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Regulation of dietary astragalus polysaccharide (APS) supplementation on the non-specific immune response and intestinal microbiota of sea cucumber *Apostichopus japonicus*

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ABSTRACT

Astragalus polysaccharide (APS) plays important roles in antibacterial, antiviral and antiparasitic activities in mammals, birds and aquatic animals. However, the relationship between non-specific immune responses and intestinal microbiota in sea cucumber (*Apostichopus japonicus*) after dietary APS supplementation has not been reported to date. Here, the effect of dietary APS supplementation on the non-specific immune response and intestinal microbial composition and species distribution of sea cucumber was explored. We found that although there was no significant effect on sea cucumber growth, the enzymatic activity and expression level of immune- and antioxidant-related genes changed after dietary APS supplementation. Furthermore, the intestinal microbial composition and species distribution of sea cucumber were different at the phylum and genus levels after dietary APS supplementation. The phyla Proteobacteria and Bacteroidetes were significantly different between the APS2 group and CK group. The results of PCA and PCoA analysis also showed that the APS2 group was significantly different compared to the other groups. Finally, analysis of the relationship between non-specific immune responses and the intestinal microbiota showed that the expression level of NF-κB was significantly correlated with intestinal microbiota at the genus level. This finding suggests that dietary APS supplementation might affect the non-specific immune response and intestinal microbiota of sea cucumber through the NF-κB signalling pathway; the appropriate added level was 800 mg/kg. Taken together, our results lay a foundation for further understanding the relationship between non-specific immune responses and intestinal microbial of sea cucumber.

1. Introduction

Sea cucumber (*Apostichopus japonicus*), which belongs to the Echinodermata phylum and Holothuroidea class, is an important commercially farmed species in China, Japan and Korea because of its high nutritional value [1,2]. In recent years, disease of sea cucumber has become a serious issue for aquaculture [3]. Bacterial disease is the most reported and most serious disease in modern aquaculture [4,5]. However, disease of sea cucumbers has generally been controlled by antibiotics and chemotherapeutics, leading to many negative impacts, such as drug resistance and environmental pollution [1,2,6]. Therefore, improving disease control and enhancing their immunity has become an urgent need in sea cucumber aquaculture.

Currently, immune enhancers are widely used in aquaculture to inhibit pathogens, improve growth and immunity, promote vitamin synthesis and digestion, and improve the structure of the intestinal microbiota [7–11]. The *Astragalus membranaceus* root, for which the active ingredient is astragalus polysaccharide (APS), has been used as an immune enhancer for thousands of years in China [12]. It is well documented that APS can enhance antibacterial, antiviral and antiparasitic activities in aquatic animals, birds and mammals [9,13–16]. APS combined with probiotics administration in feed displayed synergistic modulatory effects on immunity and intestinal microbiota in chicken [13]. Otherwise, APS enhances the immune response to avian infectious bronchitis virus vaccination [15]. Dietary intake of *A. membranaceus* root or its polysaccharides could enhance the immune responses of sea cucumber and improve its resistance to infection by *Vibrio splendidus* [16]. Shrimps and fishes fed with diets containing certain Chinese herbs could improve non-specific immunity, such as bacteriolytic activity and leucocyte function [17,18]. A recent study showed that APS stimulated key nodes in the TLR4-MyD88 dependent signalling pathway, including TLR4, MyD88, TRAF6, NF-κB and AP-1, both in

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vitro and in vivo. However, TRAM was an exception. Moreover, TRAF6 and NF-κB were not triggered by APS in gene-deficient tumour-bearing mice. Therefore, APS may modulate the immunity of host organisms through activation of TLR4-mediated MyD88-dependent signalling [19].

A wide variety of microbiota is present in the intestinal tract of humans and other animals, forming a complex intestinal microbiota that has various physiological activities, including roles in metabolism, maturation of the immune system and inhibition of pathogenic bacteria [20–22]. Immune enhancers are well known for their beneficial outcomes on health of sea cucumber and other aquatic animals through modifying the host intestinal microbiota [23,24]. Dietary β-glucan supplementation promoted the proliferation of Rhodobacteraceae of the Alphaproteobacteria class and Verrucomicrobiaceae of the Verrucomicrobiae class and reduced the relative abundance of Flavobacteriaceae of the Flavobacteria class in sea cucumber [24].

Recent studies have shown that astragalus polysaccharide can modulate effects on immunity and intestinal microbiota in chicken and mice [13,25]. However, their effects on intestinal microbiota and the regulatory immune mechanism in sea cucumber have not been reported. Therefore, this work aimed to evaluate the relationships between intestinal microbiota diversity and expression of immune-related genes after dietary APS supplementation in sea cucumber.

2. Material and methods

2.1. Experimental animals

Juvenile sea cucumbers were collected from an aquaculture farm of Qingdao in China and maintained in a recirculation system in our lab at water temperature of 15–19 °C. During the experiment, the *p*H of water was maintained at 7.8–8.2 and the salinity at 31–32‰ The sea cucumbers were fed with a commercial diet for 2 weeks to acclimate to the experimental conditions. Following a 24 h fast, sea cucumbers of similar size (9.3 ± 0.05 g) were randomly distributed into 15 tanks (50 × 30 × 60 cm) at a density of 25 sea cucumbers per tank.

