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Host Habitat as a Dominant Role in Shaping the Gut Microbiota of Wild Crucian Carp (*Carassius auratus*)

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Abstract: Current knowledge on the fish gut microbiota has largely been obtained from experiments on laboratory-reared animals. Here, the crucian carp (Carassius auratus) with a mean weight of 159.9 ± 11.4 g (mean ± SD) were collected from their natural habitats (i.e., Wuhu lake and Poyang lake, China), and the gut microbiota were analysed by using the next-generation sequencing of 16S rRNA gene. We obtained more than 430,000 high-quality reads, which constituted more than 1200 operational taxonomy units (OTUs), revealing extremely diverse microbes in the fish gut. Proteobacteria, Fusobacteria, Bacteroidetes and Firmicutes were detected as the prominent phyla (each > 1% of total abundance) within the gut microbiota, regardless of the host habitat or the gut segment (i.e., foregut vs. hindgut). Although the microbes in the hindgut were more diverse (OTU number, Shannon and Chao1; One-way Anova, $p > 0.05$) than in the foregut, the host habitat had a significant role in shaping the community structures (MRPP, ANOSIM, PERMANOVA, $p < 0.01$). Interestingly, we also detected a set of common OTUs, whereby genera *Aeromonas* and *Cetobacterium* might comprise the core gut microbiota of crucian carp.

Keywords: gut microbiota; 16S rRNA; crucian carp; habitat effects

Key Contribution: The present study provided a comprehensive analysis of the gut microbiota of crucian carp that hatched and developed in natural environments and, more importantly, highlights a strong habitat effect on the gut microbial community composition.

Citation: Li, X.; Huang, X.; Zhao, L.; Cai, W.; Yu, Y.; Zhang, J. Host Habitat as a Dominant Role in Shaping the Gut Microbiota of Wild Crucian Carp (*Carassius auratus*). *Fishes* **2023**, *8*, 369. https://doi.org/10.3390/fishes8070369

Academic Editors: Jee Eun Han and Patharapol Piamsomboon

Received: 17 May 2023
Revised: 30 June 2023
Accepted: 10 July 2023
Published: 17 July 2023

1. Introduction

A majority of endogenous microbes occur in the digestive tract of the host and facilitate their host survival in the microbial-rich environment [1,2]. In particular, the previous studies have suggested that the gut microbiota of fish plays an important role in host metabolism, immunity, and health maintenance [3–5].

As one of the oldest cultured fish species in China, the crucian carp (*Carassius auratus*) constitutes one of the most cultured species in the world. Current knowledge on the gut microbiota of crucian carp, therefore, has largely been obtained from the experiments on laboratory-reared individuals [6]. The crucian carp is an omnivorous freshwater teleost fish native to Europe and Siberia and indigenous to lakes, ponds, and slow-moving rivers [7]. It is still unclear whether the gut microbial community composition can vary with the host natural habitats.

The constituency of gut microbiota includes members of all three domains of life (i.e., Bacteria, Archaea, and Eukarya) as well as viruses. The fish gut microbiota generally consist of facultative and obligate anaerobes, which are dominated by four phyla of prokaryote (i.e., Proteobacteria, Fusobacteria, Actinobacteria, and Cyanobacteria) [8]. Moreover, genera *Aeromonas*, *Bacillus*, and *Cetobacterium* have been widely identified as the predominant members in fish gut microbiota [9,10]. Conventional techniques, such as culture-dependent

as well as culture-independent methods have been widely used to investigate the gut microbial diversity [11]. These conventional techniques-based studies on the gut microbiota, however, have been largely limited to the clone library analysis of at most a few hundred sequences per sample, thereby only detecting the most abundant microbial taxa. [12,13]. Understanding the high microbial diversity in the gut is a prerequisite to further identifying the features with potential benefits to host fish.

