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EDITED BY
Wei Huang,
Ministry of Natural Resources, China

REVIEWED BY
Li Li,
Ocean University of China, China
Chenghua Li,
Ningbo University, China

*CORRESPONDENCE
Songchong Lu
luschvip@outlook.com
Libin Zhang
zhanglibin@qdio.ac.cn

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Seasonal variations in microbial diversity and metabolite profiles of the gut of sea cucumber (*Apostichopus japonicus*)

Beini Deng[1,2,3], Xiaoshang Ru[2,3,4,5,6], Ting Wang[2,3], Chenxi Zhang[2,3], Wanhui Sun[7], Songchong Lu[1]* and Libin Zhang[2,3,4,5,6]*

[1]College of Life Sciences, Qingdao Agricultural University, Qingdao, China, [2]CAS Key Laboratory of Marine Ecology and Environmental Sciences, Institute of Oceanology, Chinese Academy of Sciences, Qingdao, China, [3]CAS Engineering Laboratory for Marine Ranching, Institute of Oceanology, Chinese Academy of Sciences, Qingdao, China, [4]Laboratory for Marine Ecology and Environmental Science, Qingdao National Laboratory for Marine Science and Technology, Qingdao, China, [5]Center for Ocean Mega-Sciences, Chinese Academy of Sciences, Qingdao, China, [6]Shandong Province Key Laboratory of Experimental Marine Biology, Qingdao, China, [7]Laizhou Municipal Bureau of Marine Development And Fisheries, Yantai, China

The sea cucumber (*Apostichopus japonicus*) is the main economic species in China and has a significant role in aquaculture. Gut microbiome composition is closely related to external environments. In this study, we identified the effects of seasonal changes on the composition and main metabolites of symbiotic microorganisms in the intestine of *A. japonicus*. We used 16S rRNA sequencing to identify the composition of symbiotic microorganisms in different seasons. Intestinal metabolites were determined using liquid chromatography with tandem mass spectrometry, which linked symbiotic microorganisms to intestinal metabolites. Analyzing changes in intestinal microbial composition across different seasons. The results showed that seasonal changes of intestinal microorganisms were significant, *A. japonicus* were infected by *Vibrio* easily in summer, Stigmasterol and sitosterol could affect the growth of body wall of *A. japonicus*. It is vital importance for *A. japonicus* that the results benefit for the growth, immunity and aquaculture.

KEYWORDS

16S rRNA, gut microbiome, metabolomics, sea cucumber (*Apostichopus japonicus*), seasonal variations

Introduction

The sea cucumber (*Apostichopus japonicus*) is highly favored by Asian and Chinese populations due to its unique flavor and taste. The aquaculture of sea cucumbers has significantly increased in recent years, and has become a major aquaculture species in China, generating huge revenues for the aquaculture industry (Ru et al., 2019). Sea cucumbers mainly ingest sediment to extract organic matter and microorganisms (Xu et al., 2015; Mohsen et al., 2018) and are colloquially known as "Ocean scavengers", therefore, they have crucial roles in marine ecosystems. The diet of sea cucumber is closely related to the variation of environment, so, the intestinal contents of sea cucumbers can reflect ecosystem characteristics and environmental changes.

Sea cucumber activities are closely related to the seasons. As a temperate species, spring and autumn are fast-growing seasons when the water temperature is 10–20°C. Thus, feeding rates increase and are concomitant with increased body weight. Aestivation begins when the water temperature reaches 20.0–24.5°C. During aestivation, sea cucumbers stop eating and intestinal degeneration occurs and activities slow down (Mitsukuri, 1903; Tanaka, 1958a; Tanaka, 1958b; Yang et al., 2015). In autumn, water temperatures are decreased and feeding activity returns to normal. In winter, water temperatures are very low and activity, growth, and metabolism of sea cucumber are decreased.

Intestinal microbiotas are associated with all aspects of host health, including metabolism and digestion, the immune system, and even behavior, while disorders or abnormal intestinal microbiota development are related to different gastrointestinal and metabolic diseases (Spor et al., 2011). The gut houses an enormous variety of microbial communities with important roles in host metabolism and immunity (Egerton et al., 2018). The gut microbiome is essential for host health and physiological functions and has important roles enhancing host digestion, regulating gut endocrine functions and neurological signaling, and maintaining host reproductive capacity (Lynch and Pedersen, 2016; Fan and Pedersen, 2021). In particular, the microbiome performs functions that the host cannot perform, including the production and regulation of metabolites which are used as metabolic substrates and signaling molecules, with major impact on host metabolism and health (Schoeler and Caesar, 2019). Thus, gut microbiome variations may lead to metabolite alterations, such as metabolites are the end products of many cell regulatory processes, significant microbiome alterations can impact responses to the external environment (Fiehn, 2020; Xing et al., 2021). Thus, joint microbiome and metabolome studies are required to evaluate and characterize host-microbiome interactions (Turnbaugh and Gordon, 2008; Visconti et al., 2019).

