Host Dependence of Zooplankton-Associated Microbes and Their Ecological Implications in Freshwater Lakes

Qianhong Wang 1,2, Zheng Hao 1,2, Ruirui Ding 1,2, Huabing Li 1, Xiangming Tang 1 and Feizhou Chen 1,2,*

1 State Key Laboratory of Lake Science and Environment, Nanjing Institute of Geography and Limnology, Chinese Academy of Sciences, Nanjing 210008, China; wqh_sdc@163.com (Q.W.); hz_ghosta@163.com (Z.H.); realdi@163.com (R.D.); hbl@niglas.ac.cn (H.L.); xmtang@niglas.ac.cn (X.T.)
2 University of Chinese Academy of Sciences, Beijing 100049, China
* Correspondence: feizhch@niglas.ac.cn; Tel.: +86-25-8688-2220

Abstract: Zooplankton is colonized by quite different microbes compared with free-living and particle-associated bacteria, serving as a non-negligible niche of bacteria in aquatic ecosystems. Yet detailed analysis of these bacterial groups is still less known, especially in freshwater lakes. To widen our knowledge of host-microbe interaction and bacterial ecosystem functions, we chose two specific populations of zooplankton, i.e., cladoceran *Moina* and copepod Calanoids, as hosts from five natural lakes, and illustrated detailed features of their associated bacteria. Through 16S rRNA gene sequencing, we found microbes colonized on Calanoids presented significantly higher α-diversity, stronger bacterial interaction and metabolic function potentials than for *Moina*. It was also notable that zooplankton-associated bacteria showed a high potential of fatty acid metabolism, which is beneficial for host’s development. Moreover, we found that zooplankton-associated microbes may exert profound effects on biogeochemical cycles in freshwater lakes, since several bacterial members able to participate in carbon and nitrogen cycles were found abundant. Overall, our study expands current understanding of the host-microbe interaction and underlying ecological dynamics in freshwater ecosystem.

Keywords: freshwater lakes; zooplankton; host; bacteria; ecosystem function

1. Introduction

Zooplankton is widespread in freshwater lakes as an indispensable component of food webs. These small aquatic invertebrates can directly prey on phytoplankton, and thus contribute to inhibiting algal bloom and keeping a clear state of lake [1–3]. Generally, zooplankton and bacteria are routinely recorded as independent study objects and free-living bacteria (FL) are assumed to be evenly distributed in the water column. Zooplankton can feed on microbial bacteria and a predation link is formed in the microbial loop [4,5]. Until recently, a new link has been formally reported, that is, zooplankton-associated bacteria (ZA), referring to those bacteria colonized on the exterior and interior parts of zooplankton bodies [6,7].

Providing a special micro-environment, zooplankton might be a biotic selector for specific microbial groups. For instance, through sloppy feeding and excretion, zooplankton can enrich the dissolved organic matter (DOC) and nutrients, for instance, ammonia in “zoosphere” [8–10]. Zooplankton also provides a habitat or a refuge for bacteria [6]. Therefore, bacterial colonization can be observed all over the body of zooplankton, especially the oral, anus, and intestine areas [11]. Previous studies also reported that both abundance and size of ZA are significantly higher than those of FL by electron microscopic observation [12]. Through enumeration of probe–positive bacterial cells, Heidelberg et al. (2002) found that Gamma–proteobacteria of ZA was 100–1000–fold higher than FL [13]. In addition, Grossart et al. (2010) suggested that cladoceran (Bosmina coregoni) and
cyclopoid (<i>Thermocyclops oithonoides</i>) ZA had different components and responded differently to nutrient levels using DGGE method [14]. Although several studies have reported the characteristics of ZA, they were mostly based on bacterial count or culture methods. Due to the technical limitation, concrete taxonomic information of ZA was not available until recent years. By Illumina sequencing technology for 16S rDNA, Samad et al. (2020) systematically compared bacterial community structure of FL, particle–associated bacteria (PA) and ZA, and significant differences were observed even at phylum level [7]. This study also demonstrated that ZA of cladocerans and copepods differed significantly. However, cladocerans or copepods are at order level consisting of diverse taxa, and the interaction between the host and microbes may differ substantially in a more subdivided level of taxonomy. Choosing specific populations of zooplankton is therefore needed to test the host dependence of ZA.

