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High-throughput sequencing of 16S rRNA amplicons characterizes gut microbiota shift of juvenile sea cucumber *Apostichopus japonicus* feeding with three antibiotics*

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Abstract Sea cucumber *Apostichopus japonicus* is an important marine economic species in Asian countries due to its profound nutritional and medicinal value. So far, with the rapid development of intensified artificial aquaculture of sea cucumbers, the use of antibiotics is still an inexpensive and dispensable way to treat pathogenic infections, especially during the nursery phase. However, there is little information on the effects of antibiotics on the intestinal microbiota of sea cucumber. Therefore an Illumina based sequencing method was used to examine the intestinal bacterial composition of juvenile *A. japonicas* following diets with three typical antibiotics (tetracycline, erythromycin, and norfloxacin) under 15, 30, and 45 d. The findings reveal that different antibiotics have distinct effects on the growth performance of juvenile sea cucumbers. However, the richness and diversity of microbiota were barely affected by antibiotics but the community composition alterations indicated that the three antibiotics exhibited their respective patterns of reshaping the intestinal bacteria of juvenile sea cucumbers. In common, the abundance of some sensitive genera with helpful functions, such as *Thalassotalea*, *Shewanella*, *Sulfitobacter*, and *Halomonas* decreased significantly with exposure to antibiotics and the abundance of multiple potential pathogenic- and suspected antibiotic-resistant microorganisms like *Arcobacter*, *Leucothrix*, and *Clostridium_sensu_stricto_1* was found increased significantly in the antibiotic groups. These results suggest that low doses of antibiotics could affect the composition of the intestinal microbiota of sea cucumbers and might increase the risk of infection of the hosts. This study could help us to explore how antibacterial compounds modify the gut microbiota of sea cucumbers and provide theoretical guidance in hatchery management by scientific antibiotic use in sea cucumber mariculture.

Keyword: gut microbiota; sea cucumber; antibiotic; 16S rRNA gene; illumina sequencing

1 INTRODUCTION

The intestine is a multifunctional organ system inhabited by trillions of bacteria. The gut provides a rich environment for supporting microbial survival, and the microbiota plays an important role in the health and well-being of host organisms. Over the last 15 years, scientists have demonstrated that gut microbiota is involved in a multitude of important physiological processes of host organisms, including nutrition absorption, energy sources synthesis, glucose and fat metabolism, immune defences, and even the lifespan regulation (Topping and Clifton, 2001; Flint et al., 2012; Tremaroli and Bäckhed, 2012; Schuijt et al., 2016; Han et al., 2017). Therefore, gut microbiota and hosts actually constitute a complex and symbiotic "super organism" to orchestrate host physiology and pathology. The alterations of the gut community could cause the imbalance of the host-microbial symbiosis and further trigger a horrifying list of diseases, including both intestinal and extra-intestinal disorders such as inflammatory bowel

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disease, asthma, metabolic syndrome, diabetes, allergies, cardiovascular disease and even obesity (DiBaise et al., 2008; Vijay-Kumar et al., 2010; Carding et al., 2015; Stiemsma and Turvey, 2017).

Alterations of a gut microbiota composition can be resulted from various environmental factors, such as diets, pathogens, toxins, and drugs. Among them, the antimicrobial agents are the most dramatic and complex factor and have recently attracted much attention. As the most influential innovation of the 20th century, antibiotics are widely and heavily used for public health and agriculture all over the world (Van Boeckel et al., 2014). Due to the lack of reasonable supervision and weak public consciousness, antibiotic misuse or abuse is widespread. This situation is especially severe in China, for example, a survey showed that more than 162 million kg of antibiotics were consumed in 2013, and almost half were used in agriculture applications (Zhang et al., 2015). Due to their high water solubility and low bioavailability, the multitude of unabsorbed antibiotics not only pose a serious ecological threat to the environment but also severely disturb the gut homeostasis of organisms and lead to the emergence of antibiotic-resistant strains (Li et al., 2015). Previous studies showed that antibiotic addition was also closely linked to the gut microbiota community shift of aquatic organisms. Antibiotics addition caused a reduction in the gut bacteria number and diversity in Atlantic halibut (*Hippoglossus hippoglossus*) larvae and few culturable bacteria were recovered after the treatment (Verner-Jeffreys et al., 2004). Navarrete et al. (2008) found that the gut microbiota of Atlantic salmon in the oxytetracycline-treated group was characterized by a lower diversity and consisted only of *Aeromonas*, which was proven as an antibiotic-resistant strain. In the study of grass shrimp following exposure to low doses of oxytetracycline, *Vibrio alginolyticus* showed significant positive growth, whereas other bacterial species abundance declined over time (Uyaguari et al., 2009). In zebrafish and *Gambusia affnis* fish, studies also showed lasting effects on mucosal microbiomes following antibiotic exposure (Carlson et al., 2017; Zhou et al., 2018).

The sea cucumber *Apostichopus japonicus* is an important marine economic species in Asian countries due to its profound nutritional and medicinal value (Liao, 1997). With the rapid development of intensified artificial aquaculture of sea cucumbers, large amounts of antibiotics were used to treat pathogenic diseases and promote growth, especially during the nursery phase (Wang et al., 2007). However, despite the widespread use of antibiotics in sea cucumber industries, the effects of antibiotics on the bacterial ecology have received little attention. The objective of this research was to study the effects of three typical antibiotic compounds on the gut bacteria of juvenile sea cucumbers by high-throughput sequencing of 16S rRNA amplicons. The study could help us to explore how antibacterial compounds modify the gastrointestinal microbiota of sea cucumbers and provide theoretical guidance in hatchery management by scientific antibiotic use in sea cucumber mariculture.

