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Research Article

Pleurotus pulmonarius Stalk Waste Extract Supplementation Modulates Serum Proteins Level in Red Hybrid Tilapia *Oreochromis* sp. upon PAMPs Challenge

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ABSTRACT

Utilization of functional feeds to enhance fish health and disease resistance has become common practice in aquaculture over the past few decades. This study investigated the effects of hot water extract (HWE)-supplemented feed on serum protein expression in red hybrid tilapia following PAMPs challenge. Fish were fed with a basal or HWE-supplemented diet for 30 days and subsequently injected with lipopolysaccharide (LPS), polyinosinic: polycytidylic acid (poly(I:C)), or PBS (control). Proteomic analysis revealed differential expression of key immune-related proteins like immunoglobulin μ heavy chain and apolipoprotein A-II, which were significantly higher in the HWE-supplemented group upon LPS challenge. On the other hand, C-type lectin domain-containing protein, immunoglobulin μ heavy chain, hemopexin, serotransferrin, α2-HS-glycoprotein-like, inter-alpha-trypsin inhibitor heavy chain H3-like, L-rhamnose-binding lectin, and vitellogenin were lower in the HWE-supplemented diet group as compared to the basal diet group post-poly(I:C) challenge, while plasma protease C1 inhibitor and apolipoprotein Eb increased. Functional classifications analysis revealed that most of the DEPs found were involved in the cellular process, biological regulation, metabolic process, and response to stimulus. LC-MS identified bioactive metabolites in HWE, which may contribute to its immunomodulatory effects. These findings suggest that HWE supplementation modulates immune protein expression and enhances disease resilience in tilapia, offering a sustainable strategy for improving aquaculture productivity.

Key words: Functional feed, metabolite, *Pleurotus pulmonarius*, proteomic, red hybrid tilapia

INTRODUCTION

Recently, there has been an increasing demand for fish as a nutritious and valuable food source for human consumption. The production of fish is anticipated to grow in the coming years as it contributes to food security as the global population increases. In aquaculture, several important factors need to be considered for efficient production, such as nutrition, welfare, and health management (Raposo de Magalhães *et al*., 2020; Moyo & Rapatsa, 2021). Fish often suffer from various stressors in aquaculture systems, like poor water quality, high stocking density, and the spreading of infectious diseases (Baleta *et al*., 2019; Wanja *et al*., 2020; Mohd Khatib & Jais, 2021). Therefore, improving fish's health status and disease resistance in the early development stage could enhance the production and food quality in the aquaculture industry (Assefa & Abunna, 2018; Samat *et al*., 2020).

Over the past decades, using functional feeds to enhance fish's health status and disease resistance has become a common practice in aquaculture (Reverter *et al*., 2021; Onomu & Okuthe, 2024). Functional feeds are beneficial to the health and physiological state of an organism beyond the basic requirement of nutrition. Including functional ingredients in diets improved the health status of diverse species of fish like common carp, salmon, and tilapia (Onomu & Okuthe, 2024). In addition, functional feeds can act as a preventive measure for both infectious and non-infectious diseases in aquaculture to reduce the usage of antibiotics and chemotherapeutants, which may create concern about consumer liability and environmental contamination (Mohan *et al*., 2019). To date, various functional ingredients are being used in aquaculture, including probiotics and prebiotics supplements, immunostimulants, nucleotides, phytogenic, and pigments (Zuberi *et al*., 2024). Among these, immunostimulants such as mushroom polysaccharides are gaining more attention due to the well-known properties like antioxidant, antimicrobial, antiviral, and immunostimulatory effects (Van Doan *et al*., 2019; Mohan *et al*., 2019).

In the previous studies, *Pleurotus pulmonarius* stalk waste extract supplemented feed was shown to provide ameliorative

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effects on the immune system of tilapia, as well as to improve the growth performance and survival rate under stressed conditions (Ahmed *et al*., 2017a; Ahmed *et al*., 2017b; Ching *et al*., 2021). In addition, Nile tilapia supplemented with Cordyceps spent mushroom substrate showed enhanced innate immune responses and increased resistance to *Streptococcus agalactiae* (Van Doan *et al*., 2017). Beyond their immunomodulatory benefits, incorporating mushroom stalk waste extract in fish feed reduces the feed production costs and minimizes the environmental pollution caused by the disposal of mushroom stalk waste (Ahmed *et al*., 2017a). However, the exact mechanisms by which these extracts influence immune function in fish remain unclear, particularly at the protein expression level.

In past decades, proteomics has emerged as a powerful approach to provide a deeper understanding of aquatic organisms' physiological and biological processes (Raposo de Magalhães *et al*., 2020). On top of that, comparative proteomics analysis of tissues, fluids, or cells at a specific time point enables the researchers to study the protein alteration in aquatic animals towards bacterial, viral, or parasitic infections and environmental stress (Carrera *et al*., 2020; Jitjumnong *et al*., 2025). The proteomics approach can also be used to investigate the influences of functional feeds on the metabolic process of the immune system and the underlying molecular pathways triggered by the functional ingredients (Nieman *et al*., 2019). Yet, few studies have applied proteomics to examine how mushroom stalk waste extracts modulate fish immunity.

Therefore, this study was performed to observe the changes in serum protein levels after supplementation of hot water extract (HWE) of *P. pulmonarius* stalk waste in red hybrid tilapia *Oreochromis* sp. one day after lipopolysaccharide (LPS) and polyinosinic: polycytidylic acid (poly(I:C)) challenge. LPS and poly(I:C) were used in this study to mimic bacterial and viral infections, respectively. By linking these protein-level changes to previously observed immune system improvement, this study would provide a better understanding of the regulation mechanism of *P. pulmonarius* stalk waste extracts against bacterial and viral antigens in red hybrid tilapia. In addition, while β-glucans are known immunomodulators in fish, mushroom stalk waste extracts contain other potential bioactive metabolites that remain unexplored. Since β-glucan is not the only factor that triggers immunomodulation, LC-MS analysis was carried out on the sample to identify potential metabolites that could contribute to the changes observed in the fish.

