UNIVERSITI PUTRA MALAYSIA

EFFICACY OF IMMERSION AND ORAL VACCINATION AND THEIR COMBINATION AGAINST *Aeromonas hydrophila* IN JUVENILE HYBRID RED TILAPIA (*Oreochromis* sp.)

SITI AISHAH ABD RASHIT

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SITI AISHAH ABD RASHIT

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

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CERTIFICATION

It is hereby certified that I have read this project paper entitled "EFFICACY OF IMMERSION AND ORAL VACCINATION AND THEIR COMBINATION AGAINST *Aeromonas hydrophila* IN JUVENILE HYBRID RED TILAPIA (*Oreochromis* sp.*)"* by Siti Aishah Binti Abd Rashit and in my opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course VPD 4999 – Final Year Project.

_____

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(Supervisor)

DEDICATION

"So, verily, with every difficulty, there is relief.

Verily, with every difficulty there is a relief."

-Q.S. Al-Insyirah (94:5-6)

This project paper is dedicated to Allah the Almighty, He sent the messenger, holy Prophet Muhammad (peace be upon him) whose way of life has been a continuous guidance for us. He had created me and made all circumstances possible throughout this project.

To my family,

My father, Abd Rashit Omar,

My mother, Zaileha Husin,

My siblings: Muhammad Muaz, Abd Qayyum, Abd Qawiy,

My friends,

DVM 2023,

For their endless support, morally and physically; may Allah S.W.T bless each one of you for I am able to complete this study.

ACKNOWLEDGEMENTS

All praise be to Allah the Greatest, and His blessings for the completion of this study. My humblest gratitude to the Almighty for all the opportunities, trials and strength poured out during this study.

My utmost appreciation to the people who were involved in making this final year project a great success. To my beloved parents Mr. Abd Rashit Omar and Mrs. Zaileha Husin, and my family, whom I can never forget to owe my indebtedness to them for providing all the care in the world that I ever ask for, with indefinite compassion, and who had cheered and prayed endlessly for me.

Special thanks go to my supervisor, Assoc. Prof Dr Md Sabri Mohd Yusoff for his constant inspiration, patience, and guidance. I would also like to acknowledge with well respect, Dr. Sani and Dr. Najwa for their time, support and aid.

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Special thanks to my partners during this study, Suriani Khalid and Adam Lee, my friends Syakirah, Mira El and Fatimah as I could not have completed this study without their companion.

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LIST OF ABBREVIATION

A. hydrophila	*Aeromonas hydrophila*
sp.	species
O. mossambicus	*Oreochromis mossambicus*
O. aureus	*Oreochromis aureus*
CFU/mL	Colony Forming Unit per milliliter
H_0	Null hypothesis
H_1	Alternative hypothesis
BHI	Brain Heart Infusion
rpm	Revolutions per minute
µm	Micrometre
H&E	Hematoxylin and eosin
SALT	Skin-Associated Lymphoid Tissue
PBS	Phosphate-buffered saline
FKV	Formalin-killed vaccine
TDFV	Top-dressed feed vaccine
TSA	Trypticase Soy Agar
IP	Intraperitoneal
DPX	Distyrene Plasticizer Xylene
SEM	Standard error of mean
ELISA	Enzyme-linked immunosorbent assay
°C	Degree Celsius
%	Percentage
SPSS	Statistical Package for the Social Science
p	*p*-value (significance)

ABSTRAK

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

KEBERKESANAN VAKSINASI MELALUI RENDAMAN DAN ORAL DAN GABUNGANNYA TERHADAP *Aeromonas hydrophila* PADA ANAK IKAN TILAPIA HYBRID MERAH (*Oreochromis* sp.)

oleh

Siti Aishah Binti Abd Rashit

2022

Penyelia: Prof. Madya Dr Md Sabri Mohd Yusoff

Aeromonas hydrophila adalah salah satu bakteria penting yang menjangkiti ikan tilapia hybrid merah (*Oreochromis* sp.). Vaksinasi dapat memberikan perlindungan terhadap ikan daripada wabak penyakit, namun kaedah pemberian vaksin turut memainkan peranan yang krusial dalam mengoptimumkan keberkesanan vaksin. Justeru, kajian ini dijalankan untuk mengkaji perbezaan antara kesan pemberian vaksin secara oral, rendaman, dan gabungan kedua-duanya terhadap *A. hydrophila* dalam anak tilapia hybrid merah. Sejumlah 75 anak tilapia hybrid merah dengan berat 15g ± 5g telah dibahagikan kepada 5 kumpulan mengikut kaedah pemberian vaksin, iaitu vaksinasi oral diikuti vaksinasi penggalak melalui rendaman (Kumpulan OI), vaksinasi rendaman diikuti penggalak oral (Kumpulan IO), vaksinasi oral diikuti penggalak oral (Kumpulan OO), vaksinasi rendaman diikuti penggalak rendaman

(Kumpulan II) dan akhir sekali, kumpulan yang tidak divaksinasi bertindak selaku kawalan negatif. Vaksinasi pertama bermula pada minggu 0, kemudian diikuti dengan vaksinasi penggalak pada minggu 1, dan seterusnya cabaran dengan *A. hydrophila* pada minggu 2. Pengambilan sampel kulit dan insang untuk histopatologi dilakukan selepas cabaran untuk menilai tindak balas mukosa. Ketebalan epidermis, bilangan tisu limfoid yang berkaitan dengan kulit (SALT), dan saiz sel inflamasi pada insang dianalisis. Apabila dibandingkan dengan kumpulan kawalan, ketebalan epidermis dan bilangan SALT menunjukkan perbezaan yang tidak signifikan antara semua kumpulan. Namun, saiz sel inflamasi pada insang menunjukkan perbezaan yang signifikan ($p<0.05$) dalam kumpulan II. Justeru, membuktikan vaksinasi dan penggalak secara rendaman menjadi faktor terbesar kepada tindak balas immuniti mukosa. Perbandingan antara semua kumpulan menyokong hipotesis bahawa tindak balas immuniti mukosa adalah berkait dengan kaedah pemberian vaksin yang berbeza.

Katakunci: tilapia, *Aeromonas hydrophila*, vaksinasi oral, vaksinasi rendaman

ABSTRACT

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

EFFICACY OF IMMERSION AND ORAL VACCINATION AND THEIR COMBINATION AGAINST *Aeromonas hydrophila* IN JUVENILE HYBRID RED TILAPIA (*Oreochromis* sp.)

by

Siti Aishah Binti Abd Rashit

2022

Supervisor: Assoc. Prof Dr Md Sabri Mohd Yusoff

Aeromonas hydrophila is one of the important bacteria infecting the hybrid red tilapia (*Oreochromis* sp.). While vaccination is a crucial protection of fish against disease, the route by which the vaccine is administered also plays a dependent role in optimizing its efficacy. Therefore, this study aims to differentiate the effects of oral vaccination, immersion vaccination, and their combination against *A. hydrophila* on juvenile hybrid red tilapia. A total of 75 juvenile hybrid red tilapia of 15g ± 5g were divided into 5 groups, which were categorized into types of administration routes, namely oral vaccination with immersion booster (group OI), immersion then oral booster (IO group), oral then oral booster (group OO), immersion then immersion boosters (group II), and finally an unvaccinated group as a control group. The first vaccination started at week 0, followed by a booster vaccination at week 1, then a challenge with live *A. hydrophila* at week 2. Skin and gill samples were collected post-

challenge for histology to assess the mucosal immune responses. The thickness of epidermis, number of skin-associated lymphoid tissue (SALT), and inflammatory cells size in gills were assessed. Compared to control group, the epidermis thickness and number of SALT were insignificantly different in all groups. However, size of gills' inflammatory cells in II group were significantly (p<0.05) increased post-challenge. This shows that immersion-immersion vaccination was most attributable to the mucosal immune response. The comparison between these groups supports the hypothesis that there is a correlation between mucosal immune response and different vaccination routes.

Keywords: tilapia, *Aeromonas hydrophila*, oral vaccination, immersion vaccination

1.0 INTRODUCTION

1.1 STUDY BACKGROUND

In Malaysia, tilapia has become one of the staple fish dishes, with production scattered across the country and a strong domestic market for the species (Fitzsimmons, 2006). The increasing demand for freshwater supply in many countries may create a need for research into aquaculture in brackish water and seawater. Tilapia might be the best candidates as they can grow and reproduce in very high salinity (Abdel-Fattah, 2006). Abdullah et al. (2013) also stated that tilapia is one of the freshwater species that is grown rapidly and capable of living in a poor water environment, referred as "aquatic chicken" that is easy to breed with simple hatchery technology.