2 MATERIAL AND METHOD

2.1 Animal preparation

Two hundred forty healthy juvenile sea cucumbers (average weight of 2.57±0.3 g) were collected from the coast of Yantai, Shandong Province, China, in March 2017. After temporary rearing for 3 d without feeding, the juveniles were randomly divided into four groups for experimentation. All juveniles were fed with a formula feed of 1.5% of their body weight every day, and three experimental groups had three typical antibiotics (tetracycline, erythromycin, and norfloxacin) added as a 2% weight of formula feed, while the control group had nothing but formula feed added. Tetracycline (CAS: 60-54-8), erythromycin (CAS: 114-07-8) and norfloxacin (70458-96-7) were purchased from MeiLun Biotechnology Co. Ltd., Dalian, China. The mortality of sea cucumbers was observed, and dead ones were removed every day; the weight of all individuals was noted at each time of sampling. From these data, the survival rate (SR), body weight gain (BWG) and special growth rate (SGR) were calculated according to the following formulas:

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

$$\mathrm{BWG}(\%)=\frac{W_t-W_0}{W_0}\times 100,$$

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

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

2.2 Sample collection and DNA extraction

Ten individuals of each group were sacrificed at 15, 30, and 45 d after the experiment started, and the intestinal contents of sea cucumbers were aseptically dissected and stored in liquid nitrogen. The gut sample information of juveniles is given in Table1. Genomic DNA was extracted from individual intestinal contents using a FastDNA® Spin Kit for Soil (MP Biomedicals, USA) according to the manufacturer's protocol. DNA concentration and purity were monitored on 1% agarose gels. According to the concentration, DNA was diluted to 1 ng/μL using sterile water.

2.3 PCR amplicon and product quantification

The 16S rRNA V4 and V5 regions were amplified by specific primer combination of 515F-907R (515F: 5′-GTGCCAGCMGCCGCGGTAA-3′ and 907R: 5′-CCGTCAATTCMTTTRAGTTT-3′) with barcode (Chen et al., 2016; Zhang et al., 2016b). All PCRs were carried out in a total volume of 30 μL (15 μL Phusion® High-Fidelity PCR Master Mix (New England Biolabs, MA, USA), 0.2 μmol/L forward and reverse primers and 10 ng template DNA) as follows: 98°C for 1 min; 30 cycles of 98°C for 10 s, 50°C for 30 s, 72°C for 60 s; and a final elongation step at 72°C for 5 min. Samples with bright bands between 400 bp and 450 bp were extracted and mixed in equidensity ratios and then purified with a GeneJET™ Gel Extraction Kit (Thermo Scientific, NY, USA).

2.4 High-throughput sequencing

Sequencing libraries were generated using a TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) following the manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific, NY, USA) and Agilent Bioanalyzer 2100 system. At last, the library was sequenced on an Illumina HiSeq 2500 platform and 250 bp paired-end reads were generated.

2.5 Bioinformatics data analysis

Raw tags were first merged by paired-end reads using the FLASH tool (V1.2.7, http://ccb.jhu.edu/software/FLASH/) (Magoč and Salzberg, 2011) and then filtered under specific filtering conditions to obtain high-quality clean tags according to the QIIME (V1.7.0, http://qiime.org/index.html) quality controlled process (Caporaso et al., 2010). After removing the chimaera sequences by using the UCHIME algorithm (UCHIME Algorithm, http://www.drive5.com/usearch/manual/uchime_algo.html), the effective tags were finally generated (Edgar et al., 2011).

Sequence analyses were performed by Uparse software to assign OTUs (operational taxonomic units) using the average neighbour algorithm at a 97% similarity level. Furthermore, each representative sequence was annotated using taxonomic information based on the RDP3 classifier algorithm in the Green Gene Database. QIIME software was used to calculate alpha diversity: Good's coverage was used to characterize the sequencing depth, the observed species were used to estimate the number of unique OTUs found in each sample, Chao 1 and ACE were used to identify community richness, and the Shannon index and Simpson index were used to identify community diversity. And Beta diversity analysis was carried out to compare the microbiota community composition in different samples depending on QIIME calculates including the unweighted pair-group method with arithmetic means (UPGMA) clustering and principal coordinate analysis (PCoA). All sequences in this study were deposited into the NCBI database with GenBank accession nos. SPR130109.

Table 1 Sample information of sea cucumbers in this study

Group	Sample name	Sampling time
Control (D)	D	In the initial stage of the control group
Tetracycline Treatment (T)	T1	15 d after tetracycline treatment
	T2	30 d after tetracycline treatment
	T3	45 d after tetracycline treatment
Norfloxacin Treatment (N)	N1	15 d after norfloxacin treatment
	N2	30 d after norfloxacin treatment
	N3	45 d after norfloxacin treatment
Erythromycin Treatment (H)	H1	15 d after erythromycin treatment
	H2	30 d after erythromycin treatment
	H3	45 d after erythromycin treatment

2.6 Statistical analysis

Differences between SR, BWG and SGR results were subjected to one-way analysis of variance followed by LSD's multiple range test by using SPSS 18.0 software (SPSS Inc, Chicago, USA) and the significance in microbial diversity and abundance among the different antibiotic sea cucumber was tested by Student's *t*-test using R software, and the statistical significance was set at α=0.05.

