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The effects of benzo[*a*]pyrene on the composition of gut microbiota and the gut health of the juvenile sea cucumber *Apostichopus japonicus* Selenka

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ABSTRACT

The gut microbiota is essential for health and physiological functions in the host organism. However, the toxicological evaluation of environmental pollutants on the gut microbiota is still insufficient. In the present study, the juvenile sea cucumber *Apostichopus japonicus* was exposed for 14 days to Benzo[*a*]pyrene (BaP), which is a model polycyclic aromatic hydrocarbon (PAH), at four different concentrations (0, 0.5, 5, and 25 μg/L). We analyzed the intestinal microbial community of *A. japonicas* using 16S rRNA gene amplicon sequencing. Our results demonstrate that BaP exposure caused alterations to the microbiome community composition in sea cucumbers. At the phylum level, *Planctomycetes* were significantly more abundant in BaP exposure groups at 14 d compared with the control group, and the abundance of *Proteobacteria* and *Bacteroidetes* increased while the abundance of *Firmicutes* decreased following BaP exposure. At the genus level, multiple beneficial and autochthonous genera declined in the BaP treatment groups compared to the control, including *Lactococcus*, *Bacillus*, *Lactobacillus*, *Enterococcus*, *Leuconostoc* and *Weissella*; however, a bloom of alkane-degrading bacteria was found in BaP-exposed guts and included *Lutibacter*, *Pseudoalteromonas*, *Polaribacter*, *Rhodopirellula* and *Blastopirellula*. Furthermore, histological morphology, enzymatic activity and gene expression analysis revealed that BaP exposure also negatively impacted gut structure and function and presented as inflammation or atrophy, oxidative stress and immune suppression in sea cucumber intestines. Collectively, these findings provide insights into the toxic effects of BaP exposure on *A. japonicas* associated with intestinal microbiota and health.

1. Introduction

Trillions of commensal bacteria reside in animal guts and constitute a multifunctional system called the gut microbiota. As an integral part of an animal organ that has coevolved with the host to constitute a complex and symbiotic relationship, it is increasingly recognized that the gut microbiota plays crucial roles in many biological processes, including energy metabolism and storage, immune system modulation, neurotransmission and even behavior regulation [1–4]. According to previous studies, alterations of the bacterial community composition, or dysbiosis, are closely associated with digestive or nondigestive disorders such as inflammatory bowel disease, obesity, allergies and diabetes [5]. The perturbations of the gut microbiota can be caused by various environmental factors such as diet and drugs. And concerns regarding the effects of environmental contaminants on the gut microbiota of animals are growing rapidly due to the serious situation of environmental pollution [6]. Many studies suggest that exposure to environmental contaminants can alter the composition of the gut microbiome and lead to disorders of energy metabolism, nutrient absorption, and immune system function or the production of other toxic symptoms [7–11].

Among the listed environmental contaminants, polycyclic aromatic hydrocarbons (PAHs) are an important class of persistent organic pollutants (POPs). PAH contaminants are attracting renewed attention due to their carcinogenic, mutagenic and toxic effects on various species [12]. PAHs have been widely detected in aquatic ecosystems throughout the world, notably in the sediments of estuaries and coasts, and they are mainly derived from petrogenic pollution, anthropogenic combustion, crude oil spillage and industrial or urban discharges [13–16]. Unfortunately, many of these areas are important mariculture zones in China, which indicates that PAHs could pose a threat to aquatic organisms, especially benthic organisms [17–19]. It was reported that

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Table 1
Growth performance of juvenile sea cucumbers under different concentrations of BaP treatment.

Index	Time	Control	Treat Groups of BaaP		
			0.5 μg/L	5 μg/L	25 μg/L
SR(%)	7d	100 ± 0	99.21 ± 0.79	98.41 ± 1.04	96.83 ± 1.85
	14d	100 ± 0	89.58 ± 0.79*	90.32 ± 1.86**	90.32 ± 1.85**
SPG(%/d)	7d	0.50 ± 0.03	−2.38 ± 0.24**	−3.65 ± 0.20**	−0.46 ± 0.26
	14d	0.55 ± 0.06	−1.32 ± 0.13**	−3.00 ± 0.28**	−0.73 ± 0.10**

Data are mean ± SE. Student's t-test was used to detect the significant differences between the control and the BaaP treatment groups, * represents $p < 0.05$, ** represents $p < 0.01$.

the concentration of PAHs ranged from 3.61 μg/L to 98.21 μg/L in the seawater of the Sanggou Bay, Yellow Sea, China [20].

The sea cucumber *Apostichopus japonicas* (Selenka) belongs to the phylum *Echinodermata*, class *Holothuroidea* and is one of the most important marine economic species in Asian countries due to its great medicinal and nutritional value [21,22]. Given the increasing market demand and the decrease of natural resources, sea cucumber culture industries have rapidly developed [23]. Sea cucumbers are mainly cultured in ponds and coastal shallow waters, and these areas are easily polluted by environmental contaminants such as PAHs. Benzo[*a*]pyrene (BaP), which is one of the well-studied carcinogenic PAHs, is widely used as a reference compound in studies on the toxicity of PAHs in natural communities [24]. The toxic effects of BaP on mollusks, crustaceans and fishes have been well studied, and it has been shown that BaP has adverse effects on growth [25,26], antioxidant and detoxification defenses [27,28], energy metabolism, neurotoxicity, osmotic regulation [29] and reproduction [30]. However, the toxic effect of PAHs on sea cucumber has rarely been studied [31]. In the present study, BaP was selected as a model PAH to investigate the gut microbiota and gut function response of sea cucumber after PAH exposure. The results could provide valuable information for elucidating the detoxification mechanism of BaP toxicity in sea cucumbers and risk assessment of BaP exposure on sea cucumbers.

2. Materials and methods

2.1. Animal culture and benzo[a]pyrene exposure

Healthy juvenile sea cucumbers with an average weight of 5.36 ± 0.14 g were purchased from Oriental Ocean Technology Co., Ltd, (Yantai, China) and were then transported to the lab using an ice box. They were acclimatized for 7 d in 300 L aquariums with aerated seawater prior to the experiment (salinity: 30‰, pH: 7.8–8.3, temperature: 16 ± 2 °C). All of the juveniles were fed with a formula feed of 1.5% of their body weight after the feces and residual food was siphoned every evening. After acclimatization, the sea cucumbers were randomly allocated to four groups exposed to different concentrations (0, 0.5, 5, and 25 μg/L) of BaP, and each treatment had three replicates with 30 individuals in each 40 L tank. The BaP (96% purity, Sigma-Aldrich, Saint-Quentin Fallavier, France) was dissolved in acetone first and then proportionately diluted with sea water to ensure the final concentration of acetone in the seawater was lower than 0.001% in all replicate tanks (the pre-experiment results and previous study all indicated that the acetone concentration of 0.001% had no side effect on sea cucumbers) [31]. During the experiment, the juveniles were routinely managed as the acclimatization period and sea water was renewed daily. Fifteen individuals of each group were sacrificed at 1d, 7 d and 14 d after exposure. The intestine tissue and intestinal contents of the sampled sea cucumbers were aseptically dissected and stored in liquid nitrogen for later analysis. Additionally, the body weight and survival rate of sea cucumbers was also recorded at each sampling time. The survival rate (SR) and special growth rate (SGR) were calculated according to the following formulas:

$$SR(\%) = \frac{S_t}{S_0} \times 100$$

$$SGR(\%) = \frac{\ln W_t - \ln W_0}{t} \times 100$$

Noted: S_t: survival numbers of the sea cucumber at the end of the experiment; S_0: survival numbers of sea cucumbers at the beginning of the experiment; W_t: wet weight of sea cucumbers at the end of the experiment; W_0: wet weight of sea cucumbers at the beginning of the experiments.

