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Intestinal microbiota and immune related genes in sea cucumber (*Apostichopus japonicus*) response to dietary β-glucan supplementation

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ABSTRACT

β-glucan is a prebiotic well known for its beneficial outcomes on sea cucumber health through modifying the host intestinal microbiota. High-throughput sequencing techniques provide an opportunity for the identification and characterization of microbes. In this study, we investigated the intestinal microbial community composition, interaction among species, and intestinal immune genes in sea cucumber fed with diet supplemented with or without β-glucan supplementation. The results show that the intestinal dominant classes in the control group are Flavobacteriia, Gammaproteobacteria, and Alphaproteobacteria, whereas Alphaproteobacteria, Flavobacteriia, and Verrucomicrobiae are enriched in the β-glucan group. Dietary β-glucan supplementation promoted the proliferation of the family Rhodobacteraceae of the Alphaproteobacteria class and the family Verrucomicrobiaceae of the Verrucomicrobiae class and reduced the relative abundance of the family Flavobacteriaceae of Flavobacteria class. The ecological network analysis suggests that dietary β-glucan supplementation can alter the network interactions among different microbial functional groups by changing the microbial community composition and topological roles of the OTUs in the ecological network. Dietary β-glucan supplementation has a positive impact on immune responses of the intestine of sea cucumber by activating NF-κB signaling pathway, probably through modulating the balance of intestinal microbiota.

1. Introduction

The animal intestine harbors complex communities of microbes, which play a critical role in the host health. Intestinal microbes benefit the host by improving digestion of nutrients or protecting against pathogenic bacteria infection [1]. Recent years, a large amount of genetic information has provided deep insight into the gut microbiota with rapid development of metagenomics and high-throughput sequencing. The strong association between diet and health is well accepted. Diet with prebiotic has served as functional feed in aquaculture with a beneficial outcome on growth and immunity. β-glucan serves as a prebiotic for its resistance to digestion, fermentability, and selectivity in promoting the growth or activity of beneficial bacteria such as Bifidobacterium spp. and Lactobacillus spp [2,3]. However, most studies on intestinal microbiota focused on "species" richness, and some studies about interaction among intestinal microbiota were observed in human [4,5], while no report about interactions among intestinal microbiota is found in aquatic animals. Trillions of bacteria in intestine, interacting with each other, form complicated networks and accomplish systems functions through the flow of energy, matter, and information. Thus, it is important to explain the network structures and the underlying mechanisms, which are the essential parts of ecology.

In addition, previous studies have demonstrated that dietary β-glucan can enhance the innate immune response of aquatic animals [6,7], while the regulatory mechanism of immune system is still unclear. The NF-κB signaling pathway is the most important way of non-specific immunity, and nuclear transcription factor (NF-κB) plays a central role in regulating non-specific immune response [8]. In mammals, the NF-κB family of transcription factors contains five members: RelA (p65), RelB, c-Rel, NF-κB1 (pl05/p50) and NF-κB2 (pl00/p52). The NF-κB family has been reported in some aquatic

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animals including sea cucumber [9,10]. In addition, Lysozyme (lys), an efficient natural antimicrobial enzyme, is one of the most important humoral factors in aquatic animals, which can effectively damage cell walls of pathogens invading the body and plays an important role in the innate immune defense.

Sea cucumber, *Apostichopus japonicus* Selenka, is among the most economically important holothurian species in China. Previous studies have described the use of β-glucan on sea cucumber and found that dietary they could promote the growth performance and enhance the immune response in coelomocyte and intestine [7,11]. However, their effects on intestinal microbiota and regulatory mechanism of immune system have not been reported. Therefore, the aim of this study is to evaluate the effects of dietary β-glucan supplementation on the intestinal microbiota and expression of intestinal immune related genes (*Aj*-p105, *Aj*-p50, *Aj*-rel and *Aj*-lys) in sea cucumber.

2. Material and methods

2.1. Experimental animals

Disease-free juvenile sea cucumbers were obtained from Lian He Yuan Jian Farm (Qingdao, China). Sea cucumbers were cultured in a 1000 L fiberglass tank and fed with a commercial diet of sea cucumber (Great seven Bio-Tech, Qingdao, China) for 15 d to acclimate to the experimental conditions. Following a 24 h fast, sea cucumbers of similar size (4.67 ± 0.06 g) were randomly distributed into 10 aquaria (53 × 28 × 34 cm, 50 L) at a density of 10 sea cucumbers in each aquaria.