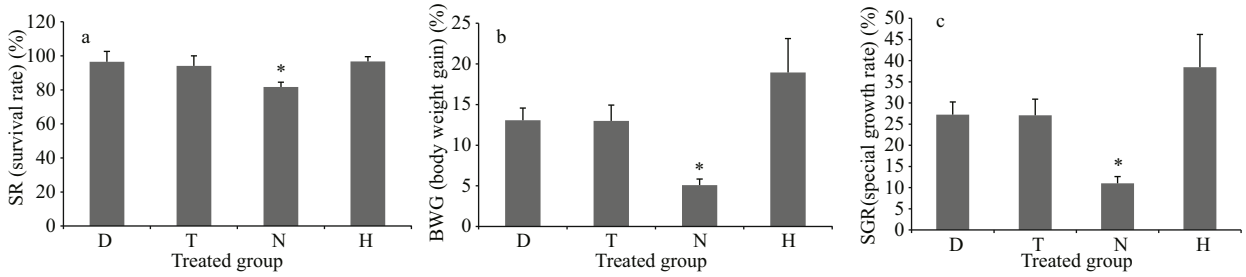

Fig.1 Effects of antibiotic dietary treatments on the growth performance of juvenile sea cucumbers

a. survival rate; b. body weight gain; c. special growth rate. Comparisons between the four groups were made by one-way ANOVA following the LSD multiple-comparison test, and asterisk (*) represents $P<0.05$. The capital letter D, T, N, and H represents control group, tetracycline treatment group, norfloxacin treatment group, and erythromycin treatment group, respectively.

Table 2 Illumina high-throughput sampling depth, richness and diversity index of juvenile sea cucumbers intestinal bacteria in control and antibiotic treatment groups

Sample	Sampling depth		Richness		Diversity	
	Mean sequences	Goods_coverage	Chao	ACE	Shannon	Simpson
D	66 804±8 004	0.997 7±0.000 58	362.5±58.1	370.5±53.2	3.1±0.27	0.8±0.07
T1	64 125±1 461	0.997±0.001	423.8±127.77	439.2±120.18	3.7±0.18	0.8±0.04
T2	66 020±11 704	0.997±0.001	414.2±126.86	437.9±143.16	3.3±0.33	0.8±0.07
T3	63 993±2 926	0.998±0.000 58	256.5±58.19	257.1±50.52	3.0±0.04	0.8±0.01
N1	56 364±2 311	0.997±0.000 58	344.0±107.53	369.6±105.38	3.0±0.19	0.7±0.01
N2	51 506±3 202	0.996±0.000 58	567.1±125.99	591.2±126.82	3.7±0.33	0.8±0.04
N3	55 526±5 294	0.997±0.002 1	430.3±401.39	460.8±410.29	3.2±0.76	0.8±0.04
H1	64 479±9 766	0.995±0.000 58	653.3±82.37	688.9±78.96	4.4±0.55*	0.9±0.06
H2	52 869±14 682	0.998±0.001	325.6±110.11	355.2±101.99	3.3±0.43	0.7±0.11
H3	73 001±13 473	0.999±0.001	352.5±257.69	355.8±266.00	3.2±0.73	0.8±0.04

Abbreviations are the same as in Table 1. Data are expressed in means±SD. Student's *t*-test was used to test the diversity indices. Asterisk (*) represents $P<0.05$. No significant difference was found in Chao, ACE, and Simpson index.

3 RESULT

3.1 Dietary antibiotic and growth performance of sea cucumber juvenile

The SR, BWG, and SGR were measured to assess the effect of antibiotic treatments on the growth performance of juvenile sea cucumbers. The results showed that the SR varied from 81.67% to 96.67% in the four groups (Fig.1a). The SR of the norfloxacin dietary group was significantly lower than those of other groups ($P<0.05$), and the SR of the erythromycin group was the highest (96.67%), but no significant difference was observed. The BWG and SGR in the four groups (three antibiotic groups and control group) varied from 5.09% to 18.95% and 11.03% to 38.47%, respectively (Fig.1b, c). Similar to SR, the BWG and SPG of the norfloxacin group were significantly lower ($P<0.05$) and the erythromycin group was higher than those of the other groups with no significant differences. These results indicated that different antibiotics had different effects on the growth performance of juvenile sea cucumbers.

3.2 Illumina sequencing

A total of 1 844 059 optimized reads were obtained from the 30 gut content samples of juvenile sea cucumbers (200 412 reads of the control group and 1 643 647 reads of the other three antibiotic treated groups) by Illumina sequencing. The optimized read numbers for each sample ranged from 37 704 to 87 337 with a mean average of 61 469±9 856. The average length of the reads was 253 bp. The mean sequences of each group are listed in Table 2. In addition, these data revealed that the goods' coverages of all samples were ≥99.5% (Table 2), which indicated that the sequencing depth was sufficient to cover the microbial diversity in all samples.

