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Variance Analysis of Intestinal Microbial Diversity of the Noble Scallop (*Chlamys nobilis*) under Enrofloxacin Exposure

Jiawei Hong[1,2], Mingqiang Chen [1,2], Zhenghua Deng[1,2], Youning Li[1,2] and Yu Wang[1,2]*

[1]Tropical Aquaculture Research and Development Center, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Sanya, 572018, China
[2]Key Laboratory of South China Sea Fishery Resources Exploitation and Utilization, Ministry of Agriculture, Guangzhou, 510300, China

Jiawei Hong and Mingqiang Chen contributed equally to this work.

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JH conceived the idea and wrote the manuscript. MC and YL helped in field experimental work. ZD assisted in data analysis. YW supervised the manuscript.

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***Chlamys nobilis*, microbial diversity, enrofloxacin, 16S rDNA**

ABSTRACT

To investigate the possible impact of enrofloxacin (ENR) on noble scallop (*Chlamys nobilis*), we quantitatively evaluated the microbial shifts in the intestine of noble scallop in response to enrofloxacin treatments at different dosages (0, 5, and 10mg/L ENR) using 16S rDNA gene sequencing. A total of 11 phyla comprising 76 genera were detected. At the phylum level, the relative abundance of Proteobacteria increased (from 34.96% to 77.31%) with the increasing of enrofloxacin exposure dosage. The dominant position of Tenericutes was replaced by Proteobacteria, and in parallel the proportion of Tenericutes slumped to 3.85%. At the genus level, the relative abundance of *Mycoplasma*, dropped down from 58.38% to 3.85%, and *Vibrio* increased (from 15.23% to 40.8%) to become the dominant genus. The hierarchical clustering heat map analysis and principal component analysis (PCA) showed that the microbial community of the high dosage group (10 mg/L) was clearly different from the other two groups. Overall, enrofloxacin at high dosage of 10 mg/L significantly altered the community diversity of noble scallop. This study characterized the variation regularity of the intestinal microbial of the noble scallop in response to enrofloxacin treatment. These results, provide a comprehensive acquaintance with intestinal microecosystem of the noble scallop and contributes to a reasonable use of enrofloxacin treatment on noble scallop.

INTRODUCTION

The intestine is the most important digestive organ, and hosts a large amount of microbes with complex structures. Microbial community is an indispensable part of the host (Pérez *et al*., 2010; Abid *et al*., 2013; Cahenzli *et al*., 2013), which maintains a dynamic balance in the intestine and plays an important role in nutrition metabolism, regulating immune function and resisting pathogens (Macpherson *et al*., 2004; Sekirov *et al*., 2010; Martiny *et al*., 2015). Intestinal microbes help maintain the barrier function of the intestinal mucosal system (Hecht *et al*., 1999; Cho *et al*., 2012). In case this balance is destroyed, the host will be more susceptible to various diseases due to the intestinal community disorders (Ring *et al*., 2003). Therefore, maintaining the stability of intestinal microbial community structure is an important factor to avoid the occurrence of bacterial diseases (Round *et al*., 2009).

The noble scallop *Chlamys nobilis* Reeve (Pectinidae, Pterioida), widely distributed in Japan, Indonesia and the Southern Sea of China, is cultivated as an important economic mollusk in China (Qiu *et al*., 2007; Zheng *et al*., 2010). As invertebrates, shellfish do not have specific immune system to resist the infection of various pathogens that reduce the impact of environmental stress (Anderson, 1988). The normal intestinal community structure and function are particularly important for scallops. Studies on the diversity of microbiota in the intestinal tract have been greatly developed by the high throughput sequencing technology. The method makes great improvement in the depth and breadth of microbial diversity analysis in animals, and allows for an insight into understanding of the structure and function of intestinal microbial community (Turnbaugh *et al*., 2009; Caporaso *et al*., 2012;

* Corresponding author: winterrose@163.com
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Ye *et al*., 2014; Zhang *et al*., 2015). Therefore, we want to investigate the intestinal microbial diversity of noble scallop using this technology so that it can give us an another view on this problem.