2.2. Feed ingredients and diet formulation

Five experimental groups, with isoproteic (20% crude protein) and isolipidic (3% crude lipid) diets, were formulated as shown in Table S1, and the 0 g/100 g of APS added group served as the control. All ingredients were ground into a fine powder through 200 mm rate and thoroughly blended. Pellets (2.0 ± 2.0 mm) were made automatically using a pellet-making machine and dried in a ventilated oven at 40 °C for approximately 12 h. Then, the feeds were packed in double plastic bags and stored at −20 °C until use.

The experiment lasted for 60 days. At the termination of the experiment, four sea cucumbers of each replicate were randomly selected and dissected. The coelomic fluid was obtained from the coelom with 1 ml syringes and put into 1.5 ml Eppendorf tubes for enzymatic assay. The intestinal tract and respiratory tree from each sea cucumber was removed and frozen at −80 °C until further analysis. The intestinal content from four sea cucumbers was collected. Samples were frozen at −80 °C until further analysis.

2.3. Calculation of growth and survival

Growth performance in terms of weight gain rate (WGR), specific growth rate (SGR) and Visceral wall ratio (VWR) was calculated as follows:

$$WGR\ (\%) = (M_t\text{-}M_0)/M_0 \times 100\%;$$

$$SGR\ (\%.\ d^{-1}) = 100 \times (LnW_t\text{-}LnW_0) / t;$$

$$VWR\ (\%) = \text{Visceral weight/ Body wall weight} \times 100\%$$

where W_0 and W_t were the initial and final body weights (g) of sea cucumber, respectively; t was the duration days of the experiment.

2.4. Enzymatic assay

Coelomic fluid samples were thawed on ice and centrifuged at 4 °C and 3000 g for 20 min. Then, the supernatant solution was transferred to new 1.5 ml Eppendorf tubes and stored at 4 °C for the determination of immune- and antioxidant-related enzymes. All enzymatic assays were conducted within 24 h of extraction. The enzymatic activity of lysozyme (LZM), acid phosphatase (ACP), alkaline phosphatase (APK), catalase (CAT), and superoxide dismutase (SOD) and malondialdehyde (MDA) content were measured by commercial kits from Nanjing Jiancheng Bioengineering Institute of China.

2.5. RNA extraction and real-time quantitative polymerase chain reaction (qRT-PCR)

Total RNA from the respiratory tree of sea cucumber was extracted and reverse-transcribed to cDNA by PrimeScript™ RT reagent Kit (Takara, Japan). PCRs were run in triplicate using the following conditions: 10 min at 95 °C followed by 40 cycles of 30 s at 95 °C, 15 s at 60 °C, and 10 min at 72 °C. The quantitative primers of reference genes (*β-actin*) and immune- and antioxidant-related genes in this experiment are listed in Table S2. Data were quantified using the $2^{-\Delta\Delta Ct}$ method based on Ct values. Values were considered to be significant at $P < 0.05$.

2.6. DNA extraction and 16S RNA gene sequencing

Total genomic DNA from the 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 V4–V5 region of the bacterial 16S RNA gene was performed by a biotechnology company (Shanghai Biozeron Biotech. Co., Ltd) using barcoded fusion primers 515F: 5′-GTGCCAGCMGCCG CGG-3′ and 907R: 5′-CCGTCAATTCMTTTRAGTTT-3'. 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 into equimolar concentrations and sequenced with an Illumina MiSeq platform.

2.7. Intestinal microbiota diversity analysis

Paired-end sequence reads were merged using FLASH [26], and quality filtering of the raw tags was performed under specific filtering conditions to obtain high-quality clean tags according to the QIIME (V1.7.0) quality control process [27]. Sequence analysis was performed using Uparse software (v7.0.1001); sequences were assigned to the same operational taxonomic units (OTU) if they had greater than or equal to 97% similarity [28]. For each representative sequence, the Greengenes Database was used based on an RDP 3 classifier (Version 2.2) algorithm to annotate taxonomic information [29]. Phylogenetic relationships between OTUs and dominant species differences among sample groups were performed using multiple sequence alignments with MUSCLE software (Version 3.8.31) [30].

Alpha diversity was used to analyse the complexity of species diversity. This analysis used the Chao1, Shannon and Simpson indices. The microbial flora from intestinal tract samples of sea cucumber were calculated by QIIME (Version 1.7.0) and displayed by R software (Version 2.15.3). Beta diversity analysis was used to evaluate differences in species complexity. Beta diversity using both weighted and unweighted UniFrac was calculated with QIIME software (Version 1.7.0). Cluster analysis was preceded by principal component analysis (PCA), which was applied to reduce the dimension of the original

variables using FactoMineR and ggplot2 in R software (Version 2.15.3). Principal coordinate analysis (PCoA) was used to visualize complex, multidimensional data. A distance matrix of weighted or unweighted UniFrac among samples was transformed to a new set of orthogonal axes. The maximum variation factor was demonstrated by the first PCoA and the second maximum by the second PCoA and so on. The unweighted pair-group method with arithmetic means (UPGMA) clustering was performed as a type of hierarchical clustering method to interpret the distance matrix using average linkage, and it was performed with QIIME software. The Venn diagram of differential analysis at the genus level was produced by TBtools software [31].

2.8. Statistical analysis

One-way analysis of variance was used to test the significance of growth performance, activities of immune- and antioxidant-related enzymes, expression level of immune- and antioxidant-related genes and microbial diversity among the different groups after dietary APS supplementation of sea cucumber using the statistical software SPSS (Release 23.0). The level of significance was $P < 0.05$.

