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Metabolomics analysis for skin ulceration syndrome of *Apostichopus japonicus* based on UPLC/Q-TOF MS*

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Abstract Skin ulceration syndrome (SUS) is the main diseases affected the development of sea cucumber (*Apostichopus japonicus*) culture industries. To better observe the changes in the sea cucumber *A. japonicus* with SUS and understand the pathogenesis of the disease, activities of superoxide dismutase (SOD), catalase (CAT), and level of malondialdehyde (MDA) in coelomic fluid were detected using the Assay Kit and metabolites in the body wall were assessed using ultra-performance liquid chromatography and quadrupole-time of flight mass spectrometry. The results indicated that level of MDA was increased during SUS compared with healthy individuals (P<0.01), but activities of SOD and CAT were reduced (P<0.05). In metabolomics analysis, metabolites, such as adenosine, choline, betaine aldehyde, palmitic acid, and taurine, were found to be upregulated and 2-oxoadipic acid, anthranilic acid (vitamin L1), thioetheramide-PC, cholesterol-3-sulfate, and pentadecanoic acid were downregulated (VIP>1 and P<0.1). Pathway enrichment analysis indicated most enrichment of KEGG pathways were mainly related to energy metabolism, immunity, and osmoregulation such as ABC transporters, glycine, serine and threonine metabolism, tryptophan metabolism and neuroactive ligand-receptor interaction. Our study reflected the difference in enzyme activity and metabolites between *A. japonicus* with SUS and those without, which will provide reference data for investigating SUS.

Keyword: *Apostichopus japonicus*; skin ulceration syndrome (SUS); metabolome; differential metabolites

1 INTRODUCTION

Sea cucumber (*Apostichopus japonicus*) is an important aquaculture species, with high nutritional and medicinal value. Since the 1980s, aquaculture of sea cucumber has rapidly developed, becoming one of the most important aquaculture species in China (Yu et al., 2014). In China, the annual production of sea cucumber was up to 1.74×10^5 t in 2018 (Zhang et al., 2019), but diseases caused by bacteria, virus, and protozoa bring enormous economic loss to aquaculture, which is one of the major factors limiting the productivity and development (Li et al., 2012). In addition, skin ulceration syndrome (SUS) is the most serious of all diseases due to its high infectivity and mortality of 90%–100% (Deng et al., 2008). *Vibrio splendidus* is a widely recognized pathogen responsible for SUS outbreaks (Liu et al., 2010). Therefore, *V. splendidus* was used in this study. Currently, SUS in *A. japonicus* has been investigated from multiple aspects. For example, Zhang et al. (2013) studied SUS related miRNA targets using a transcription technique and Lv et al. (2019) analyzed SUS with proteomics. To fully understand the characteristics and pathogenesis of the disease, *A. japonicus* with SUS were analyzed using metabolomics in this study.

Metabonomics is a qualitative and quantitative analysis of all metabolites in the whole organism, tissue, or single cell to find target differential

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metabolites under specific physiological cycles or conditions (Yan et al., 2019). There have been multiple studies using metabolomics. For example, Wikoff et al. (2009) analyzed the influence of intestinal flora on blood metabolites in mammals using metabolomics. Sabatine et al. (2005) used metabolomics to find labeled metabolites for myocardial ischemia disease. Metabolomics has been applied in the investigation of *A. japonicus*, including metabolic responses to stresses such as high temperature and hypoxia (Huo et al., 2019) and heat stress on the intestinal tract (Xu et al., 2017).

Recently, ultra-performance liquid chromatography (UPLC) and quadrupole-time of flight (Q-TOF) mass spectrometry (MS) has been widely applied in metabolomics studies due to its high sensitivity, good repeatability, high limit of detection, handling capacity, and chromatographic resolution. For example, Wang et al. (2008b) analyzed the constituents in the rat plasma after oral administration with *Yin Chen Hao Tang* (a classical traditional chinese medicine formula), Yin et al. (2006) investigated metabolomics in the intestinal fistula, Gu et al. (2019) determined the progestin residues in fish by UPLC/Q-TOF MS. In the present study, we investigated SUS in the sea cucumber *A. japonicus*, by assessing metabolites in the body wall using UPLC/Q-TOF MS and using the Assay Kit to detect the activities of superoxide dismutase (SOD), catalase (CAT), and level of malondialdehyde (MDA) in coelomic fluid. The results will help us to understand SUS, and provide reference data for investigating its characteristics and pathogenesis.

