INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
20–0540/202x/00–0–000–000
DOI: 10.17957/IJAB/15.1613
http://www.fspublishers.org

Full Length Article

Pyrosequencing of the 16S rRNA Gene Elucidated the Diet and Age-related Association of the Intestinal Microbial Community in *Ailuropoda melanoleuca* (Giant Panda)

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Received 04 April 2020; Accepted 03 August 2020; Published _____

Abstract

Giant Panda (*Ailuropoda melanoleuca*) possesses a digestive system of typical carnivores while they consume a distinctive bamboo diet. The patterns of age-related variations in the intestinal microbiota of Pandas are not well understood, especially the impact of different diet structures at different growth stages on their microbiota. In this study, pyrosequencing of the 16S rRNA gene was performed by the IonS5TMXL platform to investigate the microbiotas of 23 Pandas, which included all typical growth stages and diet structure. It was shown that the basic structure of intestinal microbiota in Pandas was similar to each other regardless of their ages, but the relative abundance of dominant bacteria varied significantly with age. Alpha diversity showed a variable trend with age. Notably, the relative abundance of *Streptococcus* reached a peak in the adult Pandas. Further analysis proved that the diet had a significant impact on the intestinal microbiota of Pandas. During weaning, when they consumed a large amount of bamboo with high fiber, the diversity of intestinal microbiota decreased significantly. Interestingly, *Streptococcus*, the dominant bacteria in Pandas, was positively correlated with food fiber content, although it is not a typical fiber-digesting bacterium. The increase of *Streptococcus* in adult individuals may be related to the utilization and metabolism of carbohydrates, which needs to be confirmed by further detailed studies. Overall, our study provides insight into the intestinal microbial ecology of Panda, which will enhance our knowledge of the relationship between their native microbiota and host physiology.

Keywords: Giant pandas; Intestinal microbiota; IonS5TMXL sequencing; Age; Diet

Introduction

The animal gastrointestinal (GI) tract has a complex microbial community that plays a vital role in the maintaining the health of the host (Costello *et al.* 2009; Khan *et al.* 2019). Pathogenic microorganism have been a serious threat for domestic and wild animals (Rahman and Mohsin 2019; Kalhoro *et al.* 2019). The intestinal microbiota have a crucial role in getting nutrients from diets, thus influencing the host nutrition, metabolism, and body development (Turnbaugh and Gordon 2009). In addition, it can prevent the colonization of pathogens in the GI tract and is essential for mucosal homeostasis, intestinal maturation, and full functions (Chow *et al.* 2010; Hu *et al.* 2018).

As a flagship species of wildlife conservation, giant Panda is a highly endangered species in the IUCN red list (2016) that attracts worldwide attention (Swaisgood *et al.* 2016). Giant Pandas belong to the family Ursidae in terms of phylogenetic classification, which have the digestion characteristics of both carnivores and omnivores (Arnason *et al.* 2007; Krause *et al.* 2008). The most amazing thing about giant Pandas is that they consume a lot of cellulose-rich bamboos every day, but they have a short and simple gastrointestinal digestive system of typical carnivores (Xue *et al.* 2015). Several studies have analyzed the intestinal microbiota of giant Pandas by multiple methods, which has led to a preliminary understanding of both structural and functional features of their gut microbiota (Zhang *et al.*

To cite this paper: Huang X, N Wang, MA Mehmood, MK Saleemi, T Deng, H Wu, Y Ke, H Zhu, (202x). Pyrosequencing of the 16S rRNA gene elucidated the diet and age-related association of the intestinal microbial community in *Ailuropoda melanoleuca* (Giant Panda). *Intl J Agric Biol* 00:000–000

1995; Li *et al.* 2010; Williams *et al.* 2016; Guo *et al.* 2019; Yao *et al.* 2019).

The average life expectancy of Pandas in captivity is 25 years and their sex maturation time is around 6–7 years. In captivity, there is an adaptive succession of the diet structure with the growth and development of Pandas. Usually, under the age of one, they are fed on whole milk. After one year, a certain amount of bread, bamboo leaves, bamboo shoots, apples, carrots, and calcium gluconate/zinc are added in the milk-based diet gradually (Luo 2014). After 2 years, they are gradually settled into a dietary structure consisting of bamboo stalks and shoots (mainly), bread, apples, and carrots. With further aging, the intake of bamboo stalks and bamboo shoots in the diet is continued to increase. As Pandas grow old, just like any other animal, their digestive and metabolic functions start to deteriorate. Resultantly, the proportion of bamboo in their diet is altered, replaced with bamboo leaves and bamboo shoots which are relatively easy to digest, at this stage additional vitamins and sources of calcium are added to the diet (Zhang *et al.* 2017). However, little is known about patterns of age-related changes in the intestinal microbiota of pandas, especially the impact of different diet structures at different growth stages on the intestinal microbiota of Pandas.