3. Results

3.1. Growth performance

The growth performance of weight gain rate (WGR), specific growth rate (SGR) and Visceral wall ratio (VWR) of sea cucumber after dietary astragalus polysaccharide supplementation are shown in Fig. S1. Our results showed that there were no significant differences between any of the added different density gradient APS groups and the control group, suggesting that dietary APS supplementation had a non-significant effect on the growth performance of sea cucumber.

3.2. Activities of immune- and antioxidant-related enzymes

The activities of immune- and antioxidant-related enzymes of sea cucumber changed after dietary APS supplementation. The results showed that the enzymatic activity of immune-related enzymes increased after dietary APS supplementation (Fig. 1A to C). The enzymatic activity of lysozyme (LZM) was significantly increased between the APS2, APS3 or APS4 and CK groups ($P < 0.05$), while the enzymatic activity of LZM in the APS1 group was not significantly different from the CK group ($P > 0.05$) (Fig. 1A). The similarity regularity was present in the enzymatic activity of acid phosphatase (ACP) after dietary APS supplementation (Fig. 1B). The alkaline phosphatase (APK) activity was significantly increased in the APS1, APS2 or APS3 group compared with the CK group, while the APK activity of the APS4 group was not significantly different from the CK group (Fig. 1C). The activity of antioxidant enzymes was reduced after dietary APS supplementation (Fig. 1D to F). The enzymatic activity of catalase (CAT) was significantly reduced between the APS1, APS2 or APS3 and CK groups (Fig. 1D). The similarity regularity was present in the enzymatic activity of malondialdehyde (MDA) activity after dietary APS supplementation (Fig. 1F). However, the enzymatic activity of superoxide dismutase (SOD) was not significantly different after dietary APS in comparison with the CK group (Fig. 1E).

3.3. Expression level of immune-related genes

The effects of dietary APS supplementation on the expression level of immune- and antioxidant-related genes of the respiratory tree in sea cucumbers was also explored. The results showed that the expression level of lysozyme (LZM) was significantly different between the APS3 group and CK group, and the expression level of lectin (LEC) was significantly different between the APS2 group and CK group ($P > 0.05$) (Fig. 2A and B). However, the expression level of superoxide dismutase (SOD) and catalase (CAT) was significantly different between the APS4 group and CK group ($P < 0.05$), while the expression level of SOD and CAT was not significantly different between the APS1, APS2 or APS3 group and the CK group ($P > 0.05$) (Fig. 2C and D). The NF-κB family has 5 members in mammals, including NFKB1 (p105), NFKB2 (p50), RelA (p65), RelB and Rel. However, there are only 3 members (p105, p50 and Rel) of the NF-κB family in sea cucumber. The expression level of p50 and p105 after dietary APS supplementation revealed a similar phenomenon to that of SOD and CAT (Fig. 2E and F). We also found that the expression level of Rel was significantly different between the APS2 or APS4 group and CK group (Fig. 2G). Our results suggest that APS might regulate sea cucumber immunity through the NF-κB signalling pathway.

3.4. OTU clusters and alpha diversity of microbiota in intestinal contents

To explore the diversity of intestinal microbiota of sea cucumber after dietary APS supplementation, the intestinal microbial composition and species distribution of sea cucumber were investigated by 16s rRNA sequencing. The samples from the intestine of sea cucumber were analysed by assigning OTUs at the 97% identity level. Our results showed that the Chao1 and Simpson indices of alpha diversity were

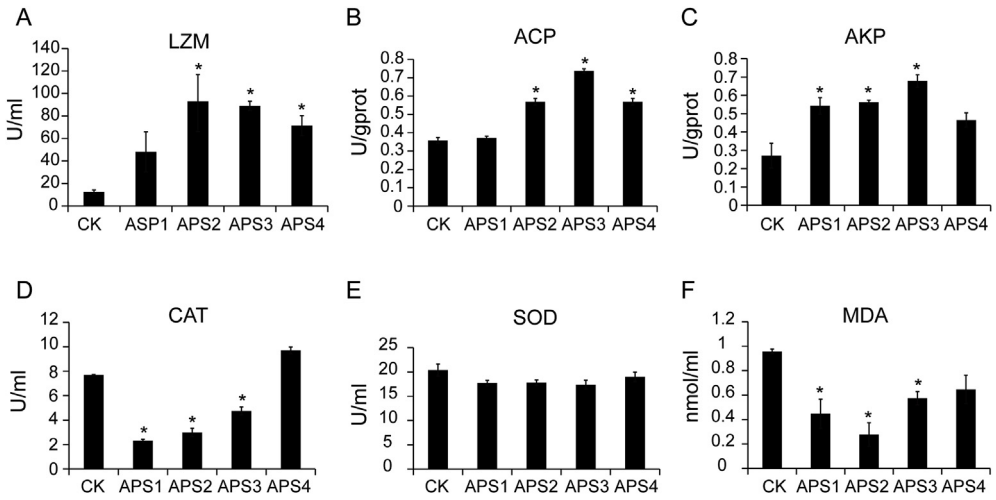

Fig. 1. Activities of immune- and antioxidant-related enzyme content in coelomic fluid of sea cucumbers fed diets with different levels of astragalus polysaccharide (APS).

