

Full Length Article

Composition and Diversity of Intestinal Microbiota of Sea Cucumber *Apostichopus japonicus* from Different Habitats

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Abstract

The intestinal flora of the sea cucumbers *Apostichopus japonicus* Selenka were detected by 16S rRNA sequence. The sea cucumbers were collected from Zhangzidao Island (ZZD), Dandong (DD) and Zhuanghe (ZH) at the Yellow Sea, Huludao Island (HLD) at the Bohai Sea, and Lvshun (LS) at the confluence of the Yellow Sea and the Bohai Sea. Proteobacteria was found to be the predominant phylum, while dominant genera were different from different habitats. *Halomonas* and *Shewanella* were the top two genera for groups at the Yellow Sea and the Bohai Sea, and genus *Bacteroides* was dominant at the confluence of the Yellow Sea and the Bohai Sea. The results of LS might be influenced by the peculiar geographical environment of the confluence. Moreover, some beneficial bacteria such as lactic acid bacteria and *Bifidobacterium* were detected with the proportion from 0.58% to 4.66% from all the samples. These results exhibit the relationship between intestinal flora of healthy sea cucumbers and their habitats, which may become directions for probable probiotics development and utilization. © 2018 Friends Science Publishers

Keywords: *Apostichopus japonicus*; Bohai sea; Composition; Diversity; Intestinal microbiota; Sea cucumber; Yellow sea

Introduction

Intestinal microbiota are essential for host's growth and survival, which play a crucial function in health and growth of the host, and is closely related to the physiological process of nutrition metabolism, biologic barrier, and immune defense (Postler and Ghosh, 2017; Wang *et al.*, 2017a). The intestinal microbial community are influenced substantially by host factors and external factors such as habitat and diet (Bletz *et al.*, 2016; Chen *et al.*, 2017). The intestinal microbiota of human (Qin *et al.*, 2010), mice (Yang *et al.*, 2017) and fish (Haygood and Jha, 2016) were extensively researched for its important role.

Apostichopus japonicus Selenka, a temperate sea cucumber, distributes widely throughout Northwest Pacific (Hamel and Mercier, 2013), which belongs to Echinodermata, Holothuroidea, Aspidochiroia. Sea cucumbers occupy the top taxonomic position of invertebrates, therefore come to the sustained attention as an evolutionary linkage between invertebrates and vertebrates (Zhou *et al.*, 2014). The animal is regarded as a model of visceral regeneration because of the digestive tract regeneration after evisceration (Ortiz-Pineda *et al.*, 2009; Mashanov and Garcia-Arraras, 2011), and can generate a variety of biosynthesis (Wang *et al.*, 2014; Li *et al.*, 2016).

Therefore, sea cucumbers become a research focus as well as commercially valuable aquatic animals, which annual production of sea cucumbers (fresh weight) exceeded **200,969 tons in China in 2014** (Han *et al.*, 2016). Thus *A. japonicus* was chased after prosperously as a vital fraction of the fishery resource in China (Chen, 2005). At present, intestinal microbiota is a growing preoccupation within *A. japonicus* research. Total prokaryotic cell counts in the intestine of *A. japonicus* were obtained by direct cell counting (Enomoto *et al.*, 2012). The intestinal flora transformation within foregut, hindgut or ambient sediment were characterized (Amaro *et al.*, 2009; Gao *et al.*, 2014) how dietary supplements impact on intestinal microbiota have been researched extensively (Chi *et al.*, 2014; Yang *et al.*, 2015). Intestinal bacteria was also proved, in some degree, contributed to the growth gap of body size of the animal (Sha *et al.*, 2016; Yamazaki *et al.*, 2016). But there have been a few research about the intestine colonizing microflora of sea cucumbers obtained from different habitats.

In this article, the intestinal flora of sea cucumbers *A. japonicus* from different habitats were analyzed by high-throughput sequencing based on 16S rRNA, the composition and diversity of intestinal microbiota were characterized, and candidate probiotics were excavated.

The statistics collected in this paper would enhance the comprehension of bacteria flora, and broaden the insight of probiotics exploitation on *A. japonicus* fishery.

