UNIVERSITI PUTRA MALAYSIA

EVALUATION OF MUCOSAL-ASSOCIATED LYMPHOID TISSUES (MALTs) IN RESPONSE TO POST-KILLED VACCINE VERSUS NATURAL INFECTION AGAINST STREPTOCOCCOSIS (Streptococcus agalactiae) IN RED HYBRID TILAPIA (Oreochromis sp.).

ADAM LEE BIN RUSLI LEE

Ip
FPV 2022 87

EVALUATION OF MUCOSAL-ASSOCIATED LYMPHOID TISSUES (MALTs) IN RESPONSE TO POST-KILLED VACCINE VERSUS NATURAL INFECTION AGAINST STREPTOCOCCOSIS (*Streptococcus agalactiae*) IN RED HYBRID TILAPIA (*Oreochromis* sp.).

ADAM LEE BIN RUSLI LEE

A project paper submitted to the

Faculty of Veterinary Medicine, Universiti Putra Malaysia

In partial fulfilment of the requirement for the

DEGREE OF DOCTOR OF VETERINARY MEDICINE

Universiti Putra Malaysia

Serdang, Selangor Darul Ehsan

September 2022

CERTIFICATION

It is hereby certified that we have read this project paper entitled "Evaluation of mucosal-associated lymphoid tissues (MALTs) in response to post-killed vaccine versus natural infection against streptococcosis (*Streptococcus agalactiae*) in Red hybrid tilapia (*Oreochromis* sp.).", by Adam Lee Bin Rusli Lee and in my opinion, it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirements for the course of VPD 4999 – Final Year Project

---

ASSOC. PROF. DR MD SABRI MOHD YUSOFF,

DVM, MVSc, PhD (UPM)

Associate Professor,

Department of Veterinary Medicine

Universiti Putra Malaysia

(Supervisor)

DR. SHARINA OMAR

DVM, PhD (University of Leicester, United Kingdom)

Lecturer

Department of Veterinary Medicine

Universiti Putra Malaysia

(Co-supervisor)

ACKNOWLEDGEMENT

First and foremost, I would like to express my utmost praise and gratitude to the Almighty God for granting me the health, energy and time to complete this project. With His blessings, I am able to complete my final year project with the aid and guidance of many others.

Next, I would like to thank my supervisor, Assoc. Prof. Dr. Md Sabri Mohd Yusoff and my co-supervisor, Dr. Sharina Omar. Thank you for all the knowledge, encouragement and effort poured unto me. I would also like to personally thank Dr. Sani Bashir and Dr. Nurnajwa Atiqah in aiding me in the process of preparing the vaccine and implementing the project. Thank you for sharing your experiences with me and correcting my mistakes. Not to forget, Ms. Latifah and Ms. Aisyah from the Histopathology Department, Dr. Fuad and Encik Azmi from the Aquatic Health Unit (AHU), UPM.

I would like to be grateful for how blessed I am to have a wonderful and supportive family which never once doubted me nor disregard my effort and accomplishments. My beloved parents, Sarah Koh Binti Abdullah and Rusli Lee Bin Abdullah, have always showered me with love more than I could possibly wish for. The love and care received has been one of my motivations to continue to pursue greatness in life. I am forever in debt to my brothers, Muhammad Lee and Aiman Lee for treating me with delicious meals and purchasing items that make me happy. To my FYP mates, Suriani and Aishah, thank you for your help. Special thanks to Ani Noraqilah in being one of my best friends and being an emotional support to me, and thank you to all of DVM22, DVM23 and friends, whether

online (especially Discord and gaming buddies, Edzra Aradzy and Abdurrahman) or offline. All of you were a great support.

As a wise sharpshooter once said, "If you're not a good shot today, don't worry. There are other ways to be useful."

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
CFU/mL	Colony-forming unit per millilitre
FKB	Formalin-killed bacteria
GALT	Gut-Associated Lymphoid tissue
GIALT	Gill-Associated Lymphoid Tissue
H&E	Haematoxylin & Eosin
NALT	Nasopharynx-Associated Lymphoid Tissue
PBS	Phosphate-buffer solution
rpm	Rotation per minute
SALT	Skin-Associated Lymphoid Tissue
S. agalactiae	*Streptococcus agalactiae*

TSA	Tryptic soy agar
TSB	Tryptic soy broth

ABSTRAK

Abstrak daripada kertas projek yang dikemukan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada VPD 4999 – Projek ilmiah tahun akhir

PENILAIAN TISU LIMFOID YANG BERKAITAN DENGAN MUKOSA (MALTs) BERIKUTAN VAKSIN PASCA-BUNUH MELAWAN JANGKITAN SEMULA JADI TERHADAP STREPTOKOKOSIS (*Streptococcus agalactiae*) DALAM TILAPIA HIBRID MERAH (*Oreochromis* sp.).

Oleh

Adam Lee Bin Rusli Lee

2022

Penyelia: Prof. Madya Dr Md Sabri Mohd Yusoff

Streptococcus agalactiae merupakan salah satu patogen utama yang menyebabkan streptokokosis. Streptokokosis mempengaruhi pelbagai hidupan akuatik yang memudaratkan industri akuakultur di seluruh dunia. Kajian ini bertujuan untuk menilai tisu limfoid yang berkaitan dengan mukosa (MALTs) dalam tilapia merah hibrid (*Oreochromis* sp.) berikutan vaksin pasca-bunuh melawan jangkitan semula jadi melalui kaedah rendaman terhadap jangkitan *S. agalactiae*. 60 tilapia diasingkan secara rawak kepada 4 kumpulan; Kumpulan A, Kumpulan B, Kumpulan C dan Kumpulan 1. Kumpulan A berfungsi sebagai kumpulan kawalan. Untuk Kumpulan B, ikan-ikan telah