MATERIALS AND METHODS

Preparation of Hot Water Extract (HWE)

Pleurotus pulmonarius stalk waste (MSW) was obtained from Arra Mushroom Sdn Bhd, Negeri Sembilan, Malaysia. All the inert elements from MSW were removed. The MSW was washed, sliced into individual stalks, and oven-dried at 60°C for 4 hr. The dried material was then ground into a fine powder using a Waring blender (Waring Commercial, New Hartford, USA) and stored at 4°C until needed. For hot water extraction, the MSW powder was boiled in distilled water for 5 hr at a 1:20 (w/v) ratio. After cooling to room temperature, the mixture was filtered twice using a linen cloth. To eliminate any unwanted sediment, the filtered solution was centrifuged at 10,000 ×g for 10 min. The supernatant was then collected and freeze-dried to produce the lyophilized mushroom extract, referred to as hot water extract (HWE). The dry matter content of HWE was 91.77%. The extract contained 42.91% carbohydrate, 24.05% crude protein, 0.51% crude lipid, 3.15% crude fibre, and 19.95% crude ash (Ching *et al*., 2021).

Preparation of experimental diets

Local fish meal, soybean meal, rice bran, and maize bran were used as protein sources, while palm oil was used as a lipid source in the experimental diets. The diets were formulated using Winfeed version 2.8 software (Winfeed Limited, Cambridge, UK) to contain approximately 34% crude protein and 6% crude lipid (Table 1). All ingredients were thoroughly mixed and made into stiff dough by progressively adding distilled water. The dough was then processed through a mini pelleting machine (KCM, Y132M-4, Xinrui, Fujian, China) to form pellets with a diameter of 0.3 cm. These pellets were oven-dried at 45°C for 48 hr, cooled to room temperature, and sealed in polypropylene bags for storage at 4°C until future feeding trials. The basal diet, without supplementation, served as the experimental control. The HWE-supplemented diet was created by adding 10 g/kg of HWE to the diet. All experimental diets were provided to the fish within one month of formulation.

Experimental fish

Red hybrid tilapia (*Oreochromis niloticus* × *O. mossambicus*) were sourced from the Aquaculture Resource Center, Hulu Langat, Selangor, Malaysia. Before beginning the tests, the fish were acclimatized to natural environmental conditions in the Aquatic Laboratory, Institute of Biological Sciences, Universiti Malaya, for two weeks and fed with commercial feed. A total of 240 fish with an initial mean weight of 17.85 ± 0.79 g were placed in 100 L tanks filled with dechlorinated tap water, with 20 fish per tank. The fish were grown under a natural photoperiod throughout the experiment. A YSI probe meter (YSI Inc., Ohio, USA) was used to measure dissolved oxygen (over 5.0 mg/L), pH (6.5 - 7.5), and water temperature (26.0 ± 1.0°C). The total ammonia-nitrogen level and nitrite level were measured using an ammonia and nitrite high-range portable photometer (Hanna Instruments, Rhode Island, USA), and the levels were kept below 0.8 mg/mL and 1.9 mg/mL, respectively, during the experimental period. Each experimental tanks were equipped with a water pump (H6350, Shanda Aquarium, Guangdong, China) connected to a filter box with a flow rate of 20 L/min. Aeration diffusers were supplied in each tank for dissolved oxygen circulation, and continuous faecal material syphoning was maintained throughout the trial. Approximately 30% of the water was changed every two days to sustain the water quality.

Experimental design

After a two-week acclimation period, the fish were randomly allocated into two diet groups. One group received a basal diet, while the other group was fed a diet supplemented with HWE. All the fish were fed twice daily (morning and evening) at a rate of 3% of their body weight for a duration of 30 days. On the 30th day of feeding, fish were divided into three treatment groups

within their feeding regime. They were collected at random and intraperitoneally challenged with 100 µL of phosphate buffer saline (PBS) containing either 8 mg/kg LPS, 8 mg/kg poly(I:C), or PBS alone using a 25 G needle (Terumo, Tokyo, Japan). Six treatment groups were established, each with two replicates of 20 fish: (1) Basal diet + PBS challenged fish; (2) Basal diet + LPS challenged fish; (3) Basal diet + poly(I:C) challenged fish; (4) HWE-supplemented diet + PBS challenged fish; (5) HWE-supplemented diet + LPS challenged fish; (6) HWE-supplemented diet + poly(I:C) challenged fish.

Table 1. Formulation and proximate composition of the experimental diets (g/kg).

	Basal diet (BD)	Hot water extract supplemented diet (HWE)
Ingredients		
Fish meal	300.00	300.00
Maize meal	145.60	142.50
Soybean meal	343.30	339.30
Rice bran	165.00	162.00
Palm oil [a]	27.20	27.60
DCP [b]	10.00	10.00
Vitamin premix [c]	4.00	4.00
Mineral premix [d]	5.00	5.00
Hot water extract	-	10.00
Proximate composition (% dry matter basis)		
Dry matter	92.00	91.80
Moisture content	8.00	8.20
Crude protein	35.66	35.89
Crude lipid	5.78	5.83
Carbohydrate	41.18	41.92

[a]Palm oil, Malaysian Palm Oil Industries, Malaysia.

[b]DCP, Dibasic calcium phosphate dihydrate, Nutri Vet Trading, Malaysia.

[c]Vitamin premix supplied the following per 100 g diet: β-carotene 96.26 mg; Vitamin D3 9.68 mg; Menadione sodium bisulfite trihydrate (K3) 45.83 mg; Thiamine-nitrate (B1) 57.75 mg; Riboflavin (B2) 192.37 mg; Pyridoxine-hydrochloride (B6) 45.83 mg; Cyanocobalamine (B12) 0.07 mg; D-Biotin 5.78 mg; Inositol 3212.83 mg; Niacine (nicotinic acid) 769.73 mg; Calcium panthothenate 269.49 mg; Folic acid 14.40 mg; Choline chloride 7869.30 mg; ρ-Aminobenzoic acid 383.25 mg.

[d]Mineral premix supplied the following per kg diet: $MgSO_4$ 5070 mg; Na_2HPO_4 3230 mg; K_2HPO_4 8870 mg; $C_6H_5FeO_7$ 1100 mg; $C_6H_{10}CaO_6$ 12090 mg; Al $(OH)_3$ 10 mg; $ZnSO_4$ 130 mg; $CuSO_4$ 4 mg; $MnSO_4$ 30; $Ca(IO_3)_2$ 10 mg; $CoSO_4$ 40 mg.

