpulan; ikan yang divaksin dan juga yang tidak divaksin, di mana setiap sampel diambil pada minggu 0, 2, 4, 6, 8 dan 10. Ikan yang divaksin diberikan vaksin pada minggu 0, 2 dan 4. Tiga blok sampel setiap minggu daripada setiap satu kumpulan telah dipotong, diwarnakan dengan pewarna Hematoksilin dan Eosin (H&E), dan dilihat di bawah mikroskop cahaya untuk mengukur luas, jumlah limfosit, dan ketumpatan GALT dalam setiap satu kumpulan. Secara umumnya, kedua-dua kumpulan menunjukkan kehadiran GALT bermula seawal minggu 0 sehingga minggu 10, di mana nombor GALT adalah lebih tinggi dan signifikan ($p < 0.05$) di dalam kumpulan ikan yang divaksin berbanding kumpulan kawalan sepanjang tempoh kajian. Kedua-dua kumpulan menunjukkan tiada perbezaan yang signifikan ($p > 0.05$) di dalam luas GALT daripada minggu 0 hingga minggu 10. Walaubagaimanapun, jumlah limfosit adalah signifikan ($p < 0.05$) dan lebih tinggi di dalam kumpulan ikan yang divaksinasi berbanding kumpulan kawalan antara minggu 0 dan minggu 10. Walaupun kedua-dua kumpulan menunjukkan peningkatan yang konsisten dalam jumlah limfosit, kumpulan ikan yang divaksinasi menunjukkan ketumpatan GALT yang lebih signifikan ($p < 0.05$) dan tinggi berbanding kumpulan kawalan sepanjang tempoh kajian. Oleh itu, ikan yang divaksin dengan vaksin *V. harveyi* berasaskan makanan mampu untuk merangsang pembangunan GALT yang lebih baik berbanding kumpulan ikan yang tidak diberi vaksin langsung dalam jangka masa 10 minggu, menghasilkan kadar perlindungan yang lebih baik terhadap vibriosis.

Kata kunci: vibriosis; *Vibrio haveyi*; vaksin oral; siakap putih

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfillment of the course VPD 4999 – Project.

THE EFFECT OF FEED-BASED VACCINATION AGAINST VIBRIOSIS ON STIMULATION OF THE GUT-ASSOCIATED LYMPHOID TISSUE (GALT) IN ASIAN SEABASS (*LATES CALCARIFER)*

By

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2022

Supervisor: Associate Professor Dr. Annas Salleh

Vibriosis is an important disease in cage-cultured fish worldwide, including Malaysia. Currently, vaccination is regarded as the way forward in controlling the disease. Vaccine administration via injection is laborious, thus we designed a feed-based vaccine from formalin-killed *Vibrio harveyi*. This study seeks to determine the effect of administration of this vaccine on gut-associated lymphoid tissue (GALT) in Asian Seabass in field setting. Archived paraffin blocks of gut samples from a previous study conducted in Pulau Ketam, Selangor, were used. The samples consisted of two groups; vaccinated, and non-vaccinated groups, which were sampled on week 0, 2, 4, 6, 8, and 10. Vaccination was administered to the vaccinated group on week 0, 2, and 4. Blocks from three fish per week for each group were sectioned, stained with haematoxylin and eosin and viewed under a light microscope to measure the area, lymphocyte number, and cell density of GALT in these groups. In general, both the control and vaccinated groups showed the presence of GALT starting from week 0 until week 10, where the GALT number was significantly ($p < 0.05$) higher in the vaccinated group than the control group throughout the course of study. Both groups showed no significant ($p > 0.05$) difference for the GALT area from week 0 until week 10. However, the lymphocyte numbers were significantly ($p < 0.05$) higher in the vaccinated group than control group between week 0 and week 10. Although both groups showed consistent increase in lymphocyte count throughout the study, the vaccinated group showed significantly ($p < 0.05$) higher GALT density than the control group throughout the study. Therefore, the feed-based *V. harveyi* vaccine elicited better GALT development in the vaccinated fish compared to non-vaccinated fish within the span of 10 weeks, thus resulting in a better protection rate against vibriosis.

Keywords*:* vibriosis; *Vibrio harveyi*; oral vaccine; Asian seabass

1.0 INTRODUCTION

1.1 Background of study

The development of aquaculture in Malaysia and throughout the world is skyrocketing due to its heavy demand to produce almost half of the world's food fish. According to the Food and Agriculture Organization of the United Nations (FAO), 214 million tonnes of fisheries and aquaculture production were recorded in 2020, in which 178 million tonnes of it consists of aquatic animals, contributed mainly from the flourishing aquaculture practices throughout the world, especially in Asia. Ever since 1961, the global demand for fisheries products has increased at an average of 3.0 percent, which surpasses the global population growth rate of 1.6 percent (FAO, 2022). The human consumption of aquaculture products has doubled from an average of 9.9 kg per capita in 1960s to 20.2 kg per capita in 2020 (FAO, 2022). A 15 percent increase in consumption of aquatic foods is expected in the year 2030 as higher purchasing power, urban sprawl, as well as dietary changes are bound to happen in the future (FAO, 2022).

Vibriosis is an infection by the bacterium from the genus Vibrio. In fish, it is most commonly caused by *Vibrio harveyi, V. parahaemolyticus, V. alginolyticus,* and *V. anguillarum*. The disease is one of the most economically important diseases in the world (Mohamad et. al, 2022), and more prevalent in marine fish as compared to freshwater fish, especially the Asian seabass (*Lates calcarifer*) population in many marine aquaculture farms in Malaysia (Ina-Salwany et al., 2019). Mortality rate due to vibriosis could reach up to 80% and morbidity rate of up to 100% in susceptible fishes.

Due to the antibiotic restriction use in aquaculture and the concern of antimicrobial resistance, vaccination is regarded as the main method of prevention of the disease (Ina-Salwany et al., 2019). Recently, an oral feed-based vaccine has been developed which allows for an easier administration route, cheaper, less stressful method, and allows a no direct contact setting between the worker and fish compared to the common vaccination delivery by injection. This vaccine was proven to provide protection against vibriosis in the laboratory and field situations (Mohamad et. al, 2021). Researchers have recently proven that parts of the mechanism of feed-based vaccines involve serum antibody, skin lysozyme, and improved efficacy of peritoneal macrophages (unpublished data). Since the vaccine requires oral intake and digestion, it is likely that it stimulates the gut-associated lymphoid tissue (GALT), which is known to be an important component of mucosal immunity.

1.2 Problems statement

The effect of oral feed-based vaccination against *Vibrio* spp. infection for the development of GALTs is still poorly understood. To comprehend this matter, antigenic stimulation from a vaccine is required to drive the humoral response and development of GALT within the dome epithelium. However, GALT development following an oral vaccination raises questions. How effective is vaccine given orally in stimulating the immune response? The observable physical characteristics changes of GALT remains a matter of speculation in Asian seabass against vibriosis. Also, in order to successfully develop an oral vaccine, one must fully understand the mechanism of the said vaccine for it to work and be proven effective against the disease.

1.3 Justification of the study

Although it has been proven that the vaccine could provide excellent protection, as well as improved growth rate of Asian seabass in the field, but, the exact mechanisms of protection has not been well-explored. The outcome of this research will provide a better understanding on the mechanism of protection of oral feed-based vaccines against vibriosis.