In this work, fish were collected from Wuhu lake and Poyang lake (China), which have been previously identified as the natural habitats for crucian carp. Given the great distance between these two lakes, we expected to observe significant habitat effects on the gut microbial communities. Specific aims of this study were (i) to reveal the diversity of microbes in the fish gut and (ii) to analyze whether the microbial community structures are host habitats dependent.

2. Materials and Methods

2.1. Sample Collection

Crucian carps were collected from two lakes (Poyang Lake and Wuhu Lake) in the Yangtze River basin (Figure 1) by using fishing nets. No specific permissions were required for the sampling activity, and the present study did not involve any endangered or protected species. All fish were captured in the lake area, where no artificial food was provided. Three fish individuals were sampled in each lake (Table 1), and all fish individuals were managed under the same protocol, which was performed with the approval of the Animal Care and Use Committee of the Institute of Hydrobiology, Chinese Academy of Sciences (IHB/ LL/2021006) [12,14]. Briefly, the fish were dissected under sterile conditions. The harvested gut was aseptically divided into two segments (i.e., foregut and hindgut) as previously described [15]. The gut contents were carefully collected into a sterile centrifuge tube, flash frozen at −20 °C, and subsequently transported to the laboratory within 6 h.

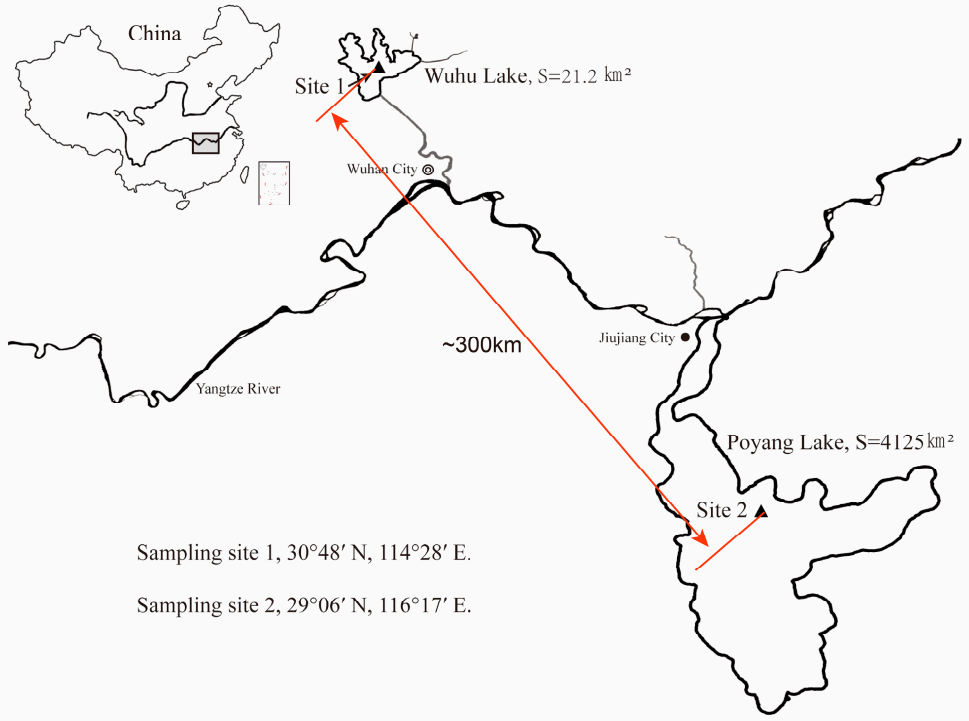

Figure 1. The large scale on the geographic distance (nearly 300 km) between the Wuhu lake (Wuhan, China) and Poyang lake (Jiujiang, China) where the crucian carps were collected for microbial sampling.

Table 1. Biological information of the sampled fish individuals.