direndam sekali selama 1 minit dalam *S. agalactiae* yang telah dibunuh dalam formalin (FKB). Untuk Kumpulan C, ikan-ikan telah divaksinasi sekali dan diberi dos penggalak seminggu selepas vaksinasi pertama, 1 minit untuk setiap sesi. Untuk Kumpulan 1, ikan-ikan telah direndam sekali dalam 2L larutan triptik soya (TSB) yang mempunyai *S. agalactiae* (10^9 CFU/mL) selama 15 minit. Petanda klinikal dan kematian dalam ikan-ikan dipantau sepanjang kajian dijalankan. 5 ekor ikan daripada setiap kumpulan dikorbankan seminggu selepas proses rendaman masing-masing dan sampel kulit dan insang diambil untuk analisis histologi. Analisis statistik ketebalan epidermis kulit dan keluasan sel-sel inflamasi insang menunjukkan bahawa tiada perbezaan signifikan ($p>0.05$) untuk tisu limfoid dalam mukosa insang dan kulit merentasi kumpulan. Hal ini menunjukkan bahawa vaksinasi dan jangkitan semula jadi melalui kaedah rendaman menunjukkan tiada perbezaan signifikan dari segi perkembangan tisu limfoid dalam lapisan mukosa insang dan kulit ikan.

Keywords: Tilapia hibrid merah, *Streptococcus agalactiae*, vaksin rendaman, ketebalan epidermis kulit, tisu limfoid yang berkaitan dengan mukosa (MALTs)

ABSTRACT

Abstract of the project paper presented to the Faculty of Veterinary Medicines in partial for the course VPD 4999 – Final Year Project

EVALUATION OF MUCOSAL-ASSOCIATED LYMPHOID TISSUES (MALTs) IN RESPONSE TO POST-KILLED VACCINE VERSUS NATURAL INFECTION AGAINST STREPTOCOCCOSIS (*Streptococcus agalactiae*) IN RED HYBRID TILAPIA (*Oreochromis* sp.).

by

Adam Lee Bin Rusli Lee

2022

Supervisor: Assoc. Prof. Dr Md Sabri Mohd Yusoff

Streptococcus agalactiae is one of the most important pathogens of streptococcosis. Streptococcosis affects various aquatic life causing harm to the aquaculture industry worldwide. This study aimed to evaluate the mucosal-associated lymphoid tissues (MALTs) in Red hybrid tilapia (*Oreochromis* sp.) in response to a post-killed vaccine versus natural infection by immersion methods against *S. agalactiae* infections. 60 tilapia fingerlings were randomly divided into 4 groups; Group A, Group B, Group C and Group 1. Group A served as a control group. For Group B, fish were immersed once for 1 minute in formalin-killed bacteria (FKB) of *S. agalactiae*. For Group C, fish were vaccinated once and boosted 1 minute per session one week after the first vaccination. For Group 1, the fish were immersed once for 15 minutes in 2 L of tryptic soy broth (TSB) containing *S.*

agalactiae (10^9 CFU/mL). The fingerlings were monitored for clinical signs and mortality throughout the study. Five fish from each group were sacrificed one week after their respective immersion and skin and gill samples were taken for histological analysis. Statistical analysis of skin epidermal thickness and gill inflammatory cell area showed that there was no significant difference ($p>0.05$) in lymphoid tissue in gills mucosal and skin between the groups. Vaccination and natural infection by the immersion method were found to show no significant difference in the development of lymphoid tissue in the gill mucosal and the skin of the fish.

Keywords: Red hybrid tilapia, *Streptococcus agalactiae*, immersion vaccine, skin epidermal thickness, mucosal-associated lymphoid tissues (MALTs)

1.0 INTRODUCTION

1.1 Study Background

Streptococcus agalactiae is one of the most common bacterial diseases in tilapia, and cost loss in production every year in the industry (Huang *et al.*, 2019). *Oreochromis* sp. (hybrid red tilapia) is an important and commercially valuable fish species for aquaculture worldwide. Fish death associated with streptococcosis due to *S. agalactiae* or *S. iniae* are usually illustrated as septicemia, meningoencephalitis, disorientation, ulcers, lethargy, and exophthalmia (Hayat *et al.*, 2021). Therefore, this important global issue warrants better control procedure to prevent economic losses.

Vaccination remains one of the best ways to prevent streptococcosis in fish. Vaccines are currently available in a variety of formulations, including oral, immersion, and injection administration, with injection being the most common method of administration. Gut-associated lymphoid tissue (GALT) in fish functions similarly to tonsils and Peyer's patches in mammals by producing and recruiting lymphocytes in response to mucosal antigenic stimulation. The ability of antigens to induce lymphocyte aggregation and an immune response in the mucosal regions, including the formation of certain anti-vaccine antibodies, has been used to assess the effectiveness of oral vaccines (Hayat *et al.*, 2021). GALTs are known to be one of the major mucosal-associated lymphoid tissue (MALT) in a teleost, along with other lymphoid tissues such as skin-associated lymphoid tissue (SALT), gill-associated lymphoid tissue (GIALT) and nasopharynx-associated lymphoid tissue (NALT). Therefore, in this project, the development of MALTs is used as an indicator of vaccine efficacy post-vaccination and also of natural infection of *S. agalactiae*.

Immersion is a good way to immunise small fish, but results can be vague (Huang *et al.*, 2019). This means that there is limited research into immersion vaccination in fish and results from immersion vaccination may not be as effective or show as many effects as other vaccine administration methods. Therefore, assessment the immersion vaccination results for this project will be beneficial to the tilapia industry.

1.2 Objectives

The objectives of this study were:

1. To observe the development of mucosal-associated lymphoid tissues (MALTs) in the skin and gills by observing the thickness of the mucosal layer of the gills and skin and the presence of MALTs in tilapia (*Oreochromis* sp.) upon killed vaccination and natural infection against *Streptococcus agalactiae*.

2. To compare the development of mucosal-associated lymphoid tissues (MALTs) in the skin and gills by observing the thickness of the mucosal layer of the gills and skin and the presence of MALTs in tilapia (*Oreochromis* sp.) between killed vaccination and natural infection of *Streptococcus agalactiae*.

1.3 Hypothesis

The hypothesis of this study were:

H_o: Killed vaccines and natural infections with *Streptococcus agalactiae* do not stimulate the development of mucosal-associated lymphoid tissue (MALT) in the skin and gills of tilapia.