This study was meant to investigate the composition of the microbial community in the gastrointestinal tract of Pandas with a special focus to identify the impact of diet and aging. Microbiota of 23 Pandas, which included all typical growth stages and diet structure, was evaluated by sequencing the 16S rRNA gene of the intestinal microbial community using the IonS5TMXL platform. This study would enhance our understanding of the intestinal microbiota of Panda, for further updating knowledge of the relationship between intestinal microbiota and animal health. Moreover, it would facilitate making a better strategy for Panda conservation and breeding.

Materials and Methods

Experimental design and sampling

The samples were collected from 23 Pandas located in the Dujiangyan Breeding and Wild Breeding Research Center, Chengdu Giant Panda Breeding Research Base. To reveal the influence of diet structure on intestinal microbiota, Pandas were separated into three groups based on the diet structure: DY group included 7 Pandas (before weaning) at the age of 0.5–1.5 years, which fed on the milk-based diet. DA group included 12 Pandas aged 2.5–18.5 years which fed on a high-fiber diet consisting of bamboo stalks and shoots, bread, apples, and carrot. DO group included 4 Pandas aged 25.5–27.5 years which were fed on a low-fiber diet which removed high-fiber bamboo stalks from their diet and added some extra vitamins and calcium. Fresh fecal samples were collected in the morning/afternoon feeding time. Once upon animal defecation, the fecal samples were placed in sterile plastic bags and frozen in liquid nitrogen containers immediately. The samples were then transported to the lab and stored at –70°C until further analyses.

Ethics statement

All experiments which involved animals in this study were strictly subjected to all procedures per the animal protection law of the People's Republic of China (October 26, 2018). Protocols for animal trials were approved by the Care and Use of Laboratory Animals of the Animal Ethics Committee of China Conservation and Research Center for the Giant Panda (Dujiangyan, China) (Approval No.20180212) and Key Laboratory of State Forestry and Grassland Administration on Conservation Biology of Rare Animals in the Giant Panda National Park.

DNA extraction and sequencing

To avoid environmental contamination, the inner part of fecal samples (70–80 mg) was carefully acquired by sterile tweezers. CTAB/SDS method was employed for total genome DNA extraction (Griffith *et al.* 2009). DNA concentration was measured by NanoDrop ND-1000 (NanoDrop Technologies) Spectrophotometer and DNA purity was examined on 1% agarose gels. PCR amplification was carried out by using the 515f/806r primer set (515f: 5'-GTG CCAGCMGCCGCGGTA A-3', 806r: 5'-XXX XXXGGACTACHV GGGTWT CTA AT-3') with a 6-bp error-correcting barcode unique to each sample. PCR reactions were performed with Phusion® High Fidelity PCR Master Mix (New England Biolabs). The purified amplified products were sent to Novogene Bioinformatics Technology Co., Ltd. (Beijing, China) for sequencing the V4 hypervariable region of the 16S rRNA gene. Detailed information for sequenced samples is shown in Table S1.

Bioinformatics and statistical analyses

The primer sequences were removed from the single-end reads which were later quality-filtered using recommended parameters of the Cutadapt (v. 1.9.1) quality-controlled process (Martin 2011). The chimera sequences (Haas *et al.* 2011) were removed by using Silva reference database (Christian *et al.* 2013) and UCHIME algorithm (Edgar *et al.* 2011). The obtained clean reads were then analyzed by Uparse software (Uparse v. 7.0.1001). Sequences with $\geqslant$97% similarity were grouped into the same OTUs. Taxonomy assignment of representative sequence for each OTU was performed based on the Silva Database and Mothur algorithm (Edgar 2013). Multiple sequence alignment analysis was conducted using the MUSCLE Version 3.8.31 (Edgar 2004).

Alpha diversity analysis included Shannon and Simpson index. Unweighted/weighted Unifrac distances and Bray-Curtis distances were calculated for Jackknifed beta

diversity analysis. Principal Coordinate Analysis (PCoA) and Non-Metric Multi-Dimensional Scaling (NMDS) was constructed based on these distances (Lozupone and Knight 2005). The alpha diversity values were also compared using Wilcoxon Rank Sum Test. Bray-Curtis distance-based similarity analysis was used for the significance test of beta diversity differences between groups. The linear discriminant analysis coupled with effect size (LEfSe) was employed to determine microbial taxa featured in different groups (Segata *et al.* 2011). The functional profiles from metagenomic 16S rRNA data were predicted using Tax4Fun. The student's *t*-test was used to identify pathways having substantial differences in abundance between groups.

