



Dietary Carbohydrate and Protein Levels Affect the Growth Performance of Juvenile Peanut Worm (*Sipunculus nudus*): An LC-MS-Based Metabolomics Study

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The peanut worm (*Sipunculus nudus*) is an economically important fishery resource in China. To determine how dietary carbohydrate and protein levels affect the growth performance of juvenile *S. nudus* and identify the mechanisms underlying observed patterns, five isoenergetic and isolipidic diets with different levels of carbohydrate and protein were formulated and fed to juvenile *S. nudus*; the experimental groups were referred to as EG1, EG2, EG3, EG4, and EG5, respectively. After 90 days of feeding, *S. nudus* had significantly lower survival rates when fed D5 compared with other diets ($P < 0.05$), and the highest survival rate was observed in EG2 individuals. The weight gain rate and specific growth rate were significantly higher in EG2 compared with the other groups ($P < 0.05$). Metabolomic profiling using liquid chromatography–mass spectrometry revealed 83 significantly differential metabolites (POS: 59; NEG: 24), which were identified via an in-house MS2 database. Pathway analysis indicated that the significantly different metabolites were involved in 22 metabolic pathways (POS: 9; NEG: 13), including tyrosine, phenylalanine, and tryptophan biosynthesis; phenylalanine metabolism; D-glutamate and D-glutamine metabolism; proline and arginine metabolism; aspartate, alanine, and glutamate metabolism; and aminoacyl-tRNA biosynthesis. These analyses implied that the biosynthetic capabilities of juvenile *S. nudus* were greater in the EG2. The results of this research enhance our understanding of the effects of dietary carbohydrate and protein levels on the growth performance of juvenile *S. nudus*.

Keywords: *Sipunculus nudus*, growth performance, metabolomics, LC-MS, carbohydrate, protein

INTRODUCTION

Peanut worm (*Sipunculus nudus*) is an important resource in China and is often referred to as the “cordyceps of the sea” because of its high medicinal and nutritional value (Shen et al., 2004; Luo et al., 2016; Zhang et al., 2018). It is cultured along beaches and obtains nutrients from surface sediments (Adrianov and Maiorova, 2010). *S. nudus* is widely distributed along the coast

of southern China, including the Zhanjiang, Guangdong and Beihai, Guangxi (Du et al., 2008, 2009). Wild-collected seeds are commonly used for the culture of *S. nudus*; however, excessive harvesting of the seeds can rapidly deplete wild seed resources (Du et al., 2009). Artificial breeding can be used to mitigate the exploitation of wild resources. Many studies have been conducted on the population genetics (Du et al., 2008, 2009) and reproductive biology (Wang et al., 2005; Cao et al., 2020; Yang et al., 2020; Zhang J. W. et al., 2021) of *S. nudus*. Given that growth performance is greatly affected by diet quality, studies of the nutritional requirements, and artificial feed of juvenile *S. nudus* could aid its artificial breeding, enhance its aquaculture, and promote the restoration of wild populations. *S. nudus* has a simple body structure, and it possesses the digestive tract and the posterior renal tube that is bathed in coelomic fluid but has no liver and pancreas. Coelomic fluid is a complex internal environment system, where many physiological and biochemical processes are completed (Xian and Huang, 2011). And coelomic fluid was mainly involved in catalytic activity and metabolic processes, most of which were associated with carbohydrate and protein metabolism (Cao et al., 2021).

The metabolic profiles of animals vary with diet quality. Metabolomics provides a “snapshot” of the metabolites and is particularly suitable for metabolic studies (Cappello et al., 2017, 2018). It is commonly used to quantify low-molecular-weight metabolites, such as sugars, lipids, and amino acids, through various tools such as gas chromatography–mass spectrometry, nuclear magnetic resonance, and liquid chromatography–mass spectrometry (LC–MS; Venter et al., 2018; Yang et al., 2021; Zhang J. B. et al., 2021). Several studies have used this approach to assess the effects of nutrient levels (Jin et al., 2015; Yang et al., 2019a,b), food shortages (Tuffnail et al., 2009; Baumgarner and Cooper, 2012), nutrient supplementation (Wagner et al., 2014; Andersen et al., 2015), and the substitution of nutrients (Cheng et al., 2016; Yang et al., 2018b) in aquatic animals.

Incorporation of the optimal level of carbohydrate and protein in diets helps improve feed efficiency and growth rate in aquatic animals. The aim of this study was to determine the optimum balance of dietary carbohydrates and proteins for juvenile *S. nudus* and compare its metabolomic responses when fed two diets differing in dietary carbohydrate and protein levels using LC–MS-based metabolomics. The results of this study aid our understanding of the metabolomic responses of juvenile *S. nudus* to different diets and provide new information on its nutritional requirements.

