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# Transcriptome profiling of *Penaeus vannamei* hepatopancreas infected with WSSV following feeding diet containing ulvan

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**Abstract**

The present study aimed to elucidate the mechanism of protection provided by dietary ulvan as observed in previous studies. Thirty shrimps (3.35 ± 0.08 g average body weight) were stocked in six polyethylene tanks (5 shrimps tank$^{-1}$, treatments were in three replicate/tank) and were fed either a diet containing no ulvan (control) or containing ulvan at 1 g kg diet$^{-1}$ for 35 days at 10% body weight twice daily. At the termination of the feeding, the shrimps were subjected to a white spot syndrome virus (WSSV) challenge test via intramuscular injection of the viral inoculum. Shrimps were sacrificed after 24 h of exposure, and the hepatopancreas was excised for total RNA extraction for transcriptome profiling. Biological validation of the RNA-seq results was also performed for 10 immune-related genes (6 up- and 4 down-regulated genes). A comparison of the ulvan group with the control group revealed that 69 differentially expressed genes (DEGs) were significantly up-regulated, whereas 640 were significantly down-regulated. 184 DEGs between the control and ulvan-treated groups were classified into six KEGG categories of Metabolism (145 DEGs), Organismal systems (16), Human disease (4), Genetic Information Processing (10), Cellular Processes (2), and Environmental Information Processing (7). The 145 DEGs under Metabolism were distributed to Level 2 subcategories as carbohydrate metabolism (59 DEGs), global and overview maps (44), energy metabolism (27), and amino acid metabolism (15). All candidate immune-related genes (67) were down-regulated except for 5 genes. The validation experiment showed proportionality of gene expressions of the qPCR and of those in the assembled transcriptome, justifying the acceptability of the RNA-seq results. In conclusion, data from the present study provided mechanisms for protecting the white shrimp by dietary ulvan against WSSV infection.

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## Introduction

Pacific white shrimp, *Penaeus vannamei* is the most extensively cultured crustacean globally because of its high yield and low salinity requirement (Zhou et al., 2012). In the last decades, viral diseases such as the white spot syndrome virus (WSSV) have seriously threatened the shrimp aquaculture industry causing high mortality and huge economic loss. WSSV could reach 100% cumulative mortality within 3-10 days (Lightner et al., 1996). To remedy the situation, antibiotics and chemotherapeutic agents have been used. Still, they are not recommended in shrimp farming due to their ill effects on consumers and their increasing threat of producing antibiotic-resistant bacteria (Parmar & Yusufzai, 2018). Managing shrimp health with natural substances is a wise choice for shrimp farmers. For example, seaweed extracts are generally recommended for use in aquafeeds to promote growth, enhance nonspecific immunity, and control various diseases in aquatic animals (Wang et al., 2017; Mohan et al., 2019; Akbary & Aminikhoei, 2018).

*Ulva intestinalis* is an essential source of the water-soluble polysaccharide known as ulvan, which accounts for approximately 38–54% of dry seaweed weight (Mohan et al., 2019; Chandini et al., 2008; Usman et al., 2017). Ulvan is mainly composed of rhamnose, xylose, glucuronic and iduronic acid and is mostly distributed in repeating disaccharide units. The disaccharide units are formed by L-rhamnose 3-sulfate linked to a D-guluronic acid residue (ulvanobiuronic acid unit A); an L-iduronic acid residue (ulvanobiuronic acid unit B); a D-xylose 4-sulfated residue (ulvanobiose unit A); or a D-xylose residue (ulvanobiose unit B) (Venugopal et al., 2019; Robic et al., 2009a,b; Vera et al., 2011). Ulvan from *Ulva intestinalis* consists of glucose (6.81 ± 0.94%), xylose (4.15 ± 0.11%), and rhamnose (25.84 ± 0.80%) (Klongklaew et al., 2021) similar to the composition of those from other *Ulva* species. However, the biochemical components of ulvan may mainly depend on the seaweed species, collection season, growing area, geographical differences, environmental factors, and extraction methods (Venugopal et al., 2019; Lahaye & Robic, 2007).

In our previous studies, we demonstrated that ulvan promoted growth (Serrano & Declarador, 2014) and prolonged the survival of *Penaeus monodon* larvae upon WSSV challenge test, 77% higher total hemocyte count (THC), higher respiratory burst (RB) activity, higher phenoloxidase (PO) activity than the control group fed diet without ulvan (Declarador et al., 2014; Lauzon & Serrano, 2015). In the Pacific white shrimp, shrimps fed with a diet containing ulvan extract from *Ulva intestinalis* exhibited higher THC, RB, and PO values than those with no ulvan (Lauzon & Serrano, 2015). Ulvan resulted in moderate disease resistance against *Vibrio harveyi* in *P. monodon* and *Vibrio parahaemolyticus* in *Penaeus vannamei* (Ge et al., 2019; Selvin et al., 2011). The present study aimed to evaluate the transcriptomic response of the Pacific white shrimp *Penaeus vannamei* in the hepatopancreas during WSSV infection following a 35-day feeding of a diet containing ulvan extracted from *Ulva intestinalis*.

