Fly-over phylogeny across invertebrate to vertebrate: The giant panda and insects share a highly similar gut microbiota

Ran Yao, Qinlong Dai, Tonggui Wu, Zhisong Yang, Hua Chen, Guoqi Liu, Yudong Zhu, Dunwu Qi, Xu Yang, Wei Luo, Xiaodong Gu, Xuyu Yang, Lifeng Zhu

College of Life Sciences, Nanjing Normal University, Nanjing, China
Sichuan Liziping National Nature Reserve, Shimian, China
Shimian Research Center of Giant Panda Small Population Conservation and Rejuvenation, Shimian, China
East China Coastal Forest Ecosystem Long-term Research Station, Research Institute of Subtropical Forestry, Chinese Academy of Forestry, Hangzhou Zhejiang, China
Sichuan Academy of Giant Panda, Chengdu, China
Mingke Biotechnology Co., Ltd., Hangzhou, China
Chengdu Giant Panda Breeding Center, Chengdu, China
Chengdu Xinagai Information Technology Co., Ltd., Chengdu, China
Sichuan Station of Wildlife Survey and Management, Chengdu, China

ABSTRACT

Many studies highlight that host phylogeny and diet are the two main factors influencing the animal gut microbiota. However, the internal mechanisms driving the evolution of animal gut microbiota may be more complex and complicated than we previously realized. Here, based on a large-scale meta-analysis of animal gut microbiota (16s RNA gene data from approximately 1,800 samples; 108 metagenomes) across a wide taxonomic range of hosts, from invertebrate to vertebrate, we found high similarity in the gut microbial community (high proportion of Gammaproteobacteria (Pseudomonas)) of invertebrate insects and vertebrate bamboo-eating pandas (giant panda and red panda), which might be associated with their plant-eating behavior and the presence of oxygen in the intestinal tract. A Pseudomonas strain-level analysis using 108 metagenomes further revealed that the response to either host niches or selection by the host might further lead to host-specific strains (or sub-strains) among the different hosts congruent with their evolutionary history. In this study, we uncovered new insights into the current understanding of the evolution of animals and their gut microbiota.

1. Introduction

Numerous studies have shown that host phylogeny and diet are the two main factors influencing the animal gut microbiota[1–3]. Several of these studies have also found some convergence in gut microbial community and function across different animals but within the same animal phylum[4,5]. For example, in meat-eating mammals, the gut microbiota is enriched in genes coding for enzymes involved in protein utilization[4]. Myrmecophagous species (anteaters, aardvarks, and aardwolves) display a convergence in the gut microbial community, even though they belong to phylogenetically distant lineages representing different mammalian orders[5]. These studies emphasized that both diet and phylogeny drive the evolution of the mammalian gut microbiota, with cases of convergence in composition and function. Still, there are also examples of phylogenetic inertia. A study on wild primate gut microbiota showed that the effects of host phylogeny on both gut microbial composition and function is much stronger than that of host diet[6]. A separate study found that bats harbor bird-like gut microbiota, indicating that the evolution of animal gut microbiota could potentially be associated with physiological adaptations to flight[7]. Therefore, the internal mechanisms driving the evolution of animal gut microbiota may be more complicated than previously realized. A large-scale meta-analysis of animal gut microbiota across host phylogeny, from invertebrate to vertebrate, might uncover new insights into our understanding of the evolution of animals and their gut microbiota.

The giant panda and red panda are typical herbivorous carnivores, and their primary diets are bamboos[8]. Many studies have shown the evolutionary adaptations of the giant panda to a
bamboo diet, including the evidence from morphology, physiology, and the symbiotic gut microbiota[9–11], with the gut microbiota studies having focused on the composition and functions involved in cellulose and hemicellulose digestion[11–13]. Specialized bamboo-eating animals include not just pandas but also many insect species[14]. Many insect gut microbiotas have evolved and adapted to a herbivorous diet[15,16]. Therefore, we hypothesized that invertebrate insects and vertebrate bamboo-eating pandas might harbor gut microbial communities that are highly similar in composition and function.