The gut microbiome is highly plastic and is altered throughout life by environmental factors (Goodrich et al., 2014; Goodrich et al., 2016; Xie et al., 2016; Visconti et al., 2019). Microorganisms in organism have critical roles; however, relatively few studies have focused on intestinal microorganism and metabolite links and their relationship with seasonal changes, therefore, we investigated sea cucumber microbiota and metabolism across different seasons using 16S rRNA and liquid chromatography with tandem mass spectrometry (LC-MS/MS). Additionally, using Kyoto Encyclopedia of Genes and Genomes (KEGG) resources, we analyzed different molecular pathways to explore metabolic differences. Our aims were to; (1) identify seasonal variations of intestinal microorganisms, (2) examine the effects of seasonal variations of sea cucumber microbiota and metabolite on immune of sea cucumbers, (3) explore the effects of seasonal variations of sea cucumber microbiota and metabolite on growth of sea cucumbers.

Materials and method

Experimental animals

Sea cucumbers were collected from Rongcheng Swan Lake (Weihai City, Shandong Province, China, 122°59'E, 37°36'N) in November, 2019 (autumn, the seawater temperature was 16.8° C); January, 2020 (winter, the seawater temperature was 1.6°C); April, 2020 (spring, the seawater temperature was 14.1°C); and June, 2020 (summer, the seawater temperature was 23.2°C). Sea cucumbers were weighed approximately 150–200 g. Sea cucumbers were euthanized by decollation, the whole sampling process was aseptic. The intestinal contents from ten individuals, during each season, (40 individuals in total), were used for sampling, 16S rRNA sequencing, and metabolomic analysis. Animals were frozen in liquid nitrogen and stored at −80°C.

Microbial *16S rRNA* sequencing and analysis

Microbial DNA was extracted using HiPure Stool DNA Kits (Magen, Guangzhou, China) according to manufacturer's protocols. The *16S rDNA* hypervariable V3–V4 region was amplified by polymerase chain reaction (PCR) (95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 60°C for 45 s, and 72°C for 90 s, with a final extension of 72°C for 10 min) using primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 806 R (5'-GGACTACHVGGGTATCTAAT-3'). PCR was performed in 50 μL reaction volumes and contained TransGen High-Fidelity PCR SuperMix (TransGen Biotech, Beijing, China), 0.2 μM forward and reverse primers, and 5 ng template DNA.

Clean tags were clustered into operational taxonomic units (OTUs) at ≥ 97% similarity using the UPARSE pipeline (version 9.2.64). Chimeric tags were removed using the UCHIME algorithm to generate effective tags for further analysis. Tag

sequences with the highest abundance were selected. Taxonomic classifications were performed in BLAST (version 2.6.0) (Altschul et al., 1990) and representative OTU sequences were searched against the National Center for Biotechnology Information 16S ribosomal RNA database (http://www.ncbi.nlm.nih.gov) (version 202101) for cluster analysis.

Metabolite profiling

Approximately 50 mg intestinal contents were weighed into a clean microtube. After, 1000 μL extract solvent (acetonitrile-methanol-water in a 2:2:1) was added, the sample was vortexed for 30 s, homogenized at 45 Hz for 4 min, and sonicated for 5 min in an ice-water bath. The homogenate and sonicate circle were repeated three times, followed by incubation for 1 hour at −20°C, and centrifugation at 12000 rpm for 15 min at 4°C. The supernatant was transferred to LC-MS/MS vials and stored at −80°C until MS analysis.

LC-MS/MS analyses were performed using an UHPLC system (1290, Agilent Technologies) on a UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 μm) coupled to a Q Exactive system (Orbitrap MS, Thermo). Mobile phase A comprised 0.1% formic acid in water for positive, and 5 mmol/L ammonium acetate in water for negative. Mobile phase B was acetonitrile. MS raw data (.raw) files were converted to mzML format using ProteoWizard, processed in R package XCMS (version 3.2), and included retention time alignment, peak detection, and peak matching. Data were then filtered using the following criterion: sample numbers contain a metabolite was less than 50% all sample numbers in a group. Normalization to an internal standard (Roberts et al., 2012) for each sample was also performed.