2 METHOD

2.1 Ethics statement

The sea cucumbers *A. japonicus* here are commercially cultured animals. All the works were conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

2.2 Animal

Adult sea cucumber *A. japonicus* individuals (weight: 96.91±10.56 g) were collected from Dalian Xinyulong Aquaculture Company (39°20′N, 122°20′E), Liaoning, China in May 2019. Forty sea cucumbers were acclimatized in two tanks (200 cm× 50 cm×50 cm) with aerated, filtered water (16 °C, salinity 30) for 7 days before use and were fed a commercial diet (Peng Anyuan Marine Food Co., Ltd., Yantai, China) once a day. Uneaten feed was removed daily during the acclimation and experimental periods. The acclimatized animals were then subjected to artificial infection.

2.3 Artificial infection and sample collection

Vibrio splendidus used in infection experiments was obtained from the Key Laboratory of Mariculture & Stock Enhancement in Northern China Sea, Ministry of Agriculture and Rural Affairs, Dalian Ocean University. The bacteria were cultivated in 2216E liquid medium (Tryptone 5 g/L, yeast extract 1 g/L, $C_6H_5Fe{\cdot}5H_2O$ 0.1 g/L, pH 7.6) with 150 r/min at 28 °C for 24 h and centrifuged at 5 000×*g* for 5 min to harvest the bacteria. For the challenge experiment, live bacteria were re-suspended in filtered seawater and adjusted to 1×10^{10} CFU/mL (The data obtained were statistically evaluated with Probit Analysis method (SPSS 20.0) and the 50% lethal concentration (LC50; 10 d; 0.2 mL) for sea cucumber *Apostichopus japonicus* exposed to the *V. splendidus*, was 4.35×10^{9} CFU/mL in our pretest.). After 7 days of acclimation, all animals were randomly divided into the normal group and the SUS group with 20 sea cucumbers in each group. And the normal group was injected with 0.2-mL 0.9% saline and the SUS group was injected with 0.2-mL 1×10^{10} CFU/mL of *V. splendidus*. 36 h after the injection, the animals shook their heads and their mouths swelled. Three days after the infection, most animals showed small patches of ulcer. A week after the infection, all the animals exhibited several deep and extensive white ulceration and displayed evisceration. We selected eight sea cucumbers with white skin ulceration and eight healthy sea cucumbers for further experiments. According to previous data (Jiang et al., 2009; Yang et al., 2016), white skin ulceration in sea cucumbers was considered the most important marker in identifying SUS and the coelomic fluid of sea cucumber is the main site of immune response. For each sampled individual, the coelomic fluid was collected immediately, split into five 2-mL tubes and one tube stored at -80 °C for osmolarity measuring. The remaining coelomic fluid was centrifuged at 800×*g* for 10 min at 4 °C, the resulting supernatant was collected and used as coelomic fluid supernatant for detecting SOD, CAT activities and MDA content (Jiang et al., 2017). Some pieces of body wall (3 g in all) were separated and stored at -80 °C before metabolite extraction.

2.4 SOD and CAT activities and MDA content

The coelomic fluid supernatant was taken at -80 °C and then dissolved at 4 °C. After dissolving at 4 °C, the activities of SOD, CAT, and MDA were detected using an Epoch microplate reader (BioTek, Winooski, VT, USA) with the SOD Assay Kit (A001-3-2, Jiancheng, Nanjing, China), CAT Assay Kit (A007-1-1, Jiancheng, Nanjing, China), and MDA Assay Kit (A003-1-2, Jiancheng, Nanjing, China) (Wang et al., 2020; Zhou et al., 2020).

The SOD activity was assayed in water-soluble tetrazolium-1 (WST-1) method. One unit of SOD activity was defined as the amount of enzyme required for 1-mg tissue proteins in 1 mL of a reaction mixture SOD inhibition rates to 50% as monitored at 450 nm. CAT activity was admeasured by using ammonium molybdate colorimetric method, which was measured at 405 nm. One unit of CAT activity was defined as 1-mg tissue proteins consumed 1-μmol H_2O_2 per second. We used thiobarbituric acid (TBA) method to determine MDA content at 532 nm.

2.5 Osmolarity measurements

After the coelomic fluid thawed at 4 °C, 50 μL of coelomic fluid was taken into a centrifuge tube. Osmomat 030 (Gonotec, Berlin, Germany) was used to detect the osmotic pressure of the sample. Each sample was measured three times and the average value was taken. One unit of osmotic pressure was defined as 1-mmol solute is dissolved in 1-kg solvent.

2.6 Sample preparation for metabolomic analysis

Samples were taken at -80 °C and then 80 mg of every sample was weighed, homogenized in 200-μL water using a FastPrep-24 5G homogenizer (MP, USA). After vortexing, 800 μL of methanol/acetonitrile (1∶1, v/v) was added to the samples. The samples were subjected twice to 30 min cryogenic sonication treatment followed by vortexing for 60 s. Then let it stand for 1 h at -20 °C and centrifuged at 14 000×*g* for 20 min at 4 °C in a 5430R ultracentrifuge (Eppendorf, Germany) to obtain the supernatant. The supernatant was freeze-dried and stored at -80 °C before further analysis.

