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Comparative study on the gut microbiotas of four economically important Asian carp species

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Gut microbiota of four economically important Asian carp species (silver carp, *Hypophthalmichthys molitrix*; bighead carp, *Hypophthalmichthys nobilis*; grass carp, *Ctenopharyngodon idella*; common carp, *Cyprinus carpio*) were compared using 16S rRNA gene pyrosequencing. Analysis of more than 590,000 quality-filtered sequences obtained from the foregut, midgut and hindgut of these four carp species revealed high microbial diversity among the samples. The foregut samples of grass carp exhibited more than 1,600 operational taxonomy units (OTUs) and the highest alpha-diversity index, followed by the silver carp foregut and midgut. Proteobacteria, Firmicutes, Bacteroidetes and Fusobacteria were the predominant phyla regardless of fish species or gut type. Pairwise (weighted) UniFrac distance-based permutational multivariate analysis of variance with fish species as a factor produced significant association (P<0.01). The gut microbiotas of all four carp species harbored saccharolytic or proteolytic microbes, likely in response to the differences in their feeding habits. In addition, extensive variations were also observed even within the same fish species. Our results indicate that the gut microbiotas of Asian carp depend on the exact species, even when the different species were cohabiting in the same environment. This study provides some new insights into developing commercial fish feeds and improving existing aquaculture strategies.

Asian carp, gut microbiota, feeding habit, pyrosequencing, *Hypophthalmichthys molitrix*, *Hypophthalmichthys nobilis*, *Ctenopharyngodon idella*, *Cyprinus carpio*

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INTRODUCTION

The silver carp (*Hypophthalmichthys molitrix*), bighead carp (*Hypophthalmichthys nobilis*) and grass carp (*Ctenopharyngodon idella*) are known as the "Four Major Domesticated Fish" in China (Billard and Berni, 2004). The common carp (*Cyprinus carpio*) have been cultivated in China for over 2,500 years (Jeney and Jian, 2009). In 2013, the production of these four economically important Asian carp species reached 14.97 million tons in China (silver carp, 3.85 million tons; bighead carp, 3.02 million tons; grass carp, 5.07 million tons; and common carp, 3.03 million tons), accounting for 53.38% of the annual output of all freshwater-cultured fish (Fishery Bureau of the Ministry of Agriculture, 2014). These carp species are traditionally raised together to maximize the utilization of different trophic and spatial resources (Wang et al., 2016). In order to further optimize the polyculture, it is essential to study their feeding habits in the polyculture system. As previous studies have shown, one approach for elucidating animal feeding habit is to examine the gut mi-

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crobial community; studies on humans, mice and chicken have successfully correlated the gut microbial community to the host's physiology (Hooper et al., 2001; Qin et al., 2010; Torok et al., 2008; Zhang et al., 2010). Thus, understanding the gut microbiota of these carp species would certainly provide valuable insights into their feeding habits and perhaps help improve aquaculture strategies.

The Asian carp species differ in their natural feeding habits (Billard and Berni, 2004). Silver carp and bighead carp are primarily filter feeders (plankton-feeding); while silver carp preferentially consumes phytoplankton, bighead carp tends to consume zooplankton. Grass carp are herbivorous and therefore also used to control weeds. Common carp are omnivorous but prefer insects or benthic worms.

Fish gut microbiota have been investigated extensively using traditional culture-dependent or microscopy methods (Aguilera et al., 2013; Al-Hisnawi et al., 2015; Ghosh et al., 2010; Pearce et al., 2003). The majority of microbes found in fish guts have not been identified due to the inability to cultivate those microorganisms in the laboratory (Clements et al., 2014). The recently developed high-throughput sequencing technologies such as Illumia Miseq have revolutionized the characterization of the microbial community, especially on the basis of their 16S rRNA gene sequences (Park et al., 2016; Sun et al., 2015). A number of recent studies that mainly focused on the gut microbiota of mammals and zebrafish (Marungruang et al., 2016; Ranjan et al., 2016; Roeselers et al., 2011) have described the use of these technologies.

To the best of our knowledge, only a few studies have compared the gut microbiota of cultured Asian carps using the 16S pyrosequencing methods (Li, J., et al., 2014; Li, T., et al., 2015; Li, Z., et al., 2015). The principal aim of this study was to characterize and compare the gut microbiota of these species. Furthermore, knowing the gut microbial community can help better understand the natural feeding habits of each carp species since gut microbiota composition can be appropriately related to their natural feeding habits.

RESULTS

Microbial complexity in fish guts

A total of 594,412 quality-filtered sequences were obtained from 35 samples, with the number of sequences per sample ranging from 2,587 to 40,024 (Table S1 in Supporting Information). The microbial complexity in the fish guts was estimated based on alpha-diversity indices (OTU number, abundance-based coverage estimator (ACE), Chao1 and Shannon indices). The number of OTUs covered 51%–79% and 55%–83% of the richness estimated by the ACE and Chao1 indices respectively based on subsampled data (Table 1). For foregut samples, the alpha-diversity indices of grass carp were the highest (ACE, 986±130; Chao1, 952±98; and Shannon index, 5.8±0.2 based on subsampled data), and were significantly higher than those of common carp and bighead carp (Welch's t test, $P<0.05$). For midgut samples, the quality-filtered sequence was highest in the grass carp when compared to that of common carp ($P<0.05$). Significant difference in diversity indices was only observed between bighead carp and common carp as per the Shannon index (Table 1). No significant difference was observed among hindgut samples of different species. Similar trends in microbial diversity were also observed in the rarefaction results which had been summarized by gut types and the number of known assigned taxa (Figure S1 in Supporting Information). As shown, the greatest number of taxa at different phylogenetic levels were detected in grass carp foregut and silver carp midgut, followed by silver carp foregut and hindgut.

Microbial community similarity between gut samples

For the phylogenetic comparisons of the gut microbial communities, we analyzed their 16S rRNA gene sequence data sets using the UniFrac metric. NMDS plots were created to compare the overall composition of the gut microbiota, although no clear clustering pattern was observed (Figure 1A). Only a limited number of gut samples were clustered according to the host species. However, certain similarity trends in the gut microbial community were still observed, with several bighead carp and silver carp samples forming two distinct clusters. In addition, most bighead carp gut samples were closer to the common carp, and grass carp samples were closer to silver carp samples (Figure 1A). Furthermore, despite the shared environment of the hosts, the overall composition of the gut microbial communities varied substantially among, and even within, the species (Figure 1B). To determine possible host species effect, permutational multivariate analysis of variance were performed with the fish species as a factor. Similar to the NMDS ordination, significant differences in the gut microbial communities were observed between bighead carp and grass carp, bighead carp and silver carp, and common carp and grass carp ($P<0.05$ for all comparisons).