Although tilapia is known to be a hardy fish, continuous exposure to pathogens in cages during a given culture period would be detrimental to the fish (Najiah et al., 2012). Tilapia production has increased significantly over time from the ecosystem, but globally this sector has faced few health risks, being impacted by emerging and re-emerging bacterial diseases causing severe losses to the economy in the industry (Adikesavalu et al., 2017; Vásquez-Machado et al., 2019).

Vaccination against bacterial pathogens is used to prevent this problem by inducing adaptive host immunity (Sommerset et al, 2005). However, fish vaccination has not really taken off in Asia as this new concept is not well known and understood among farmers and professionals (Mishra, 2017). Vaccination can be administered through different routes such as injection, immersion, spray or orally. Oral vaccination

would be the ideal vaccination method as it consumes less labour and low cost (Zamri et al., 2014).

The efficacy of administration depends on the studies evaluating the immunity induced by vaccination in the host. This experimental study was carried out to compare the efficacy of immersion and oral vaccination and their combination against *A. hydrophila* in juvenile hybrid red tilapia. The result of this study will be used to determine the best vaccination route for large-scale production. Besides that, the immune response induced by the host against the bacteria is assessed by the production of the mucosal immune system which includes the thickness of the epidermis, the number of skin-associated lymphoid tissue (SALT), and the size of the inflammatory cells in the gills from the experimentally infected animal.

1.2 JUSTIFICATION

This study will reveal the information regarding the mucosal immune response between the different vaccination methods, namely immersion and oral vaccination and their combination. This will assist in determining the choice of vaccination delivery that is more cost-effective to practice in the field to better protect fish from the pathogen.

1.3 OBJECTIVE

Objectives of this study were:

1. To differentiate the effect of immersion vaccination and oral vaccination and their combination of formalin-killed bacterin (*A. hydrophila*) in juvenile hybrid red tilapia (*Oreochromis* sp.).
2. To assess the mucosal immune response in the gill and skin-associated lymphoid tissue after each type of vaccination route; immersion, oral and both immersion and oral vaccination in juvenile hybrid red tilapia (*Oreochromis* sp.) against *A. hydrophila*.
3. To observe the histopathological changes of epidermis thickness, gills and skin-associated lymphoid tissue (SALT).

1.4 HYPOTHESIS

Hypotheses of this study were:

H_o: There is no significant difference in mucosal immune response and different routes of vaccination with formalin-killed bacterin (*A. hydrophila*) in juvenile hybrid red tilapia.

H_a: There is a significant difference in mucosal immune response and different routes of vaccination with formalin-killed bacterin (*A. hydrophila*) in juvenile hybrid red tilapia.

2.0 LITERATURE REVIEW

2.1 HYBRID RED TILAPIA (*Oreochromis sp.)*

Hybrid red tilapia is not a true species of tilapia. They are produced from selected tilapia species of the genus *Oreochromis*, which have an attractive red coloration as a result of continuous selective breeding (Mohamad et al., 2021). It is a species of freshwater fish belonging to the Cichlidae family, of which only a few species from this family are important in terms of commercial and farming, including the Nile tilapia (*Oreochromis niloticus*), the Blue tilapia (*O. aureus*) and various crosses of red hybrids of the first two species with *O. mossambicus* (Mozambique tilapia) (Shelton and Popma, 2006). According to Teh Si Win (2010), these tilapia have many characteristics that favour the aquaculture industry, including a relatively short culture period (about 6 months) to reach market size (600-900 g), a high tolerance to poor water quality, and a high stocking densities, and high productivity rates.

Tilapia is the second most farmed fish in the world, and its production rate has increased over the past decade due to its suitability for aquaculture, good marketability, and stable prices (Wang and Lu, 2016.) A study by Zhang et al. (2018) stated that tilapia has been regarded as the most resilient fish, which is more disease-resistant compared to the other cultured species, but since the development of intensive culture system for culture purposes, the incidence of disease due to bacteria, parasites, nutrition and fungus had increased (Carlos et. al, 2014). Bacterial species caused by Gram-negative and Gram-positive bacteria are likely to cause more problems in tilapia production than other pathogens (Shoemaker et al., 2006)

2.2 *Aeromonas hydrophila (A. hydrophila)*

A. hydrophila of the Aeromonadaceae family is a Gram-negative, facultatively anaerobic, oxidase-positive Bacillus bacteria (Papa et al., 2014). It exists naturally in numerous types of freshwater bodies as well as other environments. In the aquaculture world, *A. hydrophila* is considered as one of the aetiological agents of Motile Aeromonas Septicaemia (MAS) (Hasan et al., 2006). It is also a food-borne pathogen that can cause zoonotic diseases (Guz and Kozinska, 2004). Basri et al. (2020) also noted that aeromoniasis is a common bacterial disease affecting tilapia culture, particularly involving *A. hydrophila.* The isolated colonies of *A. hydrophila* appear yellowish opaque, round, convex, smooth-edged and semi-translucent on tryptic soy agar (TSA) medium (Nahar et al., 2016). *A. hydrophila* is also emerging as a worldwide disease in the human population.

Infection with *A. hydrophila* concurrent with environmental stressors such as overcrowding, handling, transportation and sudden changes in water temperature, the clinical signs of *A. hydrophila* infection can range from sudden death in healthy fish to loss of appetite, aberrant swimming patterns, pale gills, abdominal distension and skin ulceration (Paloma et al., 2017). In addition, the external clinical signs of the affected tilapia make it unmarketable. Motile Aeromonas Septicaemia (MAS) has chronic characteristics lasting for a several weeks, during which the mortality rate gradually increases, and the cumulative mortality can be high (Plumb and Hanson, 2011; Zhang et al., 2016). It can cause local pathology of the host tissue. The host response takes the form of tissue proliferation, degeneration and inflammation (Stratev et al., 2015).

2.3 VACCINATION IN FISH

Vaccination is the administration of agent-specific or disease-causing microorganisms, but relatively harmless as it consists of weakened or killed forms of microbes, its toxin or one of its surface proteins (Mishra, 2017), antigenic components that can be induced in vaccinated individuals' protective immunity against the relevant infectious agent. Vaccines are recognized as crucial tools to prevent and control fish diseases. Commercially available fish vaccines have increased in recent years, but there are still many diseases for which vaccines are not available or cases where existing vaccines do not execute well (Fish Vaccine, 2020). Dong et al. (2020) stated that the most common vaccine administrations in fish farms are through injection, oral, and immersion.

However, most tilapia farmers prefer to vaccinate by immersion or orally due to lower cost and ease of application (Hoare et al., 2021). Oral vaccination is the most practical method as it has no fish size limitations and requires minimal infrastructure and specialised skills for effective implementation (Zamri et al., 2014). However, in an aquatic environment, due to water conditions and a large number of fish, a significant amount of vaccine is required for effective vaccination. In injection vaccination, it offers a stronger protective effect compared to oral vaccination and immersion vaccination (Shoemaker et al., 2006). Injection vaccination allows multiple combinations in a single vaccine and ensures that all fish have achieved the correct dose of vaccine (Mishra, 2017), but requires special skills, labour extensive and is expensive.

2.5 ORAL VACCINATION

Oral vaccination represents the easiest way to administer antigen for vaccination as it is less stressful, suitable for both small and large fish, and has no side effects (Mutoloki et al., 2015). For oral vaccination to be effective, the antigen is either incorporated into the feed or encapsulated in a capsule or lipid that protects the vaccine components from destruction by the fish's digestive tract enzymes (Klesius, Evans, Shoemaker & Lim, 2006). In a study by Dhar et al. (2014), he stated that few oral vaccines have been registered in the aquaculture industry, possibly due to their poor performance as a result of antigen degradation in the fish stomach before reaching the second segment of the intestine where absorption occurs.

The effectiveness of the oral vaccine depends on the nature of antigens, formulation, and dosage to improve the stimulation of protective immunity. With oral vaccination, it is often difficult to determine the dose at an individual level (Mutoloki et al, 2015). Despite its poor ability to induce immunity as a primary vaccination, oral vaccination as a booster dose had shown a significant result by extending the protection (Ballesteros et al., 2014).

Oral immunoprophylaxis serves its purpose as protection. However, protection by oral immunisation has proven inconsistent. Digestive degradation has been implicated as a factor in this inconsistency, since antigenic integrity must be retained until the immunogen reaches the distal intestine, which has been identified as an immunologically active part of the gastrointestinal tract involved in the uptake of antigens (Dalmo & Horne cited in Bikramjit Ghosh et al., 2015).

Disadvantages of vaccination by the oral route include antigen degradation during gastric transit, decreased structural integrity and absorption in the second part of the intestine known as the main site for antigen absorption, and vaccine formulation inability to stimulate protective immunity for improvement (Mutoloki et al., 2015). The protection conferred by oral immunisation has been shown to be inconsistent, with digestive degradation believe to be the main cause of this inconsistency (Bikramjit et al., 2016).