Sample preparation

One day after the challenge, blood was drawn from the caudal peduncle by using a 22 G needle connected to a 1 mL syringe (Terumo). The blood samples were stored in vacutainer tubes containing a clot activator and left to clot for 1 hr at room temperature. The serum was then separated by centrifuging the tubes at 2000 ×g for 15 min. The collected serum was immediately stored at -80°C until further analysis. Before MS analysis, albumin depletion of serum samples was carried out using AlbuSorb™ Albumin Depletion Kit (Biotech Support Group, New Jersey, USA) following the manufacturer's protocol. The protein concentration of the albumin-depleted samples was then determined using the Bradford assay.

In solution, trypsin digestion

Serum samples (145 µg protein per sample) with three biological replicates from each treatment group were subjected to reduction and alkylation according to Abdul Satar *et al*. (2021). To digest the samples, 15 µL of 200 ng/µL trypsin solution (Sigma-Aldrich) was added to each sample, and digestion was performed overnight at 37°C. The reaction was halted by adding a drop of concentrated acetic acid to the solution. The digested sample was then concentrated using a CentriVap Benchtop Vacuum Concentrator (Labconco, Kansas City, USA). Subsequently, the sample was reconstituted in 50 µL of 0.1% formic acid and filtered through a Minisart 0.2 µm regenerated cellulose membrane filter (Sartorius Stedim Biotech).

Mass spectrometry

The LC-MS/MS analyses of serum samples were carried out using LTQ Orbitrap Velos coupled with EASY-nano liquid chromatography (EASY-nLC) II system (Thermo Scientific). Separation of the tryptic-digested peptides was achieved using an analytical column, EASY-column™ C18-A2 (10 cm, I.D. 75 µm, 3 µm; Thermo Scientific) coupled with a pre-column, EAST-column™ C18-A1 (2 cm, I.D. 100 µm, 5 µm, Thermo Scientific) at a flow rate of 0.3 µL/min, and the injection volume of the sample was 10 µL. The columns were equilibrated according to Abdul Satar *et al*. (2021). Mass spectra of the peptides were recorded using Xcalibur Ver. 2.1 (Thermo Scientific) with a mass tolerance threshold of 5 ppm.

The eluted peptides were electro-sprayed into the mass spectrometer with a spray voltage of 2.3 kV and a capillary temperature of 200°C. In the proteomic approach, peptides were detected using full scan mass analysis at a resolving power of 60,000 (m/z 400, full width at half maximum (FWHM); 1 sec acquisition) within a mass range of m/z 300 - 2,000. Data-dependent MS/MS analyses were triggered by the eight most abundant ions from the parent mass list of predicted peptides, while singly charged or unassigned charge states were rejected. The MS/MS analysis was done via collision-induced dissociation (CID) with normalized collision energy of 35%, a resolution of 60,000, an isolation width of 2 Da, an activation q of 0.25, an activation time of 50 ms, and a charge state of 2.

Protein identification

The MS/MS data analysis was conducted using PEAKS Studio Software V7.5 (Bioinformatics Solution Inc., Waterloo, Canada) according to Adebayo *et al.* (2022) with some modifications. SPIDER was utilised to detect further mutations in the candidate peptides in order to identify more proteins. The generated peptide mass list was searched against the Uniprot (Actinopterygii) database (Taxon ID: 7898; 4,579,536 sequences). To increase the confidence level, the peptide-spectrum matches (PSM) results were filtered at 1% false discovery rate, a 50% *de novo* confidence rate, and each protein must have at least one unique peptide.

Label-free quantification

Semi-quantitative label-free analysis was used to compare serum proteins of red hybrid tilapia from different experimental diet groups after being challenged with LPS and poly(I:C). Using the sample from the basal diet with PBS injection group as a reference, the peak areas of each identified peptide were normalised using total ion chromatograms. The relative quantification of each sample was detected by performing the expectation-maximisation algorithm of the Quantification Module in PEAKS. Proteins that contained at least 1 unique peptide with a fold change over 1.5 and significance over 13 (P<0.05) were accounted as the differentially expressed proteins (DEPs).

Bioinformatics analysis

Blast2GO V6 (Biobam Bioinformatics) was used for the functional annotation of DEPs to their biological process, molecular function, and cellular component distributions based on Gene Ontology (GO) categories (Götz *et al*., 2008). Classification of DEPs was conducted using WEGO 2.0 (Web Gene Ontology Annotation Plotting, Ye *et al*., 2018).

LC-MS metabolites analysis

For LC-MS analysis, HWE was dissolved in Milli-Q water (10 mg/mL) and analysed in triplicate using an Agilent 1290 Infinity LC system coupled to an Agilent 6520 Accurate-Mass Q-TOF mass spectrometer with a dual ESI source (Agilent, Santa Clara, USA) following Thiyagarasaiyar *et al.* (2021).

Metabolites Identification

Data was processed using Agilent Mass Hunter Qualitative Analysis B.07.00. The compounds were identified from the Search Database Metlin_AM_PCDL-N-170502.cdb, with the parameters as follows: match tolerance, 5 ppm; positive ions, H^+, Na^+, K^+, NH^{4+}; negative ions, H^-, Cl^-; charge state range, 1 to 2.

RESULTS

Differentially expressed serum protein

From the protein identification analysis, a total of 168 serum proteins were identified in the basal diet and HWE-supplemented diet groups. Among the list of DEPs obtained, particular attention was given to those directly or indirectly involved in the immune responses. Six identified proteins were differentially expressed in LPS challenge groups compared to control (PBS challenge groups) (Table 2). In contrast, poly(I:C) challenge induced more pronounced immune alterations, with a total of 22 DEPs identified (Table 3). Comparatively, poly(I:C) triggered a stronger immune response, as evidenced by the higher number of immune-related DEPs.

Table 2. List of DEPs identified from basal diet and HWE-supplemented diet groups upon LPS challenge.

No.	Accession	Description	-10lgP	Coverage (%)	#Peptides	#Unique	Avg. Mass
Immune Response Protein							
1	tr|A0A669CZ49|A0A669CZ49_ORENI	Immunoglobulin μ heavy chain	16.17	16	10	7	50988
Lipid Metabolism and Transport Protein							
2	tr|I7I7L9|I7I7L9_ORENI	Apolipoprotein A-II	14.93	82	20	3	16102
3	tr|A0A668RZ24|A0A668RZ24_OREAU	Sex hormone-binding globulin	13.91	7	6	6	43560
Blood Coagulation							
4	tr|I3JR01|I3JR01_ORENI	Prothrombin	14.61	13	5	5	70214
5	tr|I3K9D3|I3K9D3_ORENI	Fibrinopeptide A	14	14	12	1	107309
Cell Adhesion Protein							
6	tr|A0A669DM41|A0A669DM41_ORENI	Cadherin-1	15.99	9	9	8	97061

Table 3. List of DEPs identified from basal diet and HWE-supplemented diet groups upon poly(I:C) challenge.