To further compare the gut microbiota of the four species, the shared and unique OTUs were analyzed through Venn diagrams (Figure 2). Pairwise comparison was performed among all species by considering the shared OTUs as those present in at least 30% of the samples of each species, and the unique OTUs as those only present in more than 30% of the samples within one species. The number of shared OTUs varied from 6 to 48 and unique OTUs varied from 48 to 137, and only 40 OTUs were common for all fish species (Figure 2A). These 40 common OTUs corresponded to 48.34%, 46.62%, 29.82% and 34.98% of the sequences in bighead

Table 1 Summary of species richness estimators of gut samples[a)]

Sample type and number	Fish species	Sequences passed quality check	ACE**		Chao1**		Shannon**	
			Full data***	Subsampled data***	Full data	Subsampled data	Full data	Subsampled data
Foregut (12)	*H. nobilis*	11,375±5,782	950±319	560±47^{a}	903±285	534±55^{a}	4±0.6^{a}	3.9±0.6
	C. carpio	20,720±6,747	561±58^{a}	418±63^{b}	563±67^{a}	367±53^{b}	2.5±0.5^{b}	2.5±0.6^{a}
	C. idella	23,119±5,805	1,603±131^{b}	986±130^{ac}	1,619±103^{b}	952±98^{ac}	6±0.2^{ac}	5.8±0.2^{b}
	H. molitrix	15,782±2,576	1,245±345	714±145	1,202±273	735±135	5.1±0.2^{ad}	4.9±0.2^{c}
Midgut (12)	*H. nobilis*	17,155±7,178	683±190	431±124	687±204	387±100	3.2±0.4	3.2±0.3^{a}
	C. carpio	7,241±3,433^{a}	524±102	436±45	552±90	469±35	4.8±0.4	4.8±0.4^{b}
	C. idella	21,960±2,682^{b}	816±122	458±97	810±128	427±67	4±0.4	3.9±0.4
	H. molitrix	10,117±3,858	1,181±627	740±179	1,131±570	791±236	4.9±0.9	4.8±0.9
Hindgut (11)*	*H. nobilis*	23,412±8,030	919±83	504±21	874±109	479±7	3.1±0.1	3±0.1
	C. carpio	11,506±7,428	589±108	465±42	600±91	445±34	4.1±0.3	4±0.3
	C. idella	29,326±8,332	778±166	506±81	781±194	457±80	3.3±0.8	3.2±0.8
	H. molitrix	9,638±549	740±279	638±225	744±308	595±250	4.3±1.9	4.2±1.9

a) F, foregut; M, midgut; H, hindgut. Standard error of the mean (SE) was used for data description (mean±SE). *, The DNA extraction or PCR amplification were not successful in one hindgut sample. **, The species richness estimators (abundance-based coverage estimator (ACE), Chao1 and Shannon) were calculated under 3% distance cutoff. Significant difference (indicated by letters, Welch's *t* test) was calculated within each sample type (e.g. foregut, midgut, hindgut) according to fish species. ***, "Full data" means the sequences that passed quality check, "Subsampled data" means the original sequences randomly subsampled from each kind of fish gut sample.

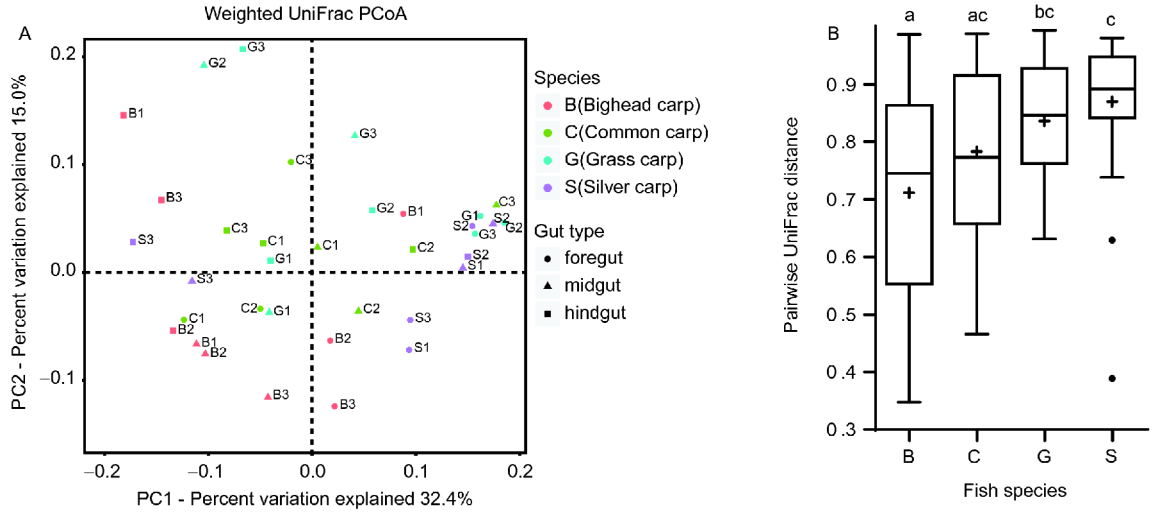

Figure 1 Phylogenetic dissimilarity of gut microbiota among the four Asian carp species. A, A non-metric multidimensional scaling (NMDS) ordination of (weighted) UniFrac distance showing the dissimilarity of the gut microbial communities. B, Pairwise Unifrac distances within each species. Pairwise community distances were also determined using the weighted UniFrac algorithm. For the boxplot, letters above fish species indicate significant differences.

carp, common carp, grass carp and silver carp respectively. By increasing the cutoff for shared and unique OTUs to 50%, the number of shared and unique OTUs apparently decreased to 1–21 and 10–75 respectively (Figure 2B). The number of common OTUs decreased to 12 and corresponded to 36.02%, 33.57%, 20.18% and 27.82% of the sequences in bighead carp, common carp, grass carp and silver carp respectively.

