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Short communication

The gut microbiota community and antioxidant enzymes activity of barramundi reared at seawater and freshwater

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ABSTRACT

To understand the physiological responses of euryhaline fish to reared salinity, the gut microbiota composition and antioxidant enzymes activity of same batch barramundi *Lates calcarifer* reared in two extreme salinity condition (seawater and freshwater) were studied, under laboratory condition. The gut bacterial composition was analyzed using high-throughput Illumina sequencing. Acid phosphatase (ACP), catalase (CAT), glutathione peroxidase (GSH-Px), lipid peroxide (LPO), lysozyme (LZM), malonaldehyde (MDA), peroxidase (POD), and superoxide dismutase (SOD) were used as biochemical indicators. The reared salinity did not change the major composition of barramundi gut bacteria under circulating aquaculture system. *Proteobacteria*, *Firmicutes* were the most phylum in barramundi gut microbiota community, and *Exiguobacterium*, *Citrobacter*, *Acinetobacter*, *Pseudomonas* were the dominate genus. CAT and ACP activity in barramundi liver were found significantly different between freshwater and seawater group. GSH-Px, LZM, POD, SOD activity and MDA, LPO levels were not significantly affected by reared salinity. This study is the first high-throughput analyses of the gut microbiota diversity in barramundi from same batch of siblings reared under two extreme salinity condition. And the findings in the present study can be instructive to the management of animal health in barramundi circulating farming activities, and further euryhaline fish gut microecology research.

1. Introduction

Gut microbiota, composed of a diverse and vast population of microorganisms, plays an important role in the nutrient digestion, immune responses and disease resistance [1–3]. The composition of gut microbial community could be affected by many factors such as stress and nutritional status, and thus lead to the changes in immune functions for the hosts [4,5]. And the selectivity for bacterial proliferation in gut was greatly affected by gut environments, depending on a functional equilibrium with the host, environment and dietary factors [1,6]. Fish gut is more likely to be affected by the surrounding environment compared to mammals, and the gut microbial composition changes more rapidly and significantly [7]. Furthermore, physiological responses would happen when the environment changed, to cope with appeared stress. The increased production of reactive oxygen species (ROS) will induce oxidative stress, and antioxidant defense systems were developed to copy with oxidative stress [8,9]. The activities of antioxidant enzymes are the potential indicators of oxidative stress, and expected to change due to external factors such as pH, salinity [10,11]. Briefly, both gut microbial composition and enzymes activity were affected by host, environment and dietary factors [12]. Salinity is one of the most common factors in aquatic environment, affecting physiological status in fish culturing. However, the information on the physiological responses of fish from same batch to environmental salinity is rare. Further research should be conducted to investigate the effect of reared salinity on fish, even for the same fish siblings.

The barramundi *Lates calcarifer* (Bloch) is a euryhaline teleost found in tropical and subtropical estuarine areas and native to the Indo-Pacific region. It is a highly valued commercial and recreational species for its flesh taste and rapid growth [8]. The barramundi can tolerate all levels of salinity from fresh to seawater allowing them to be cultured in both environments [13]. Thus, it is the suitable species for external factors research in aquatic environment.

In the present study, the same batch barramundi siblings were reared in seawater and freshwater circulating aquaculture system respectively from juvenile, and fed with the same commercial pelleted.

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The gut bacteria and enzymes activity were characterized under natural conditions. This study aimed to characterize the gut bacteria and enzymes activity features of fish under two extreme salinity condition (seawater and freshwater). And the findings of this study can be instructive to the management of animal health in aquaculture, and euryhaline fish intestinal microecology research.

2. Materials and methods

2.1. Animals and treatment

Barramundi *Lates calcarifer* (Bloch) from same batch of siblings, were obtained from Tropical Aquaculture Research and Development Center, South China Sea Fisheries Research Institute (Hainan, China). On 40 days post hatching, fish were divided into two rearing groups. One group was reared in the aquarium supplied continuously with circulating seawater (32 psμ, 31 °C) in triplicates, and another group were reared in the aquarium supplied continuously with circulating freshwater (1 psμ, 31 °C) in triplicates. The oxygen was maintained at 7.20 mg/L through an inflation pump in each aquarium. During the experimental period, fish were fed with the commercial pelleted diet (TZU-Feng Aquaculture Supplies CO., LTD., composition was as follows: 460 g kg^{-1} crude protein, 120 g kg^{-1} crude fat, 30 g kg^{-1} crude fibre, 160 g kg^{-1} crude ash, 40 g kg^{-1} calcium and 30 g kg^{-1} phosphorus) twice daily.