MATERIALS AND METHODS

Experimental Diets and Rearing

Five isoenergetic and isolipidic diets (D1, D2, D3, D4, and D5) were formulated with different levels of carbohydrate and protein based on previous studies (Zhang et al., 2011, 2012; Xu et al., 2013). Proteins were obtained from fish meal, *Spirulina platensis* powder, yeast powder, and soybean meal; carbohydrates were obtained from starch and kelp powder; and lipids were obtained from fish oils, average crude lipid level was 8.71%. The formulated

diets were ground into desirable particle sizes (180 mesh). The ingredients and proximate composition of the formulated diets are shown in **Supplementary Table 1**. The amino acid profiles of the formulated diets are shown in **Supplementary Table 2**. D1–D5 were fed to animals (mean total weight: 1.55 ± 0.25 g) in five groups: EG1, EG2, EG3, EG4, and EG5, respectively; each experimental group had three replicates, and each replicate (1 m²) had 120 animals. The experiment was run for 90 days. During the experiment, the juvenile peanut worms were fed twice at 8:00 and 20:00 per day, and up to 3% of their total biomass each time. About 20% of the water volume was renewed daily. The concentration of dissolved oxygen in the water was 5.00 mg/L, the water salinity was 30, and the temperature ranged from 24.1–29.7°C, ammonia-N was <0.5 mg/L, and pH was around 7.4–7.9.

The protocols for measuring crude protein, crude lipid, ash, and amino acid of the formulated diets were detailed by Wang et al. (2016), carbohydrate values were calculated following the methods of Xia et al. (2015), gross energy values were calculated following the methods of Xie et al. (2017).

Survival Rate and Growth Performance

The total number and total weight of juvenile *S. nudus* in each group were determined at the beginning and end of the experiment. Total weight was obtained using an electronic balance. Survival rate, weight gain rate (WGR), and specific growth rate (SGR) were calculated following the methods of Xia et al. (2015).

Sample Collection

After the 90-day culture period, eight *S. nudus* from each group were randomly selected and coelomic fluid from juvenile *S. nudus* was extracted following the methods of Yang et al. (2020), immediately placed in liquid nitrogen, and stored at –80°C.

Metabolite Extraction and LC–MS/MS Analysis

A 100- μ L sample was pipetted into an Eppendorf tube, and 400 μ L of extract solution (acetonitrile: methanol = 1: 1) containing an isotopically labeled internal standard mixture was added. Metabolite extraction followed the methods of Yang et al. (2021).

A UHPLC system (Vanquish, Thermo Fisher Scientific) with a UPLC BEH Amide column (2.1 mm \times 100 mm, 1.7 μ m) and a Q Exactive HFX mass spectrometer (Orbitrap MS, Thermo) was used for LC–MS/MS analyses. The analysis was carried out with an elution gradient as described by Yang et al. (2021). The flow rate was 0.5 mL/min; the auto-sampler temperature was 4°C; the column temperature was 30°C; and the injection volume was 2 μ L.

MS/MS spectra were acquired using a QE HFX mass spectrometer through the information-dependent acquisition mode in Xcalibur software (Thermo Fisher Scientific). The software continuously evaluates the full-scan MS spectrum in this mode. The sheath gas flow rate was 30 Arb; the Aux gas flow rate was 25 Arb; the capillary temperature was 350°C; the full

MS resolution was 60,000; the MS/MS resolution was 7,500; the collision energy was 10/30/60 in NCE mode; and the spray voltage was 3,600 V (positive, POS) or -3,200 V (negative, NEG).