2.7 UHPLC-Q-TOF MS spectrometry analysis

The metabolic profiling analysis of the tissues was conducted on an Agilent 1290 Infinity UPLC system (Agilent, USA) with an ACQUITY UPLC BEH Amide column (1.7 μm, 2.1 mm×100 mm) under constant flow rate of 0.3 mL/min. The column temperature was set to 25 °C. The mobile phase consisted of aqueous ammonium acetate (25 mmol/L)/ammonia (25 mmol/L) (A) and acetonitrile (B). The gradient elution was: 0–1 min, 95% B; 1–14 min, 95–65% B; 14–16 min, 65%–40% B; 16–18 min, 40% B; 18–18.1 min, 40%–95% B; and 18.1–23 min, 95% B. A Triple TOF 5600 mass spectrometer (AB SCIEX, USA) was used to detect the metabolites eluted from the column, and the MS was performed to analyze the supernatant in positive and negative ion mode. The electrospray ionization (ESI) source operation parameters were: ion source gas1 (Gas1): 60, ion source gas2 (Gas2): 60, curtain gas (CUR): 30, source temperature: 600 °C, ion spray voltage floating (ISVF) 60, curtain gas (CUR): 30, source temperature: 600. The electrospray: 1 000 Da, product ion scan *m/z* range: 25–1 000 Da, TOF MS scan accumulation time 0.20 s/spectra, product ion scan accumulation time 0.05 s/spectra. MS/MS data were obtained from an information-dependent acquisition (IDA), operating in a high-sensitivity mode. Declustering potential (DP): ±60 V (positive and negative ion mode), collision energy: 35±15 eV, and exclude isotopes within 4 Da, candidate ions to monitor per cycle: 6 was used in information-dependent acquisition.

All MS data were conducted with three analytical replications (Zhao et al., 2017).

2.8 Data processing

The initial MS data were converted into extensible markup language (mzXML) format using the ProteoWizard, and then the xml cryptographic message syntax (XCMS) program was used for peak alignment, retention time correction, and peak area extraction. The method of MS/MS data matching and accurate mass matching (<25×10^{-6}) to retrieve a self-built database from the laboratory was used to identify and authenticate the metabolite structure.

The analysis methods included the principal component analysis (PCA) and orthogonal partial least-squares discriminant analysis (OPLS-DA). All data were subject to preprocessing by Pareto scaling before analyses (Yan et al., 2019). The online databases Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/pathway.html) (Kanehisa et al., 2015) were used to identify possible metabolic pathways.

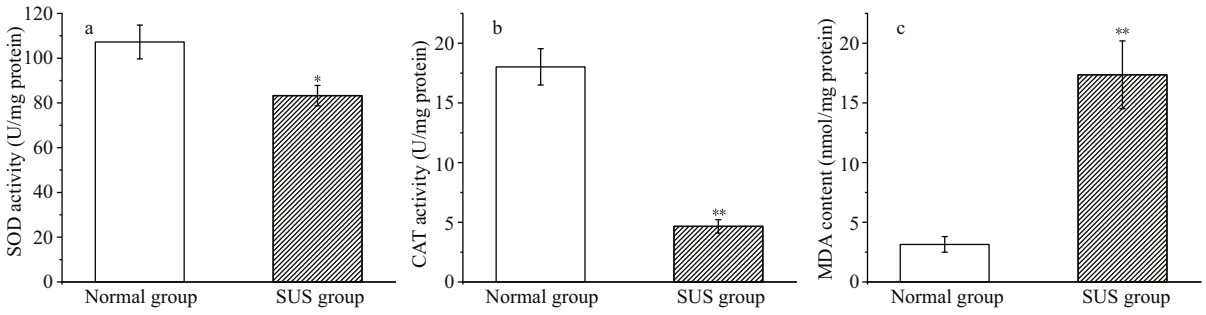

Fig.1 Influence of SUS on SOD activity (a), CAT activity (b) and MDA content (c) in coelomic fluid of *A. japonicas*

Compared with normal group, * indicates significant differences (P<0.05), ** indicates highly significant differences (P<0.01).

2.9 Statistical analysis

For the metabolomics analysis, Student's *t*-test was conducted to select the differential metabolites based on the variable importance in the projection (VIP) values>1 and the *P*-value<0.1 (Hao et al., 2018). For the SOD, CAT activities and MDA content analyses, all samples were analyzed and data were averaged, and the results are expressed as the mean±SD. Significant differences (P<0.05) and highly significant differences (P<0.01) for each variable were detected using Student's *t*-test. Statistical analysis was performed using Excel (Microsoft, USA) and SPSS 20.0 (IBM, USA).