Results

Metadata and sequencing

In total, 1,557,721 high-quality reads with an average of 67,727 reads per sample using the IonS5TMXL platform Single-End sequencing of 16S rRNA gene amplicons after filtering were obtained. The overall effective rate of quality control was 95.38%. These sequences were allocated to 782 operational taxonomic units (OTUs) based on 97% similarity. Among them, 782 (100.00%) OTUs were assigned to the Silva132 database; 98.59% OTUs were assigned to phylum level; 97.19% OTUs were assigned to Class level, 93.22% OTUs were assigned to Order level; 87.60% OTUs were assigned to Family level; 61.76% OTUs were allocated to Genus level; 21.36% OTUs were allocated to Species level. The original 16S rRNA sequence data were deposited in the Genome Sequence Archive (Wang *et al.* 2017) of BIG Data Center (Zhang *et al.* 2017), Beijing Institute of Genomics (BIG), Chinese Academy of Sciences (Accession NO. CRA002404).

Microbial community composition and its dynamic change related to age in the panda GIT

Regardless of their ages, the microbial community of Pandas was predominated by phylum *Firmicutes* (69.096%), *Proteobacteria* (4.995%) and *Cyanobacteria* (3.119%). The phylum *Firmicutes* was mainly composed of genus *Streptococcus* (48.242%) and *Lactobacillus* (5.996%). The phylum *Proteobacteria* mainly consisted genus *Stenotrophomonas* (3.271%) and *Aeromonas* (1.724%). At Species-level, the dominant bacteria belonged to *Streptococcus gallolyticus* subspp. *Macedonicus* (37.428%), *Clostridium disporicum* (12.620%), and *Lactobacillus faecis* (2.203%) (Fig. 1). As shown in the Venn petal diagram (Fig. 2A), a total of 109 OTUs were identified as core OTUs shared by Pandas of all age groups. The unique OTUs for Pandas of different age groups were 12, 68, 41, 5, 2 and 65, respectively. Meanwhile, the relative abundance of dominant bacteria varied significantly with age (Fig. 2 and Fig. S1). For Alpha diversity, the diversity index including Simpson and Shannon index, both showed a downward trend and then an upward trend along with age. The Alpha diversity of intestinal microbiota in adult Pandas (6.5–8.5 years) was the lowest among all age groups (Fig. 2B and C). For dominant bacteria, the relative abundance of phylum Firmicutes showed a rising trend and then a descending trend along with age, while *Proteobacteria* showed a clear downward trend and then an upward trend. At the genus level, there was a notable trend that the relative abundance of *Streptococcus* was shown to be increased at first and then was decreased. The relative richness of *Streptococcus* reached a peak in the adult Pandas at age of 6.5–8.5, which was comprised of 80% of the total microbiotas in Pandas (Fig. 2D, E and Fig. S2).

Influence of diet structure on intestinal microbiota of pandas

Microbial community richness (alpha diversity) was assessed by Shannon and Simpson index. As shown in Fig. 3A and B, Shannon index of the DY group was meaningfully higher than that of the DO group (p=0.0356) by the Wilcoxon Rank Sum Test. There was a meaningful difference in the Simpson index between the DA-DY group (P = 0.0358). However, there were no substantial variances in Shannon and Simpson between DA-DO, DO-DY groups (P > 0.05). Observed species also showed no difference between DA-DO, DO-DY, DA-DY group (P > 0.05). Overall, the diversity of intestinal microbiota in Pandas was shown to be decreased significantly during the transition from a milk-based diet to an adult bamboo-based diet. In response to the higher proportion of bamboo-fiber in the Panda diet, the diversity of intestinal microbiota was shown to be increasing, but no significant differences were observed in statistical terms (DA-DO, Shannon-Wilcox, P= 0.1636).

