



Influence of Light/Dark Cycles on Body Color, Hepatopancreas Metabolism, and Intestinal Microbiota Homeostasis in *Litopenaeus vannamei*

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In aquatic animals, the light/dark cycle acts as an important biological factor that influences the entire life cycle. Until present, evidence regarding the regulation of physiological metabolic process under different light/dark cycles is limited in *Litopenaeus vannamei*. In this study, we mainly investigated the effects of different light/dark cycles (12 h light/12 h dark, 0 h light/24 h dark) on the hepatopancreas metabolism and intestinal microbiota homeostasis in *L. vannamei* using multiomics techniques. One interesting finding was that the body color of *L. vannamei* became darker after dark treatment for 8 weeks. Further hepatopancreas transcriptome analysis identified down-regulated genes involved in regulating nutrition metabolism, body-color formation, diurnal rhythm, immune function, hormone levels, and posttranslational modifications. The intestinal microbiota analysis showed that dark treatment-induced alterations in intestinal bacterial abundances in *L. vannamei*, such as decreased ($P < 0.05$) relative abundance of *Formosa*, *Demequina*, *Lutimonas* and increased ($P < 0.05$) relative abundance of *Ruegeria*, *Vibrio*, *Actibacter*, *Roseovarius*, *Ilumatobacter*, and *Kriegella* at the genus level. The microbiota functional analysis demonstrated that the dark treatment mainly increased susceptibility of pathogens, decreased nutrition metabolism, and influenced circadian rhythm. This study indicated for the first time that constant darkness treatment darkened the body color and altered hepatopancreas metabolism and intestinal microbiota homeostasis in *L. vannamei*, which might give potential clues for improving the productive capacities by changing light/dark cycles in shrimp farming.

Keywords: light/dark cycles, multi-omics techniques, microbiota homeostasis, circadian biological rhythm, crustacean, physiological metabolism

INTRODUCTION

Most species have developed endogenously driven circadian rhythms in physiology and behavior that are attuned to changes in the daily 12 h light/12 h dark cycle (Hussain and Pan, 2015; Schilperoort et al., 2020). In other words, the photoperiod profoundly influences the circadian rhythm of biochemical, physiological, and behavioral processes in almost living organisms (Wright et al., 2013; Dannerfjord et al., 2021). Epidemiological

studies and substantial experimental evidence have repeatedly reported that changes in the light/dark cycles have been closely associated with various metabolic disorders (Rutter et al., 2002; Maury et al., 2010). In aquatic animals, the light/dark cycle acts as an important biological factor that influences the entire life cycle from embryonic development to sexual maturation (Downing and Litvak, 2001; Migaud et al., 2010; Arambam et al., 2020). In recent years, growing evidence has shown that the effects of the light/dark cycle on various species are diverse, some have a natural preference for the dark environment while some have an improved physiological state under high-light intensity (Serra et al., 1999; Ruchin, 2006; Li et al., 2021b; Wei et al., 2021). Therefore, it is a promising research direction for further studies.

The circadian nature of physiology and behavior is regulated by a circadian clock that is located in the suprachiasmatic nucleus (SCN) of the hypothalamus (Dannerfjord et al., 2021). Apart from the SCN, the tissues are capable of generating multiple circadian biological rhythms (Hastings et al., 2018; Pett et al., 2018). Earlier studies have demonstrated the mechanism of dark-light-adaptation changes in crustaceans compound eyes (Migaud et al., 2010). Nevertheless, our understanding regarding the influential mechanism underlying the photoperiods on other tissue is unknown in crustaceans and needs to be investigated further. Currently, the relationship between the light/dark cycle and intestinal microbiota has aroused widespread concern (Peyric et al., 2013; Fortes-Silva et al., 2016; Kim et al., 2019). Furthermore, different light/dark cycles could affect growth, digestibility, and physiological metabolism in fish (Leiner and MacKenzie, 2001; Li et al., 2021b). The crustacean hepatopancreas is an important organ constituting the main site for nutrient digestion, absorption, and metabolism (Li et al., 2021a). Therefore, the hepatopancreas and intestine can be the potential targets for studying the responsive mechanism of shrimp in response to different light/dark cycles.

The Pacific white shrimp *Litopenaeus vannamei* is considered a paramount aquaculture species, accounting for more than 70% of the total global shrimp production worldwide (Jiao et al., 2020). *L. vannamei* starts benthic life soon after the mysis stage or the early stage of postlarvae. Currently, the effects of different light/dark cycles on larval survival and growth performance have been studied in *L. vannamei* and *Macrobrachium rosenbergii* (Sanudin et al., 2014; Wei et al., 2021). Until present, evidence regarding the regulation of physiological metabolic process under different light/dark cycles is limited in *L. vannamei*. Thus, in this study, we mainly investigated the effects of different light/dark cycles (12 h light/12 h dark, 0 h light/24 h dark) on the hepatopancreas metabolism and intestinal microbiota in *L. vannamei* using multiomics techniques. Moreover, body color was also measured. This study might provide an important theoretical basis and reference for further understanding the influence of light/dark cycles on the body color, hepatopancreas metabolism, and intestinal microbiota in shrimp.

MATERIALS AND METHODS

Animals and Design of 8-Week Feeding Experiment

All experimental procedures complied with the Standard Operation Procedures (SOPs) of the Guide for Use of Experimental Animals of Ningbo University.

Litopenaeus vannamei juvenile was obtained from Ningbo Marine Fishery Science and Technology Innovation Base and temporarily reared in a concrete pond with running aerated water ($28 \pm 2^\circ\text{C}$, 21.7–23.5 ppt salinity, 4.32–5.5 mg/L dissolved oxygen) prior to the feeding experiment.

Figure 1 shows the experimental protocol. A total of 180 shrimp (approximately 0.72 g per individual) were randomly divided into two groups, namely, the natural light group (12 h light/12 h dark) and dark treatment group (0 h light/24 h dark), with three replicates per group. A total of 30 shrimp were reared in a 100-L cylindrical fiberglass tank, considered as one replicate. After the 8-week feeding trail, body color was captured by digital cameras and then analyzed by the Just Color Picker 5.5 software. Hemolymph samples of six shrimp in each tank were collected from the pericardial cavity and placed into 1.5 ml centrifuge tubes overnight at 4°C before centrifugation (1,811 g, 10 min). The supernatant was mixed as one replicate and collected for the biochemical analysis. The hepatopancreas of six shrimp in each tank was collected rapidly and sterilely, mixed as one sample, and frozen in liquid nitrogen. Similarly, the intestinal content of six shrimp in each tank was collected through a sterile operation, placed into one sterile Eppendorf centrifuge tube, and immediately frozen in liquid nitrogen. Later, intestine and hepatopancreas samples were sent to Hangzhou Mingke Biotechnology Co., Ltd., China for further 16S rDNA-based microbiota and transcriptome analysis, respectively.

Analytical Testing

Hemolymph Biochemical Analysis

Hemolymph triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL) and high-density lipoprotein cholesterol (HDL), glucose (GLU), glutamic-pyruvic transaminase (ALT), glutamic oxalacetic transaminase (AST), albumin (ALB), and total protein (TP) were determined using Selectra ProM Clinical Chemistry System (ELITech, Sees, France). The parameters were estimated using enzymatic spectroscopic methods on dedicated system packs according to the conventional laboratory protocols. This system has been calibrated, and quality controls were duly maintained before measurement. An independent-sample *t*-test was used to compare the index. The data was expressed as mean \pm SD. $P < 0.05$ was considered statistically significant.