H_A: Killed vaccines and natural infection of *Streptococcus agalactiae* stimulate the development of mucosal-associated lymphoid tissue (MALT) in the skin and gills of tilapia.

1.4 Justification

The justification of this study were:

The efficacy of oral vaccine administration against streptococcosis is well documented, however, there is a limited study available specifically for *S. agalactiae* infection for the immersion vaccine. Therefore, this study aims to determine the development of the mucosal-associated lymphoid tissue (MALT) in skin and gills of tilapia (*Oreochromis* sp.) following post-vaccination and also during natural infection of *Streptococcus agalactiae*.

2.0 LITERATURE REVIEW

2.1 Red hybrid tilapia

Tilapia or also known as mango fish, *Oreochromis* sp. belonging to the cichlidae family. They are freshwater fish that are available and popularly cultivated in the aquaculture industry around the world, but they are actually native to Africa. El-Sayed (2006) stated that many parts of the world, tilapia are introduced primarily for purposes such as food storage, aquatic weed control, recreational fishing, and research use. Characteristic of these fish are laterally compressed, deep bodies with long dorsal fins. He *et al.* (2018) reported that in human diet, tilapia are known as "aquatic chicken", and are particularly favored by pregnant women and children.

Tilapia is considered a suitable fish for farming in the aquaculture industry for several reasons. The tilapia fish have several great characteristics such as rapid growth, highly reproductive capacity, tolerance to a variety of environmental conditions, stressors, and diseases, and they can adapt to artificial feeds directly after yolk-sac absorption (El-Sayed, 2006). Zhang *et al.* (2018) also reported that tilapia was considered to be the hardiest fish capable to withstand and be more resistant towards diseases as compared to other cultured fish species. Based on these characteristics, they are easy to breed and sell to all parts of the world. In addition, the Food and Agriculture Organization (FAO, 2013) reported that there has been increased yield in terms of production value through the success of genetically improved fish tilapia (GIFT), which all male or monosexual tilapia with the characteristics of wide-body conformation.

2.2 Streptococcosis

Streptococcus agalactiae is deemed to be one of the main causes of streptococcosis in tilapia. These Gram-positive bacteria that cause red boil disease in tilapia have led to a decline in the progress of the aquaculture industry, resulting in remarkably reduced profits and financial losses for farmers and cultivators. In 1997, there was a reported case of *S. agalactiae* infection in tilapia, and now streptococcosis is reported to have affected many tilapia rearing cages in various locations such as Kenyir, Pedu and Pergau Lakes. An overall mortality rate of 60-70% has been reported in this population (Siti-Zahrah *et al.*, 2005). Hayat *et al.* (2021) stated that some of the conditions caused by streptococcosis that can lead to death in fish are septicemia, meningoencephalitis, disorientation, ulcers, lethargy and exophthalmia. Other signs may include erratic swimming pattern and also sudden death.

Streptococcosis in tilapia can cause certain damage to the fish's internal organs. This can be determined by necropsy of the infected fish. Zamri *et al.* (2010) found at necropsy of the streptococcosis-infected fish, the visceral organs were swollen and congested, specifically the liver, gills, kidney and spleen. There was also softening of the brain, and edematous lesions were observed in some cases. Many of the major blood vessels of the affected fish were congested where vasculitis related to bacterial colonies. When these symptoms are present, fish may have a hard time to survive, which contributes to the high mortality rate from streptococcal infections.

2.3 Vaccination

Vaccination is one of the methods that can be applied in the aquaculture industry to prevent major problems such as streptococcosis. These applications of vaccines include methods of administration such as injection, immersion or vaccine mixed with feed orally administered. Hayat *et al.* (2021) noted that vaccine efficacy can be demonstrated by lymphocyte aggregation and mucosal immune response in response to the presence of antigen. The production of specific antibodies against the vaccine is included in the statement. Immersion vaccination of fish can be performed by immersing the fish in water mixed with vaccine antigens. Bøgwald and Dalmo (2019) stated that the dip vaccination is performed in about one or more minutes, and this process is rapid since the fish are dipped in water containing a high dose of vaccine antigens, while the bath vaccination, takes longer than the fish placed in water that has a lower concentration of vaccine antigens.

In immersion vaccination, efficacies range from low to moderate in most cases, and it is quite challenging to determine the reasons why some vaccines show high efficacies while others show low efficacies. When conducting immersion vaccination trials, many factors that affect vaccine efficacy must considered, including dosage of vaccine, time of immersion, soluble antigen absorbed during immersion immunization of fish, adjuvant performance, temperature, size, and the age of fish, osmolarity, prime-boost strategy, replicative vs. non-replicative vaccines, and how to implement the experimental pathogen challenge (Bøgwald and Dalmo, 2019).

3.0 MATERIALS AND METHOD

3.1 Fish and experimental condition

A total of 60 red hybrid tilapia fingerlings were transported from Beranang tilapia farm, Selangor. They were acclimatized in a tank for a week. 4 aquarium tanks were cleaned and disinfected prior to the project implementation. 15 fish were placed in each of the 4 tanks according to their groups, namely Group A, Group B, Group C and Group 1. The water was dechlorinated, and filters and aerators were installed in the tanks. Water quality was monitored throughout the study. Fish were fed commercial pellet twice daily throughout the study.

3.2 Bacterial and growth condition

Streptococcus agalactiae stock were cultured onto tryptic soy agar (TSA) plates and incubated at 37°C for 24 hours. The following day, the bacterial colonies formed on the cultured TSA plates were identified with the characteristics of small, white to gray raised colonies with a smooth edge and smooth surface. The colonies were further tested with Gram stain and upon examination, there were Gram-positive cocci appearing in pairs or in short chains, suggestive of *S. agalactiae*.