2.5 IMMERSION VACCINATION

Immersion vaccination of fish is the most common method, particularly in younger animals. In immersion vaccination, this can be done by dip or bath, with dip vaccination, fish are immersed in a solution containing a highly concentrated vaccine, while bath vaccination, fish are immersed in a longer duration of time in low concentrated vaccine solution. In other words, the immersion vaccination acts on the ability of the mucosal surface of the fish, which had the ability to recognize the pathogen and evoke an immune response by the action of antibody-secreting cells present in the skin and gill epithelium (Mishra, 2017).

In addition, it leads to uptake by the skin, gills, and intestines (after drinking), which subsequently triggers local responses. Mucosal administration of antigens offers the most feasible approach to immunisation of small fish. It also specifically targets the stimulation of mucosal immunity in fish, which is the first line of defence against most pathogens (Rambout & Gomez, cited in Bikramjit Ghosh et al., 2015).

3.0 MATERIALS AND METHOD

3.1 FISH AND EXPERIMENTAL CONDITION

75 juvenile red hybrid tilapia (*Oreochromis* sp.) with an average weight of 15 g ± 5 g were obtained from Shah Alam, Selangor. They were acclimatized in the tank for a few days to accustom them to the new environment before being divided into 5 groups. Group OI, group IO, group OO, and group II served as a positive control and the group control as a negative control. Prior to the experiment, all aquariums were cleaned and disinfected, then filled with approximately 70 litres of dechlorinated water for each aquarium. Continuous aeration was continuously provided throughout this study using an automated aerator. The fish were fed with commercial feed pellets twice a day and the water were changed twice a week. Water quality and temperature were monitored throughout the study and water tests was performed using the Freshwater Master Test Kit to test for pH, ammonia, nitrite, and nitrate.

3.2 BACTERIAL ISOLATE AND GROWTH CONDITION

The isolate of *A. hydrophila* was acquired from an outbreak of *Aeromonas hydrophila* infection at Kenyir Lake, Terengganu in 2013. Brain Heart Infusion (BHI) agar was prepared before the isolate was then subcultured onto the BHI agar plate and incubated at 37^{o}C overnight.

3.3 PREPARATION OF FORMALIN-KILLED VACCINE (FKV)

A loop of *A. hydrophila* colony from a BHI agar plate was further inoculated into BHI broth and incubated in a shaker incubator at 30°C for 24 hours. Growth of bacteria was observed by the presence of a cloudy appearance. The cultures were then centrifuged at 10,000 rpm at 16°C for 3 minutes and the supernatant removed leaving the pelleted cells. The pellets were then washed three times with phosphate-buffered saline (PBS) before being treated with buffered formalin at a concentration of 0.5% formalin in phosphate-buffered saline (PBS) and kept at 4°C overnight. Then the bacteria were diluted to 1 x 10^9 CFU/ml (determined by serial dilution) as stock vaccine used for feed-based vaccine preparation and immersion vaccination. The solution was cultured in TSA as negative growth indicates successful inactivation of bacteria.

3.4 PREPARATION OF TOP-DRESSED FEED VACCINE (TDFV)

500 mL FKV was sprayed directly onto 500 g commercial fish feed pellet and mixed thoroughly. The mixture was then dried up at 30°C for 24 to 48 hours in the incubator to be used as an oral vaccine in this study. A sample of the feed was taken and subjected to Gram-staining to observe the presence of an inactivated cell in the feed due to the presence of Gram-positive cocci bacteria.

3.5 PREPARATION OF *A. hydrophila* FOR CHALLENGE

The bacteria were subcultured into BHI broth and incubated in shaker incubator at 30°C for 24 hours. Growth of bacteria was observed by the presence of a cloudy appearance. The cultures were then centrifuged at 10,000 rpm at 16°C for 3 minutes and the supernatant removed leaving the pelleted cells. The pellets were then washed six times with phosphate-buffered saline (PBS). The last concentration of live *A. hydrophila* was recorded as 1×10^9 CFU/mL and was used immediately for the challenge experiment.

3.6 EXPERIMENTAL DESIGN

The study was conducted over a period of seven weeks. The first vaccination took place at week 0. At week 0, groups OI and OO received the first vaccination orally twice daily with TDFV for three consecutive days at a rate of 2% of bodyweight. Groups IO and II received the first vaccination by immersion in FKV for 30 seconds. At week 1, groups OI and II were boosted by immersion, while groups IO and OO were boosted orally twice daily with TDFV for three consecutive days at a rate of 2% of bodyweight. The group control served as a negative control group in which no vaccination was administered. The following week at week 2, all groups including the control group were challenged with 0.1 mL of 1×10^9 CFU/mL *A. hydrophila* by intraperitoneal (IP) injection. Post-challenge, all fish were observed for mortality and samples from three fish from each aquarium were collected. The skin and gill samples were subjected to histopathologically to assess the mucosal immune response.

3.7 HISTOLOGY

All skin and gill samples were placed in 10% buffered formalin fixatives at a ratio of 1:10 for 24 hours. The samples were then trimmed into the cassette to a size of 1 cm × 1 cm before being fixed in 70% alcohol, drained, and processed overnight in the tissue processing stage. The aim of pre-embedding is to infiltrate tissue samples with paraffin and replace the water content of the tissue with this wax material. Pre-embedding is a sequential process consisting of dehydration of tissues in increased concentrations of alcohol solutions, and subsequent gradual replacement of alcohol by a paraffin solvent. As a result, the tissue sample was dehydrated with the graded series of alcohol and washed paraffin solvent (xylene), then the tissue sample was completely infiltrated with paraffin. Tissue samples were retrieved at the end of the processing program (automates are usually run overnight to start the embedding process the next morning). The samples were then embedded in paraffin wax. After cooling and trimming, the samples were sectioned at 4 µm, dried and stained with haematoxylin & eosin (H&E), according to the Harris haematoxylin and eosin (H&E) staining protocol. The glass slide was mounted using distyrene plasticizer xylene (DPX) after the staining process and dried overnight. Finally, the slides were viewed under the microscope, and the average size of the inflammatory cells in the gills was measured using the FIVE Image Analyzer (Olympus, Japan), while the number of SALT was measured using the Image analyser NIS-Elements D. 3.2 (Nikon, Japan).

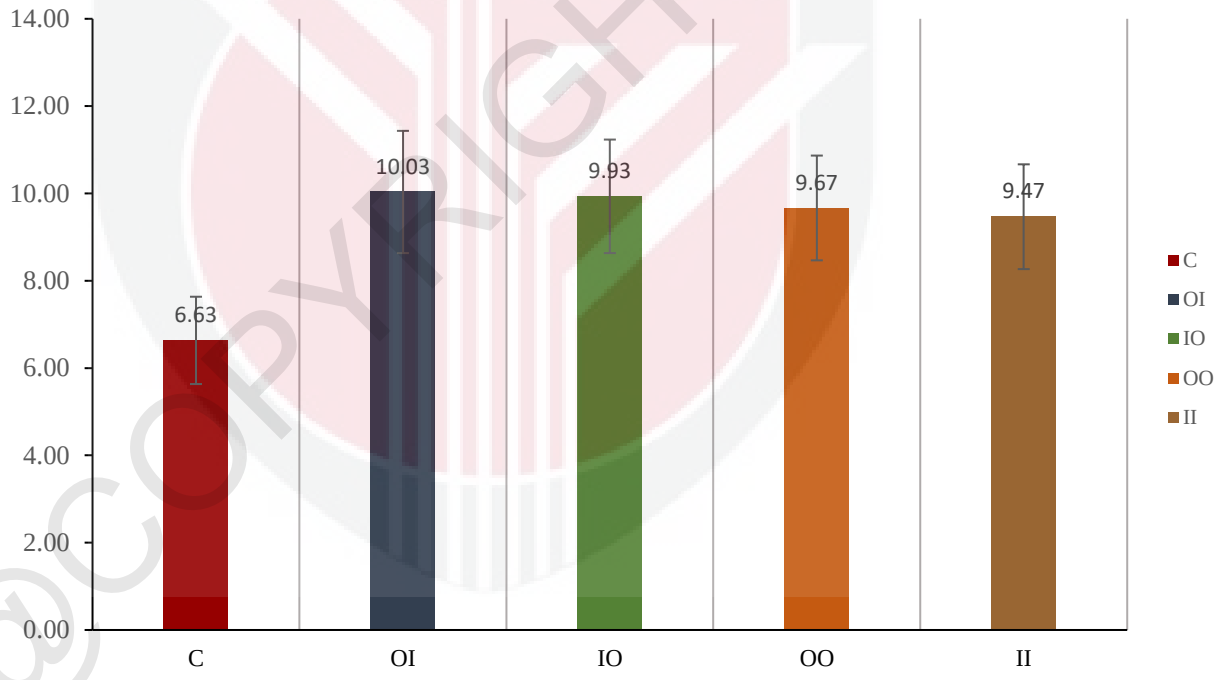
3.8 STATISTICAL ANALYSIS

Data were checked for normality using the Shapiro-Wilk test of normality (IBM SPSS Statistics Version 25). The mean ± standard error of mean (SEM) of epidermal thickness, the number of SALT, and the size of the inflammatory cells in the gills were compared using the Kruskal-Wallis test. The significance value was at $p<0.05$. The measured values determined were the average thickness of the epidermis, the number of SALT, and the size of the inflammatory cells in the gills.