3 RESULT

3.1 Activities of SOD and CAT, and level of MDA

Activities of CAT and SOD, MDA content in coelomic fluid were detected in the normal and SUS group. As shown in Fig.1, the activities of CAT and SOD were reduced (P<0.05) compared with the normal group but the level of MDA was increased (P<0.01).

3.2 Osmolarity value

There were significant differences in the osmolarity values of coelomic fluid collected from two groups of the study sea cucumbers (Fig.2). Differences were also detected following Student's *t*-test (P<0.01).

3.3 Analysis of the body wall metabolites using UHPLC-Q-TOF/MS

The total ion chromatography (TIC) results of the normal and SUS groups are presented in Fig.3. In total, 579 metabolites were identified, and some differences in terms of peaks heights were observed between two groups. The differences in the metabolites of the body wall can be discriminated by the pattern recognition method, such as PCA and OPLS-DA

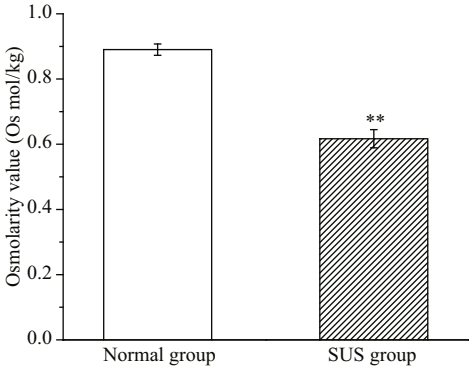

Fig.2 The osmolarity values (Os mol/kg) for two groups of the sea cucumber

Compared with normal group, ** indicates highly significant differences (P<0.01).

models. As shown in Fig.4b, the OPLS-DA scores plot for all groups is excellently clustered and grouped without any overlap. R^2X, R^2Y, and Q^2 are 0.494 cum, 0.991 cum, and 0.604 cum. In permutation testing (Fig.4c), the values of R^2 and Q^2 were 0.888 6 and -0.137 5, indicating good repeatability and predictability of the model.

3.4 Screening of the differential metabolites

A total of 10 differential metabolites were finally detected (VIP>1 and P<0.1; Fig.5 & Table 1) , the levels of metabolites, including adenosine, choline, betaine aldehyde, palmitic acid, and taurine were upregulated, compared with the normal group, whereas, 2-oxoadipic acid, anthranilic acid (Vitamin L1), thioetheramide-PC, cholesterol 3-sulfate, and pentadecanoic acid were down-regulated.

3.5 Enrichment pathway of differential metabolites

The screened differential metabolites were input into the KEGG database, and KEGG pathway enrichment analysis was performed via Fisher's precise test. All enrichment pathways are illustrated in Fig.6. Moreover, the enrichment of KEGG