Hepatopancreas Transcriptome Analysis

Total hepatopancreas RNA was extracted from tissues using TRIzol reagents (Invitrogen, Waltham, MA, United States) and then determined its quality and quantity as previously described (Jiao et al., 2020). Subsequently, mRNA was isolated

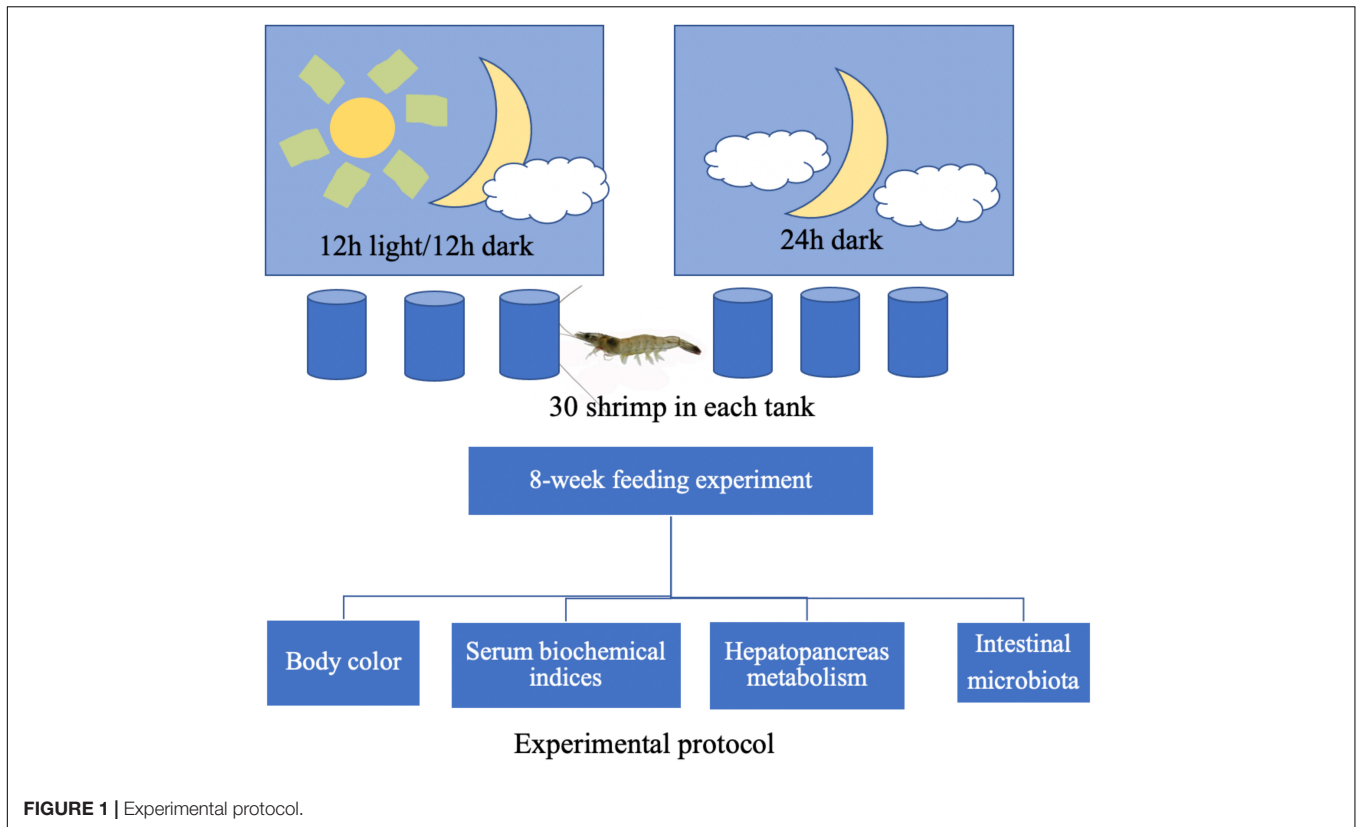


FIGURE 1 | Experimental protocol.

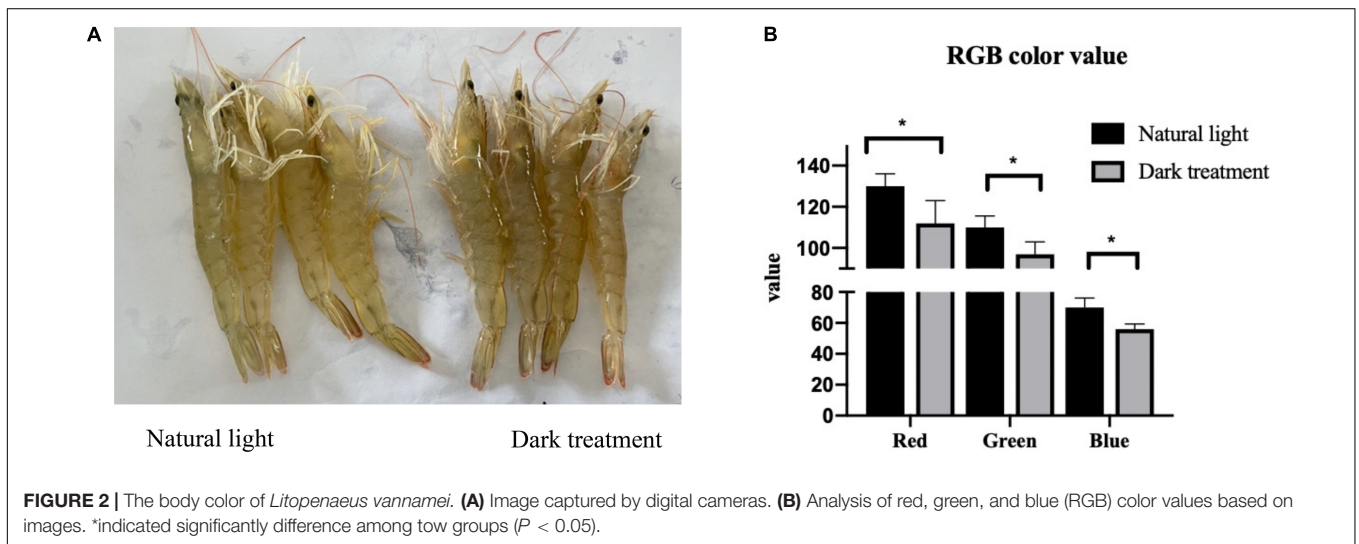


FIGURE 2 | The body color of *Litopenaeus vannamei*. (A) Image captured by digital cameras. (B) Analysis of red, green, and blue (RGB) color values based on images. *indicated significantly difference among tow groups ($P < 0.05$).

and fragmented for cDNA synthesis. The sequencing library was constructed and sequenced by HiSeq 2500 platform (Illumina, San Diego, CA, United States).

The raw paired-end reads were trimmed and quality controlled for obtaining clean data. Later, clean data were assembled *de novo* with Trinity and searched to identify proteins to retrieve their functional annotations. The expression value of each transcript was calculated using fragments per kilobase

of exon per million mapped reads method, while the software package RSEM was used for quantifying gene and isoform abundances. P -value < 0.05 and $|\log_2(\text{fold change})| \geq 1$ were identified as the threshold for significance of gene expression differences. In addition, the functional-enrichment analysis including GO and KEGG were performed by Goatools and KOBAS online analysis to identify differential expression genes (DEGs) at Bonferroni-corrected P -value ntial.

