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ISSN 0792 - 156X

PUBLISHER:

The Society of Israeli Aquaculture and Marine Biotechnology (SIAMB)

The Israeli Journal of Aquaculture - Bamidgeh, IJA_71.2019.1538, 7 pages

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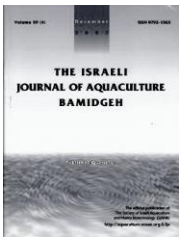

Salinity Effect on Intestinal Microbiota in Golden Pompano *Trachinotus Ovatus* (Linnaeus, 1758)

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Keywords:*Trachinotus ovatus*; salinity; intestinal microbiota; 16s rRNA

Abstract

Golden pompano (*Trachinotus ovatus*) is a commercially important marine fish which is widely cultured in the coastal area of South China. Salinity is one of the most important environmental factors influencing the intestinal microbiota. The aim of this study was to investigate the intestinal microbiota of juvenile golden pompano reared at different salinities. In this 56 day experiment, juveniles were reared at 5, 15, 25 and 35‰ salinities. Based on ACE, Chao1 and Shannon, Simpson diversity indices, salinity did not influence the bacterial richness and diversity in golden pompano intestine. The data indicated that the dominant phyla in golden pompano intestine reared at each salinity group were *Proteobacteria*. The principal coordinates analysis (PCoA) indicated that the bacterial communities were not clustered, and the bacterial communities were very different in the salinity groups. The findings showed that golden pompano have specific intestinal microbiota in different salinity environments. However, the intestinal microbiota in golden pompano were strongly influenced by salinity and were also affected by the phylogeny of golden pompano. Further studies are needed to explore the relationship between the intestinal microbiota and growth of golden pompano under different salinities.

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Introduction

The intestinal tract is home to trillions of microbial cells known as intestinal microbiota. Maintaining functional, stable intestinal microbiota is important to the host's health, by performing many beneficial functions to the host such as balancing the immune response, absorbance of nutrients, and maintaining homeostasis (Hooper and Macpherson, 2010). The mutually beneficial interactions between host and microbes are shaped by eons of coevolution taking place in all creatures (Chung et al., 2012). Different host species are colonized by different assemblages of microbial populations (Lay et al., 2005). In addition to the host phylogeny, additional factors such as lipid sources (Zhang et al., 2014a) and environment (Zhang et al., 2016) can also influence intestinal microbiota composition, and consequently have adverse health effects to the host. Variation in intestinal microbiota composition in fish is correlated with habitat salinity, trophic level, and possibly taxonomy of the fish species (Sullam et al., 2012). Factors influencing intestinal microbiota, especially those specific to aquatic animals, have been studied. Aquatic animals such as fish are known to constantly experience changes in environmental conditions such as salinity, temperature, pressure and factors that may cause stress.

Euryhaline fish can adapt to a broad range of environmental salinities. How the microbiota in these host species respond to environmental changes is an important question, not only to researchers in aquaculture physiology but also to the fish industry. The digestive tract is known to harbor complex assemblages of microbiota, that maintain a functional, stable microbial community. This is important to the host's health but can be constantly influenced by various environmental factors (Yang et al., 2018). The shift in the intestinal microbiota with respect to the change of salinity is likely attributed to the environmental selection for microbes which could grow better under high or low salinity, and the host response to salinity stress and subsequent stress exerted on the intestinal microbiota (Zhang et al., 2014a; Zhang et al., 2016). The intestine is one of the major osmoregulatory organs and can express genes involved in salinity acclimation in fish (Wong et al., 2014), however the influence of salinity changes on the intestinal microbiota, which are important in the host, remains unknown (Shannon, 1948). Findings on the effect of salinity on intestinal microbial communities can provide an insight into differences in growth conditions, explain the influence of salinity on host physiology, and demonstrate the impact of environmental factors on intestinal microbiota. However, the relationship between salinity, intestinal microbiota, and growth conditions of aquatic animals remains unclear.

Golden pompano, *Trachinotus ovatus* belongs to the Carangidae family. It is distributed in tropical and subtropical areas of Southeast Asia and the Mediterranean Sea. Due to its delicious taste and rapid growth, golden pompano is one of the most important marine fish commercially cultured in South China (Sun et al., 2013; Tan et al., 2016). Information on the life cycle of golden pompano, and several key issues related to larval rearing, such as food and feeding, the development of the larval digestive system and weaning, have been successfully addressed (Ma et al., 2015). Studies have shown that the intestine is inhabited by diverse bacteria which have a mutual relationship with the host and play an important role in growth (Verner-Jeffreys et al., 2003). The effect of salinity on intestinal microbiota however has not been reported. In the present study, groups of golden pompano were reared at different salinities to assess the effect on intestinal microbiota. An Illumina-based high throughput sequencing method was used to analyze the biodiversity and composition of the intestinal microbiota, and the association between salinity and intestinal bacterial profile of golden pompano.

