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RNA-seq revealed the signatures of immunity and metabolism in the *Litopenaeus vannamei* intestine in response to dietary succinate

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ABSTRACT

The intestine is important for nutrition, metabolism and immunity. Succinate (SA) plays a vital role in the physiological homeostasis of animal intestines. However, the effects of dietary SA on the intestinal immunity and metabolism in shrimp are not clear. In this study, we investigated the immune and metabolic responses in the intestine of *Litopenaeus vannamei* that were fed diets consisting of different levels of SA: 0 g/kg (Con) and 10 g/kg (SA) for 56 days. The results from a RNA-seq analysis identified 6005 differentially expressed genes (DEGs), including 2728 upregulated genes and 3277 downregulated genes, which were grouped into 312 pathways. The DEGs were most enriched in pathways related to protein synthesis and amino acid metabolism, including "ribosome", "aminoacyl-tRNA biosynthesis", "pyrimidine metabolism", and "arginine and proline metabolism"; additionally, carbohydrate and lipid metabolism pathways were also activated. A large number of immune-related genes were associated with mucus barrier modification, antimicrobial activity, pathogen attachment and recognition, antioxidant activity, and apoptosis. The expression patterns of several candidate genes involved in the immune response and nutrition metabolism were detected by qPCR. This study provides insight into the transcriptomic modulating mechanisms associated with intestinal immunity and the metabolism of *L. vannamei* in response to the intake of dietary SA.

1. Introduction

The Pacific white shrimp *Litopenaeus vannamei* is an important aquatic species of great economic value worldwide. Currently, diseases occur frequently, causing serious economic losses to shrimp farmers. Intestinal immunity serves as a front-line defense against diseases and affects the overall health of the shrimp. Shrimp live in a water environment that is rich in pathogens and toxic substances; thus, the intestinal immunity of the shrimp is necessarily affected by environmental stress and pathogen infection [1]. Additionally, the intestine of the shrimp harbors a large number of microbial communities, including some with opportunistic pathogens, which also greatly affects host health [2]. A disorder in intestine immunity places the host at risk for contracting diseases [3]. Therefore, it is crucial to improve the intestinal immunity of shrimp.

Succinate (SA) is an important intermediate in the citrate cycle, which is at the crossroads of several metabolic pathways [4], and can be produced through the fermentation of dietary fiber by intestine microbes [5]. In mitochondria, SA contributes to adenosine triphosphate (ATP) generation for energy supply [6]. In addition to its metabolic roles, SA can accumulate in immune cells, act as a signal to induce inflammation and cancer and can have both protumor and antitumor effects [7,8]. Additionally, SA can regulate the stabilization of hypoxia inducible factor-1 (HIF-1α) [4,5] and the formation and elimination of reactive oxygen species [9], and it can block the growth and proliferation of colon cancer cells [10]. In terms of intestinal physiological homeostasis, SA can serve as an acidulant and antimicrobial agent by acidifying the intestinal environment and suppressing pathogenic bacteria [11]. Furthermore, SA has been shown to modulate intestinal gluconeogenesis [5,12] and inflammation [13]. Diets that are rich in SA can improve plasma glucose and body weight and control the activation of adipose tissue thermogenesis [14,15]. Overall, SA has important roles in metabolism and immunity, which affect the health of an organism and are particularly important for maintaining the physiological homeostasis of the intestine.

In our previous studies, we found that dietary SA promoted growth and enhanced the immune function of *L. vannamei*, which enabled their defense against ammonia-induced stress [16]. Therefore, we

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hypothesize that SA can also regulate the intestinal immunity and metabolism in shrimp; however, the relevant molecular regulatory mechanisms remain unknown. To characterize the mechanistic components and identify the candidate immune and mechanism-related genes, in this study, we explored the effects of dietary SA intake on transcriptomic responses in the intestine of *L. vannamei*. The results of this study provide a greater understanding of the molecular mechanism of *L. vannamei* in response to the intake of dietary SA.

2. Materials and methods

2.1. Diet preparation

The source of the SA (S108852) in this study was purchased from Aladdin Co., LTD (Shanghai, China). Two experimental diets were prepared, and they differed in SA content: 0 g/kg (control) and 10 g/kg (SA). The feed formula is given in Table S1. The diet was prepared by the method of Duan et al. [16]. The nutritional ingredients for two experimental diets, including the moisture, crude protein, crude lipid and ash content, were measured according to the standard method recommended by the Association of Official Analytical Chemists (AOAC) (1995) [17].

2.2. Shrimp culture conditions, feeding trial and sampling

The rearing of *L. vannamei* and the experiments were carried out at the Shenzhen Base, South China Sea Fisheries Research Institute of Chinese Academy of Fishery Sciences (Shenzhen, China). The juvenile shrimp were obtained from a culture pond at the Shenzhen Base, and the average weight of each shrimp was 3.1 ± 0.1 g. The shrimp were temporarily cultured in a 500 L fiberglass tanks for 7 days, and the tanks contained sand-filtered and aerated seawater. The following water quality parameters were detected using a portable multi-parameter meter (YSI, USA): salinity 30‰, pH 8.4, temperature 30 ± 0.5 °C. One-third of the water was changed daily. The shrimp were fed daily an amount of formulated pellets (Haida Feed, Jieyang, China) that was equal to 5% of their body weight.