Microbial community compositions

The phylogenetic classification of the gut sample sequences revealed 26 different phyla. The sequences that could not be classified into any known group were assigned as "Unclassified". The 35 gut samples examined showed highly dissimilar 16S rRNA profiles of relative abundance even at the phylum level (Figure 3), indicating that even within a fish

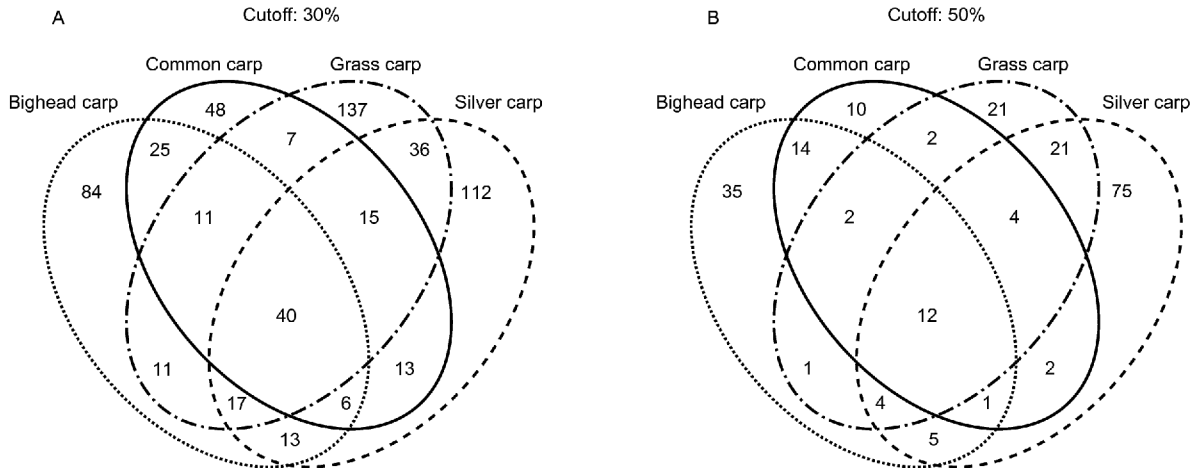

Figure 2 Unique and shared OTUs in the four Asian carp gut samples. Venn diagram showing the number of shared and unique OTUs among the gut samples of these four fish species at 30% (A) and 50% (B) cutoff level. The definitions of the shared and unique OTUs are given in the Materials and Methods section.

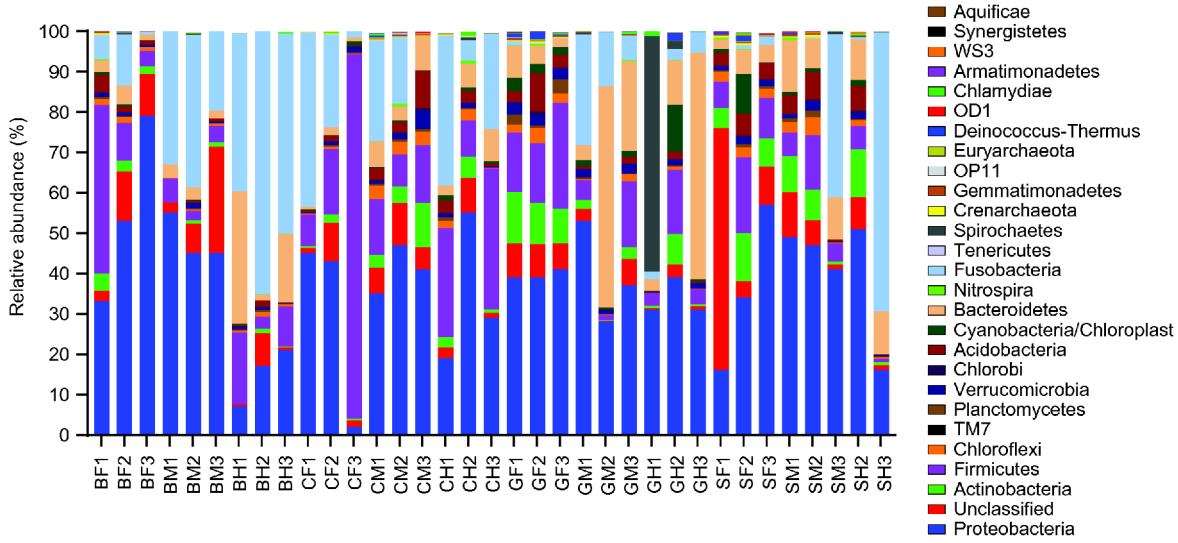

Figure 3 The relative abundance of microbial phyla in different communities. Sequences that could not be assigned at phylum level were marked as "Unclassified". Lanes BF1-3, BM1-3 and BH1-3 represent three bighead carp foregut, midgut and hindgut samples respectively; lanes CF1-3, CM1-3 and CH1-3 represent three common carp foregut, midgut and hindgut samples respectively; lanes GF1-3, GM1-3 and GH1-3 represent three grass carp foregut, midgut and hindgut samples respectively; lanes SF1-3, SM1-3 and SH1-3 represent three silver carp foregut, midgut and hindgut samples respectively.

gut sample, 0.31%–59.95% of the total sequences could not be assigned to known microbial phyla (see Table S2 in Supporting Information for more detail). Proteobacteria were one of the most dominant phyla, representing 1.66%–79.01% of the total sequences in each sample (bighead carp, 39.45% ± 22.23%; common carp, 35.30%±16.36%; grass carp, 37.54% ±7.41%; and silver carp, 38.93%±15.74%). The relative abundance of another dominant phylum Firmicutes accounted for 10.84%±12.53%, 24.56%±26.27%, 11.19%± 8.32%, and 8.22%±5.66% in bighead carp, common carp, grass carp, and silver carp respectively. Bacteroidetes were identified in all fish gut samples (bighead carp, 7.63%± 10.65%; common carp, 4.23%±2.97%; grass carp, 18.32%± 21.95%; and silver carp, 8.02%±3.58%), while Fusobacteria were only absent in one sample of common carp, accounting for 29.31%±21.26%, 19.40%±15.14%, 14.17%±26.17%, and 6.46%±8.91% of the sequences of bighead carp, common carp, silver carp, and grass carp respectively. Other microbial phyla that each made up >1% of total sequences included Actinobacteria (0.04%–12.76%), Cyanobacteria/ Chloroplast (0–11.67%), Acidobacteria (0–9.51%), Spirochaetes (0–58.21% and highly abundant in two samples of grass carp), Chloroflexi (0–0.97%), and Verrucomicrobia (0–4.56%). Rare phyla, namely Planctomycetes, Deinococcus-Thermus, Chlamydiae, Gemmatimonadetes, Nitrospira, Chlorobi, Crenarchaeota, OD1, Armatimonadetes, Eur-

yarchaeota, Aquificae, OP11, WS3, Tenericutes, TM7 and Synergistetes were observed sporadically at low abundance (making up 1.73% of the total sequences) in some of the fish gut samples.