To examine the beta diversity among different diet groups, unweighted/weighted Unifrac distances and Bray-Curtis distances were calculated to evaluate the dissimilarities in the structure of the microbial community. Principal coordinate analysis and Non-Metric Multi-Dimensional Scaling were employed to visualize the distances (Fig. 3C, D and E). It was shown that although DY and DA groups had some intersection in space, samples in the same group clustered together separately, which indicated that the intestinal microbiota of each group had their characteristics under a specific diet. The spatial distribution of individuals in the DO group was more discrete, which indicated that the intestinal microbiota of elderly Pandas varied greatly, and the characteristics of its microbiota were relatively unstable. The differences in community membership among different diet groups were proved to be statistically significant by analysis of similarity (ANOSIM, DA-DY, r=0.405, P=0.009; DO-DY, r=0.3294, P=0.036; DO-DA: r=0.8287, P=0.001).

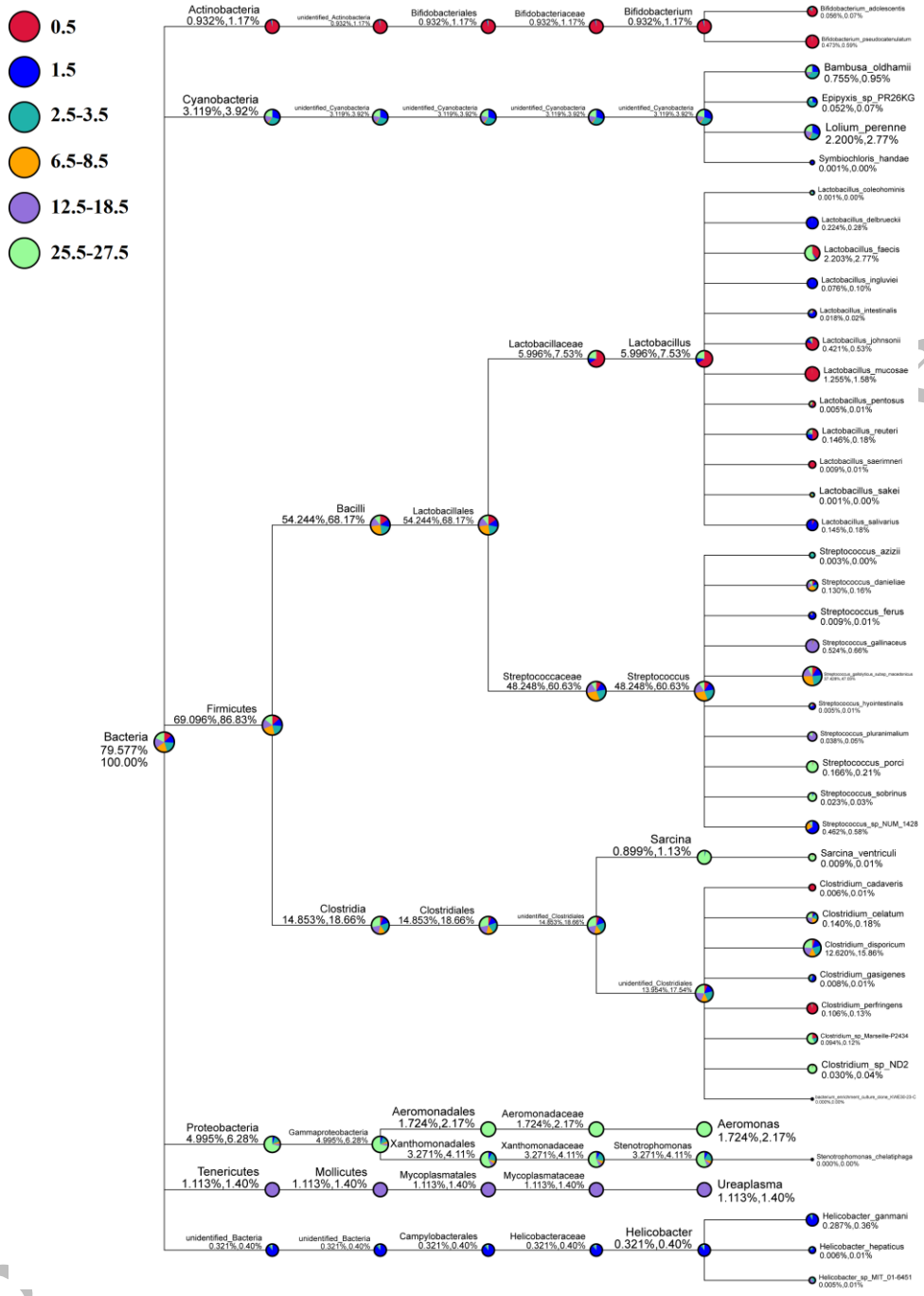

Fig. 1: Specific species taxonomy tree analysis of the microbial community of pandas in different ages

