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Zheng Yan, Hairui Wang, Xiaoyan Liu, Le Wang, Xing Chen, Dingzhen Liu, Shibin Yuan

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The metabolic function of giant panda (*Ailuropoda melanoleuca*) gut microbiota decreased compared with other mammals

Zheng Yan [1,2,3], Hairui Wang[3], Xiaoyan Liu[2], Le Wang[1], Xing Chen[2], Dingzhen Liu[2,*]

Shibin Yuan[1,*]

[1]Key Laboratory of Southwest China Wildlife Resources Conservation (Ministry of Education), China West Normal University, Nanchong, 637001, CHN

[2]Key Laboratory for Biodiversity and Ecological Engineering of Ministry of Education, Department of Ecology, College of Life Sciences, Beijing Normal University, Beijing, 100875, CHN

[3]Chengdu Research Base of Giant Panda Breeding, Sichuan Key Laboratory of Conservation Biology for Endangered Wildlife, Chengdu, 610081, CHN

[*]Corresponding author: Dingzhen Liu, E-mail: dzliu@bnu.edu.cn, Shibin Yuan, E-mail: yshibin1020@cwnu.edu.cn

Abstract

Varied responses of the diverse gut microbiota impact the host nutrient metabolism and disease immunity. The reason and the impact behind the low diversity level of gut microbiota giant panda are yet to be explored. The current study is an attempt to highlight the impact of intestinal type and diet on gut microbiota diversity of giant pandas. A multi-species comparison (n=73) was conducted to analyze the effects of different diets and intestinal type on gut microbiota function of giant panda using PICRUSt method. The gut microbiota of giant panda was significantly lower than herbivores and carnivores provided with same diets and intestinal type respectively. Moreover, giant panda exhibit significantly reduced microbiota on high-fiber bamboo

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culm diet than bamboo shoot and leaf. The low diversity of gut microbiota constraint nutritional metabolic function of giant panda including rate of energy and amino acid metabolism. These results showed that the short intestinal tract and high fiber diet diminishes the gut microbiota diversity and metabolic function of giant pandas.

Keywords: Giant panda, Gut microbiota, PICRUSt, 16S rRNA gene, Gene function

Introduction

Gut microbiota (GM) refers to the assembly of complex and dynamically changing microbial community inhabits the gut, and closely associated with nutrient metabolism and immunity of the host (Kelly et al. 2005; Ley et al. 2006). A highly diverse gut microbiota generally confers benefit to host health (Cotillard et al. 2013). Previous research demonstrated that giant panda (*Ailuropoda melanoleuca*) harbor significantly lower gut microbiota diversity than other herbivores, carnivores and omnivores mammals (Xue et al. 2015; Guo et al. 2020). The factors contributing this constraint in the microbiota diversity is however remain unknown, nor its impact on the giant panda nutrient metabolism.

The giant panda, a typical vegetarian carnivore specie feed exclusively on high-fiber bamboo. (Dierenfeld et al. 1982; Viswanathan 2010). The anatomy of the giant panda's gastrointestinal tract is similar to other clades of carnivores with short intestinal tract and not used to ferment fibers (Dierenfeld et al. 1982; Guo et al. 2018b). Evidence predicated the transition of giant pandas from omnivorous into bamboo-eating species in the mid-Holocene (Han et al. 2019) albeit it still belongs to the Ursidae family (Krause et al. 2008; Liu et al. 2008). Comparative analysis of panda with in other clades of omnivores and herbivores revealed that the gut microbiota of pandas constituted

significantly lower abundance of cellulase genes and higher abundance of hemicellulase genes (Zhang et al. 2018). Therefore, the low gut microbial diversity of giant pandas is proposed to be associated with intestinal tract and dietary interventions. Yet sound evidences are highly missing. It is an effective method to investigate the effects of intestinal and nutritional factors on gut microbiota on the basis of dietary interventions in multiple animal clades (Muegge et al. 2011; Sanders et al. 2015; Vital et al. 2015). Recently, comparison of giant panda with other species of carnivores, omnivores and herbivores has been the focus of the research, yet the data available is not sufficient, especially for herbivores (less than 4 animal samples) (Zhu et al. 2011; Xue et al. 2015; Guo et al. 2020). The current study is an attempt to unravel the factors behind the low gut microbiota diversity in the panda by comparing them with other herbivores, omnivores and carnivores (21, 11, 7 individuals respectively). The potential functions of the gut microbiota repertoire were theoretically tested by PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Langille et al. 2013). The results will broaden the understanding and knowledge pertaining the giant panda research, and put insight into the transition of diet and evolution in the giant panda.

