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Comparative analysis on microbial community associated with different gastrointestinal regions of wild northern snakehead *Channa argus* Cantor, 1842*

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Abstract Microbial communities in different gastrointestinal regions (stomach, foregut, midgut, and hindgut) of the northern snakehead *Channa argus* (Cantor, 1842) were compared by polymerase chain reaction and partial 16S rDNA sequencing. A total of 194, 140, 212, and 122 OTUs were detected in the stomach, foregut, midgut, and hindgut, respectively. Significant differences were found in the Sobs, ACE, Shannon, and Simpson indices among samples (P<0.05). The gastrointestinal microbial community of *C. argus* consisted predominantly of Proteobacteria with either *Halomonas*, *Shewanella*, *Plesiomonas*, or *Sphingomonas*. Fusobacteria, Firmicutes, and Bacteroidetes also existed in the gastrointestinal tracts. However, significant differences were found in the compositions of microbial community among the four regions (P<0.05). Cyanobacteria and Spirochetes were significantly higher in the midgut and hindgut (P<0.05). Fusobacteria and Firmicutes were dominant in the hindgut and foregut, respectively (P<0.05). Proteobacteria was the lowest in the hindgut (P<0.05). At genus level, *Cetobacterium* and *Plesiomonas* were significantly higher in the hindgut than in the other three samples (P<0.05). *Clostridium* and *Prevotella* were the highest in the midgut (P<0.05). *Halomonas*, *Shewanella*, and *Sphingomonas* were the highest in the foregut (P<0.05). *Paracoccus* and *Vibrio* were the highest in the stomach. Several genera were only detected in certain regions, as follows: stomach, *Paracoccus* and *Vibrio*; foregut, *Halomonas*, *Shewanella*, and *Sphingomonas*; midgut, *Clostridium* and *Prevotella*; and hindgut, *Cetobacterium* and *Plesiomonas* (P<0.05). At the species level, *Acinetobacter rhizosphaerae* was only detected in the stomach. *Prevotella copri* and *Clostridium perfring* were not detected in the foregut and midgut, respectively, whereas *Prevotella copri* and *Faecalibacterium pra* were not detected in the hindgut. These findings provide valuable information on the microbial community in each gastrointestinal region of *C. argus*. Moreover, this study indicated that microbial community was not only related to rearing environment but also to the physico-chemical characteristics of each gastrointestinal region.

Keyword: northern snakehead (*Channa argus* Cantor, 1842); 16S rDNA; microbial community; gastrointestinal region; physico-chemical characteristic

1 INTRODUCTION

The northern snakehead *Channa argus* (Cantor, 1842) is becoming one of the most popular cultured fishes in China because of its high food and medicinal value. The annual output of *C. argus* was nearly 500 000 tons in 2015 (Yuan and Zhao, 2016), and the culture area for this fish is continuously expanding. Culturing *C. argus* is a good development prospect. The trash fish is the main feed source of *C. argus*. The application of formulated feed has been rapidly gaining more attention because of the limited supply of trash fish and risks in disease infection caused by this fish. However, the lower utilization has restricted the promotion of formulated feed in the *C. argus*

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culture. Extensive studies have focused on the utilization of formulated feed because of the important role of microorganisms in the maintenance of gastrointestinal homeostasis. Studies on the gastrointestinal microbiota of aquatic animal have also shown that the microbial community is vital for the growth, nutrition, and health of the host (Standen et al., 2015; Day et al., 2016). Among the functions of the intestinal microorganism, the contribution to the nutrition utilization of fish has attracted great attention. For example, Sugita et al. (1997) found that microbes in some freshwater fish could produce amylase, which is involved in the digestion of starch in the host intestine. Ramirez and Dixon (2003) isolated anaerobic bacteria from the gastrointestinal tract of angelfish (*Pterophyllum scalare*), oscar (*Astronotus ocellatus*), and southern flounder (*Paralichthys lethostigma*), which could secrete acid and alkaline phosphatases, esterases, and lipids. Generally, microorganisms have different functions in the digestive enzyme secretion (Ragauskas et al., 2006; Zhang et al., 2006). For example, Bacteriodetes was reported to produce cellulase, whereas Proteobacteria and Firmicutes were considered to produce protease (Godoy-Vitorino et al., 2012; da Cruz Ramos et al., 2016). The importance of intestinal microbial structure and function, especially the potential contribution to nutrition utilization of host, has been increasingly recognized (Chaiyapechara et al., 2012; Li et al., 2012). Thus, the intestinal microbial communities associated with the various intestinal regions should be determined, because the different intestinal regions possess distinct digestive functions. However, few studies have focused on this difference.