Statistical analysis

Microbiota analyses incorporated abundance, species, α-diversity indices and LEFSe. The abundance statistics of each taxonomy was visualized using Krona (Cheung et al., 2010) (version 2.6). Chao1 and Shannon indices were calculated in QIIME (Caporaso et al., 2010) (version 1.9.1). Species comparison between groups was calculated by welch's t-test in R project Vegan package (version 2.5.3). Biomarker features in each group were screened by LEFSe software (version 1.0), taking Linear Discriminant Analysis (LDA)>3 as a threshold.

For metabolomic analyses, we performed principal component analysis (PCA), analyzed KEGG pathways. T-tests were used as a univariate analysis for screening differential metabolites. P values from t-tests < 0.05 and VIP ≥ 1 were considered differential metabolites between two groups. Metabolites were mapped to KEGG pathways for pathway and enrichment analysis. The calculated *p*-value was gone through FDR correction, taking FDR ≤ 0.05 as a threshold. Pathways meeting these conditions were defined as significantly enriched for differential metabolites.

Results

Gut microbial profiles of *A. japonicus* across different seasons

Gut microbiota composition varied greatly with seasons. The dominant phyla were Planctomycetes and Proteobacteria, as the results shown, these phyla are complementary (Figures 1A, B). It's worth noting that it is higher than other seasons that the relative abundance of Cyanobacteria, Verrucomicrobia and Bacteroidetes in spring (Figure 1B). According to the PCA which revealed the relationship of intestinal microbes among different seasons, the results showed that the compositions of autumn and winter are similar, however, the compositions among spring, summer and autumn are different significantly (Figure 1C).

The statistics results showed that four phyla of intestinal microbiota were significantly different in spring and summer, and four phyla were significantly different in autumn and summer (Figures 2A, B). Notably, the mean abundance of Proteobacteria was higher in summer than in autumn. Thus, LEFSe analysis further on this phenomenon between summer and autumn. Then, we found that *Vibrio* populations spike in summer, significantly higher than in autumn (Figure 3).

Gut microbial diversity in different seasons

Alpha-diversity indices reflected the diversity and richness of symbiotic microbiota in each season, incorporated Chao1 and Shannon indices. Chao1 indices reflect species abundance in samples; thus, symbiotic microbiota abundance was poor in summer, (Figure 4A). Simpson indices reflect species richness, we can learn that the highest richness was in autumn, but the richness was relatively low in spring (Figure 4B). The phenomenon of richness has significant seasonal trends. It rises from spring to autumn, and then falls until the following spring.

Metabolite variations across different seasons

To further understand gut microbe metabolic interactions, LC-MS/MS was used for metabolic profiling. Among the 26,136 identified metabolites, 13,116 were tested in positive ion mode (POS) and 13,020 in negative ion mode (NEG). From PCA, we could learn that the relationships among seasons (Figure 5). It is