3.3 Formalin-killed bacteria (FKB) preparation

The isolated *S. agalactiae* from the TSA plates were further subcultured into tryptic soy broth (TSB) in conical flasks and were placed in a shaker incubator to be incubated at 30°C for 24

hours. The solution was then standardized to the concentration of 10^9 CFU/mL using the serial dilution method. 0.5% formalin was added into the bacterial suspension and allowed to stand for 24 hours to kill the bacteria. The solution was cultured onto TSA and incubated overnight to ensure no growth of bacteria was observed. The solution was then centrifuged using a centrifuge machine at 10,000 rpm for 3 minutes. The supernatant of the centrifuged solution was removed, and the centrifugation process was repeated. Phosphate-buffer solution (PBS) was added into the formalin-killed *S. agalactiae* in the falcon tube and suspended using a vortex machine until homogenized. The suspended solution was centrifuged again to wash the bacteria from the remaining formalin, and this process was repeated for six times.

3.4 Immersion vaccine preparation

A laboratory glass bottle was prewashed with 70% alcohol for 5 minutes before proceeding with three 3-minute washes using a sterile PBS solution. The prepared vaccine was then poured into the sterilized laboratory glass bottle for proper storage and transportation.

3.5 Preparation of immersion solution containing live *Streptococcus agalactiae* for natural infection

The isolated bacteria from TSA were subcultured into TSB and incubated in a shaker incubator for at 30°C 24 hours. The 2-liter solution was assayed using the standard plate count method and the final concentration of live *S. agalactiae* was 1.79×10^9 CFU/mL.

3.6 Experimental design

60 juvenile tilapia were randomly divided into 4 groups; Group A, Group B, Group C and Group 1. Group A served as a control group and did not participate in any immersion in vaccine or solution containing live *S. agalactiae*. Group B was immersed once in formalin-killed bacteria (FKB) of *S. agalactiae* for 1 minute. For Group C, the fish were vaccinated once and later boosted one week after the first vaccination with 1 minute for each session. For Group 1, the fish were immersed in the 2 L TSB solution containing live *S. agalactiae* for 15 minutes. Clinical signs and mortality of the fish were monitored and recorded throughout the study. 5 fish were sacrificed from each group using the pithing method, while skin and gill samples were taken one week after their respective immersion procedure. The samples were then processed for histological analysis.

	Week 0 & 1	Week 2	Week 3	Week 4
Group A	Vaccine Preparation	X		
Group B		Vaccine	X	
Group C		Vaccine	Booster	X
Group 1			Natural Infection	X

X = Sampling

Table 3.6.1: Table showing the vaccination administration, natural infection and also sampling process based on the weeks of study.

3.7 Histology

Five fish from each group were sacrificed using the pithing method one week after their respective immersion. The skin of each fish was incised to approximately 5 mm thickness in the dorsolateral region and gill samples were taken by cutting the gill arch. Skin and gill samples were followed by 24 hours fixation in 10% formalin. The skin samples were then processed using a decalcifying procedure that used 10% nitric acid as the decalcifying agent. This process was done to remove the minerals and calcium salts from the cartilaginous bones and scales. After the decalcification process was completed, the tissues were trimmed to smaller sizes. The trimmed skin tissues and also the gill samples were placed into tissue cassettes before being labeled with their respective groupings. The cassettes were then placed on a cassette holder or mold before being cast with paraffin wax. This process was called embedding and served to prepare the samples for the later sectioning process. After the paraffin wax had hardened, the samples were sectioned using the microtome machine and the tissue ribbons were placed into a water bath before being transferred onto glass slides. The slides were allowed to dry overnight before staining with the H&E stain.

3.8 Bacterial isolation and Gram staining

There were 2 deaths in the fish from Group 1 (naturally infected group) and the rest were euthanized one week after the immersion challenge. Some fish also showed signs of an erratic swimming pattern that coincided with streptococcosis. Brain, kidney and eye samples were taken from the fish for bacterial isolation and identification. Samples were cultured on TSA agar incubated at 37°C for 24 hours. The bacterial colony grown on the plates was morphologically

similar to *S. agalactiae* and they were further detected using Gram stain. Gram staining observed Gram-positive, paired, or short-chain cocci, suggestive of *S. agalactiae*.

3.9 Statistical analysis

IBM SPSS Statistics version 27.0 for Windows 10 was used for statistical analysis of the results of this study and tested at the 5% significance level. The thickness of the skin epidermis of all groups and the gill inflammatory cell location was measured throughout the study. The average value was recorded and analyzed using one-way ANOVA.

4.0 RESULTS

4.1 Thickness of epidermis

In this study, immunization by immersion vaccination of formalin-killed *S. agalactiae* in Red hybrid tilapia resulted in insignificant changes (p>0.05) in epidermal thickness in the control, the single-vaccination, and subsequent booster groups, and the natural infection group.