The diversity in the intestinal microbial community may be affected by several factors, such as species specificity (Xiong et al., 2015), nutritional status (Rico et al., 2016), environmental conditions (Wan et al., 2015; Fan et al., 2016), developmental stages, and complexity of fish digestive system (Zarkasi et al., 2016). However, the compositions of the microbial communities in the different intestinal regions were also speculated to be related to their respective intestinal physiological functions (Johnson et al., 2008; Stanley et al., 2014). Therefore, 16S rDNA gene-based analyses were conducted to investigate the microbial communities along the gastrointestine of *C. argus*. The interaction between the gastrointestinal function and the microbial community was elucidated.

2 MATERIAL AND METHOD

2.1 Sample collection

A total of 10 healthy *C. argus* with an average weight of approximately 200 g were captured from Gaoyou lake, Yangzhou, Jiangsu Province, China in September 2015. Then, the fish were transferred to the laboratory and rinsed with 70% ethanol to reduce contamination. The fish were dissected immediately using a pair of sterile scissors, and the contents of the gastrointestinal tracts were squeezed out. The gastrointestinal tract was divided into four regions, namely, stomach, foregut, midgut, and hindgut. The two ends of each part were clamped to prevent contamination. The mucosa from each part (approximately 2 cm) was collected for analysis. All samples were divided into three replicates, collected in Eppendorf tubes, and then immediately stored in liquid nitrogen until DNA extraction.

2.2 DNA extraction

Total bacterial DNA was extracted from samples using QIAamp DNA stool mini kit (QIAGEN, cat#51504) according to the manufacturer's instructions. Then, the final quantity and quality of the DNA was evaluated by fluorescence quantitative detection and agarose gel electrophoresis. A standard concentration of 10 ng/μL was prepared for each sample for all PCR assays.

2.3 PCR amplification and sequence processing

The V3–V4 gastrointestine and water samples were amplified and sequenced on the bacterial 16S rDNA gene to determine the microbial diversity. The primers for the PCR reaction were 515 F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806 R (5′-GGACTACHVGGGTWTCTAAT-3′). The purified amplicons were sequenced on an Illumina MiSeq PE250 sequencer using pair-end method at the Beijing Genomics Institute (Shenzhen, China).

2.4 Data analysis

Raw 250 bp paired-end sequence reads were combined using FLASH v1.2.11 with the default settings. The 16S rDNA operational taxonomic units (OTUs) were selected from the combined reads using USEARCH GLOBAL clustered at 97% identity. Then, OTU representative sequences were taxonomically classified using Ribosomal Database Project Classifier v.2.2 trained on the Greengenes

Table 1 Operational taxonomic units (OTUs) and alpha diversity statistics of microbial sequencing in *Channa argus* gastrointestinal tract (mean±S.D. of three replications)

Sample	Reads		Tag	OTU	97% similarity				
	Raw reads	Valid reads			Sobs	Chao	ACE	Shannon	Simpson
Stomach	41 261	39 259	38 860	194±44^{ab}	140±8^{a}	155±21	164±8^{ab}	2.49±0.09^{b}	0.17±0.01^{bc}
Foregut	41 448	39 321	38 959	140±29^{a}	212±10^{b}	221±36	236±16^{bc}	2.61±0.06^{b}	0.11±0.00^{a}
Midgut	41 104	39 159	38 819	212±36^{b}	122±5^{a}	142±26	136±11^{a}	2.14±0.07^{a}	0.20±0.01^{c}
Hindgut	41 193	39 194	38 899	122±37^{a}	194±11^{b}	201±48	203±13^{b}	2.53±0.06^{b}	0.14±0.01^{ab}