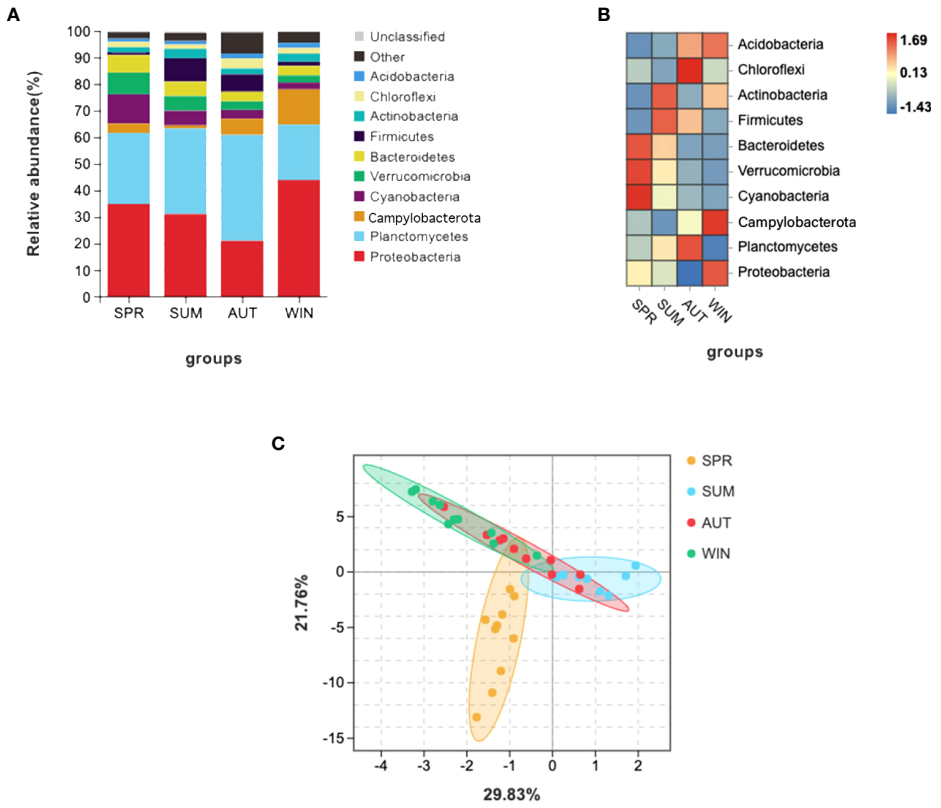

FIGURE 1
16S RNA summary and principal component analysis (PCA) of gut microbiota in *A. japonicus*. **(A)** Relative abundance (%) of the main phyla in the gut microbiota. **(B)** Heatmap showing the dynamic abundance of gut microbiota from *A. japonicus* across different seasons. **(C)** PCA of OTU levels.

interesting that these PCA results are similar with the PCA analysis of microbiota. The principal component of metabolites of autumn and winter are similar, however, the principal component of metabolites among spring, summer and autumn are different significantly.

Differential metabolites were further analyzed using KEGG annotations (VIP > 1, $P < 0.05$). We analyzed three secondary metabolic pathways: amino acid, carbohydrate, and lipid metabolism between summer and spring, and these pathway between summer and autumn (Figure 6). Our results showed that protein digestion and absorption exhibited the most significant differences between summer and autumn (Figure 6). Pathways of biosynthesis of unsaturated fat acids, primary bile acid biosynthesis, and mineral absorption were more significant, while sulfur metabolism exhibited the most significant differences between summer and spring (Figure 7). We also analyzed differences in metabolites among summer, spring, and autumn. Metabolites in summer were significantly higher than those in spring and autumn and mainly included benzene and substituted derivatives (phenylacetaldehyde, L-formylkynurenine, and phenylethylamine), steroids and steroid derivatives (cholesterol sulfate), carboxylic acids and derivatives (L-isoleucine), and glycerophospholipids (lysoPC).

Discussion

Variations in gut microbiota in *A. japonicus* across different seasons

According to the results, we find that the microbiota has seasonal variations in the gut of *A. japonicus*, and traits are significant. Variations of spring, summer and autumn are obviously different. According to Chao1 index, the richness in samples of species is lowest in summer and increased gradually from autumn. It is closely that this phenomenon related to the life history of *A. japonicus*. The reason is that *A. japonicus* entered aestivation in summer, the intestine degenerated, stop feeding, thus, the microbial contents in intestinal tract decrease sharply (Mitsukuri, 1903; Tanaka, 1958a; Tanaka, 1958b; Yang et al., 2015). In autumn, *A. japonicus* recovered feeding, thus, the abundance of gut microbiota increased. According to the

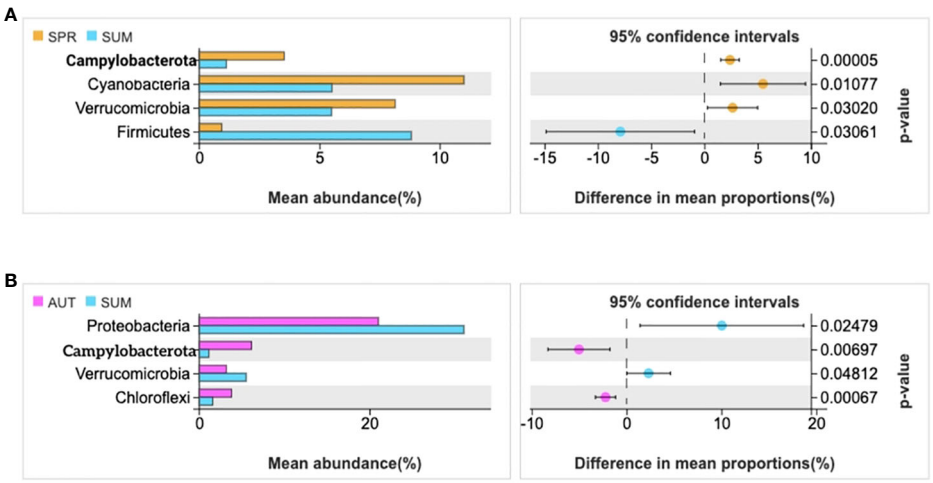

FIGURE 2
Statistical examination of intestinal microbial diversity of A. japonicus across different seasons ($P \leq 0.05$). **(A)** the statistical examination between spring and summer. **(B)** the statistical examination between summer and autumn.