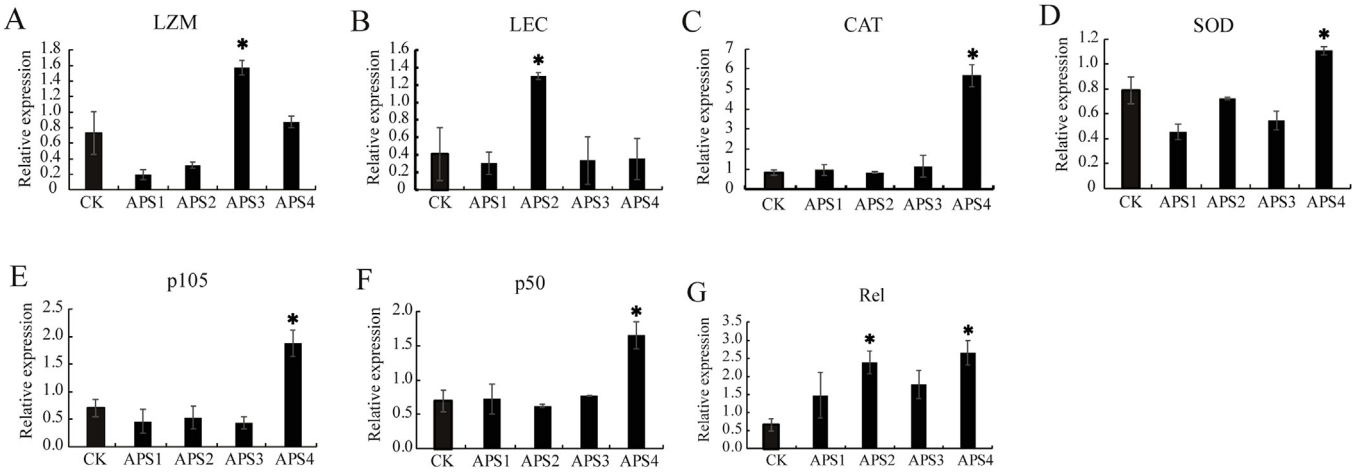

Fig. 2. The gene expression level of immune- and antioxidant-related genes in the respiratory tree of sea cucumbers fed diets with different levels of APS.

Table 1
Intestinal microbial diversity indices of sea cucumber after dietary APS supplementation.

Sample	OTU	Chao1	Shannon	Simpson	Coverage
CK	607	1063	3.31	0.14	0.99
APS1	387	555	3.17[a]	0.11	0.99
APS2	640	779	4.12	0.05	0.99
APS3	504	653	3.71	0.07	0.99
APS4	479	617	3.62	0.09	0.99

[#]OTU, Chao1, Shannon and Simpson indices had a similarity of 0.97 between reads.
[a] Difference significant at the 0.05 level.

reduced after dietary APS supplementation, but there were no significant differences between them. However, the Shannon index of alpha diversity was significantly reduced between the APS1 group and CK group (Table 1).

The intestinal microbial composition and species distribution of sea cucumber were different after dietary APS supplementation at the phylum and genus levels. The dominant populations of sea cucumber intestinal microbiota at the phylum level were Proteobacteria and Bacteroidetes. The other phyla with a relative abundance > 1% were Planctomycetes and Verrucomicrobia (Fig. 3A). When the diversity of intestinal samples of the dietary APS supplementation and control groups were compared, there were no differences in the levels of Planctomycetes and Verrucomicrobia, while Proteobacteria and Bacteroidetes were significantly different between the APS2 group and CK group ($P < 0.05$) (Fig. 3B). The dominant populations of sea cucumber intestinal microbiota at the genus level were *Lutibacter*, *Pelagicola* and *Sedimentitalea*, with relative abundances > 5% (Fig. 4A). The results of difference analysis showed that there were many significantly different genera after dietary APS supplementation, wherein 87 genera were different between the APS2 group and CK group, of which 52 genera were specific to the ASP2 group (Fig. 4B). The genera of *Rhodobacteraceae_Unclassified*, *Rhodobacteraceae_Uncultured* and *Halocynthiibacter* were increased in the APS2 group, while the genera of *Lutibacter*, *Flavirhabdus*, *Algibacter*, *Shewanella* and *Aquibacter* were reduced (Fig. 4C). The results suggest that dietary APS supplementation can affect the intestinal microbial composition and species distribution of sea cucumber.

3.5. Beta diversity of bacteria after dietary APS supplementation

β diversity analysis was employed to assess differences between the dietary APS supplementation groups and control group. The result of hierarchical clustering based on distance matrix of beta diversity showed that the 3 samples of APS2 were clustered together, while the samples of APS1, APS3, APS4 and CK were clustered together. There was no separation between them, suggesting that the intestinal microbial composition and species distribution of the APS2 group were significantly different than the other groups (Fig. 5). Therefore, we performed Principal Component Analysis (PCA) and principal coordinate analysis (PCoA) of weighted UniFrac distances and unweighted pair groups with arithmetic mean algorithm (UPGMA) to compare the diversity of each intestinal microbiome sample. The first and second principal coordinates accounted for 66.05% and 13.69% (Fig. 6A) and 81.43% and 9.63% (Fig. 6B), respectively. There was no significant overlap between the APS2 group and others (Fig. 6). The result of non-parametric factorial Kruskal-Wallis (KW) sum-rank test of LEfSe also