Materials and methods

Raw data processing and OTU assigning

The 16S rRNA sequencing data of feces were retrieved from 27 captive giant pandas fed on shoot, leaf and culm, from our previous study (Yan et al. 2021), and 7 wild giant panda fecal samples data were generated by referring to the open released database including clone library (Zhu et al. 2011). The 16S rRNA gene pyrosequencing data was generated from 7 carnivorous, 11 omnivorous, and 21 herbivorous animal

76 fecal samples (Table 1) (Muegge et al. 2011; Zhu et al. 2011). A total of 73 individual's
77 gene gut microbiota data was included in current study. The obtained sequences were
78 aligned and merged with sequences from Greengenes database, and the operational
79 taxonomic units (OTUs) were delineated at 97% sequence identity by using UCLUST
80 in QIIME (Edgar 2010). We excluded 4 wild pandas sequence datasets with less than
81 500 raw reads per sample to avoid the loss of too much data during subsequent analysis,
82 and conducted comparative analysis on 69 samples.

83 Table 1 16S rRNA gene datasets used from mammals fed different diets

Number	Common name	Genus/Species	Diet	Gut physiology	number of species
1	Bush dog	*Speothos venaticus*	C	SG	1
2	Spotted hyena	*Crocuta crocuta*	C	SG	1
3	Lion	*Panthera leo*	C	SG	2
4	Polar bear	*Ursus maritimus*	C	SG	1
5	Short-beaked echidna	*Tachyglossidae aculeatus*	C	SG	1
6	Southern three-banded armadillo	*Tolypeutes matacus*	C	SG	1
7	Springbok	*Antidorcas marsupialis*	H	FF	1
8	Speke's gazelle	*Gazella spekei*	H	FF	1
9	Bighorn sheep	*Ovis canadensis*	H	FF	2
10	Transcaspian urial sheep	*Ovis orientalis*	H	FF	1

11	Reticulated giraffe	*Giraffa camelopardalis reticulata*	H	FF	1
12	Okapi	*Okapia johnstoni*	H	FF	2
13	Visayam warty pig	*Sus cebifons*	H	FF	1
14	Red kangaroo	*Macropus rufus*	H	FF	1
15	Rock hyrax	*Procavia capensis*	H	FF	2
16	Black and white colobus	*Colobus guereza*	H	FF	1
17	European rabbit	*Oryctolagus cuniculus*	H	HF	1
18	Horse	*Equus equus*	H	HF	1
19	Grevy's zebra	*Equus grevyi*	H	HF	1
20	Black rhinoceros	*Diceros bicornis*	H	HF	1
21	Western lowland gorilla	*Gorilla gorilla*	H	HF	1
22	Sumatran orangutan	*Pongo pygmaeus abelii*	H	HF	1
23	African elephant	*Loxodonta africana*	H	HF	1
24	Capybara	*Hydrochaeris hydrochaeris*	H	HF	1
25	Spectacled bear	*Tremarctos ornatus*	O	SG	1
26	American black bear	*Ursus americanus*	O	SG	1

27	Goeldi's marmoset	*Callimico goeldii*	O	SG	1
28	Hamadryas baboon	*Papio hamadryas*	O	SG	2
29	Chimpanzee	*Pan troglodytes*	O	SG	2
30	Black lemur	*Eulemur macaco macaco*	O	SG	1
31	Ring-tailed lemur	*Lemur catta*	O	SG	1
32	White-faced saki	*Pithecia pithecia*	O	SG	1
33	Prevost's squirrel	*Callosciurus prevostii*	O	SG	1

Abbreviations: C: carnivorous; H: herbivorous; O: omnivorous; FF: foregut fermenter; HF: hindgut fermenter; SG: simple gut. Sequencing region: 16S rRNA V2. Obtained from published data (Muegge et al. 2011; Zhu et al. 2011).