After acclimation, the shrimp were divided into two groups (Con and SA) and fed with the control and experimental feed, respectively. There were three replicate tanks for each group, and each tank contained 40 shrimp. The culturing conditions for the shrimp was the same as it was during the acclimation period. The shrimp in every tank were fed three times per day at 07:00, 12:00 and 18:00 for 56 days. The shrimp were fed close to satiation, and uneaten feed and feces were cleared from the tanks in a timely manner. At 56 days, the whole intestines of six shrimp from each tank was collected individually and the feces removed. Then, the six intestines were combined into one sample and snap-frozen in liquid nitrogen for transcriptome analysis.

2.3. RNA extraction, library construction and sequencing

Total RNA was extracted from the intestine using TRIzol® Reagent (Invitrogen, USA), and genomic DNA was removed using DNase I (TaKaRa, China). The RNA quality was detected using a 2100 Bioanalyzer (Agilent) and quantified using a ND-2000 spectrophotometer (NanoDrop Technologies). A high-quality RNA sample (1 μg) was used to construct a sequencing library. Transcriptome sequencing libraries were constructed using a NEBNext Ultra™ RNA Library Prep Kit for Illumina (NE, USA). All the procedures of the experiment were carried out according to the instructions from the manufacturer of the corresponding equipment used. After quantification by TBS380, paired-end libraries were sequenced with an Illumina HiSeq X Ten system.

2.4. De novo assembly and functional annotation

Raw paired-end reads were trimmed, and quality control procedures were used to screen the clean reads by Trimmomatic (Version 0.39) with default parameters. After filtering the clean reads, RNA de novo assembly was carried out with Trinity software (http://trinityrnaseq.sourceforge.net/) [18]. All the assembled transcripts were annotated with a Basic Local Alignment Search Tool (BLASTX) against the NCBI protein nonredundant (NR), Swiss-Prot, Cluster of Orthologous Groups (COG), Gene Ontology (GO), and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. Sequence similarity was set to the typical cutoff *E*-value of < 1e-5. The GO annotations of the unique assembled transcripts were determined for biological processes, molecular functions and cellular components using the BLAST2GO program (http://www.blast2go.com/b2ghome) [19]. Metabolic pathway annotations were determined using the KEGG database (http://www.genome.jp/kegg/) [20].

2.5. Differential expression analysis and functional enrichment

Differentially expressed genes (DEGs) between two groups (SA vs Con) were identified, and the expression level of each transcript was calculated according to the reads per kilobase of exon per million mapped reads (RPKM) method. The DEGs and the abundance of their isoforms were quantified using RSEM (http://deweylab.biostat.wisc.edu/rsem/) [21]. A differential expression analysis was performed with R statistical package software Empirical analysis of Digital Gene Expression in R (EdgeR, http://www.bioconductor.org/packages/2.12/bioc/html/edgeR.html) [22]. Additionally, a GO functional enrichment analysis was performed to identify the DEGs that were significantly enriched according to GO terms using Goatools (https://github.com/tanghaibao/Goatools). A KEGG pathway analysis was performed to identify the DEGs that were significantly enriched in the metabolic pathways using KOBAS 2.0 (http://kobas.cbi.pku.edu.cn/home.do) [23]. The significance of the GO and KEGG analyses were set at a Bonferroni-corrected *P*-value < 0.05.

2.6. Validation of the RNA-seq profiles by qPCR

To validate the differentially expressed genes identified by RNA-seq, 28 genes with different expression patterns and differential involvement in immune responses were selected for qPCR confirmation using the same RNA samples analyzed used for the transcriptome profiling. The *β-actin* gene of *L. vannamei* was used as an internal control to verify the successful reverse transcription and to calibrate the cDNA template. The gene-specific qPCR primers were designed with Primer Premier 5.0 software (Table S2), and the efficiency was evaluated with an amplification plot and a melting curve. Total RNA (1 μg) was reverse transcribed to cDNA using a PrimeScript™ RT reagent kit (TaKaRa, China) according to the manufacturer's protocol. Real-time RT-qPCR was conducted in a LightCycler 480 system using a SYBR® Premix Ex Taq™ II kit (TaKaRa, Japan). The qPCR method was previously described by Duan et al. [16].

2.7. Statistical analysis

From the analysis of the relative expression levels of the DEGs, all data are shown as the mean ± SE. All statistical analyses were performed using one-way analysis of variance (ANOVA; SPSS v22.0). Overall differences among multiple comparisons were subjected to a Duncan test. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Sequencing, de novo assembly, and gene annotation

Six intestine samples of *L. vannamei* from the Con and SA groups were sequenced with an Illumina HiSeq platform, and 310841824 total raw reads and 46626273600 raw bases were generated, which were submitted to the Sequence Read Archive (SRA) (accession: PRJNA564708). After removing reads containing adapters or ploy-N and low quality reads, 260935584 total clean reads and 37409922860 clean bases were generated. The total clean reads ratio was 77.87%–90.38% (Table S3). Trinity generated approximately 66657 transcripts and 52129 unigenes. The unigene lengths ranged from 200 bp to 36279 bp with a mean length of 757 bp and an N50 of 838 (Table S4). The number of unigenes that exceeded 2000 bp was 2658 (Fig. S1).

The 52129 unigenes were annotated by alignment with public databases including the NR, Swiss-Prot, GO, COG, and KEGG databases. A total of 4941 unigenes were found in all the databases, and the highest percentage of unigenes were annotated with the NR database (20401, 39.14%) (Fig. S2). According to COG terms, the 16949 matched unigenes were clustered into 25 categories, with the largest number in the "amino acid transport and metabolism" cluster, and the lowest number in the "nuclear structure" cluster (Fig. S3). Of the 7460 matched unigenes with a GO annotation, the largest subcategories of biological processes were the "cellular process" (GO:0009987) and "metabolic process" (GO:0008152) subcategories; the cellular components included "cell" (GO:0005623) and "cell part" (GO:0044464); and the molecular functions included "binding" (GO:0005488) and "catalytic activity" (GO:0003824) (Fig. S4). The 14049 matched unigenes were involved in 283 different KEGG pathways, of which the largest category was "metabolic pathways" (ko01100), followed by "biosynthesis of secondary metabolites" (ko01110), "microbial metabolism in diverse environment" (ko01120), and "carbon metabolism" (ko01200) (Fig. S5).

