IMMUNOCHARACTERISATION OF LYMPHOID ORGANS IN BROWN-MARBLED GROUPER, *Epinephelus fuscoguttatus* (Forsskål, 1775)

By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Master of Science

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Aquaculture in Malaysia has been expanded and developed to be one of the economic potentials of the country. The grouper industry in Malaysia however, has been hindered by massive issues associated with infectious diseases. As a consequence, understanding immunology in this commercially important species is vital. The current study aimed to give essential insight into the differences in immunological robustness between these lymphoid organs in the humoral or cellular functional studies. Tissue leukocytes were isolated from brown-marble grouper's lymphoid organs (spleen, head kidney, and guts) (*Epinephelus fuscoguttatus*). Leukocytes isolated were used to profile the phenotypic characterization of leukocytes by flow cytometric scattering profile and immunofluorescent staining of $CD8\alpha^{+}$, humoral functional studies like respiratory burst assay, lysozyme assay, and myeloperoxidase assay. Immunofluorescent staining of $CD8\alpha^{+}$ demonstrated that the gut had a significantly higher CD8α+ cell population (4.44 ± 0.53%) and upon dividing the CD8α cells based on size, the grouper spleen possessed a significantly higher percentage of large $CD8\alpha^{+}$ cell size (2.89 ± 0.43%). Meanwhile, the gut had the most abundant of small-sized $CD8\alpha^{+}$ (3.33 ± 0.45%). On the other hand, among the tested humoral and cellular functional assays, activated gut resident leukocytes recorded strong humoral immune reactions and cellular immune reactions. In humoral immune reaction, the gut has strong robustness in both respiratory burst and myeloperoxidase assay with (1.01 ± 0.05) and (120.11 ± 26.62) respectively. In addition, the gut showed a higher immune reaction in cellular immune reactions of phagocytosis assay with (3.97 ± 01.24%). As for the humoral and cellular immune reactions that will further promote pro-inflammatory responses, such as lysozyme assay and lymphoproliferation assay grouper gut showed weaker immune robustness compared to the head kidney and spleen. In lysozyme assay, head kidney and spleen were more robust with (0.36±0.03) and (0.31±0.35) respectively. Overall, the present study demonstrated that different grouper lymphoid organs have varying immune responses robustness depending on the tested immune parameter. According to the result, as a mucosal lymphoid organ, the grouper gut had more robust immune responses upon activation than the systemic lymphoid organs. This can be observed in both assays of phagocytosis and respiratory burst and myeloperoxidase assay.

However, the gut showed weaker immune responses in the lymphoproliferation and lysozymes assay. This indicated that the grouper gut has weaker robustness in the immune reactions that can trigger more pro-inflammatory responses. This might be a beneficial mechanism to maintain intestinal homeostasis and to protect the resident gut commensal microbiota. The current study gives valuable insight into the differences in the immune functionality and robustness of different lymphoid organs. These findings can facilitate future research to choose the suitable lymphoid organs to be assayed for the selected functional tests, which in turn enable accurate evaluation of the target treatment in grouper.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENCIRIAN KEIMUNAN ORGAN-ORGAN LIMFOID DALAM IKAN KERAPU HARIMAU, *Epinephelus fuscoguttatus* (Forsskål, 1775)

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Akuakultur di Malaysia telah berkembang, membangun dan berpotensi menjadi salah satu sumber ekonomi penting negara. Walau bagaimanapun, perkembangan industri akuakultur kerapu yang mapan telah terhalang dengan pelbagai masalah terutama dalam keberjangkitan penyakit berjangkit. Oleh itu, pemahaman dalam imunologi bagi spesies kormesial ini amalt lah penting. Kajian semasa bertujuan memberi gambaran penting mengenai perbezaan kekuatan imunologi antara organ limfoid di dalam kajian fungsi humoral atau selular. Tisu leukosit dari organ limfoid kerapu harimau seperti limpa, ginjal dan usus diasingkan dan diekstrak. Leukosit yang diperolehi kemudian digunakan dalam memprofil ciri fenotipik leukosit dengan menggunakan sitometri aliran dan pewarnaan leukosit menggunakan keimunopendarfluoran CD8α, kajian fungsi humoral seperti uji kaji ledakan respitori, lisozim dan *myeloperoxidas*e. Pewarnaan keimunopendarfluoran $CD8\alpha^+$ menunjukkan bahawa usus mempunyai populasi sel $CD8\alpha^+$ yang jauh lebih tinggi (4.44 ± 0.53%) dan setelah pembahagian sel $CD8\alpha^+$ berdasarkan ukuran, limpa memiliki peratusan yang lebih tinggi bagi sel $CD8\alpha^+$ berukuran besar (2.89 ± 0.43%). Sementara itu, usus mempunyai jumlah peratusan tinggi bagi $CD8\alpha^+$ berukuran kecil (3.33 ± 0.45%). Sebaliknya, antara pengujian fungsi humoral dan selular yang diuji, leukosit daripada usus mencatatkan reaksi imun humoral dan selular yang kuat. Usus menunjukkan tindak balas imun humoral yang kuat dalam dua uji kaji ledakan respitori dan *myeloperoxidase* dengan masing-masing (1.01 ± 0.05), (120.11 ± 26.62). Di samping itu, usus menunjukkan tindak balas imun yang lebih tinggi dalam reaksi imun selular dari uji kaji fagositosis dengan (3.97 ± 1.24%). Walau bagaimanapun tindak balas imun humoral dan selular yang akan meningkatkan tindak balas pro-radang, seperti uji kaji lisozim dan limfoproliferation usus kerapu menunjukkan ketahanan imun yang lebih lemah berbanding dengan ginjal dan limpa. Dalam ujian lisozim, ginjal dan limpa lebih kuat dengan masing-masing (0.36 ± 0.03) dan (0.31 ± 0.35). Secara keseluruhan, kajian ini menunjukkan bahawa organ limfoid kerapu harimau yang berlainan mempunyai kekuatan tindak balas imun yang berbeza-beza bergantung pada parameter imun yang diuji. Menurut hasilnya, usus kerapu, sebagai organ limfoid mukosa, mempunyai tindak balas imun yang paling kuat ketika diaktifkan daripada organ limfoid sistemik. Ini