The alpha and beta diversity analysis

Referred to the variation in sequencing results of different samples, a corrected sequencing depth was adopted. The OTU abundance matrix of all the samples unified to the minimum sequencing depth, and the rarefied to the consistent sequencing depth. Flattened data is analyzed to maximize consistency and reliability depicting in both alpha and beta diversity. For comparative evaluation of diversity, all samples in the OTU abundance matrix were randomly re-sampled at the minimum sequencing depth of 90% to adjust the disparities caused by sequencing depth. Furthermore, QIIME (Quantitative Insights in to Microbial Ecology) software was used for calculation of the diversity index for each sample (Caporaso et al. 2010). QIIME pipeline is used to plot the data obtained from UniFrac distance Principal coordination analysis (PCoA) (Thongboonkerd and Saetun 2007).

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100

101 **Prediction of microbial metabolic function**

102 The PICRUSt was applied to predict the metabolic function of gut microbiota
103 inferred from KEGG pathway database (http://www.genome.jp/kegg/pathway.html)
104 (Langille et al. 2013). Existing gene sequencing data were compared with known
105 metabolic functions in the microbial reference genome database to predict the metabolic
106 functions of gut microbiota. The variation of 16S rRNA gene copy number among
107 different species was also considered in the prediction process, and the species
108 abundance data in the original data were corrected to make the prediction results more
109 accurate and reliable (Langille et al. 2013).

110

111 **Results**

112 **The difference of gut microbiota composition by 16S rRNA sequencing**

113 According to 97% sequence similarity, the obtained sequences were compared with
114 Greengenes database and divided into 1790 OTUs. The gut microbiota composition
115 was significantly different between the giant panda and other herbivores, omnivores
116 and carnivores. The α-diversity index showed that the gut microbiota richness and
117 diversity of captive and wild giant pandas were not significantly different, yet both were
118 significantly lower than other herbivores, carnivores and omnivores (both $P < 0.05$).
119 The herbivores exhibit the highest gut microbiota richness and diversity than
120 omnivorous animals ($P < 0.01$). There found no significant difference between
121 omnivorous and carnivorous animals (Fig. 1).

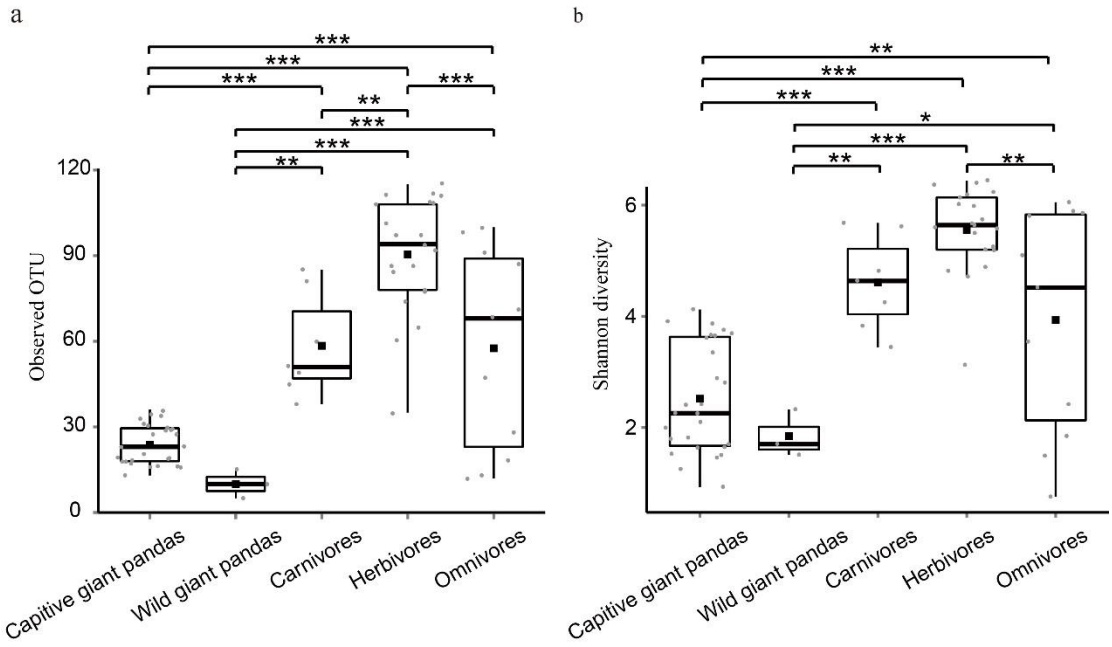

Fig. 1. Comparison of α-diversity of gut microbiota in captive, wild giant pandas and other herbivorous, omnivorous and carnivorous. * P <0.05，** P <0.01，*** P <0.001