Shannon index, the seasonal variations of intestinal microbial richness were more obvious, it gradually increased from spring to autumn and began to decrease after reaching its maximum in autumn. The change trend of Shannon index is opposite to Chao1, this is the same as the change trend of microorganisms in the environment. This trend maybe relates to the change of sea water nutrient. According to previous research in Weihai sea area (Shandong Province, China), the content of inorganic nitrogen (DIN) in summer is lower than in spring and autumn, DIN changes were positively correlated with Chao1 changes, the N/P of nutrients in autumn is higher than summer and spring (Fang et al., 2012), which is positively correlated with Shannon index. The change trend of microbe in the intestine of *A. japonicus* is consistent with the change of nutrient in the environment. Microbial richness in the gut of the sea cucumber was highest in the autumn, due to the high nutrient composition in seawater in autumn, which promoted the growth of a variety of microbiota (Bunse and Pinhassi, 2017).

From our analysis of intestinal microbial diversity across different seasons, we observed Proteobacteria maintained dominant positions. Proteobacteria is the largest bacterial flora population in aquaculture systems, and the most abundant phylum in the trench sediment and water samples (Herlambang et al., 2021). Thus, Proteobacteria of gut of *A. japonicus* may come from environment. Previous studies have shown that, Proteobacteria is also dominant bacteria in sea urchin (Hakim et al., 2016), and this bacterium has major roles in nitrogen and carbon cycles in aquaculture environments (Wiegand et al., 2018). According to previous research on the rice-fish farming system which investigated the effects of several classes of Proteobacteria in nutrient cycle, such as, Alphaproteobacteria members are involved in the nitrogen cycle (Newton et al., 2011), Gammaproteobacteria help modulate excess nitrate (Fernandes et al., 2014; Herlambang et al., 2021). At the same time, this bacterium is related to sulfur-related processes, from the study of metabolites, it can be found that the sulfur metabolism pathway is indeed subjected to some changes.

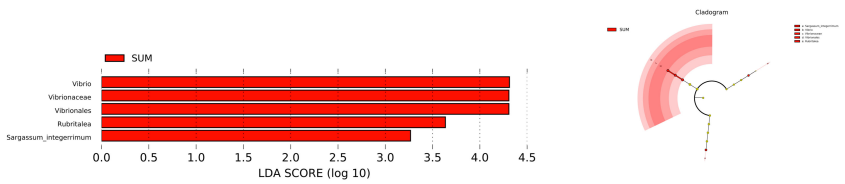

FIGURE 3
LEFSe analyzed the species of gut microbiota of A. japonicus between summer and autumn (LDA>3).

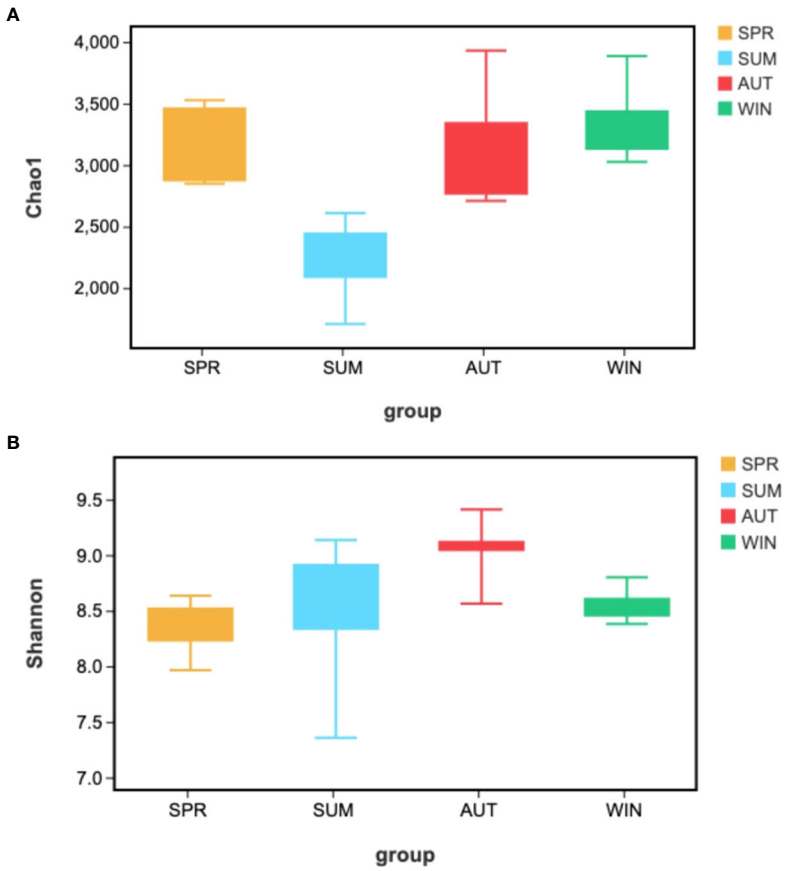

FIGURE 4
Kruskal-Walli's rank sum test analyzed Alpha-diversity difference analysis of gut microbiota from A. japonicus across different seasons by **(A)** Chao1, **(B)** Shannon.