2.2. Tissue sections for microscopic analysis

Nine sea cucumbers were collected from each treatment group after experiment started 7d and 14d and segments of the foregut were fixed in Bouin's fixative for 24 h. The samples were dehydrated in different concentrations of ethanol (70%, 75%, 85%, 95% and 100%) followed by a transparency treatment with xylene, and then they were embedded in paraffin wax. Then, 5 μm sections were transversely sliced and stained with hematoxylin and eosin (H&E). Stained slides were observed under an optical microscope (Olympus DP72, Japan), and the thickness of the intestinal layers was measured by DP2-BSW software using the distance-measuring tool.

2.3. Enzyme analysis

The intestine tissues of four juvenile sea cucumbers from each treatment group after experiment started 1d, 7d and 14d were weighed and homogenized with 0.8% physiological saline and centrifuged at 2500 × *g* at 4 °C for 15 min, and the supernatant was collected for enzyme analysis. A series of antioxidant and immune enzyme activities were assayed, including phosphatase (ACP) activity, alkaline phosphatase (AKP) activity, superoxide dismutase (SOD) activity and malondialdehyde (MDA) content, and they were determined using assay kits (Nanjing Jiancheng Institute, China) according to the manufacturer's instructions. One unit of ACP and AKP activity was defined as the amount of enzyme required as 1 mg of phenol is liberated at 37 °C (U/mg protein/30 min); one unit of SOD activity was defined as the amount of enzyme required when the inhibition rate reached 50% in a 1 mL reaction system at 37 °C (U/mg protein/min); the MDA concentration was measured by the thiobarbituric acid-reactive substance assay and was expressed in μmol per milligram protein. The results were recorded on a microplate reader (Epoch, BioTek, USA) according to the instructions of the manufacturer.

2.4. Quantitative real-time PCR

For gene transcription analysis, total RNA was extracted from the four juvenile sea cucumber intestinal tissues from each treatment group after experiment started 1d, 7d and 14d using RNAiso Plus reagent following the manufacturer's instructions (TaKaRa, Dalian, China), and samples were treated with RNase-free DNase (TaKaRa, Dalian, China). The RNA integrity was confirmed by electrophoresis on 1% agarose

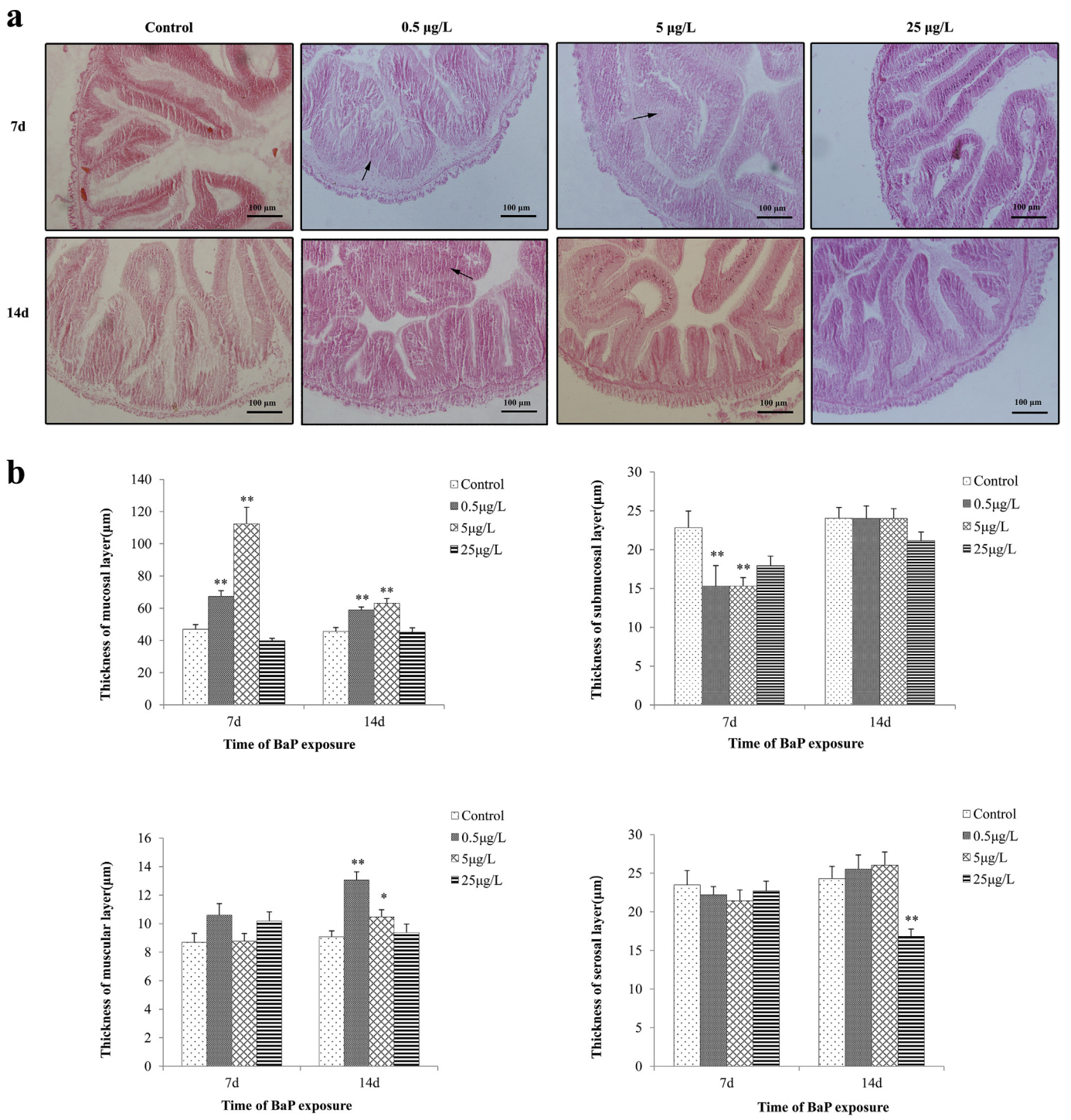

Fig. 1. Histological morphology of the intestine of sea cucumbers treated with BaP. (a) Representative hematoxylin and eosin histology of the foregut of *A. japonicas* in control and BaP exposed groups at three concentrations of 0.5, 5, 25 μg/L for 7d and 14d. Scale bar: 100 μm. The black arrows indicate the swollen mucous layer in BaP exposed groups. (b) Changes in the thickness of four intestinal layers. Student's t-tests were used to detect significant differences between the control and BaP exposed groups, * represents $p < 0.05$, and ** represents $p < 0.01$. The values indicate mean ± SE (N = 9).