3.2. Identification and functional annotation of the DEGs

A total of 6005 genes were differentially expressed in response to the intake of dietary SA, including 2728 upregulated genes and 3277 downregulated genes (Fig. 1A and B). Gene expression patterns of the SA and Con groups were mainly clustered and showed a distinct pattern with a noticeable cluster that was highly upregulated or a noticeable cluster that was downregulated (Fig. 1C). Then, the DEGs were analyzed for GO enrichment. In total, 80 GO terms included 49 (61.25%) biological processes, 4 (5.00%) cellular components, and 27 (33.75%) molecular functions. Of these, "cellular process" (GO:0009987), "metabolic process" (GO:0008152) and "single-organism process" (GO:0044699) were the most well-represented terms among biological processes; "cell" (GO:0005623) and "cell part" (GO:0044464) represented a large proportion of cellular components; and "binding" (GO:0005488) and "catalytic activity" (GO:0003824) were the most enriched terms among molecular functions (Fig. 2). In addition, we obtained the gene information from other significantly enriched GO subcategories using directed acyclic graph (DAG) analysis, such as "glutamine biosynthetic process" (GO:0006542), "RNA phosphodiester bond hydrolysis, endonucleolytic" (GO:0090502), and "anion transport" (GO:0006820) in the biological processes subcategory (Fig. S6); "RNA polymerase complex" (GO:0030880), "DNA-directed RNA polymerase complex" (GO:0000428), "nuclear DNA-directed RNA polymerase complex" (GO:0055029), and "box C/D snoRNP complex" (GO:0031428) in the cellular components subcategory (Fig. S7); "glutamate-5-semialdehyde dehydrogenase activity" (GO:0004350), "O-phospho-L-serine:2-oxoglutarate aminotransferase activity" (GO:0004648), "phosphotransferase activity, carboxyl group as acceptor" (GO:0016774), "endoribonuclease activity" (GO:0004521), and "endoribonuclease activity, producing 5′-phosphomonoesters" (GO:0016891)" in the molecular functions subcategory (Fig. S8).

The DEGs were further analyzed by KEGG pathway analysis. Of the top 30 most enriched pathway terms, all pathways were upregulated. Of these pathways, the largest category was associated with a translation function, including "ribosome" (ko03010) and "aminoacyl-tRNA biosynthesis" (ko00970); the second category was associated with amino acid metabolism, including "pyrimidine metabolism" (ko00240) and "arginine and proline metabolism" (ko00330) (Fig. 3). Additionally, other DEGs were identified to be potentially associated with carbohydrate metabolism pathways, including "pyruvate metabolism" (ko00620), "glycolysis/gluconeogenesis" (ko00010), "citrate cycle" (ko00020), "propanoate metabolism" (ko00640), and "butanoate metabolism" (ko00650) (Table 1); pathways related to lipid metabolism, "fatty acid degradation" (ko00071), "sulfur metabolism" (ko00920), "glycerophospholipid metabolism" (ko00564), "glycerolipid metabolism" (ko00561), and "fatty acid biosynthesis" (ko00061) (Table 1); pathways related to immune system, including "Epstein-Barr virus infection" (ko05169), "PI3K-Akt signaling pathway" (ko04151), "toxoplasmosis" (ko05145), "antigen processing and presentation" (ko04612), and "peroxisome" (ko04146) (Table 2).

3.3. Identification of the DEGs related to the immune response

Based on the above analysis, many DEGs related to the immune response of the shrimp were also identified based on the NR database annotations, which provide a greater understanding of the physiological response of the *L. vannamei* intestine induced by the intake of dietary SA. These immune-related DEGs were mainly clustered into 5 key categories: "mucus barrier modification", "antimicrobial", "pathogen attachment and recognition", "antioxidant", and "apoptosis" (Table S5). The expression trends of these immune-related DEGs were different. For example, the mucus barrier modification genes, such as the mucins (*Muc*), collagens (*COL*), and laminins *(Lam*), were upregulated. Antimicrobial genes, such as anti-lipopolysaccharide factor (*ALF*), crustin (*Crus*), crustacyanin, lysozyme (*Lys*), and serine proteinase (*SP*), were upregulated, while serpin 3a (*Serp*), Kruppel-like factor 2 (*Klf2*), low affinity immunoglobulin epsilon Fc receptor isoform X4 (*FCER4*), and C1q-binding protein (*C1qBP*), were downregulated. Pathogen attachment and recognition genes, such as c-type lectin (*C-Lec*) and hemolymph clottable protein (*CP*), were upregulated, while integrin alpha 5 (*ITGA5*) and proclotting enzyme (*PE*) were downregulated. Antioxidant genes, namely, heat shock proteins (*HSP21*, *HSP31*, and *HSP70*), ferritin 2 (*Fer*), thioredoxin reductase (*Trx*), zinc metalloprotease (*Met*), catalase (*CAT*), superoxide dismutase (*SOD*), glutathione peroxidase (*GPx*), glutathione S-transferase (*GST*), and peroxiredoxin (*Prx*), were upregulated, while only *HSP10* was downregulated. Apoptosis genes, such as inhibitor of apoptosis protein (*IAP*) and *p53*, were upregulated.