## Materials and Methods

*WSSV Inoculum Preparation*

White spot syndrome virus (WSSV), courtesy of the SEAFDEC Aquaculture Department in Tigbauan, Iloilo, Philippines, was used as the primary WSSV isolate and was passaged once in *Penaeus vannamei* before the inoculum preparation. Tissues from infected shrimp underwent a polymerase chain reaction (PCR) test to confirm the infection. The WSSV inoculum was prepared based on the method of Callinan et al. (2013). Gill and muscle tissue from infected shrimp were aseptically dissected and placed on ice, and homogenized manually using polyethylene pestles in sterile phosphate buffered saline at a 1:10 w/v ratio. The suspension was then centrifuged at 8000 rpm for 10 min. The clear supernatant was passed through a 0.20 µm membrane filter before inoculation.

*WSSV inoculation and virus titer determination*

In a separate experiment before the feeding trial, the virus was WSSV stock produced for the challenge experiment in a later period. A Southeast Asian Fisheries Development

Council-Aquaculture Department (SEAFDEC-AQD) isolate of WSSV from naturally-infected *Penaeus vannamei* was used. This virus isolate was passaged once also in the white shrimp. A gill suspension was obtained and was diluted $10^{-1}$ in phosphate-buffered saline (PBS) pH 7.4, and 10 µl were injected intramuscularly into WSSV-free *Litopenaeus vannamei* to amplify the virus. The inoculated shrimp were collected at 48 h post-inoculation (hpi) and were frozen at −70°C until use. Muscle tissues from these shrimps were analyzed by one-step PCR to confirm WSSV infection. A $10^{-1}$ suspension was made in PBS and centrifuged (3000 × g at 4°C for 20 min). The supernatant was centrifuged (13000 × g at 4°C for 20 min), filtered (0.45 µm), and aliquoted for storage at −70°C. The total volume was 250 ml.

To determine the virus infection titers, three experiments were conducted with shrimp (4.14 g ± 0.08 average body weight, ABW, n=30) inoculated with 10 µl·g$^{-1}$ of a 10-fold serial dilution of WSSV. Five shrimp were used per dilution. Moribund and dead shrimp were processed for the detection of WSSV infection. These experiments were terminated at 120 hpi. The virus infection titers ($SID_{50}$ ml$^{-1}$) and mortality ($LD_{50}$ ml$^{-1}$) were calculated using the method of Reed & Muench (1938). Briefly, data from infected and uninfected shrimp at each dilution were recorded. Totals were calculated for infected shrimp from the lowest to the highest concentration and uninfected shrimp in the inverse direction to obtain the percentage of infected shrimp for each dilution. The 2 dilutions with the nearest percentage above (a) and below (b) 50% were used to calculate a proportional distance (50%−b)/(a−b), which was added to the log10 of the highest dilution below 50% to determine the 50% endpoint of infection ($SID_{50}$ ml$^{-1}$) according to the volume inoculated. The computed $LD_{50}$ ml$^{-1}$ was $10^{6.38}$, while the computed virus infection titer was $10^{5.5}$ ($SID_{50}$ m$^{-1}$).

*Feeding trial- feeding with the control and ulvan-treated diets and WSSV challenge test*

The feeding trial proper was performed in which three replicate groups of fish per dietary treatment were used. Thirty shrimps (ABW=3.35 ± 0.08 g) were stocked in six 16L polyethylene tanks (5 shrimp tank$^{-1}$), acclimated for 3 days and fed with the experimental diets (Table 1) for 35 days at 10% body weight daily, divided into twice daily. At the termination of the feeding trial, the shrimps were subjected to a WSSV challenge test via intramuscular injection of the viral inoculum prepared as 10 $^{6.38}$ ml$^{-1}$ $LD_{50}$ at 10 µl·g$^{-1}$ shrimp. Shrimp were sacrificed after 24 h of exposure, and the gills and hepatopancreas were aseptically dissected for DNA (for 1-step PCR verification of WSSV) or RNA extraction for the RNA-seq analysis.

Water quality was monitored throughout the entire feeding. Average temperature, pH, salinity, and ammonia were 25.16°C ± 0.03, 8.16 ± 0.01, 25.0 ppt ± 0.00, 0.17 ± 0.01ppm, respectively.

**Table 1** Composition of the experimental diets.

| *Ingredients* | *Control diet(g)* | *Ulvan diet(g)* |
|---|---|---|
| Peruvian fish meal | 20.00 | 20.00 |
| Shrimp meal | 34.00 | 34.00 |
| Soybean meal | 21.00 | 21.00 |
| CMC | 3.48 | 2.48 |
| Vitamin mix | 1.00 | 1.00 |
| Mineral mix | 1.00 | 1.00 |
| BHT | 0.02 | 0.02 |
| Lecithin | 0.50 | 0.50 |
| Cod liver oil | 4.00 | 4.00 |
| Starch | 15.00 | 15.00 |
| Ulvan | 0.00 | 1.0 |
| TOTAL | 100.00 | 100.00 |

*RNA isolation, cDNA library construction and RNA-seq*

Total RNA was extracted using Trizol plus reagent kit (Invitrogen, USA), according to the manufacturer's instructions. The degradation and contamination of the extracted RNA

were monitored using gel electrophoresis on a 1% agarose gel and spectrophotometer (Nanodrop). The extracted RNA was put in desiccator tubes containing RNA stable (Brand, place) and were allowed to dry for 24 h and were sent to Novogene Bioinformatics Technology Co. Ltd., Beijing, China) for further quality assessment (Qubit and Agilent 2000), cDNA library construction, and RNA-seq. The libraries included 6 gigabases (GB) of each sample.