The experimental procedure was complied with the standards of Institutional Animal Care and Use Committee guidelines [14]. All experiments were conducted in line with the principles and guidelines for the care and use of live fish and the guidelines for animal experimentation approved by the Animal Experimental Council (AEC/NRIFS) of the National Research Institute of Fisheries Science, Fisheries Research Agency.

2.2. Experimental design and sampling

After five months rearing, 30 healthy fishes (430 ± 20 g) were selected randomly from each group respectively for sampling and further experiments. Approximately, ten healthy fishes from each replica were randomly picked up for gut content collection and enzyme activity measurement. The fishes were anaesthetized with overdose tricaine methanesulfonate (MS-222, Sigma-Aldrich, St. Louis, MO, USA) before dissection. Then, the fish were dissected with sterile scissors. For gut microbiota composition experiment, the intestinal contents were carefully collected and preserved into 1.5 mL sterile centrifuge tube and stored at −80 °C until DNA extraction. For enzyme activity measurement experiment, the liver from each fish were collected and preserved into 5 mL sterile centrifuge tube and stored at −80 °C until enzyme activity measurement.

2.3. DNA extraction

Samples were prepared for genomic DNA extraction using EZNA Stool DNA Kit (Omega Bio-tek), according to the manufacturer's instruction. DNA concentration and quality were checked using Qubit 3.0 Fluorometer (Invitrogen, US) and agarose gel electrophoresis.

2.4. Amplicon generation and library preparation

The purified DNA (20–30 ng) was used to generate amplicons. The V3 and V4 hypervariable regions of prokaryotic 16S rDNA were selected for generating amplicons and taxonomy analysis. A panel of proprietary primers were designed to detect the V3 and V4 variable regions in bacteria and Archaea16S rDNA.

The V3-V4 region of bacteria 16S ribosomal RNA genes was amplified by PCR using the forward primers containing the sequence "CCTACGGRRBGCASCAGKVRVGAAT" and reverse primers containing the sequence "GGACTACNVGGGTWTCTAATCC". At the same time, indexed adapters were added to the ends of the 16S rDNA amplicons to generate indexed libraries ready for downstream NGS sequencing on Illumina Miseq.

PCR amplifications were performed in triplicate in a 25-μL mixture containing 2.5 μL TransStart Buffer, 2 μL dNTPs, 1 μL of each primer, and 20 ng template DNA. The thermal cycling program was performed as follows: 94 °C for 3 min, followed by 24 cycles at 94 °C for 5 s, 57 °C for 90 s, 72 °C for 10 s and a final extension at 72 °C for 5 min. The PCR products were examined using 1.5% agarose gel, and then excised and purified using the QIAquick Gel extraction kit (Qiagen, Hilden, Germany) according to the manufacture protocol.

2.5. Bacterial 16s rRNA gene sequencing and analyses

The purified PCR products were used for library preparation and high-throughput sequencing. DNA libraries concentrations were validated by Qubit3.0 Fluorometer. Quantify the library to 10 nM, DNA libraries were multiplexed and loaded on an Illumina MiSeq instrument according to manufacturer's instruction (Illumina, San Diego, CA, USA). Sequencing was performed using PE250/300 paired-end, image analysis and base calling were conducted by the MiSeq Control Software (MCS) embedded in the MiSeq instrument.

The QIIME data analysis package was used for 16S rRNA data analysis [15]. After sequencing, the paired-end reads (forward and reverse reads) were joined, assigned to samples based on barcode and truncated by cutting off the barcode and primer sequence [16]. The quality filtering on joined sequences was performed and the sequences that did not fulfill the following criteria were discarded: sequence length < 200 bp, no ambiguous bases, mean quality score ≥ 20. Then the sequences were compared with the reference database (RDP Gold database) using UCHIME algorithm to detect chimeric sequence, and then the chimeric sequences were removed.

The effective sequences were used in the final analysis. Sequences were grouped into operational taxonomic units (OTUs) using the clustering program VSEARCH (1.9.6) against the Silva 128 database preclustered at 93% sequence identity. The Ribosomal Database Program (RDP) classifier was used to assign taxonomic category to all OTUs at a confidence threshold of 0.8. The RDP classifier uses the Silva 128 database that has taxonomic categories predicted to the species level [15].