No.	Accession	Description	-10lgP	Coverage (%)	#Peptides	#Unique	Avg. Mass
		Immune Response Proteins					
1	tr|I3KZX7|I3KZX7_ORENI	C-type lectin domain-containing protein	32.15	12	2	2	18635
2	tr|A0A669CZ49|A0A669CZ49_ORENI	Immunoglobulin μ heavy chain	15.61	38	16	8	50988
3	tr|A0A3P9BYI6|A0A3P9BYI6_9CICH	Inter-alpha-trypsin inhibitor heavy chain H3-like	15.07	4	3	3	99196
4	tr|I3KS22|I3KS22_ORENI	L-rhamnose-binding lectin SML	15.2	46	11	8	24570
5	tr|F6MDM1|F6MDM1_ORENI	Plasma protease C1 inhibitor	16.05	11	3	3	67334
6	tr|A0A669DHY5|A0A669DHY5_ORENI	Vitellogenin	13.7	38	64	2	184995
		Acute Phase and Inflammatory Proteins					
7	tr|A0A669C6E6|A0A669C6E6_ORENI	α2-HS-glycoprotein-like	14.62	24	8	2	36568
8	tr|A0A668VV43|A0A668VV43_OREAU	Apolipoprotein Eb	16.08	35	12	12	32485
9	tr|A0A3Q4N4J6|A0A3Q4N4J6_NEOBR	Leucine-rich alpha-2-glycoprotein	13.2	10	2	2	37422
		Iron and Heme Binding Protein					
10	tr|A0A668S9K0|A0A668S9K0_OREAU	Hemopexin	14.66	35	32	29	44969
11	tr|A0A0E3JRK1|A0A0E3JRK1_ORENI	Serotransferrin	25.86	56	50	4	75108
12	tr|A0A669E4X1|A0A669E4X1_ORENI	Hemoglobin subunit αB-like	19.06	57	10	8	15474
		Metabolic Protein					
13	tr|A0A671R668|A0A671R668_9TELE	α-1,4-glucan phosphorylase	16.47	3	1	1	96367
		Blood Coagulation					
14	tr|A0A668VGH7|A0A668VGH7_OREAU	Carboxylic ester hydrolase	14.47	6	3	3	61579
15	tr|A0A668T6W0|A0A668T6W0_OREAU	Fibrinopeptide A	27.17	26	18	1	75979
16	tr|I3JNT9|I3JNT9_ORENI	Kininogen-1	14.18	14	5	1	40285
17	tr|I3JR01|I3JR01_ORENI	Prothrombin	14.47	13	5	5	70214
		Cell Cycle and Signaling Protein					
18	tr|A0A671M1I1|A0A671M1I1_9TELE	Cell division cycle 5-like protein	17.21	3	1	1	75192
19	tr|A0A3Q1IEL0|A0A3Q1IEL0_ANATE	Odorant receptor 131-2-like	13.34	3	1	1	38078
20	tr|A0A059T2C6|A0A059T2C6_PAROL	P2X purinoceptor 7 isoform X1	13.1	2	1	1	65310
		RNA Binding Proteins					
21	tr|A0A673CLD3|A0A673CLD3_9TELE	Pumilio RNA-binding family member 1	13.38	1	1	1	127032
		Stress Response Protein					
22	tr|I3J3H7|I3J3H7_ORENI	Putative antifreeze type 4 protein	19.7	53	9	9	14116

In the protein profile heatmaps, protein expression levels are represented by a green-red colour scale, where green indicates lower expression and red indicates higher expression. As illustrated in Figure 1, the level of DEPs was lower upon LPS challenge in both basal and HWE-supplemented diet groups. Immune-related proteins such as immunoglobulin μ heavy chain and apolipoprotein A-II showed higher baseline expression in the HWE-supplemented diet group than in the basal diet group under PBS conditions. Whilst after the LPS challenge, the level of both proteins was lower, with a significantly lower protein level in the basal diet group as compared to the HWE-supplemented diet group.

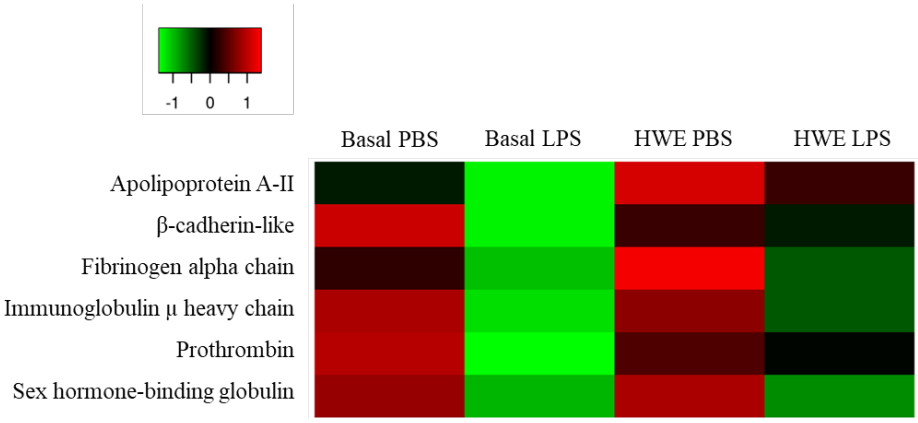

Fig. 1. Heat map for DEPs identified from basal diet and HWE-supplemented diet groups upon LPS challenge.

On the other hand, more DEPs were identified between both experimental diet groups upon poly(I:C) challenge (Figure 2). Under the control treatment (PBS challenge), immune-related proteins such as C-type lectin domain-containing protein, vitellogenin, and plasma protease C1 inhibitor showed lower levels after the supplementation of HWE. A higher protein level of immunoglobulin μ heavy chain, serotransferrin, inter-alpha-trypsin inhibitor heavy chain H3-like, and L-rhamnose-binding lectin was observed in the fish serum obtained from the HWE-supplemented diet group after PBS challenge.