Fish ID	Sampling Lake	Weight/g	Body Length/cm
P1	Poyang	167.6	14.2
P2	Poyang	150.2	13.3
P3	Poyang	161.5	13.8
W1	Wuhu Lake	142.0	12.2
W2	Wuhu Lake	168.2	14.4
W3	Wuhu Lake	170.3	14.8

2.2. DNA Isolation

DNA was isolated as described previously with certain modifications [16]. Briefly, the samples were mixed with 1200 µL lysis buffer (0.50% Sodium Dodecyl Sulfate (SDS); 10.00 mmol/L Tris-HCl, pH 8.0; 10.00 mmol/L EDTA, pH 8.0; 100.00 mmol/L NaCl; and 50 µg/mL RNase A) and stored at 37 °C for 1 h, followed by incubation at 55 °C for 12 h after the addition of 6 µL proteinase K (0.10 mg/mL). The lysates were extracted using an equal volume of phenol/chloroform/isoamyl alcohol (25/24/1, *v/v/v*) and subsequently precipitated with a double volume of pure ethanol and one-tenth volume of 3 mol/L NaCl. After washing with 70% ethanol, the purified DNA was stored at −20 °C.

2.3. PCR and Sequencing Methods

The 16S rRNA gene primer set 515f (5′-GTGCCAGCMGCCGCGGTAA-3′)/806r 5′-GGACTACHVGGGTWTCTAAT-3′) (without barcode) targeting the V4 region of 16S rDNA was used for PCR amplification because this primer set was available for both bacteria and archaea [17]. Briefly, the 25 µL PCR reactions were performed in triplicate for each sample using the following conditions: 1 min at 94 °C, 25 cycles of 20 s at 94 °C, 25 s at 53 °C, and 45 s at 68 °C, with a post-amplification extension of 10 min at 68 °C. Subsequently, the PCR products were purified using AGENCOURT® AMPURE® XP, and approximately 10 µL of the purified DNA was applied as a template for a second PCR amplification. The primer set 515f/806r (with different barcodes) was used again, and 25 cycles were performed. The amplicons were visualized on 1% agarose gels stained with ethidium bromide and followed by quantification using Molecular Probes PicoGreen® on a FLUO star OPTIMA reader. Approximately 200 ng of amplified DNA from each sample was combined in a sterile tube and visualized using agarose gel electrophoresis (90 V, 2 h). The purified DNA was re-quantified using PicoGreen and sequenced on a MiSeq platform (Illumina, San Diego, CA, USA) at Majorbio Bio-Pharm Technology Co. Ltd., (Shanghai, China) according to the manufacturer's protocol. The operational taxonomic units (OTUs) were identified (97% cut-off) according the method of Zhou et al. [18]. All of the sequences were matched through the Ribosomal Database Project (RDP) [19].

2.4. Statistical Analysis

The OTU table generated through 16S rRNA gene sequencing was further analysed by the following statistical methods after all samples were rarefied to the same sequence depth (n = 18,314 sequences) by random subsampling to correct for differences in sequencing depths: (a) alpha diversity of gut microbial community was estimated by alpha-diversity indices, they are Number of taxa (OTU number), Shannon indices and Chao1; (b) Unweighted pair-group average (UPGMA) clustering analysis based on the Simpson index; (c) Bray–Curtis distance-based non-metric multidimensional scaling (NMDS) analysis [20]; (d) nonparametric tests including multiple-response permutation procedure (MRPP) [21], analysis of similarity (ANOSIM) [21], and permutational multivariate analysis of variance (PERMANOVA) [22] to compare community dissimilarity; (e) Welch's t-test (confidence interval method: Welch's inverted, $p < 0.05$) for two samples having possibly unequal variances was performed to identify whether the OTU exhibited significant difference in abundance between samples [23]; (f) Venn diagram reveals the common OTUs of gut microbiota. All the statistical analyses were performed in R 3.6.1 [24].

3. Results

3.1. Characterization of the Gut Microbiota

A total of 436, 552 high-quality reads were obtained from the 12 gut content samples, with the number of sequences ranging from 18,314 to 64,108 per sample (Table S1). A total of 1261 OTUs were retrieved, which could be classified into 17 phyla (Table S2). More than 50% of the detected OTUs were assigned to phylum Proteobacteria, followed by Firmicutes (9.75%), Actinobacteria (7.14%), and Bacteroidetes (6.74%). Only one OTU could not be classified into any known group and was assigned as 'Unclassified'.