gels, and the RNA concentration was measured by micro-spectrophotometer NanoDrop 1000 (Thermo, USA). The first strand cDNA was synthesized with a PrimeScript™ RT reagent kit with gDNA Eraser (TaKaRA, Dalian, China), starting with 500 ng total RNA in each reaction. The gene expression patterns of several important immune-related genes, including *lysozyme (LZM), C type lectin (CLEC), NF-kB1 (p105)* and *glutathione S-transferase (GST),* were determined by quantitative real-time PCR (qPCR) on an Applied Biosystems StepOne Real-time PCR System (Applied Biosystems, USA). The primers of the above genes were designed using Primer 3 online (http://frodo.wi.mit.edu/) (Table S1), and the *β-tubulin (TUBB)* gene was used as a reference control gene as in a previous study [36]. The qPCR was conducted in a total volume of 20 μL using a SYBR Green® real-time PCR assay (SYBR PrimeScript™ RT-PCR Kit II, TaKaRA), including 10 μL of SYBR Green Master Mix, 0.8 μL of each forward and reverse primer (10 μM), 2 μL of diluted cDNA, 0.4 μL ROX Reference Dye (50 ×) and 6 μL RNase-free water) as

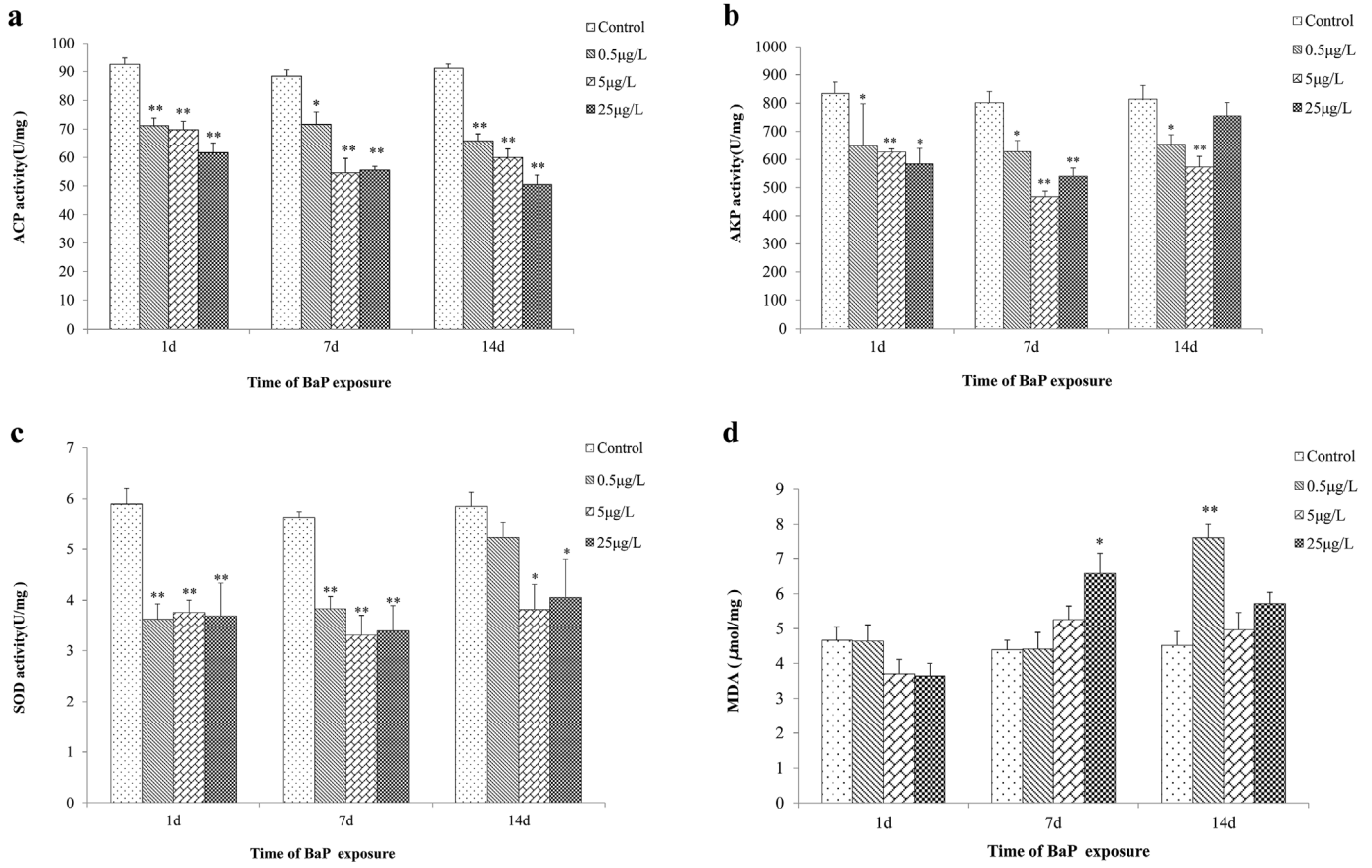

Fig. 2. Influence of BaP on the immune-related and antioxidant enzymes. (a) phosphatase (ACP) activity, (b) alkaline phosphatase (AKP) activity, (c) superoxide dismutase (SOD) activity, and (d) malondialdehyde (MDA) content. Student's t-tests were used to detect significant differences between the control and BaP exposed groups, * represents $p < 0.05$, and ** represents $p < 0.01$. Data were presented as the mean ± SE (N = 4).

follows: 95 °C for 30 s, 40 cycles at 95 °C for 5 s, and 60 °C for 30 s. A melting curve analysis of the amplification products was performed to confirm that the unique PCR product was amplified and detected. Cycle threshold (Ct) values were recorded for further analysis.

2.5. Microbiota analysis by 16S rRNA gene sequencing

2.5.1. DNA extraction

Genomic DNA was extracted from three individual intestinal contents of each treatment group after experiment started 7d and 14d using a FastDNA® Spin Kit for Soil (MP Biomedicals, Solon, GA, USA) according to the manufacturer's protocol. The DNA concentration and purity were monitored on 1% agarose gels. Then, the DNA was diluted to 1 ng/μL using sterile water according to the measured concentration.

2.5.2. 16S rRNA gene amplification and sequencing

The V4–V5 fragment of the 16S rRNA gene was amplified with a specific primer combination of 515F (5′-GTGCCAGCMGCCGCGG TAA-3′) and 907R (5′- CCGTCAATTCMTTTRAGTTT-3′) with a barcode, and the mixtures of the PCR products were purified with a Gene JET™ Gel Extraction Kit (Thermo Scientific, NY, USA). Then, the sequencing libraries were generated using a 48 reaction Ion Plus Fragment Library Kit (Thermo Scientific, NY, USA) following the manufacturer's recommendations, and they were sequenced on an Ion S5™ XL platform.