4.0 RESULTS

4.1 EPIDERMIS THICKNESS

The thickness of the epidermis differs insignificantly ($p>0.05$) in all groups. However, all vaccinated groups (Group OI, Group IO, Group OO, Group II) generally have a higher number compared to the unvaccinated (Group Control), even insignificant. Therefore, we can conclude that all types of vaccination routes elicit some mucosal immune response compared to the unvaccinated control group.



Graph 1: *Bar graph showing the thickness of the epidermis for the control group (C), oral-immersion group (OI), immersion-oral group (IO), oral-oral group (OO), and immersion-immersion group (II).*

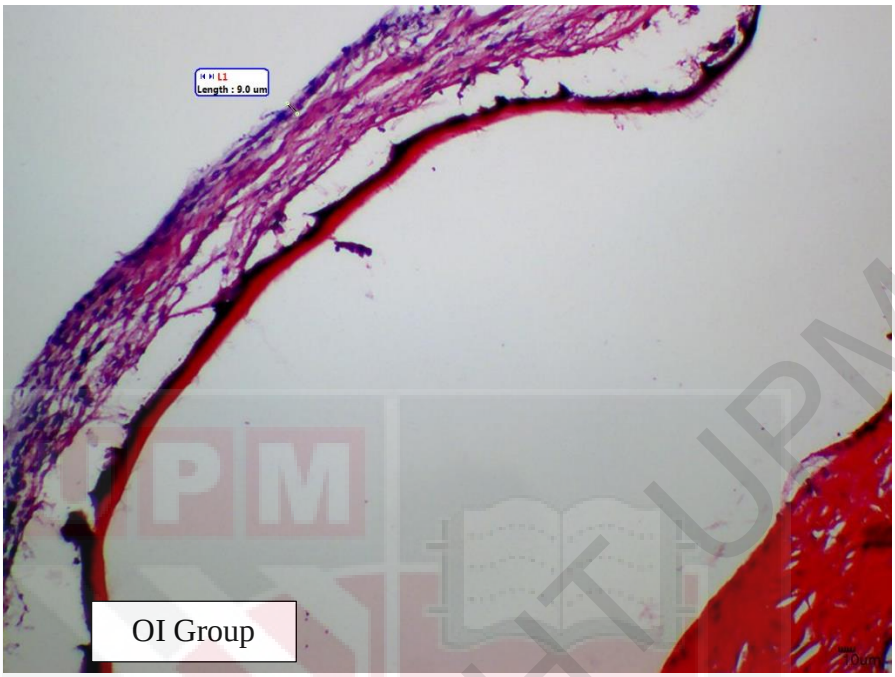


Figure 1

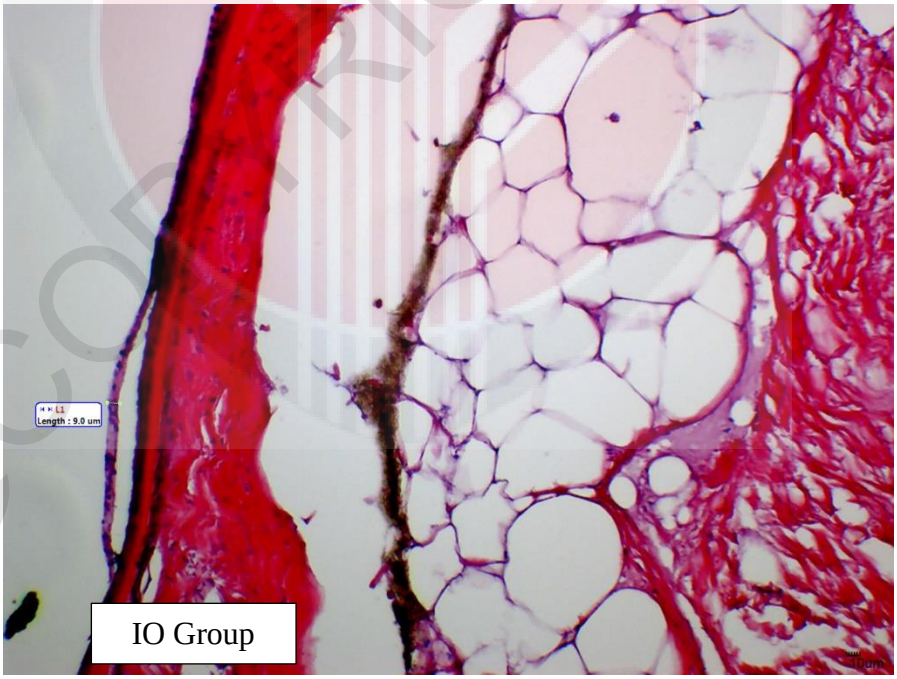


Figure 2

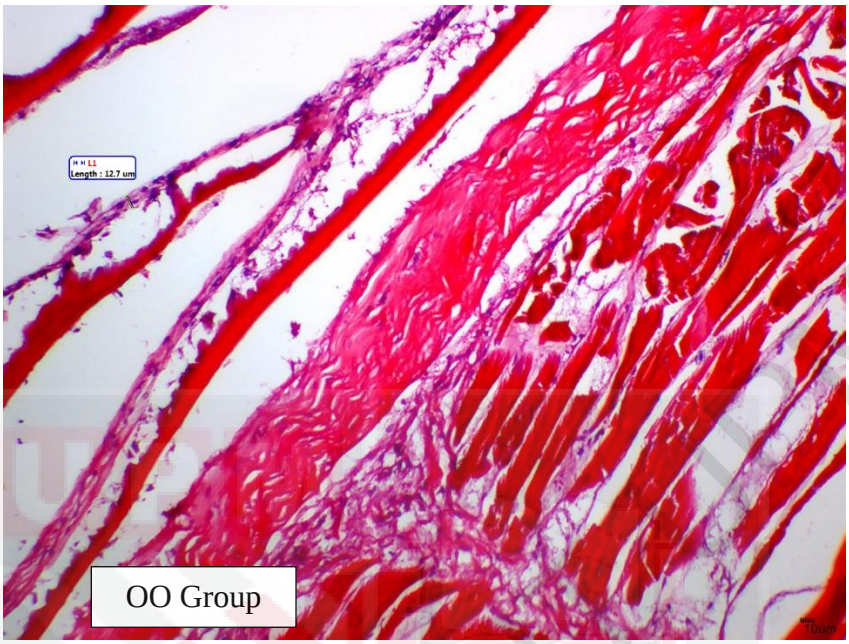


Figure 3

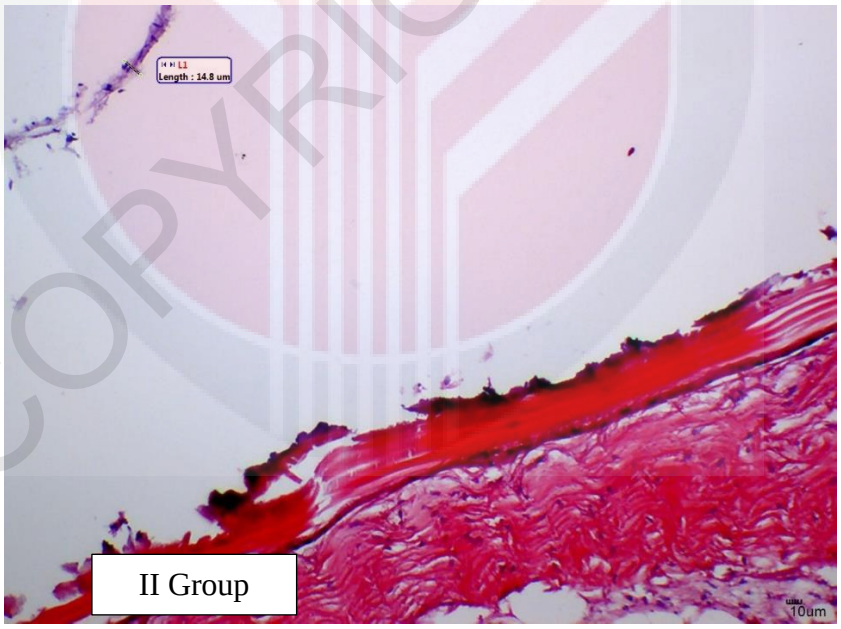


Figure 4

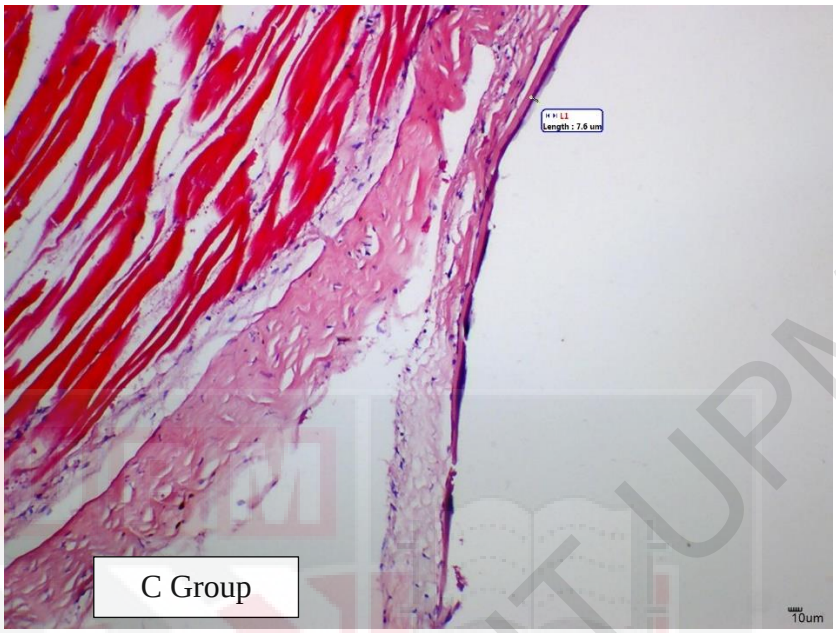
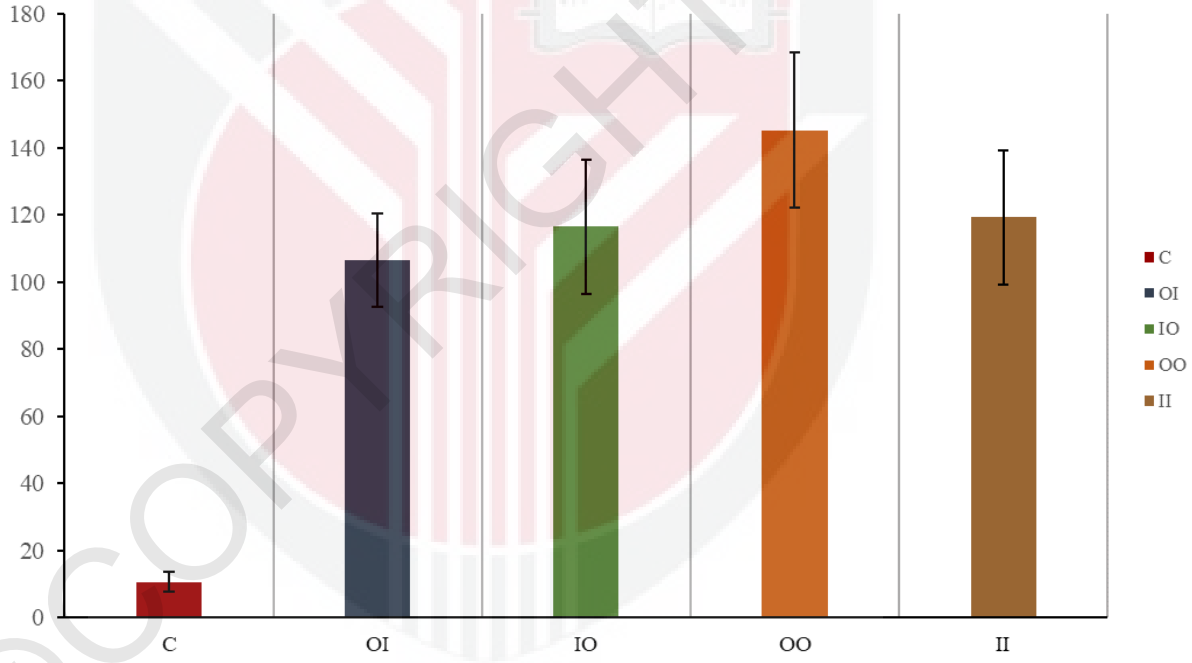


Figure 5

Figure 1-5: *Example of the thickness of the epidermis of oral-immersion group (OI), immersion-oral group (IO), oral-oral group (OO), immersion-immersion group (II) and control group (C), measured with FIVE Image Analyzer (Olympus, Japan), (H&E, 10 µm).*

4.2 NUMBER OF SKIN-ASSOCIATED LYMPHOID TISSUE (SALT)

The number of SALT is insignificantly (p>0.05) different in all groups. However, all vaccinated groups (Group OI, Group IO, Group OO, Group II) generally have a higher number compared to the unvaccinated (Group Control), even insignificant. Therefore, we can conclude that all types of vaccination routes elicit some mucosal immune response compared to the unvaccinated control group.



Graph 2: *Bar graph showing the number of SALT for control group (C), oral-immersion group (OI), immersion-oral group (IO), oral-oral group (OO), and immersion-immersion group (II).*