1.4 Objectives of the study

This research aims to evaluate the changes in the GALT morphometrics of Asian seabass fed with the oral feed-based vaccine in order to better understand the mechanism of protection elicited by the vaccine.

1.5 Hypothesis

i. **Null:** The oral feed-based field vaccination is able to incur more changes on the GALT morphometrics of Asian seabass.

ii. **Alternate:** The oral feed-based field vaccination is not able to incur more changes on the GALT morphometrics of Asian seabass.

1.6 Significance of the study

The findings of this study will explain the effects of the oral feed-based vaccination against vibriosis to the development of GALT in Asian seabass (*Lates calcarifer*). Therefore, it provides an additional basis for testing the most effective strategy for prevention and adoption of better measures in the control of Vibriosis in aquaculture in the future.

2.0 LITERATURE REVIEW

2.1 Asian Seabass (*Lates calcarifer*)

Locally known as *siakap putih* in Malaysia, one of the most important cultured marine fish species in Southeast Asia (SEA) countries is the Asian Seabass (*Lates calcarifer*) (Ayson et al., 2014). It is a member of the Latidae family. Asian seabass can be found in the waters of the tropical Indo-West Pacific region, which includes the whole SEA countries, Taiwan and stretches to Papua New Guinea and Northern Australia (Vij et al., 2014). Asian seabass is a catadromous fish, meaning that they spend most of their lifetime in freshwater and migrate to the marine water only to breed. This migration is possible as Asian seabass is known to have an incredible tolerance to a wide range of salinities (Moore and Reynold, 1982).

Figure 1: Asian Seabass (Lates calcarifer) (image source: FAO, 2022).

The fish can grow up to 200 cm, with the common size ranging from 25-100 cm. Asian seabass can reach a body weight of up to 3.0 kg in a year; and in an optimum environment can reach a maximum weight of 60 kg (Rao et al., 2013; Hassan et al., 2020b). It is favorable to fish farmers due to its hardiness and tolerance to a wide range of environment, fecund female, fine flavored meat and fast growth rate (Hassan et al., 2021).

2.2 *Vibrio* spp.

Vibriosis is an infection by the bacterium from the genus *Vibrio*, a motile Gram-negative, straight rod bacteria with polar flagella. It can be found as saprophytes in freshwater, seawater, and soil, in which they thrive at a temperature of 37°C, pH range of 7.0-7.5 and a high salinity of 20-40 ppt (Supardi & Sukamto, 1999). Although quite a number of *Vibrio* species are halophilic in nature where salt is essential for its development, it has also been reported that it can still thrive in freshwater environments due to its high adaptability (Sudheesh et al., 2012).

Although *Vibrio* infection was known to exist as early as 1718 (Colwell & Grimes, 1984), it was not identified as *Vibrio* spp. until much later. The organism *Bacillus anguillarum* was reported to cause an epizootic among migrating European eels in 1817, in which Canestrini (1893) later reported it as the first verified *Vibrio* infection in fish. Almost two decades later, a bacteria known as *V. anguillarum* was discovered and given the blame for disease epidemics in European eels along the Swedish coast (Bergman, 1909). Red disease outbreaks caused high mortality rates among European eels in Danish waters as demonstrated in a study by Bruun and Heiberg (1932). The disease was later thought to be caused by *V. anguillarum* because of the pathophysiology of the disease and the pathogen's traits being similar.

There are two main syndromes caused by *Vibrio* spp; (1) the classical vibriosis and (2) gastroenteritis. Classocial vibriosis often shows signs such as restlessness in infected fishes, presence of ulcerations throughout the skin surface, fin rot and pigmentation of the body. Apart from these, the liver, kidney and spleen will usually be enlarged, hemorrhagic and also congested (Zhang et al., 2014). As for the gastrointestinal vibriosis, its signs were noticed post-infections from *V. harveyi* and *V. alginolyticus*. Ascites is the main gross lesion as stated by Liu et al. (2004), and alterations in the eyes, with initial signs of eye opacity and exophthalmia are occasionally noticed as well (Saad & Atallah, 2014).

2.2.1 Epidemiology

Vibrio are highly motile Gram-negative, rod-shaped bacteria with presence of polar flagella at one end. It can be found worldwide and is ubiquitous in freshwater,estuarine and marine environments (Baker-Austin et al., 2017). Most *Vibrio* spp are facultative pathogens, saprophytic and halophilic in nature, and can withstand a wide range of environments. Its high tolerance to salinity has made it an excellent bacteria to thrive in both freshwater and marine environments, and thus able to cause infection to wide ranges of host animals. Among many species of bacteria, *Vibrio* species are those considered as one of the fastest growing bacteria, which responds practically instantly to favorable conditions such as warm temperature, salinity, and high level of dissolved oxygen (Baker-Austin et al., 2020; Abioye et al., 2021). In aquaculture, some of the common *Vibrio* species that are of veterinary and economic importance are *V. harveyi, V. parahaemolyticus, V. alginolyticus,* ad *V. anguillarum*.

Vibrio is not only commonly isolated from vertebrates and invertebrates, but also from aquatic plants and water columns as documented in a study by Chase et al. (2015). Due to the highly adaptable *Vibrio* spp., it can infect a wide range of animal hosts. It is more prevalent in marine fish as compared to freshwater fish, causing vibriosis, one of the most economically important diseases affecting Asian Seabass (Mohamad et al, 2022). Many factors such as the type and age of the host, exposure time and the strain virulency affect the development of clinical signs in infected fishes (Liu et al., 2016). Vibriosis in juvenile fishes usually has a more rapid onset which results in a sudden death without obvious clinical signs. Contrary to that, vibriosis tends to be in the chronic form in older fishes where obvious skin ulcerations and pigmentation are observed (Alexopoulos et al., 2011). A study by Dong et al. (2017) revealed that the liver appeared congested and hemorrhagic, and in another study by Mohi et al. (2010), kidneys of *V. harveyi*-infected tiger puffer (*Takifugu rubripes*) were congested with presence of granulomatous lesions. However, infections from different *Vibrio* species cannot be confirmed just by its clinical signs and histopathological changes alone since many common lesions are shared in different *Vibrio* species.

Environmental factors, primarily the water temperature, salinity and pH level all play important roles in the distribution of *Vibrio*. The risk of developing vibriosis outbreaks increases in optimal environment conditions that favors the multiplication of *Vibrio* spp (Givens et al., 2014). The most important criteria for *Vibrio* to proliferate are water temperature and salinity as documented by Takemura et al. (2014). It was reported that during warmer months, the population of *Vibrio* is significantly higher as compared to any other months (Johnson et al., 2012). In another study, Izumiya et al. (2017) found that the number of *Vibrio* was directly proportional to the water temperature, which explains why more vibriosis outbreaks occur in temperate countries. Even so, changes in temperature in tropical climates are less likely to affect the prevalence of *Vibrio* in these regions. This may be due to the fact that only a small change of temperature occurred, brief study intervals that had been done, and fragmented model of study. However, Vezzuli et al. (2015) showed that temperature does not significantly affect the abundance of *Vibrio* spp. in the Adriatic Sea, Italy. Instead, it was the presence of high nutrient concentrations in the seawater which enabled the *Vibrio* to maintain its population throughout the cold seasons.