To estimate and compare the bacterial richness and diversity difference among these groups, the proportions of OTUs were calculated by the ACE and

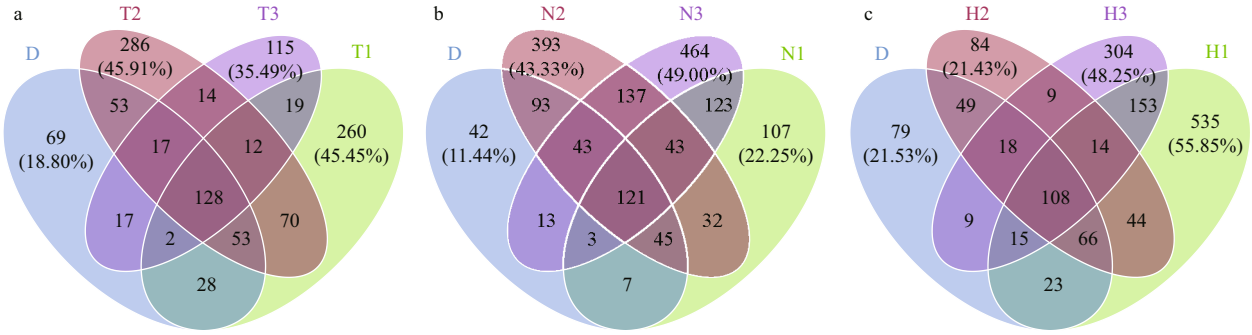

Fig.2 Venn diagram representing unique and shared OTUs between the antibiotic groups and control group

The percentages in the Venn diagram indicate the ratios of the sequences that are associated with the OTUs in the total sequences in each group.

Chao1 indices and the Shannon and Simpson diversity indices. As shown in Table 2, the ACE and Chao1 values have no significant differences between the antibiotic treatment groups and the control group, but data shows the richness indices in all three antibiotic groups increase first and then decrease with the elongated treated time. For diversity, a similar trend of Shannon and Simpson diversity indices were also observed in antibiotic treatments, and the H1 group had significantly higher levels of diversity indices (P<0.05) than the other groups (Table 2).

Venn diagrams reflected the distribution of OTUs among different treatment groups. As shown in Fig.2a, 128 OTUs were shared among the control group (D) and tetracycline groups (T), and 69, 260, 286 and 115 uniquely OTUs were observed in the four groups, which accounted for 18.80%, 45.45%, 45.91%, and 35.49% of all sequences in the D, T1, T2 and T3 groups, respectively. For the control group (D) and norfloxacin groups (N), there were 121 common OTUs shared in the four groups, and 42, 107, 393 and 464 unique OTUs were found with the percentages of 11.44%, 22.25%, 43.33% and 49.00% of all sequences in the D, N1, N2 and N3 groups, respectively (Fig.2b). In comparison with the control group (D), 108 OTUs were shared in erythromycin groups (H), and the unique OTU numbers of the D, H1, H2, and H3 groups were 79, 535, 84 and 304, which accounted for 21.53%, 55.85%, 21.43% and 48.25% of all its sequences, respectively (Fig.2c). Overall, these results suggested that the dietary supplementation of different antibiotics in sea cucumbers led to the selection of different unique microbial populations.

3.3 Effects of antibiotic treatment on the intestinal bacterial composition

Thirty-four different bacteria phyla were identified from the gut contents of all juvenile sea cucumber samples. From each group of sea cucumbers, the phylum Proteobacteria made up the dominant majority of all sequences (mean relative abundance, 95.78%±3.57%), and Firmicutes (1.43%±0.61%), Bacteroidetes (1.16%±1.43%), Fusobacteria (0.92%±1.24%), and Actinobacteria (0.18%±0.049%) were also detected in the gut microbiota of sea cucumbers. As shown in Fig.3 and Table S1, the sea cucumber gut bacterial assemblages were relatively stable at the coarse phylum level in control and antibiotic groups. But there were some significant differences in intestinal microorganisms, like the significant decreasing proportions of Proteobacteria was observed in the antibiotic treatment groups (N2, H1 and H2 group) compared to the control group (P<0.05), and some phyla exhibited more abundant within the gut of *A. japonicus* diet with antibiotics, including significant increasing percentages of Firmicutes (N3 and H3) (P<0.05), and some phyla emerging only in antibiotic diet sea cucumbers, such as Acidobacteria (N2) and WD272 (H1) (P<0.05).

At the species level, a total of 509 genera were identified in the microbiota of sea cucumber gut contents. Three antibiotic dietary treatments induced an obvious alteration of species composition in sea cucumber gut microbiota. Compared to the control group, the relative abundance of some genera began to decline but others began to increase, even with some new species emerging in the antibiotic treatment groups. As Table 3 shows, a decreased ratio of *Vibrio*, *Thalassotalea*, *Tropicibacter*, *Polaribacter*, *Shewanella*, *Sulfitobacter*, *Halomonas*, *Dinoroseobacter*, *Lewinella*, *Ruminiclostridium_9*, *Neiella*, *Blautia*, *unidentified_Rhodobacteraceae*, and *Pseudopelagicola* were seen in different antibiotic treatment groups, especially in the middle and late treatment stage. Conversely, the relative abundance of different genera in antibiotic treatments has risen by varying degrees at different treatment times, for example, some genera increased at the

Table 3 The top 25 discriminative taxa between control and antibiotic diet sea cucumbers at the genus level