At the genus level, the sequences from the 35 samples represented 595 genera. The proportion of sequences that could not be classified into any known genera ranged from 4.02% to 80.63% in different samples. These unclassified sequences, representing 31.42% of the total sequences, were placed into 130 taxa above the genus level. The representation of unclassified microbial phylotypes varied significantly among the samples (Figure S2 in Supporting Information).

A total of 18 genera and 12 other taxa (each >0.5% of the total sequences; Figure 4) constituted more than 70.28% of the total sequences in our dataset. As shown in Figure 4, *Cetobacterium* (bighead carp, 28.51%±20.77%; common carp, 19.34%±15.11%; grass carp, 5.79%±8.77%; and silver carp, 13.49%±25.18%), *Aeromonas* (bighead carp, 13.84%±12.86%; common carp, 12.04%±12.47%; grass carp, 10.79%±9.46%; and silver carp, 9.91%±13.98%), and the Unclassified (bighead carp, 7.90%±8.17%; common carp, 5.27%±3.73%; grass carp, 4.19%±3.33%; and silver carp, 12.65%±19.45%) were the most dominant genera inhabiting the four fish species. *Cetobacterium* varied most in abundance (P<0.05; Kruskal-Wallis) between fish species and was highly abundant in the bighead carp hindgut (also see Table S3 in Supporting Information; average: 50.25%) whereas *Aeromonas* was highly abundant in bighead carp midgut (Table S3 in Supporting Information; average: 27.88%). Furthermore, Unclassified Vibrionaceae (12.39%±16.36%) and *Neorickettsia* (2.13%±4.55%) significantly dominated in the bighead carp (P<0.005; Kruskal-Wallis) more than the other species. The Unclassified Firmicutes and *Yersinia* were more abundant in common carp (P<0.005 only for *Yersinia*; Kruskal-Wallis), especially in the foregut (average: 26.70% and 8.93%, respectively). *Brevinema*, *Bacteroides* and Unclassified Prevotellaceae were unique to the grass carp (P<0.05; Kruskal-Wallis, except for *Brevinema*) while no genus that was uniquely abundant in silver carp was observed in our datasets. Moreover, Unclassified Porphyromonadaceae was also more abundant in bighead carp and grass carp, *Clostridium* XI and *Clostridium sensu stricto* were significantly more abundant in bighead carp and common carp (P<0.001; Kruskal-Wallis) and Acinetobacter was significantly more abundant in grass carp and silver carp (P<0.001; Kruskal-Wallis). *Streptophyta* was detected in all foregut samples, with highest abundance in silver carp (average: 3.50%), followed by grass carp (1.78%), bighead carp (0.45%) and common carp (0.05%). Other prominent genera, including *Escherichia/Shigella*, *Spartobacteria genera incertae sedis*, Unclassified Betaproteobacteria, Unclassified Gammaproteobacteria, Unclassified Proteobacteria, and *Pseudomonas* were detected in almost all gut samples, and in total accounted for 4.41% of the total sequences.

Cyanobacteria/Chloroplast

Cyanobacteria/Chloroplast was one of important phyla responsible for the differences seen in the microbiota of different samples. Sequences affiliated with Cyanobacteria/Chloroplast were further retrieved and analyzed, and 63 OTUs were detected. Phylogeny trees using representative sequences of these OTUs (Figure S3 in Supporting Information) indicated that 17 OTUs (OTU63, *Chlorarachniophyceae*; OTU289, *Chlorophyta*; OTU10961, *GpI*;

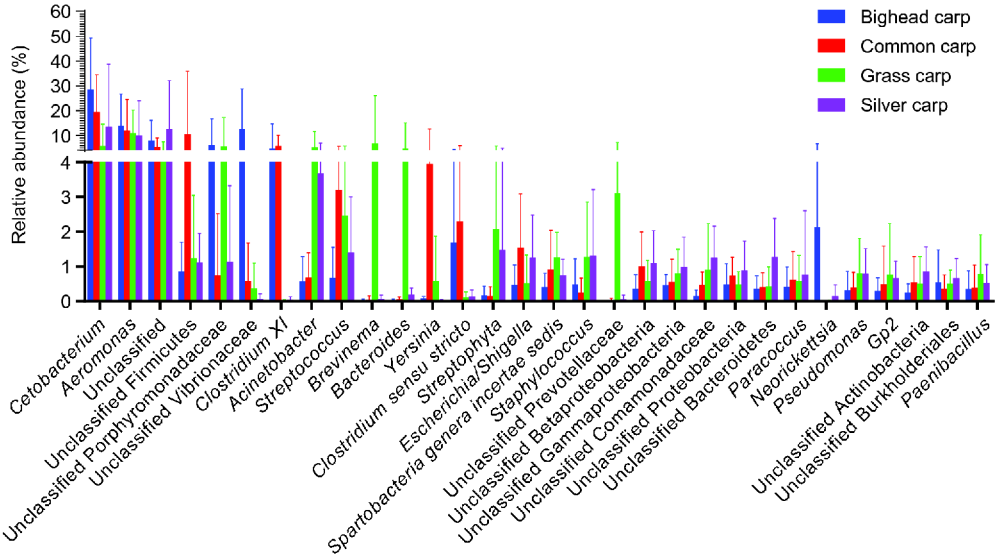

Figure 4 Relative abundance of the dominant genera (each constituting >0.5% of the total sequences) in the intestinal contents of bighead carp, common carp, grass carp and silver carp.

OTU2008, 7181, 3724, 7672, *GpIIa*; OTU9192, *GpXIII*; OTU2, 1489, 1907, *Streptophyta*; OTU4985, Chloroplast; OTU5365, 4198, 6039, 7733, 7990, Cyanobacteria;) were uniquely present in grass carp samples, 12 OTUs (OTU2685, 5845, 8425, *Bacillariophyta*; OTU6054, 6721, 845, 431, *Chlorophyta*; OTU4038, *Cryptomonadaceae*; OTU8378, *GpIIa*; OTU2330, *GpVI*; OTU8208, 3816, *Streptophyta*) were present only in common carp samples, 10 OTUs (OTU6735, *Bacillariophyta*; OTU485, *Chlorophyta*; OTU2391, *GpI*; OTU6199, *GpIIa*; OTU5218, *GpIV*; OTU10443, *GpV*; OTU6325, *Streptophyta*; OTU2179 1360, 3825, Cyanobacteria) were present only in silver carp samples, and only one OTU (OTU2825, Cyanobacteria) was uniquely detected in the bighead carp samples.