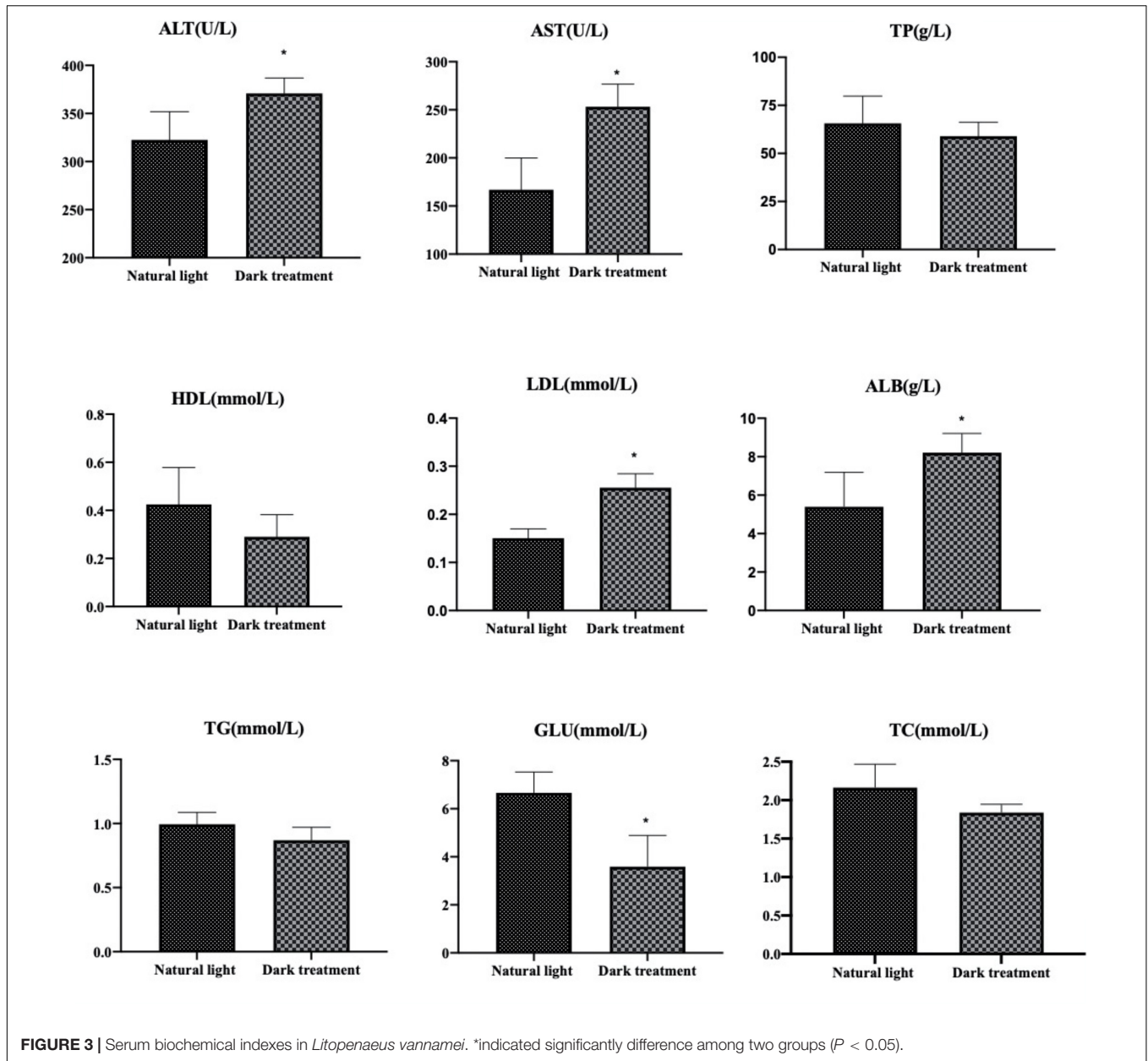


TABLE 1 | Summary of the RNA sequencing data.

Sample	Clean Data (bp) ^a	Clean reads No. ^b	Mapped-reads ^c	Mapped-rate ^d	Error rate (%) ^e	Q20 (%) ^f	Q30 (%) ^g
Natural light-1	7004876400	46699176	41837467	89.59%	0.0295%	98.02%	95.07%
Natural light-2	6725887800	44839252	40091343	89.41%	0.0294%	98.03%	95.13%
Natural light-3	6792226200	45281508	40452059	89.33%	0.0293%	98.07%	95.20%
Dark treatment-1	6689464800	44596432	40204107	90.15%	0.0294%	98.13%	95.21%
Dark treatment-2	7119960900	47466406	42760160	90.09%	0.0297%	98.00%	94.91%
Dark treatment-3	7003066500	46687110	42273076	90.55%	0.0297%	98.14%	95.21%

^aHigh-quality sequenced base number;

^bHigh-quality sequenced read number;

^cNumber of reads that mapped to the reference genome;

^dTotal mapped/clean reads;

^ePercentage of the error bases;

^fPercentage of sequenced bases with Qphred > 20 (error rate < 1%);

^gPercentage of sequenced bases with Qphred > 30 (error rate < 0.1%).

Intestinal Microbiome Analysis

Total intestinal genome DNA was extracted using CTAB/SDS method and measured its quality using a NanoDrop ND-2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The V4-V5 region of the 16S rRNA gene was amplified using primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 907R (5'-CCGTCAATTCCTTTGAGTTT-3') with the unique barcode for PCRs. Sequencing libraries were generated on an Illumina MiSeq platform. Paired-end reads were later harvested for further bioinformatics analysis as previously described (Jiao et al., 2021). Briefly, alpha diversity indexes, Simpson, Chao, Shannon, and Goods coverage were calculated with QIIME (Version 1.7.0), and rarefaction curves were displayed with GraphPad Prism 5. The microbial community distribution of all samples was visualized based on community composition information at the taxonomic levels. An independent-sample *t*-test was used to compare the microbial community distribution between two groups. The functional analysis of 16S rRNA genebased microbial community samples was predicted using the PICRUSt algorithm and further analyzed statistically by metagenomic profiles (STAMP) (version 2.1.3) package. Linear discriminant analysis Effect Size (LEfSe) analysis shows differentially abundant genera as biomarkers, which was determined using Kruskal-Wallis test ($P < 0.05$) with a linear discriminant analysis (LDA) score > 3.5 .

RESULTS

Body Color of *Litopenaeus vannamei*

Figure 2 shows the effects of different light/dark cycles on body color in *L. vannamei*. As shown in **Figure 2**, compared with the natural light group, the body color of *L. vannamei* became darker after dark treatment for 8 weeks. The result of the measurement

of RGB color value showed that each parameter (i.e., red, green, and blue) value was decreased ($P < 0.05$) in constant dark treatment compared with the natural light group.

Serum Biochemical Indexes

Figure 3 shows the effects of different light/dark cycles on serum biochemical indexes in *L. vannamei*. As shown in **Figure 3**, compared with the natural light group, constant dark treatment significantly increased ($P < 0.05$) the serum levels of ALT, AST, LDL, and ALB and decreased ($P < 0.05$) serum glucose levels in *L. vannamei*. The serum levels of TP, HDL, TG, and TC were not influenced ($P > 0.05$) by the constant dark treatment.