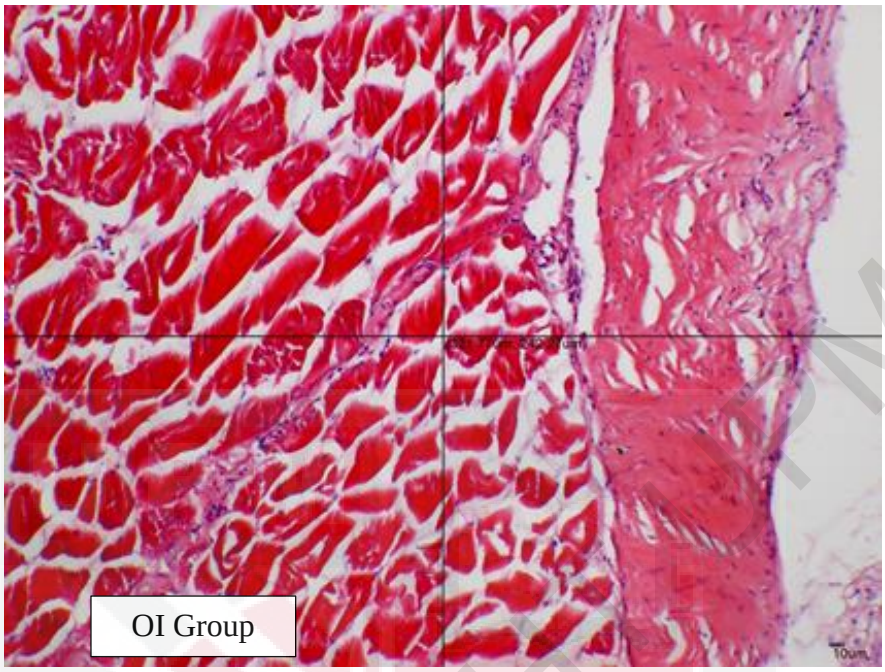


Figure 6

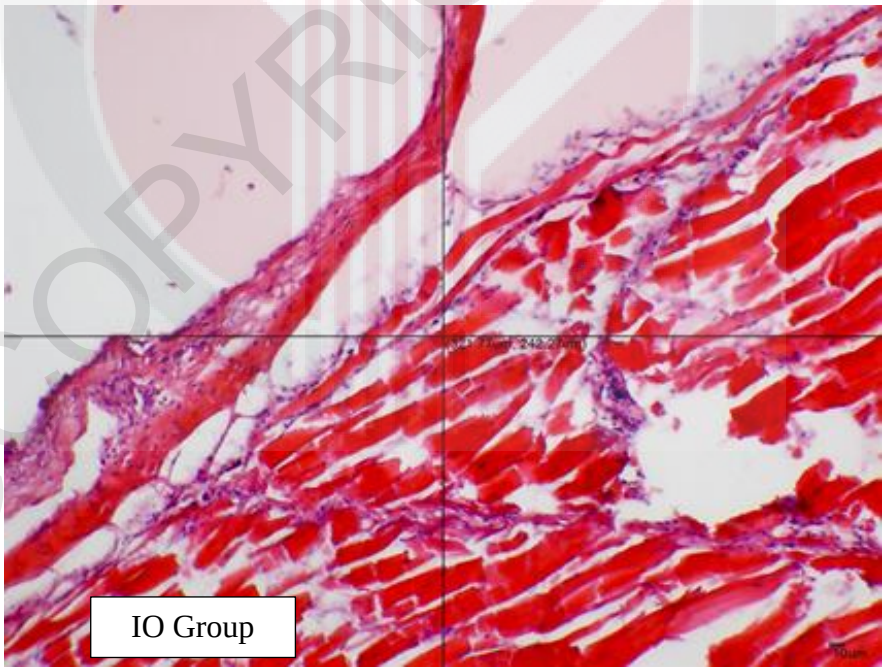


Figure 7

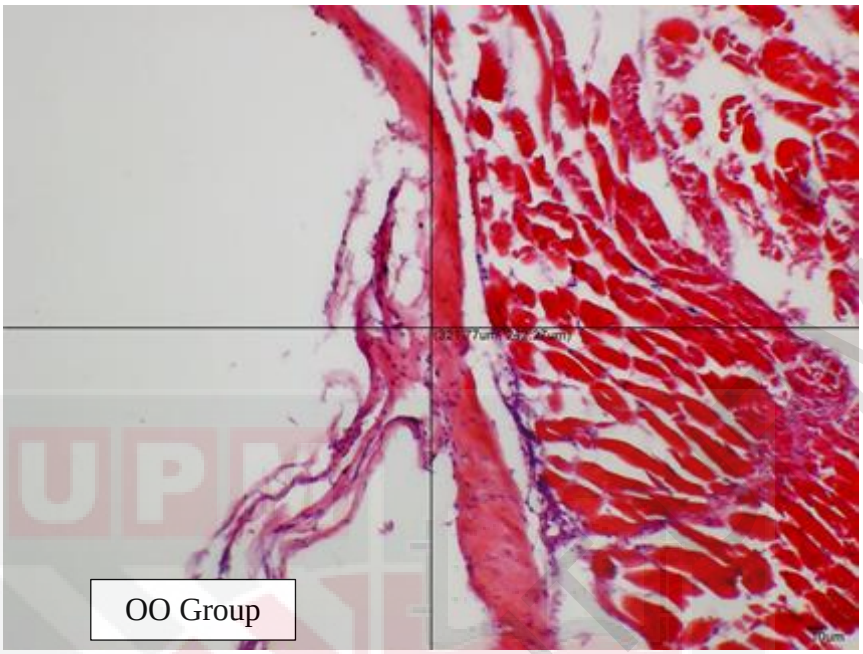


Figure 8

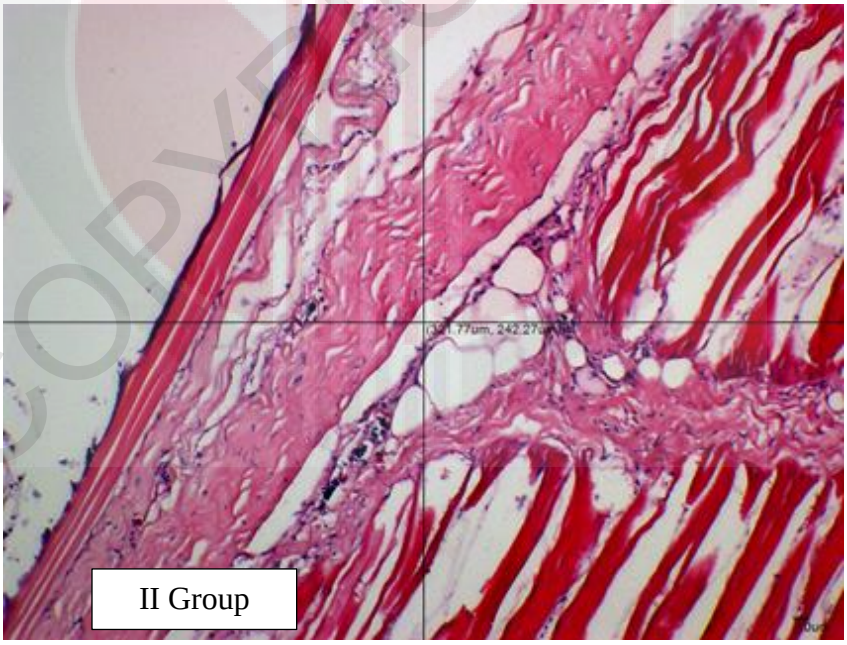


Figure 9

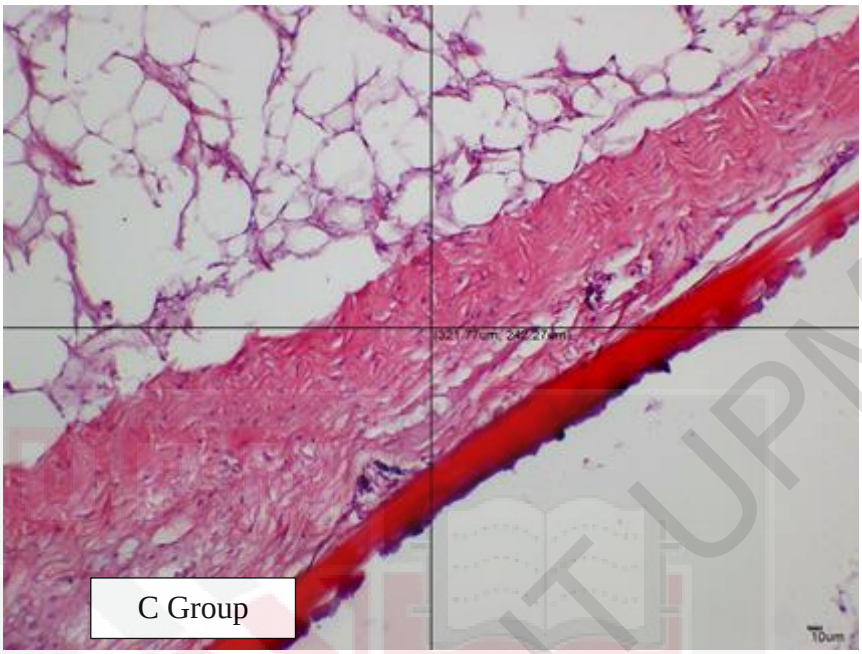
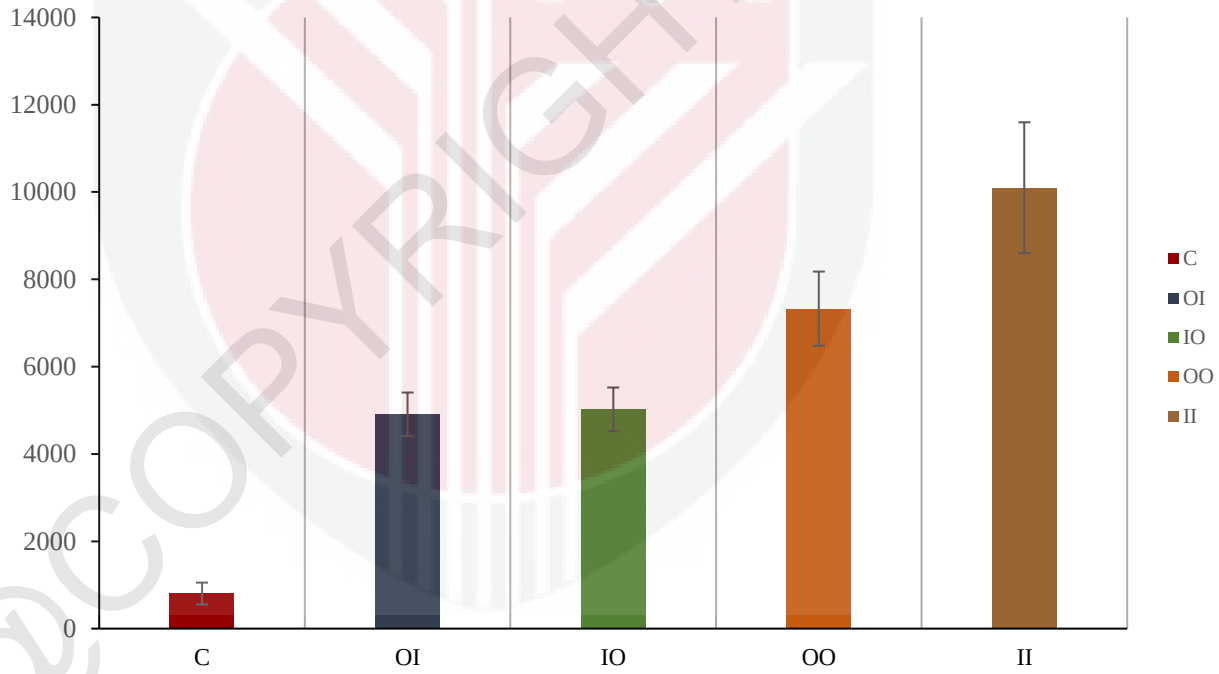


Figure 10

Figure 6-10: *Example of the number of SALT of oral-immersion group (OI), immersion-oral group (IO), oral-oral group (OO), immersion-immersion group (II) and control group (C) counted using Image analyser NIS-Elemens D. 3.2 (Nikon, Japan), (H&E, 10 µm).*

4.3 THE SIZE OF INFLAMMATORY CELLS IN GILLS

The size of the inflammatory cells in the gills was significantly ($p<0.05$) different in all groups. Group II are significantly ($p<0.05$) different when compared to Group Control which has lower average size of inflammatory cells in gills. All vaccinated groups (Group OI, Group IO, Group OO, Group II) has higher average size of inflammatory cells in gills with Group II having the highest average size. Thus, immersion route of vaccination has significantly affected the mucosal immune response of the size of inflammatory cells in gills.