Upon poly(I:C) challenge, C-type lectin domain-containing protein, immunoglobulin μ heavy chain, leucine-rich alpha-2-glycoprotein, vitellogenin, hemopexin, and serotransferrin showed lower levels in both experimental diet groups. Whilst the protein level of α2-HS-glycoprotein-like, inter-alpha-trypsin inhibitor heavy chain H3-like, and L-rhamnose-binding lectin was higher in the basal diet group but lower in the HWE-supplemented group following poly(I:C) challenge. Plasma protease C1 inhibitor and apolipoprotein Eb, on the other hand, showed a higher level after one day of poly(I:C) challenge in both experimental diet groups.

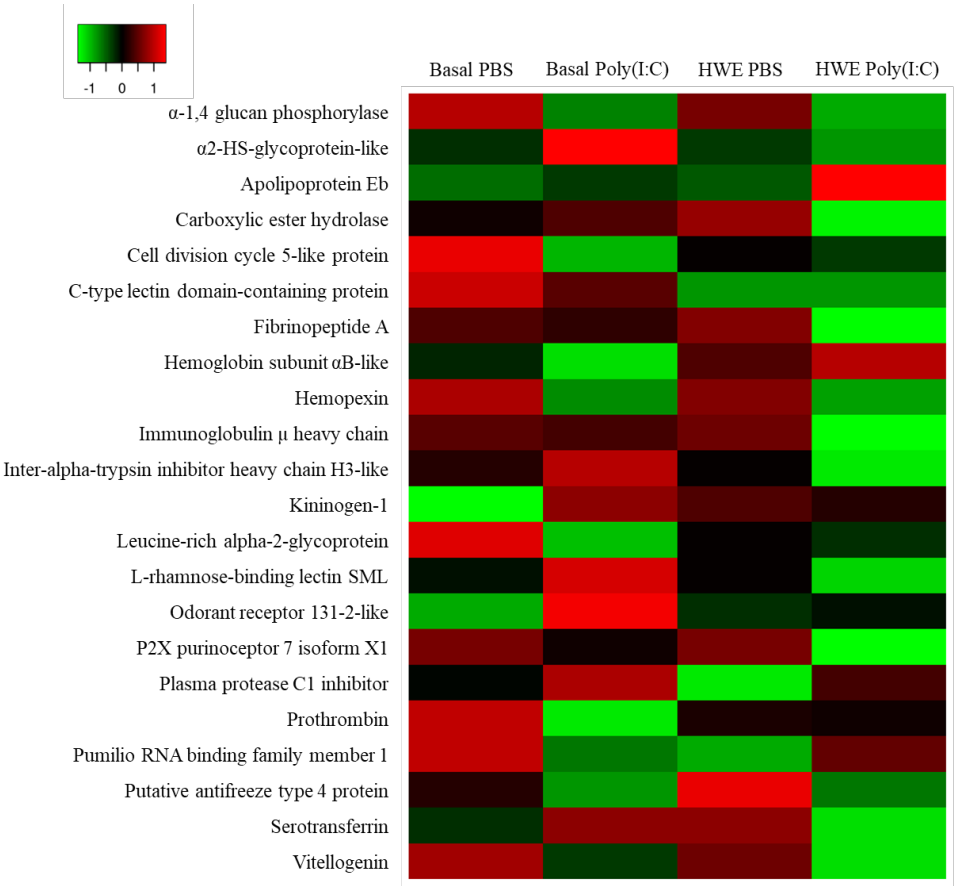

Fig. 2. Heat map for DEPs identified from basal diet and HWE-supplemented diet groups upon poly(I:C) challenge.

Gene ontology classification of DEPs

The identified proteins were categorised by Gene Ontology (GO) terminologies, namely biological process, molecular function, and cellular component. As shown in Figure 3, the DEPs identified in the experimental diet groups upon LPS challenge were observed to be involved in response to stimulus (33.3%), cellular process (16.7%), cellular component organization or biogenesis (16.7%), developmental process (16.7%), biological adhesion (16.7%), metabolic process (16.7%) and immune system process (16.7%). In terms of molecular function, 16.7% of the DEPs were shown to have catalytic activity.

On the other hand, the most prominent molecular function identified in the poly(I:C) challenge group was binding, where 22.7% of the DEPs fall into this category (Figure 4). The second prominent molecular function detected was the molecular function regulator, followed by catalytic and transporter activity. As for the biological process, the largest proportion of DEPs in poly(I:C) challenge groups was involved in cellular processes (22.7%). The metabolic process, biological regulation, and response to stimulus were the most abundant biological processes after cellular processes. Whilst the immune system process accounted for 9.1% of DEPs in the poly(I:C) challenged group.

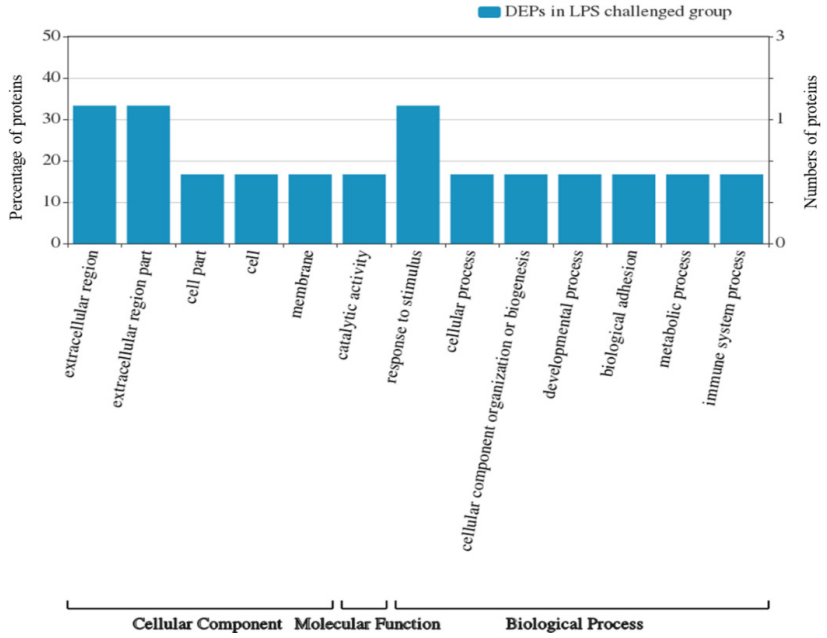

Fig. 3. GO of the DEPs identified from the basal and HWE-supplemented diet groups upon LPS challenge.