In terms of relative abundance, only 6 phyla were identified as predominant (each > 1% of the total abundance): Proteobacteria (43.68%), Firmicutes (34.13%), Fusobacteria (15.82%), Actinobacteria (2.65%), Bacteroidetes (1.11%), and Acidobacteria (1.08%).

At the genus level (Table S3), *Aeromonas* occupied 16.99% of the total abundance and was the most abundant genus in the gut bacterial community, followed by *Cetobacterium* (9.76%), *Chitinibacter* (8.15%), *Fusobacterium* (6.06%), and *Catellibacterium* (4.61%). In addition, *Pseudomonas* (1.65%), *Clostridium* XI (1.39%), *Clostridium sensu stricto* (1.30%), *Vibrio* (1.23%), and *Serratia* (1.09%) were also above the 1% threshold of total abundance.

3.2. Microbial Diversity Varied with Host Habitats

In terms of OTU number, Shannon and Chao 1, the microbiota in the gut samples collected from Poyang Lake were generally more diverse (One-way ANOVA, $p >> 0.05$) than those from Wuhu Lake (Table 2). This habitat effect on microbial diversity, however, was also influenced by the gut segment, where the microbiota were more diverse (One-way ANOVA, $p >> 0.05$) in foregut of the fish from Wuhu Lake (except for Shannon; Table 2). Moreover, the gut microbiota were more diverse in the hindgut regardless of the host's habitat (OTU number, Shannon and Chao1; One-way ANOVA, $p > 0.05$). Similar trends in microbial diversity were also observed among the rarefaction curves and the number of taxa assigned (Figure S1A,B).

In total, we detected 19 dominant OTUs (each > 0.5% of total abundance) (Figure 2), most of which were abundantly present in the gut of the crucian carp collected from Poyang Lake. Only two OTUs (OTU_518, *Aeromonas* and OTU_1491, *Cetobacterium*) were significantly different in terms of the relative abundance (Figure 2; $p < 0.05$) and were more abundant in the gut of the crucian carp collected in Wuhu Lake.

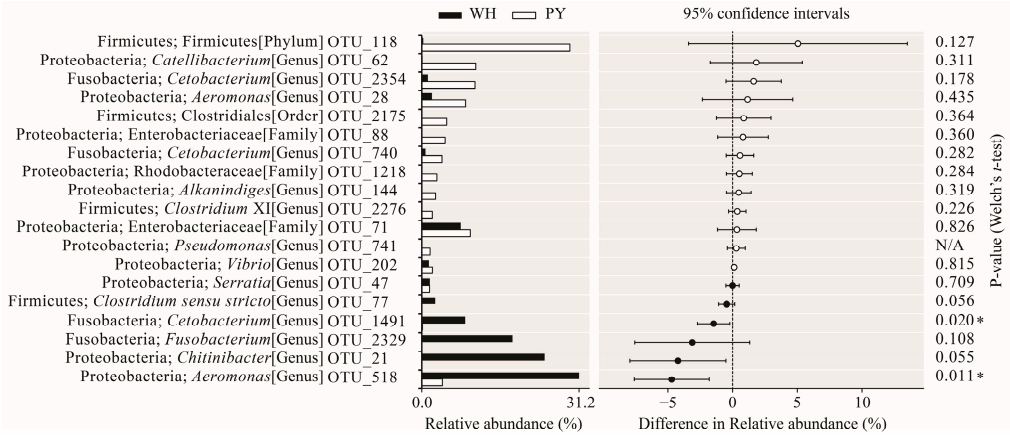

Figure 2. Extended error bar plot showing the difference in relative abundance of OTUs between the gut microbial communities from the fish in different habitats. OTUs overrepresented in the gut of the crucian carp from Wuhu Lake have a negative difference between relative abundances. OTUs overrepresented in the gut of the crucian carp from Poyang Lake have a positive difference between relative abundances. Only OTUs occupied more than 0.5% of total abundance are shown. Stars indicate statistically significant (*—$p < 0.05$) community structuring. Samples collected from Wuhu Lake and Poyang Lake are indicated by WH and PY.