Sequences were rarefied prior to calculation of alpha and beta diversity statistics. Alpha diversity indexes were calculated in QIIME from rarefied samples using the Shannon and Simpson index for measuring diversity and the ACE and Chao index for richness.

2.6. Enzyme activity measurement

The frozen liver was thawed, weighted and homogenized for enzymatic assays, using a glass homogenizer on ice in 0.2 M NaCl (w/v) [17]. The homogenate was centrifuged at 13,000 g for 10 min at 2 °C. Then, the aqueous supernatant was collected and incubated in the enzyme substrate and read on a spectrophotometer (UV-1800BPC, LiuYi Biotechnology co., Ltd, China) at a specific wavelength. Enzyme activities were tested in triplicate.

Acid phosphatase (ACP, E.C. 3.1.3.2), Catalase (CAT, E.C. 1.11.1.6), Glutathione peroxidase (GSH-Px, E.C. 1.11.1.9), lysozyme (LZM, E.C. 3.2.1.17), Peroxidase (POD, E.C. 1.11.1.7), Superoxide dismutase (SOD, E.C.1.15.1.1) activities and Lipid peroxide (LPO), Malonaldehyde (MDA) levels were measured and used as biochemical indicators. All enzymes activity in liver was measured with commercial kits, and conducted according to the manufacturer's instructions.

ACP activity was measured using an acid phosphatase activity assay kit (Catalog No. A060-2), and detected according to the red shades at 520 nm. CAT activity was measured using a catalase activity assay kit (Catalog No. A007-1). CAT can decompose H_2O_2 and this reaction can be quickly suspended by adding ammonium molybdate. The rest of

H_2O_2 combined with ammonium molybdate to produce a pale-yellow complex compound, which was detected at 405 nm. GSH-Px activity was measured using a glutathione peroxidase activity assay kit (Catalog No. A005). In the assay, glutathione peroxidase could catalyze the reaction of reduced glutathione to hydrogen peroxide. LZM activity were determined was measured using a catalase activity assay kit (Catalog No. A050-1), and determined by turbidimetry assay. POD activity was assayed using a peroxidase assay kit (Catalog No. A084-1), POD can catalyze the reaction of hydrogen peroxide, and the enzyme activity of peroxidase was obtained by measuring the change of absorbance at 420 nm. SOD activity was measured using a superoxide dismutase activity assay kit (Catalog No. A001-1), and determined by the xanthine oxidase method (hydroxylamine). LPO level was measured using a lipid peroxide assay kit (Catalog No. A106), and determined by the thiobarbituric acid reacting substance method. MDA level was measured using a malonaldehyde assay kit (Catalog No. A003-1).

The CAT, GSH-Px, LZM, POD, SOD activity and MDA level were expressed as units per milligram of protein (U/mg prot). The ACP activity was expressed as gram of protein (U/g prot), and MDA equivalents in μmol/g prot. Soluble protein of crude enzyme extracts was quantified by the bicinchoninic acid method [18], using the bicinchoninic acid protein assay kit (Catalog No. A045-4).

2.7. Statistical analyses

The results were analyzed by independent *t*-test at 5% significance level using SPSS version 18.0 (SPSSInc, Chicago, IL, USA). Results were considered statistically significant when probability (*P*) values were less than 0.05.

3. Results

3.1. Statistical analysis of sequences

A total of 708,978 effective tags were obtained in freshwater group with 349 bp average length, while 622,516 effective tags with 407 bp average length in seawater group. In this study, 2175 OTUs were defined based on a similarity greater than 93% with average of 242 OTUs in freshwater group, while 1872 OTUs were defined based on a similarity greater than 94% with 242 OTUs averagely in seawater group. The estimators of community richness (ACE and Chao) and diversity (Shannon) are shown in Table 1, and there was no significant difference ($P > 0.05$). Results from the analysis of alpha diversity metrics showed that the microbial richness and diversity were not altered significantly between freshwater and seawater group ($P > 0.05$), although there was sign of reduction in seawater group.