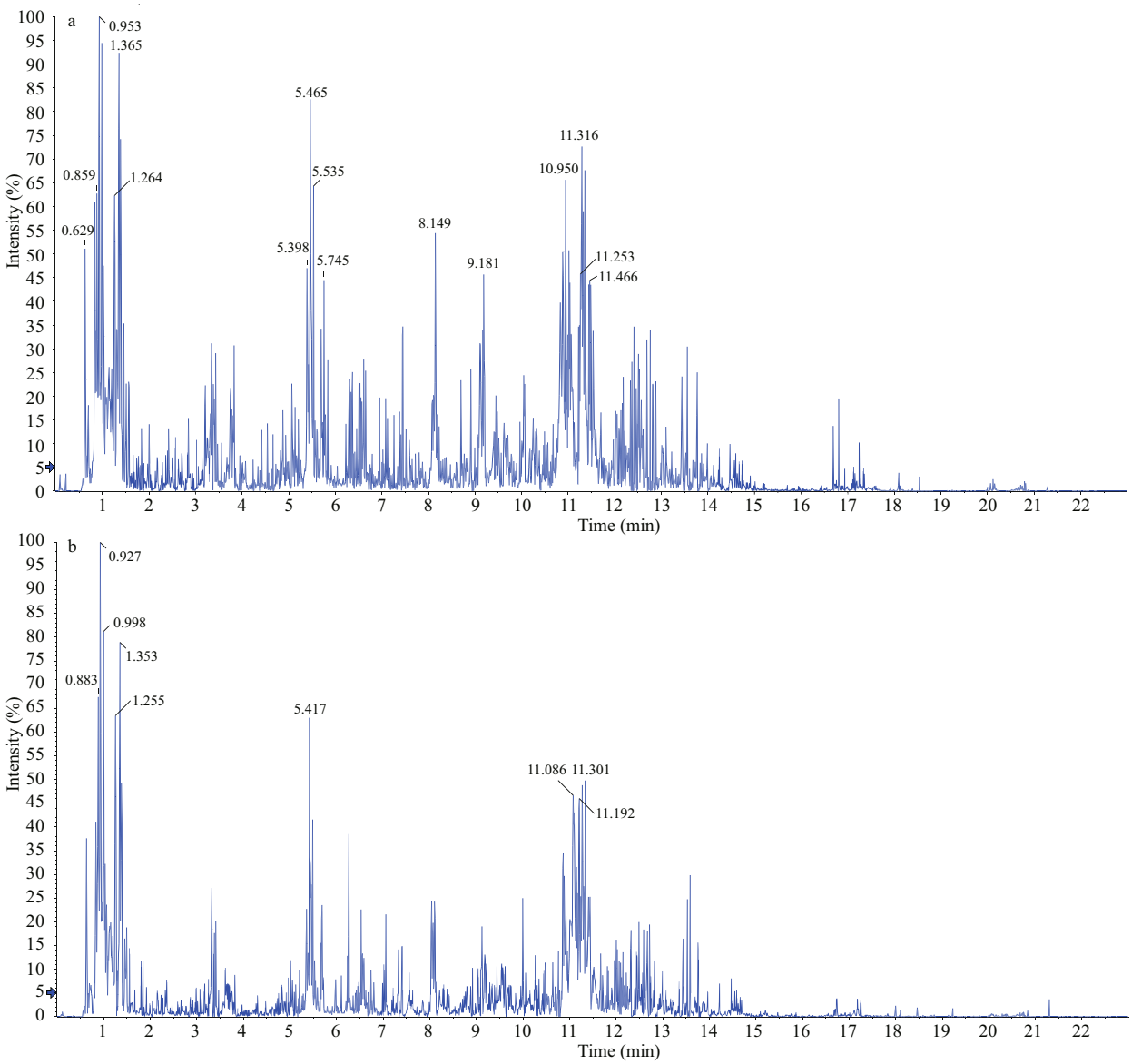

Fig.3 Total ion current (TIC) chromatograms of the samples

a. normal group; b. skin ulceration syndrome group.

Table 1 Differential metabolites between two groups

	Adduct	Description	VIP-value	Fold change	*P*-value	*m/z*	rt (s)	Variation trend
ESI-	(M-H)-	Cholesterol 3-sulfate	3.658 639	0.483 451	0.004 869	465.305 6	64.328	↓
	(M-H_2O-H)-	2-oxoadipic acid	33.213 715	0.573 283	0.009 766	141.016 9	614.925	↓
	(M-H)-	Palmitic acid	3.592 334	1.915 237	0.020 445	255.233 4	181.078	↑
	(M-H)-	Pentadecanoic acid	2.457 688	0.667 988	0.069 602	241.217 9	75.514	↓
ESI+	M+	Choline	8.581 632	6.912 428	0.000 511	104.106 2	476.263	↑
	(M+H)+	Anthranilic acid (Vitamin L1)	4.322 050	0.327 407	0.001 416	138.054 4	582.055	↓
	(M+H)+	Betaine aldehyde	4.738 921	5.527 293	0.008 677	102.090 6	655.628	↑
	(M+H)+	Taurine	2.159 773	1.744 736	0.058 615	126.021 2	558.934	↑
	(M+H-H_2O)+	Adenosine	1.262 519	3.661 323	0.091 357	250.092 5	160.738	↑
	(M-H+2Na)+	Thioetheramide-PC	4.675 371	0.484 850	0.098 385	780.552 1	210.225	↓

Compared with normal group, ↑ indicates upregulated metabolites and ↓ indicates downregulated metabolites.