Table 2. Summary of species richness estimators of different fish gut samples.

Fish Habitats	Gut Segment	Total Sequences Passed Quality Check	Number of Taxa (OTU Number)	Shannon	Chao1
Wuhu lake	Foregut	88,401	214.3 ± 43.0	2.1 ± 0.2	285.8 ± 33.0
Wuhu lake	Hindgut	91,029	216.0 ± 58.3	2.6 ± 0.5	306.1 ± 83.2
Poyang lake	Foregut	110,828	197.3 ± 77.8	2.9 ± 1.0	267.1 ± 77.1
Poyang lake	Hindgut	146,294	280.3 ± 188.3	3.0 ± 0.8	318.5 ± 193.3

3.3. Microbial Community Dissimilarity between Gut Content Samples

The UPGMA clustering showed that the gut microbial communities clustered into two groups according to the host habitats (Figure 3A). This trend in community dissimilarity was also observed by the NMDS (Figure 3C) and was estimated by the significance test based on the Jaccard distance (Table 3; MRPP, ANOSIM and PERMANOVA, $p < 0.01$). Furthermore, the microbial community dissimilarity between the foregut and the hindgut of crucian carp was not significant (Table 3). Similar trends were also observed when the dissimilarity analysis and correlation were performed between the foregut and hindgut of each fish individual (Table S4).

We also detected substantial variation on community composition (Figure 3B). As one of the most abundant phyla, Proteobacteria, respectively, represent 67.00% and 45.80% of total abundance of gut microbiota in terms of host habitats. Fusobacteria accounted for 25.90% of the total abundance in samples from Wuhu Lake, while they only occupied 9.20% of the total abundance for Poyang Lake. Interestingly, Firmicutes was more abundant in the gut of the crucian carp in Poyang Lake than (i.e., 27.50% vs. 3.00%). Similar trends between host habitats (Poyang Lake vs. Wuhu Lake) were also observed for Bacteroidetes (2.40% vs. 1.60%), Actinobacteria (5.00% vs. 0.40%), Cyanobacteria/Chloroplast (2.40% vs. 0.03%), and Acidobacteria (2.40% vs. 0.03%).