Moreover, the captive giant pandas and other feeding species were found clustered together when only presence/absence of gut micrbiota species were taken into account rather than their abundance. This indicates that species with different feeding habits exhibit distinct microbial communities (Fig. 2a). Referred to the gut microbial abundance, samples from the shoot, leaf and culm bamboo diet clearly clustered into three groups in the PCoA, the distance between the leaf and the shoot group was similar, but the distance from the culm group was longer (Fig. 2b). The gut microbiota of pandas feeding purely on culm was similar to wild giant pandas and four omnivores: the spectacled bear (*Tremarctos ornatus*), the American black bear (*Ursus americanus*), the Prevost's squirrel (*Callosciurus prevostii*) and the black lemur (*Eulemur macaco macaco*). Interestingly, the gut microbiota greatly differed between the giant panda and

herbivores (Fig. 2 a&b) although it is a vegetarian species. In general, the gut microbes of captive pandas were markedly different from those of other mammals due to their distinct diet in captivity.

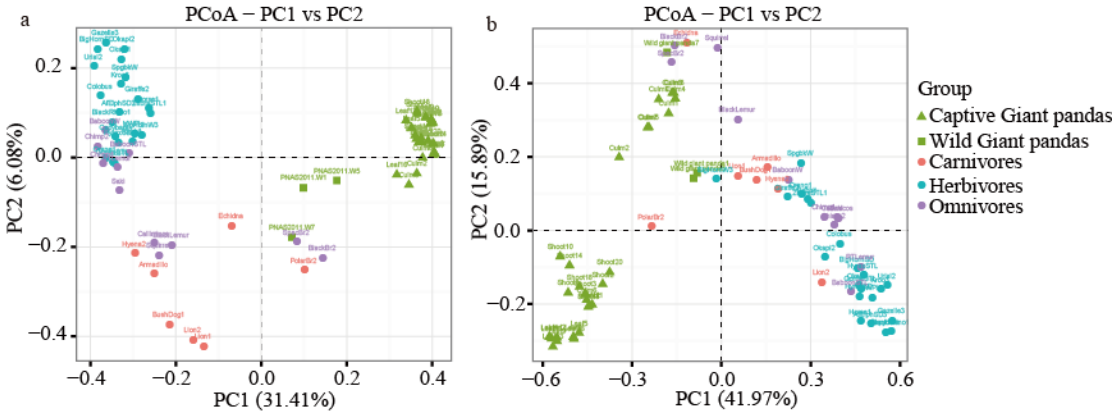

Figure 2. Principal coordinate analysis (PCoA) score plots based on the (a) unweighted and (b) weighted UniFrac distances.

Predict gene function of gut microbiota by PICRUSt

We used PICRUSt to predict 16S rRNA gene sequences in KEGG functional spectrum database. The giant Panda represented similar gut microbiota abundance to other animal in lipid metabolism at the second level of the metabolism pathway, but exhibited lower abundance in energy metabolism, amino acid metabolism, enzyme family, cofactors and vitamins (Fig. 3). In carbohydrate metabolism, captive giant pandas had the lowest abundance, but data was non-significant (Fig. 3). The gut microbiota of giant panda showed the lowest abundance of immune hemostasis by annotation at the second level of the organismal systems pathway, which is significantly lower than herbivores (P <0.001, Fig. 4).