Because of the non-hygienic and stressful conditions in aquaculture facilities, the risk of bacterial infections is high and leads to frequent use of antibiotics (Sapkota *et al*., 2008). However, the most common side effect of antibiotics is its impacts on intestinal microecology (Kim *et al*., 2012). Shifts in the composition of intestinal community induced by the excessive use of antibiotics may cause disorder in the ecological balance between microorganisms and host, and allow for the proliferation of pathogens and cause infection (Kim *et al*., 2012; Li *et al*., 2017). Many investigations have been conducted to study the intestinal community of many organisms in recent years (Ley *et al*., 2006; Han *et al*., 2010; Chen *et al*., 2015, 2016, 2018) , such as crap, golden pompano, chicken and human, but few studies about the shifts of intestinal microbial in animals after exposure of mollusks to antimicrobials, have been reported. Enrofloxacin is an efficient broad-spectrum antimicrobial against a lot of bacterial diseases (Sarkozy, 2001; Committee for Medicinal Products for Veterinary Use (CVMP), 2007), and it's one of the most used antimicrobials in aquaculture. In the present study, thus, the intestinal microbial diversity of noble scallop was analyzed by high throughput sequencing in response to different dosages of enrofloxacin, with the aim of characterizing their variation regularity, and suggest an appropriate therapeutic regimen for bacterial infections of noble scallop.

MATERIALS AND METHODS

Animals

Experimental noble scallops (shell length 71.71±0.57 mm, shell height 72.71±0.51 mm, shell width 24.61±0.56 mm, and wet weight 64.65±1.56 g) were collected from a local farm in Xincun Town, Lingshui, China, and transported to Tropical Fisheries Research and Development Center, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Science, temporary reared for two week following the management method described by Handa (2016).

Experiment design and sample collection

The enrofloxacin crystal was dissolved in 3 fiberglass tanks with 400 L volume equipped with sand filtered seawater. The concentrations of enrofloxacin in each group were determined according to the HPLC (high-performance liquid chromatography) method proposed by Fang *et al*. (2012). The following three treatments (including the control) were used: 0 mg/L ENR, 5 mg/L ENR, 10 mg/L ENR. Animals were deprived food during the experimental period, and the experiment lasted for 24h. After 24 hours immersion, three scallops were randomly collected from each tank and marked as follows: 0 mg/L group (CA group includes: CA1, CA2, CA3), 5 mg/L group (CB group includes: CB1, CB2, CB3), and 10 mg/L group (CC group includes: CC1, CC2, CC3). The intestine was removed with scissors and tweezers sterilized by alcohol lamp and rinsed with sterile 0.85% (*w*/*v*) saline solution. Samples were immersed in 75% ethanol for 3min and then rinsed sterile saline solution for 3 times. The intestinal contents were put in 1.5mL sterile freezing tubes, and were immediately transferred to store at -80 °C for later DNA extraction.

DNA extraction

The total DNA was extracted from the intestinal contents using the E.Z.N.A DNA Kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's protocol. Qubit 3.0 fluorescent photometer and agarose gel electrophoresis were used to detect the content and quality of DNA.

PCR amplification and 16S rDNA library construction

The V3-V4 hypervariable region of bacterial 16S rDNA gene was amplified by PCR using the specific primer (forward primer: 5'-CCTACGGRRBGCASCAGKVRVGAAT-3', reverse primer: 5'-GGACTACNVGGGTWTCTAATCC-3') designed by Illumina MiSeq platform. Sequencing adapters were added to the terminal of PCR products to facilitate the later Miseq sequencing. All PCR amplifications were performed in triplicate at 25 μL reactions mixture containing: 2.5 μL of TransStart buffer, 2 μL of dNTPs mixture, 1μL of each primer, 20 ng of template DNA. The thermal cycling program was performed as follows: initial denaturation at 94°C for 3 min, 24 cycles of denaturation at 94°C for 5s, annealing at 57°C for 90s, extension at 72°C for 10s, and a final extension at 72°C for 5min. The quality of amplified PCR products was checked by electrophoresis in 1.5% (*w*/*v*) agarose gel, then separated and purified with the Quick Gel Extraction Kit (Qiagen, Hilden, Germany). Purified PCR products were used for gene library construction and high-throughput sequencing.