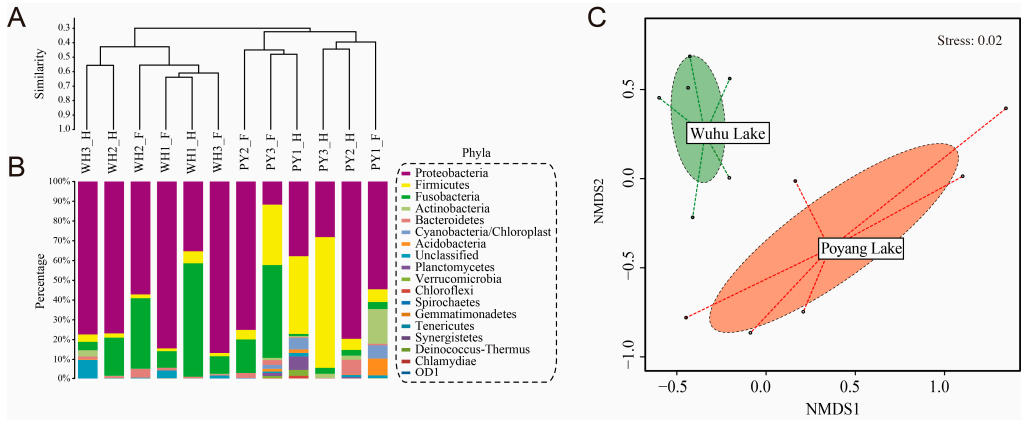

Figure 3. The community composition and dissimilarity analysis. (**A**) UPGMA clustering of 16S rRNA gene based on Simpson similarity index reveals that the gut content samples cluster by sampling location. WH_F and WH_H, respectively, indicate the microbial community in the foregut and hindgut of crucian carps from Wuhu Lake. PY_F and PY_H, respectively, indicate the microbial community in the foregut and hindgut of crucian carps from Poyang Lake. (**B**) Histogram on the end of each branch shows the gut microbial community structure on phylum level, and the relative abundance of each phylum is shown. (**C**) NMDS plot showing the gut microbial communities clustered according to the host habitats. Pairwise community distances were determined using the Bray–Curtis method.

Table 3. Dissimilarity test showing the differences in gut microbiota between host's habitat and between gut segments.

		Jaccard Distance-Based Test		Bray–Curtis Distance-Based Test	
		WH vs. PY (Host Habitat)	**Foregut vs. Hindgut (Gut Segment)**	**WH vs. PY (Host Habitat)**	**Foregut vs. Hindgut (Gut Segment)**
MRPP	*Delta*	0.840	0.895	0.745	0.825
	P	0.005	0.246	0.008	0.252
ANOSIM	*R*	0.585	0.113	0.585	0.113
	P	0.007	0.152	0.002	0.151
PERMANOVA	*F*	2.507	1.123	3.481	1.231
	P	0.008	0.272	0.004	0.218

Abbreviations: WH, Wuhu Lake; PY, Poyang Lake.

3.4. Identification of a Common Gut Microbiota

The Venn diagram showed that a total of 73 OTUs were shared by different host habitats, as well as the different gut segments (Figure 4A). By plotting the ranked abundance of OTUs according to the occurrence, we found that the 73 common OTUs was extremely abundant (Figure 4B) and represented 59.40% of the total abundance. The RDP classifier analysis revealed that the 73 common OTUs comprised the 33 genera from phyla Proteobacteria, Firmicutes, Fusobacteria, Cyanobacteria/Chloroplast, Bacterioidetes, and Actinobacteria (Table S5). *Aeromonas* was the most abundant in the shared microbial community, followed by *Fusobacterium* and *Cetobacterium* (Table S5).

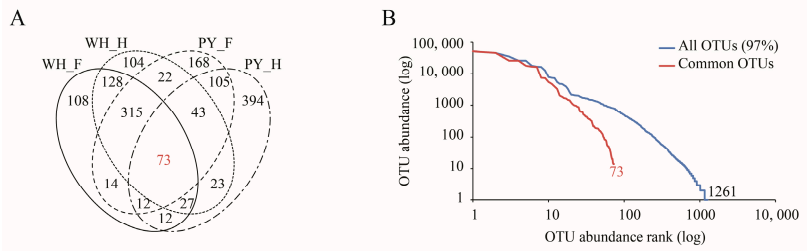

Figure 4. Deep sequencing of 16S rRNA genes reveals the common OTUs in the gut microbiotas of the crucian carps. (**A**) Venn diagram showing the distribution of all microbial OTUs (97%) detected in the gut of crucian carp. WH_F and WH_H, respectively, indicate the microbial community in the foregut and hindgut of crucian carps from Wuhu Lake. PY_F and PY_H, respectively, indicate the microbial community in the foregut and hindgut of crucian carps from Poyang Lake. (**B**) Rank abundance plot showing all OTUs (blue line) and 73 common OTUs (red line) ranked according to their abundance in the combined data set.

4. Discussion

The gut is a major organ for fish interacting with the external environment [25]. The dense bacterial community in the fish gut has been confirmed as helpful for the host in maintaining gut integrity, in strengthening immunity system, and in contributing to digestive processes [26,27]. The gut microbiota of fish contains a diverse and vast population of microorganisms [28]. To develop an effective strategy for promoting and sustaining the health of cultured species, we should first understand the gut microbiota of fish in natural environments [10].