It has been demonstrated that microbes play an important role in maintaining normal development stages of zooplankton, e.g., survival, growth and reproduction, in studies about <i>D. magna</i> [15–17]. Survival rate of bacteria–free daphnids even reduced to 0 [18]. Recently, a few studies demonstrated that ZA also impacted biogeochemical cycling in aquatic environments. For laboratory–reared <i>Daphnia</i> and marine copepods, ZA played a part in dissolved organic carbon (DOC) transfer, phosphorus metabolism, and nitrogen fixation [19–22]. Notably, Gamma–proteobacteria was proved to have nitrate respiration ability, which provide novel microbial hot spots for pelagic denitrification [23]. Nevertheless, little is known about the potential benefits of ZA on the host and its biogeochemical contributions in natural freshwater lakes due to a more changing environment than culture and marine conditions. Thus, understanding the ZA microbiome will not only shed light on host–microbiota interaction, but also can help to widen our knowledge with the direct or indirect effects of zooplankton on the lake’s ecosystem functioning. Although sporadic studies have reported characteristics of ZA in various aspects, earlier studies were biased by bacterial count or culture methods. Additionally, phylogenetic information of ZA was mostly available for laboratory–cultivating <i>Daphnia</i> (a model genus of zooplankton) and marine copepods. However, for freshwater lakes, detailed analysis is still less for these attached bacteria, especially concerning specific host and natural environments.

In this study, we used 16S rRNA sequencing technology to clarify the taxonomic and functional information of ZA community. Specific copepod and cladoceran populations were chosen, i.e., <i>Moina</i> and Calanoids, since they densely distributed in nearly all freshwater habitats in the study area. The aim of these studies can be described as follows: (1) What is the bacterial community structure associated with common zooplankton populations in natural freshwater lakes? (2) Whether ZA differs from different hosts and how ZA responds to environmental factors? (3) Whether ZA shows similar metabolism potentials? The results from this study might give insight into the community characteristics of ZA from definite taxa of the host and their ecological implications to an aquatic environment.

2. Materials and Methods

2.1. Sampling Sites, Zooplankton and Water Samples Collection

Views of the five shallow lakes located in northeast region of China (Heilongjiang and Jilin province), including Lake Xiaoxingkai (XXK), Xihulu (XHL), Qianliujia (QLJ), Qingken (QK), and Chagan (CG) are shown in Figure 1 and detailed information of the samples are shown in Table S1. All field sampling work was conducted in July 2019; thus a similar thermal condition and high species diversity could be obtained.

For each sampling site, latitude, longitude, water depth (WD), and Secchi depth (SD) were recorded on board. Temperature (T), pH, salinity (Sal), dissolved oxygen (DO), redox potential (ORP), and water turbidity (NTU) were acquired through a water measuring instrument (YSI 6600, Yellow Springs, Ohio, USA). Mixed layers of water samples
were collected to do physicochemical and FL analysis. Meanwhile zooplankton were collected via multiple tows with a plankton net (64 μm mesh, 0.2 m mouth diameter), and immediately taken back to obtain ZA samples, avoiding great changes of microbiota.

![Figure 1. Geographical location and abbreviation of five freshwater lakes. Lakes shown are Xiaoheik (XXK), Qingken (QK), Xihu (XHL), Qianliujia (QLJ), and Chagan (CG).](image)

Sterilized ultrapure water, petri dishes, and dissecting needle were prepared to pick up ideal crustaceans. Under a microscope, *Moina* and Calanoids were gently picked and removed to respective petri dishes filled with sterilized water. Undamaged, actively swimming adult female crustaceans were picked out. Zooplankton was allowed to clear their guts for 1–2 h to eliminate any food–associated bacteria. After gut clearance, zooplankton was rinsed with sterilized water 3–4 times to remove loosely attached bacteria and then placed into 2 mL sterile centrifuge tubes with 13–15 similar individuals in each sample. By sampling from five lakes, a total of 10 samples, i.e., 130–150 individuals were therefore picked for each taxon of zooplankton. Meanwhile, for each site, 200–500 mL 5 μm filtered water sample (to remove particle–associated bacteria) was subsequently filtered through a 0.22 μm membrane (Millipore, Billerica, MA, USA) to obtain FL, and were then placed into 2 mL sterile centrifuge tubes. Finally, ZA and FL samples were preserved below −20 °C until further microbial analysis.