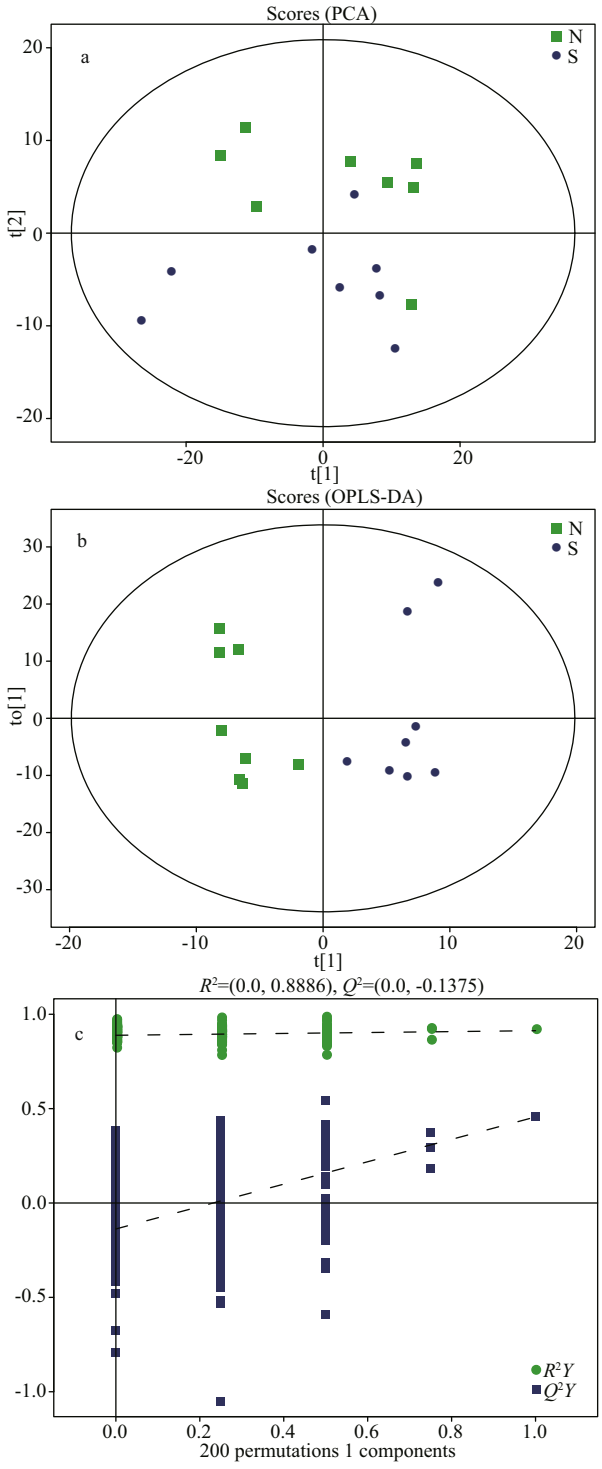

Fig.4 PCA (a) and OPLS-DA (b) score plots of normal group (square, green), skin ulceration syndrome group (round, blue), permutation test of OPLS-DA model (c)

pathways was mainly related to amino acid metabolism (glycine, serine, and threonine metabolism, Tryptophan metabolism and Taurine and hypotaurine metabolism) between two groups.

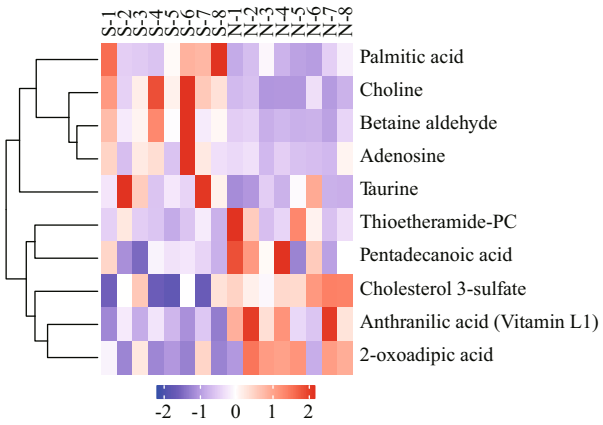

Fig.5 Hierarchical clustering analysis for SDMs

The relative metabolite level is depicted according to color scale. Red indicates upregulation, and blue indicates downregulation. The S represents SUS group, and the N represents normal group.

4 DISCUSSION

4.1 Analysis of SOD and CAT activity and level of MDA

It has been reported that sea cucumber recognizes, inhibits, kills, and eliminates pathogens by immune factors in the organism (mainly enzymes) due to the lack of a specific immune system (Wang et al., 2008a).

SOD and CAT are important antioxidant enzymes in invertebrates (Roch, 1999). In this experiment, the activities of SOD and CAT in the SUS group were significantly reduced compared to the normal (P<0.05). Lv et al. (2019) analyzed *A. japonicus* with SUS using proteomics and also found that levels of the two enzymes were downregulated. It indicated that the effects of SOD and CAT in sea cucumber with SUS were blocked, the capability of scavenging free radicals declined, and the defense response was reduced, which might be detrimental to the organism.

MDA can damage the function of tissues and cells, indicating lipid peroxidation and impaired cell function (Cossu et al., 2000). In this study, the MDA level was significantly increased in the SUS group compared with the normal group (P<0.01), which was the same as the change of MDA level when *A. japonicus* is under hypoxic stress (Huo et al., 2018). The results demonstrated that lipid peroxidation in *A. japonicus* with SUS increases with severe cell injury.

Overall, the activities of CAT and SOD were reduced (P<0.05) compared with the normal group but the level of MDA was increased (P<0.01). These results indicated that SUS changed the non-specific immunity of the organism.