The present study provided an appropriate opportunity to estimate the high microbial diversity in the gut of crucian carp and, more importantly, defined the common OTUs that are shared by the crucian carps [29–32]. The gut microbiota of the crucian carp is numerically dominated by phyla Proteobacteria, Firmicutes, Bacteroidetes and Fusobacteria regardless of differences in host habitats and differences in dietary conditions [29,32]. The relative abundances of these dominant phyla, however, can vary with the changes in

dietary condition, probiotics, and environmental chemical compoment [2,10,32]. Moreover, these bacterial phyla have also been detected within other teleost fish gut as the dominant groups [3,15,33], suggesting that these specific bacterial phyla are especially well adapted for the environment within the gut. In particular, Proteobacteria were the most dominant phyla in the gut microbiota of the crucian carp [29], and were also abundantly present in the gut of the crucian carps that living in the Dongting Lake [30]. Nevertheless, the Proteobacteria seems likely to be absent or occupy very low abundance in the gut of cultured fish individuals or mammals [34–38].

By using the high-throughput sequencing technologies, we detected that the gut microbiota significantly varied with the host's habitat. Indeed, the host habitat effects on gut microbiota were not unique to crucian carp and have also been observed in grass carp and zebrafish [30,36]. Furthermore, a study on the freshwater fishes in the Dongting Lake also revealed important effects of host habitats on the fish gut microbiota [30]. More importantly, the host habitat effect on gut microbiota has also been observed between the different artificial aquaculture systems [31]. The causes of these observed variations on gut microbiota remain unknown as they could include the different characteristics in the respective local environment such as water chemistry, diet composition, and the spectra of infectious microorganisms/viruses [9,32]. Further studies on these possible factors (including water microbiome, gut content analysis, etc.) would contribute to a better understanding of gut microbiome modulation.

Collaborating with the results in the present study, this was likely to constitute an important inference that the host habitats may play a more significant role in governing gut microbiota than fish species under the specific context. Moreover, the variations in gut microbial communities were more significant among the foregut samples than between the hindgut samples, suggesting that the reaction of gut microbiota in response to the changes in host habitat varies throughout the gut.

5. Conclusions

Given the potentially high biodiversity, it is important to apply next-generation sequencing technology to assess the gut microbial community composition in the fish that inhabit natural environments. The results are helpful for researchers investigating aspects of nutrition metabolism that could be influenced by the gut microbiota of crucian carp. In addition, our findings underscore the need to identify the selective pressures governing microbial community assembly within the gut of fish. Further study on the gut microbiota of crucian carp should be focused on the assembling process that governs the gut microbial community composition.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes8070369/s1, Figure S1: Microbial complexity based on rarefaction curves and taxonomic assignment results of the pooled sequences; Table S1: Bacterial diversity indices in each intestinal sample; Table S2: Microbial community composition at phylum level; Table S3: The microbial community composition on genus level.; Table S4: The Bray–Curtis distance and correlation (R^2) between the foregut and hindgut microbial community in each individual fish; Table S5: Classification of the common OTUs.

Author Contributions: Conceptualization, J.Z.; methodology, X.L.; data curation, X.H. and X.L.; writing—original draft preparation, X.L. and W.C.; writing—review and editing, L.Z. and Y.Y. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the National Natural Science Foundation of China (No. 31801984), and the Foundation of State Key Laboratory of Water Resources and Hydropower Engineering Science, Wuhan University (No. 2020NSG02), Key Projects of Science and Technology Research from the Education Department of Hubei Province (D20221002) and Key R&D project of Hubei Province (No. 2021BCA129).

Institutional Review Board Statement: All the protocols and procedures involving crucian carp were approved by the Animal Care and Use Committee of the Institute of Hydrobiology, Chinese Academy of Sciences (IHB/ LL/2021006).

Informed Consent Statement: Not applicable.

Data Availability Statement: The raw read sequences obtained from sequencing were deposited in the Sequence Read Archive (SRA) under BioProject accession number PRJNA988942 (SUB13582038) and will be released after 1 September 2024. The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

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