Materials and Methods

Sampling and Processing

Five groups of clinically-healthy sea cucumbers were collected from their habitats near the suburbs of Liaoning province, China during April 13-19, 2016. The sea cucumbers were collected from Zhangzidao Island (ZZD), Dandong (DD) and Zhuanghe (ZH) at the Yellow Sea, and from Huludao Island (HLD), at the Bohai Sea, and from Lvshun (LS), at the confluence of the Yellow Sea and the Bohai Sea, China. Three sea cucumbers which weigh 150 to 200 g were collected from each spot. The body wall of the sea cucumber was cut with a sterile scissor after wiping the body surface with sterilized normal saline, and then the entire digestive tract was removed from the coelomic. The intestinal tract was collected by dislodging the stomach, and squeezing out the intestinal contents. A 5 mL injector without needle was used to simply rinse the digestive tract with normal saline, and visible contents were removed as softly as possible to avoid disturbing the intestinal mucosa. The digestive tracts were frozen in liquid nitrogen rapidly and then stored at -80°C for further analysis.

Sequencing and Data Analysis

The extraction and sequencing of 16S rRNA gene including V4 hypervariable region was then performed on an Illumina HiSeq platform (Novogene Bioinformatics Technology Co., Ltd). Sequences analysis was performed by Uparse software (Edgar et al., 2011). Sequences with $\geq 97\%$ similarity were assigned to the same OTUs. Multiple sequence alignment were conducted using the MUSCLE software (Edgar et al., 2011) to construct phylogenetic relationship in different samples (groups). Alpha diversity, beta diversity and cluster analysis were calculated with QIIME (Version 1.7.0) and displayed with R software (Version 2.15.3).

Results

Sequencing Results and Quality Control

The 16S rRNA V4 regions were assessed by the Illumina HiSeq sequencing platform, a total of 1,105,924 raw tags of rRNA gene sequences were assessed assembled. After trimming barcodes and primers and filtering low quality tags, 952,005 qualified tags were gained. These tags contained an average length ranged from 250 bp to 258 bp, approximated to the length of 16S rRNA V4 region. 899,541 effective tags were obtained after removing mitochondria and chloroplast. 845,333 tags, with an average of $56,355.5 \pm 10,778.7$ tags per sample were clustered into operational taxonomy units (OTUs).

Intestinal Bacterial Communities

OTU cluster: The effective tags were clustered into 27,117 OTUs (at an average of $1,808 \pm 827$ OTUs of each sample) at a distance of 0.03. Over 80% of tags were detected into genus level, while less than 38% of tags were detected into species level.

Alpha Diversity Analysis

The rarefaction curves based on the OTUs showed that all the intestinal sample of group DD, ZZD, LS, ZH and HLD tended to approach the saturation plateau (Fig. 1). Samples were rarefied to the lowest read number within the tags (39,502).

Alpha diversity of the gut bacterial communities differed in five groups. These five groups had the Good's coverage over 98.5% on an average (Table 1), which indicated that roughly two additional OTUs would be expected for every 100 additional sequenced reads. The Shannon and Simpson index decreased in group LS, ZH, HLD, DD and ZZD in community diversity order, and the Chao1 and ACE value decreased in group LS, ZH, HLD, ZZD and DD in community richness order. **More species were observed in group LS with greater community diversity and richness as well as the polygenetic diversity.** On the contrary, the lowest community diversity was estimated in group ZZD, and the lowest community richness was estimated in group DD.

Beta Diversity Analysis

Different multiple variation statistical analyses were used to identify the relationships as well as the differences among samples from different location with different raring mode. **The PCA plot (Fig. 2a) showed differences between group LS and the other four groups, and the differences within the group LS were also observed.** The Unweighted UniFrac-based 2D PCoA plot showed a similarity between the group ZH and the group HLD (Fig. 2b).

According to the clustering tree (Fig. 2c), the distances of samples were within 0.30, indicating that all intestines shared a high similarity rate in their bacterial structure. Shorter distance found in comparison with group ZZD between group DD and the other groups, and in comparison with group ZH between group HLD and the other groups. Group LS cannot clustered together with the others.