2.5.3. Bioinformatic data analysis

Raw reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence and then filtered according to the Cutadapt quality control process and the UCHIME algorithm to obtain high-quality clean reads [32,33].

Sequence analyses were performed by Uparse software to assign OTUs (operational taxonomic units) using the average neighbor algorithm at a 97% similarity level, and for each representative sequence, the Silva Database was used based on the Mothur algorithm to annotate the taxonomic information [34,35]. QIIME software was used to calculate the alpha diversity through 6 indices including Observed-species, Chao1, Shannon, Simpson, ACE and Good-coverage. 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 was conducted by QIIME software. Principal coordinate analysis (PCoA) was performed to obtain the principal coordinates and visualize from the complex and multidimensional data. A distance matrix of weighted Unifrac among the samples was obtained before they were transformed to a new set of orthogonal axes, by which the maximum variation factor is demonstrated by the first principal coordinate and the second maximum factor by the second principal coordinate and so on. PCoA analysis was displayed by the WGCNA package, stat packages and ggplot2 package in R software.

2.6. Statistical analysis

All of the results are presented as the mean ± standard error (SE). The Student's t-test was used to independently compare each group treated with BaP and the control group. All of the statistical analyses were conducted using SPSS software (version 18.0, Chicago, IL, USA). A value of $P < 0.05$ was accepted as the criterion for statistical significance.

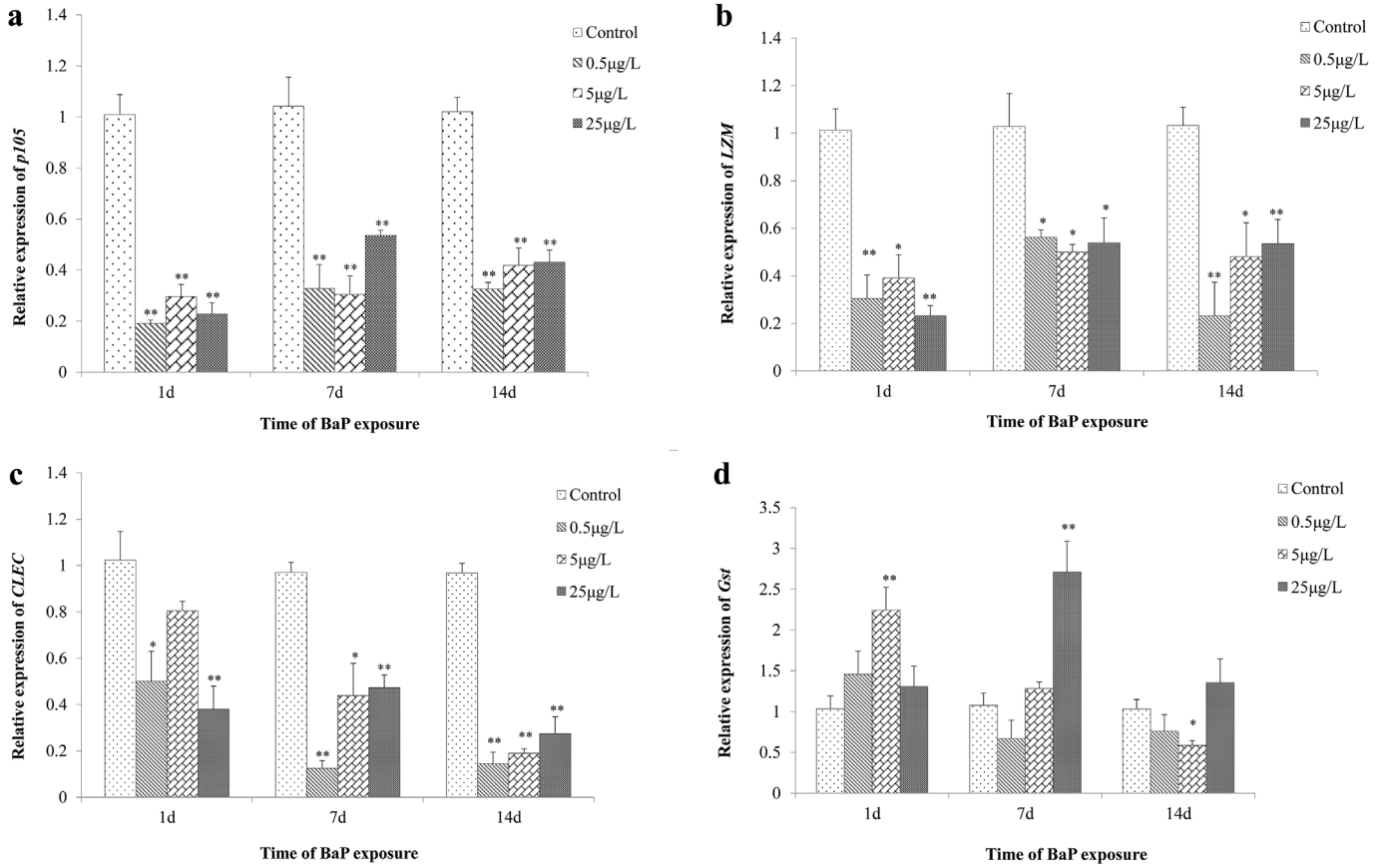

Fig. 3. Influence of BaP on the relative mRNA expression of immune-related genes.(a) *NF-kB1 (p105)*, (b) *lysozyme (LZM)*, (c) *C type lectin (CLEC)*, (d) *glutathione S-transferase (GST)*. Student's t-tests were used to detect significant differences between the control and BaP exposed groups, * represents $p < 0.05$, and ** represents $p < 0.01$. Data were presented as the mean ± SE (N = 4).

Table 2
A Illumina high-throughput sampling depth, richness and diversity index of bacterial community in the gut of sea cucumber under different concentrations of BaP treatment.

	Control	Treat Groups of BaP					
		0.5 μg/L		5 μg/L		25 μg/L	
	CT	L7d	L14d	M7d	M14d	H7d	H14d
Sampling Depth							
mean sequences	72,223	76,488	76,751	76,316	70,054	62,856	72,507
OTUs	579	719	853	585	658	416	601
goods_coverage	99.87%	99.83%	99.87%	99.83%	99.83%	99.87%	99.83%
Richness Estimators							
Chao1	366.40 ± 25.20	495.82 ± 23.35**	530.59 ± 19.46**	434.47 ± 9.76	475.43 ± 53.48*	297.31 ± 28.64	413.61 ± 12.89
ACE	371.89 ± 22.89	496.59 ± 24.44**	517.49 ± 13.55**	450.05 ± 6.33	486.18 ± 53.07*	304.13 ± 25.65	427.31 ± 13.68
Diversity Estimators							
Shannon	3.83 ± 0.81	4.75 ± 0.26	4.93 ± 0.40	4.98 ± 0.44	4.71 ± 0.68	3.18 ± 0.62	3.97 ± 0.17
Simpson	0.77 ± 0.09	0.88 ± 0.02	0.86 ± 0.04	0.90 ± 0.04	0.87 ± 0.06	0.70 ± 0.09	0.80 ± 0.01

Data are mean ± SE. Student's t-test was used to detect the significant differences between the control and the BaP treatment groups, * represents $p < 0.05$, ** represents $p < 0.01$.