The concentrations of total nitrogen (TN) and total phosphorus (TP) were measured using unfiltered water samples by UV–Vis spectrophotometer [24]. The 0.2 μm filtered water samples were used to measure the concentrations of dissolved organic carbon (DOC), orthophosphate (PO43−), ammonium (NH4−N), nitrite (NO2−N), and nitrate (NO3−N) with standard methods [24]. The chlorophyll a (Chl a) was extracted with ethanol and then measured through spectrophotometer [25].

2.2. Bacterial DNA Extraction and High-Throughput Sequencing of 16S rRNA Gene Amplicons

The bacterial DNA extraction was performed using the ALFA–SEQ Advanced Water DNA Kit following the manufacturer’s instructions. The V4 hypervariable region of prokaryote 16S rRNA genes were amplified using the primers 515F (5′-GTGCGCCACMCGCCGCGTA–3′) and 806R (5′-GAGACTACHVGTTATCTAAT–3′). The polymerase chain reaction (PCR) mixtures contained 3 μL diluted DNA template, 10 μM of each primer, 25 μL of 2× Premix Taq, 50 ng DNA, and nuclease–free water was added to a final volume of 50 μL. The PCR mixtures were subsequently quantified, pooled and purified (detailed procedure is documented in “Materials and Methods” of Supplementary Materials). A database was then constructed with
standard operating procedure of “NEBNext Ultra II DNA Library Prep Kit for Illumina® (New England Biolabs, Ipswich, MA, USA) and sequenced on the Illumina Nova 6000 Platform (Guangdong Magigene Biotechnology Co., Ltd., Guangzhou, China).

Subsequently, paired-end raw reads were filtered using fastp software (version 0.14.1, https://github.com/OpenGene/fastp, 13 October 2020) and paired-end clean reads were obtained with cutadapt software (https://github.com/marcelm/cutadapt/, 16 October 2020). Using usearch–fastq_mergepairs (V10, http://www.drive5.com/usearch/, 20 October 2020), paired-end clean reads were spliced to obtain raw tags. Raw tags were filtered using fastp software to obtain clean tags. Subsequently, Operational Taxonomic Units (OTU) were clustered based on 97% similarity of the sequences with UPARSE software and denoised using qiime2 (version 2016.11.0) [26–28]. The sequences which own the highest abundance in each OTU were taken as the representative sequences for each OTU and were then assigned in the Silva database (version 132, https://www.arb-silva.de/, 30 October 2020) using usearch–sintax (version 10.0.240). The threshold was set as 0.8, and the OTUs were assigned to different hierarchical taxa. Chloroplast and mitochondria were removed using the de novo method through UCHIME, and effective OTU tables were finally obtained and standardized by PICRUSt software (version 1.1.2) [29]. The green gene ID of each OTU was then compared with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (05 January 2021, https://www.kegg.jp/) to obtain KO and pathway information about the potential function of the microbes. The abundance of each function was calculated based on the abundance of OTUs. The complete nucleotide sequence datasets were deposited in the Sequence Read Archive (SRA) under BioProject PRJNA725992.

2.3. Statistical Analysis

Non-parametric Kruskal–Wallis test was conducted in Statistical Package for the Social Sciences (SPSS) 24 software to test the differences in OTU diversity among Moina– and Calanoid–associated bacteria, and the significance was valid when \( p < 0.05 \). The average value was calculated for each lake and each group of zooplankton microbiome, therefore, consisted of five replicates. Analysis of similarities analysis (ANOSIM) was also used to evaluate significant differences in two ZA communities. When R value was closer to 1, two bacterial groups are totally different. Conversely, when two groups were similar, R value was closer to 0. Further, divergence in two bacterial communities were presented by principal co-ordinates analysis (PCoA) in R software (version 4.0.3).