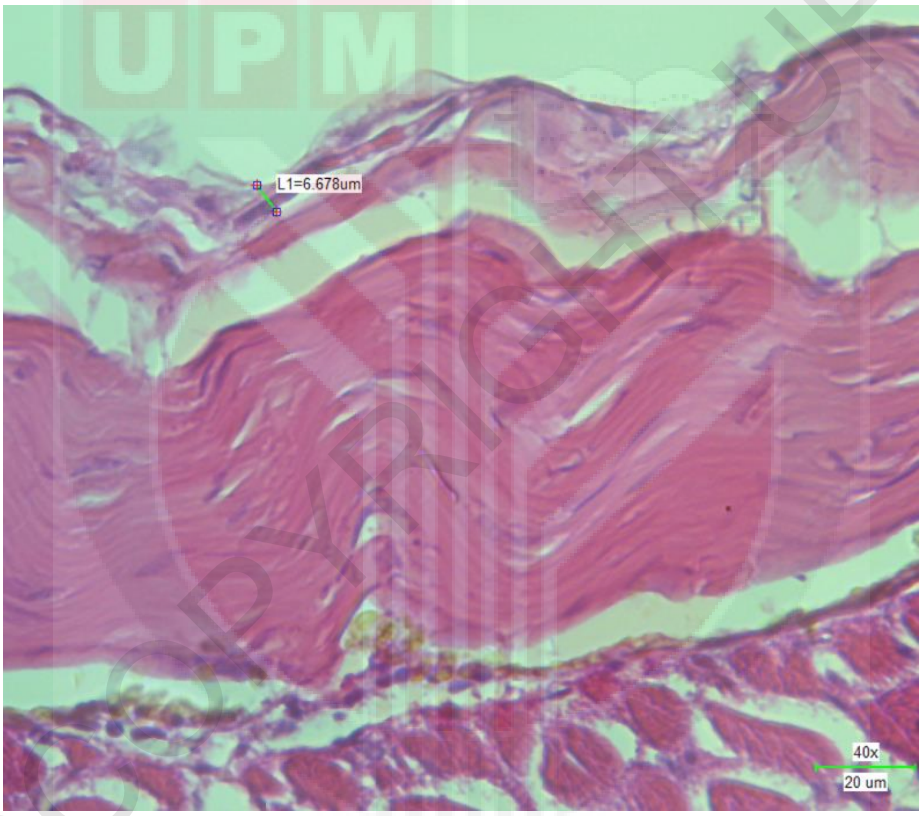

Figure 4.1.1: Thickness of skin epidermis for Group A. Photograph was captured at 40× magnification (scale bar: 20 µm).

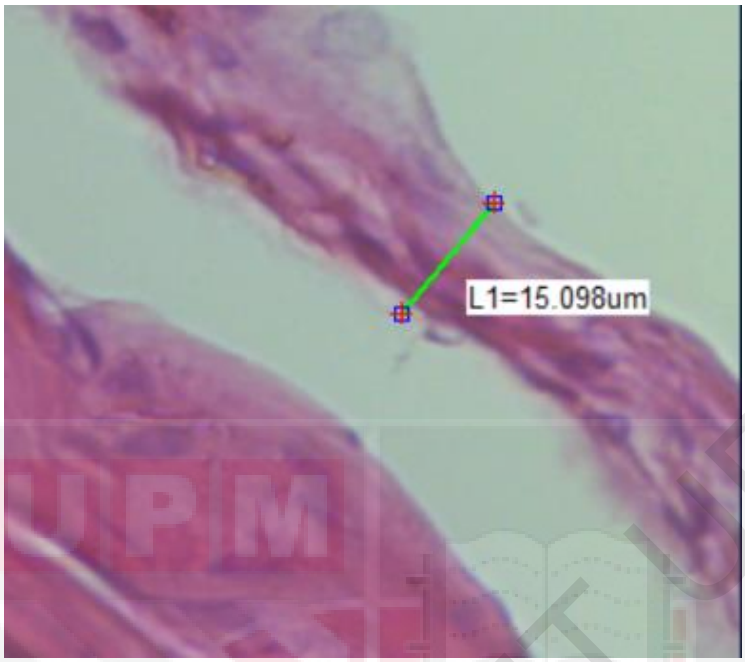

Figure 4.1.2: Thickness of skin epidermis for Group B. Photograph was captured at 40× magnification (scale bar: 20 μm).

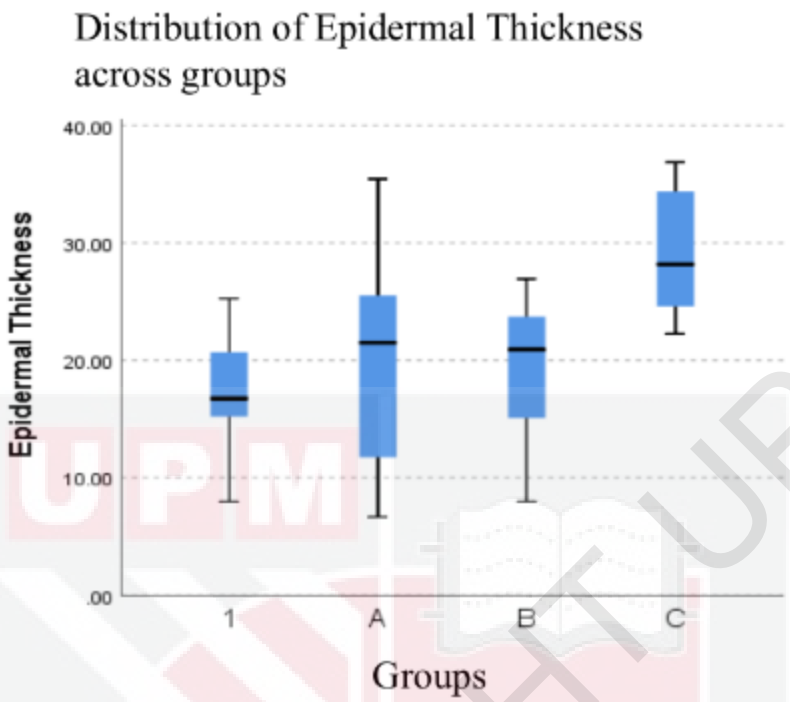

Figure 4.1.3: Distribution of thickness of skin epidermis in Group A (control), Group B (single dose), Group C (double dose), and Group 1 (natural infection) was monitored one week after their respective vaccination.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Epidermal Thickness is the same across categories of Slides.	Independent-Samples Kruskal-Wallis Test	.125	Retain the null hypothesis.
2	The distribution of Gills Inflammatory Cells Size is the same across categories of Slides.	Independent-Samples Kruskal-Wallis Test	.339	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .050.

Figure 4.1.4: Based on a statistical analysis of ANOVA, the Kruskal-Wallis test showed that there is no significant effect (p>0.05) for the epidermal thickness and gill inflammatory cell area between the groups.

4.2 Gill inflammatory cells area

In this study, immunization by immersion vaccination of formalin-killed *S. agalactiae* in Red hybrid tilapia resulted in insignificant changes (p>0.05) in the gill inflammatory cell area for the control, single-vaccination, subsequent by booster groups, and the natural infection group.