Regarding the presence of *Vibrio* spp. within the environment of those who work closely in aquaculture, human vibriosis can be acquired through consumption of raw seafood or contaminated water sources. Some *Vibrio* species such as *V. cholerae, V. parahaemolyticus,* and *V. vulnificus* are able to cause diseases in the human population. Few of the recorded symptoms in humans caused by the highly pathogenic *V. vulnificus* are cellulitis and septicaemia (Vinh et al., 2006), necrotizing fasciitis, limb amputation and terminal septicaemia in immunocompromised individuals (Williams et al., 2014). The rate of vibriosis-associated diseases in the future is expected to rise due to the rising global temperature (Brehm et al., 2020).

2.2.2 Pathogenesis

Water is the primary transmission mode of *Vibrio* spp. as documented by many studies. The bacteria are able to gain entrance to the host through the openings in the gill, epidermis cut and gastrointestinal tract. Out of all these, opening on the skin surface serves as the primary mode of infection (Frans et al., 2011). The mucosal layer covering the outer surface of the skin acts as a physical and chemical barrier against pathogen invasion; therefore, injury to the skin where openings are made available, the risk of infection from *Vibrio* spp. or any other pathogens is more likely to occur. Also, the biological role of mucus of the skin in which the presence of immunoglobulins and enzymes such as lysozyme, lectins and lysins are able to obstruct *Vibrio* spp. from gaining entrance to the fish host (Chen et al., 2008). In fish farming practices, the skin injury is most likely due to net structure or farm facilities, where high stocking density and cannibalism of some fish species further increases the risk of epidermis cut. Other than epidermis cut, the gill also serves as one of the openings for the invasion from *V. vulnificus* (Callol et al., 2015), *V. anguillarum* and *V. harveyi* (Cardinaud et al., 2014). Not only due to the fact that the water is contaminated with *Vibrio* spp, the development of vibriosis is also highly correlated with the presence of physical stressors (water temperature and salinity) and biological stressors (stocking density and presence of other macro- or micro-organism) (Huicab-Pech et al., 2016) that impairs the immune system of the fish. Vibriosis in fish can also develop after the ingestion of *Vibrio* spp. For instance, the natural reservoirs of *V. cholera*, copepods and chironomids, may be ingested by fishes, and these fishes later serve as vector where they are eaten by bigger predatory animals such as bigger fishes or migratory birds (Senderovich et al., 2010).

After a successful invasion of the *Vibrio* spp. via any of the portals of entry, the bacteria will first multiply within the subcutaneous tissues, gill filaments, or in fish gut accordingly, before spreading to the muscle tissues causing tissue necrosis (Liu et al., 2016), and gaining access to the bloodstream causing septicaemia, and later on spread to other organs such as the liver, spleen and kidney (Noga, 2010). Clinical signs such as erratic swimming behavior, skin ulceration and hemorrhages, and drop scale can be seen. The fish will eventually die due to septicaemia or from a severe secondary bacterial infection due to the introduction of other opportunistic organisms through the skin ulcerations.

2.2.3 Economic Importance

2.2.3.1 Direct Impacts

Infectious diseases such as vibriosis could easily disrupt the performance of cultured Asian seabass due to its rapid farming intensification (Hutson, 2013). Two of the most common *Vibrios* that are to blame for vibriosis outbreaks in Malaysia are *V. harveyi* and *V. alginolyticus*, leading to high mortality rates (Dong et al., 2017). The economic repercussions of vibriosis on cultured marine fish are still hard to come by due to lack of documentation. However, it is widely accepted that fish mortality due to vibriosis is directly contributing to economic loss. In the shrimp industry, vibriosis outbreaks can be so severe that it can cause a significant drop in its production (Tran et al., 2014), and was thought to have cost the Asian shrimp industry USD 1 billion in losses (GAA, 2013). Also, vibriosis outbreaks alone causes the aquaculture of Asian seabass in Pulau Ketam, Selangor, Malaysia to cost RM48 million in losses in 1990 (Chan, 1997). A recent study by Norhariani et al. (2019) concluded that vibriosis in cultured Asian seabass required an average cost of RM 0.99 (USD 0.24)/fish. The cost of controlling and preventing vibriosis was not calculated as part of the study, though.

2.2.3.2 Indirect Impacts

Even if vibriosis does not significantly cause high mortality rates, it could still impair the productivity of a fish farm by slowing down the fish growth rate. The presence of *Vibrio* is linked with the slow growth rate of green grouper as demonstrated by Sun et al. (2009a), in which another study by Cano-Gomez et al. (2009) stated that stress and inappetance are few of the factors that are responsible for it. As a consequence, extra cost and time are needed for the fish to reach its market weight, while inevitably causing a drop in the production rate. Other than that, the development of external lesions due to vibriosis such as skin ulceration and hemorrhages (Bellos et al., 2015) has made their market value drop as well. Fish becomes less appealing to buyers and might as well end up not being sold. Not to forget the local communities that rely mostly on aquaculture as their only means of support, fish diseases such as vibriosis could make them no longer able to continue it. A study by Somga et al. (2002) showed that the Philippines experienced 75% decrease in household income due to the occurrence of vibriosis outbreaks in small-scale grouper cultures.

2.3 Common *Vibrio* species in fishes

2.3.1 *Vibrio harveyi*

Most aquatic species around the world, especially in the Asia continent, are susceptible for an infection from the pathogenic *Vibrio harveyi*. Due to the flourishing aquaculture practices particularly in Asia and South America, the organism is now regarded as one of the most important pathogens in marine invertebrates (Austin B. & Zhang, X. H., 2006). A study by Santhyia et al. (2015) showed that *V. harveyi* causes a more severe disease in shrimp (*Penaeus* spp.) farming systems in SEA countries including Malaysia, India, Thailand and the Philippines. Apart from that, a cultured Japanese abalone farm suffered mass mortalities due to the organism (*Sulculus diversicolor supratexta*; Nishimori et al. 1998), in which the infected abalone was unable to remain stick on to the surfaces resulting in drowning to death to the bottom of the culture cage.

2.3.2 *Vibrio alginolyticus*

Plenty of commercially important fish species, such as the European seabass, are susceptible to bacterial infection from *Vibrio alginolyticus*. This organism can be found almost everywhere in marine aquatic environments due to its halophilic nature and is able to disrupt the marine ecosystem when the conditions are favorable to it. Several authors have demonstrated that *V. alginolyticus* is able to cause high mortality outbreaks in a variety of fish species such as grouper, trout, sea bream, and European seabass (Moustafa et al., 2014; Lee, 1995; Zorrilla et al., 2003; Eissa et al., 2017). It is also reported that *V. alginolyticus* is capable of infecting freshwater Nile tilapia in Saudi Arabia as demonstrated in a study by Al-Sunaiher et al. (2010). Common clinical signs exhibited by infected fish include hemorrhagic septicemia together with diffused hemorrhages on the outer skin surfaces (Roberts, 2012).