The vast amount of gut microbiota data across different vertebrate phyla that have been published over the past decade, including the studies by our group on the gut microbial composition and function of the bamboo-eating pandas[11–13], provided us an opportunity to investigate this hypothesis. To gain a large data set from insects, we collected samples from different habitats (e.g., bamboos, reeds, and shrubs). Therefore, we tested this hypothesis based on the meta-analysis of the gut microbial composition and function across host phylogeny from invertebrates to vertebrates.

2. Materials and methods

2.1. Sample collection

We collected 270 insect samples from bamboo, shrub, and forest habitats in Jiangsu, Anhui, Zhejiang, Guangdong, Fujian, and Sichuan provinces (Table S1). The insect samples belonged to 14 families and two mixed groups (Table S1). All instruments and materials were sterilized prior to sampling. The insects were frozen after sampling and then shipped to the lab on dry ice. Each insect was dissected to collect the gut contents in 2-ml aseptic centrifuge tubes. Due to the lack of enough gastrointestinal content in a single insect, the gut contents from five individuals were pooled as one insect sample for DNA extraction. The majority of the 270 insect samples were pooled samples, with the exception of the four samples from Cerambycidae. The insect samples collected from the bamboo habitats were treated as insectBam group. The remaining insect samples, collected from the non-bamboo habitats, were treated as “Insect” group in this study. We also collected 73 samples from the main dietary plants (bamboos and other plants) of these insects (Table S1). Although there are many published metagenomes from wild giant pandas, we also collected three fresh feces from the wild giant pandas at the LiziPing National Nature Reserve in Xiaoxiangling Mountains for deep-metagenomic analysis. All plant samples and fresh feces were frozen upon collection and then shipped on dry ice to the laboratory for analysis.

3. DNA extraction and metagenomic sequencing

The Fast DNA Spin Kit for feces (MP, Ohio, USA) was used to extract microbial DNA from the gut contents and fecal samples once they were thawed at room temperature. The Fast DNA Spin Kit for Soil (MP, Ohio, USA) was used to extract microbial DNA from the plant samples. We used the primers [515F: 5’-GTGCTACGGGTTATCCTAAT-3’; 806: 5’-GACTACHVGGGTWTCTAAT-3’] to amplify the V4 region of the bacterial 16S ribosomal RNA gene [51]. The polymerase chain reaction thermocycling conditions were: 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s, with a final extension step at 72 °C for 10 min. High-throughput sequencing of amplicons was performed using the Illumina MiSeq platform (Paired-end and about 250 bp for read length (PE250), and about 30,000 reads per sample). Sequencing was performed by Mingke Biotechnology Co., Ltd. (Hangzhou, China).

Twelve samples (seven from the insect samples, two from the bamboo samples, and three from the giant panda fecal samples) was used for deep metagenomic sequencing. Metagenomic shotgun sequencing libraries were prepared and sequenced at Mingke Biotechnology Co., Ltd. For each sample, 1 μg of genomic DNA was used with Illumina’s TruSeq for library preparation. Libraries were sequenced using the Illumina HiSeq 4000 (Paired-end and about 150 bp for read length (PE150), and about 3,500,000 reads per sample).

4. 16S rRNA gene-based sequence analysis

We combined our 16S rRNA gene Miseq data (343 samples) in this study with the published 16S rRNA gene Miseq data (1,474 samples) for the Meta-analysis. In order to decrease the bias caused by the difference in the DNA extraction kit from different studies, most of the published data came from our labs using the same 16S rRNA gene region. The data used in the meta-analysis represented the typical phyla of the vertebrates (from fishes to mammals). Details of the published data are shown in Table S2, including our previous data (in wild giant pandas, (Gpwild)[12], wild red pandas (Rpwd)[12], Père David’s deere (Mulu)[17], wild frogs (Frog)[18], bighheaded cars (Fish)[19], and soil [20]) and the published data from other research groups (wild meat-eating carnivorans (CAR or CA)[21], wild birds (Bird)[22], wild primates (Primate)[6], wild lizards (Lizard)[23], captive black bears (Bbear) [24], and soils[23]).