Data standardization and hierarchical cluster analysis were conducted via vegan package (version 2.5–7) based on Bray–Curtis distance. The \( \alpha \)-diversity indexes, i.e., Chao1 index and Shannon diversity index, of each sample were calculated using usearch–alpha_div (V10, http://www.drive5.com/usearch/, 02 January 2021) at the same sequence depth. The Chao1 index reflects the number of species, and the Shannon index also shows the evenness, that is, the homogeneity of the species [30,31]. To test which environmental parameters explained the variance of ZA, the environment parameters were fitted onto an ordination plot using Redundancy Analysis (RDA) with Bray–Curtis distance matrix generated from the OTUs. The relationships between the ZA diversity and the environmental factors were examined with Pearson correlation analysis, and then analyzed by vegan package in R software. Linear discriminant analysis Effect Size (LEfSe) was performed to define indicator species, namely significant different OTUs between two ZA groups.

Co–occurrence network analysis was conducted in R based on Spearman’s correlation, and correlation was assigned to 0 when \( p > 0.05 \) or \( R < 0.6 \). Visualization of bacterial community structure, \( \alpha \)-diversity indexes, co–occurrence network, etc., was achieved by pheatmap, ggplot2 packages in R 4.0.3, Gephi 0.9.2, and Origin 2018 software.
3. Results

3.1. Community Structure of Zooplankton–Associated Bacteria

A total of 1,470,676 bacterial sequences were generated from 20 ZA samples, with an average of 73,534 in each sample. Good’s coverage estimators indicated that the sizes of libraries were sufficient to cover more than 99% of the bacterial communities.

From the phylogenetic analysis, 41 bacterial phyla were detected in the 20 libraries. Proteobacteria, Actinobacteria, and Firmicutes were dominant phyla, representing 52%, 21%, and 18% of total bacterial population. For Moina ZA, those were 46%, 30%, and 22%, respectively. For Calanoid ZA, those were 58%, 12%, and 14%, respectively. ZA varied a lot among different Moina samples, whereas it showed a more similar composition in Calanoids (Figure 2).

Most sequences were assigned to 2580 OTUs at 97% identity. At genus or OTU level, the bacterial community structures of two ZA groups owned significant difference ($p = 0.034$). PCoA also demonstrated the difference of two zooplankton microbiome, with the first axis showing 30.1% of variation and the second axis of 22.2% (Figure S1). On the whole, the most abundant bacterial genus was assigned to Pseudomonadaceae (accounted for 16% of total OTU sequences). For Moina ZA, those were Brevibacterium (20%), Pseudomonadaceae (18%), and Acinetobacter (10%). Calanoid ZA mainly consisted of Pseudomonadaceae (12%), Exiguobacterium (11%), and Acinetobacter (9%) (Table S2). A heatmap also shows the relative changes in the abundance of bacterial genera among the samples (Figure S2). Of all OTUs, 851 OTUs were shared by 2 microbiomes, and accounted for 33% of the involved sequences (Figure S3).

![Figure 2](https://example.com/image.png)

**Figure 2.** Relative abundance of top 6 classes in microbes associated with Moina and Calanoids in five freshwater lakes. Samples are listed as XXK, QK, XHL, QLJ, and CG. FL, free-living bacteria; ZA, zooplankton–associated bacteria.

3.2. Significantly Different OTUs between Two Zooplankton–Associated Microbial Groups

A total of 83 significantly different OTUs, i.e., indicator OTUs, among two ZA groups were identified by LEfSe analysis, and low abundance (< 0.1%) ones were neglected. In class level, Alpha–proteobacteria was the dominant indicator group (Figure 3), with an average abundance of 12.6% in Calanoid ZA, and only 1.8% for Moina. Within Alpha–proteobacteria, Rhizobiales, Rhodobacterales, Rickettsiales, and Sphingomonadales were main indicator orders. The second dominance was Gamma–proteobacteria, mainly including Enhydrobacter, and Pseudomonas genera. In addition, Planctomycetia, Sphingobacteriaceae, and Thermomicrobia were significantly different between two ZA groups.
Figure 3. Significantly different OTUs between microbial communities associated with Moina and Calanoids in five freshwater lakes. Mean proportion of each OTU and the highest taxonomy level was listed in the left panel where “o”, “f”, and “g” respectively represent order, family, and genus. Significance (p-value < 0.05) is shown in the right panel.