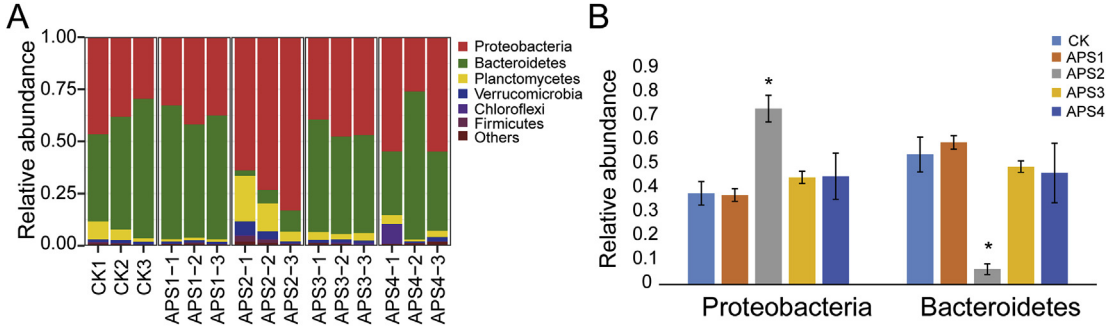

Fig. 3. The intestinal microbial composition and species diversity of sea cucumber fed diets with different concentration of APS at the phylum level. A) The relative abundance of intestinal microbes of sea cucumber fed diets with different concentrations of APS at the phylum level. B) The phylum of Proteobacteria and Bacteroidetes were significantly different between the APS2 group and CK group after dietary APS supplementation in the intestine of sea cucumber.

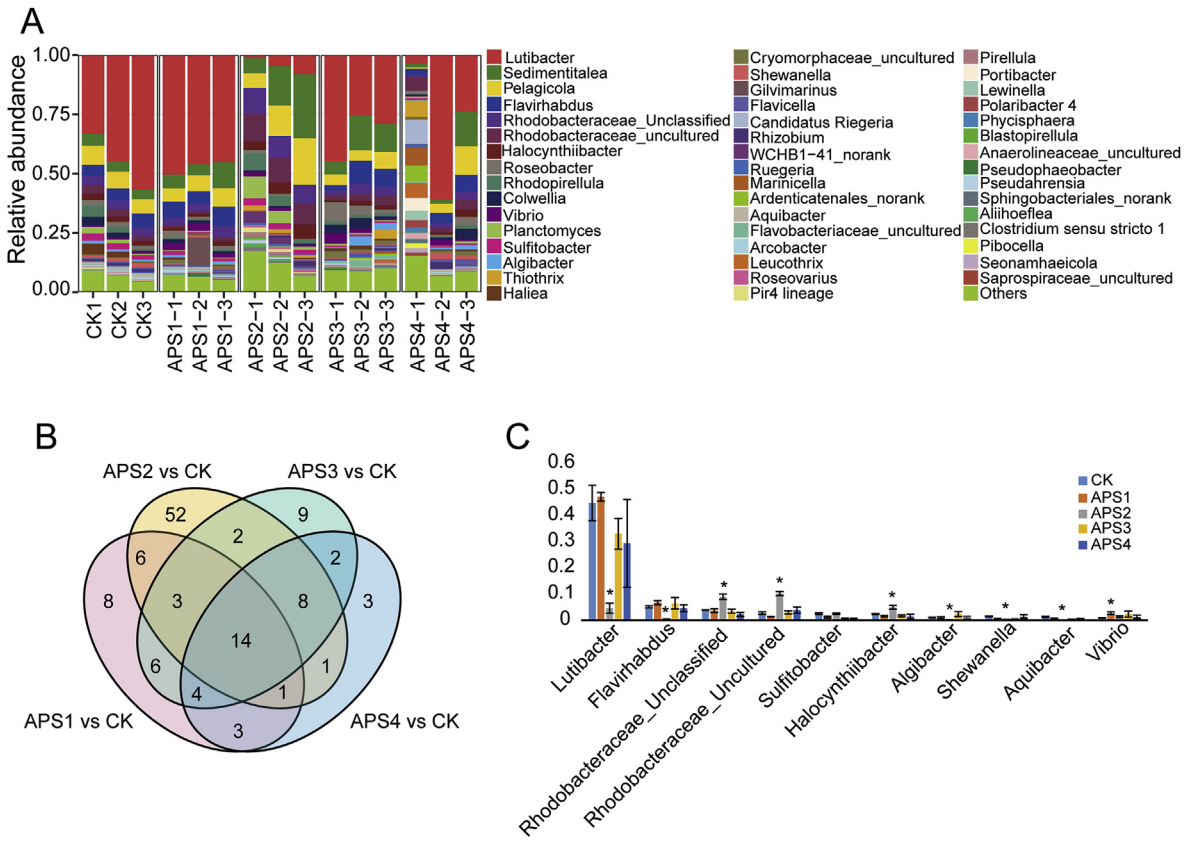

Fig. 4. The intestinal microbial composition and species diversity of sea cucumber fed diets with different concentrations of APS at the genus level. A) The relative abundance of intestinal microbes of sea cucumber fed diets with different concentrations of APS at the genus level. B) Differential analysis of the intestinal microbes of sea cucumber after dietary APS supplementation at the genus level. C) The relative abundance of ten dominant differential genera in the intestine of sea cucumber after dietary APS supplementation.

showed that there were many differences in order, family, genus or species level between the APS2 group and others (Fig. 7). Therefore, we speculate that the intestinal microbial composition and species distribution of sea cucumber are significantly different after 800 mg/kg dietary APS supplementation.