In preparation of the raw 16S rRNA gene data (this study and previously published), the search program was used to check for chimeras and to remove low-quality sequences, the flash program was used to merge matching paired-end reads[25], and the trimmomatic program was used for quality control[26]. Some of the published data used the different 16S rRNA gene regions. Thus, we used pick-up-closed-OTU methods (using closed-reference OTU clustering) to define operational taxonomic units (OTU) were defined as sharing > 97% sequence identity by searching clean sequences against the SILVA132 database[27]. A taxon summary was created using the abundance of the OTUs table in QIIME 1.9 [28]. We then calculated the pairwise unweighted unifrac and weighted unifrac distance among these samples in QIIME 1.9 [28]. The unweighted unifrac and weighted unifrac distances were used to generate NDMS (nonmetric multidimensional scaling) in the Vegan package[29] in R software, respectively. We also created a hierarchical clustering tree for these samples using unweighted unifrac distance by FastTree[30]. We used the PERMANOVA significance test (using unweighted Unifrac distance) for group-level differences in the microbial composition and to evaluate the stability of the clustering patterns in this study. Finally, we detected the significant differences in the abundance of the microbiotas among groups using the taxonomic summary table by the Kruskal-Wallis test (the significant level at 0.05). The result after testing were visualized by Cytoscape[31].

5. Strain level analysis (metagenome-assembled genomes, MAGs) based on the metagenomes

We combined the 12 metagenomes of this study with the 96 published metagenomes for the Meta-analysis (Table S3). Here, the aim was to evaluate the similarity in the strain level analysis (MAGs) in the gut microbiome between the insects and bamboo-eating pandas. Also, we selected other mammals as the control. Most of the metagenome data came from our lab. The detailed previously published data included our previous data (XXL (16 wild giant panda gut metagenomes from the Xiaoxiangling Mountains)[32], Qinling (nine wild giant panda gut metagenomes from...
the Qinling Mountains) [33], R. pxi (six wild red panda gut metagenomes from the Xiaoxiangling Mountains) [32], CA (19 meat-eating carnivoran gut metagenomes) [34], OC (10 omnivorous carnivoran gut metagenomes) [34], and HE (12 herbivore gut metagenomes) [34] and the published data from other research groups (Yaan (10 captive giant panda gut metagenomes from the Yaan Research Center) [35], Chengdu (seven captive giant panda gut metagenomes from the Chengdu Breeding Center) [13], and Qionglai (seven wild giant panda gut metagenomes from the Qionglai Mountains) [35]).

The raw reads of these 108 metagenomes were trimmed using Trimmomatic [26] to remove (1) all read <50 bp in length, (2) reads with degenerate bases (N's), and (3) all duplicates defined as sequences whose initial 20 nucleotides were identical and shared an overall identity of ≥ 97% throughout the length of the shortest read. We used MegaPhy [36] to assemble the clean reads, and Salmon was used for quality control of the contigs and to remove contigs with coverage below 60% [37].

Six of the 12 deep metagenomes (three from the wild giant pandas, two from bamboo, and one from an insect) had enough coverage for binning analysis. We used BWA [38] and Samtools [39] to map the clean reads to the contigs per metagenome. MetaBAT2 was used to obtain the contigs for each bin based on the mapping result per metagenome [40]. CheckM was used to make the quality control for each bin, and only the high-quality bins (coverage > 80%, contamination rate < 10%) were kept [41]. Then, we used BWA [38] to map the clean reads to each high-quality bin per metagenome, and we gained the clean reads for each bin per metagenome. We used Spades to assemble the clean read of each bin in each metagenome. CheckM was used as a second round of quality control for each bin, and only the high-quality bins (coverage > 80%, contamination rate < 10%) were retained [41]. This resulted in the creation of high-quality bins (putative strains) for these six deep metagenomes.

PhyloPhAIA [42] was used to construct the phylogenetic tree for these bins. We used Salmon [43] to map the clean reads to these bins and determine the TPM (transcripts per million) abundance of these bins in all 108 metagenomes. Finally, the bins were searched against the KEGG database by using diamond [44]. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways associated with each sequence were determined for these bins (strains), and each bin was annotated against the KEGG pathways.

In addition, we downloaded 58 complete Pseudomonas genomes (Table S4) (representing the different species or strains) for the meta-analysis combined with our putative Pseudomonas bins. Thus, we could gain the phylogenetic information for the metagenome-assembled genomes (MAGs) of Pseudomonas in this study. PhyloPhAIA [42] was used to construct the phylogenetic tree for the putative Pseudomonas bins. We used Salmon [43] to map the clean reads to these Pseudomonas genomes and MAGs and determine the TPM abundance of these bins in all 108 metagenomes.