3.4. Gene-act network of the DEGs

According to the interaction of genes, proteins and compounds in the KEGG database, we constructed a gene-act network (Global Signal Transduction Network). The predictive relationships among genes were not comprehensive, and the gene-act network contained relatively few genes. There was no up-down relationship in this study, so we introduced the differential genes in the network. A total of 19 DEGs were involved in the gene-act network in the SA vs Con groups: 10 were upregulated genes, and 9 were downregulated genes (Fig. 4). The relationships of the genes included activation, binding, compound, expression, and inhibition. Several genes were involved in disease and immunity (*CD23*, *P38*, *POLK*, *CREBBP*, *FCER2*, and *eEF1A*), cell proliferation (*PCNA*), or protein metabolism (*DAG1*).

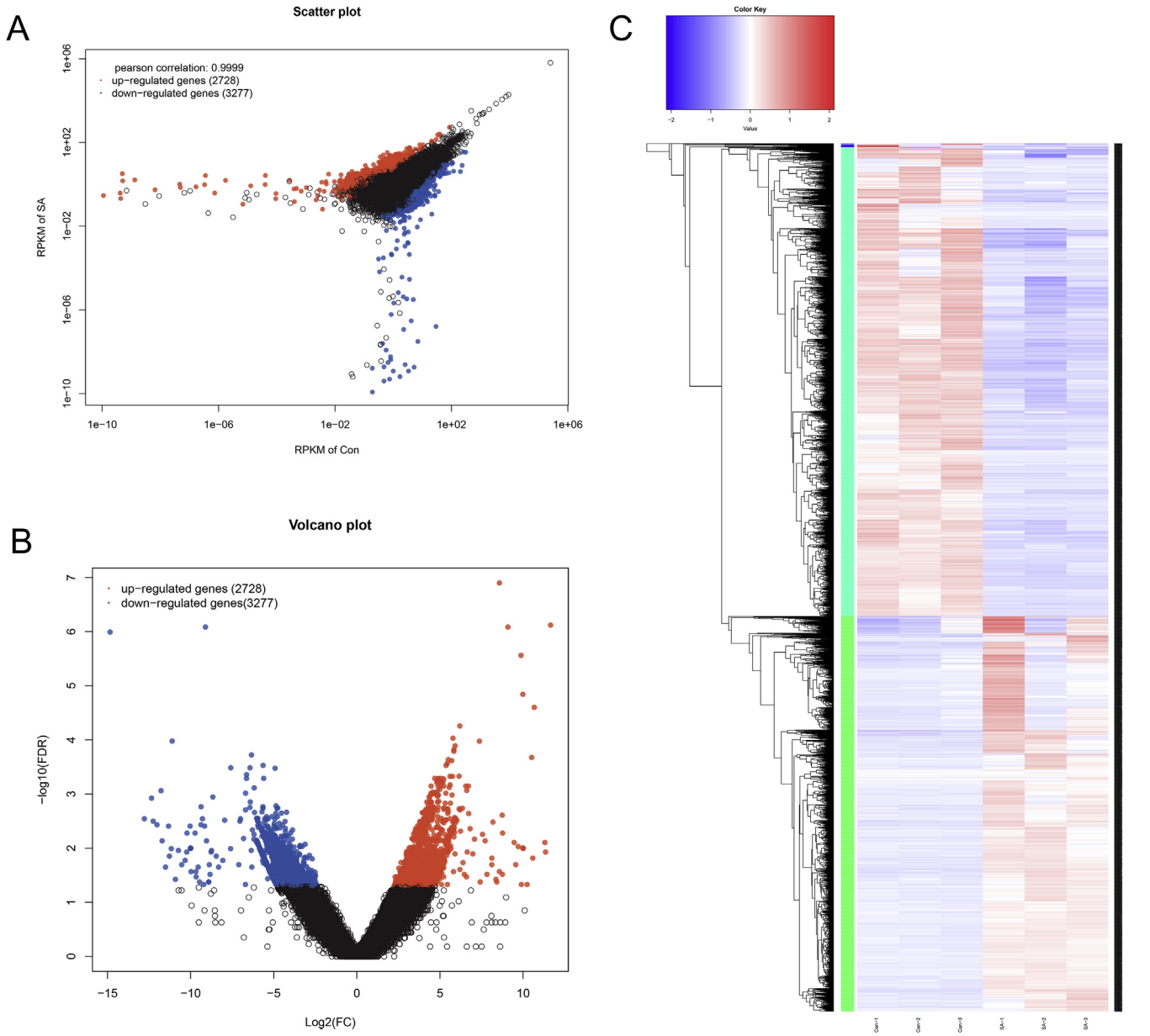

Fig. 1. Visual scatter and volcano diagram of the DEGs in the intestine of *L. vannamei* following dietary SA (SA vs Con). **(A)** Scatter plot. The x-axis and y-axis indicate the expression quantity (FPKM value) of genes in two samples, respectively. **(B)** Volcano plots. The x-axis and y-axis indicate the log2-fold change and log2-FDR of genes of two samples, respectively. Each dot represents a specific gene. The red dots represent significantly upregulated genes, the blue dots represent downregulated genes, and the black dots represent non-significantly differentially expressed genes. **(C)** Expression pattern cluster diagram of the DEGs in the intestine of *L. vannamei* following the intake of dietary SA. The x-axis and y-axis indicate the samples and genes, respectively. Colors represent gene expression ranging from blue (less expressed) to red (more expressed) corresponding to log10 FPKM values. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.5. Validation of the DEGs by qPCR

To verify the DEG expression profiles identified by RNA-seq, we randomly selected 26 genes that were involved in metabolism or immune functions for qPCR analysis. The similar patterns of DEG mRNA abundance in the qPCR analysis were generally consistent with those obtained from the RNA-seq (Fig. 5). Thus, the DEGs identified by the RNA-seq analysis were accurate.