3.3. Bacterial Richness and Diversity of Zooplankton Microbiome

The richness of Moina and Calanoid ZA were similar (470 and 380 of Chao1 index respectively, p = 0.53, n = 5) and Shannon diversity (3.09 and 1.91, respectively) were significantly higher in Calanoid ZA microbiome compared with that of Moina (Figure 4, p = 0.03, n = 5). Additionally, both indexes were significantly higher (726 of Chao1 index and 3.31 of Shannon index) in FL community (p < 0.05, n = 5).

Figure 4. Species richness and Shannon diversity index between microbial communities associated with Moina and Calanoids in five freshwater lakes.
3.4. Relationship between Environmental Parameters and Zooplankton Microbiome Composition

The relationship between the OTUs and the three parameters (DOC, TN, and pH) in the RDA plot is shown in Figure 5. The first axis of the RDA for Moina explained 33.30% of the variation in OTUs and was positively correlated with the pH and negatively correlated with DOC and TN of ambient water. The second axis was negatively correlated with DOC, TN, and pH. These three geochemical parameters explained 53.68% of the total variance. As for Calanoids, the first axis of the RDA explained 46.37% of the variation in OTUs, and was positively correlated with the DOC and TN. The second axis explained 7.00% and was negatively correlated with TN, positively correlated with DOC. These two geochemical parameters explained 53.37% of the total variance. Within these parameters, DOC and TN were identified as the most important environmental factors for shaping the microbial community structures of two microbiomes.

![Figure 5](image)

**Figure 5.** Relationship between microbial communities and environmental factors respectively associated with Moina (a) and Calanoids (b). Each symbol shows the sum of 13–15 individuals from each sample in each lake. Each significant factor ($p < 0.05$) was shown in the plot with red arrows. TN, total nitrogen; DOC, dissolved organic carbon.

3.5. Co–Occurrence Network of Zooplankton–Associated Bacteria

To discern the microbial co–occurrence patterns of the zooplankton microbiome, a network interaction was constructed based on Spearman’s correlations between OTUs (Figure 6). Network indices were calculated in the FL, Moina, and Calanoid microbiome. The resulting microbial networks consisted of 132 nodes, 4501 edges for Moina, and 222 nodes, 9588 edges for Calanoids. Network indices were also calculated for two microbiomes (Table S3). Zooplankton microbiome species, regardless of the phylum level, exhibited aggregation distributions. Positive correlations accounted for more than 90% of the total correlations in both networks.

![Figure 6](image)

**Figure 6.** Co–occurrence network among different bacterial phyla associated with Calanoids (a) and Moina (b) are respectively shown. Different phyla are marked with different colors. Connections are shown by lines.
The topological properties suggested that the co–occurrence network of Calanoid microbiome was more compact than that of Moina, as well as higher connectivity, shorter path length, and higher clustering efficiency, which are key network properties in terms of system efficiency and robustness. Average clustering coefficients were 0.831 and 0.736, respectively. Average path lengths were 1.754 and 1.82, respectively. For Calanoid microbiome, the numbers of average degree, total nodes and edges were higher than that of Moina. Average degree was usually used to reflect the complexity of networks, and the higher value indicated the lower network complexity. For each network, correlations between phyla showed different characteristics. In Calanoid microbiome network, it showed strong positive correlations between different phyla. As for Moina microbiome, strong positive correlations mainly existed in the same phylum. In addition, both ZA networks showed significantly higher connectivity than that of FL (Table S3).

3.6. Biogeochemical Relevant Microbial Groups

KEGG functional analysis showed that a total of 6439 potential bacterial functions was found in all zooplankton microbiome samples. Among those, RNA polymerase (0.41%), iron complex outer membrane receptor protein (0.40%), enoyl–CoA hydratase (0.40%), and methyl–accepting chemotaxis protein (0.32%) consist of the most abundant functions (Table S5).

For a coarser level, several KO were identified as nitrogen, carbon, and fatty acid cycling function (Figure S4). Among them, fatty acid metabolism was the most abundant function, which accounted for 2.77% in all samples. Nitrogen metabolism functions showed 0.89% potential. Especially for nitrogen reduction, the function abundance of Calanoid ZA was 23% higher than that of Moina (p = 0.018, n = 5). In addition, carbon metabolism accounted for 1.46% and relevant KO abundance of Calanoid ZA was 16% higher than that of Moina (p = 0.022, n = 5).