3.2. Taxonomic composition

There was no significant difference for the relative abundance of dominate phylum and genus between freshwater and seawater group ($P > 0.05$). A total of six phylum were detected in both freshwater group and seawater group. *Proteobacteria* and *Firmicutes* were the dominate phylum both in freshwater and seawater group, without significant difference between freshwater and seawater group respectively ($P > 0.05$). The relative abundance of *Proteobacteria* and *Firmicutes* were 56.14 ± 1.9%, 38.72 ± 2.00% respectively in freshwater group, and 54.14 ± 0.46%, 41.92 ± 0.81% respectively in seawater group (Table 2). A total of 406 OTUs were detected as core microbiota for the digesta compartments in freshwater group, and 322 OTUs in seawater group. 30 bacteria genus were detected after further analysis in freshwater group, and 39 bacteria genus in seawater group. *Exiguobacterium*, *Citrobacter*, *Acinetobacter*, *Pseudomonas*, *Escherichia-Shigella*, *Lactococcus*, *Anoxybacillus*, *Geobacillus* were the main genus (relative abundance > 1%) both in freshwater group and seawater group, without significant difference respectively ($P > 0.05$). *Exiguobacterium*, *Citrobacter*, *Acinetobacter*, *Pseudomonas* were the dominate genus, whose relative abundance > 10% both in freshwater and seawater group (Table 2).

Table 1
ACE, Chao, Shannon, Simpson and Good's coverage for 16s rRNA libraries of all samples from freshwater and seawater group for Barramundi *Lates calcarifer* (Bloch).

	Reared conditions	
	Freshwater group	Seawater group
ACE	160.53 ± 26.71	129.86 ± 7.12
Chao	160.74 ± 28.98	130.99 ± 8.13
Shannon	3.68 ± 0.15	3.52 ± 0.13
Simpson	0.84 ± 0.01	0.84 ± 0.01
Good's coverage	0.93 ± 0.12	0.94 ± 0.12

Different superscripts identify significant differences ($P < 0.05$).

Table 2
The major phylum and genus relative abundance (%) in gut bacteria of all samples from freshwater and seawater group for Barramundi *Lates calcarifer* (Bloch). Others means the sum of phylum or genus that relative abundance < 1%.

		Reared conditions	
		Freshwater group (%)	Seawater group (%)
phylum	*Proteobacteria*	56.14 ± 1.95	54.14 ± 0.46
	Firmicutes	38.72 ± 2.00	41.92 ± 0.81
	Others	1.75 ± 0.46	2.90 ± 0.74
	Unclassified	3.39 ± 1.52	1.04 ± 0.60
genus	*Exiguobacterium*	29.19 ± 0.38	30.37 ± 1.21
	Citrobacter	20.91 ± 0.81	21.48 ± 1.05
	Acinetobacter	13.69 ± 1.27	13.39 ± 0.50
	Pseudomonas	10.03 ± 1.52	9.66 ± 0.08
	Escherichia-shigella	6.40 ± 1.43	7.19 ± 1.04
	Lactococcus	2.87 ± 0.88	3.70 ± 0.18
	Anoxybacillus	3.17 ± 0.61	3.64 ± 0.57
	Geobacillus	1.08 ± 0.41	1.15 ± 0.16
	Others	7.73 ± 3.35	6.86 ± 1.86
	Unclassified	4.93 ± 2.01	2.55 ± 0.07

Different superscripts identify significant differences ($P < 0.05$) of relative abundance.

3.3. Antioxidant enzyme activity

ACP and CAT activity varied significantly between freshwater and seawater group ($P < 0.05$), while GPX, LZM, POD, SOD activity and LPO, MDA levels were without significant difference ($P > 0.05$, Table 3). ACP and CAT activity in freshwater group (0.33 ± 0.04 U/g prot, 46.44 ± 9.35 U/mg prot respectively) significantly higher than seawater group (0.20 ± 0.02 U/g prot, 111.35 ± 34.63 U/mg prot respectively).

4. Discussion

The gut microbes have great influence on the growth and development of the host, affecting the amount of energy extracted from the diet and energy harvest [3,19]. The fish microbiome revealed a rich biodiversity that predictably reacts to changing gut conditions [20]. Gut bacterial communities differ with host species. *Firmicutes* and *Bacteroidetes* were the most dominant phyla in mammals [21], and *Proteobacteria* was in fish [22]. In the present study, the gut bacteria diversity of reared barramundi from freshwater and seawater were characterized. The results indicated that *Proteobacteria* and *Firmicutes* were the most phylum of barramundi gut bacteria both in freshwater and seawater environment, and *Exiguobacterium*, *Citrobacter*,

Table 3
Effect of rearing salinity on antioxidant enzymes activity (ACP, CAT, GSH-Px, LZM, POD, SOD) and MDA, LPO levels.