Taxonomy Composition of *A. japonicus* Intestinal Flora

At the phylum level, on an average 89.3% of all tags were detected into phylum level, and an average of 26.6 ± 3.2 , 26.3 ± 6.6 , 32.3 ± 4.0 , 29.0 ± 2.6 and 34.0 ± 1.0 different phyla were detected form group ZZD, DD, ZH, HLD and LS. Ten most prominent OTUs were Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, Cyanobacteria, Acidobacteria, Fusobacteria, Gemmatimonadetes, Verrucomicrobia and Chloroflexi (Table 2).

Table 1: Estimated OTU richness and diversity indices in intestinal microbiota of fifteen *A. japonicus* samples (mean± standard deviation, n=3)

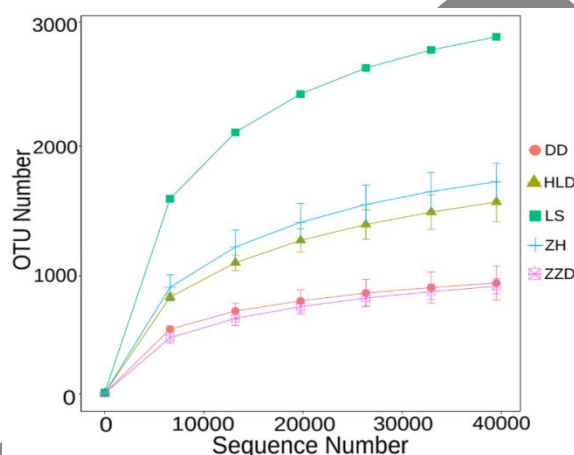
Habitat	Sample name*	Observed species	Community diversity		Community richness		Good's coverage
			Shannon	Simpson	chao1	ACE	
The Yellow Sea	ZZD	868±75	4.25±0.28	0.785±0.068	1107±35	1071±75	0.994±0.001
	DD	895±167	5.08±0.78	0.824±0.110	1072±243	1062±244	0.995±0.002
	ZH	1712±182	5.99±0.61	0.881±0.049	2067±17	2062±73	0.989±0.002
The Bohai Sea	HLD	1548±194	5.84±0.46	0.873±0.054	1976±377	1965±358	0.987±0.004
The confluence	LS	2880±30	9.59±0.10	0.995±0.002	3164±23	3226±66	0.986±0.001

*: The sea cucumbers were respectively collected from Zhangzidao Island (ZZD), Dandong (DD), Zhuanghe (ZH), Huludao Island (HLD) and Lvshun (LS), China

Table 2: Top 10 bacteria phyla isolated from 5 groups of *A. japonicus* samples (at the decrease order of group ZZD)

Taxonomy	Abundance (%)				
	ZZD*	DD	ZH	HLD	LS
Proteobacteria	72.15±6.33	66.36±12.32	67.02±3.26	68.22±2.17	36.44±4.83
Actinobacteria	6.39±5.49	3.03±0.66	3.43±1.08	3.69±1.32	7.63±1.55
Firmicutes	5.62±1.45	13.36±8.35	12.73±3.34	11.87±1.81	10.49±1.98
Bacteroidetes	2.29±0.15	6.15±2.16	4.06±1.99	5.77±3.27	10.3±0.64
Cyanobacteria	1.47±0.39	3.43±2.56	5.22±2.45	2.02±1.5	4.73±2.71
Acidobacteria	0.39±0.11	0.64±0.37	1.37±0.4	0.96±0.25	11.22±1.86
Fusobacteria	0.33±0.2	1.86±1.97	0.39±0.25	0.5±0.4	0.19±0.05
Gemmatimonadetes	0.24±0.13	0.11±0.09	0.79±0.47	0.36±0.28	5.84±0.84
Verrucomicrobia	0.18±0.11	0.11±0.08	0.43±0.1	0.35±0.01	3.33±0.64
Chloroflexi	0.16±0.08	0.19±0.04	0.44±0.07	0.24±0.09	1.25±0.25

*: Abbreviations are as in Table 1

**Fig. 1:** Rarefaction curves of observed species of *A. japonicus* at 97% similarity based on OTUs. Tags were normalized to the minimum numbers of tags (39,502) in all samples

At the class level, Gammaproteobacteria was the most predominant class, followed by Alphaproteobacteria and Epsilonproteobacteria in all five groups. All of these three classes are members of phylum Proteobacteria.