After the QC procedures, mRNA from eukaryotic organisms is enriched from total RNA using oligo(dT) beads. The mRNA was then fragmented randomly in a fragmentation buffer, followed by cDNA synthesis using random hexamers and reverse transcriptase. After first-strand synthesis, a custom second-strand synthesis buffer (Illumina) was added, with dNTPs, RNase H, and *Escherichia coli* polymerase I to generate the second strand by nick-translation and AMPure XP beads were used to purify the cDNA. The final cDNA library is ready after a round of purification, terminal repair, A-tailing, ligation of sequencing adapters, size selection, and PCR enrichment. Library concentration was first quantified using a Qubit 2.0 fluorometer (Life Technologies) and then diluted to 1 ng/μl before checking insert size on an Agilent 2100 and quantifying to greater accuracy by quantitative PCR (Q-PCR) (library activity >2 nM).

Transcriptome sequencing was carried out on an Hiseq Illumina 4000 platform that generated ~150 bp pair-end raw reads. The RNA from three replicates of the control and ulvan groups were prepared for cDNA library generation.

*Bioinformatic Analysis*

The following analysis was done: (1) De Novo Transcriptome Assembly using Trinity software. Corset was then further used to perform hierarchical clustering to remove redundancy. The longest transcripts in each cluster were selected to represent a particular unigene. (2) Gene Functional Annotation in which annotation of contigs or genes was done using 7 databases, namely Nr (NCBI non-redundant protein sequences), Nt (NCBI nucleotide sequences), Pfam (Protein family), Swiss-Prot, KOG/COG (Cluster of Orthologous Groups of Proteins, COG; and eukaryotic Orthologous Groups, KOG), GO (Gene Ontology), and KEGG (Kyoto Encyclopedia of Genes and Genome). (3) Gene Expression Analysis*,* in which *de novo* assembled transcriptome was used as the reference sequence for gene expression analysis. (4) Analysis of Differentially Expressed Genes (DEGs), to identify DEGs using DESeq software based on a negative binomial distribution wherein the *p*-values were normalized first using the *q*-value. A q-value <0.05 and |log2 (fold-change)| >1 were set as threshold to classify significant differential expression; (5) Enrichment Analysis - GO enrichment of DEGs was done using GOseq R packages and KOBAS software for KEGG Pathway Enrichment Analysis.

*Validation of DEGs by qPCR*

To confirm the transcriptome results, 10 DEGs (six down-regulated and four up-regulated) were chosen from the transcriptome data of the hepatopancreas. The *Clec, DGI, FH, Akr, Acox, IFNy, Chap, HSP60, HSP70, HSP90,* and *Sag* genes were authenticated by quantitative PCR (qPCR) using Applied Biosystems StepOnePlus™ Real-Time PCR System. Being a housekeeping gene, glyceraldehyde 3- phosphate dehydrogenase (gapdh) was used as a reference gene. The aforementioned reference gene primer was sequenced by Integrated DNA Technologies, having a forward primer sequence of 5'-CCA TGG AAT GTT CTC GGG CT -3' and a reverse sequence of 5'-AAG TAT GAC AGC ACC CAC GG -3'. Primers for all DEGs, on the other hand, were manually designed and sequenced by Kinovett Scientific Solutions Co. Primers for all DEGs, on the other hand, were manually designed and sequenced by Kinovett Scientific Solutions Co. Relative expression of genes was determined using the $2^{-\Delta\Delta Ct}$ method. The summary for primer sequences of DEGs is shown in **Table 2**.

**Table 2** Information of primers for qPCR

| *Abbreviations* | *Full names* | *Primers* | *Sequences (5′-3′)* |
|---|---|---|---|
| *Clec* | C-lectin | *Clec* | ACGCCTTCTGGCTTGGAGGAT |
| | | *Clec* | GTTCTGATTGGTTCCTCCATCGG |
| *DGI* | Drystoglycan | *DGI*-F | TGAGATCGATGGTTCCATTCTGG |
| | | *DGI*-R | ACTACATCACCATCAAGGCATACG |
| *FH* | Fumarate hydratase | *FH*-F | CCAATGGGGAAGTTCATGACGGA |
| | | *FH*-R | GCATAGCAACATCCTCAGCCAT |
| *Akr* | Aldo-keto reductase | *Akr*-F | GCTAAAATAGTGCCTGCCAACAA |
| | | *Akr*-R | TAGCCCAAGGTCTGTCTGGTGA |
| *Acox* | Acyl-coA reductase | *Acox*-F | TCTCGCCAACAATCTGCTGAAC |
| | | *Acox*-R | TCTCGGACCCAGAATTCCTCGA |
| *IFNy* | Gamma interferon | *IFNy*-F | GAACATCTCCACCTCCATGATGT |
| | | *IFNy*-R | CGGTCAAGATCCACCTGTACTAC |
| *Chap* | Chaperonin | *Chap*-F | GGCTTTAGAAGTTGTTCCTCGTCA |
| | | *Chap*-R | ACATCAACGCCAAACCACTTGC |
| *HSP60* | Heat Shock Protein 60 | *HSP60*-F | TTGGTGATCTTGGGACTGCC |
| | | *HSP60*-R | GCACTACCTGGCGAGACACTA |
| *HSP70* | Heat Shock Protein 70 | *HSP70*-F | ATGCTTCATGTCGCTCTGGACTG |
| | | *HSP70*-R | GACACAGAGCGTCTGATTGGTGA |
| *HSP90* | Heat Shock Protein 90 | *HSP90*-F | TCTGCCTCTGGCGACGAGAT |
| | | *HSP90*-R | CGCTTCTTAACCCTCTCAACGA |
| *Sag* | Surface antigen | *Sag*-F | GTTGCCACCTGAGATAGGGTTC |
| | | *Sag*-R | TTCTTCTGGCAGGTAACTCAGGT |