2.2. Experimental diets and feeding experiment

The basal diet (CT group) was formulated with marine mud (20%), red fish meal (10%) and sargasso (70%). It contains 16.1% crude protein and 0.87% crude lipid. On basis of the basal diet, the β-glucan group (PO group) diet was supplemented with 1.51 g kg^{-1} β-glucan provided by Shandong Bio Sunkeen co., Ltd (Zoucheng, China). 5 replicate aquaria per treatment. The dosage of β-glucan at the level of 1.25–2.50 g kg^{-1} was beneficial to the health of sea cucumbers [11].

Sea cucumbers fed once daily at 6:00 pm for 60 days. The feeding rates were 5% of the biomass during the feeding trial. The environmental conditions (temperature, 17 ± 1 °C; salinity, 28–30 ‰; pH, 8.0 ± 0.3; dissolved oxygen, 10 ± 0.25 mg L^{-1}) were suitable for sea cucumber.

2.3. Sample collection and processing

At the end of the experiment, four sea cucumbers of each replicate were randomly selected and dissected. The mid-intestinal tract from each sea cucumber was removed and frozen at −80 °C until further analysis. The intestinal content in hindgut from four sea cucumbers was collected and mixed. Samples were frozen at −80 °C until further analysis.

2.4. DNA extraction and 16S DNA gene sequencing

DNA of microbes in intestinal content was extracted using a PowerSoil™ DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA) according to the manufacturer's instructions. Amplification and sequencing of the V3–V4 region of the bacterial 16S DNA gene was performed using barcoded fusion primers 341F (CCTACGGGNGGCWGCAG) and 805R (GACTACHVGGGTATCTAATCC). PCR amplification was performed under the following conditions: initial denaturation at 98 °C for 30 s, 25 cycles at 98 °C for 10 s, 53 °C for 30 s and 72 °C for 30 s, and final extension at 72 °C for 7 min. The amplicons were pooled in equimolar concentration and sequenced with an Illumina MiSeq platform.

2.5. RNA extraction and real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA from intestine was reverse-transcribed to cDNA by PrimeScript ™ RT reagent Kit (Takara, Japan). A quantitative thermal cycle was used in Real-time PCR (Mastercycler® ep realplex; Eppendorf, Germany). The amplification was assayed according to Peng et al. (2014) [12]. The Real-time PCR program was as follows: 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 s, Ta Opt for 10 s (60 °C for β-actin, *Aj*-p105, *Aj*-p50, *Aj*-rel, and *Aj*-lys), and 72 °C for 20 s. The reaction was carried out with three duplicates of each sample. The quantitative primer of reference genes (*β*-actin), *Aj*-p105, *Aj*-p50, *Aj*-rel and *Aj*-lys in this experiment were selected by the study of Wang et al. (2011 and 2013) in sea cucumber [10,13] and were list in Table 1. To calculate the expression of *Aj*-p105, *Aj*-p50, *Aj*-rel and *Aj*-lys, the comparative CT method ($2^{-\triangle\triangle CT}$ method) was used, and the value stood for n-fold difference relative to the calibrator.

2.6. Bioinformatic analyses

The raw sequences were processed using the BIPES pipeline, followed by chimera sequences filtering with UCHIME. After preprocessing, OTUs were picked at 97% similarity level against green gene version 13.8 using QIIME 1.8.0 [14]. Taxonomies were assigned with uclust for each OTU. Alpha and beta diversity analyses were also performed using QIIME.

The OTU networks were constructed with a random matrix theory (RMT)-based approach, and then visualized using Cytoscape 3.0.0 according to Zhou et al. (2011) [15].

Data from RT-qPCR were subjected to a one-way ANOVA and the differences among the means were tested by Duncan's multiple-range test (SPSS 16.0). The level of significance was set at $P < 0.05$.