***Graph 3**: Bar graph showing the average size of inflammatory cells in gills for control group (C), oral-immersion group (OI), immersion-oral group (IO), oral-oral group (OO), and immersion-immersion group (II).*

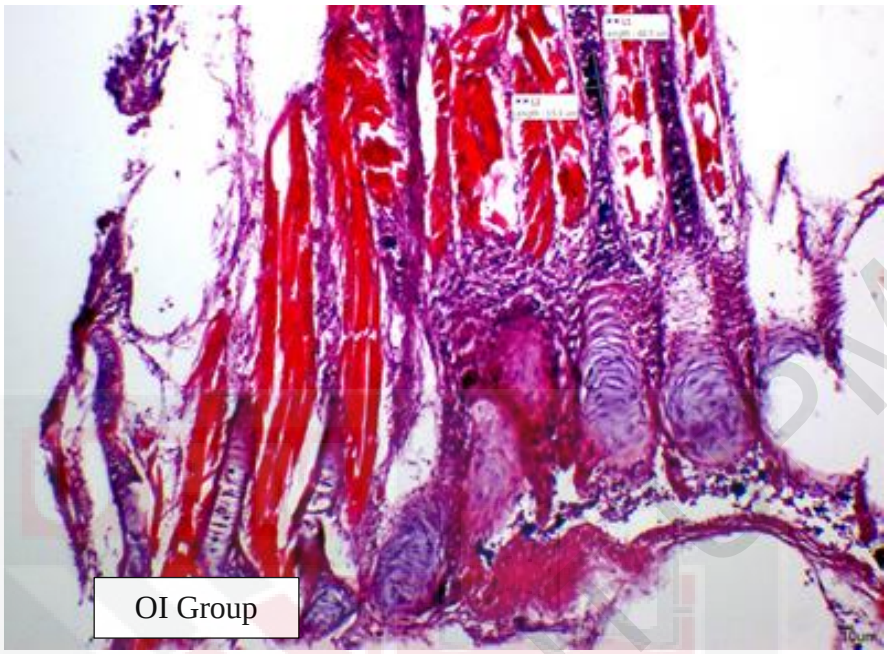


Figure 11

Figure 12

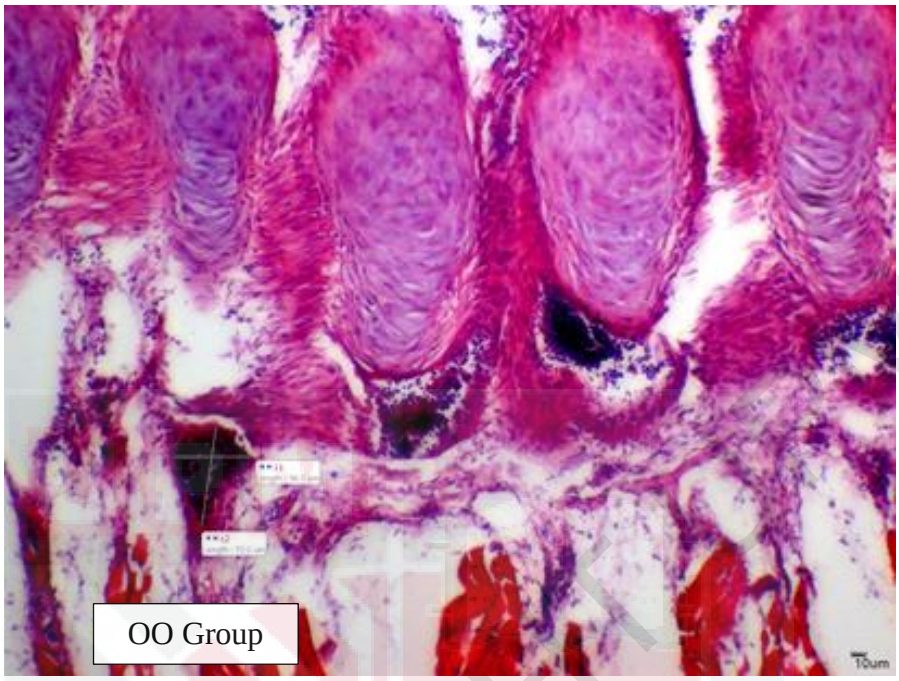


Figure 13

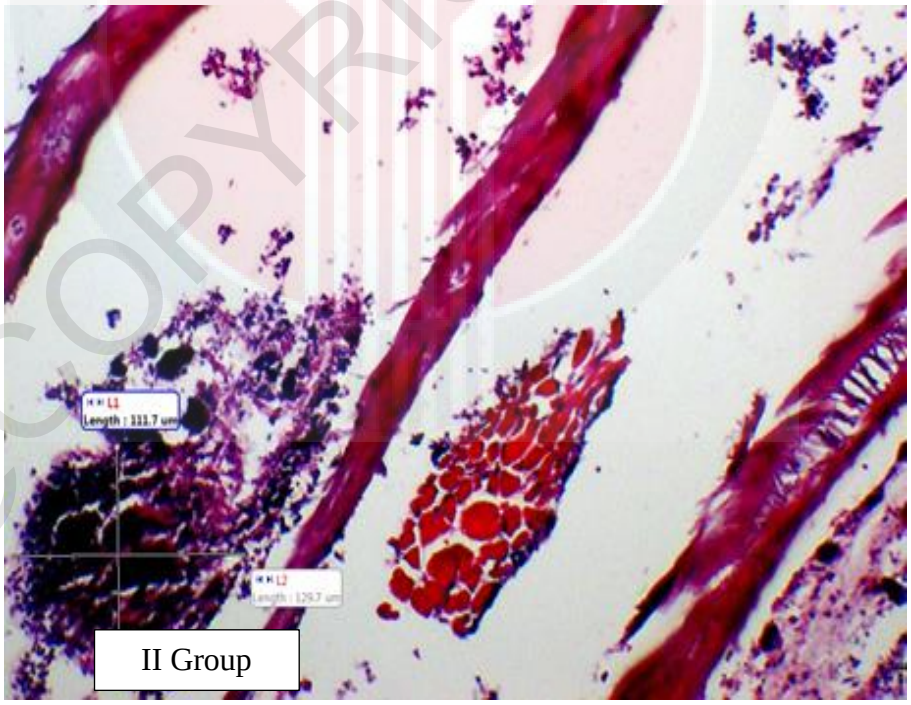


Figure 14

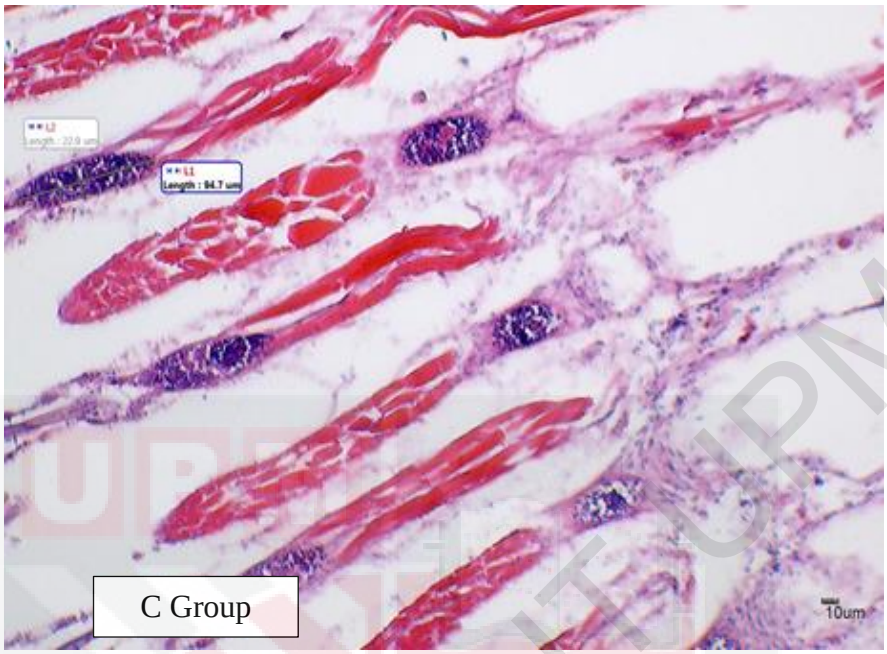


Figure 15

Figure 11-15*: Example of size of inflammatory cells in gills of oral-immersion group (OI), immersion-oral group (IO), oral-oral group (OO), immersion-immersion group (II) and control group (C), measured with FIVE Image Analyzer (Olympus, Japan), (H&E, 10 µm).*