Bioinformatics analysis

The concentration of DNA library was detected by Qubit 3.0 fluorescent photometer, The DNA library was quantified to 10 nM and then loaded samples to Illumina MiSeq device (Illumina, San Diego, CA, USA) for sequencing according to the instruction. PE 250/300

were used for pairing with ends, picture analysis and base check were performed by the MiSeq control software (MCS) attached to the MiSeq device. Paired-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence (Schloss *et al*., 2009). Pyrosequencing reads with ambiguous bases, quality score of Q≥20, and reads shorter than 200 bp were removed. Raw data were merged using Flash (version v1.2.7) and filtered by Qiime (version v1.9.1). Uchime analysis was then performed to remove chimeric clusters from the sequencing data from each sample (Caporaso *et al*., 2012). Effective data were clustered at a 97% sequence identity into operational taxonomic units (OTUs) using Uparse (version v7.0.1001) software, and taxonomic OTU assignments were accomplished by Ribosomal Database Project (RDP) Classifer (Caporaso *et al*., 2010). Representative sequences of OTUs were aligned using the Silva_128 16S rRNA database (Koetschan *et al*., 2014). Rarefaction curves were analyzed with Mothur (version v.1.30). Qiime was used to calculate the bacterial alpha diversity index, including Shannon and Simpson (diversity), abundance-based coverage estimator (Ace) and Chao1 (richness), and coverage (the Good's coverage). Beta diversity was used as a comparative analysis of microbial communities in different samples. Heatmaps were generated with the R package (Kang *et al*., 2013). UniFrac PCA was used for the principal component analysis (PCA).

Statistical analysis

Data were analyzed using the SPSS 19.0 statistical software packages. All values are presented as the means ± standard deviation (mean ± SD). The data were determined by use of one-way analysis of variance (ANOVA). The statistical significance was accepted at $P < 0.05$.

RESULTS

Microbial community richness and diversity

A total of 946,800 effective sequences were obtained from the total nine samples after processing with the number of sequences ranging from 46,421 to 124,102 per sample. The average length of effective sequences was 460.39 (Table I). The sequences were clustered into 273 OTUs (Operational Taxonomic Units) at the 97% similarity level, and the number of OTUs for each group was 233, 228, 199, respectively. The rarefaction curves tended to approach a saturation plateau with the increase of sequencing depth (Fig. 1), which indicated that the obtained sequences could commendably represent the entire microbial community in the present study. The Good's coverage of the three groups (Table II) was about 100%, which also reflected the reliability of the results.

Table I. Statistics of sequences.

Sample ID	Effective sequences	Average length (bp)
CA1	107210	455.01
CA2	115886	461.29
CA3	114061	460.94
CB1	124102	458.30
CB2	110516	462.10
CB3	46421	461.54
CC1	111091	462.13
CC2	108051	462.14
CC3	109462	460.02
Mean	105200	460.39

Table II. Microbial community richness and diversity indices of each treatment.

	Treatment		
	CA	CB	CC
ACE	173.05 ± 34.91[a]	169.72 ± 10.32[a]	131.15 ± 43.89[a]
Chao1	176.76 ± 38.01[a]	171.79 ± 12.65[a]	130.53 ± 43.66[a]
Simpson	4.00 ±0.32[a]	4.15 ± 0.6[5]a	4.37 ± 0.75[a]
Shannon	0.85 ±0.04[a]	0.88 ± 0.05[a]	0.91 ± 0.04[a]
Good's coverage	1.0 ± 0.0[a]	1.0 ± 0.0[a]	1.0 ± 0.0[a]

In the same row, values with different letter superscripts mean significant differences ($P < 0.05$). ACE and Chao 1 are used to calculate the community richness and estimate the number of OTUs in community. Simpson and Shannon are used to estimate the community diversity. Good's Coverage is the coverage of the sample libraries. The higher the coverage, the lower the probability that the sequences in the sample are not detected.

Table II presented the alpha diversity of three exposure groups. The community richness was estimated based on the alpha-diversity indices (Chao 1, and ACE index), a higher number of which represents more richness. The ACE and Chao 1 indices of three groups were173.05 ± 34.91, 169.72 ± 10.32, 131.15 ± 43.89 and 176.76 ± 38.01, 171.79 ± 12.65, 130.53 ± 43.66, respectively. In addition, the microbial community diversity was demonstrated by alpha-diversity estimations (Shannon and Simpson indexes). The higher Shannon index or the lower Simpson index means higher diversity of microbial community in the sample. The Simpson and Shannon indices of three groups were 4.00 ± 0.32, 4.15 ± 0.65, 4.37 ± 0.75 and 0.85 ± 0.04, 0.88 ± 0.05, 0.91 ± 0.04, respectively. Microbial community richness and diversity had

no significant difference among three groups ($P > 0.05$).