4. Discussion

Undoubtedly, the present study is the first RNA-seq report on aquatic animal intestines following the intake of dietary SA. The RNA-seq analysis yielded 6005 DEGs, of which 2728 were upregulated and 3277 were downregulated, clearly showing a significant impact of the dietary SA on the *L. vannamei* intestine transcriptome. According to the KEGG pathway analysis, more genes were associated with ribosomes, aminoacyl-tRNA biosynthesis, pyrimidine metabolism, arginine and proline metabolism, and Epstein-Barr virus infection, indicating that protein translation, amino acid metabolism, and the immune processes in the *L. vannamei* intestine have increased activity in response to dietary SA.

4.1. Intestinal metabolic response to dietary SA

SA is the intermediate between succinyl-CoA and fumarate, which

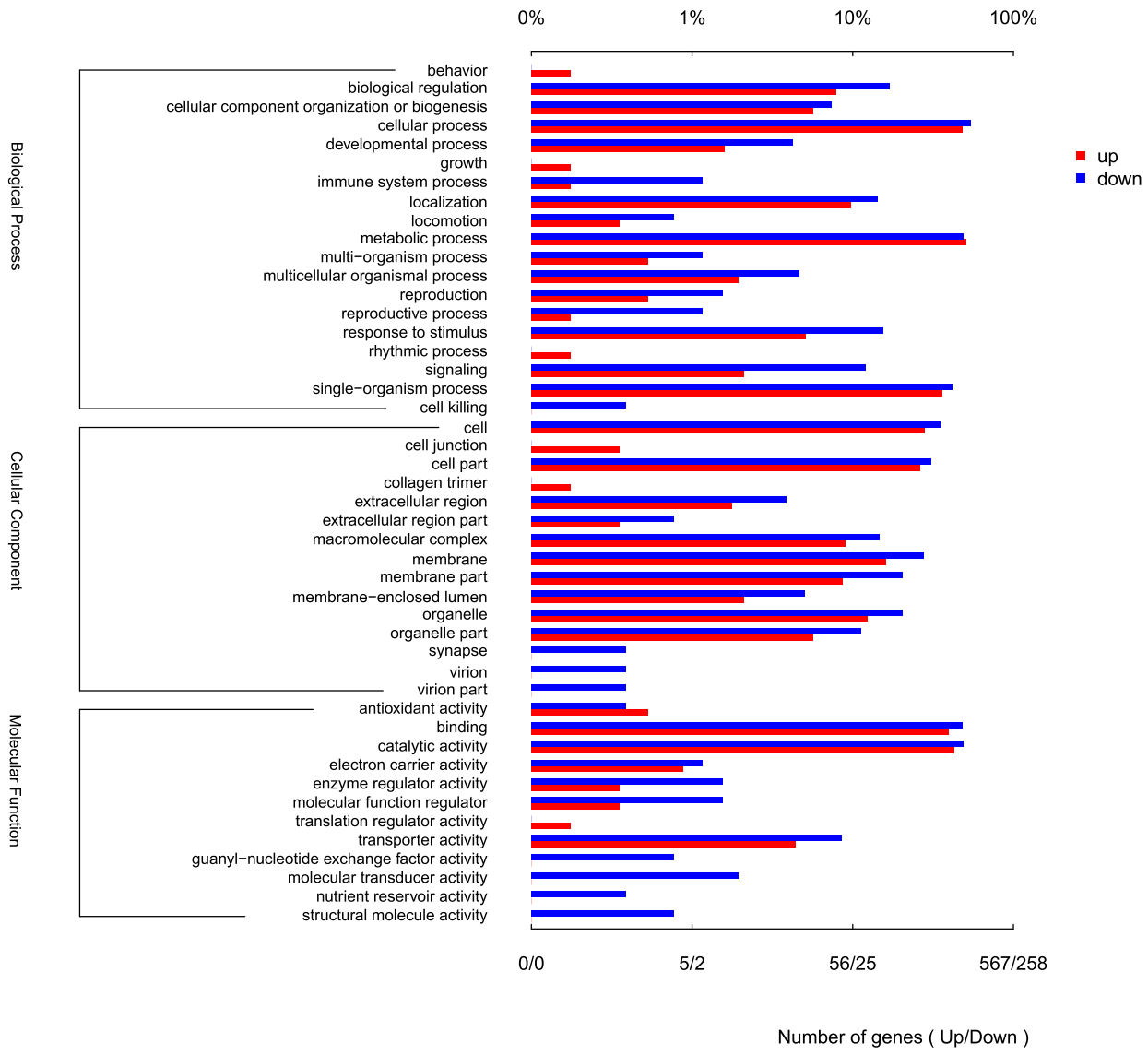

Fig. 2. The most enriched GO terms of the DEGs in the intestine of *L. vannamei* following the intake of dietary SA (SA vs Con).

can be converted to glucose, and has been proven to have signaling properties to activate intestinal gluconeogenesis [5,12]. In this study, the pathways involved in the carbohydrate metabolism-related section, including “pyruvate metabolism”, “glycolysis/gluconeogenesis”, and “citrate cycle”, were upregulated in response to dietary SA. Additionally, several genes were also significantly upregulated to promote the pathways of carbohydrate metabolism and the synthesis and decomposition of SA, including those of hexokinase (*HK*), glucokinase (*GK*), pyruvate kinase 3 (*PK3*), pyruvate dehydrogenase (*PDH*), pyruvate carboxylase (*PC*), succinate dehydrogenase (*SDH*), fumarate reductase (*FRD*), and succinyl-CoA synthetase (*SUC*). These results indicate that dietary SA can activate carbohydrate metabolism and SA metabolism.