The linear discriminant analysis effect size (LEfSe) was used to determine specific taxon that was differentially dispersed among different diet groups. A total of 13 taxa were differentially represented between DY and DA group (Fig. 3F), out of which 10 were shown to be more abundant in DY group (*e.g.*, order Enterobacteriales, family *Enterobacteriaceae*, family *Lactobacillaceae*, genus *Lactobacillus*, kingdom *Bacteria*, phylum *Actinobacteria*, class unidentified *Actinobacteria*, family *Bifidobacteriaceae*, genus *Bifidobacterium*, order *Bifidobacteriales*) and 3 were shown to be more abundant in DA group (*e.g.*, species *Streptococcus gallolyticus* subsp. macedonicus, genus *Streptococcus*, family Streptococcaceae).

To estimate the putative role of intestinal bacteria in Pandas, Tax4Fun was used to envisage the functional capabilities of the microbial community (Fig. 4). Metagenomic inference indicated that DA group harbored microbiomes with greater abundances of genes such as

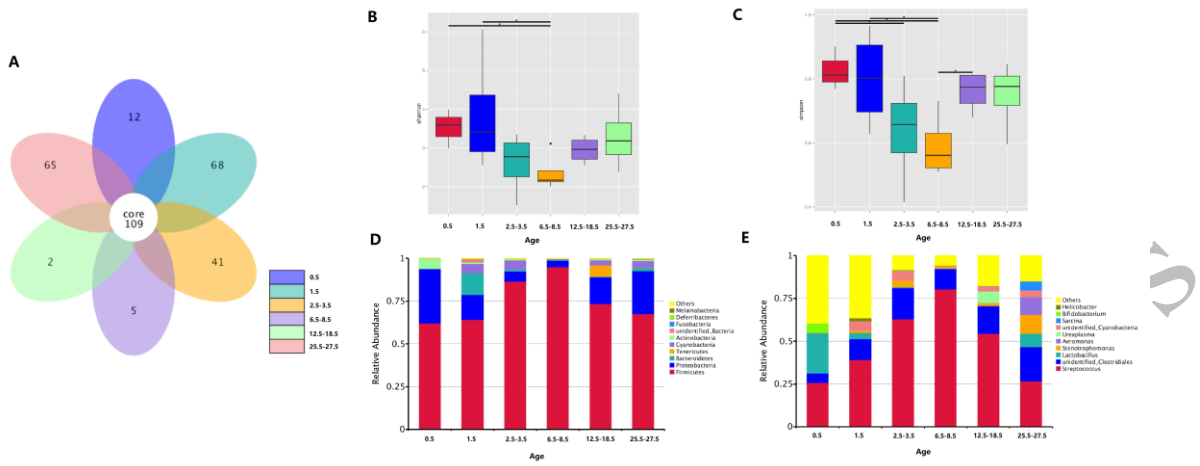

Fig. 2: Comparison of the microbial community of pandas in different ages

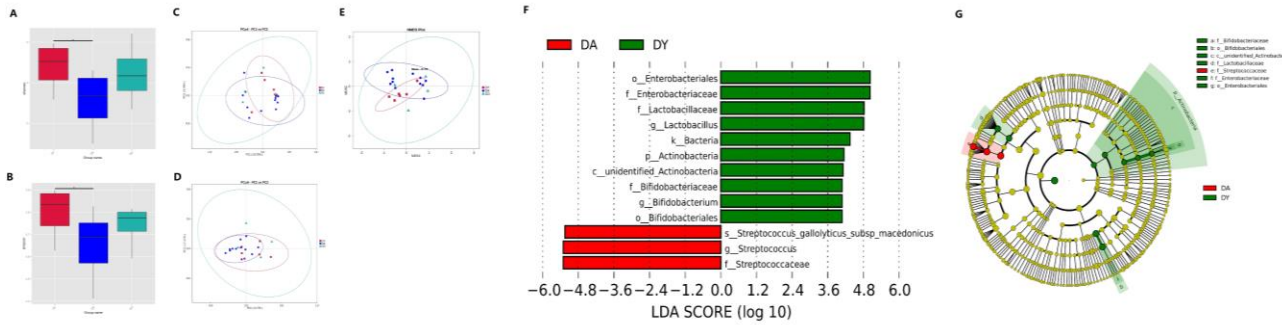

Fig. 3: Differences in the microbial community among pandas in different diet groups

amino-sugar, pyrimidine metabolism, and nucleotide-sugar metabolism, peptidoglycan biosynthesis, and degradation proteins, mismatch repair, cell cycle-Caulobacter, base excision repair, glycolipid metabolism, biosynthesis of annamycin when compared to the DY group. The abundance of genes like biofilm formation (*Escherichia coli* and *Pseudomonas aeruginosa*), signal transduction mechanisms, phenylalanine metabolism, vitamin B6 metabolism, inositol phosphate metabolism, biosynthesis of unsaturated fatty acids, and pertussis in DA group were significantly less when compared to DY group. Cell cycle-Caulobacter gene was more abundant in the DO group than in the DY group, while Signal transduction mechanisms related genes abundance in the DO group was significantly lower than that in the DY group (*t*-test, $P < 0.05$).