dapat dilihat pada ujian fagositosis, ledakan respitori dan *myeloperoxidase*. Walau bagaimanapun, usus menunjukkan tindak balas imun yang lebih lemah dalam pemeriksaan limfoproliferasi dan lisozim. Ini menunjukkan bahawa usus kerapu mempunyai kekuatan yang lebih lemah dalam reaksi imun yang mampu mencetuskan lebih banyak tindak balas pro-radang. Ini mungkin merupakan mekanisme yang bermanfaat untuk mengekalkan homeostasis usus dan melindungi mikrobiota komensal usus. Kajian semasa memberi gambaran berharga mengenai perbezaan fungsi imun dan ketahanan organ limfoid. Penemuan ini dapat memudahkan penyelidikan masa depan untuk memilih organ limfoid yang sesuai untuk dinilai untuk ujian fungsional yang dipilih, yang seterusnya membolehkan penilaian tepat terhadap sasaran rawatan pada kerapu.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the Degree of Master of Science. The members of the Supervisory Committee were as follows:

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APCs	Antigen-presenting cells
BCR	B-cell receptor
BSA	Bovine serum albumin
CO2	Carbon dioxide
Con A	Concanavalin A
CpG	5’-C-phosphate-G-3’
CTAB	Cetyltrimethylammonium bromide
DAMPs	Damage-associated molecular pattern
DAPI	4’,6-diamidino-2-phenylindo
DMSO	Dimethylsufoxide
DNase I	Deoxyribonuclease I
dsRNA	Double-stranded RNA
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorter
FAO	Food and Agriculture Organization
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FLD	Fish Lymphocystis Disease
GALT	Gut-associated lymphoid tissue
GIALT	Gill-associated lymphoid tissue
H2O2	Hydrogen peroxide
HBSS	Hanks balanced salt solution
IgA	Immunoglobulin A

IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgT	Immunoglobulin T
IgY	Immunoglobulin Y
IgZ	Immunoglobulin Z
i-IELs	Intestinal intraepithelial lymphocytes
KOH	Potassium hydroxide
LPS	Lipopolysaccharide
MAb	Monoclonal antibody
MALT	Mucosal-associated lymphoid tissue
MHC I	Major histocompatibility complex class I
MHC II	Major histocompatibility complex class II
MPO	Myeloperoxidase
MS-22	Tricaine Mesylate
NaCl	Sodium chloride
NALT	Nasopharynx-associated lymphoid tissue
NBT	Nitroblue tetrazolium
NNV	Nervous necrosis virus
O2	superoxide anion radical
O2	Oxygen
OD	Optical density
OH-	Hydroxyl radical
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffer saline

PHA-M	Phytohaemagglutin
PMA	Phorbol myristate
PRRs	Pattern recognition receptors
RBA	Respiratory burst activity
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
RSIVD	Red Seabream Iridovirus Disease
SALT	Skin-associated lymphoid tissue
SGD	Sleepy Grouper Disease
ssRNA	Single-stranded RNA
TCR	T-cell receptor
TGIV	Grouper Iridovirus Disease of Taiwan
TMB	3,3′,5,5′-tetramethylbenzidine hydrochloride

CHAPTER 1

INTRODUCTION

In past years, being the global solution to overcome the rapid depletion of wild fish stock, the aquaculture sector is the fastest-growing food sector. Aquaculture in Malaysia has been grown and developed to be one of the economic potentials to the country. Brown-marbled grouper (*Epinephelus fuscoguttatus*) is one of the popular marine cage culture candidates of high economic value in Malaysia. Brown-marbled grouper or locally known as tiger grouper is a tropical marine cultured finfish species pertaining to the family Serranidae. Overfishing and habitat destruction have driven the shifting from wild capture fishery to the grouper mariculture development in many countries including Malaysia (Froese and Pauly, 2019). According to 2018's Annual Fisheries Statistics published by the Department of Fisheries Malaysia, grouper is one of the marine aquaculture fish species that fetches the highest wholesale prices. From 2017 to 2018, there is a 31.01% of increment in the aquaculture production of grouper due to the production of hybrid grouper that more resistance to diseases. However, the industry faces stagnant productivity again in recent years as various diseases emerge and rampant the mariculture despite hybridisation has been adopted (Zhu *et al*., 2018).