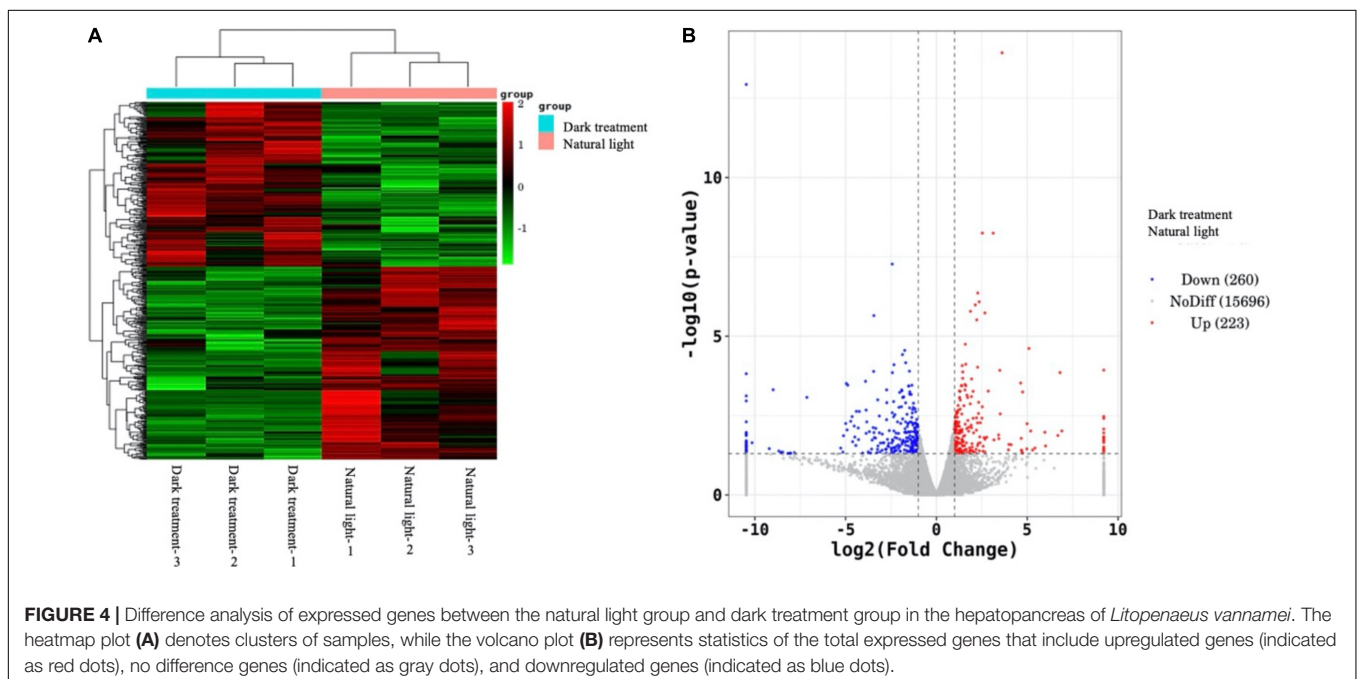
Transcriptome Analysis

Summary of RNA Sequencing

Table 1 shows the two cDNA libraries that were constructed using the hepatopancreas from the natural light group and dark treatment group. In this experiment, 136,819,936 and 138,749,948 clean reads were obtained from the hepatopancreas transcriptome sequencing in the natural light group and dark treatment group, respectively. Approximately, 89.33–90.55% of the reads were mapped onto the reference genome. The percentage of sequenced bases with Qphred > 30 is higher than 94.91%, and the average error rate was 0.0293–0.0297%. The above summary of RNA sequencing showed that good-quality sequence data were harvested for further bioinformatics analysis.

Identification of Differentially Expressed Genes

As shown in **Figure 4**, the heatmap plot (A) and a volcano plot (B) present the difference analysis of expressed genes between the natural light group and dark treatment group in the hepatopancreas of *L. vannamei*. In this study, we can see that three replicates in the natural light group (i.e., natural light-1,



natural light-2, and natural light-3) were clustered together and three replicates in the dark treatment group (i.e., dark treatment-1, dark treatment-2, and dark treatment-3) were clustered together, indicating that gene expression in the hepatopancreas of *L. vannamei* was significantly different between the two groups. Furthermore, the volcano plot identified 483 DEGs, among which 223 genes were upregulated and 260 genes were downregulated.

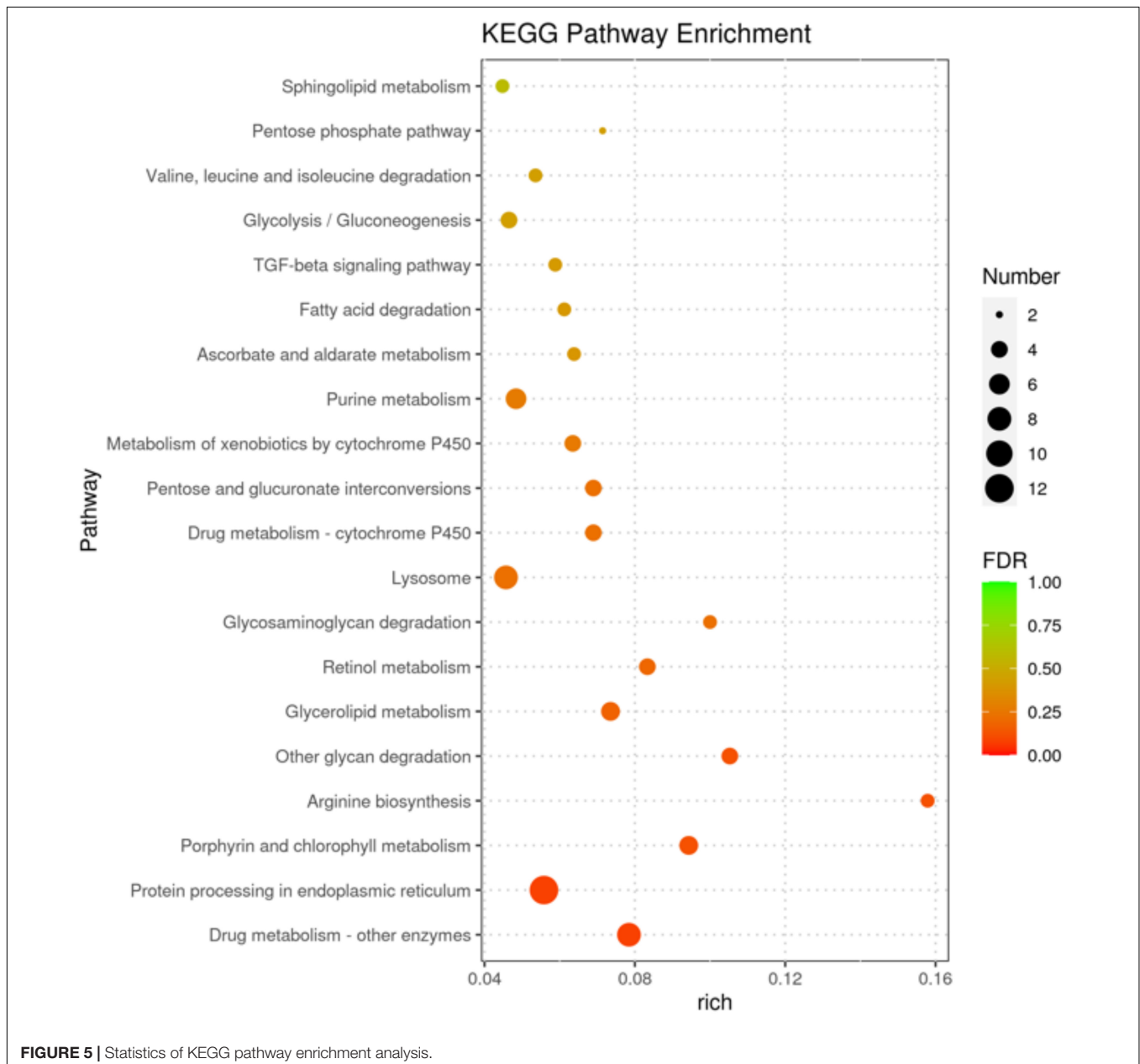
KEGG Enrichment Analysis

In Figure 5, the senior bubble chart showed the top 20 enrichment pathways, namely, protein processing in the endoplasmic reticulum, drug metabolism-other enzymes, carbohydrate metabolism (i.e., glycolysis/gluconeogenesis, pentose phosphate pathway, ascorbate and aldarate metabolism,

pentose phosphate pathway, and amino sugar and nucleotide sugar metabolism), metabolism of cofactors and vitamins (i.e., porphyrin and chlorophyll metabolism), amino acid metabolism (i.e., arginine biosynthesis and valine, leucine, and isoleucine degradation), lipid metabolism (i.e., glycerolipid metabolism, fatty acid degradation, and sphingolipid metabolism), transport and catabolism (i.e., lysosome and phagosome), and metabolism of cofactors and vitamins (i.e., porphyrin and chlorophyll metabolism and retinol metabolism).