Discussion

Several studies have systematically elaborated on the characteristics of intestinal microbiota in Giant Pandas. Ley *et al.* (2008) compared the fecal microbiota of 59 mammalian species including humans. It showed the intestinal microbial community of the Pandas was similar to that of bears, but significantly different when compared to other mammals. In another study, the fecal microbiota of the Giant Pandas, the red Pandas, and Asian black bears was compared by 454 GS FLX pyrosequencing of 16S rRNA (Li *et al.* 2015). The results showed that the intestinal microbiota of the Giant Pandas was clustered closer to those of the black bears instead of the red Pandas; even Giant Pandas shared the same diet with red Pandas. Moreover, fecal samples of 45 captive Giant Pandas were constantly collected within one year, and then investigated the large-scale structural profiling of the fecal microbiota based on 16S rRNA gene. It was indicated that the microbiota of Giant Panda was dominated by *Shigella/Escherichia* and *Streptococcus* species, not some well-known cellulose-degrading bacterium (Xue *et al.* 2015). Afterward, a shotgun metagenomic study was applied to detect the functional potential of intestinal microbiota in giant Pandas (Guo *et al.* 2018). The gut microbial community of Panda was compared with some herbivores, carnivores, and omnivore's species reported in current and early studies. The results were demonstrated that a bear-like intestinal microbiota inhabited in the Giant Panda, which was distinct from those of herbivores. Moreover, the comparative richness of genes