2.3.3 *Vibrio anguillarum*

More than 50 species of fish in temperate countries are affected by *Vibrio anguillarum* (Frans et al., 2011). The pathogenesis of fish vibriosis is widely studied by using *V. anguillarum* as the model organism (Higuera et al., 2013). This organism often causes large mortality through acute and chronic infections, and is well known to be a secondary pathogen of fish (Wang & Ma, 2019). Out of 23 known serotypes of *Vibrio* spp., only three (O1, O2 and O3) are correlated with fish mortalities (Noga, 2010). Serotype O1 is routinely isolated from Norwegian salmon (Hellberg et al., 2010) and European seabass in the Mediterranean sea (Dierckens et al., 2009). Within serotype O2, it can be further subdivided into O2a and O2b which can be distinguished through immunoelectrophoresis, dot blot, and enzyme-linked immunosorbent assays (Santos et al., 1995). In a study by Vervarcke et al. (2005), African catfish fed with *V. anguillarum* O2 antigens were able to elicit mucosal response in the skin and gut. The study showed that orally fed fish with *V. anguillarum* O2 antigens showed lower antigen levels in the serum samples when compared to those that are anally intubated with it. Although *V. anguillarum* is widely known to infect marine fish, vibriosis by this organism is also seen in a freshwater species, Ayu (*Plecoglossus altivelis*), one of the most cultured fish in the Japanese fisheries (Nagai et al., 2008).

2.3.4 *Vibrio parahaemolyticus*

Human vibriosis is primarily caused by *Vibrio parahaemolyticus* and it is the leading cause of seafood-related outbreaks in the world (Li et al., 2019). Speaking of aquaculture, in several Asian countries, the leading cause of high mortality rate among cultured penaeid shrimp is *V. parahaemolyticus*, in which the organism is known to cause an acute hepatopancreatic necrosis disease (AHPND) (De Schryver et al., 2014). Also, in tropical fish species such as yellow croaker, *V. parahaemolyticus* is known to cause a serious infection as demonstrated in a study by Liu et al. (2016) in China. It is estimated that in the near future (2046-2065) and by the end of the twentieth century (2081-2100), an increment of 40-67% and 39-86% respectively of this pathogen in oysters is expected due to the rising global temperature (Ndraha & Hsiao, 2022). All this being said, effective control measures to lessen the number, if not totally eliminate, of this organism in seafoods are required for a safer seafood consumption.

2.4 Control of Vibriosis

2.4.1 Biosecurity

Frequent disease outbreaks simply mean a failure in the biosecurity system. An effective and precise system must be applied in order to maintain a disease-free status in aquaculture facilities as it has been proven that a good biosecurity is able to successfully contain infectious diseases such as vibriosis (Lafferty et al., 2015). The use of first-rate seeds and fingerlings remain as the most crucial part in the biosecurity system. This is because decent disease management in hatcheries is unable to be achieved if the source of fingerlings is already contaminated by pathogens in the first place. Therefore, the fingerlings must be from a trusted source with a long history of excellent client feedback to avoid despair in the future. Disease transmission could also be limited through the constant use of freshwater in culture facilities as per documented by Fouz et al. (2002), where experimentally infected freshwater eels with *Photobacterium damselae* subsp. *damselae (*formerly *Vibrio damsela)*, experienced no mortality at all.

Frequent monitoring and early detection of clinical signs of fishes has a crucial role in biosecurity. Upon seeing odd behavior, obvious clinical signs, and sudden spike of mortality rate, all of these serve as an early indicator of any ongoing infectious diseases, including vibriosis. Other than that, poor hygiene of aquaculture facilities due to infrequent disinfection and dry-out procedures will only favor the spread of *Vibrio* spp and its persistence in that fish farm (Yildiz & Visick, 2009). Therefore, biosecurity is something not to be overlooked by fish farmers if a disease-free fish farm status is desired.

2.4.2 Antibiotics

Among the most commonly used antibiotics in treating *Vibrio* spp. are oxytetracycline, tetracycline quinolones, nitrofurans, sulfonamides, trimethoprim, sarafloxacin, flumequine, and oxolinic acid (Lagana et al., 2011; Rico et al., 2012; Yano et al., 2014). A 100% mortality control against *V. alginolyticus* was achieved in a study done by Rajan et al. (2001) where oxytetracycline was incorporated into fish feed at 75 mg/kg fish/ day for eight consecutive days in cultured cobia fishes infected the bacteria. It was also found that in experimentally infected Atlantic cod with *V. anguillarum*, administrating 20 mg/kg of oxolinic acid and florfenicol in fish via oral route were able to significantly reduce the mortality rate (Samuelsen & Bergh, 2014). Although the use of antibiotics was proven to be effective in many studies, the exploitation of antibiotics by fish farmers has led to an ongoing worldwide antibiotic resistance, which may make future treatments more challenging. Not only *Vibrio* strains have developed worldwide resistance at an alarming rate in the aquaculture industries (Zhu et al., 2018), the frequent use of antibiotics in aquaculture has also made those strains that are capable of infecting humans to develop resistance as well (Hernandez-Robles et al., 2016). Therefore, antibiotic treatment in the aquaculture industries around the world has been remarkably reduced (FAO, 2005; WHO, 2006).

2.4.3 Vaccination

Due to the antibiotic restriction use in aquaculture and the concern of antimicrobial resistance, vaccination is regarded as the main method of prevention of the disease (Ina-Salwany et al., 2019). At least a 99% success rate was achieved in a large scale vaccination trials against *V. anguillarum* serotype O1 on European seabass in Spain (Sommerset et al., 2005). It was also reported that in cultured seabass, sea bream and turbot, the use of inactivated vaccines could provide a rather promising protection against vibriosis (Gudding & Van Muiswinkel, 2013). Although quite a number of vaccines has been developed against vibriosis, the disease remains relatively common even in vaccinated fish which is most likely due to the highly adaptable nature of Vibrio spp.

Recently, a study by Mohamad et al. (2021) has developed a feed-based vaccine which allows for an easier administration route, cheaper, less stressful method, and allows a no direct contact setting between the worker and fish compared to the common vaccination delivery by injection. This vaccine was proven to provide protection against vibriosis in the laboratory and field situations. A mixture of palm oil as an emulsion together with a whole-cell vaccine using formalin-killed *V. harveyi* strain VH1 was made into a commercial pellet as an alternative method to the time-consuming injection and immersion technique of vaccine administration. Inactivated vaccines work best as it is proven to be effective in fish (Huang et al., 2014), hence the wide use of inactivated vaccines in aquaculture (Muktar & Tesfaye, 2016). Also, researchers have recently proven that parts of the mechanism of feed-based vaccines involve serum antibody, skin lysozyme, and improved efficacy of peritoneal macrophages (unpublished data). Since the vaccine requires oral intake and digestion, it is likely that it stimulates the GALT, which is known to be an important component of mucosal immunity. However, fish vaccination is only practical and worth investing in when the production is at a larger scale, as it requires high production cost (Yanez et al., 2014). Therefore, a feed-based vaccine that offers cross-protection, inexpensive, and easy for farmers to use should be emphasized in the future.