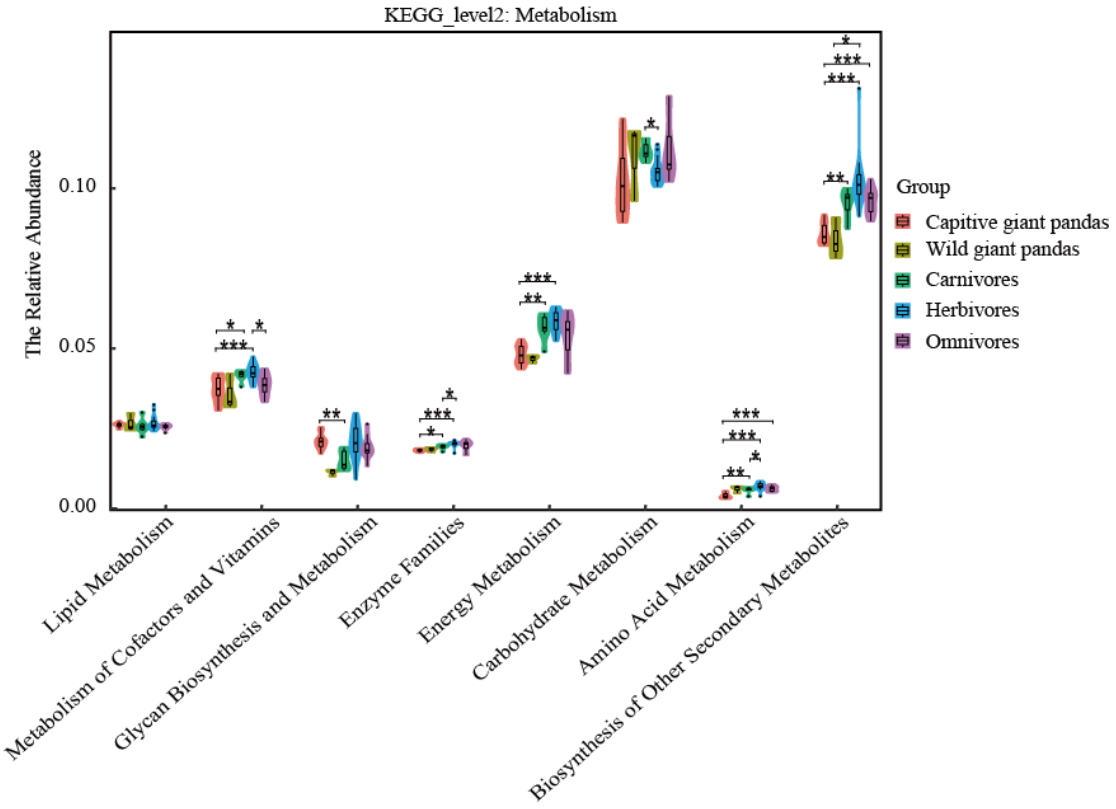

Fig. 3. The difference of predicted metabolic functions on KEGG level 2 determined by PICRUSt.

Note: The ordinate is the relative abundance of each functional group within each group, the thickness of violins reflects the density of sample data distribution. The boxplot border represents the Interquartile range (IQR), the horizontal line represents the median value, the upper and lower whiskers represent the 1.5 times IQR range beyond the upper and lower quartile, and the dot symbol represents the extreme value beyond the range. * P <0.05, ** P <0.01, *** P <0.001, Wilcoxon Rank Sum Tests.