3. Results

3.1. Richness and diversity

The number of OTUs, the Chao1, abundance-based coverage estimator (ACE) and Shannon indices obtained for all the samples in the two groups are reported in Table 2 to assess the alpha diversity of intestinal microbiota of the two groups of sea cucumbers. All those indices suggest the CT group has higher microbial diversity in their intestines, whether measured in richness or evenness. We also computed the Good's estimated sample coverage (ESC). On average, the two groups have ESC of 98.0% and 98.3%, respectively,

Table 1
Primers of internal reference and target genes.

Gene	Primer name	Primer sequences(5′-3′)
β-actin	actin-F	TTATGCTCTTCCTCACGCTATCC
	actin-R	TTGTGGTAAAGGTGTAGCCTCTCTC
Aj-p105	p105-F	GCAACACACCCCTCCATCTT
	p105-R	TCTTCTTCGCTAACGTCACACC
Aj-p50	p50-F	TCCTATCGGTCTGAATCTTCCAA
	p50-R	TTTCTTCCCTTTCTGGCTATGTTC
Aj-rel	rel-F	TGAAGGTGGTATGCGTCTGG
	rel-R	TTGGGCTGCTCGGTTATG
Aj-lys	lys-F	AGGGAGGTAGTCTGGATGGA
	lys-R	GCGCAAAATCCTCACAGGTA

Table 2
Diversity indices used in this study (n = 5).

Sample	Diversity index				
	OTUs	Chao1	ACE[a]	Shannon	ECS[b] (%)
CT[c]	4178 ± 180	11698 ± 1079	11519 ± 1033	5.22 ± 0.42	98.0 ± 0.15
PO[d]	3596 ± 642	11513 ± 1753	11313 ± 1849	4.62 ± 0.21	98.3 ± 0.15

[a] ACE: abundance-based coverage estimator.
[b] ECS: Good's estimated sample coverage.
[c] CT: CT group.
[d] PO: PO group.

indicating that roughly two additional OTUs would be expected for every 100 additional sequenced reads (Table 2).

3.2. Changes in intestinal bacterial community structure in sea cucumber

Taxonomically, 34 different bacterial phyla or groups in intestine of sea cucumber are identified. The dominant phyla in both groups are Proteobacteria, Bacteroidetes and Verrucomicrobia. Specifically, as shown in Fig. 1, CT group is enriched with classes of Flavobacteriia (49%), Gammaproteobacteria (18%), and Alphaproteobacteria (16%), while PO group is enriched with Alphaproteobacteria (40%), Flavobacteriia (17%), and Verrucomicrobiae (16%). The relative abundance of Alphaproteobacteria and Verrucomicrobiae (classified as Rhodobacteraceae and Verrucomicrobiaceae in our present study, respectively) is increased by dietary β-glucan supplementation, concurrent with reduction in Flavobacteria (classified as Flavobacteriaceae in our present study) in the intestine.

The heatmap of the bacterial distribution shows that the abundance of microbial taxa and the microbial communities in intestine of sea cucumber fed diet with β-glucan supplementation for 60 days notably differed from that in the control group (Fig. 2). In addition, Principal coordinates analysis (PCoA) of weighted and unweighted UniFrac distances also confirms the PO and CT intestinal microbiota cluster separately into two groups (Fig. 2).

3.3. Network analyses of intestinal microbiota in sea cucumber

The RMT analysis shows that the CT and the PO networks differ significantly (Fig. 3). The ecological network can naturally divide into different modules, each serving as a functional unit and performing an identifiable task [16,17]. The CT network consists of 56 modules, with 563 nodes and 1522 edges (links), while the PO network 34 modules, with 435 nodes and 1356 edges (Table 3). Among all the nodes, Flavobacteria and Alphaproteobacteria are the most abundant taxa in the CT group and PO group, respectively. In addition, 200 nodes were shared in the two networks. The modules of the CT group are much more inter-connected than those of the PO group. Additionally, there are more negative interactions (red) in the CT group than in the PO group. In brief, dietary β-glucan supplementation dramatically alters the network interactions in the CT and the PO groups.