Materials and Methods

Fish, experimental design and experimental conditions.

Experimental fish were obtained from the Tropical Fisheries Research and Development Centre, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Science, Lingshui (Hainan, China). The mean body weight of the fish was 12.07 ± 0.13 g. Fish were initially stocked into 3000 L tanks with recirculating seawater (30‰).

In the 56 day experiment, pH, oxygen, and temperature were measured daily by a HQ30d (HACH30d, Loveland, Colorado, USA). Oxygen saturation was close to 100% at all times and water flow was adjusted to keep ammonia well below critical levels. Two air stones were used in each tank to maintain dissolved oxygen close to saturation. Salinity in each rearing tank fluctuated by ±4‰ per day by adding fresh or seawater to maintain the target salinity of 5‰, 15‰, 25‰ and 35‰. All experiments were conducted in triplicate. A total of 25 fish were stocked into each 600 L tank and fish were fed commercial pellets (Hengxing, Guangzhou, China) twice daily at 07:00 and 17:00

hours to apparent satiation. During the experimental period, water temperature was maintained at 26.9-31.4°C. The pH, ammonia nitrogen, and dissolved oxygen ranged between 7.17-8.23, 0.05-0.1 mg/L, and 6.16-8.06 mg/L, respectively.

Sample collection and DNA extraction.

At the end of the feeding trial, fish were fasted for 24h before sampling, and were then anesthetized with 100 mg/L Eugenol (Shanghai Medical Instruments Co., Ltd., Shanghai, China). Intestines of five fish from each tank were aseptically dissected and the intestinal contents collected and immediately stored in liquid nitrogen until used. All experiments in this study were approved by the Animal Care and Use Committee of South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences (no. SCSFRI96-253). Total bacterial community DNA was isolated with a TIANamp Micro DNA Purification Kit (Tiangen, Beijing, China). The bacterial DNA yield was measured in a NanoDrop (Thermo Fisher Scientific, Waltham, MA, US) and qualitied by PCR amplification of the bacterial 16S rRNA genes.

Illumina high-throughput sequencing of barcoded 16S rRNA genes.

Bacterial DNA was used as the template for the 16S rRNA V4+V5 region amplification (Sun et al., 2013). The forward and reverse primers were 515F: 5'-GTGCCAGCMGCCGCGG-3' and 907R: 5'-CCGTCAATTCMTTTRAGTTT-3', respectively. Unique eight-base barcodes were added to each primer to distinguish the PCR products. The PCR reaction mixture (20 μL) contained 0.25U of PrimeSTAR HS DNA Polymerase, 10 μL of the corresponding 5×PrimeSTAR Buffer (Mg^{2+} Plus), 4 μL of dNTP Mixture (2.5 mM each), 1 μL of forward Primer (5 μM), 1μL of reverse Primer (5 μM) and 10 ng of Template DNA. The PCR program began with a 1 min denaturation step at 98^0C followed by 1 cycle, a 1min annealing step (30 s at 98^0C; 30 s at 55^0C; 30 s at 72^0C) followed by 27 cycles and a 5 min extension step at 72^0C. The PCR products were purified using an AxyPrep™DNA Gel Extraction Kit (AXYGEN, Hangzhou, China). In this experiment, 30 ng of each purified PCR product was subjected to Illumina-based high-throughput sequencing (Guangzhou Saizhe Biotechnology Co., Ltd., Guangzhou, China). The sequences obtained are available in the GenBank database (accession no. PRJNA253075).

Bioinformatics and statistical analyses.

After de-multiplexing the data and discarding certain abnormal reads, the remaining ones were converted to FASTQ format. In this study, 250bp reads were truncated at any site with an average quality score of < 20 over a 10bp sliding window. Reads <50bp were discarded. The minimum value of the overlap was 10bp when merging the reads; sequences whose barcodes did not match an expected barcode were also discarded. Chimeric sequences were determined by UCHIME (Edgar et al., 2011). Operational taxonomic units OTUs were defined with a threshold of 97% similarity by UPARSE (Edgar, 2013). Alpha diversity (ACE estimator, Chao1 estimator, observed Operational Taxonomic Index, Shannon index and Simpson index) and beta diversity (PCoA) analysis was calculated with QIIME (v1.7.0) and displayed with R software (v2.15.3). The unweighted tests were applied to determine whether two or more communities have the same structure (Lozupone and Knight, 2007). A heatmap showing the relative abundance of selected OTUs was generated using the gplots package in R, version 3.1.0. Data were reported as means ± standard errors (SE) and one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests was used to determine significant differences between treatments. All statistical analyses were performed using SPSS 22.0 (SPSS Inc., Chicago, USA). The significance level adopted was 95% ($p < 0.05$).