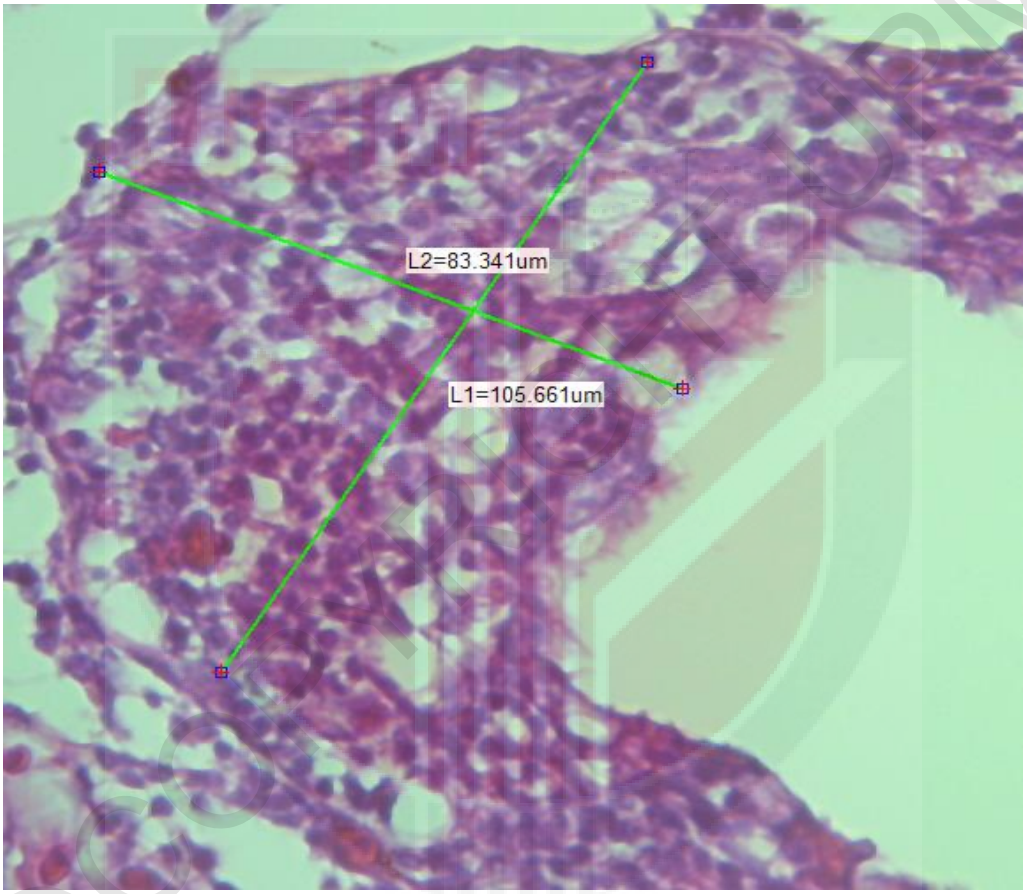

Figure 4.2.1: Gill inflammatory cells area for Group A. Photograph was captured at 40× magnification (scale bar: 20 µm).

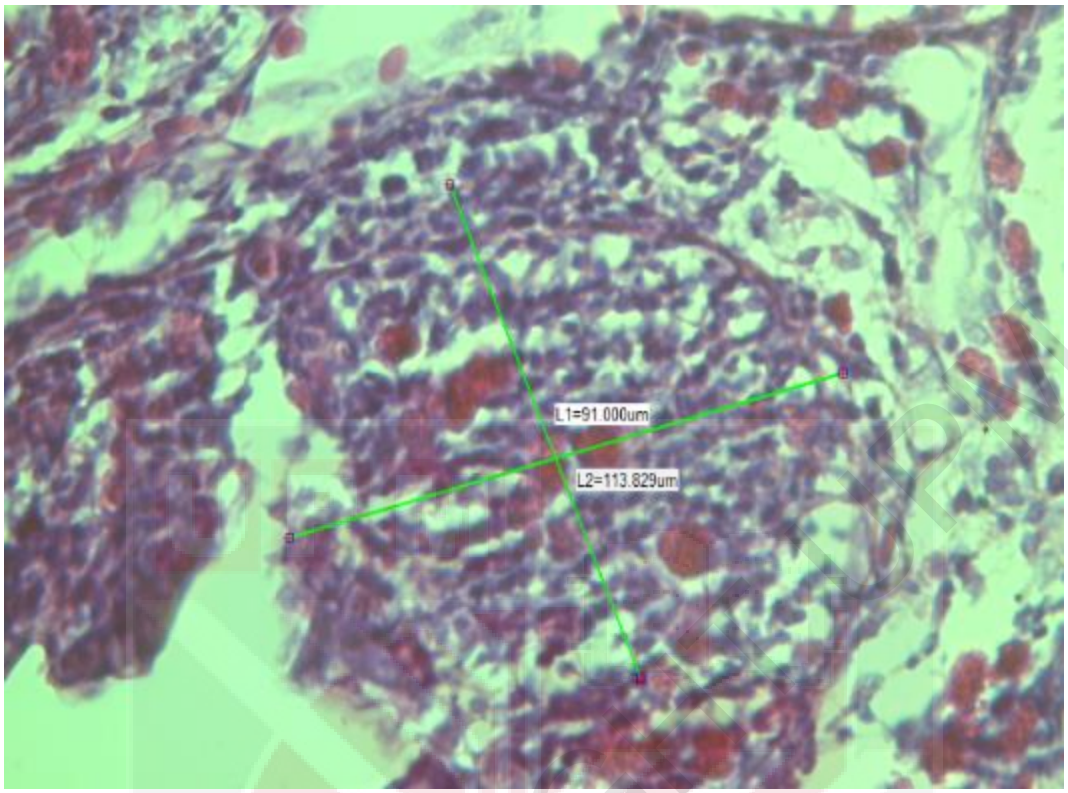

Figure 4.2.2: Gill inflammatory cells area for Group B. Photograph was captured at 40× magnification (scale bar: 20 µm).