5.0 DISCUSSION

The aim of this study is to find significant association between the mucosal immune response and different routes of vaccination with formalin-killed bacterin (*A. hydrophila*) in juvenile hybrid red tilapia. Along with that, to present the comparison of the microscopic lesions between the different vaccination routes, namely oral and immersion vaccination, and their combination. With regard to the histological basis, the comparison was made according to different groups that had different vaccination routes based on the thickness of the epidermis, the number of skin-associated lymphoid tissue (SALT), and the size of the inflammatory cells in the gills. It was found that the thickness of the epidermis and the number of SALT had an insignificant difference between all groups. Fish Vaccines (2020) states that before applying vaccination to fish, the species of the fish to be vaccinated, the status of the immune system of the fish, the environment, the production cycle and history, the course of disease, the farming technology (handling or mechanization), environment such as temperature and salinity, stress factors, nutrition, and cost benefits must be considered. However, the status of the immune system of the fish in this study, whether the fish suffer from pre-existing disease, was not confirmed prior to vaccination, thus might have influenced the results and the immune response. The protection conferred by oral immunisation has proved inconsistent with digestive degradation as the major cause of this inconsistency. Digestive degradation was caused by the acidity of the stomach because the stomach could not differentiate between food and vaccine, so the vaccine was degraded before it could reach the distal intestine, which is responsible with uptake of antigens (Bikramjit et al., 2016).

Focusing on the average size of the inflammatory size in the gills, it was found that there is a significant difference ($p<0.05$) between immersion-immersion route of vaccination, Group II and the non-vaccinated group. This shows that the immersion-immersion route was most strongly attributable to the mucosal immune response. Immersion vaccination is particularly useful for inducting of a mucosal immune response (Dhar et al., 2014). Mucosal administration of antigens offers the most feasible approach to immunising small fish. It is specifically aimed at stimulation of mucosal immunity in the fish, which provides the first line of defence against most pathogens (Rambout & Gomez cited in Bikramjit Ghosh et al., 2015). Immersion vaccination can deliver the vaccines directly to mucosal surfaces (skin and gills), which is a key entry point for numerous pathogens, and then stimulate mucosal surfaces and systemic organs to elicit an immune response against infection (Dhar et al., 2013). Consequently, Crosbie (2004) stated that protection from the gut route of administration showed that it offered less protection compared to protection from immersion or intraperitoneal injection.

However, towards the end of this study, the overall vaccinated group still generally had a thicker epidermis, higher numbers of SALT, and higher average size of inflammatory cells in the gills compared to the unvaccinated group. Thus, it can be concluded that either oral vaccination, immersion vaccination or the combination of both vaccination works well. In the hopes that the researchers will continue to study the efficacy of different vaccination routes, not only immersion and oral, but also other vaccination routes as well as for other infectious diseases, which may assist determine the best vaccination route to better protect the fish from pathogens.

6.0 CONCLUSION

The result of this study showed that there is a significant difference between immersion-immersion vaccination compared to the unvaccinated group. Overall, the immersion-immersion route, which was the Group II was most attributable to the mucosal immune response. While it only applies to the immersion-immersion route, other vaccination routes, which are oral-immersion, immersion-oral, and oral-oral group, generally elicits a stronger mucosal immune response based on the thickness of the epidermis, the number of skin-associated lymphoid tissue (SALT), and the inflammatory cells size in the gills were even insignificantly compared to the unvaccinated group. From this, it can be concluded that either oral vaccination, immersion vaccination or the combination of oral and immersion vaccination works well. However, the 100% mortality observed post-challenge ironically which vaccinated group should have a higher survival rate since its received vaccinations, resulting in a larger average size of inflammatory cells in gills and a higher number of SALT corresponds to higher protective immunity. This could be due to the inability for immune response to develop well before it could protect the fish from the disease, which might render the efficacy of the vaccine and routes of administration to be compromised. As for the recommendation, screening of the fish before the experiment should be performed as the health status of a fish can affect the result of our study.

Moreover, the duration of the study should be longer to better assess the immune response since immunity takes at least two weeks to increase. Also, increasing the sample size would help improve this study since the smaller the sample size, the higher the variability. A larger sample size would better represent the population. In future study, other samples such as gut, kidney and brain may be taken, and other analyses such as ELISA and lysosome activity could be performed to better reflect immune response.

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APPENDICES

Appendix 1: *Average number of epidermis thickness of Control (C) Group, Oral-Immersion (OI) Group, Immersion-Oral (IO) Group, Oral-Oral (OO) Group, and Immersion-Immersion (II) Group.*

Group	C	OI	IO	OO	II
Epidermis thickness	6.4	11.6	9	8.5	6
	7.5	9	12.7	12.9	7.6
	6	9.5	8.1	7.6	14.8
Average	6.63	10.03	9.93	9.67	9.47

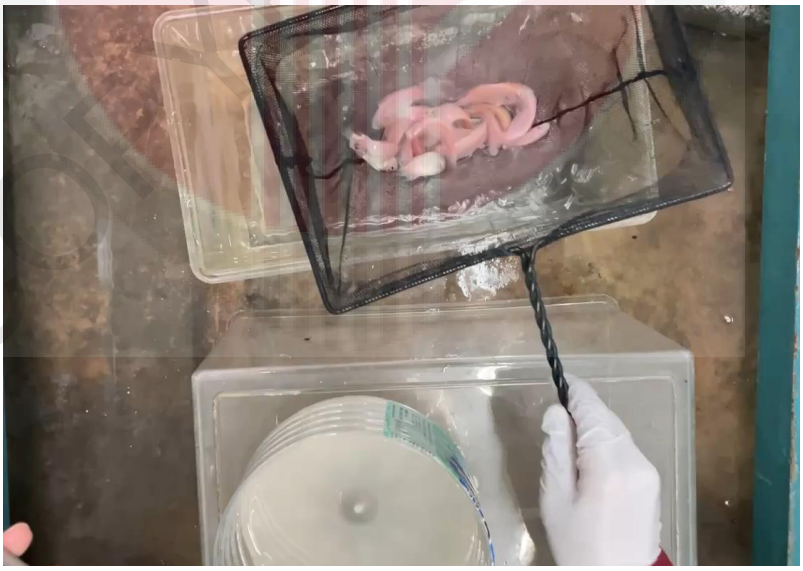
Appendix 2: *Average number of SALT of Control (C) Group, Oral-Immersion (OI) Group, Immersion-Oral (IO) Group, Oral-Oral (OO) Group, and Immersion-Immersion (II) Group.*

Group	C	OI	IO	OO	II
Number of SALT	10	0	182	140	210
	0	99	72	228	80
	22	221	96	68	68
Average	10.67	106.67	116.67	145.33	119.33

Appendix 3: *Average size inflammatory cell in gills of Control (C) Group, Oral-Immersion (OI) Group, Immersion-Oral (IO) Group, Oral-Oral (OO) Group, and Immersion-Immersion (II) Group.*

Group	C	OI	IO	OO	II
Size of inflammatory cell in gills	173	4698.34	5400.31	7813.01	14487.49
	742.05	5225.09	4712.03	7230.42	11743.44
	1496.3	4799.57	4956.3	6942.31	4064.87
Average	803.78	4907.67	5022.88	7328.58	10098.60

Appendix 4: *Procedure of oral vaccination of TDFV done twice daily for three consecutive days at a rate of 2% of bodyweight*



Appendix 5: *Procedure of immersion vaccination in FKV approximately 500 mL (1 × 10^9 CFU/mL) for 30 seconds.*

Appendix 6: *Procedure of challenge with 0.1 ml A. hydrophila (1 × 10^9 CFU/mL) through intraperitoneal injection.*

Appendix 7: *Collection of skin and gills sample.*