PDH is a key enzyme responsible for the formation of acetyl-CoA, which connects the metabolic processes of carbohydrates, lipids, and amino acids [24]. In this study, the upregulated expression of the *PDH* gene induced by dietary SA might also promote lipid and amino acid metabolism. Lipid metabolism plays a vital role in the survival and reproduction of animals [25,26]. In this study, the pathways involved in lipid metabolism, which included “fatty acid biosynthesis”, “fatty acid degradation”, “glycerophospholipid metabolism”, and “glycerolipid metabolism”, were all upregulated. The lipid metabolism genes, including short-chain specific acyl-CoA dehydrogenase (SCACD), long-chain specific acyl-CoA dehydrogenase (LCACD), fatty acid-binding protein (FABP), and fatty acid synthase (FAS), were upregulated, while acetyl-CoA carboxylase (ACC) was downregulated. These results reveal that dietary SA can regulate the lipid metabolism of *L. vannamei*.

In amino acid metabolism, five pathways, namely, “pyrimidine metabolism”; “arginine and proline metabolism”; “alanine, aspartate and glutamate metabolism”; “glycine, serine and threonine metabolism”; and “cysteine and methionine metabolism”, were activated in the *L. vannamei* intestine in response to the intake of dietary SA. Most important, glycine, serine, and threonine amino acids were involved in the stress responses of the shrimp [27–29], and arginine and proline amino acids could enhance the immune function of aquatic animals [30,31]. Hence, planning the dietary SA intake can not only increase the metabolic rate of the amino acids but can also enhance the intestinal immunity of *L. vannamei*.

4.2. Intestinal immune response to dietary SA

The intestinal mucosal immune system is an important barrier the body uses to prevent pathogen infection, the function of which mainly relies on mucus components and immune molecules [1]. As an

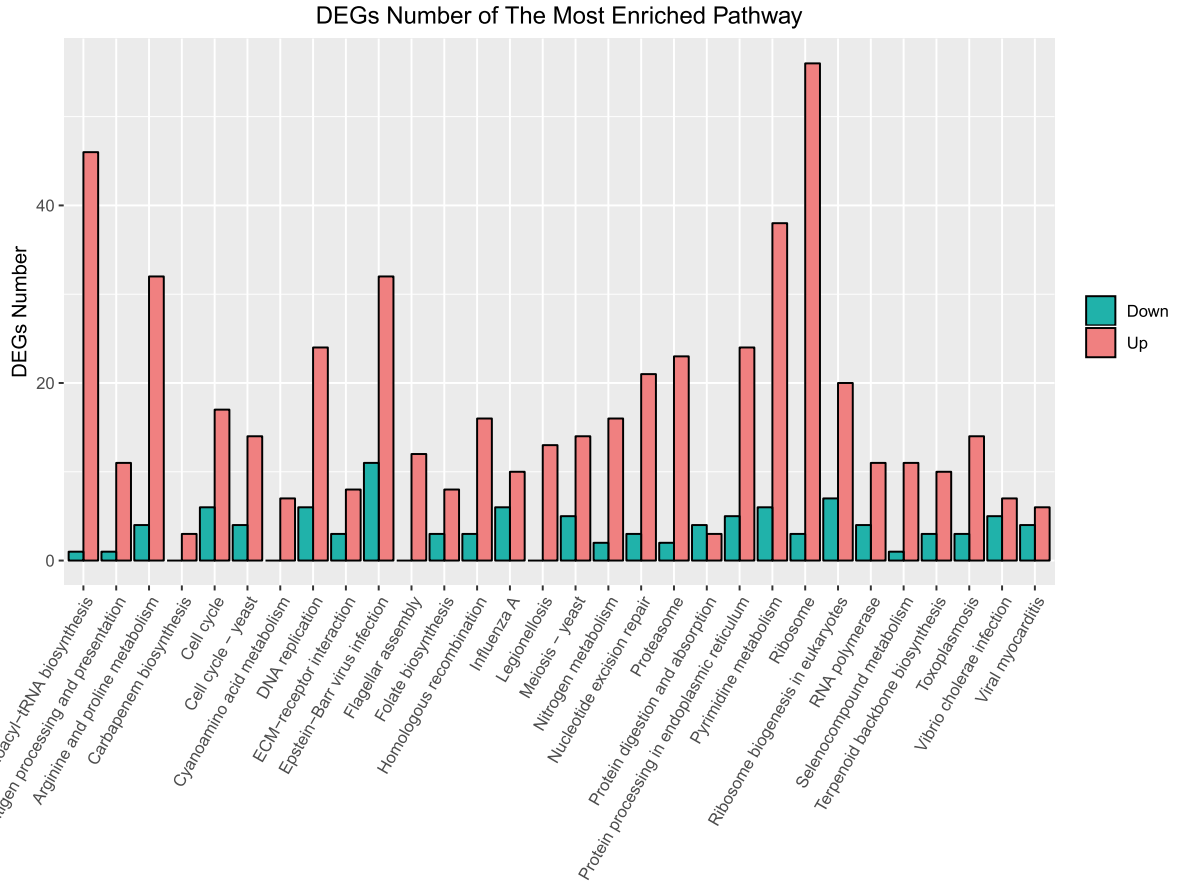

Fig. 3. The 30 most enriched pathways of the DEGs in the intestine of *L. vannamei* following the intake of dietary SA (SA vs Con).