6. Results and discussion

6.1. Insects and bamboo-eating pandas harboring highly similar gut microbiota based on 16S rRNA amplicon sequencing

Both the hierarchical clustering and NMDS, using either unweighted or weighted Unifrac distances, showed the high similarity of the gut microbial community among the samples from bamboo-eating pandas and insects (Figs. 1-2, Figure S1-S2). The PERMANOVA test (using unweighted Unifrac distance) showed a significant difference (p < 0.05) in the microbial composition among these groups, which indicated the stability of the clustering patterns in this study. For example, the majority of samples from insects (including bamboo-eating insects (InsectBam)), bamboo (bamboo and other Gramineae plants), and bamboo-eating pandas (Gpwild and Rpwild) were clustered together within the same clade (Figs. 1-2). Pairwise comparison using unweighted unifrac distance matrix between the samples from the giant pandas (Gpwild) with other groups showed further that the relatively large distance between them and the Milu, Primates, meat-eating carnivorans (CAR), lizards, and soils, and the relatively small distance to red pandas (Rpwild), non-bamboo eating insects, and insects that eat bamboo (InsectBam) (Figure S3). These findings indicated that phylogenetically distant hosts (invertebrate insects and vertebrate bamboo-eating pandas) had similar gut microbial compositions. Previous studies have shown convergence of animal gut microbiota within the same host Class [4–7]. Here, the large-scale meta-analysis shows a putative convergence pattern across a much broader host phylogeny, from invertebrate to vertebrate, bringing new insights into the current understanding of the evolution of animals and their gut microbiota.

Moreover, compared with the environmental samples, the giant panda gut microbiota had relatively higher similarity to the microbiota of bamboo and other plants as compared to those of soils (Figure S3-S6). Most bamboo samples and some plant samples were clustered with the insect samples (including InsectBam). While some plant species are the main diet of Milu, both the hierarchical clustering and NMDS analyses displayed a high dissimilarity in the gut microbial composition between Milu and plants (Figs. 1-2, Figure S1-S2). These findings indicated that plant-eating insects (including InsectBam) and the bamboo-eating pandas might harbor similar gut microbial groups derived from their dietary plants, which might be caused by the special physiological characteristics of the host. Our previous studies speculated that the brief digestion time, short digestive tract, and fast intestinal peristalsis might lead to high concentrations of oxygen that select for the growth of aerobes and facultative anaerobes (e.g., Pseudomonadaeceae from Proteobacteria) in giant pandas [12,45]. Therefore, we deduced that the relatively simple digestive system of herbivorous insects (e.g., Orthoptera and Lepidoptera) might lead to the presence of low levels of oxygen in their digestive systems [46,47], which could result in the survival of or colonization by organisms from the microbiota of their herbivorous diet (including bamboo).

7. Facultative anaerobes (Pseudomonas) might partially contribute to this similarity between the insect and panda gut microbiota based on 16S rRNA amplicon sequencing

We then investigated specific microbiota to further confirm our hypothesis that the proportion of some facultative anaerobes (e.g., Gammaproteobacteria) were high in the samples of the bamboo-eating pandas, insects (including insectBam), birds, bamboo, and other plants (Figs. 2-3). Some bacteria from Enterobacteriaceae were enriched in the insects, bamboo, frogs, and birds (Fig. 3). Additionally, some members of the Pseudomonadales were enriched in the bamboo-eating pandas and insects (Figs. 2-3). Many of the samples from bamboo-eating pandas, bamboo, and insects had high proportions of Pseudomonas (over 40 percent in many samples) (Figure S7). Moreover, the meta-analysis revealed that the gut microbiota of some animal groups harbored specialized bacteria. For example, some bacteria from Ruminococcaceae and Lachnospiraceae (phylum Firmicutes) were enriched in the primate gut microbiota, while members of the Ruminococcaceae and Bacteroidales were enriched in the Milu gut microbiota. Fusobacteria were enriched in the meat-eating carnivoran gut microbiota, which was in agreement with our previous research [34]. Alphaproteobacteria were found to be enriched in the soil samples (Fig. 3). Therefore, we speculated that the high similarity in the microbiota...
among the insects, pandas, and plants (especially bamboos) might be partially associated with the high proportion of Gammaproteobacteria (e.g., *Pseudomonas*). *Pseudomonas* is one of the main symbiotic microbial groups among plants (including bamboos) [48]. The bamboo-eating behavior of the insects and pandas and the existence of the low level of oxygen in their digestive systems might facilitate colonization by *Pseudomonas*.