Genus	Treat group of antibiotics (relative abundance %)									
	D	T1	T2	T3	N1	N2	N3	H1	H2	H3
Vibrio	70.11	47.67	58.96	55.53	59.60	47.86↓*	61.13	37.29↓*	55.06	45.58↓*
Thalassotalea	3.48	1.29↓*	2.89	2.43	2.63	16.27↑**	11.16	1.53↓*	2.06	1.56↓*
Arcobacter	4.25	4.65	6.00↑*	4.55↑*	20.63↑*	15.43	7.62	3.60	3.46	7.47↑*
Litoribacillus	0.083	0.40↑**	0.24	0.0082	0.30↑**	0.10	0.0036	2.48	0.14	0.012
Tropicibacter	0.088	0.047	0.088	0↓**	0.0027↓**	0.12	0↓**	0.023↓*	1.86	0↓**
Alteromonas	0.027	0.96	0.0091	0.0082	0.026	0.0091	0.0046	0.077↑*	0.013	0.0055
Pseudomaricurvus	0.028	0.15↑**	0.018	0.0046↓*	0.11	0.034	0.014	1.87	0.29	0.077
Polaribacter	0.12	0.068	0.14	0.0064↓**	0.015↓**	1.39	0.012↓**	0.051↓*	0.55	0.0046↓**
Leucothrix	0.15	0.82	0.18	0.028↓*	0.090	1.21↑*	0.088	0.21	0.22	0.019↓*
Shewanella	0.66	0.75	0.27	0.018↓*	0.10	0.14	0.02↓*	0.41	0.15	0.11
Sulfitobacter	0.10	0.18	0.094	0.0036↓**	0.24	0.17	0.0036↓**	0.20	0.75	0.0073↓**
Clostridium_sensu_stricto_1	0.0018	0.50	0.0055	0.02↑*	0.22	0.0027	0.027	0.22	0	0.014↑**
Maricurvus	0	0.059	0	0	0.044↑*	0	0	0.40	0.0018	0
Halomonas	0.070	0.067	0.067	0.051	0.093	0.22	0.064	0.067	0.091	0.026↓*
Dinoroseobacter	0.041	0.14	0.070	0.0046↓*	0.035	0.13	0	0.18	0.077	0.0091↓*
Owenweeksia	0.0064	0.014	0.042↑**	0.0027	0.014	0.030	0.0091	0.16	0.026↑*	0.0018
Lewinella	0.023	0.036	0.015	0↓**	0.0082	0.069	0.0036	0.047	0.16	0.0036↓**
Ruminiclostridium_9	0.0091	0.00091↓**	0.033	0.0073	0↓**	0.12	0↓**	0.029	0.0091	0.0046
Aquibacter	0	0.020	0	0	0.029↑*	0	0	0.15	0	0
Neiella	0.056	0.0091	0.13	0.0073	0↓*	0.026	0↓*	0.0027↓*	0.028	0
Alistipes	0.0064	0.0018	0.013	0.042	0↓*	0.11	0↓*	0.096↑*	0.016	0↓*
Blautia	0.014	0	0.0073	0.0091↓**	0	0.074	0.0055	0.0064	0.0036↓**	0.0064
Unidentified_ Rhodobacteraceae	0.0073	0.091	0.014	0.00091↓**	0.048	0.016	0.0027	0.021↑*	0.012	0.0036↓**
Parabacteroides	0.0036	0	0.0073	0.039	0	0.02	0.0091	0.11↑*	0.0064	0.0018
Pseudopelagicola	0.013	0.021	0.016	0.00091↓**	0.046	0.095	0.00091↓**	0.0082	0.015	0.00091↓**

Abbreviations are as in Table 1. Student's *t*-test was used to detect the significant differences between the control and antibiotic exposed groups, * represents $P<0.05$, ** represents $P<0.01$. ↑represents relative abundance up-regulated, ↓represents relative abundance down-regulated.

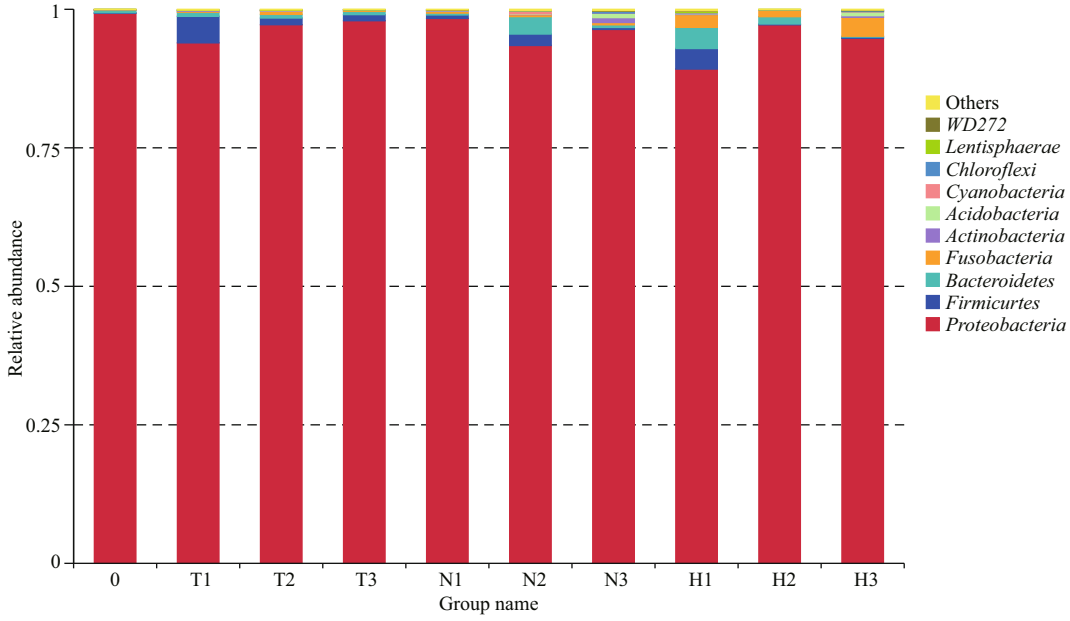

Fig.3 Microbial composition at the phylum level of gut microbiota in juvenile sea cucumbers under different antibiotic treatments