Different nodes play distinct topological roles in the network [18]. According to values of *Zi* and *Pi*, the roles of OTUs can be categorized into the following four types: peripherals, connectors, module hubs and network hubs (Fig. 4). Here, the majority of OTUs (99.2%) in the CT and PO groups are peripherals with most of their links inside their own modules. Distinct microbes play as connectors ($Pi \geq 0.62$) and module hubs ($Zi \geq 2.5$) in the CT and PO groups. No network hubs are found in these two networks. In the CT group, the connectors are Flavobacteria and Bacilli, compared to Alphaproteobacteria and Betaproteobacteria in the PO group. The module hubs are Planctomycetia and Actinobacteria, respectively, for the two groups.

3.4. The relative expression level of immune genes in the mid-intestine tissue

Dietary β-glucan supplementation had a positive impact on the immunity system of aquatic animals. In this study, the relative expression levels of *Aj*-p105 and *Aj*-p50 genes in the mid-intestine tissue of sea cucumber in the PO group are significantly higher than those in the CT group ($P < 0.05$) (Table 4). There are no significant differences in the relative expression levels of *Aj*-rel and *Aj*-lys between the CT and PO groups ($P > 0.05$).

4. Discussion

β-glucan can benefit host, including mirror carp (*Cyprinus carpio* L.) [19], pig [20], juvenile beluga (*Huso huso*) [21] and broilers [22], by selectively stimulating the growth and/or activity of one or certain microbial genera/species. This study evaluated the intestinal microbiota in sea cucumber response to dietary β-glucan supplementation. Its alpha diversity decreases with dietary β-glucan supplementation, compared with the control group. Similar result is also found in the mirror carp fed β-(1,3) (1,6)-D-glucan supplementation [19].

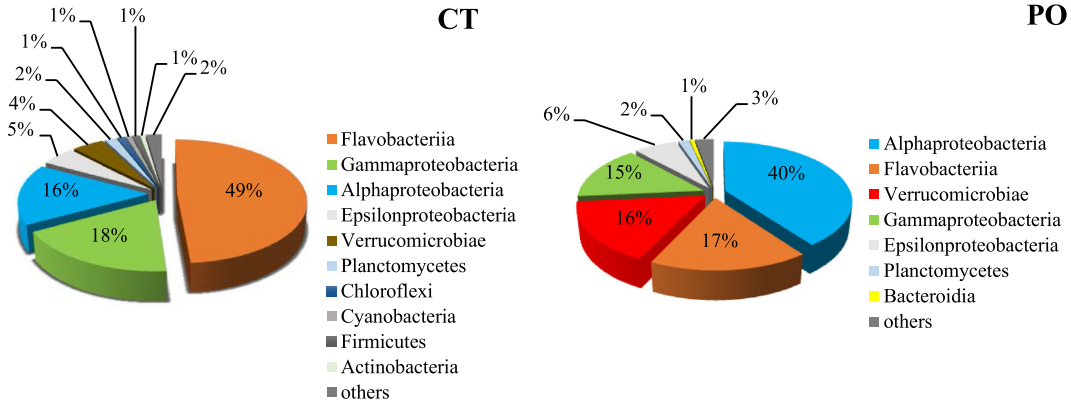

Fig. 1. Relative abundance of different bacterial classes above a cutoff value of 0.6% in intestinal microbiota. CT: CT group, PO: PO group.