## Results

*Assembled transcript and functional analysis during WSSV infection following feeding diets containing ulvan*

The statistics of the sequencing data are shown in **Table 3**, and a total of 292,181,804 raw reads were obtained. Reads with low-quality primers and a ratio of N above 10% were removed, and 283,537,5288 clean reads were obtained. For each group, 42.90 to 57.52 million clean reads were produced from 44.22 to 59.37 million raw reads, with a GC content of 51.56–53.33% for clean reads. The Q20 of all the six cDNA libraries was>97%, and the Q30 was all >89.5%. The overall *de novo* assembly was performed using the combined reads from the 6 libraries, generating a total of 113,255 unigenes. The number of unigenes in the assembled transcript and their length distribution are shown in **Tables 4** and **5**. In contrast, the proportion of successfully annotated genes in the *de novo* assembly is shown in **Table 6**.

**Table 3** Summary of reads in WSSV-infected *P. vannamei* fed either the control (no ulvan) or ulvan diet.

| *Sample* | *Raw Reads* | *Clean Reads* | *Clean* | *Error(%* | *Q20(%* | *Q30(%)* | *GC(* |
|---|---|---|---|---|---|---|---|
| Control1 | 44634798 | 43261326 | 6.49G | 0.01 | 97.26 | 93.49 | 51.8 |
| Control2 | 44220914 | 42896990 | 6.43G | 0.01 | 97.24 | 93.45 | 53.3 |
| Control3 | 45640954 | 44299252 | 6.64G | 0.01 | 97.38 | 93.69 | 52.5 |
| Ulvan1 | 50684382 | 49244868 | 7.39G | 0.01 | 97.34 | 93.66 | 51.5 |
| Ulvan2 | 59369274 | 57517094 | 8.63G | 0.01 | 97.24 | 93.48 | 51.6 |
| Ulvan3 | 47631482 | 46317998 | 6.95G | 0.02 | 95.37 | 89.49 | 52.3 |

**Table 4** Overview of the number of transcripts and unigenes in different length intervals.

| *Transcript length interval* | *200-500bp* | *500-1kbp* | *1k-2kbp* | *>2kbp* | *Total* |
|---|---|---|---|---|---|
| Number of transcripts | 44,163 | 33,451 | 20,288 | 15,402 | 113,304 |
| Number of unigenes | 44,115 | 33,450 | 20,288 | 15,402 | 113,255 |

**Table 5** Overview of the length distribution of transcript and unigenes.

| | *Min Length* | *Mean Length* | *Median Length* | *Max Length* | *N50* | *N90* | *Total nucleotides* |
|---|---|---|---|---|---|---|---|
| Transcripts | 201 | 1,049 | 619 | 15,159 | 1,681 | 443 | 118,815,357 |
| Unigenes | 201 | 1,049 | 619 | 15,159 | 1,681 | 443 | 118,802,818 |

**Table 6** The percentage of successfully annotated genes in the *de novo* assembled transcript.

| | *No. of Unigenes* | *(%)* |
|---|---|---|
| Annotated in NR | 33681 | 29.73 |
| Annotated in NT | 13822 | 12.2 |
| Annotated in KO | 15630 | 13.8 |
| Annotated in SwissProt | 29097 | 25.69 |
| Annotated in PFAM | 37923 | 33.48 |
| Annotated in GO | 38198 | 33.72 |
| Annotated in KOG | 18565 | 16.39 |
| Annotated in all Databases | 5106 | 4.5 |
| Annotated in at least one Database | 48423 | 42.75 |
| Total Unigenes | 113255 | 100 |

*Identification of DEGs in WSSV-infected shrimp previously fed a diet with Ulvan or with the Control diet*

The volcano plot showing the number and relationship between fold-change and *p*-value of up- or down-regulated DEGs is shown in **Figure 1**. The ulvan vs control groups resulted in a total of 112,546 unigenes exhibiting no significant differences; 69 were significantly up-regulated, whereas 640 were significantly down-regulated.