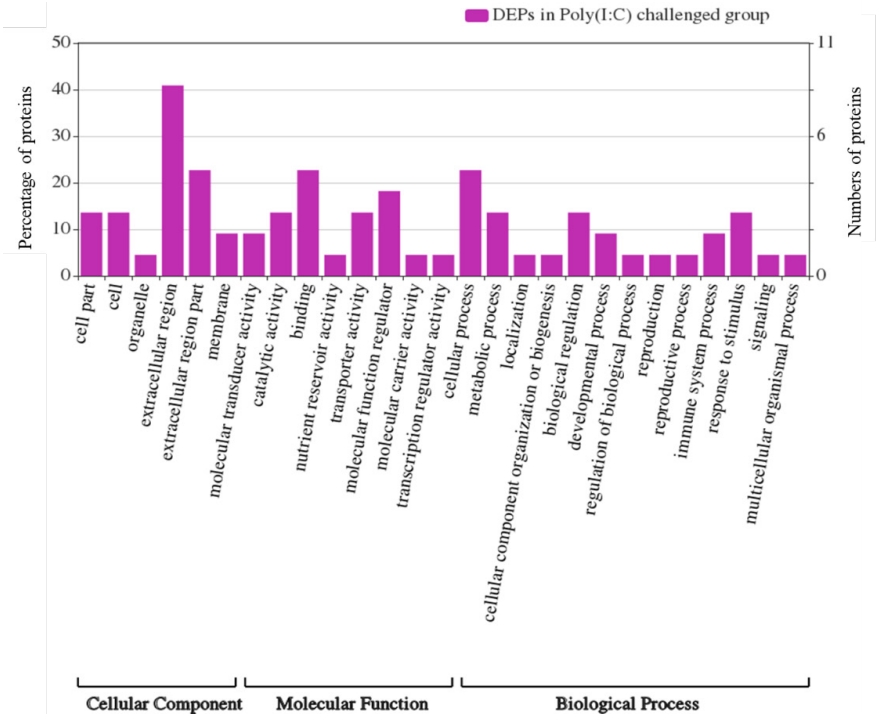

Fig. 4. GO of the DEPs identified from the basal and HWE-supplemented diet groups upon poly(I:C) challenge.

Metabolites analysis of HWE

Even though β-glucan is always associated with the immunomodulatory properties of mushrooms, the activities can also be contributed to by bioactive metabolites. Metabolite analysis was thus carried out to identify the potential bioactive metabolites in HWE that might contribute to the immunomodulation observed. These metabolites were identified by the METLIN database after HWE were subjected to LC-MS analysis. As shown in Table 4, a total of 23 metabolites were detected in HWE, where seven of the total detections have no matched identity in the database, although the compounds have MFG scores of more than 60% and Diff (MFG.ppm) values between -5 and +5. The identified compounds belonged to the groups of fatty acid, amino acid, monosaccharide, pyridine, phenol, nucleotide, and benzenoid.

Table 4. Predicted compounds in *P. pulmonarius* stalk waste extracts identified through LC-MS analysis.

No.	Base peak	Predicted compounds	Compound class	Formula	Mass
1.	136.061	(+)-threo-2-Amino-3,4-dihydroxybutanoic acid	Fatty acid	$C_4H_9NO_4$	135.053
2.	204.122	L-Glutamic acid n-butyl ester	Amino acid	$C_9H_{17}NO_4$	203.115
3.	124.039	Isonicotinic acid	Pyridines	$C_6H_5NO_2$	123.032
4.	274.093	Piscidic acid	Phenol	$C_{11}H_{12}O_7$	256.059
5.	130.049	N-Acryloylglycine	Amino acid	$C_5H_7NO_3$	129.042
6.	153.040	Xanthine	Nucleotide	$C_5H_4N_4O_2$	152.033
7.	145.050	Methylitaconate	Fatty acid	$C_6H_8O_4$	144.043
8.	221.045	3-[6-(Carboxymethylene) cyclohexa-2,4-dien-1-ylidene]-2-oxopropanate	Quinodimethane	$C_{11}H_8O_5$	220.038
9.	252.109	Deoxyadenosine	Nucleotide	$C_{10}H_{13}N_5O_3$	251.101
10.	148.061	Isoglutamate	Amino acid	$C_5H_9NO_4$	147.053
11.	162.112	4-propionyl butyric acid	Fatty acid	$C_7H_{12}O_3$	144.078
12.	137.045	Erythronic acid	Monosaccharide	$C_4H_8O_5$	136.038
13.	182.082	m-Coumaric acid	Phenol	$C_9H_8O_3$	164.048
14.	294.156	N-(1-Deoxy-1-fructosyl) isoleucine	Amino acid	$C_{12}H_{23}NO_7$	293.15
15.	268.105	Acetyltryptophanamide	Amino acid	$C_{13}H_{15}N_3O_2$	245.116
16.	166.087	Benzocaine	Benzenoid	$C_9H_{11}NO_2$	165.079

DISCUSSION

The dietary use of mushrooms and their derivatives is receiving increasing attention in aquaculture as an alternative method to boost fish immune responses and mitigate the negative effects linked to antibiotics and chemotherapeutics (Van Doan *et al*., 2019). Mushroom stalk waste, which shares the therapeutic properties of the fruiting body, has been reported to hold great potential as a cost-effective immunostimulant in aquatic species (Ahmed *et al*., 2017a; Ching *et al*., 2021). In the present study, label-free quantification was used to identify the DEPs in fish serum obtained from basal diet and HWE-supplemented diet groups following PAMPs challenge. The identification of immune-related proteins and signalling molecules during the defence response of red hybrid tilapia towards PAMPs invasion is vital to understand the effect of mushroom stalk waste extracts on the mechanisms of the immune system, which may also permit the discovery of serum protein biomarkers.