At the genus level, based on an abundance cutoff of 0.1%, 40, 52, 59.3, 60.3 and 90 genera were detected from group ZZD, DD, ZH, HLD and LS, respectively.

The predominant genus observed in group ZZD, DD, ZH and HLD were *Halomonas* (ZZD, 42.66±9.01%, DD, 36.97±18.72%, ZH, 31.73±7.52% and HLD, 31.96±10.03%), followed with *Shewanella* (ZZD, 14.34±3.14%, DD, 9.13±5.4%, ZH, 9.56±1.94% and HLD, 8.95±3.24%), while 3.72±1.02% of tags were identified to *Bacteroides* in LS.

Further, some potential probiotics such as LAB and bifidobacteria were detected from the intestinal microbiota according to our results. *Lactobacillus* spp., *Pediococcus* spp., *Leuconostoc* spp., *Lactococcus* spp., *Streptococcus* spp., *Enterococcus* spp. and *Bifidobacterium* spp. were detected at a cutoff of 0.01% (Table 3).

Discussion

Intestinal microbes play key roles in growth and immunity of the host by maintaining the microecology (Hempel *et al.*, 2012). According to de Almada *et al.* (2015), composition of the intestinal microbiota varies along with longitudinal variations in the intestine, which demonstrated that autochthonous flora in intestinal tract is more worth concerning than allochthonous flora. In our study, the intestinal contents were squeezed out to reduce the interference of transient bacteria. Then the intestinal microbiota of sea cucumber from different habitats were characterized by 16S rRNA sequencing to analyze composition and diversity.

Table 3: Relative abundance (%) of lactic acid bacteria and other candidate probiotics in the intestinal tract of *A. japonicus* samples

Taxonomy	Abundance (%)				
	ZZD	DD	ZH	HLD	LS
<i>Lactobacillus</i>	0.24	2.77	1.03	0.93	0.06
<i>Bifidobacterium</i>	3.72	0.21	0.24	0.36	0.42
<i>Streptococcus</i>	0.47	0.5	2.18	1.24	0.08
<i>Enterococcus</i>	0.17	0.8	0.79	0.69	0.01
<i>Lactococcus</i>	0.05	0.03	0.42	0.13	NF*
Total	4.65	4.32	4.66	3.35	0.58

*: NF, not found, the abundance < 0.01% was regarded as not found in the intestinal microbiota. Abbreviations are as in Table 1

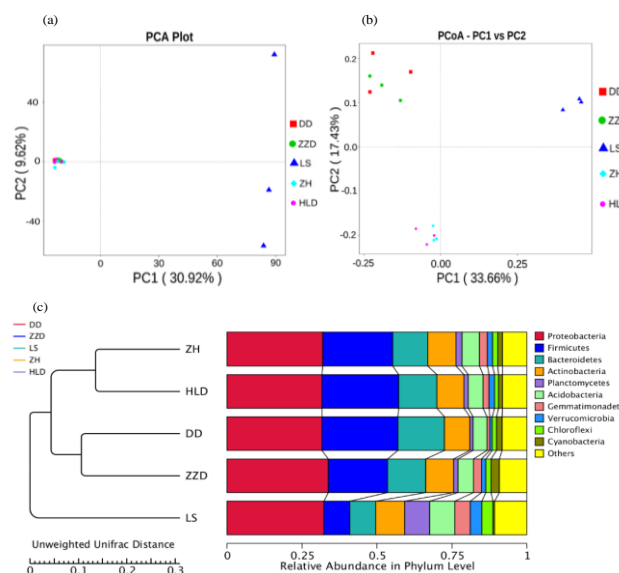


Fig. 2: Estimated beta-diversity of group DD, ZZD, LS, ZH and HLD based on OTUs analysis. (a), PCA plot. Percent variation expected of PCA were PC1 30.92%, PC2 9.62%; (b), PCoA plot. Percent variation expected of unweighted UniFrac-based PCoA were PC1 33.66%, PC2 17.43%; c, Unweighted UniFrac tree clustered by relative abundance of microbiota in samples and groups