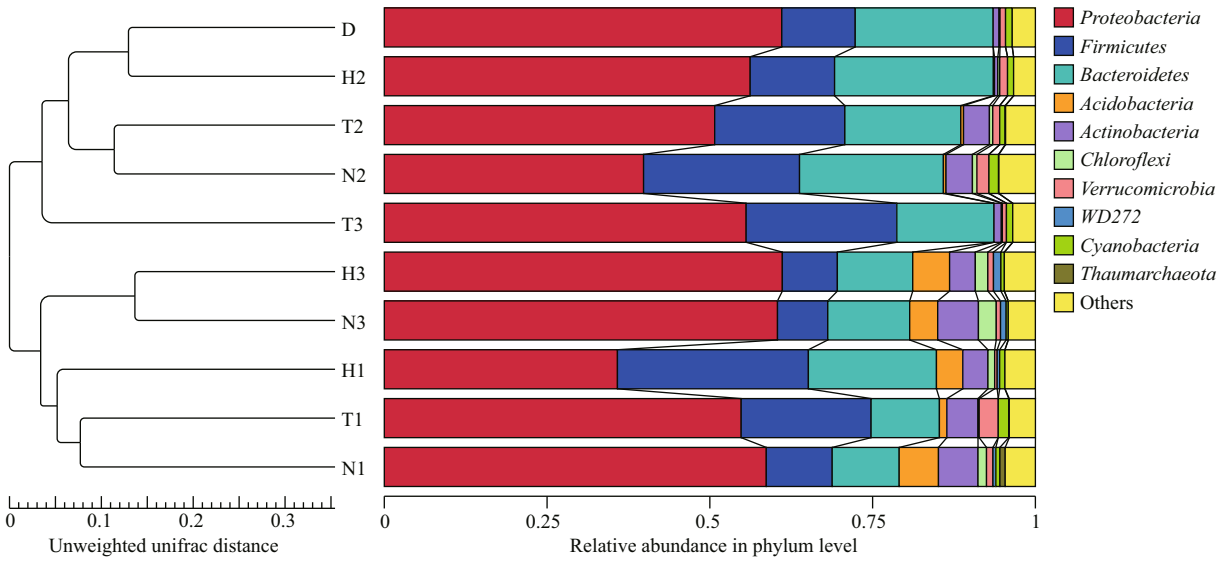

Fig.4 UPGMA clustering tree at the level of phylum between different groups

early treatment stage, like *Litoribacillus* (T1 and N1), *Alteromonas* (H1), *Aquibacter* (N1) and *Parabacteroides* (H1); and some genera tended to increase at the middle and late treatment stage, like *Arcobacter* (T2, T3, N1, and H3), *Leucothrix* (T3, N2 and H3), *Clostridium_sensu_stricto_1* (T3 and H3), and *Owenweeksia* (T2 and H2). There are some genera only been seen in the antibiotic groups, like *Maricurvus* and *Aquibacter*.

3.4 Relationships among microbiota of control and antibiotic treatment groups

A UPGMA clustering tree was constructed to analyse the similarities and differences in gut microbiota taxonomic composition among the different treatment groups (Fig.4). The results showed that the taxonomic composition of the control group (D) was closest to that under 30 d of antibiotic treatment (H2 T2 and N2), indicating that these four groups resulted in more similar community structures. Similarly, the taxonomic compositions of the groups under antibiotic treatments by 15 d (H1, N1, and T1) and 45 d (H3 and N3) were clustered into two separate groups according to antibiotic treatment time, but T3 seems to be an exception in the clustering tree. The principal coordinate analysis (PCoA) was also performed to identify relationships among samples, as shown in Fig.5. The analysis results confirmed that the D group was clustered closest to the T2 and H2 groups. However, the clustering tendency of other groups was ambiguous, probably due to large individual differences among the samples within a group.

4 DISCUSSION

Antibiotics exposure has been closely linked with the weight changes of animals. To date, low doses of antibiotics are fed to large numbers of animals as growth promoters and prophylactics all around the world (Carvalho and Santos, 2016). However, the effects of different antibiotics on weight gain of aquatic animals remain inconsistent. In our study, different kinds of antibiotics seem to have completely different effects on the growth conditions of juvenile sea cucumbers. It was observed that dietary norfloxacin induced lower weight gains of juveniles; dietary tetracycline had little effect on growth acceleration, while dietary erythromycin seemed to promote growth. Similarly, different antibiotics also have varying effects on the growth of other aquatic animals. For example, feeds supplemented with oxytetracycline and sulfamethoxazole could accelerate the weight gain of channel catfish (*Ictalurus punctatus*) and zebrafish respectively, while the dietary sulphonamides did not show any growth-promoting influence on rainbow trout (*Salmo gairdneri*), and the dietary sulfamerazine even inhibited growth of brook trout (*Salvelinus fontinalis*) (Snieszko and Wood, 1955; Sanchez-Martínez et al., 2008; Zhou et al., 2018). Actually, each class of antibiotics has different properties, the voluntary or involuntary weight changes of animals might be due to gut microbiota shift and functional modifications depending on the antibiotic class, dose, and period of exposure (Angelakis et al., 2013).