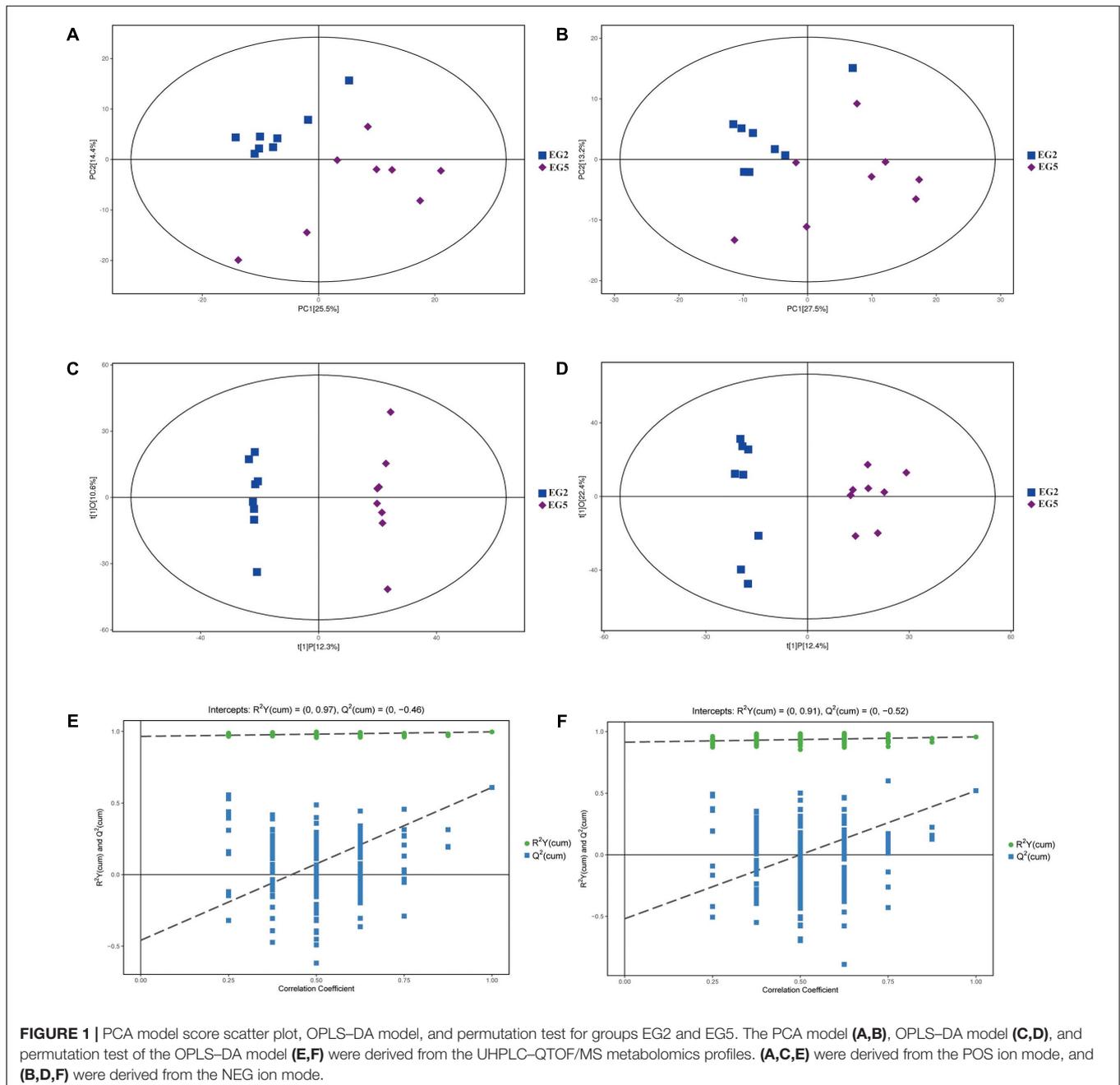
Data Preprocessing and Annotation

ProteoWizard was used to convert the raw data to mzXML format, followed by processing with an in-house program. This

TABLE 1 | Survival rate and growth performance of juvenile peanut worm *S. nudus*.

Items	EG1	EG2	EG3	EG4	EG5
Survival (%)	81.48 ± 1.70 a	82.59 ± 1.70 a	77.78 ± 2.94 a	78.15 ± 1.28 a	70.37 ± 6.79 b
Weight gain rate (%)	87.67 ± 5.19 b	117.03 ± 7.57 a	55.83 ± 2.95 c	51.23 ± 2.68 cd	45.70 ± 6.38 d
Specific growth rate (% day ⁻¹)	0.70 ± 0.03 b	0.86 ± 0.04 a	0.49 ± 0.02 c	0.46 ± 0.02 cd	0.42 ± 0.05 d

Means with the same letters within a row are not significantly different ($P > 0.05$).



program was used for extraction, peak detection, alignment, and integration and was developed using R. The cutoff for annotation was 0.3. The metabolite identification was performed via an in-house MS2 database, including metlin, pubchem, and self-built database of Biotree Biotech Co., Ltd. (Shanghai, China).