Results showed that Proteobacteria was the predominant phylum, and Gammaproteobacteria was the predominant class in all samples. These results were similar to Gao *et al.* (2014), Sha *et al.* (2016), in which Proteobacteria was the predominant phylum in the intestinal microbiota of sea cucumber *A. japonicus*. While the results were different in groups from different habitats at the genus level. *Halomonas* and *Shewanella* were the top two genera in groups ZZD, DD, ZH, and HLD, while *Bacteroides* was the dominant genus in group LS. Microflora of *A. japonicus* intestinal contests acquired different in the former studies, such as *Aeromonas*, *Bacillus*, *Enterobacter*, *Flavobacterium*, *Micrococcus*, *Pseudoalteromonas*, *Pseudomonas* and *Vibrio* (Sun and Feng, 1989; Gao *et al.*, 2014; Bogatyrenko and Buzoleva, 2016). In addition, *Halomonas* was reported only by Bogatyrenko and Buzoleva as a major microflora with a

proportion of 3.8% (2016). These results showed the high diversity of *A. japonicus* intestinal microflora contests. Further, our study differed from these results greatly. Since the intestinal contests were squeezed out, the methods we adapted might be the reason for those differences. Phylum Proteobacteria was recognized as a vital component of intestinal microflora (Li *et al.*, 2014; Hacquard *et al.*, 2015), and class Gammaproteobacteria was reported as the predominant cultivable protease-producing bacteria widespread in marine environments (Zhang *et al.*, 2015). *Halomonas* and *Shewanella* belong to the class Gammaproteobacteria, which were present in different habitats of the marine environment, such as sediments, seawater and sea ice (Wang *et al.*, 2017b). These two genera might come from surrounding sediments, and might be easier to adhere to the intestinal tract.

Results of dominant genera and clustering analysis showed a similarity among groups from the Yellow Sea and the Bohai Sea. These exhibited the stability of core intestinal flora of sea cucumbers from these two areas. In addition, group LS cannot be clustered together with the other four groups. Compared with the other four groups, greater community diversity was observed in group LS. The peculiar results of LS might be determined by the special geographical environment of the confluence. Diet remarkably affects in both the microbial community species diversity and evenness (Roura *et al.*, 2017). Habitat difference influenced the diet of sea cucumber, thus influence intestinal microflora of sea cucumber.

Furthermore, some beneficial bacteria such as LAB and *Bifidobacterium* were detected from all the samples with the proportion from 0.58% to 4.66%. Those bacteria were widely used as probiotics in aquaculture industry (Akrami *et al.*, 2015). Particularly, LAB can decrease the gut pH and improve intestinal epithelial cells defense by the secretion of short-chain fat acids (Bottacini *et al.*, 2014; de Almada *et al.*, 2015). These candidate probiotics distributed widely in sea cucumber intestine suggested potential probiotics development prospects. *Lactobacillus* was found more abundant in group DD, *Bifidobacterium* was found more abundant in group ZZD, and *Streptococcus* was found more abundant in group ZH. The abundant difference would provide a positive direction for further probiotics screening.

Conclusion

Habitat difference impact on the intestinal microflora of sea cucumber vastly. *Halomonas*, *Shewanella* and *Bacteroides* were detected dominant in the intestinal microflora. Potential probiotics lactic acid bacteria and *Bifidobacterium* is worth follow-up developing.

Acknowledgements

This work was financially supported by the Department of Education of Liaoning Province (L201602), Oceanic and Fishery Department of Liaoning Province (201510) and

Dalian Municipal Science and Technology Plan Project (2015R080).

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(Received 16 April 2018; Accepted 04 May 2018)