Means in the same column with different superscripts are significantly different (P<0.05) as determined by Tukey's test.

database. The taxon abundance of each sample was generated into phylum, class, order, family, genera, and species levels. Of the 465 611 sequences processed, 95.9% (446 639) shared more than 97% sequence identity with a reference sequence.

2.5 Statistical analysis

Differential analysis among groups was performed using the alpha diversity indices. Alpha diversity analysis, including Chao, ACE, Simpson, Shannon, and Sobs, was performed to evaluate the diversity of microbial community. The indices were calculated by Mothur (v1.31.2), and the calculation formula of each index can be referred to http://www.mothur.org/wiki/Calculators. One-way ANOVA was conducted to compare the differences among all treatments. When significant difference (P<0.05) was found, Tukey's test was used to compare the mean values between individual treatments.

3 RESULT

3.1 Effectiveness of sequencing

In this study, 41 261, 41 448, 41 105, and 41 193 raw reads were obtained for the stomach, foregut, midgut, and hindgut samples, respectively. Table 1 shows that 95% of the raw reads met the standards of quality and length. These qualified sequenced reads were classified into different OTUs based on the identity level at 97% (Table 1).

3.2 Classification and alpha diversity analysis

According to the qualified OTUs of each sample, alpha diversity was estimated by five indices, namely, Sobs, Chao, ACE, Shannon, and Simpson (Table 1). No significant differences were found in the Chao indices among samples (P>0.05), but significant differences were found in the other four indices among samples (P<0.05). Sobs was higher in the foregut and hindgut than in the stomach and midgut (P<0.05). ACE was the highest in the foregut but lowest in the midgut (P<0.05), which was opposite in the Simpson index (P<0.05). Shannon was significantly lower in the midgut than in the other three samples (P<0.05).

3.3 Community composition and species abundance analysis

The community composition of each sample is presented in Fig.1. A total of 18 phyla were detected in the gastrointestinal microbiota of the 10 healthy fish. Of these 18 phyla, the six main phyla were Proteobacteria (52.85%–76.04%), Firmicute (12.16%–29.60%), Spirochetes (0.00%–18.80%), Fusobacteria (0.15%–13.48%), Bacteroidetes (0.40%–3.11%), and Actinobacteria (0.15%–0.72%). Significant differences were found in the phyla among samples (P<0.05). The relative abundances of Cyanobacteria and Spirochetes were significantly higher in the midgut and hindgut than in the stomach and foregut (P<0.05). A total of 13.5% Fusobacteria was found in the hindgut, which was significantly higher than those in other three samples (P<0.05). Firmicutes was the highest in the foregut than in the other three samples, and Proteobacteria was the lowest in the hindgut than in the other three samples (P<0.05). Furthermore, 17 phyla were detected in the water samples, with Actinobacteria (32.67%), Bacteroidetes (26.35%), Proteobacteria (25.24%), Chlorobi (3.58%), Cyanobacteria (3.95%), Planctomycetes (3.23%), and Verrucomicrobia (2.36%) as the main phyla.