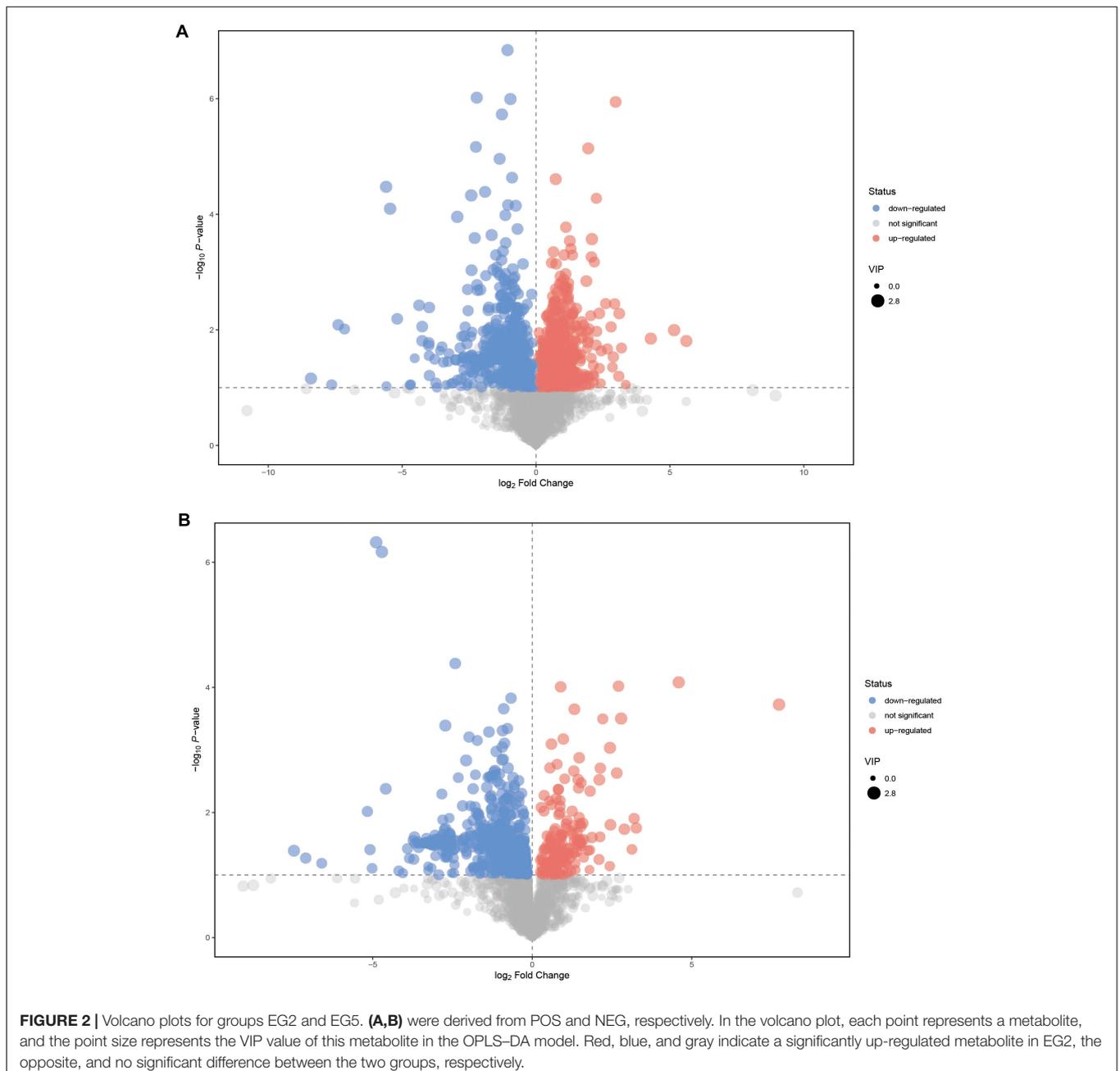
Data Analysis

The final dataset contained information such as the sample name, peak number, and normalized peak area and was imported to SIMCA16.0.2 (Sartorius Stedim Data Analytics AB, Umea, Sweden) for orthogonal projections to latent structures discriminant analysis (OPLS-DA) and principal

component analysis (PCA). Data were scaled and logarithmically transformed to minimize the effect of noise and high variance among the variables. Data were analyzed as previously described (Yang et al., 2021). In the OPLS-DA analysis, the variable importance in projection (VIP) of the first principal component in OPLS-DA analysis was obtained. Metabolites with $P < 0.1$ (Student's t -test) and $VIP > 1$ were significantly different metabolites (SDMs). In addition, the KEGG¹ and MetaboAnalyst² databases were utilized for pathway enrichment analysis.

¹<http://www.genome.jp/kegg/>

²<http://www.metaboanalyst.ca/>



Survival rate, WGR, and SGR values were determined using one-way analysis of variance, followed by Duncan's multiple comparisons test. The data were presented as mean \pm SEM; significant differences ($P < 0.05$) between variables.