Among indicator OTUs, several taxa were found to exhibit nitrogen fixation and nitrate reduction function, e.g., Rhizobiales and Pseudomonas families belonging to Alpha- and Gamma–proteobacteria, respectively (Figure 7a and 7c) [32,33]. Methane can be used as the only carbon and energy source for some species of Methylobacteriaceae, consequently involved in methane metabolism (Figure 7b). Facultative anaerobes were also marked in ZA, including but not limited to Enhydrobacter, Rhodobacterales, Thermomicrobia, and Rhodocyclus (Figure 7d). In particular, Enhydrobacter genus was previously extracted from anoxic region of eutrophic lakes. It is noteworthy that those anaerobes were 5–fold higher in Calanoid ZA (p = 0.009, n = 5). What is more, Brucellaceae and Rickettsiales were obligate parasitism to eukaryotic cells. Especially, Epulopiscium, Pasteurellales, and Cenarchaeales were previously extracted from animal intestines. Acidophilic Acetobacteraceae was observed in higher abundance in Calanoid ZA. Sphingomonadales, which are capable to degrade aromatic compounds, was also observed.
Figure 7. Reads of Pseudomonas (nitrogen reduction) and Methylobacteriaceae (carbon metabolism) associated with Moina and Calanoids from five freshwater lakes are respectively shown in (a,b). The dotted line to the right means higher read counts. Nitrogen fixing and facultative anaerobes are shown in (c,d). Lakes are listed as XXK, QK, XHL, QIJ, and CG.

4. Discussion

4.1. Community Structures of Zooplankton–Associated Bacteria in Freshwater Lakes

Zooplankton in freshwater lakes was colonized by comparatively few OTUs of bacteria. In this study, each ZA sample contained about 300 OTUs and α–diversity was lower than that of FL (Figure 4), but bacterial density was maybe 100–1000–fold higher than that of FL [13]. Besides, ZA shared similar groups with ambient water column, but in a different relative abundance (Figure 2), implying that specific bacterial groups have a disposition to colonize on zooplankton [34]. In this study, Proteobacteria, Actinobacteria, and Firmicutes were dominant phyla in two ZA groups. A similar result was also obtained for cladoceran Bosmina coregoni and copepod Thermocyclops oithonoides in German freshwater lakes [14]. Other studies also illustrated the dominant presence of these phyla, whereas their relative abundance differed. For instance, Proteobacteria and Bacteroidetes were the most dominant phyla in ZA from ocean and West Polish lakes [7]. By contrast, Firmicutes, Bacteroidetes, and Actinobacteria are dominant persistent members of ZA in copepod guts from the North Atlantic Ocean [35]. Differences may result from different hosts and physicochemical properties of their habitats. As for host factors, zooplankton was only classified into order level in studies above, which means several families or genus may co–exist within a sample. A factor of divergent life histories may not be excluded, also leading to differences [15]. In addition, gut clearance was not conducted in studies above, which means food–associated, i.e., possibly transient bacteria were also included for anal-
ysis and food concentrations, and types would significantly affect zooplankton–associated bacteria abundance [16,36,37]. Considering environmental factors, temperature (temperate and sub–arctic zones), and trophic levels were found to significantly impact ZA [14,38,39].

The bacterial community associated with the same zooplankton population (Moina or Calanoids) also exhibits differences which may result from different characteristics of their ambient environment (Figure 2 and Table S1). Previous studies have demonstrated that the assembly of the ZA shows a strong environmental effect, suggesting that environmental variables also play an important role in shaping the zooplankton microbiome [40].

4.2. Zooplankton–Associated Bacteria Exhibited Different Features Depending on the Host Traits

Although sampled from the same site, and analyzed with a common procedure, microbiota associated with different zooplankton populations displayed significant differences. Proteobacteria, especially Alpha–proteobacteria was found higher in Calanoid (copepods) ZA. It underlined the shaping role of host to ZA microbiome. It has been demonstrated that copepod– and cladoceran–associated microbes differ substantially, in which Proteobacteria proportion was found higher in copepods ZA than that of Cladocerans. In case of FL, the relative abundance of Alpha–proteobacteria was observed higher in phytoplankton–dominant (higher nutrient loading) area than that of macrophyte–dominant area [41,42]. Calanoids have a shorter gut passage time and a higher excretion rate than those of Moina, which tend to provide more nutrients and DOC to bacteria, leading to a higher proportion of Alpha–Proteobacteria [43,44].