2.5 Mucosal Immunity in fish

2.5.1 Mucosal-Associated Lymphoid Tissue (MALT)

The teleost mucosal surfaces are responsible for stimulating an active immune system, apart from being a physical barrier to multiple environmental pathogens as the first line of defense (Salinas et al., 2011). In fish, there are four mucosal-associated lymphoid tissue (MALT), namely the gut-associated lymphoid tissue (GALT), skin-associated lymphoid tissue (SALT), gill-associated lymphoid tissue (GIALT), and the recently found nasopharynx-associated lymphoid tissue (NALT). All four MALTs have similar features which includes the lack of an organized network of lymphoid structures, presence of secretory Igs and specific mucosal Ig class, as well as commensal bacteria (Salinas et al., 2011).

2.5.2 Gut-Associated Lymphoid Tissue (GALT)

Gut-associated lymphoid tissue (GALT) is one of the mucosal immune systems in teleost fish that is associated with the gastrointestinal tract. It differs among vertebrate groups due to anatomical differences of the stomach, intestinal length and the presence of pyloric caeca, intestinal loops and valves (Evans, 1998). In groups of higher vertebrates, the GALT is composed of both scattered and organized lymphoid tissues. However in fish, they lack organized GALT and hence the absence of specialized structures such as the Peyer’s patches (Rombout et al., 2010).

In fish gut, the production and recruitment of lymphocytes to the stimulation sites results in the development of GALT, in which the lymphocytes aggregate at the loci where antigen is presented in the lamina propria (Firdaus-Nawi et al. 2013). A systemic immune response is elicited in the systemic lymphoid organs (kidney and spleen) after the uptake of antigen by the enterocytes following an oral immunization (Eldrige et al., 1999). As a result, immunoglobulins (IgM) or specific antibodies are produced after the differentiation of B cells into plasma cells.

There are two main populations of immune cells that the GALT is made up from: (1) lamina propria leukocytes (macrophages, granulocytes, plasma cells and lymphocytes), (2) intraepithelial lymphocytes which mainly composed from T cells and B-cells. These cells, along with the epithelial cells, neuroendocrine cells and goblet cells work together to regulate the gut immune responses (Parra et al., 2015).

A study done by Firdaus-Nawi et al. (2011) revealed that GALT aggregation was found in the gut lamina propria following oral vaccination against *S. agalactiae* in tilapia fish. The study concluded that aggregations of GALTs and the size are directly related to the frequencies of oral vaccination.

2.5.3 Skin-Associated Lymphoid Tissue (SALT)

Being one of the primary and largest mucosal defense systems of the fish, the skin-associated lymphoid tissue (SALT) serves as the first line of defense since the skin is directly in contact with the external environment and hence the most susceptible mucosal immune organ to pathogens. Unlike the mammalian skin, the epidermis of fish is stratified and not keratinized due to the fact that the outermost layer of the skin is made up from living cells that are capable of undergoing mitosis. The mucus that is coating the skin surface is produced by the secretory cells (Malphigian cells, goblet cells, club cells and sacciform cells) that are residing within the epithelium. It is believed that Malphigian and goblet cells are also responsible in many biological roles (Salinas et al., 2011), such as having phagocytic properties (Chen et al., 2015). Also, many other molecules can be found in the mucus of the teleost skin such as lysozymes, complements, lectins, and immunoglobulins to protect the fish from invading pathogens (Nigam et al., 2012). It was also reported that the teleost skin is capable of eliciting almost similar immune responses as seen in the gut (Xu et al., 2013).

2.5.4 Gill-Associated Lymphoid Tissue (GIALT)

In teleost fish, four pairs of gill arches that are made up from numerous gill filaments serve as an efficient gas-exchange system. Not only the gills are responsible for exchanging oxygen, but also play vital roles in osmoregulation, maintenance of pH balance, waste excretion, hormonal regulation, and detoxification purposes (Maina, 2002). Due to the constant exposure of the gills to water, the gill-associated lymphoid tissue (GIALT) act as primary defense system in the gills of many fish species since there are numerous numbers of immune cells, such as the neutrophils, macrophages, and eosinophilic granulocytes (Mulero et al., 2007), as well as antibody-secreting cells (Dickerson et al., 2008) that are present within the gill.

3.0 METHODOLOGY

3.1 Samples of study

Previously acquired samples of the gut of Asian seabass (*Lates calcarifer*) from control and vaccinated groups were utilized. The gut tissue samples were previously fixed in 10% neutral buffered formalin, were sectioned at 4 µm, and stained with Hematoxylin and Eosin (H&E). In this study, three H&E-stained gut samples of both groups from every two weeks from the start of study until week 10 were retrieved from the Histopathology Laboratory, Faculty of Veterinary Medicine, UPM.

3.2 Examination of Histology Slides

All of the samples were observed under a light microscope at 20x magnification and were analyzed and evaluated for the histomorphometrics of GALT, which involves the GALT number and area, number of lymphocytes as well as the cell density. A total of 10 microscopic fields were viewed in every sample. The area of a GALT was measured using ImageJ, while the number of GALT and lymphocytes were manually counted with the aid of ImageJ. Lastly, the cell density was measured by dividing the number of lymphocytes by the GALT area, and is expressed in cell unit/μm^2 (Cu/m^2).

3.3 Statistical Analysis

The mean values of the GALT number and area, lymphocyte population and cell density between the control and vaccinated groups were analyzed and compared using an independent T test in IBM SPSS Statistics® version 26.0. Value of $p < 0.05$ is regarded as statistically significant.

4.0 RESULTS

4.1 General Histological Analysis

In general, three types of GALT distribution patterns were seen in both groups - organized, diffuse/scattered and lymphocyte-filled villi (Figures 2-4). However it was noticed that as the week increased, more scattered patterns of GALT were seen instead of the other two. Most of the GALTs were seen within the lamina propria of the intestine of the fish.

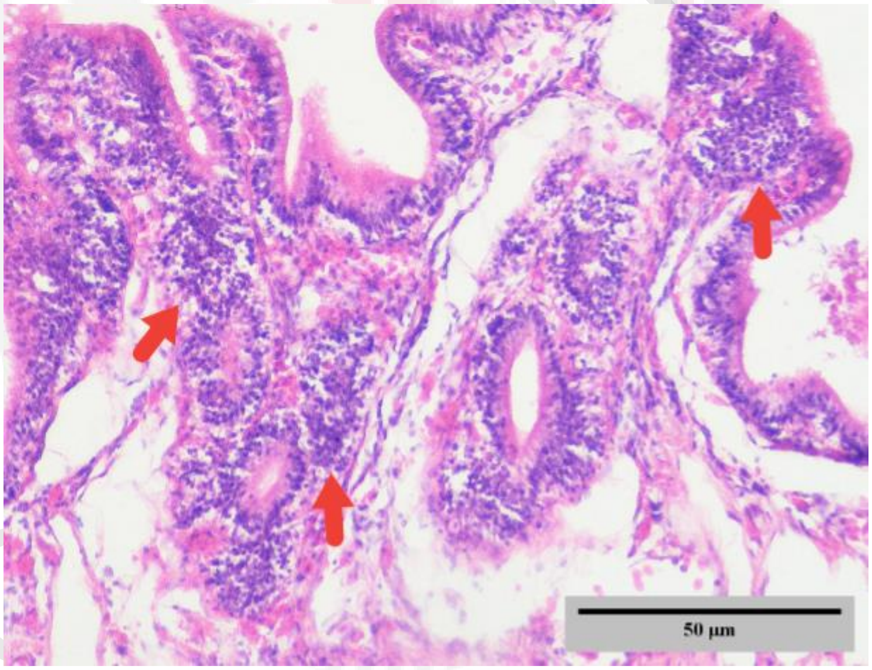

Figure 2 : Organized GALTs (arrows).