The proteomic analysis revealed more DEPs in serum following poly(I:C) challenge compared to LPS, suggesting a broader activation of immune mechanisms against viral antigens than bacterial antigens. This observation potentially reflects the need for multifaceted antiviral defences in fish, including antibody pathways and acute-phase responses. Immunoglobulin μ heavy chain was the DEP identified in both LPS and poly(I:C) challenged groups. As shown in the heat maps, red hybrid tilapia fed with an HWE-supplemented diet showed a higher level of immunoglobulin μ heavy chain as compared to the basal diet-fed fish under control treatment. This observation suggested that supplementing fish with HWE could enhance their ability to defend themselves against infections (Salinas *et al*., 2021). Likewise, administration of *Lentinula edodoes* extract and *Ganoderma applanatum* extract in the diet of rainbow trout significantly increased the serum total immunoglobulin level (Baba *et al*., 2015; Manayi *et al*., 2016). However, the protein level was lower after PAMPs challenge, which may be due to the utilisation of immunoglobulins to neutralise the antigens in the bloodstream.

A similar pattern was observed for apolipoprotein A-II, which showed a higher protein level after HWE supplementation under the control treatment (Figure 1). According to Yang *et al*. (2018), apolipoprotein A-II can induce cytokine production and inflammation in fish. Therefore, a higher level of apolipoprotein A-II in the HWE-supplemented group than in the basal diet group suggested that HWE supplementation can intensify the inflammatory response in red hybrid tilapia by producing more apolipoprotein A-II. On top of that, studies reported that *Pleurotus pulmonarius*, *Pleurotus ostreatus,* and *Pleurotus florida* supplementation in rats can increase the high-density lipoprotein cholesterol level, which is mainly comprised of apolipoprotein A (Khursheed *et al*., 2020; Amirullah *et al*., 2021; Iqbal *et al*., 2024). Nevertheless, the LPS challenge induced a lower level of apolipoprotein A-II in both experimental diet groups, but the level was much lower in the basal diet group as compared to the HWE-supplemented group. This result suggested that HWE supplementation can prevent the excessive reduction of apolipoprotein A-II when encountering LPS antigen in the bloodstream.

In comparison, more immune response proteins were differentially expressed upon poly(I:C) challenge, such as plasma protease C1 inhibitor, inter-alpha-trypsin inhibitor heavy chain H3, C-type lectin domain-containing protein, and L-rhamnose-binding lectin. Both plasma protease C1 inhibitor and inter-alpha-trypsin inhibitor heavy chain H3 showed higher levels in the basal diet group compared to the HWE-supplemented group after poly(I:C) challenge, suggesting the modulation of complement activity. As serine protease inhibitors, these proteins play an important role in the inhibition of proteins involved in the complement

system (Lord *et al*., 2020; Bavia *et al*., 2022). Additionally, inter-alpha-trypsin inhibitor can control neutrophil activation by reducing the production of reactive oxygen species and adhesion to vascular endothelial cells (Lord *et al*., 2020). Comparatively lower level of inter-alpha-trypsin inhibitor heavy chain H3 was also observed in rainbow trout liver at 48 hr post *Aeromonas salmonicida* infection (Causey *et al*., 2018). Therefore, lower levels of these proteins in HWE-fed fish suggested a stronger inflammatory response, complement activity, and neutrophil activity when encountering poly(I:C) in the bloodstream.

C-type lectin domain-containing protein, vitellogenin, and L-rhamnose-binding lectin can function as pattern recognition receptors that recognise glycan, nucleic acids, LPS, lipoteichoic acid, and other PAMPs (Mu *et al*., 2022; Scur *et al*., 2023; Shoba *et al*., 2023). These proteins can bind to bacterial and viral surface molecules, triggering agglutination as well as acting as opsonins that facilitate pathogen clearance via phagocytosis (Sun & Zhang, 2015; Bermejo-Jambrina *et al*., 2018; Mu *et al*., 2022; Wang *et al*., 2023; Miyake, 2025). Collectively, these proteins neutralise viruses, modulate inflammation, and activate the complement system. One day after the poly(I:C) challenge, the levels of these proteins were lower in the HWE-supplemented group in comparison with the basal diet group. This reduction may reflect their utilisation in binding circulating antigens and forming immune complexes, which are subsequently phagocytosed and removed from circulation, leading to lower detectable concentrations in serum.

Several acute-phase and inflammatory proteins, including apolipoprotein Eb, leucine-rich alpha-2-glycoprotein, and α2-HS-glycoprotein, were also differentially regulated in red hybrid tilapia following poly(I:C) challenge. Apolipoprotein Eb, which is upregulated during bacterial infections in fish, modulates inflammation by suppressing excessive pro-inflammatory cytokine release and reducing apoptosis, thereby improving the survival rate of fish (Feng *et al*., 2023). Leucine-rich alpha-2-glycoprotein, an inflammatory biomarker induced by cytokines, contributes to the amplification of inflammatory cascades (Sun *et al*., 2022). In the present study, higher levels of apolipoprotein Eb and lower levels of leucine-rich alpha-2-glycoprotein were observed in the HWE-supplemented group following the poly(I:C) challenge, suggesting enhanced immune modulation and protection against excessive inflammation. α2-HS-glycoprotein, on the other hand, is an anti-inflammatory protein that inhibits the TGF-β and NF-κB signalling pathways, thereby suppressing immune overactivation (Zhao *et al*., 2025). α2-HS-glycoprotein level was lower in the HWE-supplemented group after one day of challenge, consistent with its role as a negative acute phase protein, where its amount decreases during infection or inflammation (Kuleš *et al*., 2020). This result may also reflect its engagement in controlling inflammation through negative feedback mechanisms. Taken together, these observations suggest that HWE supplementation promotes a more regulated acute phase response, preventing prolonged viral mimic inflammation in fish.

Hemopexin and serotransferrin are DEPs that play important roles in metal ion homeostasis. Hemopexin binds and transports free heme and thus reduces the oxidative stress and iron loss induced by heme (Lechuga *et al*., 2022). Previous study revealed that hemopexin from Nile tilapia possesses antibacterial property by impeding the growth of *S. agalactiae* and *A. hydrophila* (Yin *et al*., 2021). Similarly, transferrin can bind to iron, creating a low-iron environment that is unfavourable for the survival of pathogenic microorganisms in fish (Asmamaw, 2016). The transferrin was also able to regulate the non-specific cytotoxic cell (NCC) signalling pathway and improve the killing activity of NCC (Huang *et al*., 2022). Lower levels of hemopexin and transferrin were detected in the HWE-supplemented group after poly(I:C) challenge as compared to the basal diet group. This may be due to the heme-bound hemopexin and iron-bound transferrin being internalized by cells through endocytosis during iron transportation, therefore resulting in a lower concentration in blood (Asmamaw, 2016; Vanacore *et al*., 2019; Lechuga *et al*., 2022).