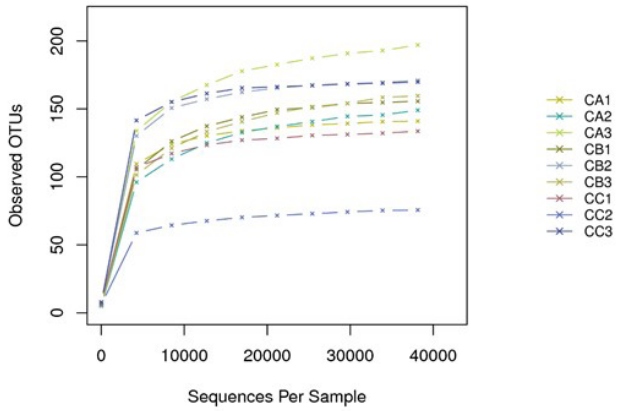

Fig. 1. Rarefaction curves of the OUTs for nine samples. Sequences were clustered at 97% sequence similarity.

To evaluate the distribution of OTUs among the different samples, the Venn diagram was made (Fig. 2), which described the shared OTUs and unique OTUs. The shared OTUs indicated the microbial community similarity, while the unique OTUs showed the microbial community difference among the samples. 40 OTUs were shared by CA and CB group, and only 8 OTUs were shared by CB and CC group. Accordingly, OTUs shared by CC and CA group decreased to 13. The decreasing shared OTUs indicated that the microbial community structure changed when the concentration of enrofloxacin up to 10 mg/L.

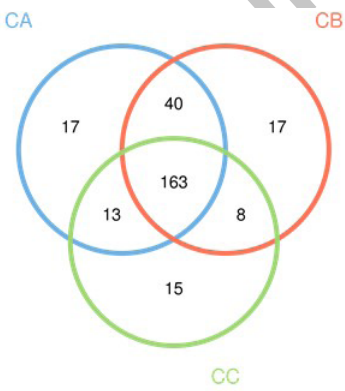

Fig. 2. OTUs distribution in community level in venn diagram.

Microbial community composition and structure

A total of 11 phyla comprising 76 genera were identified by the RDP classifier. The relative abundances of bacterial community at the phylum level are illustrated in Figure 3a. After data standardization, the relative abundance from high to low was Proteobacteria, Tenericutes, Firmicutes, Cyanobacteria, Bacteroidetes, Spirochaetae, Fusobacteria, Deinococcus-Thermus, one unclassified phylum, Actinobacteria, Gracilibacteria. In details, Proteobacteria, Tenericutes, Firmicutes, Cyanobacteria, Bacteroidetes were the five dominant phyla which, in total, accounted for 98.64%, 99.66%, and 99.38% of the entire microbial community respectively. The relative abundance of Proteobacteria increased gradually from 34.96% to 77.31% with the increased enrofloxacin concentration. As a result, the most abundant division changed from Tenericutes to Proteobacteria, and the proportion of Tenericutes decreased gradually at the same time. Cyanobacteria had the same trend with Tenericutes, while Firmicutes rose obviously until the concentration reached 10mg/L.

Figure 3b showed the top thirty most abundant genera at the genus level under different enrofloxacin concentrations and other genera were grouped as the "others". *Vibrio*, *Mycoplasma*, *Exiguobacterium*, *Nannochloropsis-oceanica*, *Citrobacter*, *Escherichia-Shigella*, Photobacterium, *Acinetobacter*, *Amphritea* and an unclassified genus were the dominant genera, which collectively represented about 92.68%, 92.1%, 84.15% of the microbial community respectively. There were some changes of the top ten dominant genera between the total microbe populations under different enrofloxacin concentrations. The control group (0mg/L) contained highest proportion of *Mycoplasma* (58.38%), which fell sharply to 3.85% at the high enrofloxacin concentration (10mg/L). With the increased enrofloxacin concentrations, *Mycoplasma* lost the leading position and was replaced by *Vibrio* (the relative abundance increased from 15.23% to 40.8%). *Exiguobacterium*, *Citrobacter*, *Escherichia-Shigella*, and *Acinetobacter* had a similar trend as *Vibrio*.

Similarities in microbial community structure

A hierarchical clustering heat map analysis was performed at the genus level based on the top 30 most abundant microbial communities across three groups (Fig. 4). The analysis displayed that the samples were segregated into two groups. One group was composed of first two enrofloxacin treatments CA and CB, and CC were assigned to the other group independently. In addition, the principal component analysis also showed a similar trend as in the hierarchical clustering heat map analysis (Fig. 5). The principal component analysis (PCA) indicated that the bacteria community in CA and CB that clustered together had a greater difference than in CC. They were also distinctly different than in PC2 than in PC1.