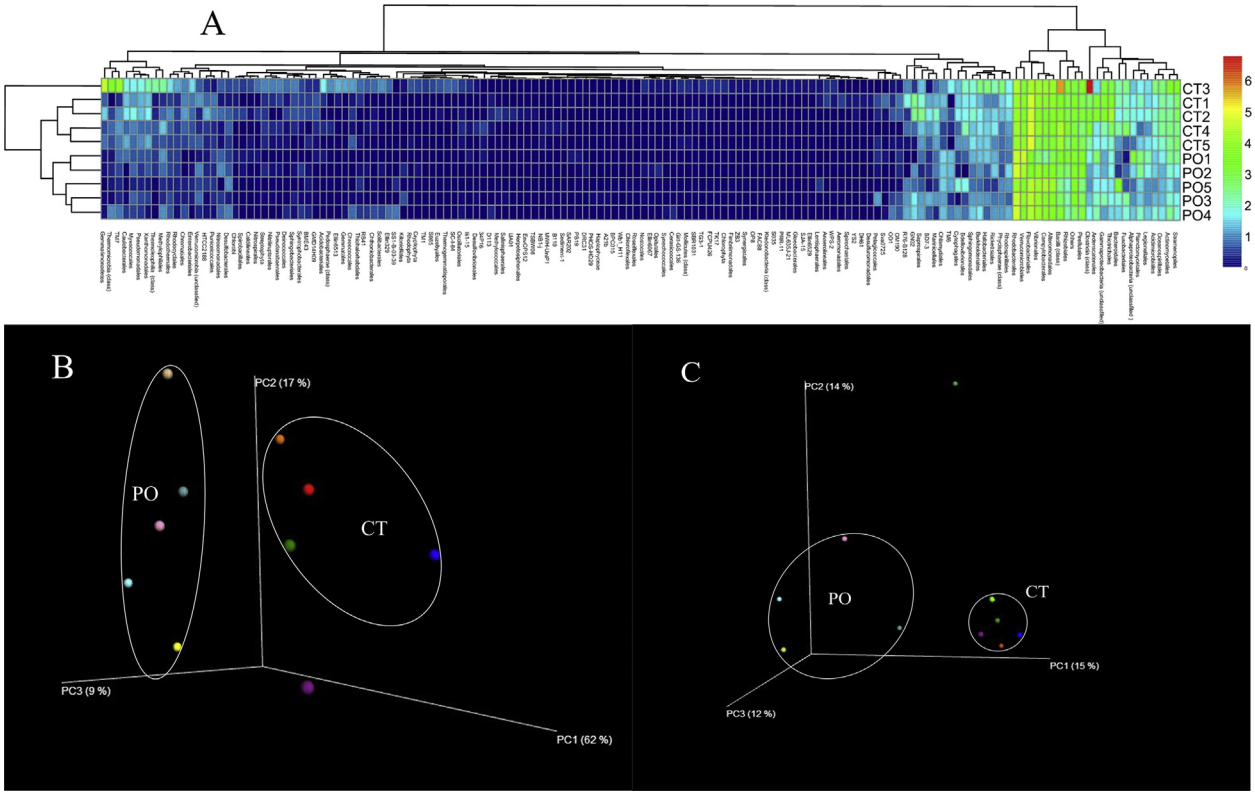

Fig. 2. Hierarchically clustered heatmap and principal coordinates analysis (PCoA) of the intestine microbial communities. A: heatmap; B: PCoA Plots based on weighted UniFrac metrics; C: PCoA Plots based on unweighted UniFrac metrics; CT: CT group, PO: PO group.

In the present study, the dominant phyla in the sea cucumber intestine in both groups are Proteobacteria, Bacteroidetes and Verrucomicrobia. At class level, the dominant bacterial groups in the CT group are Flavobacteriia, Gammaproteobacteria and Alphaproteobacteria whereas class Alphaproteobacteria, Flavobacteriia, and Verrucomicrobiae dominate the community in the PO group. Gao et al. (2014) found different dominant groups at both phylum and class levels, which likely result from differences in diet and growing environment [23]. Their sea cucumbers were fed with natural diets in the pond, as compared to artificial diet in this study. In this study, the β-glucan did not increase Bifidobacterium spp. or Lactobacillus spp., as opposed to what previous studies showed [2,3]. Instead, Rhodobacteraceae and Verrucomicrobiaceae were significantly increased, while Flavobacteriaceae was decreased. This difference is probably due to the fact that there are rare or even no Bifidobacterium spp. or Lactobacillus spp. found in the sea cucumber of either CT or PO group. Rhodobacteraceae is aquatic, photosynthetic bacteria that is potentially used as a probiotic for a high gastrointestinal transit tolerance and safe for intestinal epithelial cells of tilapias [24,25]. In contrast, little is known about