DISCUSSION

In China, bighead carp, common carp, grass carp and silver carp are the four most important freshwater fish species that are cultivated for human consumption. Their respective gut microbiotas however remain less understood (Li, X.M., et al., 2014; Mandal and Ghosh, 2013). An NMDS plot (Figure S4 in Supporting Information) suggests that their gut microbial composition is closer to that of *Siniperca chuatsi* (Yan et al., 2016), *Silurus meridionalis* (Yan et al., 2016), *Pelteobagrus fulvidraco* (Wu et al., 2010) and the domesticated *Danio rerio* (Roeselers et al., 2011) than to *Carassius cuvieri* (Li, T., et al., 2015), river caught *D. rerio* (Roeselers et al., 2011), and mammals (*Homon sapiens* (Claesson et al., 2009) and *Bos taurus* (Shanks et al., 2011)). The gut microbiota composition of the Asian carp species were further compared with other gut microbiota that have been studied till the phylum level (Figure S5 in Supporting Information). Proteobacteria was the most dominant phylum detected this study as well as in most fish gut samples in previous studies, and is mostly absent in the gut of mammals and cultured *C. cuvieri* (Li, T., et al., 2015). While all gut samples of mammals shared Firmicutes as the most dominant phylum, Bacteroidetes was highly abundant (relative abundance >10%) in grass carp and mammals (Claesson et al., 2009; Shanks et al., 2011). Euryarchaeota on the other hand was unique to the fish gut samples in our study.

Extensive intra-species variation was observed in our study, which is also the case with other vertebrate hosts such as zebrafish (Stephens et al., 2016), humans (Caporaso et al., 2011) and mice (Benson et al., 2010), suggesting a general phenomenon in adult vertebrates. Dissimilarity tests based on UniFrac (weighted) distances still demonstrated significant differences in gut microbiotas of the four carp species, suggesting a correlation between the species and the gut microbial populations. Given that the different carps were reared under the same environmental conditions, the differences in gut microbiota may not be a simple reflection of microbes in the surrounding water, but instead result from the species-specific diet, gut morphology, trophic level and phylogeny, as shown in previous studies (Gatesoupe et al., 2014; Liu et al., 2016; Ni et al., 2014; Wong and Rawls, 2012).

Cyanobacteria/Chloroplast are known to be an important food source for most fish species (Bunn et al., 2003; Currin et al., 2011). The relatively high abundances of this phylum in silver carp and grass carp seen in our study further underscores its role as a food source for these two fish species. The majority of the 16S rRNA sequences of Cyanobacteria/Chloroplast belong to the genus *Streptophyta*, which represents chloroplasts derived from ingested plant matter and is abundant in the distal gut of humans who have a largely plant based diet (David et al., 2014). In this study, *Streptophyta* was mainly observed in the midgut samples of silver carp and the hindgut samples of grass carp, indicating an incomplete digestion process in the respective gut regions. Similar incomplete digestion was also observed in invasive Asian silver carp and planktivorous gizzard shad (Ye et al., 2014). In addition, no member of the Cyanobacteria/Chloroplast phylum was shared between silver carp and bighead carp, suggesting that these two plankton-feeding species did not compete for the same type of plankton even in a polyculture.

Proteobacteria and Fusobacteria are two most abundant phyla in the gut microbiota of the four Asian carp species (Li, J., et al., 2014; Li, T., et al., 2015). Most Proteobacteria (30.96%) observed in the fish gut samples in this study belonged to genus *Aeromonas*, which includes pathogens and has been associated with fish gastroenteritis (Austin, 2011; He et al., 2017). This finding is consistent with previous studies showing that fish gut is likely a reservoir for opportunistic pathogens (Mohammed and Arias, 2015). Given that all gut samples in this study were collected from healthy fish, the potential role of *Aeromonas* in these four species is unclear but could be associated with cellulose degradation (Jiang et al., 2011; Ray et al., 2012). This possibility was supported by the abundance of *Aeromonas* in the grass carp. Almost all Fusobacterial 16S rRNA sequences detected in the fish gut samples in this study belonged to the genus *Cetobacterium*, which has been observed in the human feces samples and can ferment peptides and carbohydrates; all fish samples used in this study carried this genus (David et al., 2014). *Cetobacterium* can produce vitamin B12 (Tsuchiya et al., 2008), which is interesting given that carps do not have a dietary requirement of vitamin B12. The combination of a fermentative metabolism together with vitamin production well explains the relevance of *Cetobacterium* in the gut of these carp species.

The Firmicutes and Bacteroidetes are the most significant phyla reflecting the differences between the natural feeding

habits of the fish species. The relative abundance of Firmicutes, which potentially metabolizes dietary plant polysaccharides in the human gut, was highest in common carp (24.56%±26.27%), followed by grass carp (11.19%±8.32%) and bighead carp (10.84%±12.53%). Considering their natural feeding habits, this is not unexpected as the two major genera constituting Firmicutes (27.22%) are *Clostridium sensu stricto* and *Clostridium* XI, which include cellulose-degrading and saccharolytic (Schwiertz et al., 2002), as well as some proteolytic species (Gramignoli et al., 2012). The members of these two genera are most abundant in the bighead carp and common carp. In addition, although the proteolytic genus *Proteocatella* was present at low abundance in the grass carp, it was higher than in the other carp species (David et al., 2014). The genus *Bacteroides* of phylum Bacteroidetes dominated the midgut of grass carp followed by its hindgut. *Bacteroides* are normally mutualistic microbes and show a high degree of versatility in processing complex molecules like plant glycans to simpler ones in the gastrointestinal tract of animals, allowing the host to maximize utilization of energy (Nayak, 2010). Since grass carp are herbivorous, polysaccharide-degrading bacteria like *Bacteroides* sp. are important for their digestive system. In addition, the genus *Prevotella*, which always dominate the gut of those who consume more carbohydrates (David et al., 2014), was observed in the guts of grass carp, silver carp and bighead carp, but at a very low abundance.

In conclusion, the gut microbiotas of these four economically important Asian carp species harbored saccharolytic or proteolytic microbes, likely in response to their specific food habits. Considering the differences in their natural diets, their digestive processes are different and mainly consist of carbohydrate or amino acid fermentation. Therefore, this "food connection" between the gut microbiota and host points towards possible beneficial effects of the gut microbes on fish nutrition. However, a number of microbes detected in our study could not be classified, which suggested the possibility of novel bacteria that needs to be validated. Further studies on the gut microbiotas of the other Asian carp species can help understand the differences in their natural feeding habits.