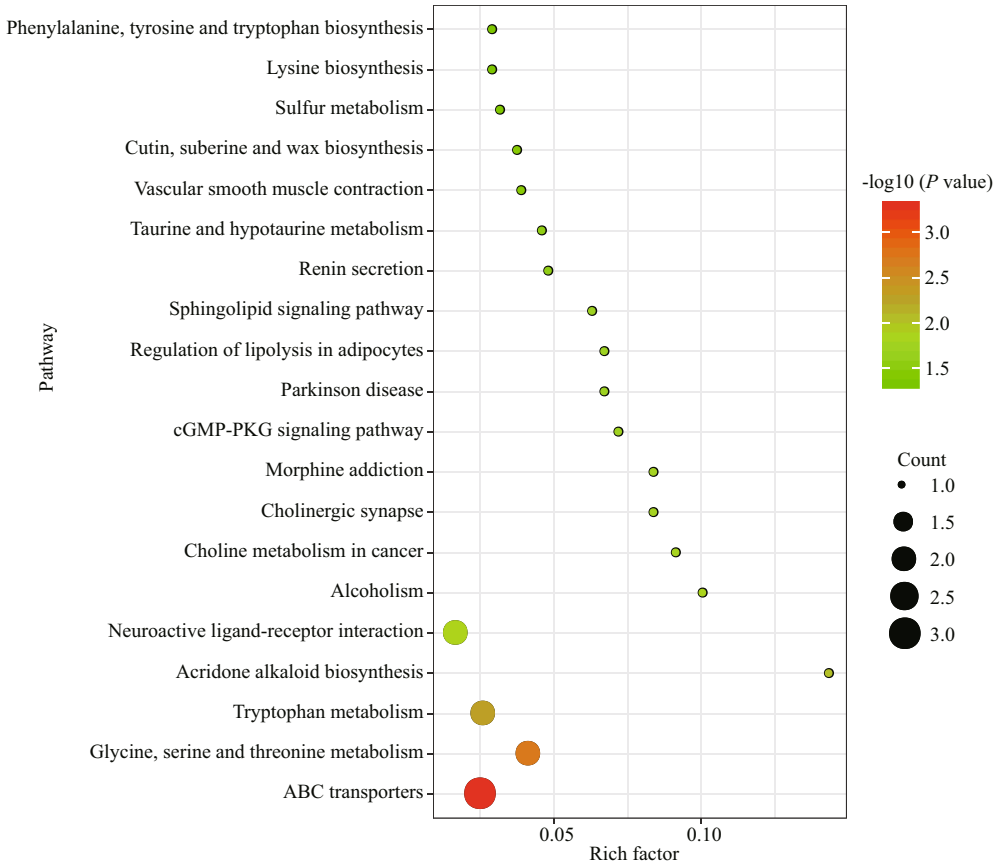

Fig.6 KEGG pathway enrichment analysis of differential metabolites between two groups

4.2 Analysis of differential metabolites

Adenosine can prevent or reduce the increased permeability of the endothelial monolayer, and further alleviate the damage to function by oxidation (Richard et al., 1998). Adenosine has been reported to alleviate injury in multiple types of animal (Lasley et al., 1990; Baldissera et al., 2018). In this study, sea cucumbers in the SUS group showed some white speaks of skin ulceration. It can be inferred that the adenosine level was upregulated (VIP>1 and P<0.1) in the SUS group in order to alleviate cell injury caused by SUS.

Glycine betaine is an organic penetrant synthesized by a two-step reaction from choline: choline → betaine aldehyde + nicotinamide adenine dinucleotide (NAD+) + → glycine betaine + dihydronicotinamide adenine dinucleotide (NADH), which plays a role in regulating osmotic equilibrium in marine invertebrates (Perrino and Pierce, 2000). Taurine is also an organic penetrant. In previous study, *V. splendidus* has been reported to cause osmoregulatory change in *Ruditapes philippinesis* (Liu et al., 2013). As showed in Fig.2, the osmolarity values of coelomic fluid collected from two groups were significantly different (P<0.01). Therefore we propose that the levels of choline, betaine aldehyde, and taurine in *A. japonicus* were upregulated (VIP>1 and P<0.1) in the SUS group compared with the normal group, causing osmoregulatory change in *A. japonicus*.

Palmitic acid has been found to cause oxidative stress in cells via damage to the normal function of mitochondrial oxidative respiratory chain (Win et al., 2015). It can be also used as an efficient proinflammatory factor to induce an inflammation-related cascade reaction (Pillon et al., 2015; Ma et al., 2018). It was reported that there was a change in the proinflammatory response and expression of immunity-related genes in SUS (Zhang et al., 2013). Thus, upregulation of palmitic acid in the SUS group might lead to an inflammatory response and oxidative stress, damaging the organism and influencing immunocompetence. The 2-oxoadipic acid is a metabolite of tryptophan decomposition (Shibata et al., 2011). Anthranilic acid (Vitamin L1) plays a very important role in the biosynthesis of tryptophan (Wiklund and Bergman, 2006). Tryptophan, as a functional essential amino acid, plays important roles in enhancing anti-oxidative and immune functions in

animals (Li et al., 2016). In this study, the levels of 2-oxoadipic acid and anthranilic acid (Vitamin L1) in the SUS group were downregulated (VIP>1 and P<0.1) compared to those in the normal group, which might influence anti-oxidative and immune functions by influencing tryptophan metabolism. Moreover, the changes of SOD, CAT activities and MDA content supports these speculations, compared with normal group, the immunocompetence of SUS group has changed. Based on available data, pentadecanoic acid can influence energy metabolism (Pfeuffer and Jaudszus, 2016). The change of energy metabolism in sea cucumber with SUS was proven (Shao et al., 2013; Zhao et al., 2017). We infer the level of pentadecanoic acid downregulated is one cause for the change of energy metabolism.