Identification of Key Differentially Expressed Genes

As shown in Table 2, key genes regulating nutrition metabolism, body color formation, diurnal rhythm, immune function,



hormone levels, and posttranslational modifications were downregulated after constant darkness for 8 weeks.

Intestinal Microbiota Analysis Characteristics of Sequencing Results

As **Figure 6A** shown, 2,61,195 high-quality reads were produced, with an average of $38,086 \pm 1,499$ in the natural light group and $48,979 \pm 9,345$ in the dark treatment group. The rarefaction curves tended toward the saturation plateau (**Figure 6B**), while the coverage estimations of Good revealed that the amounts of obtained bacterial species in the natural light group and dark treatment group were 0.9993 and 0.9997, respectively, which indicates an adequate sequencing coverage that was able to capture the diversity of the bacterial communities. OTUs, the diversity index (Shannon and Simpson), and the estimators of the community richness index (Chao) were summarized. No significant differences ($P > 0.05$) were observed in OTUs, richness index (Chao), and diversity index (Shannon and Simpson) between the natural light group and dark treatment group.

Comparison of the Intestinal Microbiota Composition

The taxonomic assignment of all sequences was used to assess the microbial composition at the phylum and genus level as summarized in **Figures 7A,C**. At the phylum level, the intestinal microbiota is mainly composed of *Bacteroidetes* and *Proteobacteria*. Compared with the natural light group, dark treatment significantly decreased ($P < 0.05$) the relative abundance of intestinal *Bacteroidetes* and increased ($P < 0.05$) the relative abundance of *Proteobacteria* at the phylum level of *L. vannamei* (**Figure 7B**). At the genus level, dark treatment significantly decreased ($P < 0.05$) the relative abundance of *Formosa*, *Demequina*, and *Lutimonas* and increased ($P < 0.05$) the relative abundance of *Ruegeria*, *Vibrio*, *Actibacter*, *Roseovarius*, *Illumatobacter*, and *Kriegella* at the intestine of *L. vannamei* (**Figure 7D**).

In **Figure 8**, we applied the LEfSe to distinguish the specific bacterial taxa that varied in relative abundance by different treatments. As more bacteria were identified from phyla to species with LDA scores > 2.0 , we raised the criteria and selected specific bacterial taxa with LDA scores > 3.5 . It could be observed that *Ruegeria*, *Vibrio*, *Actibacter*, *Simiduia*, *Illumatobacter*, *Cellvibrionales*, *Kriegella*, *Tamlana*, *Muricauda*, and *Iamia* were biomarkers in the dark treatment group, while *Formosa*, *Pseudoalteromonas*, *Lutimonas*, *Celeribacter*, *Demequina*, *Bacteroidales*, and *Haliaceae* were biomarkers in the natural light group.

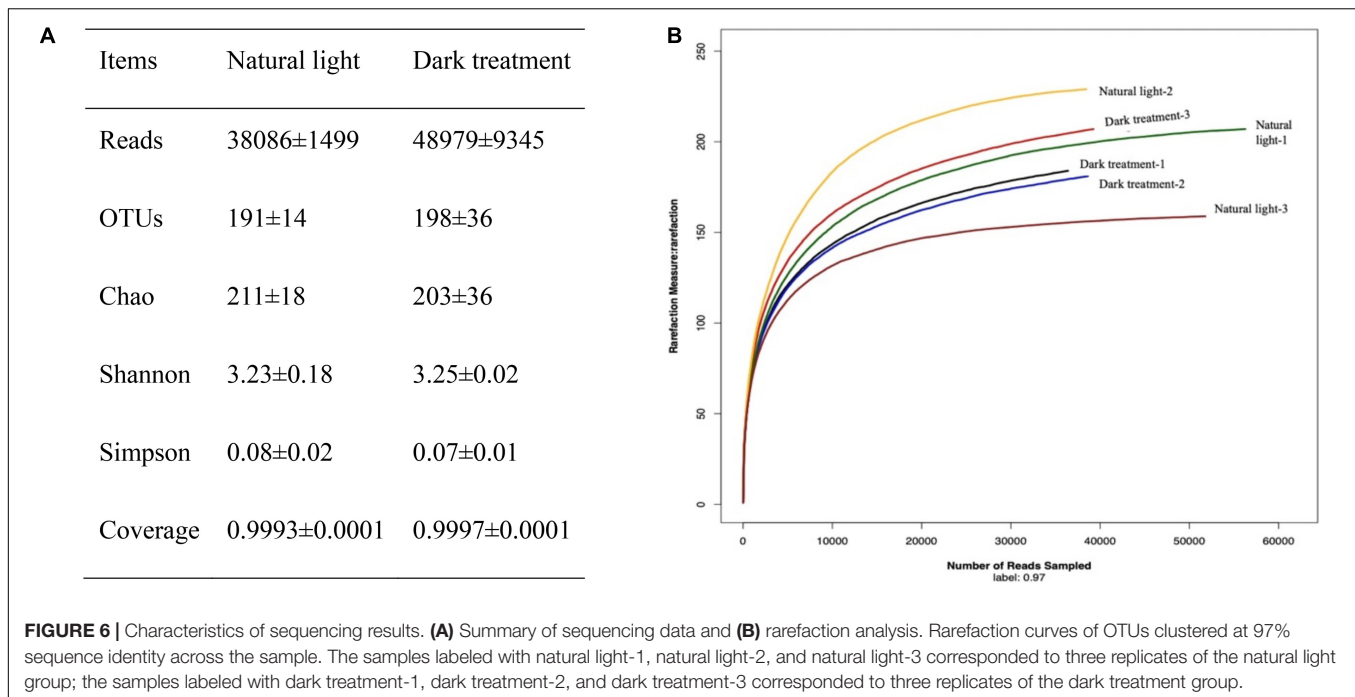
Functional Prediction of the Intestinal Microbial Community

In **Figure 9**, significant differences were observed in the KEGG pathways between the natural light group and the dark treatment group. Compared with the natural light group, the dark treatment group significantly increased ($P < 0.05$) KEGG pathways involving mainly in the two-component system, bacterial chemotaxis, phosphotransferase system (PTS), *Vibrio cholerae* infection, *Staphylococcus aureus* infection, and

TABLE 2 | Summary of significantly downregulated genes.

Pathways	Significantly downregulated genes
Nutrition digestion and absorption	Peritrophin-44-like protein
	Chitinase 1 precursor
	Glucose transporter 2
	Facilitated glucose transporter member 1-like isoform X1
	Zinc transporter foi-like
	Epithelial chloride channel protein
	osbpl1a, putative
	Eukaryotic translation initiation factor 3 subunit 12
	Phosphoglycerate mutase 2
	Sodium-dependent phosphate transporter
	Selenium-binding protein 1-A
	Putative cation-transporting ATPase 13A3
	Sodium-dependent phosphate transporter
	Facilitated glucose transporter member 8
	Chitin deacetylase 9-like protein
	Putative sodium-dependent multivitamin transporter
	Calcium-independent phospholipase A2-gamma
	Major facilitator superfamily domain-containing protein 6 isoform X1
	Sodium/glucose cotransporter 4-like
	Glucose transporter 2
	SEC14-like protein 2
	Lipoprotein receptor 2A
	Solute carrier family 22 member 3
Transient receptor potential channel pyrexia	
Heme-binding protein 2-like	
Body color	Crustacyanin subunit C
	Cryptochrome
Circadian rhythms	Aryl hydrocarbon receptor nuclear translocator-like protein 1
	Period circadian protein homolog 1
Posttranslational modifications	Circadian locomotor output cycles kaput protein
	NAD-dependent protein deacetylase Sirt4
Immune	Ubiquitin conjugation factor E4 B
	KAT8 regulatory NSL complex subunit 3-like
	Beta-1,3-glucan binding protein
	C-type lectin
Hormone regulation	Antilipopolsaccharide factor like protein
	Mutant C-type lectin
	NF-kappa-B inhibitor delta
	iodotyrosine dehalogenase 1
	Thyroid hormone receptor beta-A-like
	Pituitary homeobox x-like
	C-type allatostatin
	insulin-like receptor

biofilm formation of *Vibrio cholerae*. The dark treatment group significantly decreased ($P < 0.05$) KEGG pathways involving mainly metabolism (e.g., nitrogen metabolism, sulfur metabolism, and galactose metabolism), circadian rhythm, and biosynthesis of the siderophore group non-ribosomal peptides.