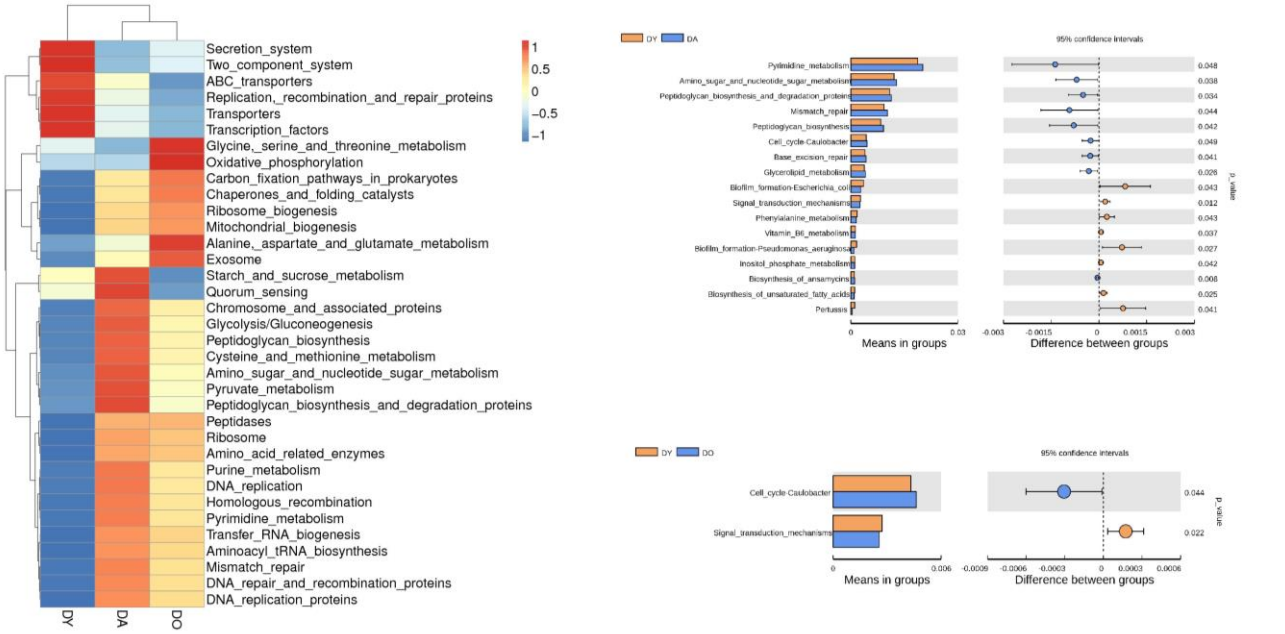

Fig. 4: Differences in microbial community among pandas in different diet groups

associated with hemicellulose- and cellulose-digestion, as well as the enrichment of enzymes involved in amino acid degradation and biosynthetic reactions pathways in Giant Panda were more close to a carnivore microbiome. Further *in vitro* experimental assay confirmed that the enzyme activities of cellulase and xylanase in Giant Panda's fecal samples were the lowest among major herbivores, which indicated that the digestive system of Giant Panda did not specifically evolve for bamboo diet (Guo *et al.* 2018). The present study has shown that intestinal microbiota of the Panda was predominant by *Streptococcus*, unidentified Clostridiales, *Lactobacillus* regardless of the age and diet. The unidentified Clostridiales may contain some of the cellulose-digesting bacterial groups (Zhu *et al.* 2011), but most of the dominant bacteria (especially *Streptococcus*, which accounts for about 50% of the intestinal microbiota) are not commonly cellulose-digesting bacteria in giant Pandas. Compared with herbivores such as ruminants, the relative abundance of *Bacteroides* and *Ruminococcus* with cellulose-digesting ability was significantly lower than that of herbivores (Ley *et al.* 2008).

As a rare wild animal in China, there are less than 1600 wild Pandas in the world, and the number of captive Pandas is only 548. Chengdu Research Base of Giant Panda harbored one of the largest captive giant Panda populations in the world. The intestinal microbiota of 23 Pandas aged 0.5–27.5 years was systematically studied in this research. There were 3–4 Pandas at each representative age point. The samples covered almost all growth stages and characteristic diet structure. The results showed that the basic structure of intestinal microbiota of pandas of different ages was similar to each other, but the diversity of the microbial community and the abundance of dominant bacteria was significantly changing with age. Xue *et al.* (2015) also compared the diversity of intestinal microbiota in adult and juvenile Pandas. They found the alpha diversities of the adult (aged 6 to 22 years) and juvenile (aged 2 to 5 years) samples were similar to each other in every sampling season. Unlike the age span of different age groups, the more detailed age group division in the present study led to the observation of a significant decline in microbial diversity in certain periods. Besides, there were significant changes in alpha diversity across seasons. In their study, seasonal variation further concealed the declining trend of microbial diversity during the die-transformation period. Zhang *et al.* (2017) also studied microbiotas of 14 captive-born Pandas and divided them into four groups: S1 (Panda fed on breast milk as diet and <2 months old), S2 (between 3 and 12 months old and no bamboo found in their feces, commercial milk as a dietary supplement), S3 (>6 months old and bamboo stems or leaves as diet), and S4 (>6 months old and bamboo shoots as diet). They only covered the age under 27 months. As we know, before 1.5 years old the Panda still mainly feeds on milk. Usually, <3 months old, Panda is fed on breast milk; between 3 and 18 months old, commercial milk as main diet (after about 6 months, they eat some bamboo shoots, stems or leaves for adaptive diet transformation); after 2.5 years old they feed on the bamboo-based diet. Thus, their S3 and S4 group were certain stages of the weaning period. Although our two experiments studied the changes in intestinal microbiota in two different age ranges, both showed that diet had a huge impact on intestinal microbiota.

For most of the animals, including carnivores, omnivores, and other herbivores, the diversity of intestinal microbiota may fluctuate before and after weaning (Favier *et al.* 2002; Klein-Jöbstl *et al.* 2014; Kumar *et al.* 2016). However, throughout the whole life span, the diversity of intestinal microbiota always increases at first and then decreases with aging. The peak diversity of intestinal microbiota often occurs in adulthood, when the digestive physiological and metabolic capacity of animals usually reaches its peak in this period (Claesson *et al.* 2011, 2012; Liu *et al.* 2017; Bermingham *et al.* 2018; Zhu *et al.* 2018). Unexpectedly, the diversity in the intestinal microbiota of the Pandas showed a descending trend and then a rising trend along with the age. The microbial diversity of intestinal microbiota in adult Pandas was the lowest among all age groups. At the same time, this period was also the period when Pandas consumed most of the food and had the highest fiber content in their diet structure, which suggested that the unusual decline in microbial diversity may be related to diet structure.