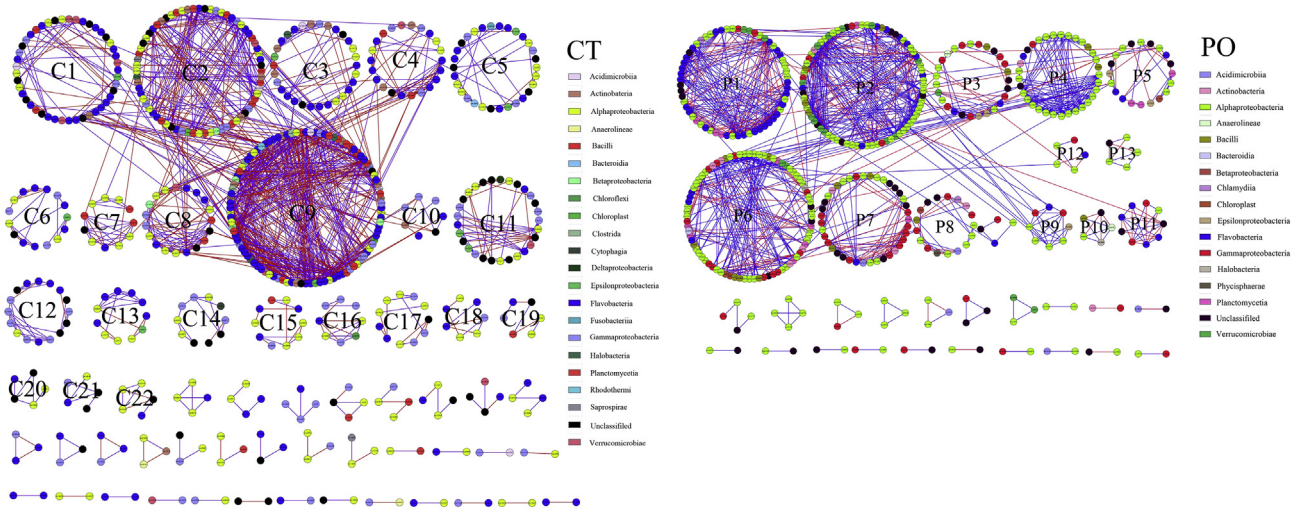

Fig. 3. The network of the intestinal microbiota in sea cucumber. The network graph with submodule structure by the fast greedy modularity optimization method. Each node indicates one OTU. Colors of the nodes indicate different major classes. A blue edge indicates a positive interaction between two individual nodes, while a red edge indicates a negative interaction. CT: CT group, PO: PO group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
The composition of the ecological network.

Index	CT	PO	Number of Shared OTUs
Acidimicrobiia	6	5	2
Actinobateria	21	10	9
Alphaproteobacteria	132	191	75
Anaerolineae	2	2	1
Bacilli	35	19	15
Bacteroidia	1	1	1
Betaproteobacteria	5	3	3
Chlamydiia	0	2	0
Chloroflexi	1	0	0
Chloroplast	3	1	1
Clostrida	3	0	0
Cytophagia	1	0	0
Deltaproteobacteria	1	0	0
Epsilonproteobacteria	8	5	3
Flavobacteria	173	53	33
Fusobacteriia	1	0	0
Gammaproteobacteria	79	56	29
Halobacteria	4	2	2
Phycisphaerae	0	1	0
Planctomycetia	8	14	4
Rhodothermi	1	0	0
Saprospirae	2	0	0
Unclassifiled	65	50	16
Verrucomicrobiae	11	20	6
Total number of OTUs	563	435	200
The number of modules	56	34	–
Total number of links	1522	1356	–

the metabolism and ecological roles of Verrucomicrobiaceae except that Verrucomicrobiaceae was found capable of degrading polysaccharide [26]. The increase of Rhodobacteraceae and Verrucomicrobiaceae by dietary β-glucan supplementation in this study suggests that they could effectively employ β-glucan as their nutrient source.

In addition to the change in microbial community composition, the supplementation of β-glucan could influence interactions among species. We show the number of modules and interconnections between modules are both notably decreased by dietary β-glucan, probably a result of negative impact of the supplementation on intestinal bacterial diversity in the PO group. From the molecular viewpoint, OTUs of the same species within a module likely share the same functions [27]. As shown in Fig. 3,