	Reared salinity	
	Freshwater group	Seawater group
ACP (U/g prot)	0.33 ± 0.04[a]	0.20 ± 0.02[b]
CAT (U/mg prot)	46.44 ± 9.35[a]	130.99 ± 8.13[b]
GSH-Px (U/mg prot)	95.23 ± 13.04[a]	106.63 ± 12.57[a]
LZM (U/mg prot)	2.35 ± 0.49[a]	3.08 ± 1.37[a]
POD (U/mg prot)	0.44 ± 0.18[a]	0.62 ± 0.15[a]
SOD (U/mg prot)	1.29 ± 0.27[a]	2.13 ± 0.82[a]
MDA (U/mg prot)	0.25 ± 0.07[a]	0.32 ± 0.16[a]
LPO (μmol/mg prot)	1.51 ± 0.24[a]	1.37 ± 0.31[a]

Note: The significance level of $P < 0.05$ was used for all statistical tests. Different superscripts identify significant differences. ACP = Acid phosphatase, CAT = Catalase, GSH-Px = Glutathione peroxidase, LPO = Lipid peroxide, LZM = Lysozyme, SOD = Superoxide dismutase, MDA = Malonaldehyde, POD = Peroxidase.

Acinetobacter, *Pseudomonas*, *Escherichia-shigella*, *Lactococcus*, *Anoxybacillus*, *Geobacillus* were the dominate genus. It was in agreement with the research results about gut microbiome of seawater reared Asian seabass from Xia et al. [7], and results about gut microbiome of freshwater reared Asian seabass from Apper Xia et al. [23]. *Firmicutes* was be involved in energy resorption, and have been demonstrated probiotic properties in fish [24,25]. *Exiguobacterium*, *Lactococcus*, *Anoxybacillus*, *Geobacillus* were belonging to *Firmicute*, and *Citrobacter*, *Acinetobacter*, *Pseudomonas*, *Escherichia-shigella* were belonging to *Proteobacteria*. It is highlighted that, there was no significant difference for relative abundance of most phylum in reared barramundi gut bacterial communities from freshwater and seawater in the present study, as well as the dominate genus. The results indicated that barramundi under different rearing salinity have similar gut bacterial communities and compositions. In addition, the alpha diversity was used for further analysis, and the results suggested rearing salinity did not affect the richness and diversity of barramundi gut bacteria. Similarly, previous study has also demonstrated that the gut microbiota of aquatic animals has not been affected significantly by living environment [26]. Furthermore, the barramundi has great adaptability to tolerate salinity changes, and all the circulating aquaculture system used in the present study were equipped with UV sterilizer to remove the bacterial in aquatic environment.

Oxidative stress is harmful for fish, induced by the increased production of reactive oxygen species (ROS). ROS levels are controlled by the collaborative action of antioxidant defense mechanisms [10]. Activities of antioxidant enzymes are the potential indicators of oxidative stress and adaptive responses to remove the excessive ROS. In the present study, only CAT and ACP activity in barramundi liver were found significantly different between freshwater and seawater group. CAT provides the first line of defense to clean up ROS [27]. ACP plays an important role in the body defense system, immune regulation, ion secretion and other important physiological functions [28]. The different activity of CAT and ACP occurred in liver from freshwater and seawater group, indicated that the different reared salinity condition affect the sensitivity of antioxidant enzyme in barramundi, even though most of tested antioxidant enzymes activity was not significantly different between freshwater and seawater group in the present study.

In summary, this study is the first high-throughput analyses of the gut microbiota diversity in barramundi from same batch of siblings reared under two extreme salinity condition (seawater and freshwater). We highlighted that reared salinity did not change the major composition of barramundi gut bacteria ($P > 0.05$) under circulating aquaculture system. *Proteobacteria*, *Firmicutes* were the most phylum in barramundi gut microbiota community, and *Exiguobacterium*, *Citrobacter*, *Acinetobacter*, *Pseudomonas* were the dominate genus (relative abundance > 10%). The sensitivity of CAT, ACP in barramundi were affected by different reared salinity condition. The findings in the present study provide an understanding gut microbiome composition and diversity in barramundi from two extreme salinity condition under circulating aquaculture system. And it can be instructive to the management of animal health in barramundi circulating farming activities, and further euryhaline fish gut microecology research.

Conflicts of interest

The authors are not currently aware of any existing conflicts.

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