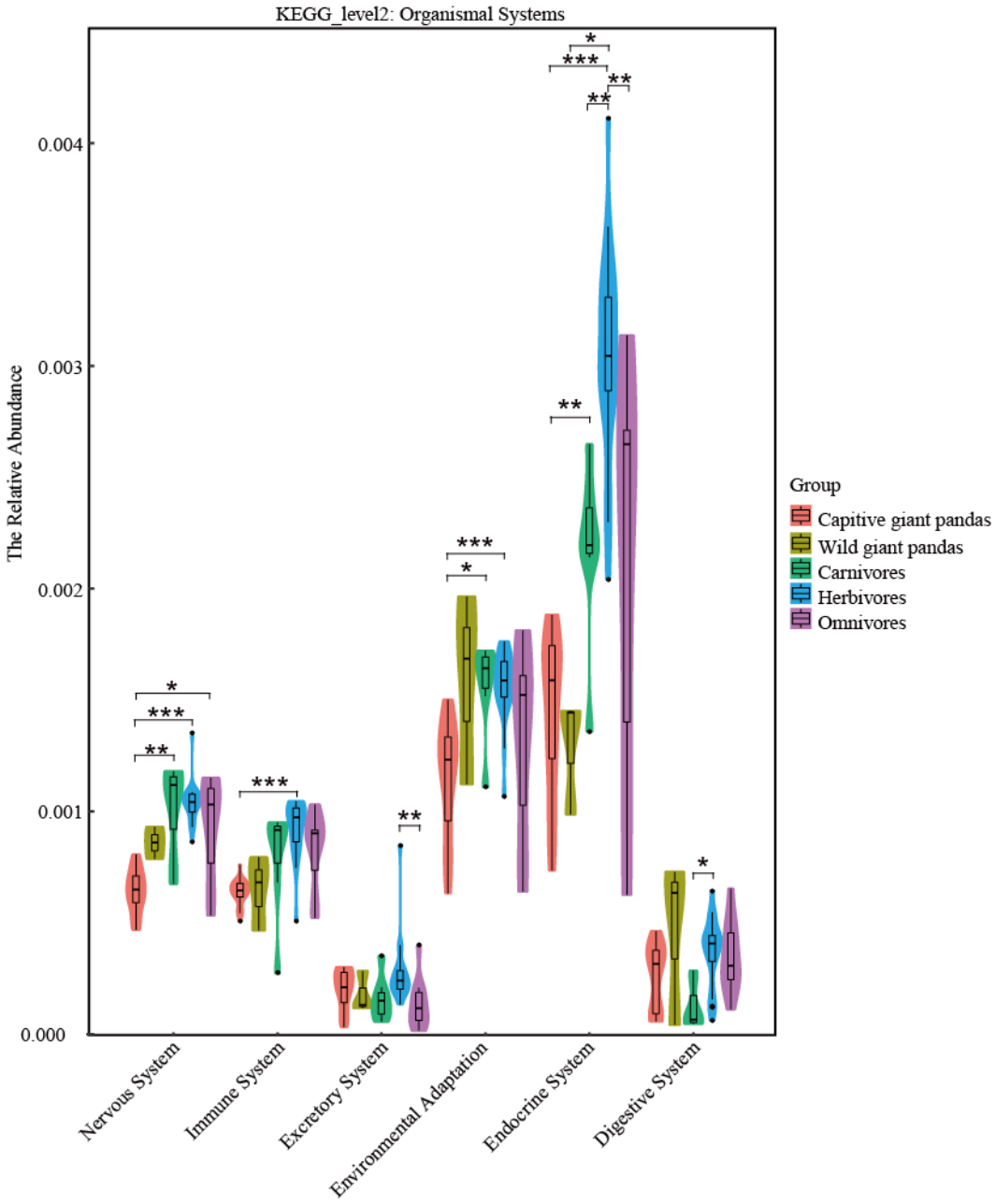

Fig. 4. The difference of predicted organismal systems on KEGG level 2 determined by PICRUSt.

Note: The ordinate is the relative abundance of each functional group within each group, the thickness of violins reflects the density of sample data distribution. The boxplot border represents the Interquartile range (IQR), the horizontal line represents the median value, the upper and lower whiskers represent the 1.5 times IQR range beyond the upper and lower quartile, and the dot symbol represents the extreme value

beyond the range. * P <0.05, ** P <0.01, *** P <0.001, Wilcoxon Rank Sum Tests.

Discussion

The findings of the current study revealed that herbivore and carnivore harbor the highest and lowest gut microbiota diversity upon treatment with different diet interventions (P<0.05, Fig. 1). These results confirm the findings of previous studies in different animals (Ley et al. 2008; Xue et al. 2015; McKenney et al. 2018). The panda constitutes a shorter intestinal track as compared to the high-fiber plant eating herbivore with a total length of 6.05 meters (3.41-7.30 meters), about 4.3 times of the body length, in contrast to 10-22 times of body length in herbivores (Raven 1936; Davis 1964; Keymer 1976). The gut microbiota diversity of the panda is significantly lower than that of herbivores species (P < 0.05, Fig. 1). Herbivores have rumen and longer intestines, endorsing abundant microbial colonization (Fan et al. 2020). The short intestinal tract of giant panda cannot adequately accommodate gut microbiota colonization, resulting in low diversity.