RESULTS

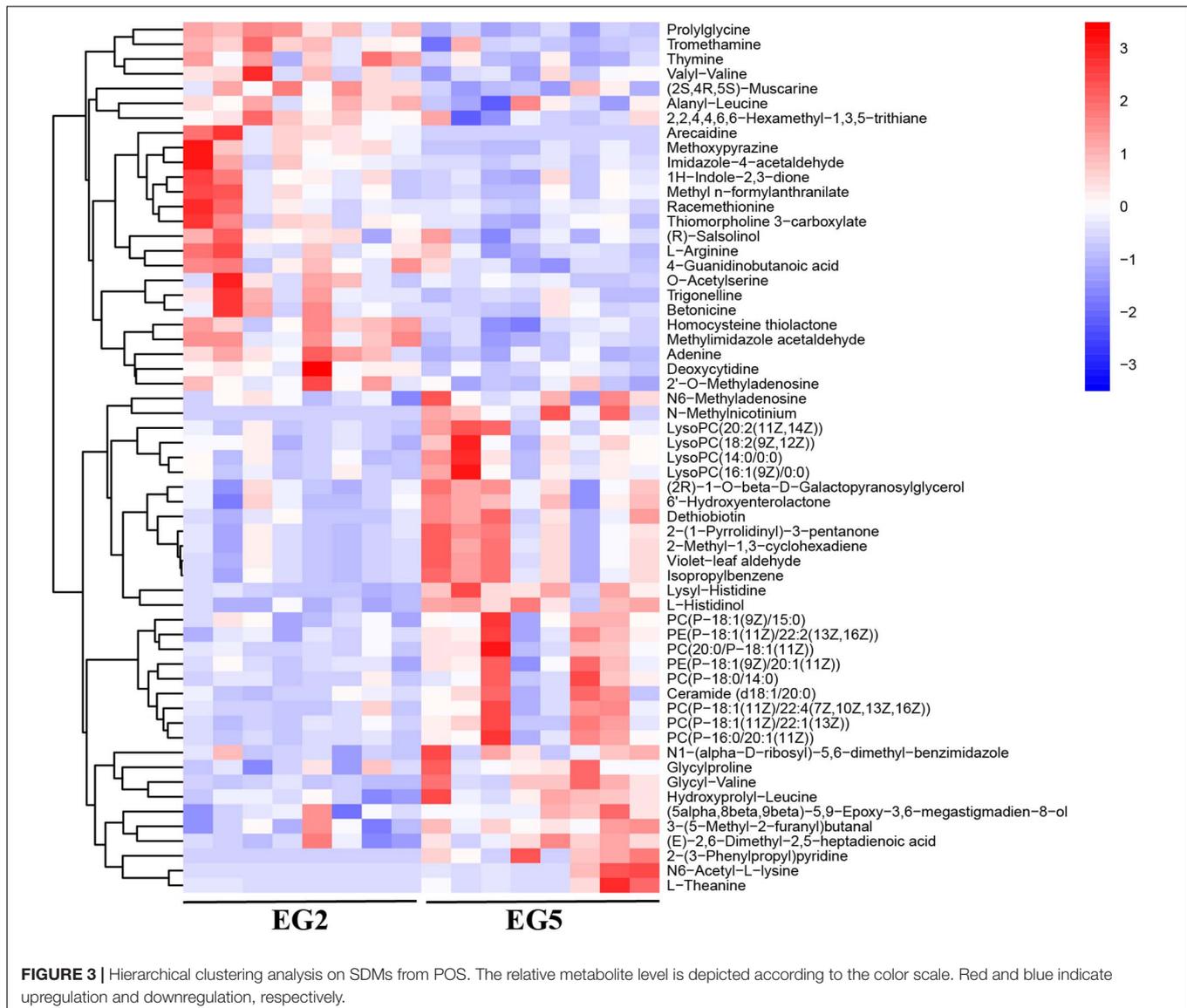
Survival Rate and Growth Performance

The survival rates of juvenile *S. nudus* ranged from 70.37 to 82.59%. EG5 *S. nudus* had significantly lower survival rates compared with *S. nudus* in other groups ($P < 0.05$, **Table 1**). Although no significant differences were observed among EG1, EG2, EG3, and EG4, the highest survival rate was observed in EG2 ($P > 0.05$, **Table 1**). Juvenile *S. nudus* in group EG2 had significantly higher WGR and SGR compared with the other groups (**Table 1**, $P < 0.05$).

General Changes in Metabolites

The peak area and peak retention time of the total ion chromatograms from all QC samples showed extensive overlap, indicating the stability of the analytical system (**Supplementary Figure 1**). A total of 4,160 positive (POS) and 3,227 negative (NEG) peaks were obtained for the coelomic fluid from juvenile *S. nudus*. These valid peaks were matched to 207 and 103 metabolites in POS and NEG, respectively, via an in-house MS2 database.

In the PCA score scatter plot, similar datasets were clustered more closely, whereas different datasets were placed further apart (**Figures 1A,B**). The values for R^2X of the PCA model between EG2 and EG5 were 0.572 (POS) and 0.574 (NEG), respectively. OPLS-DA was performed to characterize the different metabolic patterns of coelomic fluid from juvenile *S. nudus* (**Figures 1C,D**). The values for R^2X , R^2Y , and Q^2 in the OPLS-DA model of POS between EG2 and EG5 were 0.229, 0.977, and 0.609, respectively.



The values for R^2X , R^2Y , and Q^2 in the OPLS-DA model of NEG between EG2 and EG5 were 0.348, 0.957, and 0.520, respectively. A permutation test was used to avoid the transition fit in the OPLS-DA mode (Figures 1E,F). The R^2Y and Q^2 intercepts were 0.97 and -0.46 between EG2 and EG5 (POS) and 0.91 and -0.52 between EG2 and EG5 (NEG), respectively. Thus, the OPLS-DA model was not overfit and showed high stability; it was thus suitable for use in subsequent analyses.