A total of 135 genera were detected at the genus level, and the relative abundance of 10 genera was greater than 0.1% in four gastrointestinal samples (Fig.2). Moreover, significant differences were found in the community composition among samples. *Halomonas* (14.18%) was the highest in the stomach, followed by *Paracoccus* (7.86%), *Clostridium* (7.80%), and *Shewanella* (7.32%), whereas

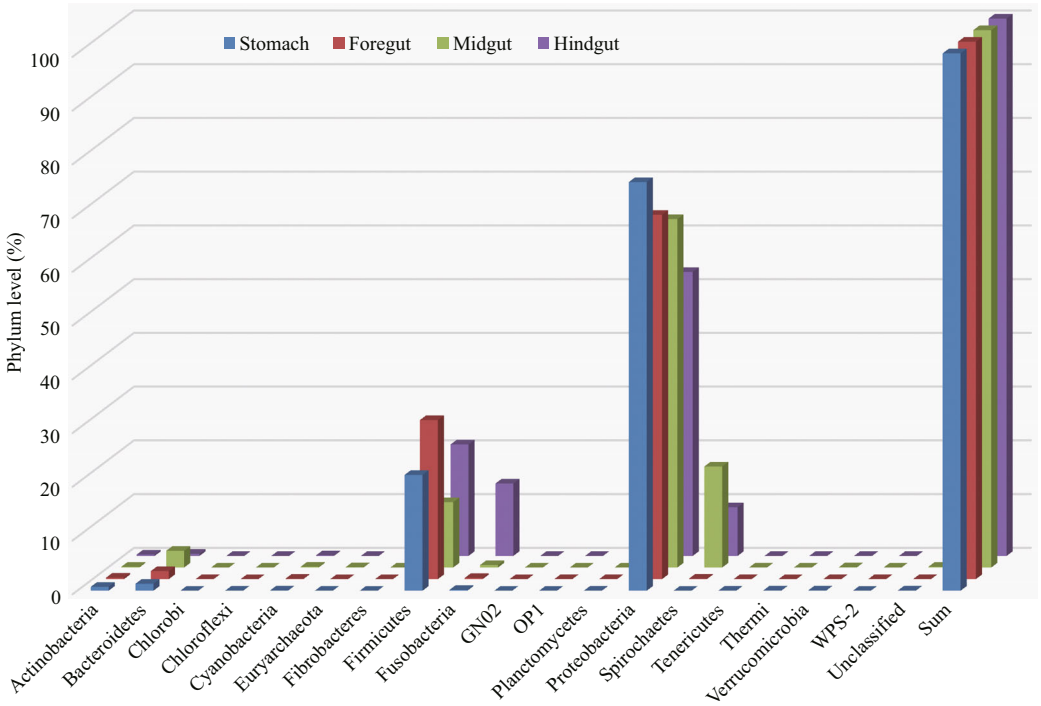

Fig.1 Community composition at phylum level of *Channa argus* gastrointestinal tract

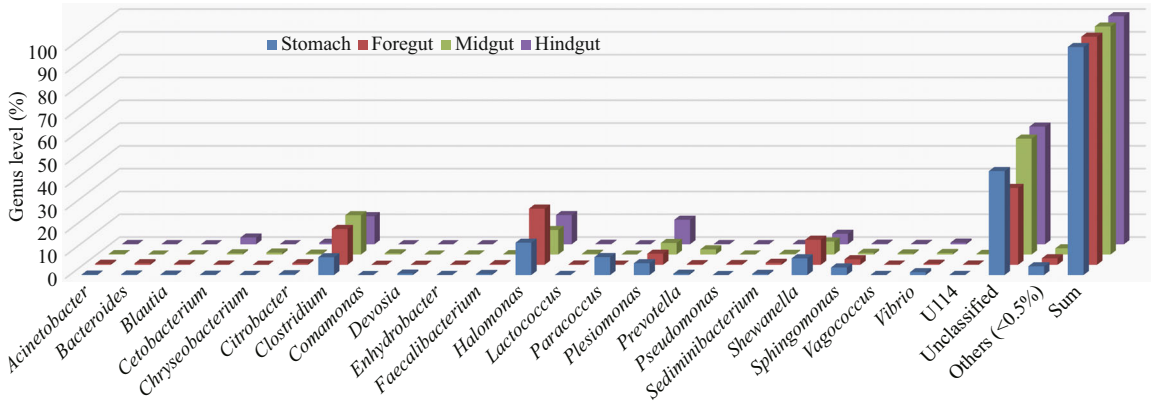

Fig.2 Community composition at genus level of *Channa argus* gastrointestinal tract