Table 1
Metabolism related pathways of the DEGs in the intestine of *L. vannamei* following the intake of dietary SA (SA vs Con).

Pathway	Pathway ID	DEGs in term (6005)	All gene in term (52129)
Carbohydrate metabolism			
Pyruvate metabolism	ko00620	33 (0.55%)	253 (0.49%)
Glycolysis/Gluconeogenesis	ko00010	23 (0.38%)	197 (0.38%)
Citrate cycle (TCA cycle)	ko00020	17 (0.28%)	187 (0.36%)
Glyoxylate and dicarboxylate metabolism	ko00630	21 (0.35%)	178 (0.34%)
Amino sugar and nucleotide sugar metabolism	ko00520	19 (0.32%)	154 (0.3%)
Butanoate metabolism	ko00650	13 (0.22%)	124 (0.24%)
Propanoate metabolism	ko00640	9 (0.15%)	128 (0.25%)
Lipid metabolism			
Fatty acid degradation	ko00071	16 (0.27%)	144 (0.28%)
Sulfur metabolism	ko00920	15 (0.25%)	91 (0.17%)
Glycerophospholipid metabolism	ko00564	11 (0.18%)	121 (0.23%)
Glycerolipid metabolism	ko00561	10 (0.17%)	66 (0.13%)
Fatty acid biosynthesis	ko00061	9 (0.15%)	76 (0.15%)
Amino acid metabolism			
Pyrimidine metabolism	ko00240	44 (0.73%)	301 (0.58%)
Arginine and proline metabolism	ko00330	36 (0.6%)	234 (0.45%)
Alanine, aspartate and glutamate metabolism	ko00250	28 (0.47%)	190 (0.36%)
Glycine, serine and threonine metabolism	ko00260	28 (0.47%)	191 (0.37%)
Cysteine and methionine metabolism	ko00270	16 (0.27%)	149 (0.29%)

Table 2
Immune related pathways of the DEGs in the intestine of *L. vannamei* following the intake of dietary SA (SA vs Con).

Pathway	Pathway ID	DEGs in term (6005)	All gene in term (52129)
Epstein-Barr virus infection	ko05169	43 (0.72%)	184 (0.35%)
PI3K-Akt signaling pathway	ko04151	22 (0.37%)	158 (0.3%)
Toxoplasmosis	ko05145	17 (0.28%)	81 (0.16%)
Platelet activation	ko04611	12 (0.2%)	93 (0.18%)
Antigen processing and presentation	ko04612	12 (0.2%)	41 (0.08%)
Peroxisome	ko04146	10 (0.17%)	133 (0.26%)

important immune component of intestinal mucus, Muc is a glycoprotein that is secreted by intestinal goblet cells to confer protection against pathogen attachment and invasion [32]. COL and Lam are components of the extracellular matrix (ECM). The balance between collagen synthesis and breakdown is an important factor that affects the recurrence of disease [33]. Lam plays an important role in the formation and stability of intestinal epithelial basement membranes [34]. In this study, the levels of *Muc* (*Muc*-5AC*, Muc-12, Muc-22*), *COL*, and *Lam* (*Lam-α* and *Lam-γ1*) mRNA expression were upregulated in the *L. vannamei* intestine in response to SA, a finding that indicates that the intake of dietary SA can induce the protein components of intestinal mucus and the basement membrane. These changes might promote the stability of the intestinal epithelium and mucosal barrier.

SA can induce the key inflammatory cytokines and IL-1β through HIF-1α signaling [35]. Pattern recognition proteins (PRPs) are beneficial for the intestinal immune system because they recognize and respond to pathogens and thus protect symbiotic bacteria in the intestine [36]. In this study, several PRP genes of *L. vannamei* were significantly altered in response to SA, but they showed different expression trends. Notably, the levels of *C-Lec* and *CP* gene expression were upregulated, while those of *ITGA5* and *PE* were downregulated, indicating that the