Functional classifications suggested that the majority of the DEPs found in this LPS challenge group were involved in the response to stimulus, and 16.7% of the DEPs were specifically involved with the immune system processes, which were immunoglobulin μ heavy chain. On the other hand, DEPs in the poly(I:C) challenge group were mainly associated with cellular processes, such as L-rhamnose-binding lectin and α2-HS-glycoprotein-like. The following biological processes were metabolic processes, biological regulation, and response to stimulus, which include apolipoprotein, hemopexin, and inter-alpha-trypsin inhibitor heavy chain H3-like. Immunoglobulin μ heavy chain and plasma protease C1 inhibitor were DEPs associated with the immune system process in the poly(I:C) challenge group. These findings highlight the tailored nature of fish immune responses to distinct pathogen types.

Overall, most of the DEPs have a lower protein level after one day of LPS and poly(I:C) challenge. This might be attributed to the fact that more proteins were being employed during the immune responses against bacterial and viral antigens, and therefore fewer proteins were found in the serum upon the PAMPs challenge. The proteome analysis revealed that HWE supplementation can regulate immune-related serum proteins in red hybrid tilapia in response to PAMPs stimulation. The bioactive compounds in HWE are believed to modulate the activity of innate immune cells, such as macrophages and neutrophils, allowing for a more effective clearance of opsonised pathogens by phagocytosis. The activation of innate immune response by HWE supplementation also allows the production of acute phase proteins, which are essential for controlling the inflammatory response when exposed to LPS and poly(I:C) in the blood circulation. The acute phase proteins with metal ion-binding properties can also reduce the availability of metal ions for pathogens, thus impeding the growth of pathogens. Furthermore, the bioactive compounds in HWE may interact with immune cells and activate the signalling pathways involved in adaptive immunity, leading to the modulation of antibody production.

Previously, we reported that HWE was largely composed of water-soluble carbohydrates and proteins, where the immunostimulatory effects of HWE on red hybrid tilapia may be attributed to the presence of β-glucans (Ching *et al*., 2021). However, there was a possibility that other types of bioactive compounds related to immunomodulation also exist in HWE besides β-glucans. The existence of active metabolites might explain the difference in the degree of immune response to previously reported E-HWE supplementation (Ching *et al*., 2022). The metabolite profile of an extract typically reveals the existence of naturally occurring, smaller-sized bioactive compounds (Coelho *et al*., 2023). Studies have shown that apart from β-glucan, mannogalactan, heteroglycan, lectins, pleurostrin, proteoglycans, terpenoids, lovastatin, and phenolic compounds present in the *Pleurotus* spp. also exhibited immunomodulatory properties (Golak-Siwulska *et al*., 2018; Van Doan *et al*., 2019; Zhao *et al*., 2020). Therefore, metabolite profiling of HWE was carried out in this study to screen for possible metabolites that

may contribute to the immunomodulatory property of HWE.

LC-MS analysis of HWE revealed several bioactive metabolites, including m-coumaric acid, piscidic acid, isonicotinic acid, methyl itaconate, xanthine, and N-(1-Deoxy-1-fructosyl) isoleucine. Piscidic acid and m-coumaric acid are phenolic compounds that possess antioxidant and antimicrobial activities (Blando *et al*., 2019; Alexandre *et al*., 2021; Sawicki *et al*., 2024). Piscidic acid can reduce the generation of reactive oxygen species by complexing Fe^{2+} (Ressaissi *et al*., 2017), while m-coumaric acid can inhibit the growth of *Streptococcus mutans* (Coyago-Cruz *et al*., 2025). Besides that, isonicotinic acid derivatives and methyl itaconate exhibit antibacterial and antiviral activities (Boltz *et al*., 2018; Popiołek *et al*., 2018; Hooftman & O'Neill, 2019; Krause *et al*., 2021). Isonicotinic acid derivatives can promote the rupture of bacterial cell walls and inhibit the replication of viruses (Boltz *et al*., 2018; Popiołek *et al*., 2018). On the other hand, methyl itaconate was shown to reduce the oxidative stress and inhibit the transcriptional responses to LPS in murine macrophages (Hooftman & O'Neill, 2019). It also plays a modulating role in murine neurons upon Zika infection (Hooftman & O'Neill, 2019).

Xanthine, a purine base, is widely used as a precursor of pharmacologically active compounds such as caffeine, theophylline, and theobromine (Bortolotti *et al*., 2021; NCBI, 2022). Xanthine possesses anti-inflammatory properties by stimulating the production of anti-inflammatory cytokines and modulating gene transcription (LiverTox, 2012). Furthermore, N-(1-Deoxy-1-fructosyl) isoleucine is an isoleucine derivative that can produce reactive oxygen species (Bernardo-Bermejo *et al*., 2019). Isoleucine serves various biological functions, including stimulating the immune system, promoting wound healing, detoxifying nitrogenous wastes, and regulating blood sugar and energy levels (Alamgir, 2018). Therefore, the presence of these bioactive metabolites in HWE was likely to be a contributing factor to the immunomodulatory effects shown in red hybrid tilapia.

Through the data obtained, HWE supplementation shows strong potential as a sustainable feed additive, modulating immune responses in red hybrid tilapia through regulating key serum proteins involved in bacterial and viral defence. These results offer practical value for fish farmers, hatchery managers, and the aquaculture feed industries seeking to improve fish health and disease resilience.

CONCLUSION

This study demonstrated that HWE supplementation to red hybrid tilapia modulates the expression of immune-related serum proteins in response to bacterial and viral antigens. The presence of bioactive compounds in HWE beyond β-glucans, such as piscidic acid, methyl itaconate, and isonicotinic acid, supports the extract's immunomodulatory potential. These findings indicate that *P. pulmonarius* stalk waste extract is a promising functional feed additive to enhance innate immunity and reduce reliance on antibiotics in aquaculture. Incorporating such bioactive-rich supplements into fish diets may improve health management and sustainability in fish farming systems. Future research should evaluate long-term immune and growth effects of HWE supplementation and validate benefits through real pathogen challenge trials. Large-scale economic feasibility assessment can also be done, as it will be crucial for successful industry adoption.

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ETHICAL STATEMENT

Ethical clearance in handling the animal for this project was obtained from the Institutional Animal Care and Use Committee, Universiti Malaya (Ethics Reference Number: S/03102019/10052019-01/R).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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