proportions of *Plesiomonas*, *Sphingomonas*, and *Vibrio* were 5.19%, 3.33%, and 1.25%, respectively. In the foregut, the order according to the relative abundance was *Halomonas* (24.63%) > *Clostridium* (15.70%) > *Shewanella* (10.98%) > *Plesiomonas* (4.85%) > *Sphingomonas* (2.41%). In the midgut, the order was *Clostridium* (17.23%) > *Halomonas* (10.75%) > *Shewanella* (5.70%) > *Plesiomonas* (5.06%) > *Prevotella* (2.26%). In the hindgut, the order was *Halomonas* (12.80%) > *Clostridium* (12.30%) > *Plesiomonas* (10.76%) > *Shewanella* (4.59%) > *Cetobacterium* (2.93%) > U114 (1.56%).

Additionally, significant differences were found in the microbial composition among samples. *Cetobacterium* and *Plesiomonas* were significantly higher in the hindgut than in the other three samples (P<0.05). *Clostridium* and *Prevotella* were the highest in the midgut (P<0.05). *Halomonas*, *Shewanella*, and *Sphingomonas* were the highest in the foregut (P<0.05). *Paracoccus* and *Vibrio* were the highest in the stomach (P<0.05). Furthermore, 154 genera were detected in the water sample. *Polynucleobacter* (7.49%), *Sediminibacterium* (6.03%), *Fluviicola* (4.70%), *Flavobacterium* (3.64%), and *Limnohabitans* (1.87%) were the main genera.

At species level, only 10 species were confirmed in the four gastrointestinal samples (Fig.3). *Acinetobacter rhizosphaerae* was only detected in the stomach, and its content was 0.05%. *Clostridium perfring* and *Faecalibacterium pra* were not detected

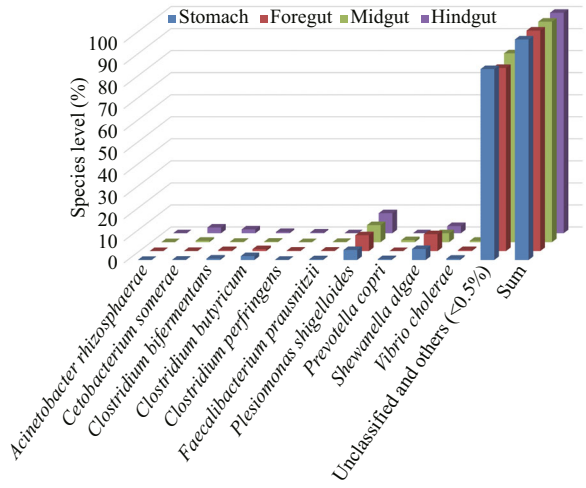

Fig.3 Community composition at species level of *Channa argus* .1

in the midgut and hindgut, respectively. *Prevotella copri* was not detected in the foregut and hindgut. The relative proportions of *Cetobacterium somerae* and *Clostridium bifermen* were significantly higher in the hindgut than that in the other three samples (P<0.05). *Clostridium butyricu* was higher in the stomach than in the other three samples (P<0.05).

4 DISCUSSION

In agreement with the results obtained in sea cucumber *Apostichopus japonicas* (Gao et al., 2014), black tiger shrimp *Penaeus monodon* (Chaiyapechara et al., 2012), farmed puffer fish *Takifugu rubripes* (Li et al., 2015), and grass carp *Ctenopharyngodon idellus* (Wu et al., 2012), Proteobacteria and Firmicute were predominant in the *C. argus* gastrointestine, which may indicate that members of Proteobacteria and Firmicute played a very important role in maintaining the microbiota balance in *C. argus* intestine (Chen et al., 2013).