Giant pandas possess a typical carnivore-like short intestinal tract, yet possess significantly lower gut microbiota diversity as compared to carnivores (P<0.05, Fig. 1). This might be related to its bamboo-eating habit. Moreover, captive giant pandas feed primarily on single part of bamboo including shoot, leaf and culm (rich in crude protein, ether extract and crude fiber respectively, P <0.05), in specific seasons (Wang et al. 2017; Yan et al. 2021). During the bamboo culm eating season, the giant panda tends to prioritize the fiber intake than that of bamboo leaf and shoot (3225.40 ± 626.34, 978.95 ± 119.90, 357.78 ± 83.17 g/d respectively) (P < 0.05) (Wang et al. 2017), yet it displayed the lowest diversity in respective season. However, a seasonal diet switch from bamboo culm eating to high protein bamboo shoot diet displayed the highest diversity (P < 0.05) in captive giant pandas (Yan et al. 2021), suggesting that high-fiber

diet causes a lower gut microbiota diversity in the giant panda. Similar results are found in the red panda (*Ailurus fulgens*) and bamboo-eating lemurs (*Hapalemur griseus*), both displayed comparatively lower gut microbial diversity than other carnivorous species (McKenney et al. 2018; Guo et al. 2020). Moreover, the bamboo-eating lemurs exhibit similar gut microbiota to giant panda than that its sister species, the ringtail lemur (*Lemur catta*) (McKenney et al. 2018). The effect of high fiber on the gut microbiota is also demonstrated in captive giant panda cubs, with their gut microbiota diversity level significantly declined after diet switch from a high-protein milk to a high-fiber bamboo diet at weaning (Guo et al. 2018a; Guo et al. 2020). Similar results are illustrated in the red panda cubs transiting diet during weaning time (Williams et al. 2018). However, a recent study on dietary transition of giant panda cubs reported that the elevated consumption of fibrous diet decreased the richness and increased the diversity of gut microbiota (Jin et al. 2021). The distinct outcome is expected owing to different dietary supplements, such as bamboo shoots, carrots and steamed bread (Jin et al. 2021). Similarly, the adult red pandas feeding predominantly on bamboo displayed lower diversity than the weaned cubs feeding on the mix diet of milk and bamboo (Williams et al. 2018). Therefore, an exclusive high-fiber bamboo diet had a significant impact on gut microbiota.

Diet fiber plays an important role in maintaining gut microbial diversity, and it is suggested to have favorable effect on the gut microbial diversity both in animals and human being (Sonnenburg and Sonnenburg 2014; Sonnenburg et al. 2016). However, all aforementioned studies were normal diet oriented and completely different from the panda diet, with an appropriate amount of fat, simple carbohydrates, and with smaller fiber composition (Sonnenburg and Sonnenburg 2014; Sonnenburg et al. 2016). The dietary switch of mice from milk (dominant) and supplementary foods to 50% bamboo

225 powder indicated a substantial increase in gut microbial diversity of mice increased, but
226 decreased when the bamboo content increased to 80% (Guo et al. 2020), suggesting
227 that a diet with excess fiber and low protein content is not conducive to maintaining gut
228 microbial diversity. Accordingly, in the high-fiber bamboo culm consumption period,
229 adequate amount of bamboo shoots should be provided to supplement protein intake to
230 maintain the diversity of gut microbes. However, giant pandas pose high digestibility
231 for fiber in high-fiber bamboo culm diet (Wang et al. 2017), and cellulose content in
232 bamboo exhibit positive correlation with the relative abundance of *Clostridium* in the
233 intestinal tract of giant pandas ($P < 0.05$) (Jin et al. 2020). Therefore, the high-fiber diet
234 steadily decreases the gut microbiota of giant pandas, but a few bacteria attempted to
235 enhance fiber digestion and improve the utilization rate of fiber for energy supply.
236 Diet shifts alters the gut microbes, which in turn affect the host health and
237 physiological functions (Zhernakova et al. 2016). The habitat of modern giant panda is
238 only a small part of the area inhabited by ancient giant panda. Carnivores, herbivores
239 and giant pandas obviously inhabited the three distinct ecological niches, with the giant
240 panda harboring the lowest gut microbial diversity, probably due to the present giant
241 panda exclusively eating bamboo (Han et al. 2019). Despite showing interest in eating
242 bamboo, the modern giant panda get 48~61% of its energy from protein, 23~39% from
243 carbohydrate and 13~16% from fat. The proportion of energy intake via protein
244 consumption is much higher than that of herbivores, and very similar to that of
245 carnivores such as lions and tigers (Nie et al. 2019). In the bamboo shoot-eating period,
246 the butyrate-producing bacterium *Clostridium butyricum* was more abundant in the gut
247 microbiota of the panda, and their body weight increases significantly. Transplant
248 experiment in the mice further proved this point (Huang et al. 2022). Thus, the bamboo
249 shoot period is the key period for the giant panda to replenish energy and nutrients