MATERIALS AND METHODS

Sample collection and DNA extraction

Since this study did not involve any endangered or protected species, no specific permissions were required for the sampling process. All procedures for the handling and euthanasia of animals were approved by the Animal Care and Use Committee of the Institute of Hydrobiology, Chinese Academy of Sciences, the Regulations for the Administration of Affairs Concerning Experimental Animals of China, and the Regulations for the Administration of Affairs Concerning Experimental Animals of Hubei Province. Three samples were collected for each fish species from the Shangshe Lake (Wuhan, Hubei, China) and their foregut, midgut and hindgut were removed as described previously (Yan et al., 2012). The gut contents were carefully collected into a sterile centrifuge tube, flash frozen at −20°C, and subsequently transported to the laboratory (Ni et al., 2012). Microbial genomic DNA was extracted from each sample using a Power Fecal DNA extraction kit (Mo Bio, USA) according to the manufacturer's instruction. The DNA was dissolved in 100 μL sterile water and stored at −20°C for further analyses.

16S rRNA gene sequencing and data analysis

A primer set (515F/806R) targeting the V4 region of the 16S rRNA gene was used for PCR amplification as described previously (Wu et al., 2015; Yan et al., 2015). Due to the quality of the extracted DNA, one hindgut sample of silver carp could not be successfully amplified (Table S1 in Supporting Information). The PCR products were purified with Agencourt® Ampure® XP (Beckman Coulter, Inc., USA) and then used as the template for the second PCR amplification using the same primer set but different barcodes. The positive amplicons were quantified using The PicoGreen dsDNA Assay Kit (Invitrogen, USA) and equal amounts of the PCR products were pooled together and sequenced using an Illumina MiSeq platform at the Institute for Environmental Genomics, University of Oklahoma. One sample failed to amplify so was not included in the final analysis.

Quality filtering, de-noising and chimera checking of the sequences obtained from pyrosequencing were conducted using the IEG's Galaxy pipeline (http://zhoulab5.rccc.ou.edu:8080) as described previously (Wu et al., 2015; Yan et al., 2015). The taxonomic assignments of the sequences were provided using the UPARSE method with 97% cutoff. The representative sequence from each operational taxonomy unit (OTU) was used to align with 16S GreenGene sequences using PyNAST, and a maximum-likelihood tree was constructed using FastTree for the subsequent phylogenetic structure analysis (Caporaso et al., 2010). The rarefaction curves were calculated for each species and the alpha-diversity indices (i.e. OTU number, Chao1 estimator, abundance-based coverage estimator (ACE), and Shannon estimator) for each individual fish was calculated using "phyloseq" package in R (R Development Core Team, 2008). The shared OTUs were derived on the basis of the OTU table generated by IEG's Galaxy pipeline.

Statistical analysis

Dissimilarities of gut microbial communities were calculated using the weighted UniFrac beta-diversity metric (Warton et

al., 2012) via *phyloseq* (McMurdie and Holmes, 2013) package in R (R Development Core Team, 2008). Non-metric multidimensional scaling (NMDS) was used to visualize the pairwise UniFrac distances among samples (Hamady et al., 2010 ; Hammer et al., 2001). Venn diagrams were generated using *limma* package (Ritchie et al., 2015) in R. Permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001) on the basis of the UniFrac distance were performed in R using the *adonis* function from the *vegan* package (Oksanen et al., 2016). To identify microbes that probably exhibited significant differences in abundance between different fish species, Kruskal-Wallis test was performed within PAST (Hammer et al., 2001).