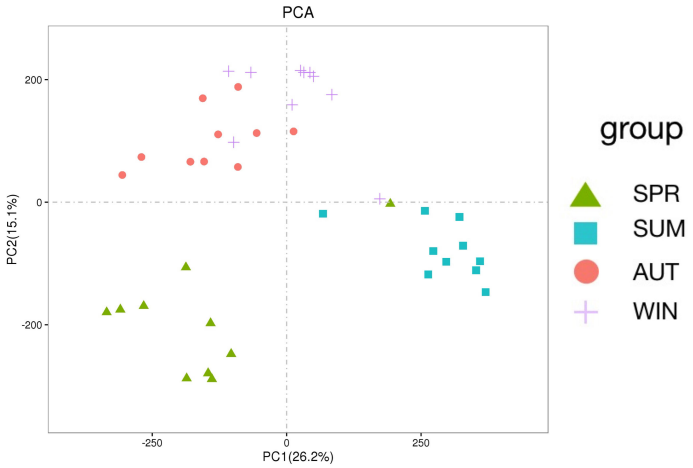

FIGURE 5
PCA shows the diversity analyses of metabolites across different seasons.

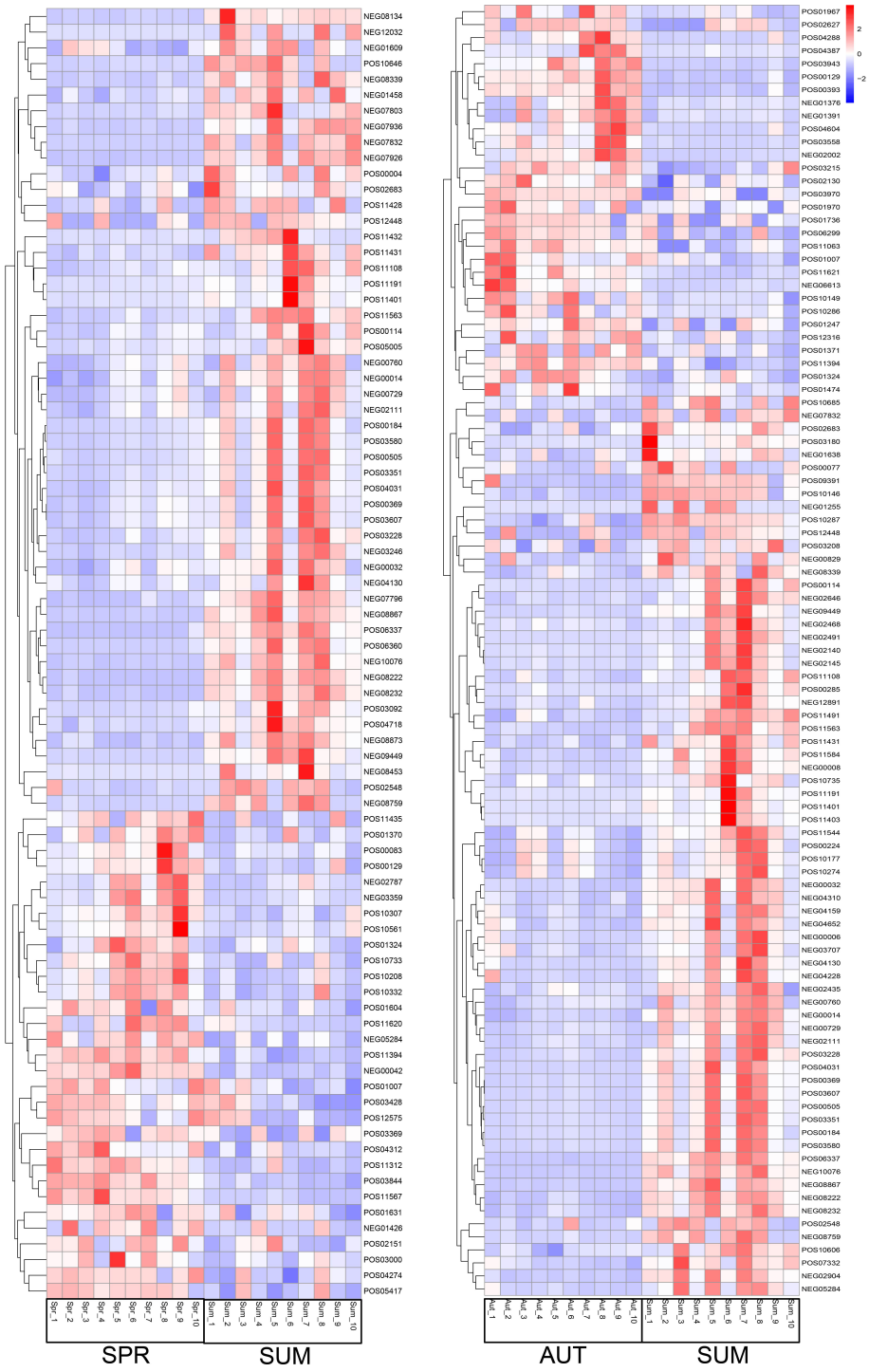

FIGURE 6
Partial metabolite heatmap of *A. japonicus* across different seasons between spring and summer (left), between autumn and summer (right).

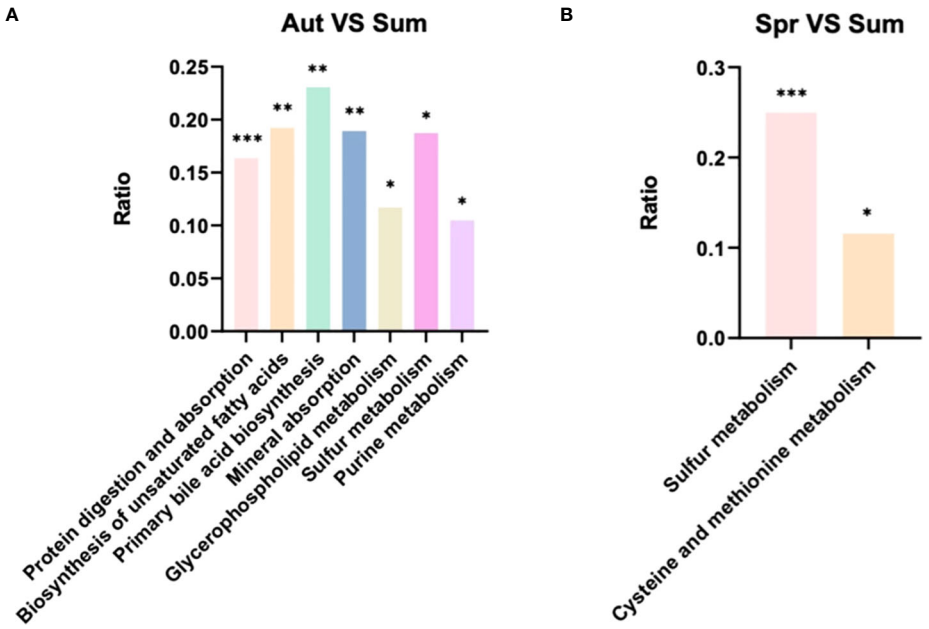

FIGURE 7
KEGG pathway analysis of level 3 differential metabolic pathways **(A)** between autumn and summer and **(B)** spring and summer.

These phenomena indicate that *A. japonicus* participate in biogeochemical cycles through microbial changes.

The effects of *Vibrio* infection on *A. japonicus*

At the species level, *Vibrio* ratios were significantly higher in summer than the other seasons, it may have been due to accelerated growth levels of aquatic *Vibrio* induced by increased temperatures. Importantly, *Vibrio* infection can lead to skin ulcer syndrome (SUS) in sea cucumbers (Yan et al., 2014). It makes us pay attention to prevent bacterial diseases caused by environmental factors in summer. The immune function of *A. japonicus* is mainly controlled by the innate immune system, which is divided into two: cellular and humoral immunity (Gross et al., 2000; Eliseikina and Magarlamov, 2002). Coelomic fluid has im portant roles in the immune processes of *A.japonicus*, the fluid contains immune cells, lysozyme, lectin and other immune active substances, it is an important medium for *A. japonicus* immune cells and immune factors. Analysis of related metabolites which had increased in summer; L-isoleucine, 3-ketosphinganine, lysoPC ((20:4 (5z, 8z, 11z, 14z)), (22:6 (4z, 7z, 10z, 13z, 16z, 19z)), (22:4 (7z, 10z, 13z, 16z)), and cholesterol sulfate. Alterations in these metabolites may be directly related to vibriosis. Isoleucine is an essential metabolite of the immune system; it provides energy for new molecule and cell biosynthesis (Calder, 2006; Nguyen et al., 2018). These observations suggest a correlation possibly exists between isoleucine and immune responses in *A. japonicus*. 3-Ketosphinganine is involved in sphingolipid metabolism. Studies have shown that sphingolipids regulate cell-cell interactions, cell adhesion, cell proliferation and migration, cell death, and other processes partly regulated by sphingolipids. It is also an inflammatory and metabolic disease, Lysosomal storage disorders and other key signaling molecules in many pathophysiological processes (Ponnusamy et al., 2012; Orsini et al., 2019; Dai et al., 2020; Quinville et al., 2021).