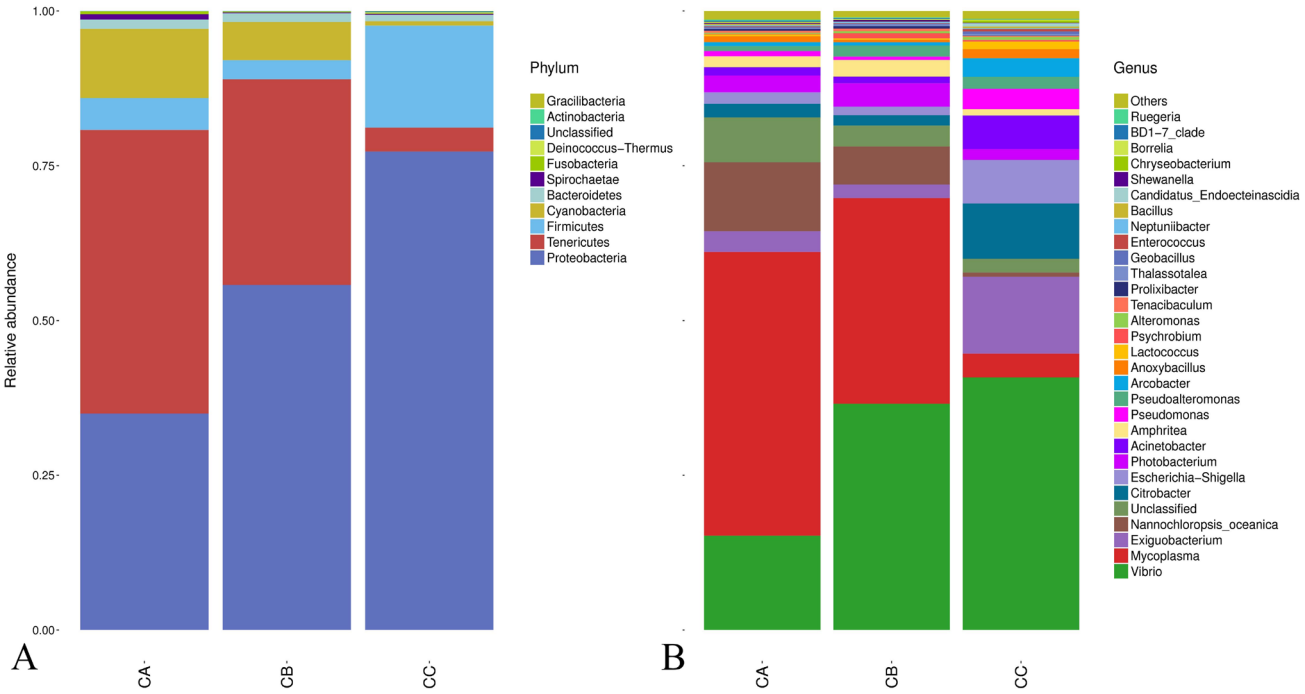

Fig. 3. Relative abundance of microbial phyla (A) and relative abundance of microbial genera (B).

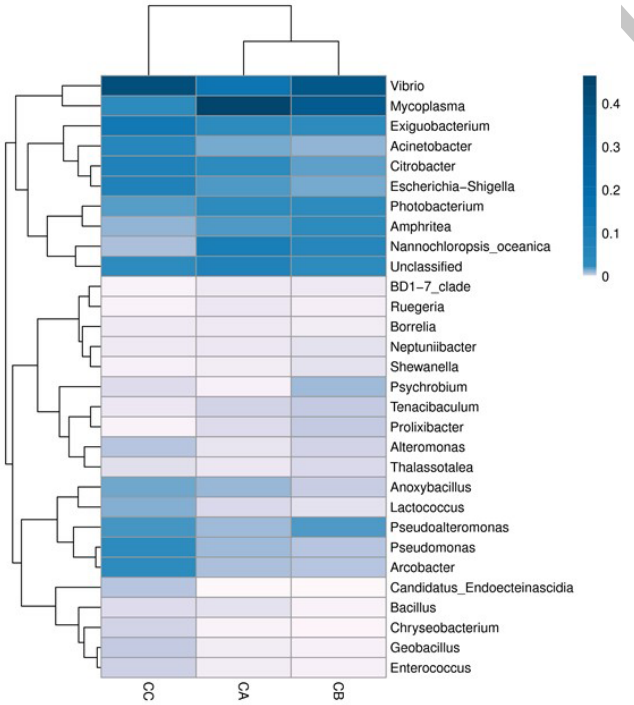

Fig. 4. The hierarchical clustering heat map analysis of the genera.