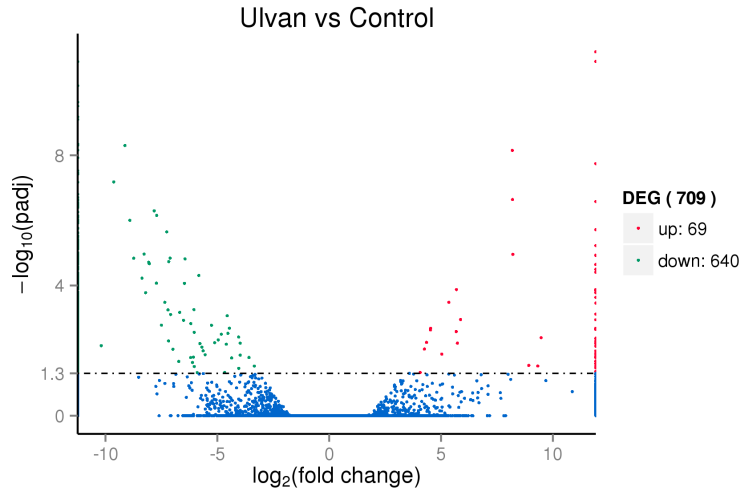


**Figure 1** Volcano plot of DEGs between ulvan and control group. Red: up-regulated; Green: down-regulated; Blue: no significant differential expression.

*GO enrichment analysis of the DEGs*

The GO database was used to annotate the DEGs further to assess their functions. All the DEGs were assigned to three major functional classes (cellular components, molecular functions, and biological processes) and 52 subcategories. There were 44 significantly enriched terms classified as a biological process (BP), including metabolic process, cellular metabolic process, single-organism metabolic process, biosynthetic process, oxidation-reduction process, etc. (**Figure 2**). Two remarkably enriched terms were categorized as molecular function (MF) catalytic activity and oxidoreductase activity; 13 other MFs were slightly but significantly enriched. The remaining 14 significantly enriched terms belonged to the cellular component (CC), including ribosome, intracellular ribonucleoprotein complex, thylakoid, thylakoid part, etc.

*KEGG enrichment analysis of the DEGs*

184 DEGs between the control group and the ulvan-added groups were classified into 41 subcategories belonging to the six KEGG categories of Metabolism (145 DEGs), Organismal systems (16), Human disease (4), Genetic Information Processing (10), Cellular Processes (2), and Environmental Information Processing (7) (**Table 7**). The 145 DEGs under Metabolism were distributed to Level 2 subcategories as carbohydrate metabolism (59 DEGs), global and overview maps (44), energy metabolism (27), and amino acid metabolism (15). Level 3 subcategories of DEGs are shown in **Figure 3**.

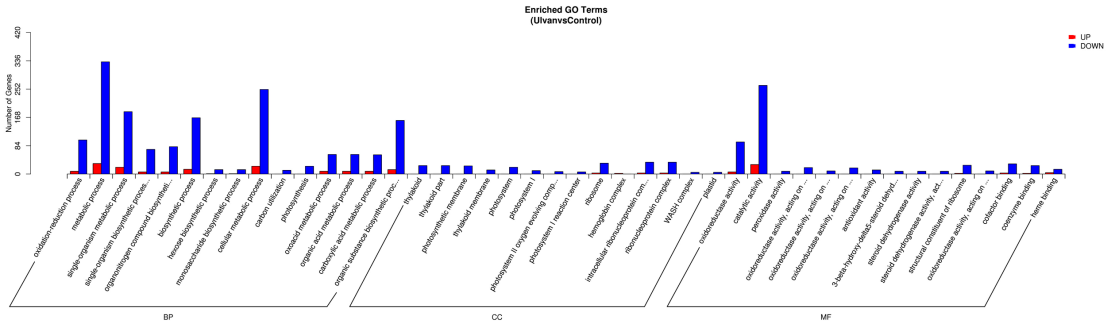


**Figure 2** Enriched GO terms when experimental groups were challenged with WSSV following the feeding of ulvan and control diet.

Among the enriched KEGG pathways that were immune-related (Level 2) were signal transduction (7 genes), folding sorting and degradation (10 genes), immune diseases (4 genes), and immune system (6 genes)(**Table 7**).

**Table 7** Most Enriched KEGG Pathways in the white shrimp after WSSV challenged following a diet of Ulvan or Control.

| Level 1 | Level 2 | Level 3 | Gene no. | P-value |
|---|---|---|---|---|
| Cellular processes | Cell growth and death | Cell cycle - Caulobacter | 2 | 0.0233742 |
| Environmental info | Signal transduction | Hippo signaling pathway - fly | 7 | 0.022295028 |
| Genetic Information Processing | Folding, sorting and degradation | Protein processing in endoplasmic reticulum | 10 | 0.037038435 |
| Human diseases | Immune diseases | Systemic lupus erythematosus | 4 | 0.018631428 |
| Metabolism | Energy metabolism | Carbon fixation in photosynthetic organisms | 18 | 6.01E-13 |
| | | Methane metabolism | 9 | 3.00E-05 |
| | Global and overview maps | Carbon metabolism | 28 | 4.17E-11 |
| | | Biosynthesis of amino acids | 16 | 3.20E-05 |
| | Carbohydrate metabolism | Glycolysis / Gluconeogenesis | 15 | 4.04E-06 |
| | | Fructose and mannose | 10 | 4.75E-05 |
| | | Glyoxylate and dicarboxylate | 8 | 0.000530852 |
| | | Pyruvate metabolism | 9 | 0.000770204 |
| | | Pentose phosphate pathway | 6 | 0.00513293 |
| | | Citrate cycle (TCA cycle) | 6 | 0.014043472 |
| | | Galactose metabolism | 5 | 0.028161381 |
| | Amino acid metabolism | Cysteine and methionine | 8 | 0.004178421 |
| | | Glycine, serine and threonine | 7 | 0.028037884 |
| Organismal system | Excretory system | Proximal tubule | 4 | 0.009910538 |
| | Immune system | Antigen processing and presentation | 6 | 0.030356273 |
| | Digestive system | Bile secretion | 6 | 0.031304849 |