Results

Illumina sequencing.

A total of 433,539 sequences were obtained for golden pompano intestinal bacteria with an average of 39,412 sequences per sample (30,910-56,807 sequences). The average length of the reads was 393bp; all sequences were identified as bacteria. Sequences with more than 97% similarity were clustered into operational taxonomic units (OTUs). To estimate and compare the bacterial diversity in each group, bacterial richness and diversity indices were calculated from the proportion of OTUs. Based on ACE, Chao1 and Shannon, Simpson diversity indices, salinity did not influence the bacterial richness and diversity in golden pompano intestine ($p < 0.05$) (Table 1).

Table 1 Illumina high-throughput bacterial diversity richness (OTUs), diversity index (Shannon and Simpson) and estimated OTU richness (ACE and Chao1) for intestinal bacterial diversity analysis of golden pompano.

Salinity	*5‰*	*15‰*	*25‰*	*35‰*
Sampling depth				
Mean sequences	32896	46510	33200	42873
Richness estimators				
ACE	518.02±115.55	627.25±30.58	1105.69±385.82	511.56±52.76
Chao1	650.32±25.17	733.40±55.73	1167.51±400.71	539.92±57.69
Diversity estimators				
Shannon	3.42±0.16	3.26±0.13	4.41±1.36	2.68±0.42
Simpson	0.81±0.00	0.76±0.03	0.79±0.10	0.61±0.09

Intestinal microbiota composition.

At the phylum level, the intestinal microbiota composition revealed the presence of *Bacteroidetes*, *Cynaobacteria*, *Proteobacteria*, *Fimicutes,* and *Tenericutes*. However, the abundance levels between the sampling sites were different, particularly for *Bacteroidetes* which was less abundant in each salinity group. The data suggested that the dominant phyla in golden pompano intestine reared at each salinity group were *Proteobacteria* (Fig 1).

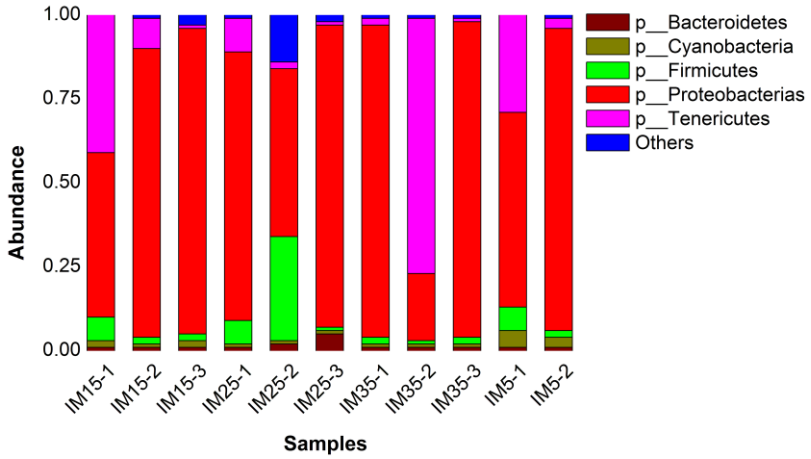

Fig. 1 Bacterial populations at phylum levels in the intestinal tract samples of four salinity levels of golden pompano. 5‰ (IM5-1, IM5-2), 15‰ (IM15-1, IM15-2, IM15-3), 25‰ (IM25-1, IM25-2, IM25-3), 35‰ (IM35-1, IM35-2, IM35-3).

Microbiota structure analysis. Principal Co-ordinates analysis (PCoA) was used to show the microbial community compositions from golden pompano reared in different salinities based on the OTU distribution. Differences in multi-dimensional data are reflected in the two-dimensional coordinate chart, wherein the more similar the community composition, the closer the samples are on the PCoA map. Compared with samples from 15‰ salinity, the intestinal microbial communities of 25‰ were relatively complex (Fig 2).