8. The comparative genomic analysis of the *Pseudomonas* genomes and MAGs revealing host-specific features based on metagenome shotgun sequencing

We obtained 51 MAGs (bins, strain level) in 6 deep metagenomes from three giant pandas, one insect, and two bamboos (Fig. 4). The phylogenetic analysis found that 23 strains belonged to the Proteobacteria, 13 strains to Bacteroidetes, seven strains to Firmicutes, five strains to Actinobacteria, and three strains to Verrucomicrobia (Fig. 4). We investigated the distribution of these 51 strains in the 108 metagenomes (12 new metagenomes from this study, 72 metagenomes from our previously published data, and 24 metagenomes from other published data). The strain level analysis confirmed the geographic pattern of the giant panda gut microbiota [49]. For example, in the gut microbiota of the Qinling giant panda population, the putative species *Clostridium cuniculi* were the dominant bacterium, and the relative abundance of the *Pseudomonas* strains was low, while *Escherichia coli* was the dominant bacteria in the captive giant panda gut microbiota. We then compared the KEGG functions for each clade of the phylogenetic tree from these 51 strains and found that clade 7, mainly composed of *Pseudomonas* and *Escherichia* strains, harbored a high number of genes involved in the amino acid metabolism as compared to strains from the other clades (Figure S8).

Moreover, we found putative host-specific features in these *Pseudomonas* strains, and those different hosts might harbor different *Pseudomonas* strains or sub-strains (Fig. 4). We then combined the *Pseudomonas* strains in this study with other published *Pseudomonas* strains and constructed a phylogenetic tree using their genomes and MAGs. The results confirmed the putative host-specific pattern of these dominant *Pseudomonas* strains among the bamboo, insect, and panda samples (Fig. 5). The dominant *Pseudomonas* strains (e.g., *Pseudomonas oryzihabitans* strain USDA-ARS-USMARC, *Pseudomonas psychrotolerans* strain PRS08-11306, and Bin35_Pseudomonas) in bamboo samples belong to a single clade. Bin4_Pseudomonas from the giant panda fecal sample and Bin9_Pseudomonas parafulva from the insect sample belong to different sub-clades within *Pseudomonas putida* clade. *Pseudomonas fragi* had the highest abundance among the 62 (four from our bins and 58 from the published genomes) *Pseudomonas* strains of these 108 metagenomes, and was mainly dominant in the fecal samples from the wild and captive giant pandas. *Pseudomonas orientalis* strain F9 was putatively mainly identified in the red panda fecal samples. Bin4_Pseudomonas strain was a common bacteria in the gut microbiota of Carnivora in this study. However, the relative abundance of most of these 62 *Pseudomonas* strains was low or rare in other mammals in this study, including CA, OC, and HE. Therefore, these findings indicated the
Fig. 2. The heatmap of the predominant microbial genera in animal and plant samples based on 16S rRNA amplicon sequencing. The detailed information is shown in Table S1-S2. Each row represented one sample. Each column represents the relative abundance in these samples for each specific genus. The tree on left is the same as in Fig. 1. In the left tree, each line represented one sample. The top tree was constructed by Bray-Curtis distance based on the abundance of the genera. The relative abundance of the genera was log_{10}-transformed.

Fig. 3. The network analysis of the microbiota in animal and plant samples based on 16S rRNA amplicon sequencing. Detailed information is shown in Table S1-S2. Differential network analysis of the relative abundance of microbiota among groups. The nodes represent the taxonomic classification, and the node (circle) size indicates the relative abundance (the mean abundance above 0.1%). Node (circle) color indicated the significant enrichment (the significant level at 0.05) of this specific group at that taxonomic level. Gray indicates no significant difference among groups. Pandas represent the bamboo-eating pandas (giant pandas and red pandas). Insects included the insect samples collected in the bamboo and non-bamboo habitats. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
host-specific features in some *Pseudomonas* strains and specialty gut microbial features in bamboo-eating pandas.