To further elucidate the impact of food structure on the intestinal microbial community, the samples from 23 Pandas were separated into three groups based on their diet structure. Both Principal coordinate analysis (PCoA) and Non-Metric Multi-Dimensional Scaling (NMDS) proved that there were significant differences in microbiota among group DY, DA, DO. Restricted by the law and regulation of China on the protection of wildlife, we were unable to design an experiment to study the impact of different diet structures on microbiotas of giant Pandas with the same age directly. Therefore, the significant differences among group DY, DA, DO are essentially the result of the combined influence of diet and age factors. Nevertheless, the intestinal microbiota of Pandas in certain stages showed a gradual change on the macro front. Most notably, there were dramatic changes in the intestinal microbiota before and after weaning and during the diet shifting of the elderly (Fig. S1B), which indicated diet, played a decisive role in shaping the intestinal microbiota.

The individuals in the DY group fed mainly on milk and the individuals in the DA group fed mainly on bamboo were clustered separately, indicating that the intestinal microbiota of each group had their characteristics under different diet structures. DY group was featured by relative higher microbial diversity and a higher proportion of bacteria in order *Enterobacteriales*., family *Enterobacteriaceae*, family *Lactobacillaceae*, genus *Lactobacillus*, kingdom *Bacteria*, phylum *Actinobacteria*, class unidentified Actinobacteria, family *Bifidobacteriaceae*, genus *Bifidobacterium*, order *Bifidobacteriales*. Consequently , genes like Biofilm formation *Escherichia coli*, signal transduction mechanisms, phenylalanine metabolism, vitamin B6 metabolism, biofilm formation *Pseudomonas aeruginosa*, inositol phosphate metabolism, biosynthesis of unsaturated fatty acids, Pertussis were more abundant in DY group. When Pandas were weaning and consumed a large amount of bamboo with high fiber, the diversity of intestinal microbiota decreased significantly. The dominant intestinal taxa also shifted into bacteria species *Streptococcus gallolyticus* subsp. macedonicus, genus *Streptococcus*, family *Streptococcaceae*. Similarly, genes like amino sugar, pyrimidine metabolism, nucleotide sugar metabolism, peptidoglycan biosynthesis and degradation proteins, mismatch repair, peptidoglycan biosynthesis, cell cycle-Caulobacter, base excision repair, glycolipid metabolism, biosynthesis of annamycin were more abundant in DA group. That is to say, the level of fiber in diet seemed to play a decisive role in the diversity of intestinal microbiota. The highest fiber content in diet led to the lowest microbial diversity in Pandas. Interestingly, *Streptococcus*, the dominant intestinal microbiota in Pandas, was positively correlated with food fiber content. However, *Streptococcus* as a common bacterial species widely existing in nature, human and animal excrement and nasopharynx of healthy people, which is not a typical fiber-digesting bacterium (Zoetendal *et al.* 2012; Bogert *et al.* 2013a, b; Bogert *et al.* 2014). At the same time, the spatial distribution of individuals in the DO group was more discrete, which indicated that the intestinal microbiota of elderly Pandas varied greatly, and the characteristics of its microbiota were relatively unstable. This may be related to the decline of immunity, and digestive and metabolic functions in elderly individuals and the structure and function of intestinal microbiota were more susceptible to external factors including diet.

Conclusion

Although the intestinal microbiota of Pandas was not predominant by fiber-digesting bacteria, the diversity and composition of intestinal microbiota were still greatly influenced by diet structure, especially bamboo fiber intake. Previous studies have also clearly pointed out that giant Pandas cannot obtain the necessary energy from cellulose. Giant Pandas mainly obtain energy through starch, hemicellulose, and pectin in bamboo (Zhang *et al.* 2018). Therefore, we speculate that the way diet affects the intestinal microbiota of Pandas is not the way that changes in bamboo cellulose intake directly change the abundance of fiber-digesting bacteria. On the contrary, the increase in fiber content significantly reduced the diversity of intestinal microbiota. The increase of *Streptococcus* in adult individuals may be related to the utilization and metabolism of carbohydrates such as simple sugars, starch, hemicellulose and pectin in bamboo (Zoetendal *et al.* 2012; Bogert *et al.* 2013) which needs to be confirmed by further detailed studies.

Author Contributions

XH designed the experimental program and participated

in the examination. NW conceived of the study, collected the experimental material and drafted the manuscript. MAM participated in drafting the manuscript. MKS collected and analyzed the raw data. TD, HW and YK collected the fecal samples and provided the information of the Giant Pandas. HZ* (Corresponding author) is responsible for this study, participated in its design and help to draft the manuscript. All authors read and approved the final manuscript.

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