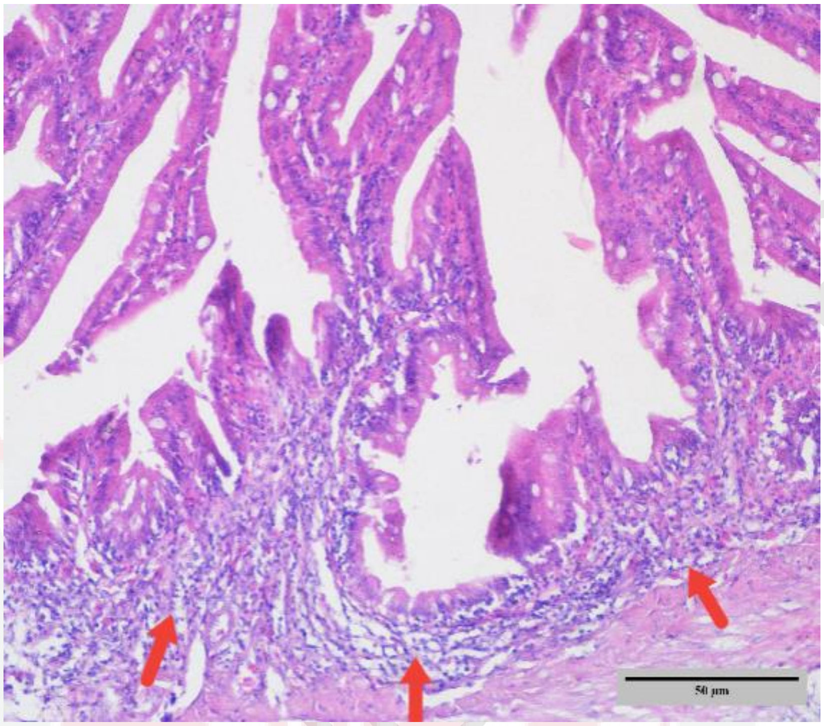

Figure 3: Diffuse/scattered GALTs (arrows).

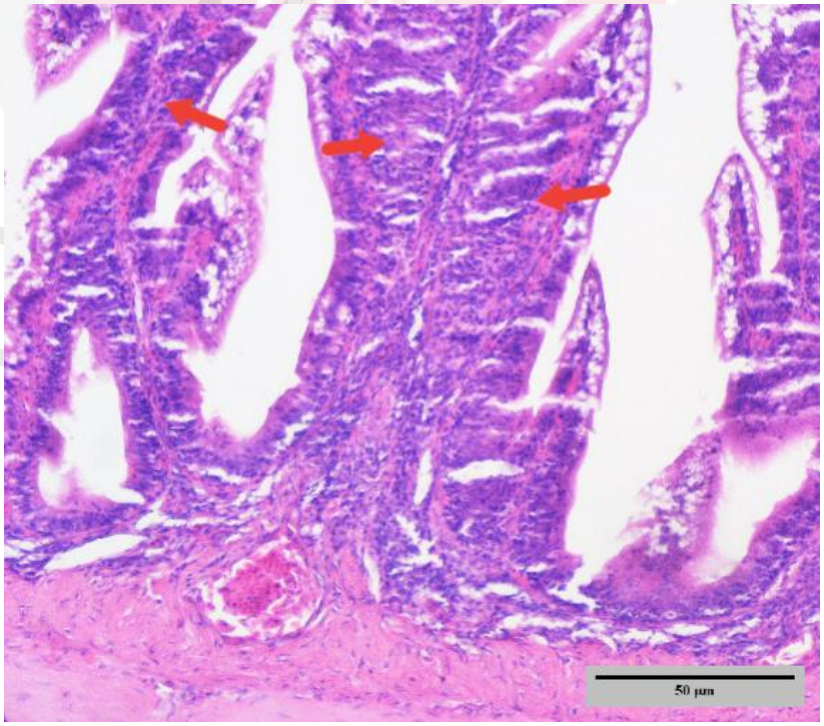

Figure 4 : Lymphocyte-filled villi GALTs (arrows).

4.2 Number of GALT

As shown in Figure 5, the presence of GALT was noticed as early as week 0 in both control and vaccinated groups, however more GALTs were seen in the vaccinated group (Figure 7). After the first booster vaccination on week 2, the mean GALT numbers of both groups increased in week 4, however it was significantly ($p < 0.05$) higher in the vaccinated group when compared with the control group (Figure 6). Both groups showed a decrease in their mean GALT numbers after week 4. Following the second booster vaccination on week 6, the mean GALT number of the vaccinated group increased consistently until week 10, but was only significantly ($p < 0.05$) higher than the control group on week 8 (Figure 5). Obvious changes in the mean GALT numbers were observed in the vaccinated group only after the booster vaccination was given.

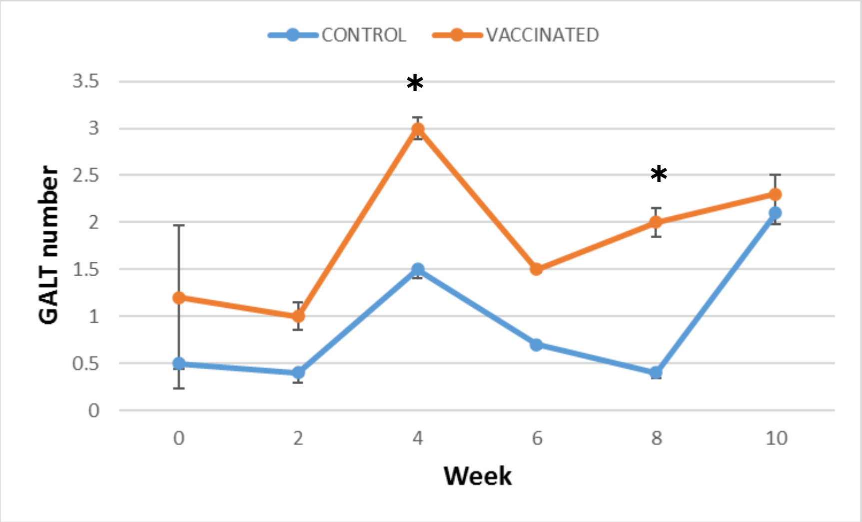

Figure 5: The number of GALTs. Asterisks (∗) indicate a significant ($p < 0.05$) difference between the two groups at the respective week.