3.6. *The relationship between immune performance and intestinal microbiota of sea cucumber*

The relationship between nonspecific immune responses and intestinal microbiota of sea cucumber was analysed through Spearman's rank correlation coefficient and Pearson correlation. The results demonstrated that the activity of LZM was strongly negatively correlated with *Shewanella* (Spearman's rank correlation coefficient: $\rho = -0.900$, $P = 0.037$) and *Aquibacter* (Spearman's rank correlation coefficient: $\rho = -1.00$, $P < 0.001$; Pearson correlation coefficient: $r = -0.981$, $P = 0.003$) (Table 2 and Table S3). Spearman's rank correlation analysis also showed that *Shewanella* was significantly correlated with the activity of MDA (Spearman's rank correlation coefficient: $\rho = 0.9$, $P = 0.037$) and AKP (Spearman's rank correlation coefficient: $\rho = -0.9$, $P = 0.037$). The correlation coefficient between the expression level of immune- or antioxidant-related genes and significantly

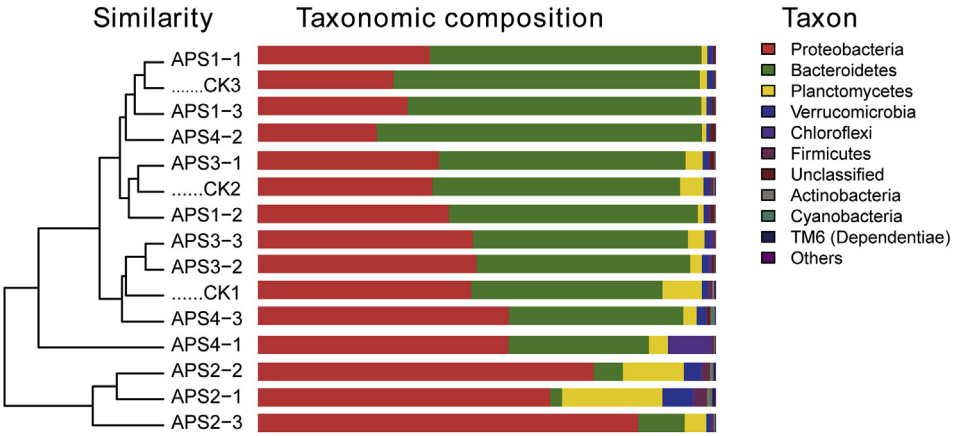

Fig. 5. Hierarchical clustering based on distance matrix analysis was used to explore the intestinal microbial composition and species diversity of sea cucumber fed diets with different levels of APS at the phylum level.

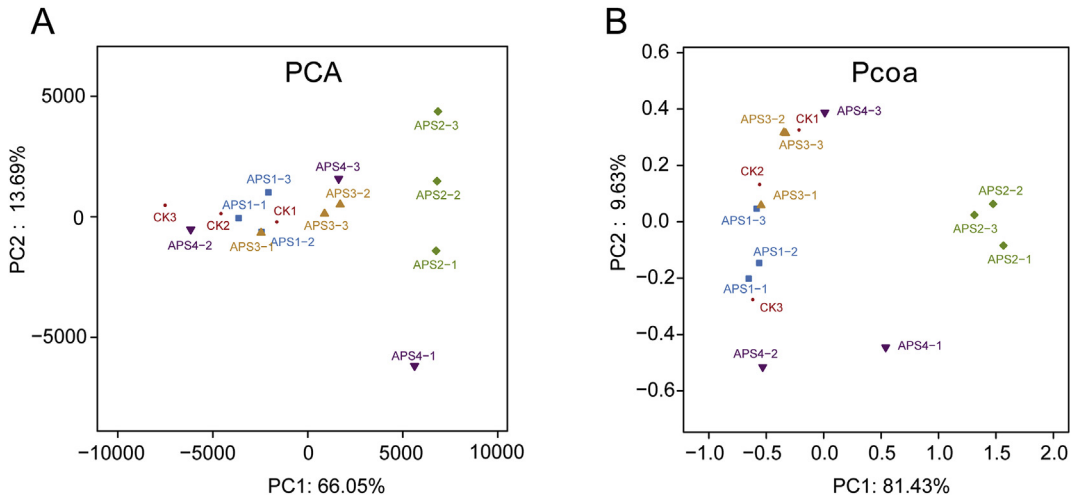

Fig. 6. Species complexity using beta diversity analysis. PCA and PCoA of weighted UniFrac distances for comparison of microbiome diversity.