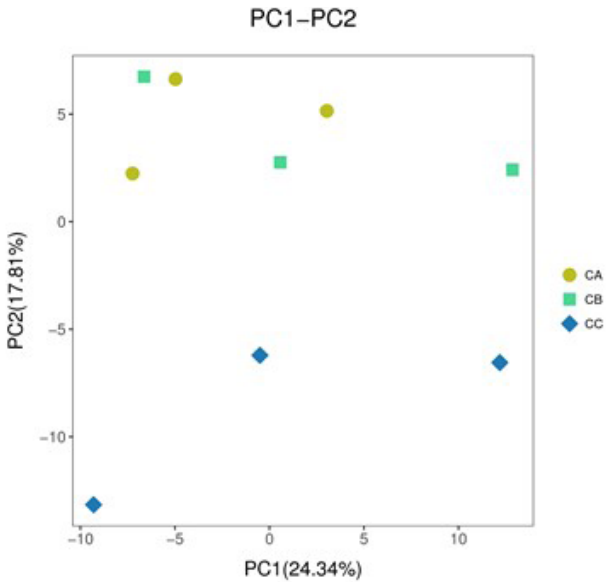

Fig. 5. Principal component analysis (PCA) of community structures.

DISCUSSION

The intestinal tract of animals hosts complex and diverse microfloras, which collectively form an important functional unit and play an important role on the growing

development of the host. Therefore, the stability of intestinal flora is of great significance for the healthy growth of the host (Ley *et al*., 2008; Nayak, 2010; Suez *et al*., 2014). When the species, amount and proportion of normal intestinal flora change abnormally, which deviate from the normal balance state, and translate into a pathological combination, that causes flora dysbiosis (Ley *et al*., 2005; Ley *et al*., 2006). At present, improper use of antibiotics has become the most common inducement of intestinal flora dysbiosis (Nord, 1990). Thus, we investigated the detailed impact of antibiotic, enrofloxacin in particular, on the intestinal microbial community of noble scallop using 16S rDNA gene sequencing.

Through sequencing results, we found that the microbial community at the phylum level was predominated by Proteobacteria and Tenericutes, which made up 34.96% and 45.86% of the control group respectively. The result was in accordance with the previous studies. Tanaka *et al*. (2004) and Nel *et al*. (2017) studied on the intestinal microbial community of cultured *Haliotis discus hannai* and *Haliotis midae*, as well as investigations on bivalves by other authors (Winters *et al*., 2011; Cleary *et al*., 2015; Rubiolo *et al*., 2018) also showed the same results. Not only bivalves, but also some fish have been studied to characterize as dominated by Proteobacteria and Tenericutes, such as rainbow trout (*Oncorhynchus mykiss*) (Wong *et al*., 2013). However, the result showed some differences with study on crustacean *Litopenaeus vannamei*, Fusobacteria and Actinobacteria also account for a large percentage except Proteobacteria and Tenericutes (Zhang *et al*., 2014). Futhermore, compared with different groups, the relative abundance of Proteobacteria increased with the increased enrofloxacin exposure dosage so that the dominant position of Tenericutes has replaced by Proteobacteria. Tenericutes are a distinctive class of bacteria that lack a cell wall, which can be pathogenic to humans (Razin *et al*., 1998). In aquatic animals, Rubiolo *et al*. (2018) suggests a tight association of Tenericutes to mussel hepatopancreass. While the role of these bacteria is still unclear, since they have been implied in pathogenesis of pacific white shrimp (Krol *et al*., 1991) and cockle (Azevedo, 1993). Thus, Tenericutes had a potential perniciousness to animals' health according to the described above. The decrease of Tenericutes in this study indicated that Tenericutes may be sensitive to enrofloxacin, we can use enrofloxacin to control the Tenericutes community according to this and decline the possible pathogenic risk.