SDMs

Volcano plots were used to visualize the SDMs; the plots revealed several significant SDMs (Figures 2A,B). In total, 83 SDMs between EG2 and EG5 (POS: 59; NEG: 24) were identified via an in-house MS2 database (Figures 3, 4 and Supplementary Table 3). Compared with EG5, 37 SDMs had higher concentrations in EG2 (POS: 25; NEG: 12; Figures 3, 4 and Supplementary Table 3).

Metabolic Pathway Analysis of SDMs

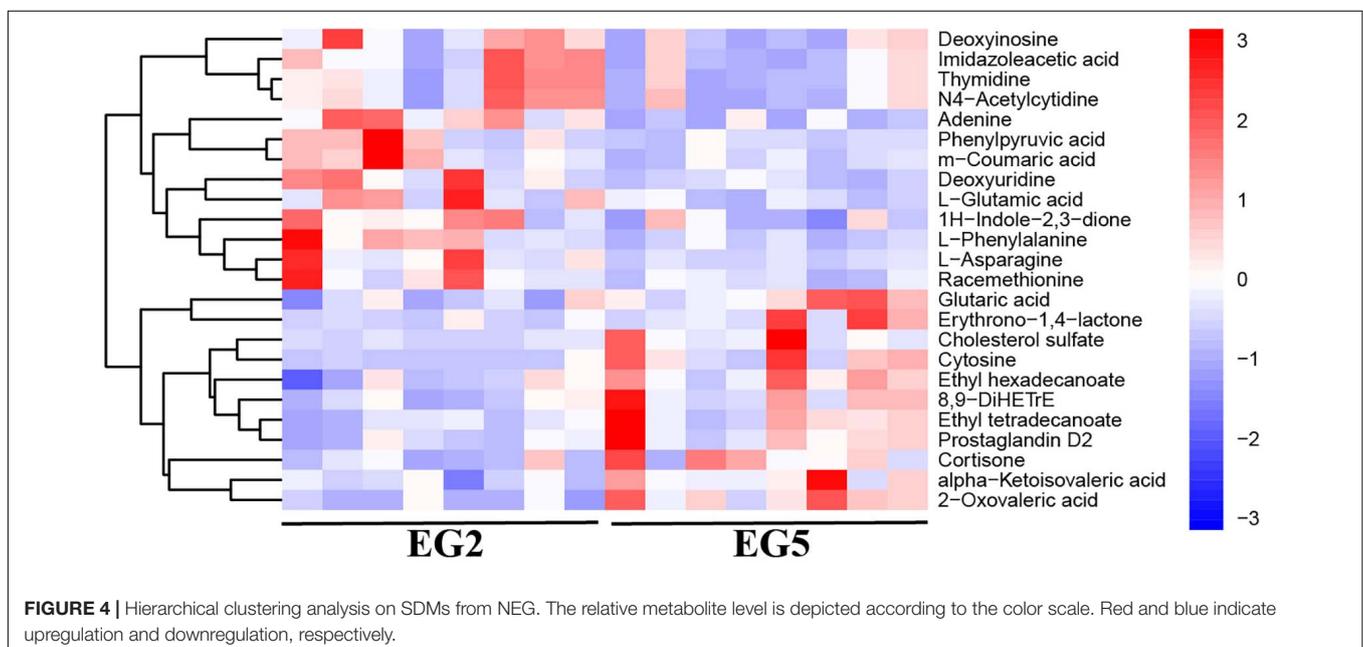
To explore potential metabolic pathways, the SDMs were imported into MetaboAnalyst 4.0. 22 metabolic pathways were identified between EG2 and EG5 (POS: 9; NEG: 13; Figure 5 and Supplementary Table 4). The metabolic pathways in POS between EG2 and EG5 identified were histidine metabolism, sulfur metabolism, riboflavin metabolism, selenoamino acid metabolism, cysteine and methionine metabolism, proline and arginine metabolism, pyrimidine metabolism, purine metabolism, and aminoacyl-tRNA biosynthesis. The relevant metabolic pathways in NEG between EG2 and EG5 identified were tyrosine, phenylalanine, and tryptophan biosynthesis; phenylalanine metabolism; D-glutamate and D-glutamine metabolism; aspartate, alanine, and glutamate metabolism; proline and arginine metabolism; and aminoacyl-tRNA biosynthesis.