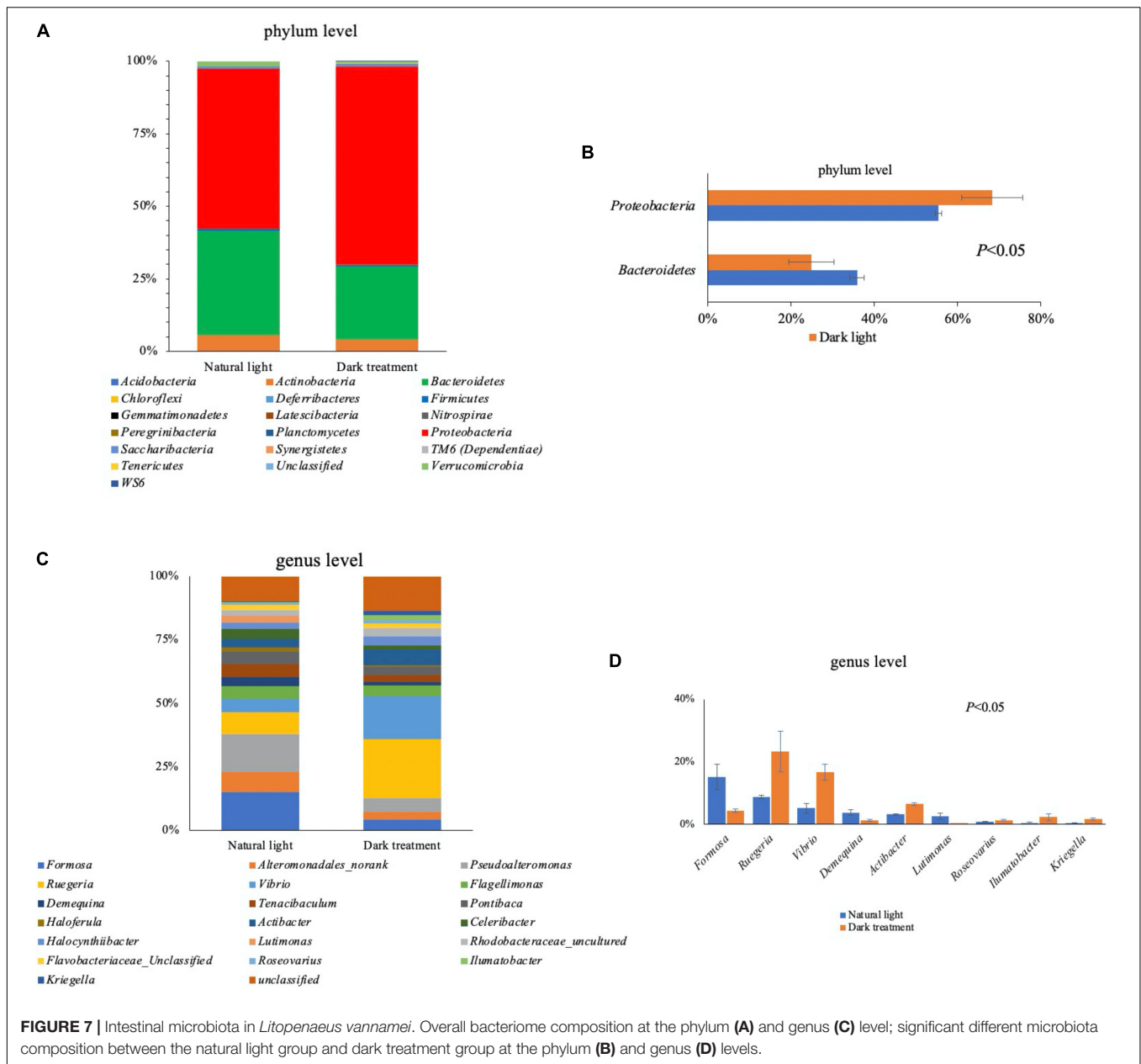
DISCUSSION

The change in somatic color in crustacea has received a great deal of attention as a conspicuous and quantifiable phenomenon related to physiological and ecological factors. Crustacean showed an ability to change coloration in response to photoperiod, which may play roles including photoprotection and enhancing camouflage in a unique marine environment (Russell and Dierssen, 2018). In our study, one interesting finding was that the body color of *L. vannamei* became darker after dark treatment for an 8-week feeding trail, which may be related to the decreased gene expression of crustacyanin subunit C (from transcriptome data). Although the molecular mechanism of the crustacean body color change is not clear, several studies have indicated that the regulation of body color can be associated with gene expression of crustacyanin (Zhao et al., 2021). Astaxanthin, one of the carotenoids found in nature, appears to be the main pigment responsible for color in crustaceans, accounting for approximately 65–98% of all the carotenoids found in shrimp species (Wade et al., 2005; Ertl et al., 2013). The stability of the highly reactive astaxanthin pigment is provided by crustacyanin, a protein that binds to the pigment to form a carotenoprotein complex, which stabilizes the pigment as well as the tertiary and quaternary structures of proteins (Wade et al., 2009). Crustacyanin subunit A and C expression levels were lower in dark shrimp than that in light shrimp (Ertl et al., 2013). In addition, it has been reported that *L. vannamei* infected with *Vibrio parahaemolyticus*, *V. fluvialis*, *V. mimicus*, *V. alginolyticus*, and *Vibrio* sp. found to take less feed and body color tended to be darker (Mastan, 2015). Later, the intestinal microbiota analysis confirmed that the relative number of *Vibrio* has been increased

after the dark treatment. Taken together, we suspected that dark treatment decreased the gene expression of crustacyanin subunit C and increased intestinal *Vibrio* number and, therefore, changed body color in *L. vannamei*. However, currently, the molecular mechanism remains unclear, which allows us to dig deeper into the possible mechanisms in the future.