3. Results

3.1. Survival rate and growth performance

The growth and survival performance of sea cucumbers during the experiment is shown in Table 1. In this study, no sea cucumber death was observed in the control group. The results indicate that the survival rate of juveniles decreased as exposure time was extended, and the 0.5 μg/L BaP treatment group had the lowest survival rate of 89.58% at 14 d. Meanwhile, BaP also seriously inhibited sea cucumber growth with significantly lower SGR of sea cucumbers exposed to BaP compared to those in the control group ($P < 0.05$), and a complete negative growth was revealed in all BaP exposure groups.

3.2. Influence of BaP exposure on the intestinal structure

To detect the influence of different concentrations of BaP exposure on the intestinal structure, foregut sections by H&E staining were examined by light microscopy, and the thickness of the layers were measured. The results indicate that after 7d of BaP exposure, the

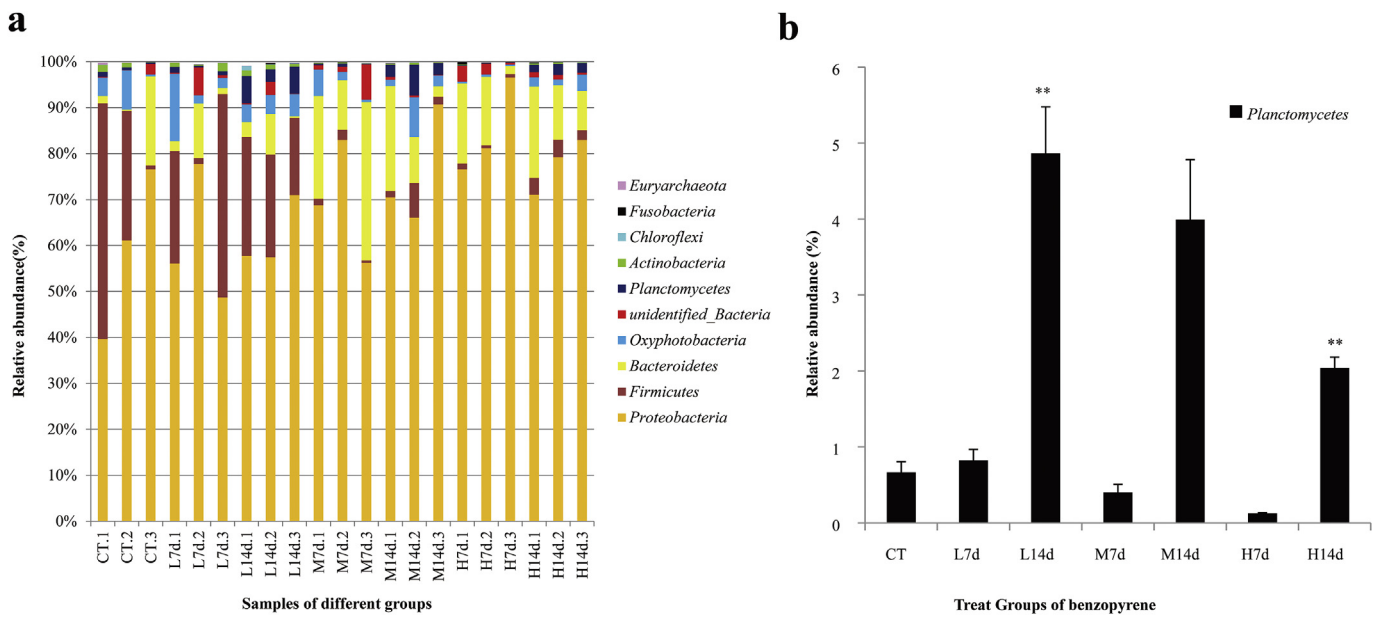

Fig. 4. Comparison of the relative abundances of major bacterial phyla between BaP exposed groups and the control group. (a) Relative abundance of the top 10 phylum level bacterial communities in different samples. (b) The significant different phylum between the control and BaP exposed groups. Student's t-tests were used to detect significant differences between the control and BaP exposed groups, * represents $p < 0.05$, and ** represents $p < 0.01$.

foregut tissues in the low and medium concentrations of BaP exposure groups (0.5 μg/L and 5 μg/L, respectively) appeared more swollen than the control group under light microscopy, while the tissue showed a light atrophy in the high concentration of BaP exposure group (25 μg/L) (shown in Fig. 1). After BaP exposure for 14d, the morphological changes began to alleviate and there was recovery in all exposure groups. Moreover, there was a significant difference in the thickness of the four intestinal layers between the control group and BaP treatment groups. The thickness of the mucosal and muscular layers increased significantly in the low and medium concentrations of BaP exposure groups (0.5 μg/L and 5 μg/L), while the serosa layer was significantly thinner in high concentration of BaP exposure group (25 μg/L) than in the control groups ($P < 0.05$).

3.3. Effects of BaP exposure on nonspecific immune reactions in the gut

The activities of enzyme ACP, AKP, SOD, and MDA contents in intestines of sea cucumbers are shown in Fig. 2. The results indicate that BaP exposure could lead to a significant decrease of ACP activity compared to the control group ($P < 0.05$) (shown in Fig. 2a). Similarly, AKP and SOD activity in the BaP treatment groups were also significantly lower than that in the control groups after 1d and 7d of exposure ($P < 0.05$, shown in Fig. 2b and Fig. 2c). However, the AKP and SOD activity seemed to recover after 14d exposure, and SOD activity even recovered to a similar level of the controls in the low concentration of the BaP exposure group (0.5 μg/L). As shown in Fig. 2d, the MDA contents in the intestine of the 0.5 μg/L and 25 μg/L BaP-treated sea cucumbers were significantly higher than the control group after 7d and 14d of exposure ($P < 0.05$).

The transcriptional expression of immune-related genes, including *LZM*, *CLEC*, *p105* and *GST,* in the intestine of *A. japonicus* is presented in Fig. 3. The relative expression levels of *LZM*, *CLEC* and *p105* were all significantly downregulated in the BaP treatment groups compared to the control group, and this significant expression inhibition continued to the end of the experiment ($P < 0.05$). In contrast, the expression levels of *GST* were significantly upregulated in medium and high concentrations of BaP exposure groups (5 μg/L and 25 μg/L) compared to the control group after 1d and 7d of exposure ($P < 0.05$).

3.4. The gut microbial community shifts exposure to BaP

3.4.1. Illumina sequencing

We assessed the gut microbiota of sea cucumbers using high-throughput sequencing of the V4–V5 region of the 16S rRNA gene. As shown in Table 2, a total of 1,521,583 optimized reads were obtained from the 21 gut content samples of juvenile sea cucumbers (216,699 reads of the control group and 1,304,914 reads of the BaP treated groups), which resulted in a total of 1285 OTUs (579 mean OTUs of the control group and 639 mean OTUs of the BaP treated groups). The Venn diagrams reflect that 379, 305 and 248 common OTUs were identified from the control and the BaP treatment groups in Fig. S1. The average length of the reads was 373 bp, and all of the sequences were identified as bacteria. In addition, these data revealed that the Good's coverages of all samples were ≥99.8% (Table 2). The rarefaction analysis showed that all of the gut content samples tended to approach the saturation plateau, which indicated that the sequencing depth was sufficient to cover the microbial diversity in all of the samples (Fig. S2). All sequences in this study were deposited into the NCBI database with GenBank accession nos. SRP158347.