DISCUSSION

The WGR and SGR of juvenile *S. nudus* increased as the level of dietary carbohydrates increased from 21.50 to 34.97% and decreasing dietary protein level from 34.07 to 24.02%. The WGR and SGR decreased with further elevation in carbohydrate level from 34.97 to 39.46% and reducing dietary protein level from 24.02 to 20.67%. Suboptimal amounts of dietary carbohydrate and protein result in the poor growth of aquatic animals (Xia et al., 2015; Guy et al., 2018; Surintorn et al., 2018; Yang et al., 2019b). Moreover, juvenile peanut worm fed with D2 (34.97% carbohydrates, 24.02% protein) got the highest survival rate. Thus, D2 was considered the most optimal diet.

Characterizing the effects of different levels of carbohydrates in the diet and exploring the mechanism underlying variation in the responses of organisms to differences in diets is essential for optimizing the diets and growth rates of aquacultural species. Metabolomics approaches are useful for providing an organism-scale overview of relevant pathways, as the metabolic pathways identified through the SDMs are those most sensitive to dietary or medical interventions (Yang et al., 2018a). Nutritional metabolomics research is a relatively new field in the context of aquaculture. LC-MS-based metabolomics analysis was performed to characterize differences in the metabolomic responses of juvenile *S. nudus* fed D2 and D5. Differences in the metabolites and changes in the coelomic fluid of *S. nudus* were also compared between these two groups. The key metabolic pathways were identified based on the common key metabolic pathways and pathways altered in the coelomic fluid.

Biosynthesis-related pathways, including tyrosine, phenylalanine, and tryptophan biosynthesis and aminoacyl-tRNA biosynthesis, were enriched. The content of amino acids involved in aminoacyl-tRNA biosynthesis, such as L-asparagine, L-phenylalanine, L-glutamic acid, and L-arginine, was higher



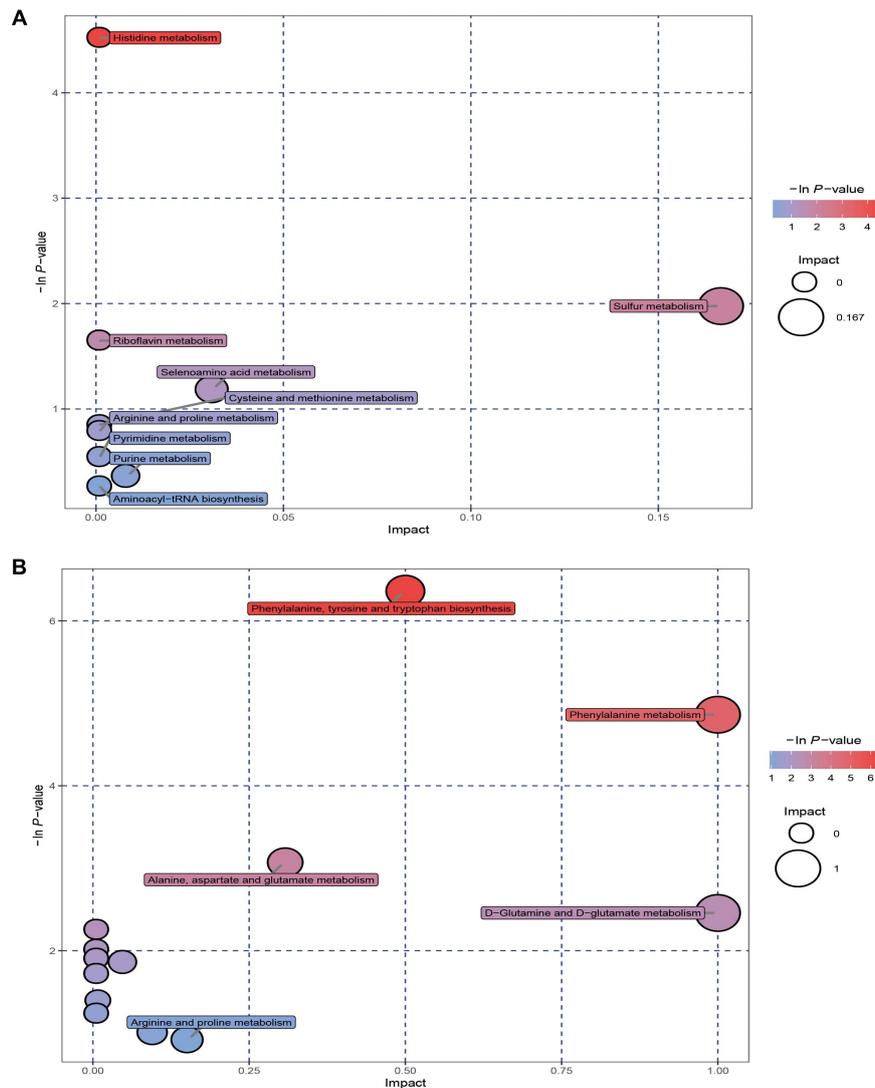


FIGURE 5 | Metabolome view map of significant metabolic pathways characterized in the coelomic fluid of *S. nudus* for groups EG2 and EG5. **(A,B)** are derived from POS and NEG, respectively. Significantly changed pathways based on enrichment and topology analysis are shown. The x- and y-axes represent pathway enrichment and pathway impact, respectively. Large sizes and dark colors represent major pathway enrichment and high pathway impact values, respectively.