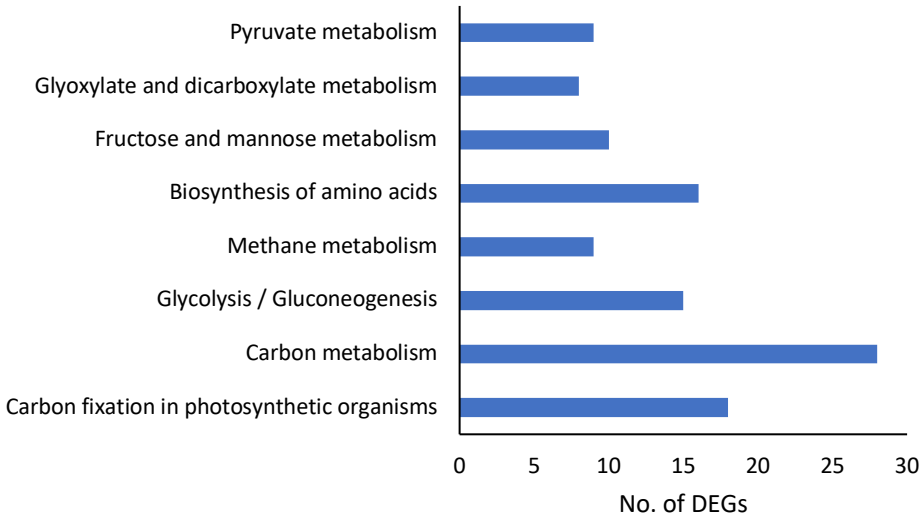


**Figure 3** Significantly Enriched KEGG pathways Ulvan v Control, following WSSV challenge.

Almost all candidate immune-related genes in the hepatopancreas of the ulvan group of Pacific white shrimp relative to the control group (fed diet with no ulvan, but also challenged with WSSV infection) exhibited down-regulation in the transcriptomic profile (**Tables 8** and **9**).

**Table 8** Comprehensive list of candidate immune-related genes after WSSV challenge following consumption of ulvan or control diet

| Gene ID | NR Description | Up-/Down-reg | padj |
|---|---|---|---|
| ***Recognition*** | | | |
| Cluster-25042.0 | hypothetical protein AK88_01740 | DOWN | 0.0032557 |
| Cluster-26701.22159 | interleukin-1 receptor-associated kinase | DOWN | 8.05E-05 |
| Cluster-27324.1 | surface antigen protein | DOWN | 0.00047366 |
| Cluster-26701.22159 | interleukin-1 receptor-associated kinase | DOWN | 8.05E-05 |
| ***Antimicrobial peptide*** | | | |
| Cluster-15960.0 | unnamed protein product CCMP3155 | DOWN | 0.0032521 |
| ***Signal transduction*** | | | |
| Cluster-26701.31321 | Thyroid peroxidase precursor | DOWN | 0.031911 |
| Cluster-26701.6772 | GF24460 | UP | 0.021103 |
| ***Serine protease and protease inhibitor*** | | | |
| Cluster-27430.0 | ATP-dependent protease | DOWN | 0.043087 |
| Cluster-35202.0 | protein phosphatase | DOWN | 0.04781 |
| Cluster-26701.50665 | Hippo | UP | 1.80E-08 |
| Cluster-26701.22159 | interleukin-1 receptor-associated kinase [ | DOWN | 8.05E-05 |
| ***Antioxidant*** | | | |
| Cluster-26701.16350 | predicted protein | DOWN | 0.024969 |
| Cluster-26701.50991 | glutathione peroxidase-like protein | DOWN | 0.032753 |
| Cluster-26701.16350 | predicted protein | DOWN | 0.024969 |
| Cluster-20313.0 | thioredoxin-dependent peroxide reductase | DOWN | 0.0034606 |
| Cluster-26701.57676 | glutathione transferase family protein | DOWN | 0.008106 |
| Cluster-26701.48471 | probable glutathione S-transferase parC | DOWN | 0.01095 |
| Cluster-16981.0 | probable glutathione S-transferase | DOWN | 0.039673 |
| Cluster-26701.53459 | probable glutathione S-transferase parC | DOWN | 0.0024446 |
| Cluster-26701.58106 | mino levulinate synthase | UP | 0.043539 |
| Cluster-26701.20929 | unnamed protein product CCMP3155 | DOWN | 0.00047366 |
| Cluster-26701.19165 | renalase | DOWN | 0.020213 |
| ***Apoptosis*** | | | |
| Cluster-26701.48786 | hypothetical protein EAI_13871 | DOWN | 0.00016614 |
| Cluster-26701.51228 | hypothetical protein X975_21388, | DOWN | 0.0011472 |
| Cluster-19658.0 | programmed cell death protein | DOWN | 0.019404 |
| Cluster-16756.0 | unnamed protein product | DOWN | 0.0092701 |
| Cluster-26701.48786 | hypothetical protein EAI_13871, | DOWN | 0.00016614 |
| Cluster-36148.0 | nucleolysin TIAR | DOWN | 0.00054968 |
| Cluster-20203.1 | splicing factor, CC1like subfamily protein | DOWN | 0.0041921 |
| Cluster-19658.0 | programmed cell death protein 5 | DOWN | 0.019404 |
| Cluster-26701.42500 | baculoviral IAP repeat-containing protein | DOWN | 0.01621 |
| ***Phagocytosis*** | | | |
| Cluster-26701.54701 | TBC1 domain family member | DOWN | 0.0057143 |
| ***Cytoskeleton*** | | | |
| Cluster-25042.0 | hypothetical protein AK88_01740 | DOWN | 0.0032557 |
| Cluster-26701.46903 | tubulin-specific chaperone A-like | DOWN | 0.035823 |
| Cluster-25042.0 | hypothetical protein AK88_01740 | DOWN | 0.0032557 |
| ***Cell adhesion*** | | | |
| Cluster-26701.44222 | connectin-like | DOWN | 7.08E-09 |
| ***Lysosome function*** | | | |
| Cluster-26701.35077 | GH14185 | DOWN | 0.0031416 |
| ***Heat shock protein*** | | | |
| Cluster-26701.28339 | high molecular weight heat shock protein | DOWN | 2.42E-07 |
| Cluster-26701.50988 | chaperone HSP90 | DOWN | 1.15E-05 |
| Cluster-26701.50987 | chaperone HSP90 | DOWN | 7.80E-05 |