The bacterial richness and diversity were determined by the ACE and Chao1 indices and the Shannon and Simpson diversity indices, respectively. As shown in Table 2, the ACE and Chao1 indices increased significantly in the 0.5 μg/L of the BaP treated groups at 7d and 14d and 5 μg/L of the BaP treated groups at 14d compared with the control subjects ($P < 0.05$). The Shannon and Simpson diversity indices have no significant differences between the BaP treatment groups and the control group.

3.4.2. Effects of BaP on the intestinal bacterial composition

Thirty different bacteria phyla were identified from all of the juvenile sea cucumber samples and the relative abundance of each phylum is shown in Table S2. At the phylum level, the phylum *Proteobacteria* made up the majority of all sequences (mean relative abundance ± SE, 69.94% ± 3.71%), and *Firmicutes* (11.53% ± 4.43%), *Bacteroidetes* (10.78% ± 2.37%), *Oxyphotobacteria* (3.48% ± 0.71%), and *Planctomycetes* (1.85% ± 0.71%) were also detected in the gut microbiota of sea cucumbers (seen in Fig. 4a). Exposure to both 5 μg/L and 25 μg/L BaP increased the abundance of

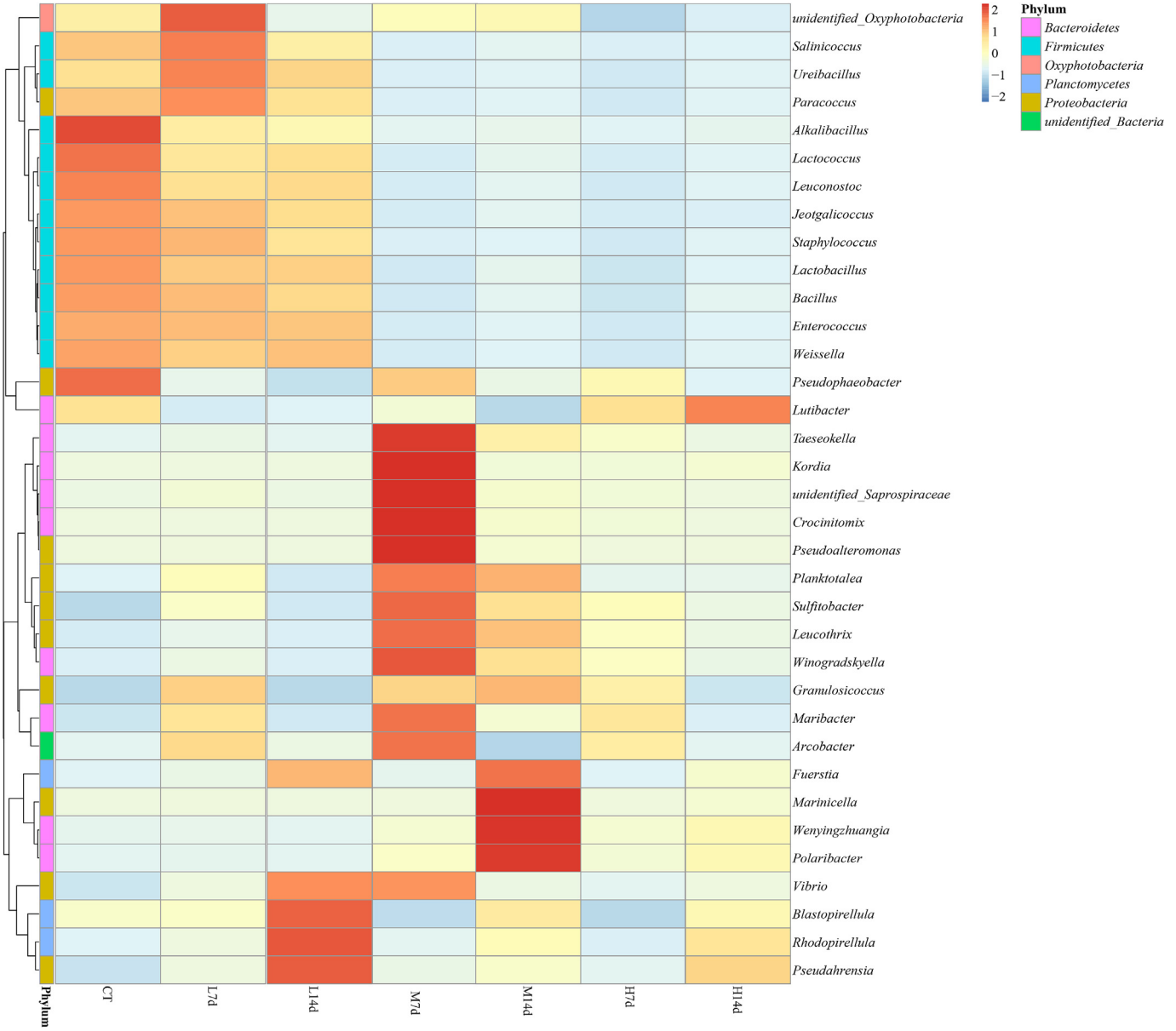

Fig. 5. A hierarchical clustering heat map at the genus level between different BaP exposed groups and the control group. The horizontal column lists the group information, and the vertical column lists the genus annotated by Silva. The heatmap plot depicts the relative abundance of each genus (vertical-axis clustering) within each group (horizon-axis clustering). The Z values, based on the abundance of genus sequences after our normalization, are indicated by color intensity with the legend indicated at the top right corner. The abbreviations are the same as in Table 2. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article)

Proteobacteria and *Bacteroidetes* in the intestine but resulted in a decrease of the abundance of *Firmicutes* (Fig. 4a). Most notably, the relative abundance of *Planctomycetes* was significantly higher ($P < 0.01$) in the BaP exposure groups compared with the control group at 14d (Fig. 4b).

At the species level, a total of 405 genera were identified in the microbiota of sea cucumber gut contents. A hierarchically clustered heatmap of the microbiota composition in the different groups of sea cucumbers is shown in Fig. 5. The abundance of the microbial taxa in the intestine of BaP-exposed sea cucumber differed remarkably from that in the control group (seen in Table S3). Compared with the control group, the relative abundance of multiple beneficial and autochthonous genera declined in the BaP treatment groups. Specifically, a decreased ratio of *Lactococcus*, *Bacillus*, *Lactobacillus*, *Enterococcus*, *Leuconostoc*, *Weissella* and *Alkalibacillus* was observed in the BaP treatment groups, especially in the medium and high concentrations of BaP groups. In contrast, the relative abundance of some oil biodegradation related genera increased in the sea cucumbers exposed to BaP such as *Lutibacter*, *Kordia*, unidentified *Saprospiraceae*, *Crocinitomix*, *Pseudoalteromonas*, *Planktotalea*, *Polaribacter*, *Rhodopirellula* and *Blastopirellula*. Meanwhile, there were more opportunistic pathogens such as *Vibrio*, *Leucothrix* and *Arcobacter* presented in the BaP treatment groups compared to the control group.