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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Aguilera, E., Yany, G., and Romero, J. (2013). Cultivable intestinal microbiota of yellowtail juveniles (*Seriola lalandi*) in an aquaculture system. Lat Am J Aquat Res 41, 395–403.
Al-Hisnawi, A., Ringø, E., Davies, S.J., Waines, P., Bradley, G., and Merrifield, D.L. (2015). First report on the autochthonous gut microbiota of brown trout (*Salmo trutta* Linnaeus). Aquac Res 46, 2962–2971.
Anderson, M.J. (2001). A new method for non-parametric multivariate analysis of variance. Austral Ecol 26, 32–46.
Austin, B. (2011). Taxonomy of bacterial fish pathogens. Vet Res 42, 20.
Benson, A.K., Kelly, S.A., Legge, R., Ma, F., Low, S.J., Kim, J., Zhang, M., Oh, P.L., Nehrenberg, D., Hua, K., et al. (2010). Individuality in gut microbiota composition is a complex polygenic trait shaped by multiple environmental and host genetic factors. Proc Natl Acad Sci USA 107, 18933–18938.
Billard, R., and Berni, P. (2004). Trends in cyprinid polyculture. Cybium 28, 255–261.
Bunn, S.E., Davies, P.M., and Winning, M. (2003). Sources of organic carbon supporting the food web of an arid zone floodplain river. Freshwater Biol 48, 619–635.
Caporaso, J.G., Bittinger, K., Bushman, F.D., DeSantis, T.Z., Andersen, G. L., and Knight, R. (2010). PyNAST: a flexible tool for aligning sequences to a template alignment. Bioinformatics 26, 266–267.
Caporaso, J.G., Lauber, C.L., Costello, E.K., Berg-Lyons, D., Gonzalez, A., Stombaugh, J., Knights, D., Gajer, P., Ravel, J., Fierer, N., et al. (2011). Moving pictures of the human microbiome. Genome Biol 12, R50.
Claesson, M.J., O'Sullivan, O., Wang, Q., Nikkilä, J., Marchesi, J.R., Smidt, H., de Vos, W.M., Ross, R.P., and O'Toole, P.W. (2009). Comparative analysis of pyrosequencing and a phylogenetic microarray for exploring microbial community structures in the human distal intestine. PLoS One 4, e6669.
Clements, K.D., Angert, E.R., Montgomery, W.L., and Choat, J.H. (2014). Intestinal microbiota in fishes: what's known and what's not. Mol Ecol 23, 1891–1898.
Currin, C.A., Levin, L.A., Talley, T.S., Michener, R., and Talley, D. (2011). The role of cyanobacteria in Southern California salt marsh food webs. Mar Ecol 32, 346–363.
David, L.A., Maurice, C.F., Carmody, R.N., Gootenberg, D.B., Button, J.E., Wolfe, B.E., Ling, A.V., Devlin, A.S., Varma, Y., Fischbach, M.A., et al. (2014). Diet rapidly and reproducibly alters the human gut microbiome. Nature 505, 559–563.
Fishery Bureau of the Ministry of Agriculture. (2014). China Fishery Statistical Yearbook. (Beijing: China Agriculture Press).
Gatesoupe, F.J., Huelvan, C., Le Bayon, N., Sévère, A., Aasen, I.M., Degnes, K.F., Mazurais, D., Panserat, S., Zambonino-Infante, J.L., and Kaushik, S.J. (2014). The effects of dietary carbohydrate sources and forms on metabolic response and intestinal microbiota in sea bass juveniles, *Dicentrarchus labrax*. Aquaculture 422–423, 47–53.
Ghosh, K., Roy, M., Kar, N., and Ringo, E. (2010). Gastrointestinal bacteria in rohu, *Labeo Rohita* (Actinopterygii: Cypriniformes: Cyprinidae): scanning electron microscopy and bacteriological study. Acta Icth Piscat 40, 129–135.
Gramignoli, R., Green, M.L., Tahan, V., Dorko, K., Skvorak, K.J., Marongiu, F., Zao, W., Venkataramanan, R., Ellis, E.C.S., Geller, D., et al. (2012). Development and application of purified tissue dissociation enzyme mixtures for human hepatocyte isolation. Cell Transplant 21, 1245–1260.
Hamady, M., Lozupone, C., and Knight, R. (2010). Fast UniFrac: facilitating high-throughput phylogenetic analyses of microbial communities including analysis of pyrosequencing and PhyloChip data. ISME J 4, 17–27.
Hammer, Ø., Harper, D.A.T., and Ryan, P.D. (2001). PAST: paleontological statistics software package for education and data analysis. Palaeontol Electronica 4, 9.
He, S., Wang, Q., Li, S., Ran, C., Guo, X., Zhang, Z., and Zhou, Z. (2017). Antibiotic growth promoter olaquindox increases pathogen susceptibility in fish by inducing gut microbiota dysbiosis. Sci China Life Sci 60, 1260–1270.
Hooper, L.V., Wong, M.H., Thelin, A., Hansson, L., Falk, P.G., and Gordon, J.I. (2001). Molecular analysis of commensal host-microbial relationships in the intestine. Science 291, 881–884.
Jeney, Z., and Jian, Z. (2009). Use and exchange of aquatic resources relevant for food and aquaculture: common carp (*Cyprinuscarpio* L.). Rev Aquaculture 1, 163–173.
Jiang, Y., Xie, C., Yang, G., Gong, X., Chen, X., Xu, L., and Bao, B. (2011). Cellulase-producing bacteria of Aeromonas are dominant and indigenous in the gut of *Ctenopharyngodon idellus* (Valenciennes). Aquaculture Res 42, 499–505.
Li, J., Ni, J., Li, J., Wang, C., Li, X., Wu, S., Zhang, T., Yu, Y., and Yan, Q. (2014). Comparative study on gastrointestinal microbiota of eight fish species with different feeding habits. J Appl Microbiol 117, 1750–1760.
Li, T., Long, M., Gatesoupe, F.J., Zhang, Q., Li, A., and Gong, X. (2015). Comparative analysis of the intestinal bacterial communities in different species of carp by pyrosequencing. Microb Ecol 69, 25–36.
Li, X.M., Zhu, Y.J., Yan, Q.Y., Ringo, E., and Yang, D.G. (2014). Do the intestinal microbiotas differ between paddlefish (*Polyodon spathala*) and bighead carp (*Aristichthys nobilis*) reared in the same pond? J Appl Microbiol 117, 1245–1252.
Li, Z., Xu, L., Liu, W., Liu, Y., Ringø, E., Du, Z., and Zhou, Z. (2015). Protein replacement in practical diets altered gut allochthonous bacteria of cultured cyprinid species with different food habits. Aquacult Int 23, 913–928.
Liu, H., Guo, X., Gooneratne, R., Lai, R., Zeng, C., Zhan, F., and Wang, W. (2016). The gut microbiome and degradation enzyme activity of wild freshwater fishes influenced by their trophic levels. Sci Rep 6, 24340.
Mandal, S., and Ghosh, K. (2013). Isolation of tannase-producing microbiota from the gastrointestinal tracts of some freshwater fish. J Appl Ichthyol 29, 145–153.
Marungruang, N., Fåk, F., and Tareke, E. (2016). Heat-treated high-fat diet modifies gut microbiota and metabolic markers in *apoe*$^{-/-}$ mice. Nutr Metab (Lond) 13, 22.
McMurdie, P.J., and Holmes, S. (2013). phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS ONE 8, e61217.
Mohammed, H.H., and Arias, C.R. (2015). Potassium permanganate elicits a shift of the external fish microbiome and increases host susceptibility to columnaris disease. Vet Res 46, 82.
Nayak, S.K. (2010). Role of gastrointestinal microbiota in fish. Aquacult