The development of sustainable aquaculture of grouper has been hampered with serious challenges due to their susceptibility and high vulnerability to diseases. Many studies reported that the intensification of aquaculture has given rise to stress level of the cultured organism and their susceptibility to disease outbreaks (Dawood *et al*., 2019). In grouper cultivation, fish were susceptible to many serious diseases such as vibriosis and grouper iridoviral disease (Shen *et al*., 2017) which occurs during grouper cultivation and serious infections which potentially can lead to large scale mortality in grouper culture (Ina-Salwany *et al*., 2019). The use of antibiotics and chemotherapeutics to treat grouper infections diseases has generated numerous issues, including high operating costs, the growth of drug-resistant bacteria, suppression of the culture organism immunity, and pollution and contamination in food and environment. (Apines-Amar *et al*., 2012; Parashar *et al*., 2020). Different from terrestrial animals, fish are constantly exposed to a potential pathogen present in the water that circulates throughout their body and may invade their epithelial barriers and exposed wounds (Salinas, 2015; Yu *et al*., 2020). In fish, lymphoid tissues act as a physical and chemical defense against high microbial loads that are present in their environment. Lymphoid organs can be classified into primary lymphoid and secondary lymphoid organs. Primary lymphoid organs where naïve lymphocytes are located are the thymus and head kidney. Meanwhile, secondary lymphoid organs in fish include mucosal lymphoid organs, the spleen, and the kidney. Mucosal-associated lymphoid organs of teleost fish are comprised of gut-associated lymphoid tissue (GALT), gill-associated lymphoid tissue (GIALT), skin-associated lymphoid tissue (SALT), and nasopharynx-associated lymphoid tissue (NALT) (Sepahi and Salinas, 2016; Kitiyodom *et al*., 2021). The aquaculture industry's rapid expansion has invigorated fish immunological field studies. Fish immune studies are inevitably necessary to evaluate a new functional feed, a culture system, an immunopropylactic product such as a vaccine, pre- or probiotics, as well as an ecotoxicological stressor. However, the availability of basic reagents and methodologies for immunological studies in grouper are limited. Previous studies of the

immune system in teleost fish have been restricted to various popular species of the temperate water system, including rainbow trout (*Oncorhynchus mykiss*) (Hoseinifar *et al*., 2020), channel catfish (*Ictalurus punctatus*) (Xu *et al*., 2019), zebrafish (*Danio rerio*) (López Nadal *et al*., 2020) and gilthead seabream (*Sparus aurata L.*) (Firmino *et al*., 2021).

Being the largest vertebrate group, fishes are of great differences physiologically. Studies have shown that different fish species and different types of lymphoid tissues elicit immune reactions in varied robustnesses (Lazado *et al*, 2020). Choosing the right lymphoid organ that is immunologically robust and sensitive is fundamental to evaluate particular biotic or abiotic stressors accurately (Kitano, 2004; Krasnov *et al*., 2020). To top it off, there is a lack of data information on brown-marbled grouper (*Epinephelus fuscoguttatus*) immunity which has been impeded and slowed down the development of immunoprophylactic approaches such as probiotics and vaccines. Therefore, the current project was carried out to accentuate the immune functionalities and characteristics of the different lymphoid organs in brown-marbled grouper, i.e. systemic lymphoid organs by using the head kidney and spleen, while the mucosal organ by using the grouper gut. The present study aims to give essential insight into the differences in immunological robustness between these lymphoid organs. Both humoral and cellular functional studies were used in this study to characterize and compare the immune profile of the lymphoid organs in grouper (*Epinephelus fuscoguttatus*).

The general objective, specific objectives and the corresponding hypotheses of this study are:

A. To accentuate the immune functionalities and characteristics of the different lymphoid organs in brown-marbled grouper. i.e. systemic lymphoid organs by using the head kidney and spleen, as well as the mucosal organ by using the grouper gut.

1. To profile the phenotypic characterization of leukocytes isolated from different lymphoid organs such as head kidney, spleen and gut.

   H_o = There is no significant difference in the phenotypic characterization between the leukocytes isolated from different lymphoid organs.

   H_1 = There are significant differences in phenotypic characterization between the leukocytes isolated from different lymphoid organs.

2. To compare humoral immune reactions of leukocytes isolated from different lymphoid organs such as head kidney, spleen and gut.

   H_o = There is no significant difference in the humoral immune reactions between the leukocytes isolated from different lymphoid organs.

   H_1 = There are significant differences in the humoral immune reactions between the leukocytes isolated from different lymphoid organs.

3. To compare cellular immune reactions of leukocytes isolated from different lymphoid organs such as head kidney, spleen and gut.

   H_o = There is no significant difference in the cellular immune reactions of leukocytes isolated from different lymphoid organs.

   H_1 = There are significant differences in the cellular immune reactions of leukocytes isolated from different lymphoid organs.

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