**Table 9** Summary of immune-related DEGs: ulvan vs control (count summary of the total number of up-and down-regulated genes)

| NR Description | No. of genes | UP-/Down-reg |
|---|---|---|
| Oxidative stress | 16 | 16 DOWN |
| Proteinases/proteinase inhibitors | 12 | 2 UP, 10 DOWN |
| Apoptotic tumor-related protein | 7 | 7 DOWN |
| Blood clotting system | 4 | 4 DOWN |
| Other immune molecules | 28 | 3 UP, 25 DOWN |
| Total | 67 | 5 UP, 62 DOWN |

### *Validation of the RNA-seq results by qPCR*

Ten DEGs (six up-regulated and four down-regulated) related to the immune pathway were selected. Compared with the control group (without dietary ulvan), the ulvan group exhibited relative expressions of *Clec, HSP60, HSP70, HSP90, Sag, Akr, Chap, DGI, FH, IFNy* from the qPCR to be proportional to their gene expression in the assembled transcriptome. Overall, the RNA sequencing results were consistent with qPCR analyses of the expression of the ten DEGs.

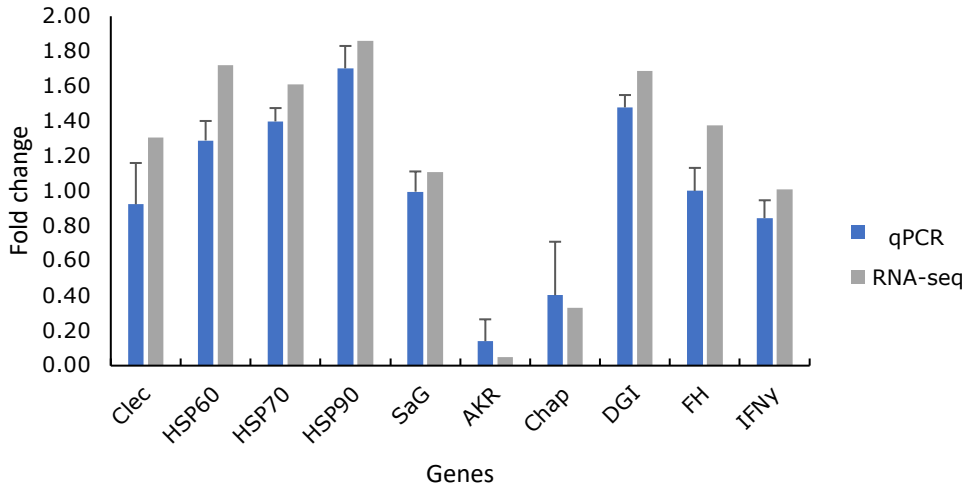


**Figure 4** Gene expression of 10 selected differentially expressed genes (six up-regulated and four down-regulated) compared with the fold-change increase/decrease in the assembled transcript following RNA sequencing.

## Discussion

We have shown previously that diets containing 1 g kg diet$^{-1}$ or more could prolong survival and promote the activation of cellular immunity, respiratory burst activity, and stimulate hemocytic degranulation and activate prophenoloxidase to become phenoloxidase both in *Penaeus monodon* (Declarador et al., 2014) and the *Penaeus vannamei* (Lauzon & Serrano, 2015) against WSSV. Additionally, ulvan has moderate effects on disease resistance against *Vibrio harveyi* in *P. monodon* and *V. parahaemolyticus* in *Litopenaeus vannamei* (Ge et al., 2019; Selvin et al., 2011). Moreover, Klongklaew et al. (2020) reported that a crude polysaccharide extract or hot water crude extract (HWCE) from *U. intestinalis* at 5 or 10 mg mL$^{-1}$ was able to inhibit WSSV and YHV infection strongly. However, the detection of the relative gene expressions of the 10 immune-related DEGs using qPCR in the validation experiment in the present study, 6 of which were found to be

up-regulated (drystoglycan, fumarate hydratase, aldo-keto reductase, acyl CoA oxidase, gamma interferon, chaperonin) demonstrating that there was a positive effect of ulvan in the protection of the shrimp against WSSV. The results of the present study agreed well with those previous studies that found that the application of marine polysaccharides results in the enhancement of the expression of various immune-related genes in shrimp (Sinurat et al., 2016; Liu et al., 2020; Lee et al., 2020; Chen et al., 2016; Klongklaew et al. (2021).