Res 41, 1553–1573.
Ni, J., Yu, Y., Zhang, T., and Gao, L. (2012). Comparison of intestinal bacterial communities in grass carp, *Ctenopharyngodon idellus*, from two different habitats. Chin J Ocean Limnol 30, 757–765.
Ni, J., Yan, Q., Yu, Y., and Zhang, T. (2014). Factors influencing the grass carp gut microbiome and its effect on metabolism. FEMS Microbiol Ecol 87, 704–714.
Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., and Wagner, H.H. (2016). vegan: Community Ecology Package. Software.
Park, S.H., Lee, S.I., and Ricke, S.C. (2016). Microbial populations in naked neck chicken ceca raised on pasture flock fed with commercial yeast cell wall prebiotics via an Illumina MiSeq platform. PLoS ONE 11, e0151944.
Pearce, D.A., Gast, C.J., Lawley, B., and Ellis-Evans, J.C. (2003). Bacterioplankton community diversity in a maritime Antarctic lake, determined by culture-dependent and culture-independent techniques. FEMS MicroBiol Ecol 45, 59–70.
Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K.S., Manichanh, C., Nielsen, T., Pons, N., Levenez, F., Yamada, T., et al. (2010). A human gut microbial gene catalogue established by metagenomic sequencing. Nature 464, 59–65.
R Development Core Team (2008) R: A language and environment for statistical computing. Vienna: R Foundation for Statistical Computing.
Ranjan, R., Rani, A., Metwally, A., McGee, H.S., and Perkins, D.L. (2016). Analysis of the microbiome: advantages of whole genome shotgun versus 16S amplicon sequencing. Biochem Biophys Res Commun 469, 967–977.
Ray, A.K., Ghosh, K., and Ringø, E. (2012). Enzyme-producing bacteria isolated from fish gut: a review. Aquacult Nutr 18, 465–492.
Ritchie, M.E., Phipson, B., Wu, D., Hu, Y., Law, C.W., Shi, W., and Smyth, G.K. (2015). limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res 43, e47.
Roeselers, G., Mittge, E.K., Stephens, W.Z., Parichy, D.M., Cavanaugh, C. M., Guillemin, K., and Rawls, J.F. (2011). Evidence for a core gut microbiota in the zebrafish. ISME J 5, 1595–1608.
Schwiertz, A., Hold, G.L., Duncan, S.H., Gruhl, B., Collins, M.D., Lawson, P.A., Flint, H.J., and Blaut, M. (2002). Anaerostipes caccae gen. nov., sp nov., a new saccharolytic, acetate-utilising, butyrate-producing bacterium from human faeces. Syst Appl Microbiol 25, 46–51.
Shanks, O.C., Kelty, C.A., Archibeque, S., Jenkins, M., Newton, R.J., McLellan, S.L., Huse, S.M., and Sogin, M.L. (2011). Community structures of fecal bacteria in cattle from different animal feeding operations. Appl Environ Microbiol 77, 2992–3001.
Stephens, W.Z., Burns, A.R., Stagaman, K., Wong, S., Rawls, J.F., Guillemin, K., and Bohannan, B.J.M. (2016). The composition of the zebrafish intestinal microbial community varies across development. ISME J 10, 644–654.
Sun, Y., Zhou, L.P., Fang, L.D., Su, Y., and Zhu, W.Y. (2015). Responses in colonic microbial community and gene expression of pigs to a long-term high resistant starch diet. Front Microbiol 6, 877.
Torok, V.A., Ophel-Keller, K., Loo, M., and Hughes, R.J. (2008). Application of methods for identifying broiler chicken gut bacterial species linked with increased energy metabolism. Appl Environ Microbiol 74, 783–791.
Tsuchiya, C., Sakata, T., and Sugita, H. (2008). Novel ecological niche of *Cetobacterium somerae*, an anaerobic bacterium in the intestinal tracts of freshwater fish. Lett Appl Microbiol 46, 43–48.
Wang, Q., Li, Z., Lian, Y., Du, X., Zhang, S., Yuan, J., Liu, J., and De Silva, S.S. (2016). Farming system transformation yields significant reduction in nutrient loading: case study of Hongze Lake, Yangtze River Basin, China. Aquaculture 457, 109–117.
Warton, D.I., Wright, S.T., and Wang, Y. (2012). Distance-based multivariate analyses confound location and dispersion effects. Methods Ecol Evol 3, 89–101.
Wong, S., and Rawls, J.F. (2012). Intestinal microbiota composition in fishes is influenced by host ecology and environment. Mol Ecol 21, 3100–3102.
Wu, L., Wen, C., Qin, Y., Yin, H., Tu, Q., Van Nostrand, J.D., Yuan, T., Yuan, M., Deng, Y., and Zhou, J. (2015). Phasing amplicon sequencing on Illumina Miseq for robust environmental microbial community analysis. BMC Microbiol 15, 125.
Wu, S., Gao, T., Zheng, Y., Wang, W., Cheng, Y., and Wang, G. (2010). Microbial diversity of intestinal contents and mucus in yellow catfish (*Pelteobagrus fulvidraco*). Aquaculture 303, 1–7.
Yan, Q., van der Gast, C.J., and Yu, Y. (2012). Bacterial community assembly and turnover within the intestines of developing zebrafish. PLoS ONE 7, e30603.
Yan, Q., Bi, Y., Deng, Y., He, Z., Wu, L., Van Nostrand, J.D., Shi, Z., Li, J., Wang, X., Hu, Z., et al. (2015). Impacts of the Three Gorges Dam on microbial structure and potential function. Sci Rep 5, 8605.
Yan, Q., Li, J., Yu, Y., Wang, J., He, Z., Van Nostrand, J.D., Kempher, M. L., Wu, L., Wang, Y., Liao, L., et al. (2016). Environmental filtering decreases with fish development for the assembly of gut microbiota. Environ Microbiol 18, 4739–4754.
Ye, L., Amberg, J., Chapman, D., Gaikowski, M., and Liu, W.T. (2014). Fish gut microbiota analysis differentiates physiology and behavior of invasive Asian carp and indigenous American fish. ISME J 8, 541–551.
Zhang, C., Zhang, M., Wang, S., Han, R., Cao, Y., Hua, W., Mao, Y., Zhang, X., Pang, X., Wei, C., et al. (2010). Interactions between gut microbiota, host genetics and diet relevant to development of metabolic syndromes in mice. ISME J 4, 232–241.

SUPPORTING INFORMATION

Table S1 Detailed fish gut sample information

Table S2 Phylum-level unclassified OTUs within domain archaea and bacteria. Only the bacterial OTU with more than 0.1% of sequences and archaea OTU are shown

Table S3 Relative abundance of bacterial genus potentially associated with host digestion

Figure S1 Microbial complexity based on taxonomic assignment results of the pooled sequences.

Figure S2 The OTU numbers of known (16S classified) and unclassified bacterial phylotypes within each gut samples.

Figure S3 Phylogenetic tree of Cyanobacteria/Chloroplast that are uniquely present in one of the four fish species gut samples. The unique OTUs are in red color and the shared OTUs in green color.

Figure S4 NMDS plot showing the microbial community differences between the gut microflora of different species (*Homon sapiens* (Claesson et al., 2009), *Bos taurus* (Shanks et al., 2011), *Carassius cuvieri* (Li, T., et al., 2015), *Danio rerio* (Roeselers et al., 2011), *Siniperca chuatsi* (Yan et al., 2016), *Silurus meridionalis* (Yan et al., 2016), *Pelteobagrus fulvidraco* (Wu et al., 2010)). The distances were determined using Bray-Curtis method with relative abundance data at phylum level.

Figure S5 Relative abundances of different phyla in the bighead carp, common carp, grass carp, silver carp and other gut systems (*H. sapiens* (Claesson et al., 2009), *B. taurus* (Shanks et al., 2011), *C. cuvieri* (Li, T., et al., 2015), *D. rerio* (Roeselers et al., 2011), *S. chuatsi* (Yan et al., 2016), *S. meridionalis* (Yan et al., 2016), *P. fulvidraco* (Wu et al., 2010)).

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