(Huang et al. 2022). The protein-rich of bamboo shoot diet will lead to significant increase in the gut microbiota richness and diversity of giant pandas (Yan et al. 2021), indicating that the nutrition source of protein depicted better utilization.

The lowest gut microbiota diversity of giant panda results in a significant variation in the in the overall gut microbial structure composition as compared to other mammals (Fig. 2). Mammals on the same feeding habits produce similar types of gut microbes, but reported differences in relative abundance (Fig. 2). Thus, gut microbiota abundance exhibited positive correlation with nutrient intake. The gut microbiota of the giant pandas rapidly reacts to the altered diet. (Yan et al. 2021), indicating that this gut microbiota can elicit rapidly proliferation according to the nutrient source and play digestive and metabolic functions.

Gut microbiota plays a potential role in maintaining energy balance (Clemmensen et al. 2017). The gut microbiota function differs significantly between the giant pandas and other mammals. The captive giant pandas possess the lowest level of energy metabolism, amino acid metabolism and enzyme spectrum of gut microbiota as compared to other animals on normal diet ($P < 0.05$, Fig. 3). However, there is reported no difference between the gut microbiota function of giant panda and other species in lipid metabolism (Fig. 3). The low intake of fat in these species indicated that fat in food is not the main source of energy. Gut microbiota stimulates the development and regulation of mucosal immune hemostasis. It plays a central role in a variety of physiological functions, including regulating inflammatory signaling and preventing infection (Pagliari et al. 2018). Low microbial diversity has been identified as a risk factor for different chronic diseases, such as intestinal inflammatory diseases, obesity and insulin resistance (Le Chatelier et al. 2013; Crovesy et al. 2017; Statovci et al. 2017; Sokol et al. 2018). Giant pandas possess reduced gut microbiota diversity (Fig.1),

speculated the low level of relative abundance of immune system function genes, which was significantly lower than that of herbivores with higher gut microbiota diversity (Fig. 4).

Spanning long evolutionary history, the host mammals and their indigenous microbial communities have co-evolved (Ley et al. 2008). Gut microbiota-host interactions contribute to the maturation of the host immune system and regulate its systemic response (D'Amelio and Sassi 2018). Endangered species that feed on bamboo such as the giant panda, bamboo lemur and the red panda have developed unique adaptation in the gut microbiota to a high-fiber diet (McKenney et al. 2018). Because the constantly changing foraging strategies of different endangered wild animals, a perspective of natural diet for raising and management of captive wild mammals require evidence based knowledge of nutrient-species relationship and to understand the detailed intricacies for their implementation. Bamboo shoots were thought to be more appropriate for giant panda than bamboo culms because of their higher crude protein and crude fat content (Wang et al. 2017). However, wild giant pandas chose bamboo culm as a food source in the proestrus and estrus period of giant pandas (Schaller et al. 1985; Taylor and Zisheng 1987; Fuwen et al. 2011; Wei et al. 2017). And it has been reported that adding crude fiber in animal diet during estrus could have productive effects such as increased gonadotrophin concentration, improve oocyte quality and maturity, and increase embryo survival (Ferguson et al. 2007; Weaver et al. 2013). Owing to protection endangered wild animals, captive institutions should broaden their understanding dietary rules and come up with the best dietary plan to raise and maintain them.

In conclusion, comparative analysis of the gut microbiota diversity level of giant panda and other diet feeding animals indicated low level of diversity in gut microbiota

of because of its short intestinal tract and high dietary fiber content. The low diversity and community richness of giant panda gut microbiota accounted for reduced level of energy and amino acid metabolism. These findings have important implications for understanding how high-fiber dietary strategies affect host-microbiome relationships.

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