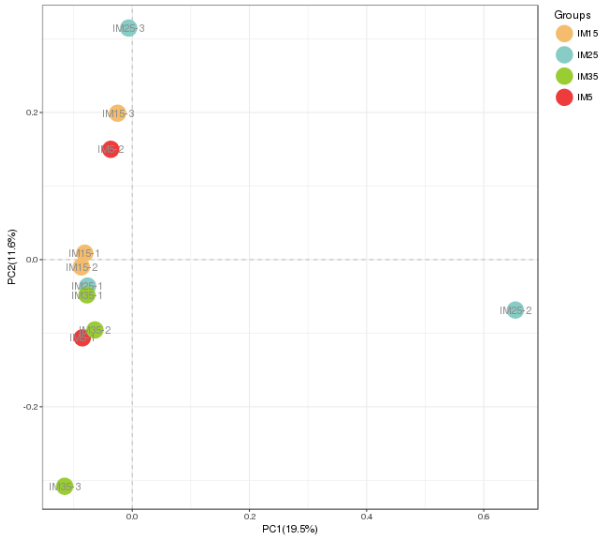

Fig.2 Principal coordinate analysis (PCoA) based on Unifrac analysis of the intestinal bacterial populations of golden pompano. 5‰ (IM5-1, IM5-2), 15‰ (IM15-1, IM15-2, IM15-3), 25‰ (IM25-1, IM25-2, IM25-3), 35‰ (IM35-1, IM35-2, IM35-3).

The (PCoA) indicated that the bacterial communities were not clustered, and the bacterial communities differed in the different salinity groups. However, the bacterial community did not cluster and the difference was great at the 25‰ salinity group. Heatmap analysis also showed a

similar with trend in the PCoA (Fig 3). The results showed large differences in the bacterial community according to each salinity group.