In previous studies, after *A. japonicus* infected by *Vibrio*, isoleucine levels are elevated and energy demands increased (Shao et al., 2013). Also, *Vibrio* infection causes oxidative stress (Nguyen et al., 2018). In our study, L-isoleucine levels in summer were significantly higher than in autumn, concomitant with increased *Vibrio* levels in summer. To ensure survival, isoleucine metabolism in *A. japonicus* may have been increased to resist *Vibrio* infection. Also, lysoPC-related metabolites increased as oxidative stress was previously shown to increase lysoPC levels (Balboa and Balsinde, 2006; Oresic et al., 2008; Ru et al., 2017; Ding et al., 2020). Immune system activation produces reactive oxygen species which directly increases oxidative damage, potentially affecting reproduction processes (Faivre et al., 2003; Alonso-Alvarez et al., 2004; Grether et al., 2004; Torres and Velando, 2007). Therefore, *Vibrio* influences in summer may have indirectly impacted *A. japonicus* reproduction in the autumn.

The effects of diet on *A. japonicus* body wall growth across different seasons

In spring and autumn, metabolism levels were significantly higher than in winter and summer. In a previous study on *Holothuria forskali*, stigmasterol and sitosterol extracts are identified in the digestive tract and body wall and were significantly higher than in other body parts (Telahigue et al., 2020). Interestingly, phytosterols are not synthesized by animals, but enter the body *via* the diet (Özyurt et al., 2013). Due to the high consumption rates of *A. japonicus* in the spring and autumn, stigmasterol and sitosterol from the environment are consumed. However, from shrimp research, sitosterol composition changed during the year, levels in shrimp muscle in spring and autumn were at their highest (Özyurt et al., 2013). Therefore, sitosterol levels altered with the seasons. Sterols in nature typically come from specific biological sources of stigmasterol and sitosterol are the main components in higher plants. Therefore, these sterols are used as markers of terrestrial organic matter input in near-shore environments (Rielley et al., 1991; Nytoft et al., 2000) which provide terrestrial higher plant debris. For marine sedimentary environments, sterols can also come from marine phytoplankton, such as some diatoms, and also Cyanobacteria and golden algae produce sterols (Volkman, 1986; Barrett et al., 1995).

By analyzing lipid content in the *A. japonicus* body wall, spring and autumn levels were higher than winter and summer (Gao et al., 2011). This phenomenon may be related to seasonal stigmasterol and sitosterol levels in the body. Also, in fish muscle, stigmasterol was confirmed as a secondary component while sitosterol was a main sterol (Özyurt et al., 2013). Phytosterols also regulate the expression of lipid regulatory genes and new fat formation (Rideout et al., 2010). Therefore, phytosterols may impact body wall growth, and the specific way of nutrition remains to be solved.

Conclusions

We showed that seasonal changes exerted a significant impact on intestinal microorganisms and metabolites in *A. japonicus*. Our research reveals the relationship between the changes of individual metabolic function of sea cucumbers caused by the changes of microorganisms in the intestine, found *A. japonicus* participated in the process of carbon, nitrogen and sulfur cycle in the ocean. The increase of *Vibrio* in summer is a serious threat to the reproduction of *A. japonicus* potentially, which should be paid more attention in aquaculture. Stigmasterol and sitosterol has a potential impact on the growth of *A. japonicus*. These materials can increase the growth of the body wall of *A. japonicus*, which is very important for aquaculture. It provides support for the intestinal immune regulation, self-metabolism, energy absorption, and body growth of sea cucumber in the future.

In this research, we have some deficiency as following, in future, we will (1) subdivide the gut microbiota in different parts of *A. japonicus*; (2) survey seasonal variations of environmental microbiota in experimental sea area; (3) design an experiment to validate effects of *Vibrio*; (4) design an experiment to verify effects of stigmasterol and sitosterol on the body wall of *A. japonicus*.

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 in the article/Supplementary Material.

Author contributions

BD performed the research and wrote the manuscript. XR conceived and designed the research. TW and CZ acquired the data, WS collected sea cucumbers, SL revised the manuscript for important intellectual content. LZ supervised this study. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The reviewer CL is currently organizing a Research Topic with the author LZ.

The remaining 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.

Publisher's note

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