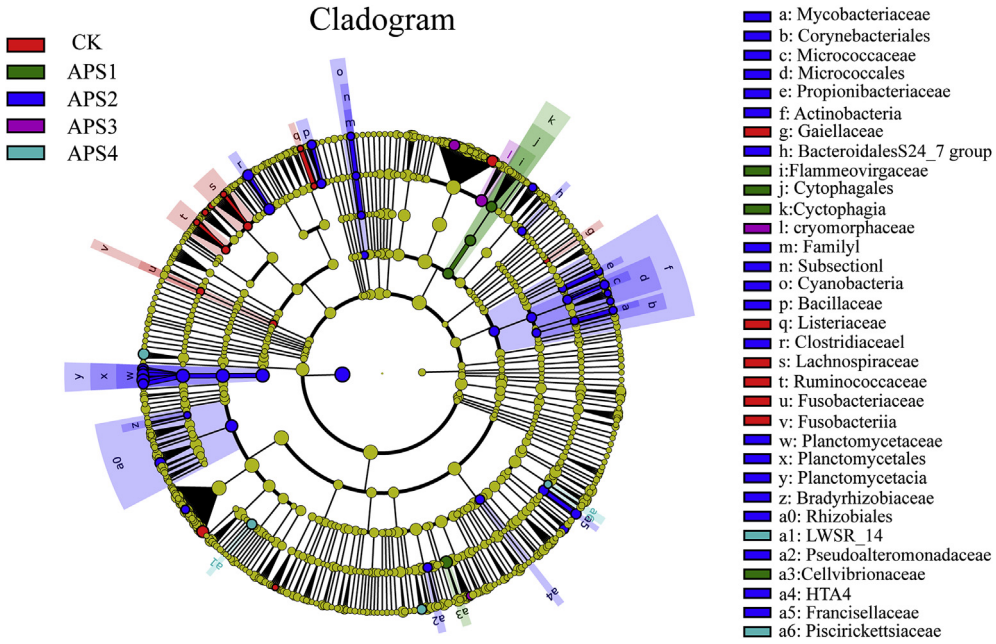

Fig. 7. Cladogram of the intestinal microbiota of sea cucumber fed diets with different levels of APS. The colour node indicates differential microbes after dietary APS supplementation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
The spearman's rank correlation coefficient between activities of immune or antioxidant related enzymes and significantly different bacteria in genus level.

		Lutibacter	Flavirhabdus	Rhodobacteraceae Unclassified	Rhodobacteraceae Uncultured	Sulfitobacter	Halocynthiibacter	Algibacter	Shewanella	Aquibacter	Vibrio
LZM	ρ	−0.800	−0.500	0.100	0.800	−0.300	0.300	−0.300	−0.900*	−1.000**	0.300
	P	0.104	0.391	0.873	0.104	0.624	0.624	0.624	0.037	< 0.0001	0.624
CAT	ρ	−0.300	−0.400	−0.500	0.300	−0.300	−0.300	0.500	0.600	0.200	−0.800
	P	0.624	0.505	0.391	0.624	0.624	0.624	0.391	0.285	0.747	0.104
MDA	ρ	0.300	0.100	−0.400	−0.300	< 0.0001	−0.300	0.700	0.900*	0.700	−0.700
	P	0.624	0.873	0.505	0.624	1.000	0.624	0.188	0.037	0.188	0.188
AMS	ρ	−0.500	−0.100	−0.600	0.500	−0.800	−0.300	0.300	−0.500	−0.700	0.300
	P	0.391	0.873	0.285	0.391	0.104	0.624	0.624	0.391	0.188	0.624
SOD	ρ	−0.100	−0.500	0.200	0.100	0.400	0.100	−0.100	0.700	0.500	−0.900*
	P	0.873	0.391	0.747	0.873	0.505	0.873	0.873	0.188	0.391	0.037
ACP	ρ	−0.564	−0.154	−0.410	0.564	−0.667	−0.103	0.205	−0.667	−0.821	0.359
	P	0.322	0.805	0.493	0.322	0.219	0.870	0.741	0.219	0.089	0.553
AKP	ρ	−0.300	0.100	< 0.0001	0.300	−0.300	0.200	< 0.0001	−0.900*	−0.800	0.700
	P	0.624	0.873	1.000	0.624	0.624	0.747	1.000	0.037	0.104	0.188

Table 3
The spearman's rank correlation coefficient between the expression level of immune or antioxidant related genes and significantly different bacteria in genus level.

		Lutibacter	Flavirhabdus	Rhodobacteraceae_Unclassified	Rhodobacteraceae_uncultured	Sulfitobacter	Halocynthiibacter	Algibacter	Shewanella	Aquibacter	Vibrio
LZM	P	−0.300	−0.100	−0.600	0.300	−0.500	−0.200	0.800	0.100	−0.200	−0.300
	P	0.624	0.873	0.285	0.624	0.391	0.747	0.104	0.873	0.747	0.624
LEC	ρ	−0.700	−0.900*	0.600	0.700	0.500	0.700	−0.300	−0.100	−0.300	−0.700
	P	0.188	0.037	0.285	0.188	0.391	0.188	0.624	0.873	0.624	0.188
CAT	ρ	−0.200	0.100	−0.900*	0.200	−1.000**	−0.800	0.200	−0.100	−0.300	0.300
	P	0.747	0.873	0.037	0.747	< 0.0001	0.104	0.747	0.873	0.624	0.624
SOD	ρ	−0.500	−0.700	−0.200	0.500	−0.100	−0.100	0.100	0.500	0.100	−0.900*
	P	0.391	0.188	0.747	0.391	0.873	0.873	0.873	0.391	0.873	0.037
p105	ρ	−0.300	−0.600	−0.100	0.300	0.000	−0.200	−0.200	0.600	0.300	−0.800
	P	0.624	0.285	0.873	0.624	1.000	0.747	0.747	0.285	0.624	0.104
p50	ρ	0.100	0.300	−1.000**	−0.100	−0.900*	−0.900*	0.500	0.300	0.100	0.100
	P	0.873	0.624	< 0.0001	0.873	0.037	0.037	0.391	0.624	0.873	0.873
Rel	ρ	−0.800	−0.600	−0.400	0.800	−0.700	−0.300	−0.300	−0.400	−0.700	< 0.01
	P	0.104	0.285	0.505	0.104	0.188	0.624	0.624	0.505	0.188	1.000