In this study, different regions of *C. argus* gastrointestinal tract shared numerous kinds of bacteria, but significant differences were found in the relative proportions among regions. On one hand, at the phylum level, compared with the hindguts, foreguts had higher proportions of Fusobacteria and Spirochaetes, but lower proportions of Bacteroidetes, Firmicutes, and Proteobacteria. The stomach had significantly higher proportions of Proteobacteria than the midgut and hindguts. On the other hand, at the genus level, results also showed differences in composition of the microbiota along the length of the gut. For example, *Halomonas* was the highest in the stomach, foregut and hindgut, but *Clostridium* was the highest in the midgut. The microbial communities originating from distinct gastrointestinal regions should be considered as separate ecosystems (Johnson et al., 2008). Differences in the compositions of microbial community in the different gut regions of host were also proved in shrimp *Litopenaeus vannamei* (Johnson et al., 2008; Shakibazadeh et al., 2009). Differences in the gastrointestinal microbial community among regions of hosts were considered to be related to the gastrointestinal physiological adaptation on the gastrointestinal condition (Johnson et al., 2008). Thus, the physico-chemical characteristics of each gastrointestinal region result in the formation of characteristic microbial community for each region. In terms of digestive physiology, different digestive enzyme activities and digestive physiologies were found along the intestinal tract (Hartviksen et al., 2014; Krogdahl et al., 2015). This result indicates that different digestive processes and digestive functions may exist in distinct intestinal regions. There is growing evidence that microbiota play functional roles in affecting the nutrition and metabolism of host, particularly the capacity to digest some nutrients (Gatesoupe et al., 2016). In this study, the higher representation of Bacteriodetes in the foregut may be related to higher cellulolytic activity, whereas the higher representation of Proteobacteria and Firmicutes may be related to higher proteolytic activity. Hindgut dominance by Fusobacteria and Spirochaetes might be related to higher carbohydrases activity (Wu et al., 2010; Godoy-Vitorino et al., 2012; da Cruz Ramos et al., 2016). This result strengthened the basic knowledge on the very close relations of intestinal-microbial function. Further studies are needed to study the effects on intestinal function induced by microbial community.

It is worthwhile to note that genus level must be taken into account in analyzing the role of gastrointestinal microbiota. For example, one of the members of Proteobacteria is *Vibrio* spp., *Vibrios* have been reported to be associated with diseases in other organisms such as coral (*V. coralliilyticus*/*V. shilonii*) (Kushmaro et al., 2001; Ben-Haim et al., 2003) and molluscs (*V. tubiashii*) (Hada et al., 1984). In the present study, *Vibrio* was 1.25% in the stomach, but 0.45%, 0.65% and 0.70% in the foregut, midgut and hindgut, respectively. Shakibazadeh et al. (2012) studied the bacterial community associated with intestines of juvenile *P. monodon* and found that the dominant genera in the intestinal tract were *Vibrio*, *Photobacterium*, or *Aeromonas*. The

content of *Vibrios* in different aquatic animals may be related to the rearing environment (Holben et al., 2002).

5 CONCLUSION

The gastrointestinal microbial community of wild *C. argus* consisted predominantly of Proteobacteria with either *Halomonas*, *Shewanella*, *Plesiomonas*, or *Sphingomonas* dominating the population. However, the relative proportions of bacteria significantly differed among the gastrointestinal regions, which indicated that the physico-chemical characteristics of each gastrointestinal region resulted in the formation of characteristic microbial community for each region. At the phylum level, compared with the hindguts, foreguts had higher proportions of Fusobacteria and Spirochaetes, but lower proportions of Bacteroidetes, Firmicutes, and Proteobacteria. The stomach had significantly higher proportions of Proteobacteria than the midgut and hindguts. At the genus level, *Halomonas* was the highest in the stomach, foregut and hindgut, but *Clostridium* was the highest in the midgut. Studies on how the gastrointestinal microbiota community acts on host nutrition utilization are necessary to provide the theoretical basis for improving the feed utilization of *C. argus*.

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