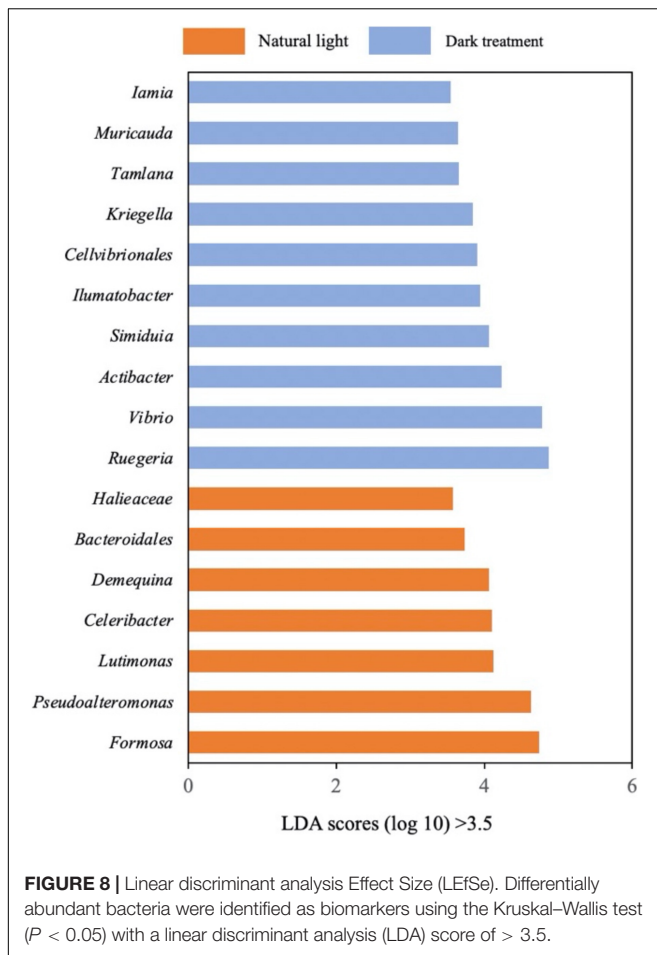
Serum enzymes such as AST and ALT are generally used in assessing liver function (Senior, 2012). We found that dark treatment elevated serum levels of AST and ALT, which are usually considered as a sign of hepatocellular damage (Panteghini et al., 1983). The transcriptomics data further revealed the influence of different photoperiods (12 h light/12 h dark, 0 h light/24 h dark) on the metabolism in *L. vannamei*. We observed that DEGs mostly involved in nutrient digestion, absorption, and transport were significantly suppressed after dark treatment for the 8-week feeding trail, which might partially explain the changed serum levels of ALB, LDL, and glucose. In detail, digestive enzymes (i.e., chitinase and chitin deacetylase 9-like protein), nutrition transport regulation (i.e., glucose transporters, ion/metal transporters, putative sodium-dependent multivitamin transporter, transient receptor potential channel pyrexia, solute carrier family 22 members, and selenium-binding protein 1-A), and lipoprotein binding (i.e., lipoprotein receptor 2A, SEC14-like protein 2, and *osbpl1a*) genes were downregulated. Similarly, Sanudin et al. (2014) reported that the ingestion rate of frozen *Artemia nauplii* under dark conditions (24 h dark) was lower than that under light conditions (24 h light) in the smallest shrimp (total length of 0.5 cm) (Sanudin et al., 2014).

Most organisms have evolved an internal circadian clock that drives circadian rhythms in metabolism, physiology, and behavior (Ko and Takahashi, 2006). The circadian clocks use a



24 h light-dark cycle as the environmental signal (zeitgeber) to establish endogenous circadian timing systems that synchronize biological functions (Roenneberg et al., 2013). In our study, we observed that circadian clock genes including cryptochrome, aryl hydrocarbon receptor nuclear translocator-like protein 1 (BMAL1), period circadian protein homolog 1, and circadian locomotor output cycles kaput protein were downregulated under constant darkness treatment in the hepatopancreas of *L. vannamei*. In similar to our data, continuous darkness decreased the mRNA levels of clock genes cryptochrome 1 (Cry1) and period 2 (Per2) in the eyestalk of *L. amboinensis* (Choi et al., 2019). In mammals, darkness exposure for 8 weeks decreased the mRNA levels of BMAL1 in the brain and muscle and decreased the mRNA levels of Per1 and Per2 in the liver (Li et al., 2020).

Molecular insights into the mechanisms of circadian rhythms have provided clues that posttranslational modifications control the circadian clock function (Gallego and Virshup, 2007). Posttranslational modifications regulate the localization, degradation, and activity of these clock genes by methylation, phosphorylation, acetylation, sumoylation, ubiquitination, etc. We observed that darkness exposure downregulated genes, which were associated with protein ubiquitination (i.e., ubiquitin conjugation factor E4 B), acetylation (i.e., KAT8 regulatory NSL complex subunit 3 and NAD-dependent protein deacetylase Sirt4), and methylation (i.e., thiopurine S-methyltransferase-like). Ubiquitin conjugation factor E4 B functions as an E4 ligase that mediates the assembly of polyubiquitin chains on substrates ubiquitinated by another E3 ubiquitin ligase

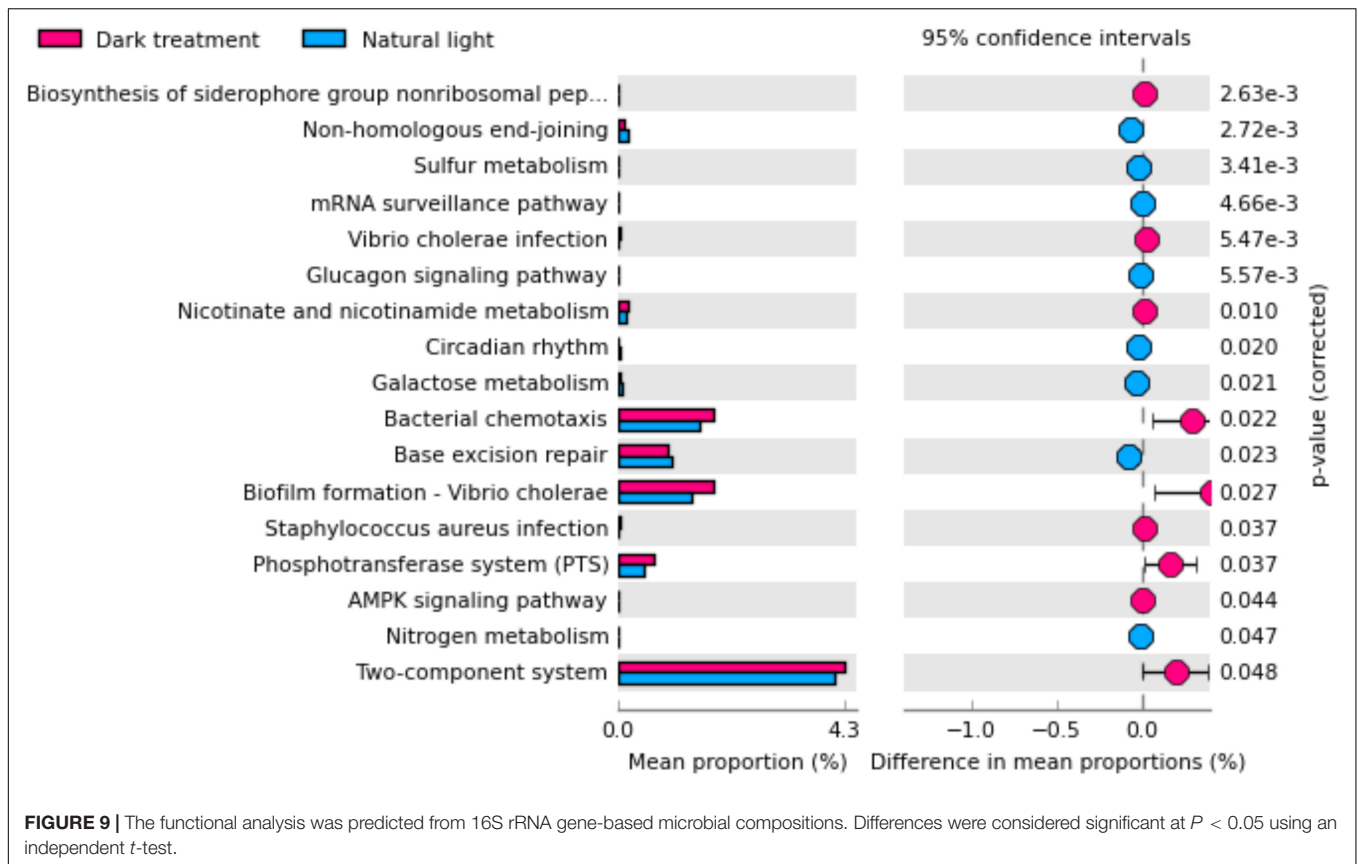


(Hatakeyama et al., 2001). NAD-dependent protein deacetylase SIRT4 is a lysine deacetylase and acts as a crucial player in maintaining insulin secretion and glucose homeostasis (Anderson et al., 2017). As part of the NSL complex, KAT8 regulatory NSL complex subunit 3 is involved in the acetylation of nucleosomal histone H4 on several lysine residues and may be involved in the regulation of transcription (Cai et al., 2010). Thiopurine S-methyltransferase-like drives methylation by directing thiopurines [6-mercaptopurine (6MP)] toward methylated entities [6-methylmercaptopurine (6MMP)] and, thus, decreases levels of active 6-thioguanine nucleotides (Urbancic et al., 2019).