3.4.3. Bacterial community analysis and comparison

The principal coordinate analysis (PCoA) of weighted UniFrac distances was performed to compare the bacterial community composition of gut microbiota samples from the BaP treatment groups. The results, which are shown in Fig. 6a, indicate that the samples from the control group and the low concentration BaP groups, the medium concentration BaP groups and the high concentration BaP groups were clustered separately into three sets by the second principal component (PC1) axis

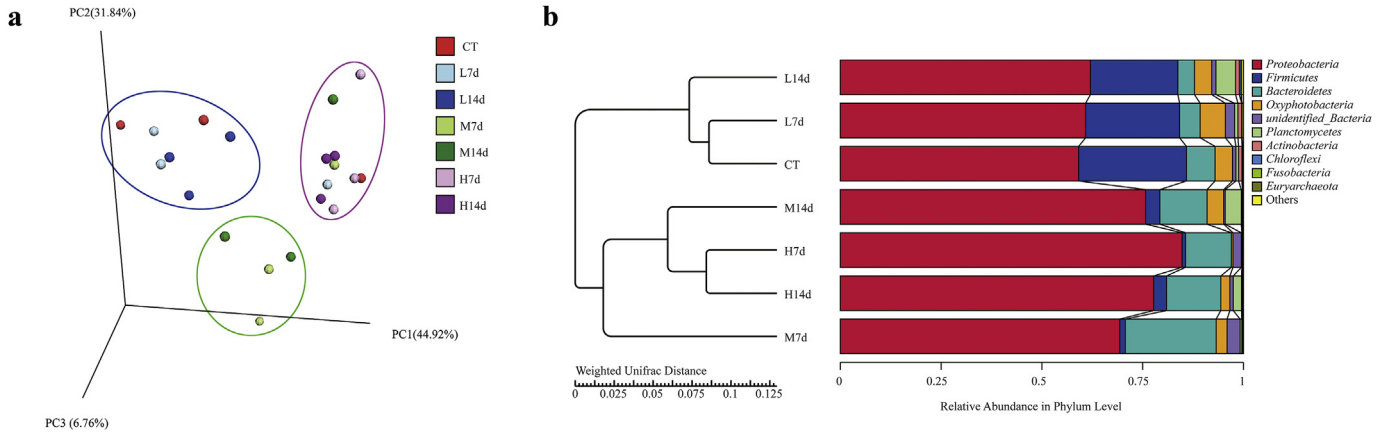

Fig. 6. Relationships among microbiota of the control and BaP treatment groups. (a) A principal component analysis (PCA) showing the separate distribution of gut microbiota composition on the plot between different BaP exposed groups and the control group. The PCA1, PCA2 and PCA3 axes explain 6.76%, 31.84% and 44.92% variation, respectively. (b) The UPGMA clustering tree at the phylum level among different groups. The abbreviations are the same as in Table 2.

and accounted for 44.92% of the total variance. Consistent with the results of the PCoA analysis, the UPGMA clustering tree also confirmed that the taxonomic composition of the control group was closest to the low concentration BaP groups (L7d and L14d), and the gut microbiota from the medium and high concentration BaP treatments (H7d, H14d, M7d and M14d) were clustered into one set in Fig. 6b.

4. Discussion

PAHs have aroused significant environmental concerns due to their mutagenicity, hepatotoxicity, teratogenicity and carcinogenicity, and benthic invertebrates have a high accumulating capability and can present as sentinel indicators of aquatic pollution [19,37]. In this study, we evaluated the effects of a typical PAH (BaP) on sea cucumber juveniles at the level of the gut microbiota and function.

In our present study, sea cucumbers exposed to BaP exhibited poorer growth performance and lower survival rates than the control group, which is consistent with previous studies of the toxicity of PAHs [25,26,38–40]. Typically, toxicants first affect the behavior, growth responses and then this leads to mortality [25]. Consistent with our results, the growth suppression of PAHs also acted on other aquatic animals, such as Chinese mitten crab *Eriocheir sinensis*, Chinook salmon *Oncorhynchus tshawytscha* and clam *Meretrix meretrix* [25,26,38]. However, the sensitivity of the aquatic animals to BaP was different, e.g., the coral *Porites astreoides* larvae was sensitive to only 10 μg/L BaP exposure [41], but more than 100 μg/L of BaP could significantly inhibit the survivorship of larval *M. meretrix* [25]. For juveniles of *A. japonicas* in our study, BaP did not seem to show high toxicity since the survival rate still reached more than 90% after 14d exposure of 25 μg/L of BaP. However, disparate results of survival rate and weight were observed in Li's study [31], which possibly due to geographical differences of the tested individuals.

Histopathologic analysis revealed that low concentration of BaP caused obvious inflammation in the intestine of juvenile sea cucumber, and high concentration of BaP resulted in intestinal atrophy at 7 d. However, the above symptoms have an obvious remission after 14d exposure. These results were consistent with those of Ribière et al. [42], who reported that moderate inflammatory signs were also observed in the ileal and colonic mucosa of mice exposed to BaP. In fact, the intestine is a major target organ for BaP toxicity and metabolism, and BaP-DNA adducts and P450 enzyme-mediated metabolism of absorbed xenobiotics have been demonstrated in the small intestine of mice that were exposed to BaP [43,44]. Since the intestinal epithelium is an important barrier in the interface between the external environment and the immune system, the impairment of the intestinal structure (especially the mucus layer) would probably damage the immune function and increase susceptibility to inflammation in the gut.

In our study, we further investigated the gut immune response of *A. japonicas* under BaP exposure. Following exposure to BaP, decreased levels of ACP and AKP activities and down regulation of the immune-related genes (*LZM, p105,* and *CLEC*) was found in the gut of *A. japonicas*, which suggests the toxic effects of BaP on sea cucumber innate immunity of the intestine. Li et al. [31] also revealed a general trend of down-regulation in expression patterns of important innate immune related genes in the body wall tissue of *A. japonicas*, with the increased BaP concentration exposure. Consistent with our results, *LZM* and *CLEC* were also observed down-regulated at all BaP concentrations in their study, however, the up-regulated expression of gene *p105* was observed at all BaP concentrations in body wall, which suggested NF-kB transcription factor might play an important role in the body wall of sea cucumbers under BaP stress. Similarly, immune suppression was also induced by BaP exposure in various aquatic animals such as crustaceans, fish and mollusks [26,45–47].