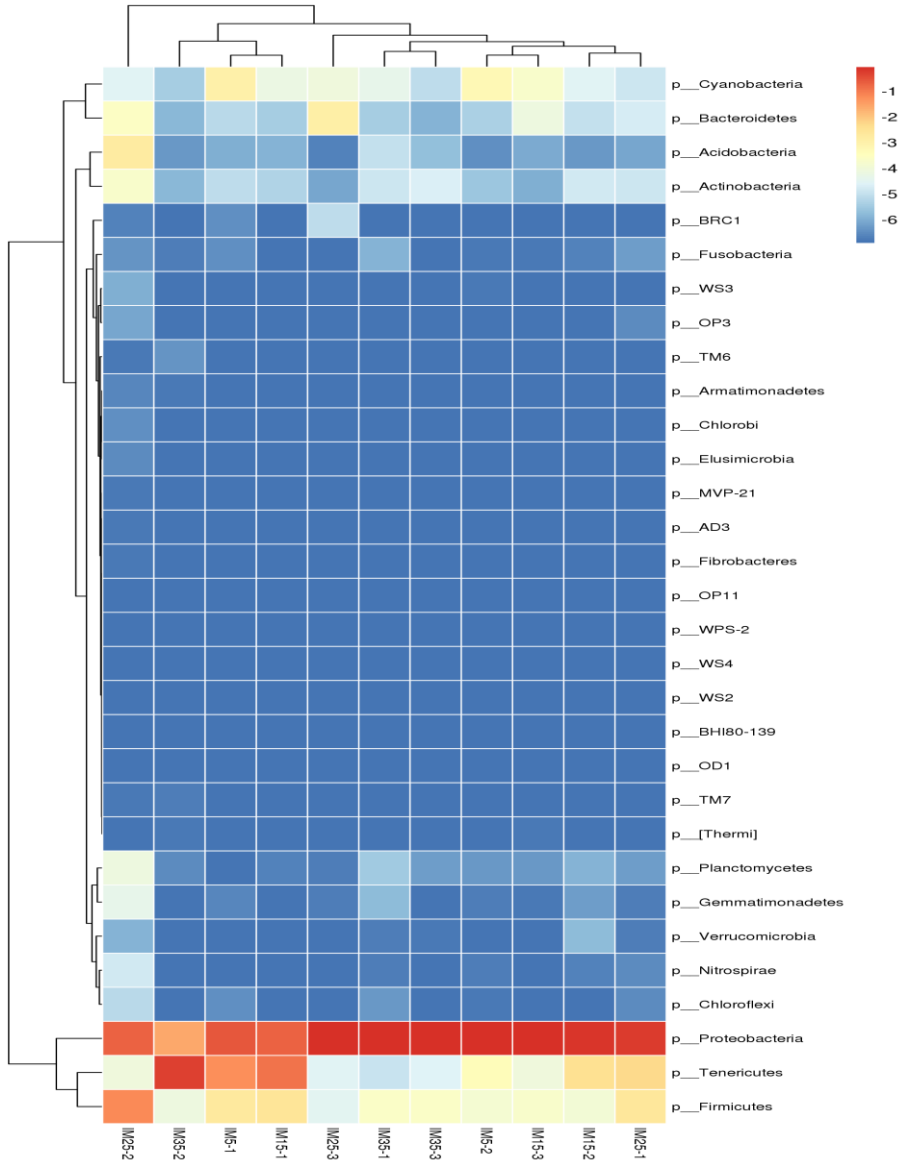

Fig. 3. Heatmap of the spot corresponds to the bacterial abundance in golden pompano intestines. The OTUs were organized according to their phylogenetic positions and the taxa of OTUs are shown on the right. The relative values of the OTUs are depicted by color intensity. 5‰ (IM5-1, IM5-2), 15‰ (IM15-1, IM15-2, IM15-3), 25‰ (IM25-1, IM25-2, IM25-3), 35‰ (IM35-1, IM35-2, IM35-3).

Discussion

Factors such as diet and living environment can affect intestinal microbiota (Zhai et al. 2017; Galley et al., 2014; Etyemez Büyükdeveci et al., 2018). Previous studies have shown that intestinal bacteria are important in growth and nutrient absorption (Wang et al., 2017). Recently, greater attention has been paid to the relationship between the host and intestinal microbiota in aquatic animals (Zhang et al., 2014b). As salinity is an important environmental factor for fish growth, the bacterial compositions in golden pompano reared at different salinities were characterized here.

Studies reported that diet and host phylogeny are the two key factors responsible for the composition of intestinal bacteria (Ye et al., 2014; Chen et al., 2018). Our results indicated the effect of salinity on intestinal microbiota composition of golden pompano. The effects of salinity on bacterial communities in living environments have been studied extensively (Lozupone and Knight, 2007). However, the influence of salinity on the intestinal microbiota of fish in various environments was only described by Sullam et al., 2012. The study showed that salinity affected the bacterial composition of fish. Our study systematically compared changes in the intestinal microbiota of golden pompano reared at different salinities. Changes in microbial community structure were observed based on the PCoA plot, indicating that salinity was also a key factor for intestinal microbiota, apart from host phylogeny. Our experimental results showed that there were certain differences within the groups, which might be related to the genetic background. At different salinity

levels, the observation of dominant microbial populations in fish populations indicated that dominant populations tolerated changes in salinity.

In our study, differences in gut microbiota structure were observed between salinity groups. The experimental data suggested that the major OTU distribution in golden pompano were *Proteobacteria* which is most common in zebrafish (Roeselers et al., 2011), grass carp (Wu et al., 2012) and *Penaeus monodon* (Rungrassamee et al., 2014). In the present study, the abundance of *Proteobacteria* in golden pompano was relatively high. This could be attributed to differences in phylogeny and salinity. Consistent with previous studies, *Proteobacteria* were dominant in *Huso dauricus* (Lv et al., 2018), *Oreochromis niloticus* and *Litopenaeus vannamei* (Zhang et al., 2016). *Proteobacteria* are the dominant phyla. Due to the limited number of studies on fish intestinal microbiota, the function of this phylum in fish intestines remains unknown. In general, our observations support the development of host phylogeny in shaping the composition of intestinal microbiota (Roeselers et al., 2011). Overall, the results showed that golden pompano reared at each salinity group had similar intestinal biota but the proportions were different.

Studies have shown that some metabolic processes of intestinal bacteria produce a variety of proteases that could significantly improve the digestive ability of fish (Pemberton et al., 1997). Therefore, it is particularly important to further explore the effects of microorganisms on the growth of golden pompano, especially for the benefits of factory farming systems.

This study will increase our understanding of the effects of salinity on bacterial community composition in golden pompano intestine and provide valuable data for the development of prevention mechanisms for golden pompano cultivation. Further studies are needed to explore the relationship between the intestinal microbiota and growth of golden pompano under different salinities. Future work needs to be carried out and should be particularly focused on revealing the relationship between symbiotic microbiota and the host.

Acknowledgments

This study was supported by China Agriculture Research System (CARS-47), China-ASEAN maritime cooperation fund, the Natural Science Foundation of Guangdong Province (2015A030313818), National Science & Technology Infrastructure platform (2018DKA30470).

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