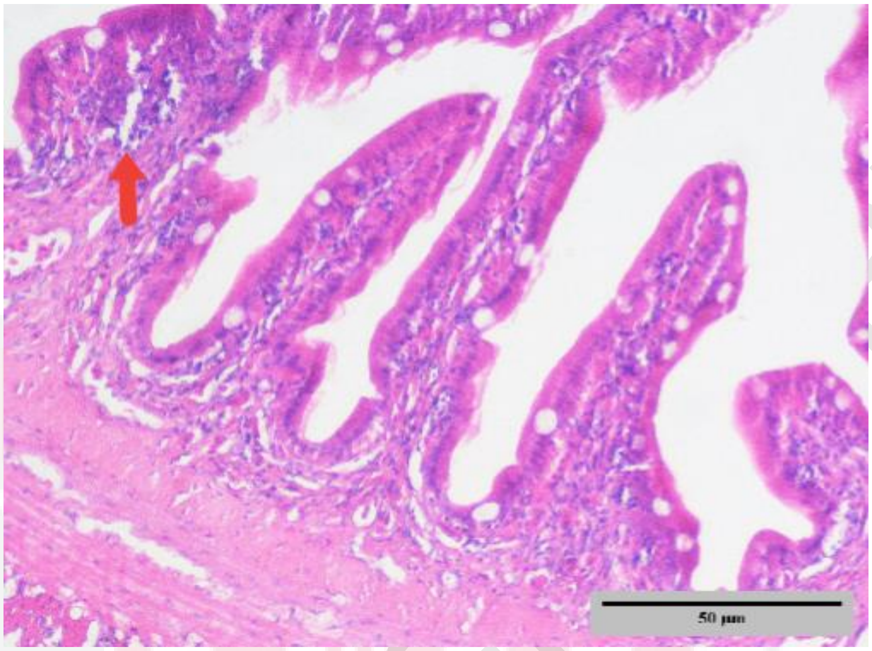

Figure 6 : Control group, Histological section of the hindgut (intestine) of Asian seabass on week 4. GALT can be seen within the intestinal villi (arrow). H&E x 20.

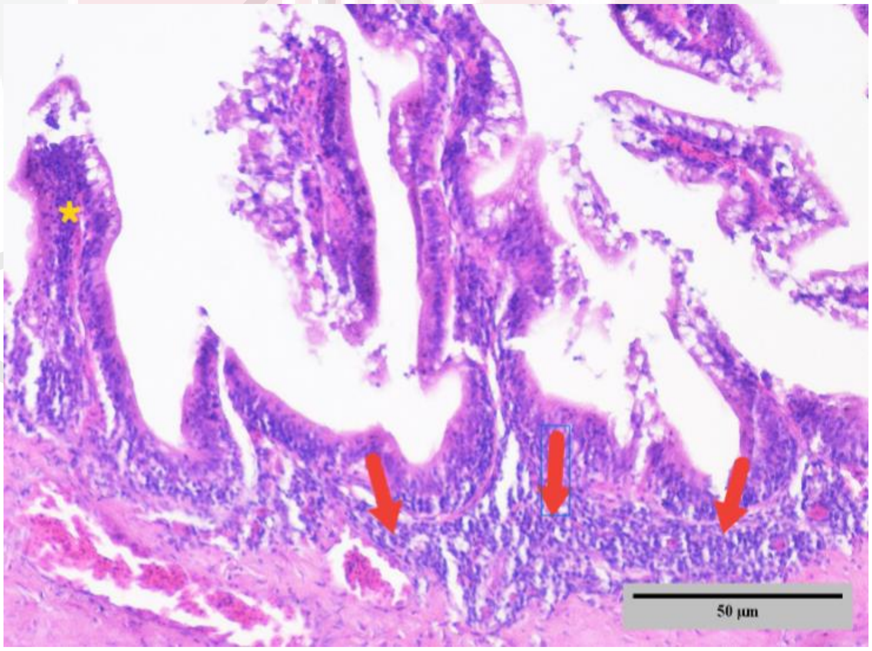

Figure 7 : Vaccinated group, Histological section of the hindgut (intestine) of Asian seabass on week 4. More GALTs can be seen within the lamina propria (arrows) and intestinal villi (asterisk). H&E x 20.

4.3 Area of GALT

As seen in Figure 8, similar sizes of GALT were observed in both groups at the start of the study. From week zero until week 10, both control and vaccinated groups showed a steady increment in the GALT area. However, the mean GALT area in the vaccinated group was bigger than the control group, but it was not significant ($p > 0.05$) throughout the study. Nevertheless, the largest GALT area 10,993.64µm^2 and 9,082.87µm^2 were recorded in the vaccinated group and 6,462.60µm^2 and 4,052.12µm^2 in the control group, both at week 10 of the study.

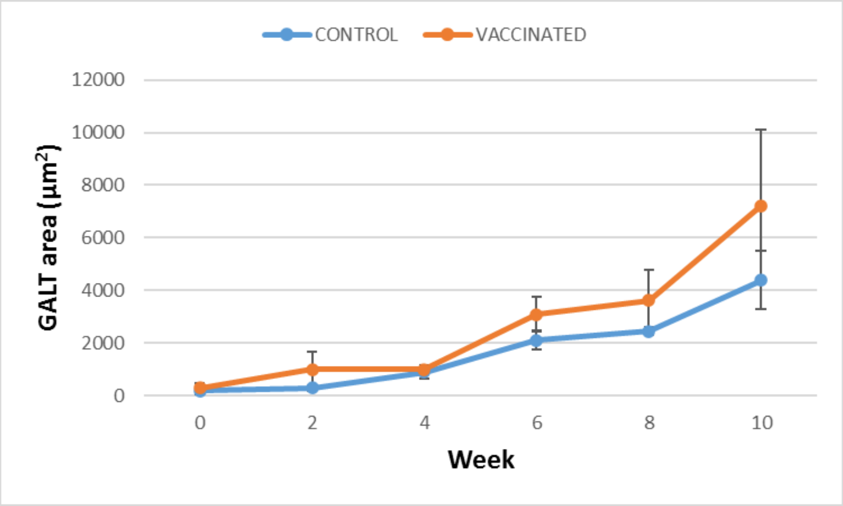

Figure 8 : The size of GALT. No significant ($p > 0.05$) difference was observed between the two groups throughout the study.

4.4 Number of Lymphocytes

Following the initial vaccination on week zero, the mean lymphocyte population in the vaccinated group showed a significant ($p < 0.05$) increase when compared to the control group in week two. After week two, the mean lymphocyte count in the vaccinated group decreased until week six, but the numbers remained significantly ($p < 0.05$) higher when compared to the control group. After the second booster vaccination on week six, the mean lymphocyte count in the vaccinated group increased tremendously and was significantly ($p < 0.05$) higher than the control group. The highest lymphocyte count of the vaccinated group was recorded on week 10. The control group showed a steady increase in its lymphocyte count, however it was significantly ($p < 0.05$) lower than the vaccinated group throughout the study period. As shown in (Figure 9).