Generally, increased host reactive oxygen species (ROS) is a common toxicological effect caused by many environmental pollutants, and the antioxidant capacity of detoxifying ROS depends on a set of antioxidant enzymes and cellular molecules [48]. In this study, the SOD activity, as a widely used marker of antioxidant defense, showed significant inhibition in all of the BaP-exposed sea cucumbers. This could signal ROS overproduction at the risk of oxidative damage to cellular macromolecules [49]. Moreover, the upregulation of *Gst* mRNA transcripts was induced by medium and high concentrations of BaP after 7d exposure, which is consistent with previous results [28]. Since GSTs are members of a protein superfamily involved in phase II xenobiotic detoxification against reactive electrophilic compounds, including a range of xenobiotics like PAHs [50], the results in our study suggest that an organism exposed to BaP might be under the pressure of oxidative damage. Furthermore, the increase of MDA content in sea cucumbers after BaP exposure indicated that enhanced lipid peroxidation was induced by an excess of ROS. This result was concordant with previous studies that BaP could also cause lipid peroxidation in marine invertebrates such as white shrimp *Litopenaeus vannamei* [27], scallops *Chlamys farreri* [51] and mussels *Mytilus galloprovincialis* [52].

Intestinal microbiota is essential for host health, and emerging evidence has shown that gut microbiota is very sensitive to environmental pollutants. Recent studies reveal that different kinds of environmental pollutants can alter the composition of the gut microbiome, which leads to gut microbiota dysbiosis and multiple potential adverse effects [53]. In our current study, BaP exposure did not seem to have an obvious impact on the richness and diversity of the intestinal flora in sea cucumbers, and there was even an increase in the bacterial richness in the low and middle concentrations of BaP-exposed sea

cucumbers. In agreement with our results, no significant difference of the bacterial richness or diversity was found in mice exposed to Bap, PCB or heavy metals when compared with those of the control group [42,54,55].

However, BaP can significantly alter the gut microbiota composition of sea cucumbers. At the phylum level, the most abundant phyla were *Proteobacteria*, *Firmicutes* and *Bacteroidetes* in the intestine in all individuals of *A. japonicas*, which is consistent with the results of María Pagán-Jiménez et al. in another sea cucumber species *Holothuria glaberrima* [56]. Furthermore, our data suggest that the medium and high concentrations of BaP-treated sea cucumbers had a substantial reduction in *Firmicutes* and an increase in *Bacteroidetes,* which is similar with the taxonomic changes in mice exposed to other pollutants such as 2,3,7,8-tetrachlorodibenzofuran (TCDF) and Cd [53,57]. Since previous studies have demonstrated that an elevated *Firmicutes/Bacteroidetes* ratio was associated with weight gain and metabolic disorders [58]. Therefore, these changes in microbiota composition suggest that the energy metabolism might be affected by BaP exposure in sea cucumbers [59]. Meanwhile, a significant increase in the abundance of the *Planctomycetes* phylum was observed in *A. japonicas* after BaP exposure for 14 d. Previous studies have reported that the *Planctomycetes* phylum bear the ability to utilize hydrocarbon compounds as source of carbon energy and to degrade alkane due to their alkane hydroxylase-related genes identified in the genomes [60,61]. These results support the potential role of the gut microbiota in BaP degradation.

At the genus level, medium and high concentrations of the BaP treatment inhibited the abundance of several beneficial genera including the widely used probiotic bacteria (*Lactococcus, Bacillus* and *Lactobacillus*) and important commensal bacteria such as *Enterococcus*, *Leuconostoc* and *Weissella.* In fact, *Weissella* and *Leuconostoc* show antimicrobial activity and saccharide metabolism abilities, which play an important role in ingested detritus digestion and immune defense [62–65]. The decrease of beneficial bacteria induced by BaP treatment was consistent with the increased inflammation and impaired immunomodulatory function of the intestine in sea cucumbers. In contrast, the abundance of *Vibrio*, *Leucothrix* and *Arcobacter*, as the common pathogenic bacteria causing severe disease outbreaks in aquaculture facilities [66–68], increased in the gut when exposed to BaP in this study. This finding suggests that BaP exposure may cause sea cucumbers to be more vulnerable to infections and this might account for the mortality in the BaP treatment.

Interestingly, the bloom of a serious of alkane-degrading bacteria was found mainly in the low and medium concentrations of BaP groups. *Lutibacter*, *Kordia*, *Polarbacter*, *Marbacter* and *Wingogradskyella* all belong to the family *Flavobacteriaceae*, which is reported as a prominent degrader of high-molecular-weight organics in the marine system [69], and various members of *Flavobacteriaceae* were widely distributed in the marine snow with phytoplankton detritus and sediments exposed to crude oil [70–72]. *Pseudoalteromonas* and *Pseudomonas* are capable of highly efficient aromatic degradation and commonly found in the oil contaminated marine environments [73]. The *Planktotalea* and *Sufitobacter*, which are affiliated with the family *Rhodobacteraceae*, were abundant in seawater with oil-containing feces and this suggests their significant role in oil spill degradation [74]. The *Sparosiraceae* family members play an important role in breaking down of complex organic compounds and are frequently used in the sludge wastewater treatment systems. *Crocinitomix* is associated with nitrogen removal in the biodegradation process. From the results, we infer that the gut microbiota may have a protective role for the host by BaP degradation and detoxification in the mild BaP exposure, which accounts for the late recovery of the intestinal structure and function in sea cucumbers. Nevertheless, this remains hypothetical and needs to be confirmed in follow-up studies.

Under BaP stress, some abnormalities in the intestinal structures and functions were observed in sea cucumber. Histopathologic analysis revealed altered inflammatory response and impaired integrity of the epithelial barrier in the intestine of sea cucumber after exposure to BaP, suggesting an increased permeability of gut bacteria [75,76]. Furthermore, suppressed immune system and disrupted oxidative homeostasis to the intestines of the sea cucumbers indicated exposure to BaP might influence the normal gut defense function. Through the assessment of these sensitive biomarkers as a whole, it is obviously that the overall health of sea cucumber intestines was severely impacted by BaP. In the meantime, BaP could significantly shift the composition of the gut microbial community in *A. japonicas*, and the dysregulation of gut microbiota may contribute to or result from an abnormal intestinal environment [76]. For example, Ribière et al. [42] reported 28 days of oral BaP exposure led to a pro-inflammatory intestinal environment in C57BL/6 mice, with a shifted gut microbiota composition of more pro-inflammatory bacterial taxa. And correlation analysis in Chen's study also found that the oxidative stress after nano-TiO2 and BPA co exposure was tightly associated with the imbalanced ratio of pathogenic *Lawsonia* and normal metabolic *Hyphomicrobium* [9]. However, there is lack of definite evidence correlating alterations in gut bacteria with the intestinal responses and functions in *A. japonicas* after exposure to BaP in the present study. Hence more study is needed to elucidate the complicated mechanisms of xenobiotic detoxification and to estimate the contribution of gut microbiota in this process.

5. Conclusions

In summary, our results indicate that BaP exposure could significantly modulate the gut microbial composition in juvenile sea cucumbers at both the phylum and genus levels. Additionally, the health and function of sea cucumber intestines was impaired by BaP as shown by alterations in histology, enzymatic activity and gene expression, which also suggests that BaP affected inflammation, oxidative stress and immune suppression following exposure. The increasing bacteria related to BaP degradation and detoxification in exposed guts indicated the protective function of symbiotic intestinal bacteria, and more mechanistic work is warranted to further elucidate the complicated interactions of gut microbiota and organic pollutants.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.07.073.

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