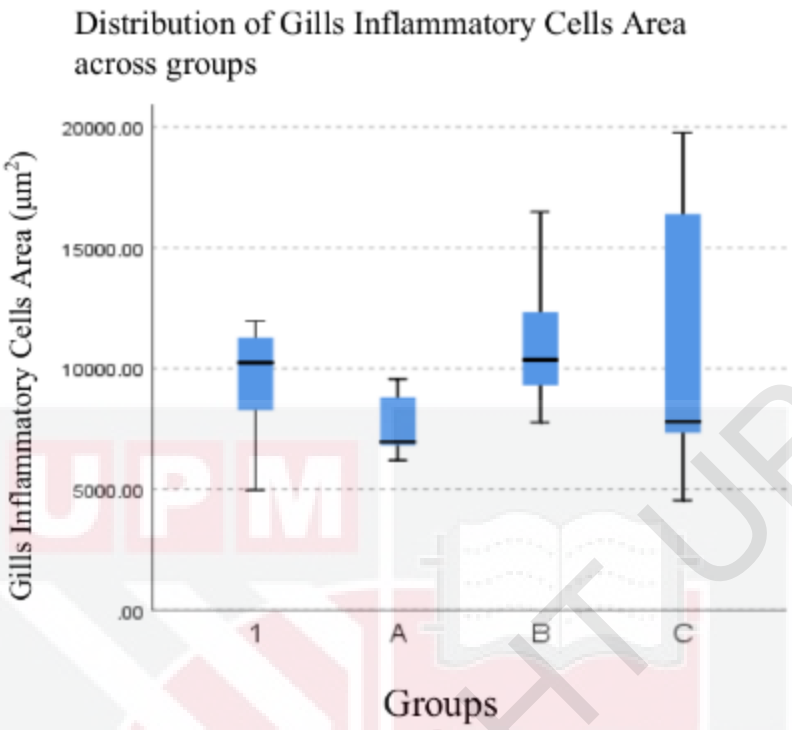

Figure 4.2.3: Distribution of gill inflammatory cells area in Group A (control), Group B (single dose), Group C (double dose), and Group 1 (natural infection) was monitored one week after their respective vaccination.

4.3 Clinical signs and mortality

Following natural infection of Group 1 with live *S. agalactiae*, the fish showed clinical signs such as a lethargic and erratic swimming pattern. The relative percent survival (RPS) for this group is 86.67% with 2 fish dying out of the 15 fish.

4.4 Bacterial isolation

Fish from Group 1 were sacrificed (euthanasia) and brain, kidney and eye samples were taken. These samples were also taken from the dead fish. After the culture process and incubation was complete, there was growth on the TSA plates revealing a pattern of small, smooth, round, and whitish colonies. Gram staining of the isolates revealed Gram-positive, paired or short-chain cocci.

Figure 4.3.1: Small, smooth, round whitish colonies that were suggestive of *S. agalactiae* on tryptic soy agar.

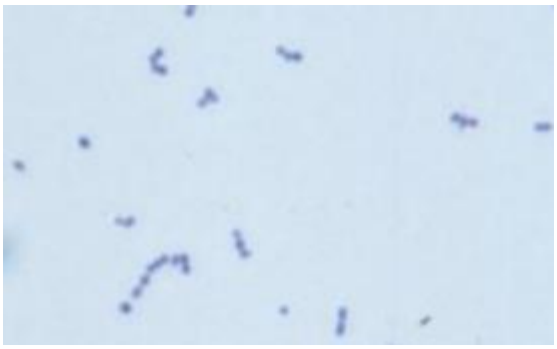

Figure 4.3.2: Gram-positive, cocci in short chains and pairs.

5.0 DISCUSSION

Mishra (2015) stated that vaccination is more optimal to prevent bacterial and viral diseases compared to using antibiotics because antibiotics can lead to safety and health issues consumers. In this study, evaluation of mucosal-associated lymphoid tissues (MALTs) in response to post-kill vaccine versus natural infection against *Streptococcus agalactiae* in Red hybrid tilapia (*Oreochromis* sp.) was determined. The method of vaccine administration and also of naturally infecting the in this study was the immersion method. This vaccination procedure can be beneficial for the tilapia fingerlings as the procedure can reduce the potential stress exerted unto the fish, such as stress of handling. This method is also superior in terms of labor compared to vaccination methods such as injection, which require more workers to administer the vaccines. One of the parameters recorded in this study was the response of skin-associated lymphoid tissues (SALTs). Skin-associated lymphoid tissues (SALTs) can directly affect changes in the skin and skin mucous cells in response to physically or chemically disturbances (Bunnoy et al., 2017). Therefore, in this study, fish skin epidermal thickness was measured to assess the response of MALTs following immersion vaccination and also natural infection.

Another parameter that has been considered to investigate the response of MALTs to vaccination and natural infection are gill-associated lymphoid tissues (GIALTs). This is due to the fact that GIALTs is one of the major mucosal-associated lymphoid tissues (MALTs) alongside with other lymphoid tissues such as skin-associated lymphoid tissues (SALT), gut-associated lymphoid tissues (GALT), and nasopharynx-associated lymphoid tissues (NALT). To determine the response of GIALTs, the area of accumulated inflammatory cells in the gill was calculated.

After each immersion, both skin and gill samples were taken for histological and statistical analysis.

This study showed that there are no significant differences in skin and gills post-vaccination and natural infection. Fish SALTs did not increase in terms of thickness and development. The accumulated inflammatory cells for GIALTs also have no development. These findings were determined by a statistical analysis performed by the Kruskal-Wallis test, and it revealed that there was no significant difference ($p>0.05$) in terms of the thickness of the epidermis and the area of the inflammatory cells in the gills for Group A, Group B, Group C and Group 1 along the study period. From these findings, we can conclude that for this particular study, the immersion method of vaccination does not actually affect the MALTs of the fish.