Alpha diversity evaluates the diversity of a community by calculating OTUs and their relative

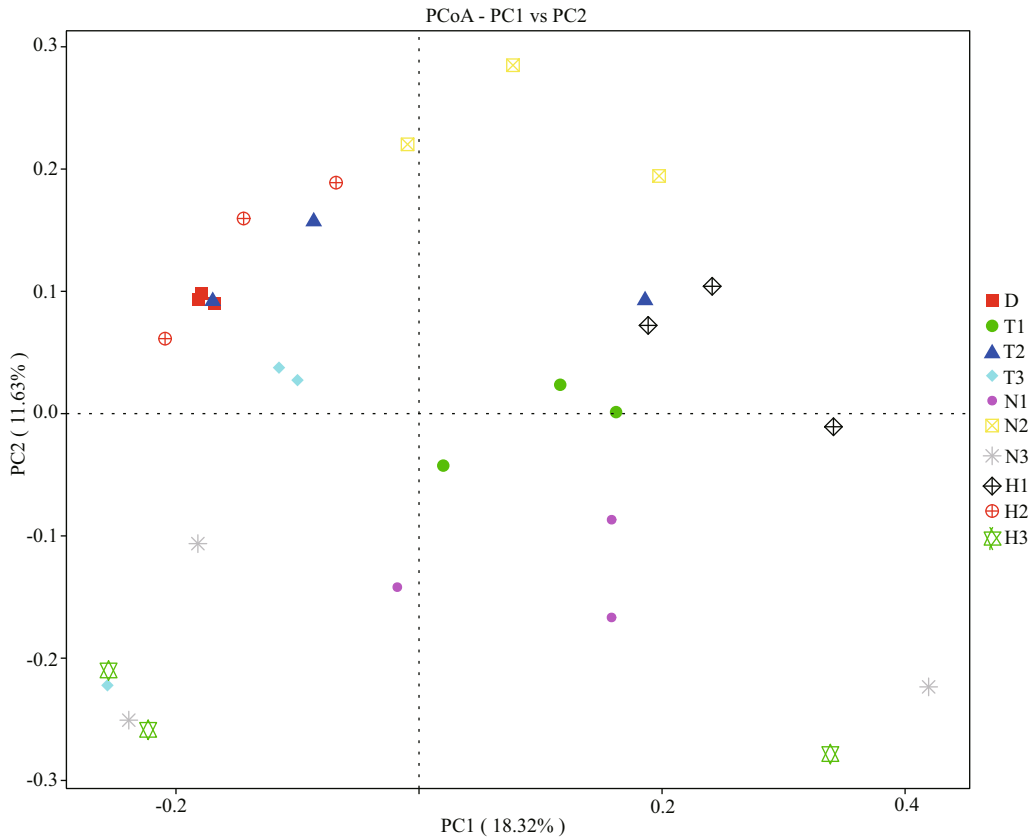

Fig.5 Principal component analysis of intestinal bacteria in the antibiotic and control groups

abundances. In our study, the Chao 1, ACE, Shannon, and Simpson indices and Venn diagrams were used to comparing the alpha diversity differences between the control and antibiotic treatment groups. The results showed that the three antibiotics did not change the microbiota species richness significantly. For gut microbiota diversity, antibiotic treatment also had little influence on the Shannon and Simpson indices, but an increase in the Simpson index was observed in the H1 group. Our results seem not to be consistent with some previous studies regarding the significant decrease in intestinal flora richness and diversity under antibiotic exposure in higher vertebrates, such as piglet and human (Panda et al., 2014; Zhang et al., 2016a). Since most of these studies have employed relatively high concentrations of antibiotics in the short term, the discrepancy between our results and the above research might be due to the difference in the antibiotic dosages administered, the treatment time and method of administration, as relatively low doses of antibiotics in the long term by diet treatment were designed in our experiments to simulate the real nursing environments of sea cucumber juveniles in the actual production condition.

As for the composition of gut microbiota in sea cucumbers, the phyla of *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Acidobacteria* were detected in our study, the data suggested that the predominant phylum was *Proteobacteria*, and our results were similar to the previous report that the composition of gut microbiota from *A. japonicas* (Gao et al., 2014; Sha et al., 2016; Wang et al., 2018; Zhang et al., 2018). Actually, the phylum *Proteobacteria* was reported as the dominant phylum in many marine invertebrates, such as marine sponges, Chinese shrimp (*Fenneropenaeus chinensis*) and grass shrimp (*Palaemonetes pugio*) (Uyaguari et al., 2009; Liu et al., 2011; Schmitt et al., 2012). In this study, the gut microbiota composition under different antibiotic treatments remained relatively stable at the phylum level, but antibiotics caused a significant decrease of *Proteobacteria* and significant increase of *Fusobacteria* of sea cucumber intestinal microorganisms. Similarly, the relative abundances of *Fusobacteria* dramatically increased in white spot syndrome virus challenged shrimp intestines and sick bald sea urchin in relation to those in healthy ones (Becker et al., 2008; Wang et al., 2019, which suggests *Fusobacteria* might be in relation to disease in these organisms, but the function of *Fusobacteria* in sea

cucumber requires a further investigation..