A marked oscillation in hormone exhibited daily rhythms under light/dark cycles (Bayarri et al., 2004; Oliveira et al., 2013). Merging evidence suggests that disturbance of the circadian rhythm of the body increases endocrine disorder risk (Gamble et al., 2014; Zimmet et al., 2019). We found that constant darkness exposure exhibited decreased several gene expressions, which was involved in hormone regulation, including insulin-like receptor, C-type allatostatin, iodotyrosine dehalogenase 1, pituitary homeobox x-like, and thyroid hormone receptor beta-A-like in the hepatopancreas of *L. vannamei*. In invertebrates, the role of the insulin pathways includes not only glucose homeostasis but also the regulation of a variety of fundamental

processes, such as growth, aging, and reproduction (Claeys et al., 2002). As the pivotal protein in any insulin family-based signaling pathway, the insulin-like receptor is responsible for mediating the signal carried by insulin-like peptides (ILPs) from the intercellular to the intracellular environment (Sharabi et al., 2016). A similar result was reported that constant darkness exposure exhibited decreased BMAL1 expression and inhibition of the NAMPT/NAD⁺/SIRT1 pathway, further suppressed GLUT4 expression, and lead to insulin resistance in the liver cells (Li et al., 2020). C-type allatostatin is named because of its potent inhibitory effect on juvenile hormone biosynthesis by the corpora allata in insects and also modulates the immune response in the hepatopancreas of mud crab *Scylla paramamosain* (Xu et al., 2020). The thyroid hormone receptors are members of the steroid receptor superfamily that mediate the action of thyroid hormone signaling in numerous tissues to regulate important physiological and developmental processes (Anyetei-Anum et al., 2018). Iodotyrosine dehalogenase 1 is a transmembrane protein involved in the encoding iodotyrosine deiodinase, and it controls the reuse of iodide for thyroid hormone synthesis (Gnidehou et al., 2004). Pituitary homeobox plays an important role in regulating the transcription of enzymes involved in steroidogenesis (Hiroi et al., 2003). Our results provide the first evidence that constant dark treatment could influence hormone regulation in the hepatopancreas of *L. vannamei*.

The involvement of the light-dark cycle as an important regulator of immune functions has been extensively described in mammals, but there is a paucity of information on the influence of this biological phenomenon in aquatic animals. Limited studies have reported that the innate immune system had a circadian rhythm based on the light-dark cycle in fish (Esteban et al., 2006; Ceballos-Francisco et al., 2020). Serum-mediated bacterial killing activities and enzyme activities associated with immune defense (i.e., lysozyme, alkaline phosphatase, and myeloperoxidase) reached the highest during the light phase in rainbow trout (*Oncorhynchus mykiss*) (Lazado et al., 2018). High-light intensity (16D:8L and 0D:24L dark/light cycles) increased lymphocyte count and improved the overall physiological state of carp (*Cyprinus carpio* L.) yearlings, while zero illumination had an inverse effect (Ruchin, 2006). We found that dark treatment significantly suppressed immune-related gene expression in the hepatopancreas of *L. vannamei*, including beta-1,3-glucan binding protein, C-type lectin, antilipopolsaccharide factor-like protein, and NF-kappa-B inhibitor delta. Beta-1,3-glucan binding protein might act as a pathogenic recognition protein to activate shrimp immune defense against invasive pathogens by the agglutination, binding, and enhancing encapsulation and phenoloxidase activity of the hemocytes (Phupet et al., 2018). C-type lectin could inhibit the proliferation of hemolymph microbiota by maintaining the expression of antimicrobial peptides in shrimp (Wang et al., 2014). An antilipopolsaccharide factor is a small protein that has been verified responsive to different pathogen challenges and with broad-spectrum antimicrobial activities (Li et al., 2015). NF-kappa-B inhibitor delta functions in the regulation of inflammatory responses and regulates the expression of IL-2, IL-6, and other cytokines



through regulation of the NF-kappa-B activity (Arnold et al., 2012). These results indicated that constant dark treatment impaired the immune function in the hepatopancreas of *L. vannamei*.

Intestinal microbiota modulates host physiological processes and plays an important role in promoting and maintaining the health of the host. Dark treatment significantly increased the relative abundance of genus *Ruegeria*, *Vibrio*, *Actibacter*, *Roseovarius*, *Ilumatobacter*, and *Kriegella* at the intestine of *L. vannamei*. It is worth noting that dark treatment promoted the proliferation of pathogenic bacteria. *Vibrio* spp. is the most typical and well-known pathogen causing vibriosis infections in aquatic animals (Arunkumar et al., 2020). As a member of the *Roseobacter* clade, *Roseovarius crassostreae* is a pathogenic bacteria of *Roseovarius* Oyster Disease and has resulted in mass mortalities of cultured *Crassostrea virginica* in the northeastern United States (Maloy et al., 2007). Furthermore, microbiota KEGG functional analysis demonstrated that dark treatment increased the abundance of pathways in *Vibrio cholerae* infection, *Staphylococcus aureus* infection, and biofilm formation of *Vibrio cholerae*. Besides, the relative abundance of *Ruegeria* was increased after the dark treatment at the intestine of *L. vannamei*. *Ruegeria* is a globally distributed gram-negative marine bacterium, which could produce the antibacterial compound tropodithetic acid and biofilm formation against several marine pathogens (Garcia et al., 2014). *Ilumatobacter* genome includes two type I polyketide synthases. The type I

polyketide synthases can catalyze the synthesis of polyketides, which are highly effective antibiotics (Zhao et al., 2019). The above findings suggested that increased *Ruegeria* and *Ilumatobacter* might help combat pathogenic bacteria. Moreover, the dark treatment significantly decreased the relative abundance of *Formosa*, *Demequina*, and *Lutimonas*. *Demequina* can help its host absorb and utilize carbohydrates by producing α -amylase, xylanase, and cellulase (Al-Naamani et al., 2015). *Lutimonas* is a strictly aerobic heterotrophic nitrifying bacterium for the degradation of ammonia (Fu et al., 2009). To date, less is known about the physiological function of *Actibacter*, *Kriegella*, and *Formosa*, which needs to be further clarified in the future.

CONCLUSION

The overall result generated from our findings indicated that constant darkness darkened body color and altered hepatopancreas metabolism and intestinal microbiota homeostasis in *L. vannamei*. Genes involved in regulating nutrition metabolism, body color formation, diurnal rhythm, immune function, hormone levels, and posttranslational modifications were downregulated after constant darkness for 8 weeks. Further intestinal microbiota analysis showed that dark treatment-induced alterations in intestinal bacterial abundances and circadian rhythm increased susceptibility of pathogens, and decreased nutrition metabolism. The results of this study

would provide an important theoretical basis and reference for further understanding of the impact of different light/dark cycles in shrimp physiological changes, which might give clues for improving the productive capacities by changing light/dark cycles in shrimp farming.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI, PRJNA761846.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics-Scientific Committee for Experiments on Animals of Ningbo University.

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AUTHOR CONTRIBUTIONS

LJF and QCZ designed and performed the experiment, analyzed the data, and drafted the manuscript. TMD helped to perform the experiment and collected samples. XYT and JJJ helped to revise the manuscript. All authors read and approved the final manuscript.

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