Alpha diversity (both of richness and Shannon diversity) of Calanoid ZA microbiome was higher than that of Moina. This may have arisen from their distinct feeding strategies. Calanoids conduct a more selective feeding mode [43]. When food particles are available, movements of the Calanoid mouthparts can capture the food and bring it to the mouth. Meanwhile, particles which are in poor quality are not captured, or voluntarily excluded after capture by their mouthparts [44]. In addition, Calanoids have a shorter gut passage time and therefore, a high excretion rate. For example, the mean release of nitrogenous products by well–fed Daphnia was estimated to be 0.76 μg/L/h, while the release of ammonia by copepods reached 1.44 μg/L/h [10,45]. Although zooplankton has a small size, their shaping effect to ambient bacteria has been shown to be significant, and thus, different gut passage time or excretion rate would exert an effect on microbes colonized on them [46]. However, Moina applies a sloppy feeding pattern, that is, they only passively obtain food through filtering all particles with certain size, no matter whether edible or not [47]. Through voluntarily hunted high–quality food, Calanoids can feed on concentrated organic matter and provide more nutrients, and Calanoid–associated bacteria could exhibit higher richness and diversity than those of Moina. Moreover, Calanoids have a larger body size and stronger swimming ability than Moina, implying bacteria may play a more important role in providing energy [48]. Beta–proteobacteria Limnohabitans was proved to share a symbiotic relationship with zooplankton, which is beneficial for high fecundity of zooplankton [49]. In this study, KEGG function analysis suggested that bacterial members of ZA were capable to degrade fatty acid (obtain short chain fatty acid) and synthetize polyunsaturated fatty acids (PUFA). FA and PUFA were important elements of zooplankton, and generally, short chains of FA are more easily utilized by zooplankton [50]. Additionally, it is necessary for zooplankton to obtain PUFA from external sources since zooplankton cannot directly synthetize them [51]. It indicated that Calanoid–associated abundant bacteria may concurrently offer nutrients to zooplankton, suggesting more bacterial members may share symbiotic relationships with zooplankton. A metagenome analysis for marine mesozooplankton also demonstrated that these associated bacteria are able to degrade high molecular weight organic matters [52].
Network analysis showed that various phyla of Calanoid ZA were more closely connected and contained more positive interactions than that of *Moina*. The topological features of two networks were also entirely different: Calanoid ZA network was more agglomerative whereas *Moina* network was more discrete. As mentioned above, $\alpha$–diversity of Calanoid microbiota was higher than that of *Moina*. It implied that ZA microbiome with high diversity often shows more interactions. An earlier study demonstrated a positive relationship between biodiversity and the functioning of ecosystems [53]. Diverse communities are predicted to be more productive than less diverse one, which means these co-existing species may positively interact to increase community performance [54,55]. What is more, competition exclusion is acknowledged to be more influential in community assembly of small spatial scales [56]. When community structure was simple (*Moina ZA*), species belonging to the same phylum will have similar competitive ability and thus are more likely to assemble, resulting in relatively discrete clustering in different phyla.

Environmental parameters that significantly correlated to bacterial composition were identified in this study. Calanoid microbiome structure was significantly correlated to DOC and TN. For *Moina*, besides those, pH was also included. Similar conclusion was also shown in an earlier study, which suggested that the cell number of *Balanus*–associated bacteria could be explained by ammonium and phosphate through multiple regression analyses [11]. Studies on freshwater zooplankton–associated bacteria have found that cladocera microbiome was more stable with change of trophic level, whereas copepod microbiome was less consistent before and after the change of environment [14]. For cladoceran Bosmina, its associated bacteria can keep $>90\%$ similarity in different treatment, while for copepod cyclopoid, only 30% was kept.

### 4.3. Potential Ecological Effects of Zooplankton–Associated Bacteria

Bacteria attached onto abiotic aggregates and phytoplankton has been increasingly investigated in freshwater lake ecosystems and proved to exert profound effects on nutrient cycling. However, the significance of bacterial attachment to zooplankton has been largely neglected, although zooplankton often grows with a great density (in case of Lake Taihu, an average of 580 individuals can be observed per liter) [57]. What is more, zooplankton–associated bacteria (ZA) were found more abundant and active, which may exhibit potentials in nutrient cycling [12,13].