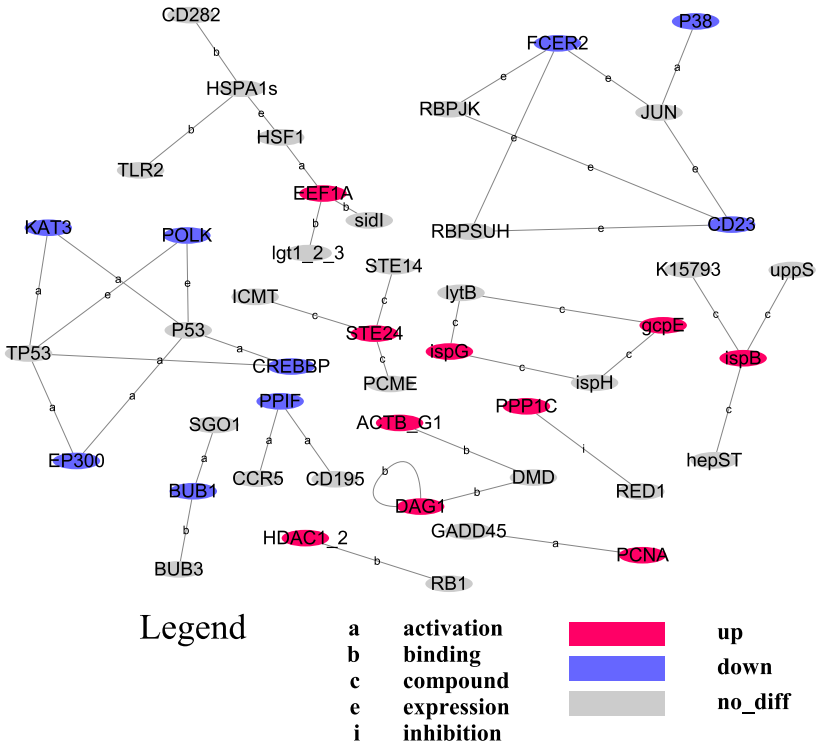

Fig. 4. Gene-act network of the DEGs in the intestine of *L. vannamei* following the intake of dietary SA (SA vs Con). The gene connections were generated from the data analysis of the KEGG pathway. The lines represent the relationships between the genes.

intake of dietary SA might induce the intestinal immune system of the shrimp undergoing pathogen attack, but the differential expression mechanisms of the PRPs need to be further studied. To resist pathogen infection, shrimp rely on their antimicrobial capabilities, including antimicrobial peptides and the prophenoloxidase (proPO) system [37,38]. In this study, the levels of antimicrobial peptides (*ALF*, *Crus*, and *Lys*) and *proPO* system (*SP*) gene expression were upregulated, while those of the serine protease inhibitors (*Serp* and *Klf2*) were downregulated, indicating that these antimicrobial molecules might be involved in the intestinal immune response to dietary SA.

Organisms rely on the antioxidant defense system to counteract oxidative stress, specifically that produced by the reactive oxygen species (ROS) during pathogen challenge and stress responses [39,40]. It was reported that SA is involved in the formation and elimination of ROS. In this study, the gene expression of antioxidant enzymes (*CAT*, *SOD*, *GPx*, *GST*, and *Prx*), heat shock proteins (*HSP21*, *HSP31*, and *HSP70*), *Fer*, *Trx*, and *Met* were all upregulated in response to SA, which indicated that dietary SA could induce the antioxidant response in the *L. vannamei* intestine and the scavenging of the ROS. Apoptosis is an autonomous and orderly cellular suicide process that is genetically programmed and can maintain the stability of the internal environment [41]. An important apoptosis-related protein, p53 can mediate apoptosis and promote resistance to cellular stress [42], while IAP functions as an antiapoptosis regulator [43]. In this study, the expression levels of the *p53* and *IAP* genes were upregulated in response to SA, suggesting that dietary SA could activate the apoptosis program in the shrimp

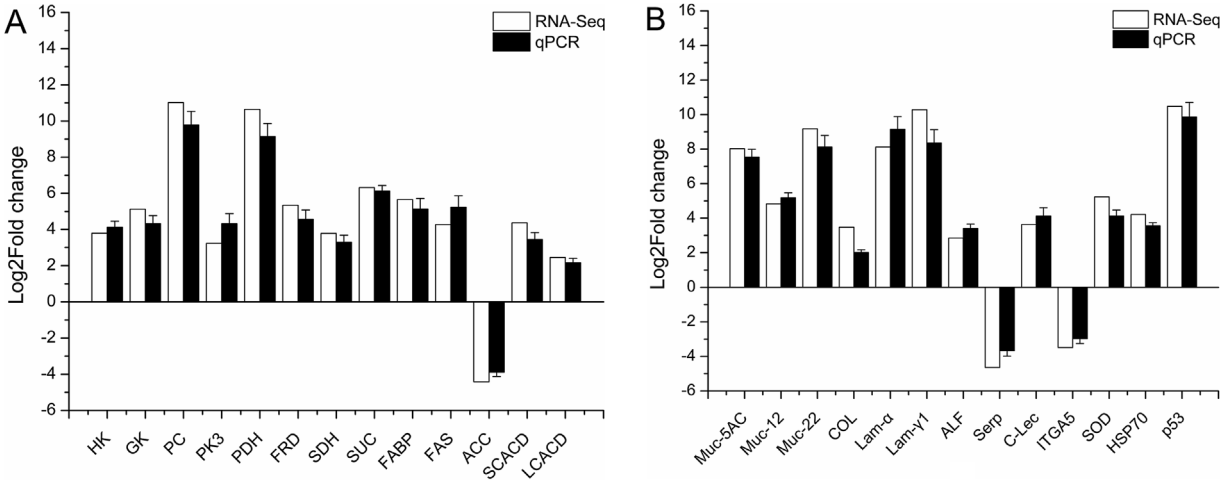

Fig. 5. qPCR verification of the DEGs in the intestine of *L. vannamei* following the intake of dietary SA. **(A)** Metabolism-related DEGs; **(B)** Immune-related DEGs. Vertical bars represent the mean ± SE ($N = 3$).

intestine to remove unnecessary, deleterious and/or aging cells and prevent the injury caused by to excessive apoptosis.

In conclusion, this study is the first to report on the metabolism and immune responses at the transcriptional level in *L. vannamei* intestine following the intake of dietary SA, and a total of 6005 genes were found to be differentially expressed. The most enriched KEGG pathways for the discovered DEGs were involved in protein synthesis and amino acid metabolism; however, pathways potentially associated with carbohydrate metabolism and lipid metabolism were also activated. Additionally, immune-related genes were identified, and those associated with mucus barrier modification, antimicrobial activity, pathogen attachment and recognition, antioxidant activity, and apoptosis indicate that the intake of dietary SA might affect shrimp intestinal immunity. Our results are expected to contribute to the understanding of the molecular mechanism underlying intestinal immunity and metabolism.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.09.074.

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