Low concentrations thioetheramide-PC can activate phospholipase A2 (sPLA2) activity (Xie et al., 2005). Recent studies suggest, sPLA2 promote several inflammatory diseases (Mallat et al., 2010) and the increase of sPLA2 was observed in aquatic organisms with *Aeromonas hydrophila* (Hu et al., 2005). The down of thioetheramide-PC may promote SUS through sPLA2 activity. The cholesterol-3-sulfate is a natural steroid and the steroid from marine echinoderms has antiviral effect (Roccatagliata et al., 1996). It can be inferred that the cholesterol-3-sulfate may also has antiviral effect in sea cucumber and the down of cholesterol-3-sulfate will promote SUS.

4.3 Metabolic pathway analysis

In this work, we used KEGG annotation for performing enrichment analyses to investigate the functional distribution of identified metabolites and the significant enrichment results of KEGG pathway were related mainly to energy metabolism, immunity, and osmoregulation between two groups.

ABC transporters and neuroactive ligand-receptor interaction were the pathways of environmental information processing. ATP-binding cassette (ABC) transporters are a large group of membrane proteins that couple transport of a substrate against a chemical gradient and energized directly by the hydrolysis of ATP (Borths et al., 2002; Kathawala et al., 2015). ABC transporters play an important role in protective function by reducing the accumulation of toxins in the body (Ejendal and Hrycyna, 2002). Studies shown that aquatic animals can efflux harmful substances out of body in time and reduce the body damage by ABC transporters (Zhou et al., 2009; Chang et al., 2012). Therefore, we infer that sea cucumber may also use ABC transporters to efflux *V. splendidus* out of body, which reduce the body damage. Neuroactive ligand-receptor interaction pathway is a signal transduction pathway composed of all ligands and receptors in cell membrane (Lauss et al., 2007). It was reported that *Eriocheir sinensis* with *Spiroplasma eriocheiris* infection characterized by trembling was associated with the neuroactive ligand-receptor interaction pathway (Wang et al., 2017). Head-shaking is one of the symptoms of sea cucumber with SUS (Zhang et al., 2006). Based on these reports, we speculate that the neuroactive ligand-receptor interaction pathway may be responsible for the sea cucumber sharks head during SUS. However, the detailed regulation role of this pathway needs to be elucidated in the future.

Glycine, serine and threonine metabolism and tryptophan metabolism were the pathways of amino acid metabolism. In marine invertebrates, amino acid is regarded as a main participant regulating energy metabolism and osmotic pressure (Viant et al., 2003). Generally, high concentrations of amino acids can be used to regulate osmotic pressure as a penetrant. When the invertebrates are exposed to various types of stress, the energy requirement is increased, which can be met by the energy generated by the oxidation of amino acids (Lv et al., 2019). This indicates that energy metabolism and regulatory capability of osmotic pressure in sea cucumber with SUS may change, which is consistent with current studies (Shao et al., 2013; Zhao et al., 2017).

5 CONCLUSION

The activities of SOD and CAT and the level of MDA in coelomic fluid in the SUS group and normal group were detected. Results indicate that the level of MDA in the SUS group was significantly increased (P<0.01), but the activities of SOD and CAT were significantly reduced (P<0.05). In addition, the non-specific immunity was changed in the sea cucumber with SUS. Levels of metabolites, such as adenosine, choline, betaine aldehyde, palmitic acid, and taurine, were upregulated. However, 2-oxoadipic acid, anthranilic acid (vitamin L1), thioetheramide-PC, cholesterol-3-sulfate, and pentadecanoic acid were downregulated (VIP>1 and P<0.1). These different metabolites are related mainly to alleviating injury, regulating osmotic pressure, and influencing immunocompetence. KEGG pathway enrichment analysis showed most enrichment of KEGG pathways are related t energy metabolism, immunity, and osmoregulation. There were significant differences in

the osmolarity values of coelomic fluid between the two groups. Therefore, this study indicates that energy metabolism, immunologic function and osmotic pressure regulation change in *A. japonicus* with SUS. Results of this study could help understand the characteristics of SUS, and provide a reference for further investigations.

6 DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this article.

7 CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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