different bacteria at the genus level was also explored. The results showed that the expression level of LEC was significantly negatively correlated with *Flavirhabdus* (Spearman's rank correlation coefficient: $\rho = -0.9$, $P = 0.037$; Pearson correlation coefficient: $r = -0.955$, $P = 0.012$); the expression level of CAT was significantly negatively correlated with *Rhodobacteraceae_Unclassified* (Spearman's rank correlation coefficient: $\rho = -0.9$, $P = 0.037$); and the expression level of p50 was significantly negatively correlated with *Rhodobacteraceae_Unclassified* (Spearman rank's correlation coefficient: $\rho = -1.0$, $P < 0.0001$), *Sulfitobacter* (Spearman's rank correlation coefficient: $\rho = -0.9$, $P = 0.037$) and *Halocynthiibacter* (Spearman's rank correlation coefficient: $\rho = -0.9$, $P = 0.037$) (Table 3 and Table S4). These results suggest that there is a close relationship between immune responses and intestinal microbiota of sea cucumber.

4. Discussion

Sea cucumber (*Apostichopus japonicus*) is an important commercially farmed species in northern China because of its high economic and nutritional value [1]. However, disease of sea cucumber is becoming increasingly serious with the rise of sea cucumber aquaculture, leading to large impacts on the sustainable development of sea cucumber aquaculture. Therefore, it is urgent to find feed additives with high immunity for sea cucumber aquaculture. It is well documented that astragalus polysaccharide (APS) can enhance antibacterial, antiviral, antiparasitic activities and modulate microbial composition and species distribution of the intestinal microbiota in aquatic animals, birds and mammals [9,13–16]. In this work, we found that APS can affect the immunity, microbial composition and species distribution of the intestinal microbiota of sea cucumber. However, the growth of sea cucumber was not significantly different among treatment groups, indicating that the growth of sea cucumber is not affected by dietary APS supplementation.

Astragalus polysaccharides (APS) are the most effective components of *Astragalus*, which is a traditional herbal medicine in China. APS has many clinical efficacies, such as antitumour, antibacterial, and antivirus activities; humoral immune response regulation; and cytokine activation [32]. Recent studies have shown that APS can improve immune responses and potentially reduce the risk of diseases, as one kind of effective immunopotentiator in Chinese mitten crab (*Eriocheir sinensis*) [33]. APS combined with probiotics administration in feed displayed synergistic modulation of immunity and intestinal microbiota in chickens [13]. APS can also be used as a safe and effective feed additive and improve the survival rate in aquaculture of Pacific white shrimp (*Litopenaeus vannamei*), but there were no significant differences between various APS diet groups and the control [34]. Dietary supplementation of APS could increase the growth performance, immune organ index and immune activity in husbandry animals, such as broilers, pig, rabbit and cattle [35]. Furthermore, dietary intake of *A. membranaceus* root or its polysaccharides could enhance the immune responses of *A. japonicus* and improve its resistance to infection by *Vibrio splendidus* [16]. In this paper, we also found that dietary supplementation of APS could affect the enzymatic activity and gene expression level of immune- and antioxidant-related genes.

Advances in 16S rRNA sequencing have revealed the intestinal microbiota to be an incredibly complex community, comprising thousands of bacterial species in human and other animals, which has various physiological activities, including roles in metabolism, maturation of the immune system and inhibition of pathogenic bacteria [21,22]. Recent studies showed that cross talk between the innate immune system and endogenous microbiota favours mutual growth, survival and inflammatory control of the intestinal ecosystem. The ability of innate immunity to distinguish between potentially pathogenic microbial components and harmless antigens by “pattern recognition receptors” (PRRs), such as Toll-like receptors (TLRs), enables cells to recognize conserved characteristic molecules present on microorganisms,

described as pathogen-associated molecular patterns (PAMPs), including lipopolysaccharides, peptidoglycans, flagellin, formylated peptides and others. In mammals, TLRs are present on intestinal epithelial cells (ECs), neutrophils, macrophages, dendritic cells (DCs) and other cells belonging to the innate immune system. Many studies have demonstrated that the intestinal microbiota can regulate the innate immune system by modulating TLR expression through PAMPs. Recognition of microbes leads to activation of nuclear factor κB (NF-κB) signalling and consequently triggers cytokine production and upregulation of immunity-related molecules [36–38]. It has been reported that astragalus polysaccharides exert immunomodulatory effects via TLR4-mediated MyD88-dependent signalling and stimulate key nodes in the TLR4-MyD88 pathway, including TLR4, MyD88, TRAF-6, NF-κB and AP-1 [18]. Interestingly, we found that dietary supplementation of APS could affect the expression level of NF-κB, intestinal microbial composition and species distribution in sea cucumber. Furthermore, there is a close relationship between the expression level of NF-κB and the intestinal microbial composition and species distribution, suggesting that the innate immune system and intestinal microbiota may cross talk after dietary APS supplementation. Furthermore, APS might stimulate immunity through the NF-κB signalling pathway in sea cucumber.

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Appendix A. Supplementary data

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