in juvenile *S. nudus* fed D2. Amino acids can be transferred to ribosomal synthetic proteins through aminoacyl-tRNA biosynthesis (Smith and Hartman, 2015). This suggested that juvenile *S. nudus* got higher synthesis protein ability when fed D2 than when fed D5. Yang et al. (2020) also surmised that *S. nudus* exhibited higher synthesis ability with higher aminoacyl-tRNA biosynthesis ability in the breeding season. Altogether, arginine is an important amino acid that is involved in a variety of biological functions like protein synthesis, urea production, metabolism of glutamic acid and proline, and synthesis of creatine and polyamines in aquatic animals (Mai, 2011; Jin et al., 2016; Qi et al., 2021). It is a precursor for creatine and nitric oxide synthesis and serves as a potent stimulant of growth hormone suggestion that it may play an important role in anabolic processes (Zhou et al., 2012; Lin et al., 2015; Wei et al., 2021).

EG2 individuals had higher arginine acid content compared with EG5 individuals, which implied that the biosynthetic capabilities of juvenile *S. nudus* were greater in the EG2. Therefore, the superior synthesis ability of EG2 relative to EG5 might explain the greater growth performance of juvenile *S. nudus* in EG2.

The oxidation of cytoplasmically produced nicotinamide adenine dinucleotide can be affected by glutamic acid. In addition, glutamic acid has an anaplerotic function in the tricarboxylic acid cycle and acts as a signaling agent between the immune system and nervous system. Glutamic acid is an essential transamination partner for glutathione synthesis, which mitigates oxidative stress (Larsson et al., 2014). The glutamic acid content was substantially higher in juvenile *S. nudus* when fed D2 than when fed D5, which may reflect the superior growth, immunity, and antioxidant status

of juvenile *S. nudus* when fed D2. Glutamic acid has also been observed to be elevated in fast-growing farmed *Haliotis midae* (Venter et al., 2018). Based on these findings, we speculate that changes in glutamic acid may affect D-glutamate and D-glutamine metabolism, as well as aspartate, alanine, and glutamate metabolism and thus explain the poorer growth performance of juvenile *S. nudus* in EG5 compared with EG2. This finding was also observed in the pearl oyster *Pinctada fucata martensii*, which was fed diets varying in carbohydrate and protein levels (Yang et al., 2019b).

Phenylalanine is an indispensable aromatic amino acid that is the sole precursor of tyrosine and is required for normal growth and metabolic processes (Zehra and Khan, 2014). When phenylalanine enters organisms, it may follow one of three pathways: conversion to tyrosine, conversion to phenylpyruvic acid, or incorporation into cellular proteins (Vockley et al., 2014). Thus, phenylalanine is an essential amino acid for aquatic organisms that enhances growth performance and plays a role in various physiological functions (Jin et al., 2015). In this study, the phenylalanine and phenylpyruvic acid content of *S. nudus* was higher when fed D2 than when fed D5, as D2 had a lower phenylalanine content compared with D5 (Supplementary Table 2). Hence, the dysfunctional phenylalanine metabolism in EG5 may in part explain the poorer growth performance of juvenile *S. nudus* fed D5 relative to EG2 individuals, which were fed D2. However, the specific mechanism underlying these patterns requires further study.

CONCLUSION

In this study, juvenile *S. nudus* fed D2 (34.97% carbohydrates, 24.02% protein) had the highest survival rate, WGR, and SGR. Comparison of the metabolic status of juvenile *S. nudus* fed D2 and D5 using an in-house MS2 database revealed a total of 83 SDMs (POS: 59; NEG: 24) between group EG2 and EG5, which were involved in 22 metabolic pathways (POS: 9; NEG: 13), including tyrosine, phenylalanine, and tryptophan biosynthesis; phenylalanine metabolism; D-glutamate and D-glutamine metabolism; aspartate, alanine, and glutamate metabolism; proline and arginine metabolism; and aminoacyl-tRNA biosynthesis. The results of this

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research enhance our understanding of the effects of dietary carbohydrate levels on the growth of juvenile *S. nudus*.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the MetaboLights repository, accession number MTBLS2887.

AUTHOR CONTRIBUTIONS

CY, QW, and YD designed the research. JH, YL, and RZ conducted the research. JH, CY, and QW analyzed the data. JH, RZ, CY, QW, YL, and YD contributed to the final writing of the manuscript. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2021.702101/full#supplementary-material>

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Conflict of Interest: Data preprocessing and annotation of metabolomics analysis were assisted by Biotree Biotech Co., Ltd. (Shanghai, China).

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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