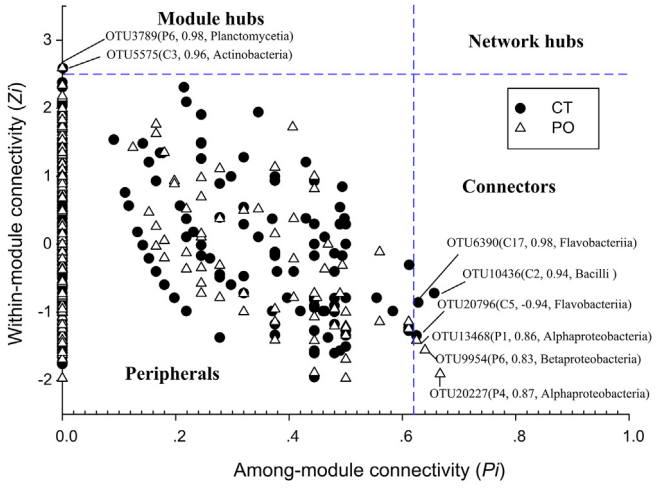

Fig. 4. *Z-P* plot showing the distribution of OTUs based on their topological roles. In parentheses are the module number, module membership, and phylogenetic associations. CT: CT group, PO: PO group.

Table 4
The relative expression of immune genes in the mid-intestine (n = 3).

Treatment	*Aj*-p105	*Aj*-p50	*Aj*-rel	*Aj*-lys
CT	1.00 ± 0.07	1.00 ± 0.07	1.00 ± 0.22	1.00 ± 0.02
PO	1.44 ± 0.04*	1.64 ± 0.08*	1.32 ± 0.06	1.34 ± 0.17

Note: CT: CT group, PO: PO group. *$P < 0.05$.

many nodes/OTUs from the same class are within the same modules in the PO network, which may imply that the function of these modules would be more stable for missing several nodes/OTUs without affecting the overall function of the module. Positive or negative interactions among different OTUs are represented in the network graph. A network connection between two OTUs in fact describes the co-occurrence of these two OTUs, which may be caused by species performing similar or complementary functions [15]. Negative interactions may indicate competition or predation among the taxa, while positive interactions signify complementation or cooperative behaviors.

The analysis of modular topological roles is important to identify key microbial groups based on the OTUs' roles in their own modules [16]. From the ecological viewpoint, peripherals may represent specialists, while connectors and module hubs generalists and network hubs super-generalists [28]. In the present study, Alphaproteobacteria is the predominant class in the PO group, two OTUs from which serve as connectors in the network, compared to two OTUs from Flavobacteria serving as connectors in the CT group. In addition, the module hubs in PO group are also different from those in the CT group. Structurally, the networks would be not affected by extinction of peripherals. Connectors and module hubs, on the other hand, play an indispensable role in the network [18]. In brief, these results suggest the dietary supplementation of β-glucan regulates the balance of intestine microbiota in sea cucumber via the change in microbial community composition and the balance of the advantages and disadvantages of various network topological characteristics for system functional stability. However, the detailed microbiota function in the ecological network needs more mechanistic studies.

In the present study, dietary β-glucan supplementation significantly induced the expression of cellular immune genes *Aj*-p105 and *Aj*-p50 genes and had a positive improvement on the expression of *Aj*-lys in the mid-intestine tissue of sea cucumber. Sea cucumber lacks adaptive immunity and relies solely on innate immune system against invading microbes. Previous studies have shown that various probiotics could act as non-specific immune factors and activate the host immune system through flagellin, LPS, peptidoglycan and the secretion of cytokines recognized by intestine mucosal cell surface receptor [29]. Dietary β-glucan supplementation can enhance the immune response of sea cucumber by activating the NF-κB signaling pathway through notably increasing potential probiotics such as Rhodobacteraceae. After activation, NF-κB migrates into the nucleus and induces the expression of many target genes including lysozyme gene [30]. Indeed, the relative expression of target gene *Aj*-lys in mid-intestine is increased by dietary β-glucan supplementation. However, the mechanism of dietary β-glucan supplementation on modulation of innate immune responses through intestinal microbiota alteration in sea cucumber is still undefined and requires further investigation.

In conclusion, our study demonstrates the supplementation of β-glucan can modulate the intestinal microbial community in sea cucumber by promoting the proliferation of intestinal Rhodobacteraceae and Verrucomicrobiaceae, change network interactions and topological roles of the OTUs in the ecological network, and has a positive improvement on the immune response in the intestine.

Author contributions

Conceived and designed the experiments: X.L.T. Performed the experiments: G.Y., M.P. Analyzed the data: G.Y., Z.J. X, S.L. D. Wrote the paper: G.Y., Z.J. X.

Conflict of interest

None.

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