In fact, zooplankton–associated bacteria may exert an effect on biogeochemistry in lakes, since nitrogen, carbon, fatty acid, etc., metabolism function potentials were found in zooplankton microbiome and account for a predominant proportion. Bacterial groups owning specific metabolism function were also identified within 83 indicator OTUs. Among them, Methylobacteriaceae is a bacterial family using methanol and methane as the unique carbon and energy source, and even reached 11% relative abundance within Calanoid ZA samples (average of 4%). Moreover, zooplankton–associated bacteria were found more active in carbon metabolism, indicating a potential effect of these bacteria on the carbon cycling in lakes [19].

It has also been suggested that nitrogen–fixing anaerobes was found associated with marine copepods [58], and Gamma–proteobacteria was proved to exhibit nitrate respiratory ability in the North Arctic Ocean [22]. In this study, nitrogen fixation and nitrate reduction bacteria were also found throughout all ZA samples, such as Rhizobiales and Pseudomonas, suggesting a potential role in the carbon cycles of freshwater lakes.

Bacteria attached onto different hosts presents different functions. For instance, anaerobic environment within copepod guts and fecal pellets may favor anaerobic bacteria growth and produce different reactions compared with aerobic cladoceran guts. In this study, facultative anaerobes were found 5–fold higher in Calanoid ZA than that of *Moina*. Moreover, with higher diversity and stronger bacterial interactions, Calanoid ZA shows higher metabolism ability referring to fatty acid, carbon and nitrogen cycles, indicating
that host-dependent bacterial community structure also leads to different metabolism ability.

5. Conclusions

Our study illustrated different features of microbes colonized on two common crustaceans, i.e., cladoceran *Moina* and copepod Calanoids in freshwater lakes. We found Calanoids-associated bacteria exhibit higher diversity, stronger interaction, and metabolism function potentials than that of *Moina*. We further interpreted these discrepancies combined with contrast physiological traits of the hosts. Aerobic or anaerobic micro-environments in guts, selective or filtering feeding strategies, different gut passage time, and swimming ability determine the habitat and nutrients supplement to microbes, and further impact their community structure and function. Therefore, we emphasized different physiological mechanisms may mediate the shaping role of host to microbes. It is also worth noting that zooplankton-associated bacteria presented a high potential of fatty acid metabolism, which is beneficial for host’s development, implying more microbes may share a symbiotic relationship with zooplankton. Moreover, several bacterial members participating in carbon and nitrogen cycles were identified in this study, such as Bradyrhizobiaceae (nitrogen-fixing), Enterobacteriaceae (nitrate reduction), and Methylobacteriaceae (carbon metabolism).

**Supplementary Materials:** The following are available online at www.mdpi.com/xxx/s1, Materials and Methods, Figure S1: Divergence in two bacterial communities were presented by principal co-ordinates analysis (PCoA), Figure S2: Heat map of top 30 OTUs among microbes associated with *Moina* and Calanoids in five freshwater lakes, Figure S3: Shared OTUs between microbial groups associated with *Moina* and Calanoids in five freshwater lakes, Figure S4: Nitrogen, methane, and fatty metabolism gene counts across all samples associated with *Moina* and Calanoids from five freshwater lakes, Table S1: Description of bacterial and environmental samples, FL, free-living bacteria, ZA, zooplankton-associated bacteria, Lake Xiaoxingkai (XXK), Xihulu (XHL), Qianliujia (QLJ), Qingken (QK), and Chagan (CG), Table S2: Taxonomic information of the top 10 OTUs in the *Moina*-, Calanoid-associated microbes, and FL, Table S3: Co-occurrence network attributes of *Moina* - and Calanoid-associated microbes, Table S4: Species information of zooplankton in five freshwater lakes, Table S5: Top 4 potential bacterial functions of zooplankton-associated bacteria.

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**Data Availability Statement:** The complete nucleotide 40 sequence datasets that support the findings of this study have been deposited in the Sequence Read Archive (SRA) under BioProject PRJNA725992.

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