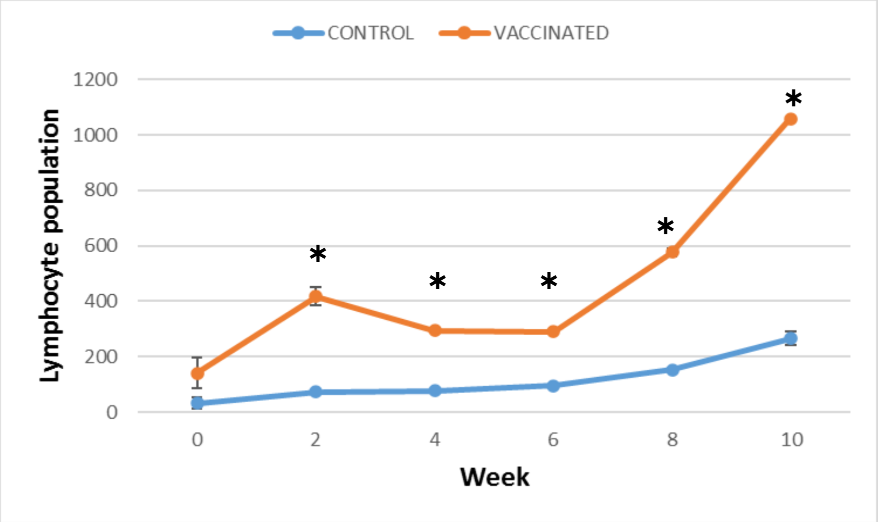

Figure 9 : The lymphocyte population. Asterisks (∗) indicate a significant ($p < 0.05$) difference between the two groups on the respective week.

4.5 Cell Density

From week zero until week eight, the mean cell density in the vaccinated group showed an increasing pattern, before it slightly decreased in week 10. When the mean cell density in both groups was compared between week two and week eight, the vaccinated group showed a significantly ($p < 0.05$) higher density than the control group. In the control group, the mean cell density increased from week zero until week four, before it decreased until week eight, and increased again in week 10. The highest cell density was recorded on week eight and week 10 in the vaccinated and control group, respectively. As shown in (Figure 10).

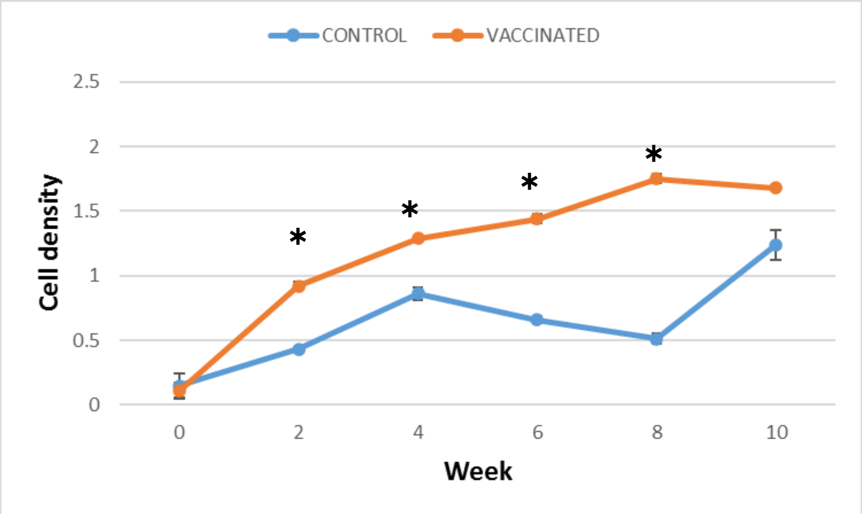

Figure 10 : The density of GALT. Asterisks (∗) indicate a significant ($p < 0.05$) difference between the two groups on the respective week.

5.0 DISCUSSION

The efficacy of a mucosal vaccine and evaluation of antigen uptake from the gut lumen can be understood better through gut histological analysis (Islam et al., 2009). Following antigen uptake in the gut lumen, there will be stimulation on of lymphocyte recruitment to the stimulation site where the antigen is presented, which leads to the development of GALT that plays a vital role in fish mucosal immunity (Nur-Nazifah et al., 2014; Kahieshesfandiari et al., 2019).

In the present study, histological analysis revealed that the oral field-based feed vaccination was able to stimulate the development of GALT, increase the GALT area, and also showed a significantly higher lymphocyte population in the vaccinated group of Asian seabass. Overall, the findings in this study was relatively similar to that of Firdaus-Nawi et al. (2013) who studied about the effect on mucosal activity and degree of protection elicited by the adjuvant feed-based vaccination of *Streptococcus agalactiae* against streptococcosis in red hybrid Tilapia.

Upon analyzing the gut histological samples from week 0 until week 10, a larger GALT area was recorded in the latter week that is most probably due to the fusion of smaller, newly formed GALTs. The mean difference of GALT area between the two groups was not significant most probably due to the small sample size as it does not represent the true population size, as well as the short study period, since the long-term effect of the oral field-based vaccination was not able to be evaluated, especially after the second booster vaccination on week six.

Despite not having any antigen stimulation from the oral feed-based vaccine, the control group still showed an increase in their lymphocyte population because this study was conducted in a field setting. The fishes were still exposed to other stressors such as changes in water temperature and pH, high stock density, as well as introduction of other microorganisms from the water into the fishes that can stimulate lymphocyte recruitment. Furthermore, water quality is an essential factor in aquaculture for fish health, in which a drop in water quality will induce more stress to the fish, and eventually the occurrence of disease outbreaks. Poor water quality allows disease outbreaks even with the lowest concentration of infectious pathogens (Zamri-saad et al., 2004). Also, the water quality of a fish farm can be affected by the environmental condition that surrounds the farm, which is correlated to the fish stress levels (Amal et al., 2008). This explains why in field setting, fishes are always exposed to stimulants that can induce the recruitment of lymphocytes.

Fish farmers favor oral vaccines due to its ease of use, allows mass vaccination in a short period of time, less stressful to the fishes, time-efficient, and less laborious (Le Breton, 2009). The only issue is that in the fish stomach, the enzymes could denature the antigen from the oral vaccine and thus provide poor protection against the disease. However, oral vaccines are still capable of stimulating GALT development despite the lesser degree of protection when compared to when the vaccine is administered through the injection route (Firdaus-Nawi et al., 2013). Therefore, the need for further research to figure out possible ways of protecting the oral vaccines against the stomach enzymes is crucial for better immune response when developing an oral vaccine.

6.0 CONCLUSION

To conclude, the oral-feed based field vaccination was able to provide a better protection against vibriosis through the development of GALTs within the gut lamina propria of the vaccinated Asian seabass. In addition to that, the administration of oral booster vaccinations was able to further stimulate a more obvious GALT development within the gut of the vaccinated Asian seabass.

7.0 RECOMMENDATIONS

In the future, we suggest that the study utilizes larger samples as well as to increase the study period. Larger sample size would definitely increase the data accuracy and be a better representation of both the control and vaccinated groups. By lengthening the study period, the GALT development between the two groups would be more distinct and easier to compare and therefore provide stronger evidence to support the effectiveness of the oral-feed based field vaccination. Also, other parameters should be included as well such as the clinical signs of fishes, mortality rates, gut Igm levels and gut lysozyme activities. By comparing the clinical signs and mortality rates between the control and vaccinated groups, the obvious effects of the oral feed-based vaccination are able to be evaluated. As for the gut IgM levels and lysozyme activities, the increment of these two parameters will reflect the ability of the oral vaccine to elicit both innate and humoral responses. And finally, is to conduct a post-vaccination challenge study on the vaccinated fishes. By doing so, the long-term protection against vibriosis is able to be evaluated as well as to see if an immune response could be achieved, protecting the fishes from the disease.

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