On the other hand, Proteobacteria is the most unstable among the four main phyla (Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria) in the intestinal microbiota (Faith *et al*., 2013). The selective pressure driven by dysbiosis can influence the stability of the microbial community and impair resistance to colonization, then Proteobacteria subsequently take the opportunity to make an expansion. Accordingly, an increased prevalence of Proteobacteria can be a marker for an unstable microbial community and a potential diagnostic criterion for disease (Shin *et al*., 2015). As previous studies illustrated, the expansion of intestinal Proteobacteria, at a deep level, reflects disturbances in metabolic and the innate immune response (Carvalho *et al*., 2012; Fei and Zhao, 2013). Therefore, our result suggests that the relative abundance of Proteobacteria increased after enrofloxacin treatment are evidence of an unstable microbial community of noble scallop, which will place the noble scallop in possible danger of affecting its physiological metabolic and making invasion by exogenous pathogens.

At the genus level, the most highly represented genus was *Mycoplasma*, which accounted for more than 50% of total detected OUTs. *Mycoplasma* is the smallest and simplest prokaryote so far has been found. It can infect people and other mammals and cause pneumonia and other diseases, such as well-known pathogens, *Mycoplasma pneumonia* and *M. gallisepticum* (Aceves *et al*., 2018). In some cases, they become intracellular pathogens, but under appropriate environmental conditions most remain a benign member of the host's microbiome (Brown *et al*., 2005). Although *Mycoplasma* was often reported to be pathogenic, it should play a positive role on noble scallop because it's abundant in intestine of ordinary individuals. Thus, its specific function to noble scallop needs further studies.

Our study also revealed that, with the increased enrofloxacin concentrations, the relative abundance of *Mycoplasma* dropped down and made space for *Vibrio*. *Vibrio* is the main genera of Proteobacteria. It was also detected on penaeid shrimp (Dempsey *et al*., 1989) and yellow catfish (Wu *et al*., 2010). Interestingly, *Vibrio*, which often acts as pathogen, increased obviously under the enrofloxacin treatment. The result is different from that done by Dethlefsen *et al*. (2008). Dethlefsen infected the body with *Vibrio* in advance, so the difference is likely due to the discrepant initial amount of *Vibrio*. Also, *Vibrio* is one of the most common opportunistic pathogens in marine environment and organisms. Its pathogenicity is greatly influenced by the physiological state of host and ambient water quality (Flick, 2007). In gram-negative bacteria, *Vibrio* has extremely powerful capacity of secreting extracellular protein (enzyme) (Marcello *et al*., 1996). The combine of extracellular enzyme products and hemolytic factors can destroy the cell membrane, mitochondria, endoplasmic reticulum and other cell endomembrane system in the body, resulting in the metabolic disorders of material and energy (Ghannoum *et al*., 2000).

Enrofloxacin have an action on bacterial topoisomerase as consequence of inhibiting DNA replication. It is reported a broad-spectrum antimicrobial, which is efficient on most gram-negative and gram-positive bacteria including *Vibrio* (Wang *et al*., 2005; Yu *et al*., 2014; Trouchon and Lefebvre, 2016), but on the contrary, it brought excessive growth of *Vibrio* as showed above. Given that *Vibrio* has such high pathogenicity, we should pay more attention to the boom of *Vibrio* after the use of enrofloxacin according to this study, and choose the better dosage of enrofloxacin to avoid the possible adverse effects behind.

At last, similarities in entire microbial community structure between groups were given by principal component analysis (PCA) and the hierarchical clustering heat map analysis. In PCA, PC1 and PC2 explained 42.15% of the variation of microbial community composition in total. the analysis supported that control group and low enrofloxacin dosage group were clustered together and clearly separated from the high enrofloxacin dosage group. The hierarchical clustering heat map analysis had the same result with principal component analysis. These results indicated that the high dosage of enrofloxacin (10 mg/L) had significant impact on the intestinal microbial community structure of noble scallops.

CONCLUSION

In summary, 10 mg/L enrofloxacin significantly affected the intestinal microbial community structure of noble scallop, which may bring potential dangerous to host health. Therefore, the low concentration of enrofloxacin (5mg/L) can be used as a safe dosage for noble scallop treatment without causing adverse effects to its intestinal flora structure. Our research provides initial guidance for the use of enrofloxacin in noble scallop, and helps to formulate appropriate treatment plans with taking the impact on intestinal flora into account. Finally, we hope to make contribution to eliminate or reduce flora disorders caused by the abuse of antibiotics and the spread of drug-resistant strains.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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