In the meanwhile, a complex alternation of bacteria composition was observed at the genus level. On the one hand, the antibiotic treatments induced the lower frequencies of *Vibrio*. Previous studies showed that some genera of *Vibrio* were the principle pathogens in sea cucumbers, such as *V. cyclitrophicus* and *V. splendidus*, which were closely associated with skin ulcer syndrome and viscera rejection syndrome (Deng et al., 2009). Antibiotic treatment can eliminate susceptible pathogen microorganisms such as *Vibrio*, which could help prevent the infection of diseases (Uyaguari et al., 2009). Nonetheless, innocent sensitive bacteria with host-helpful functions were also killed during this process, such as *Shewanella*, *Sulfitobacter*, *unidentified_Rhodobacteraceae*, and *Halomonas*. Previous research reported that *Halomonas* was part of the autochthonous microbiota of sea cucumber and showed polysaccharide degrading abilities (starch and carboxymethyl cellulose), which has played an important role in the digestion of ingested detritus and fermentation products supply for the host (Zhang et al., 2013). Existing studies have shown that *Rhodobacterales* could protect animals from pathogenic bacteria and serve as an energy source to encourage the growth of host by secreting polyhydroxybutyrate (PHB) (Yamazaki et al., 2016). *Shewanella* and *Sulfitobacter* have been practically applied in aquaculture with a high probiotics efficiency, which could produce antibiotics and improve innate immunity and disease resistance (Sharifah and Eguchi, 2012; Jiang et al., 2013). The reducing numbers of beneficial bacteria induced by antibiotic treatment undoubtedly affected the community structure stability and normal function of sea cucumbers.

On the other hand, as antibiotics can eradicate susceptible microorganisms, this gave space for the opportunists to overgrow and dominate the niche, especially for the antibiotic resistant strains and opportunistic pathogens. In our study, we observed the appearance of several suspected pathogenic or suspected antibiotic resistant bacteria in the antibiotic groups, especially at the middle and late treatment stage. For instance, a significant sharp increase of *Arcobacter* was detected in all three antibiotic groups (P<0.05). Indeed, members of *Arcobacter* are considered to be zoonotic and enteropathogenic in both animals and humans (Collado and Figueras, 2011). Similarly, the relative abundance of *Leucothrix* was observed to increase significantly in antibiotic groups (P<0.05), which are typical saprophytic and pathogenic species (Loo et al., 2011). Coincidentally, Navarrete et al. (2008) and Austin and Al-Zahrani (1988) found that oxytetracycline treatment induced the appearance of *Aeromonas* populations as common pathogens of fish in the intestinal microbiota of Atlantic salmon and rainbow trout. It is worth noting that *Clostridium_sensu_stricto_1* also increased significantly in T3 and H3, and many *Clostridium* members were reported as antibiotic resistant bacteria (Gutiérrez-Salazar et al., 2011). Research has confirmed that the antibiotics exposure of bacteria populations in the laboratory could effectively select and enrich resistant strains and species, even at subtherapeutic doses (Jakobsson et al., 2010; Toprak et al., 2012; Carlson et al., 2017). However, the suspected pathogenic or antibiotic resistant bacteria in our study need to be validated in the follow-up experiments.

In our study, distinct emerging bacteria were observed in each antibiotic group, probably due to the results of gut microbiota reshaping and selecting under respective antibiotic stress. In fact, mammalian experiments showed that perturbations of microbiota induced by antibiotic treatment can lead to long-term change, including impairing colonization resistance and weaker immuno-functions, which in turn increase the risk of host infection (Becattini et al., 2016). Our latest research also shows that the mortality was higher in antibiotically treated sea cucumbers after challenge with *Vibrio splendidus* than the control ones and lower immune-related parameters were found in antibiotic feeding juveniles (Zhao et al., 2019). However, since we got the bacterial composition information based on sequencing, the exact pathogenic information of these bacteria still needs further investigation. In addition, the multiple samples similarity tree showed that H1, T1, and N1 were aggregated, and H2, T2, N2, and D formed relatively tight clusters. These data indicated that the microbiota shift induced by antibiotics was also influenced by medicine exposure time; in other words, individuals under three different antibiotics treatments tended to have similar taxonomic compositions at the same treatment time.

5 CONCLUSION

By studying the gut microbiota shifts in juvenile sea cucumbers under three typical antibiotic medicines treatments using high-throughput sequencing of 16S rRNA amplicons, we found that

the three antibiotics have distinct effects on the growth performance of juvenile sea cucumbers: norfloxacin treatment inhibited the growth, erythromycin treatment promoted the growth, and tetracycline treatment had no obvious effects on the growth. However, the richness and diversity of microbiota were barely affected by the antibiotics but changes of the community composition indicated that the three antibiotics had their own patterns of the intestinal bacteria in juvenile sea cucumbers. The disappearance of some sensitive microorganisms with helpful functions and the appearance of multiple potential pathogenic and antibiotic resistance microorganisms after antibiotic treatment suggested that the shifted gut microbiota of sea cucumbers might increase the infection risk in the hosts. These results provide a basis to identify the relationship between the antibiotic treatments and the intestinal microbiota taxonomic composition of marine benthic invertebrates. Future research will focus on the consequences of the complex taxonomic, metabolic, and immune-modulating effects of antibiotic treatments.

6 DATA AVAILABILITY STATEMENT

The datasets during and/or analyzed during the current study available from the corresponding author on reasonable request.

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Electronic supplementary material

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