Almost all the immune-related genes in the present study were down-regulated after laboratory-induced WSSV infection following feeding diets containing ulvan. It has been observed that signaling pathways are more likely susceptible to be stimulated by WSSV in crustaceans (Yang et al., 2018; Zhong et al., 2017; Shi et al., 2018). The down-regulation of immune-related genes, including the signaling pathways in the present study, indicated that the white shrimp was fairly protected against WSSV by the dietary ulvan.

A big part of all enriched KEGG pathways identified in the ulvan group vs the control group (both groups were challenged with WSSV) in the present study were related to metabolism. This indicates that the metabolic rate was enhanced by the dietary ulvan in the white shrimps. The up-regulated, metabolism-related genes in the ulvan group were those belonging to carbohydrate, energy, and amino acid metabolism. Genes for the enzyme that promoted glycolysis and oxidative phosphorylation have been described to increase their expression during WSSV infection (Wang et al., 2007). It was hypothesized that the genes in these pathways could contribute to fulfilling the high energy requirements for virus replication. Additionally, Cabrera-Stevens et al. (2022) suggested that the overexpression of these genes in the pathways of glycolysis and oxidative phosphorylation may facilitate energy-dependent biological processes used by the host immune defense against WSSV.

In the present study, the serine/threonine-protein kinase gene was up-regulated in the ulvan group. Liu et al. (2011) found that the serine/threonine protein kinase increased its expression in response to WSSV infection. Some serine/threonine protein kinases are associated with the regulation of the apoptosis process (Cross et al., 2000), a mechanism activated in shrimp hemocytes to enhance protection against WSSV infection (Sahul Hameed et al., 2006). It could be the case that dietary ulvan resulted in an increased expression of this protein kinase as part of its action on protecting *P. vannamei* against WSSV infection in the present study.

Heat shock proteins (HSPs) are ubiquitous and are the most abundant and highly conserved multigene superfamily found in all organisms (Kregel, 2002). They play essential roles in protein chaperoning (Mayer & Bukau, 2005), developmental processes (Christians et al., 2003), and tolerance mechanisms to chemical, physical and pathophysiological stress (Rinehart et al., 2007). HSP60 and HSP70 are two forms of HSPs studied in crustaceans concerning innate immune responses and environmental stress (Huang et al., 2011); they are involved in crustacean muscle atrophy. In the present study, all three HSP genes, including HSP90, were downregulated following feeding the ulvan diet suggesting that the muscle protein degradation was reduced. In normal conditions, these HSPs are significantly induced in the premoult relative to the intermoult phase, as observed in American lobster claw muscle (Spees et al., 2003). We hypothesize that the down-regulation of these HSPs in the white shrimp fed ulvan was a form of counteracting WSSV infection.

On the question of which organ/tissue is appropriate to study transcriptomic responses of shrimps concerning functional diets, Cabrera-Stevens et al. (2022) stated that the muscle of shrimp allows for novel insights about the physiological and nutritional status that improves resistance against viral infection and provides valuable insights on growth and nutrient metabolism, including a diversity of signaling pathways and immune-related pathways (Dai et al., 2017; Li et al., 2020). These same authors explained that hemocytes participate in humoral immunity by synthesizing and releasing immune molecules as antibacterial peptides, lectins, and crustins. They have been intricately associated with phagocytosis, apoptosis, encapsulation, and nodule formation (Cui et al., 2020). In contrast, the hepatopancreas is the primary tissue or organ involved in humoral immunity

and cellular immunity in shrimp and plays a vital role in the immune system (Jiravanichpaisal et al., 2006; Zhong et al., 2017; Yang et al., 2018). The epithelial cells of the hepatopancreas are significant resources of immune molecules, such as lectins, hemocyanin, ferritin, antibacterial and antiviral proteins, proteolytic enzymes, and nitric oxide (Roszer et al., 2014). Additionally, the hepatopancreas is involved in excretion, molting, lipid and carbohydrate metabolism, and diverse metabolic activities, including synthesis and secretion of digestive enzymes, absorption of nutrients, synthesis of plasma proteins such as hemocyanin and lipoproteins (oxygen and lipid transporters and components of the defense system), and storage of energy reserves (Sanchez-Paz et al., 2007). These observations could explain the transcriptomic responses of the white shrimp towards WSSV infection following feeding on ulvan in the present study, which were largely and remarkably metabolic- and nutrient-storage-related but still reflected responses of the immune-related genes.

In conclusion, the present study provided a suggested mechanism for the protection provided by ulvan extracted from *Ulva intestinalis* as observed in previous studies in our laboratory. This was achieved by transcriptomic analysis of the hepatopancreas, which showed that during WSSV infection following consumption of a diet containing ulvan, almost all immune-related genes were down-regulated, successfully counteracting the stimulation effect of the viral infection. Those metabolic pathways involving amino acids, carbohydrates, and energy were largely up-regulated to facilitate energy-dependent biological processes used by the host immune defense against WSSV.

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