*Pseudomonas fragi* and *Pseudomonas putida* have been isolated from many different types of environmental sources, including water, plants, milk, meat, and humans (clinical). *Pseudomonas* is one of the main symbiotic bacterial genera of plants (including bamboos), which may be beneficial for plant growth, but could also be plant pathogens [48]. Plants produce many types of secondary metabolic compounds, including phenolics and terpenoids [50] and *Pseudomonas* strains often play important roles in the degradation of these compounds [51,52]. The relationship *Pseudomonas* and host plants are one of the successful symbiotic systems in evolutionary history [48]. The predominant *Pseudomonas* strains (such as *Pseudomonas fragi*, *Pseudomonas putida*, and *Pseudomonas orientalis* strain F9) in pandas or insects have also been isolated from many environmental sources, including plants, but are rare in other mammals, including other Carnivora species. In the past, the main dietary plants of the giant pandas were not only bamboos but also other plants [53]. Given the relatively high similarity of microbial communities between insects and bamboos and between pandas and bamboo, we speculated that the colonization of *Pseudomonas* in the insect and panda intestines might be associated with their plant-eating behavior and relatively simple digestive systems (low-level of oxygen concentration) during their long evolutionary history. The response to host niches (within the intestines) or selection by host niches might further lead to host-specific features in *Pseudomonas* strains. Some *Pseudomonas* strains, such as *Pseudomonas putida*, can produce lipases and proteases that take part in dairy and meat spoilage [54,55]. The ancestors of giant pandas were meat-eating carnivorans [56,57]. Considering *Pseudomonas* strains (*Bin4_Pseudomonas*, within the *Pseudomonas putida* clade) were common strains among carnivorans (bamboo-eating pandas, CA, and OC groups), we deduced that this feature in the gut microbiota might be one of the ‘imprints’ from the coevolution between the Carnivora and their gut microbiota.

In addition, here, most of the data came from our lab, and we treated the data from the raw data based on the same pipeline. However, the potential limitations of a meta-analysis in this study (e.g., 16S rRNA gene sequences) were associated with the difference in the DNA extraction kit, hypervariable region of the 16S rRNA, and other systematic problems in high-throughput sequencing. One study mentions that cross-platform meta-analysis will deal with inherent protocol and technology biases, such as DNA extraction kits and specific errors of the sequencing platforms [58]. Moreover, the different 16S rRNA gene variable
regions harbor the difference in informative power to detect phylogenetic clades, which will further impact relative OTU abundances\[58\]. Although the pattern found in this study was clear, we still need to be cautious due to the putative limitation of the meta-analysis.

9. Conclusion

Here we found a high similarity in the gut microbial communities (high proportion of Gammaproteobacterial *Pseudomonas*) of invertebrate insects and the vertebrate giant pandas, which might be associated with their plant-eating behavior and the special feature (the presence of oxygen) of their digestive tracts. Base on *Pseudomonas* strain analysis, we discovered that the response to host niches or the selection by host might further lead to host-specific strains (or sub-strains). We uncovered two ‘imprints’ in the giant panda gut microbiota during their long co-evolutionary history. First, *Pseudomonas putida* (e.g., *Bin4_Pseudomonas*), which can be associated with the proteins and lipids (food spoilage), was a common main *Pseudomonas* strain among the Carnivora, and might be associated with their current or ancestral meat-eating behavior. Second, some *Pseudomonas* strains (e.g., *Pseudomonas fragi*) found to be widely distributed among plants were the dominant *Pseudomonas* in the giant panda gut microbiota, which might be explained by a less restricted herbivorous diet (not just bamboo) in the past.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contribution

LZ conceived the project. RY, QD, YZ, WL, XG, XY, and XY collected the samples. RY performed the experiments. RY, HC, ZY, TW, DQ, GL analyzed the data. ZL wrote the manuscript. All authors approved the final version of the manuscript.

Data availability

The raw data of 16S rRNA gene sequences have been submitted to NCBI (PRJNA750668). The raw data of metagenome sequences have been submitted to NCBI with the accession number PRJNA750776.
Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.csbj.2021.08.025.

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