The relative percent survival rate (RPS) for Group 1 (natural infection) is 86.67%, while for other groups all fish survived. From this we can conclude that the immersion method for naturally infecting *S. agalactiae* in this particular study is not as effective in infecting the fish with streptococcosis. The mortality rate for streptococcosis in this study (13.33%) is comparatively lower than the mortality rate reported in other studies. He *et al.* (2018) reported that *S. agalactiae* is considered to be one of the natural pathogens affecting tilapia, and the mortality rate ranges from 30% to 100%. One of the possibilities that may contribute to this low mortality rate in the naturally infected group is through repeated subculture of bacteria. This can reduce the pathogenicity of the bacterial sample itself, since *Streptococcus* sp. is dynamic. Zamri *et al.* (2010) stated that the difference in lesion pattern for streptococcosis between natural infection and experimental

infection is that natural infection results in acute systemic infection causing sudden death, while experimental infection results in subacute and chronic infection. This could also explain the low mortality rate in this study, as the immersion method for natural infection in this project may not mimic naturally occurring *S. agalactiae* infection.

The immersion vaccination in this study may be considered less effective in making changes to the skin and gills MALTs. One of the possible reasons that may influence this result is that the optimal mucosal immune response and the maximum amounts of antibodies produced are reached around 14 days post-vaccination. At 21 days, the immune response approaches baseline immunity again, and the booster shot is usually administered. At 28 days, the immune response is usually at its peak and more developed compared to the first vaccination. However, in this study, the vaccination interval may be too short to properly develop the immune system and MALTs. The memory cells cannot develop during this duration either. There are also several other factors that can affect the efficacy of the vaccination procedure. Factors such as stress applied to the fish can cause immunosuppression and these can include handling, transport, and crowding. These factors may reduce the efficacy of the vaccine. Therefore, we can conclude that the fish in this study are not significantly protected by the immersion method due to the short vaccination interval and duration.

6.0 CONCLUSION

The result of this study showed that there was no significant difference (p>0.05) with respect to MALTs in the control group, Group A, vaccinated groups, Group B (single dose) and Group C (vaccine and booster) and naturally infected group, Group 1 in skin and gills of fish. Based on this project, we can conclude that immersion vaccination according to this study cannot induce sufficient immune response in tilapia. Other vaccine delivery methods such as oral feeding and injection should be studied and compared to the immersion method to further determine which is the most efficient to enhance the vaccination program in tilapia and in the aquaculture industry worldwide.

7.0 RECOMMENDATIONS

One of the recommendations that can be made for this project is to determine the health status of the fish before starting the project. Next, specimens of larger or adult tilapia can also be used for this study, since streptococcosis affect all ages of tilapia. Further study should also include a challenge program to determine the immune response of the vaccinated fish. This is done to ascertain the effect of immersion vaccination on the mucosal immunity of the fish compared to the control group. External stresses such as handling, and transport should be reduced to ensure that the fish maintain a stable immune status throughout the study. *S. agalactiae* can also be detected with other diagnostic methods such as ELISA and PCR to further confirm the antibodies in the skin and gills to enhance the relationship between the result and the mortality rate to draw a better conclusion.

REFERENCES

Bøgwald, J., & Dalmo, R. A. (2019). Review on immersion vaccines for fish: An update 2019. *Microorganisms*, *7*(12), 627. https://doi.org/10.3390/microorganisms7120627

Bunnoy, A., Na-Nakorn, U., & Srisapoome, P. (2019). Probiotic effects of a novel strain, *Acinetobacter* KU011TH, on the growth performance, immune responses, and resistance against *Aeromonas hydrophila* of bighead catfish (*Clarias macrocephalus* Günther, 1864). Microorganisms *7*(12), 1–30. https://doi.org/10.3390/microorganisms7120613

Defeng Zhang, Xiaoli Ke, Lihui Liu, Maixin Lu, Cunbin Shi & Zhigang Liu (2018). *Streptococcus agalactiae* from tilapia *(Oreochromis* sp.) transmitted to a new host, bighead carp *(Aristichthys nobilis),* in China. Aquacult Int 26:885–897.

El-Sayed, A.F.M. (2006). Tilapia culture. In *Tilapia Culture* (Issue January). https://doi.org/10.1079/9780851990149.0000

FAO. (2013). FAO Fisheries & Aquaculture National Aquaculture Sector Overview (NASO). Retrieved from http://www.fao.org/fishery/countrysector/naso_malaysia/en

Hayat, M., Mohd Yusoff, M. S., Samad, M. J., Abdul Razak, I. S., Md Yasin, I. S., Thompson, K. D. and Hasni, K. (2021). Efficacy of feed-based formalin-killed vaccine of *Streptococcus iniae* stimulates the gut-associated lymphoid tissues and immune response of red hybrid tilapia. *Vaccines*, *9*(1), p. 51. https://doi.org/10.3390/vaccines9010051

He, Y., Huang, J.-L., Wang, K.-Y., Chen, D.-F., Geng, Y., Huang, X.-L., Ou-Yang, P., Zhou, Y., Wang, J., Min, J., & Lai, W.-M. (2018). Pathogenicity of *streptococcus agalactiae* in *oreochromis niloticus*. *Oncotarget.* https://doi.org/10.18632/oncotarget.23551

Huang, T., Li, L. P., Liu, Y., Luo, Y. J., Wang, R., Tang, J. Y. and Chen, M. (2019). Spatiotemporal distribution of *Streptococcus agalactiae* attenuated vaccine strain YM001 in the intestinal tract of tilapia and its effect on mucosal associated immune cells. *Fish & Shellfish Immunology*, *87*, 714–720. https://doi.org/10.1016/j.fsi.2019.02.003

Mishra V., (2017). Fish Vaccination: Introduction., Delve Publishing LLC., New York

Siti-Zahrah A, Padilah B, Azila A, Rimatulhana R, Shahidan H (2005) Multiple streptococcal species infection in cage-cultured red tilapia, but showing similar clinical signs. In: *Proceedings of the Sixth Symposium on Diseases in Asian Aquaculture*. MG Bondad-Reantaso, CV Mohan, M Crumlish, RP Subasinghe, Eds., Colombo, Sri Lanka, pp. 332-339.

Zamri-Saad, M., Amal, M. N. A., & Siti-Zahrah, A. (2010). Pathological changes in red tilapias (Oreochromis spp.) naturally infected by *streptococcus agalactiae*. *Journal of Comparative Pathology*, *143*(2-3), 227–229. https://doi.org/10.1016/j.jcpa.2010.01.020