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Diet, habitat environment and lifestyle conversion affect the gut microbiomes of giant pandas

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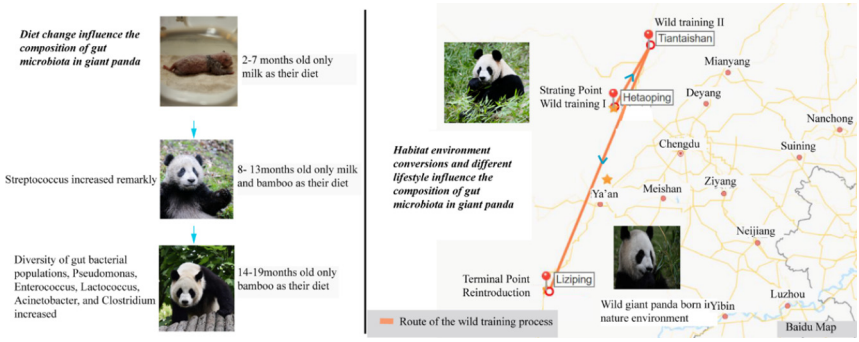
HIGHLIGHTS

- Diet conversion, habitat and lifestyle changes could influence gut microbiota remarkably in GP.
- High fiber diets significantly increased the diversity of gut bacterial populations of GP.
- *Streptococcus*, *Pseudomonas*, *Enterococcus*, *Lactococcus*, *Acinetobacter*, and *Clostridium* contribute to lignocellulose digestion.
- GM structure in reintroduced GPs converged to that of wild pandas.

GRAPHICAL ABSTRACT



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ABSTRACT

Gut microbiota (GM) are important for the health of giant pandas (GPs), in addition to the utilization of bamboo in their diets. However, it is not fully understood how diet, habitat environment and lifestyle contribute to the composition of GM in GP. Consequently, we evaluated how dietary changes, habitat environment conversions and lifestyle shifts influence the GM of GPs using high-throughput sequencing and genome-resolved metagenomics. The GM of GPs were more similar when their hosts exhibited the same diet. High fiber diets significantly increased the diversity and decreased the richness of gut bacterial communities alone or interacted with the age factor ($p < 0.05$). The abundances of *Streptococcus*, *Pseudomonas*, *Enterococcus*, *Lactococcus*, *Acinetobacter*, and *Clostridium* significantly increased during diet conversion process (Non-parametric factorial Kruskal-Wallis sum-rank test, LDA > 4). Reconstruction of 60 metagenome-assembled-genomes (MAGs) indicated that these bacteria were likely responsible for bamboo digestion via gene complements involved in cellulose, hemicellulose, and lignin degradation. While habitat environment may play a more important role in shaping the GM of GP, lifestyle can also greatly affect bacterial communities. The GM structure in reintroduced GPs notably converged to that of wild pandas. Importantly, the main bacterial genera of wild GPs could aid in lignin degradation, while those of reintroduced GPs were related to cellulose and hemicellulose digestion. *Streptococcus*, *Pseudomonas*, *Enterococcus*, *Lactococcus*, *Acinetobacter*, and *Clostridium* may contribute to

Abbreviations: GM, gut microbiota; GP, giant panda; CCRCGP, China Conservation and Research Center for the Giant Panda; OMD, only milk diet; MBD, milk and bamboo diet; OBD, only bamboo diet; OTU, operational taxonomic unit; MAGs, metagenome assembled genomes; KEGG, Kyoto Encyclopedia of Genes and Genomes; CAZys, carbohydrate-active enzymes; PCoA, Principal Coordinates Analysis; AAs, auxiliary activities; GHs, glycoside hydrolases; CEs, carbohydrate esterases; LDA, Linear Discriminant Analysis; LEfSe, linear discriminant analysis coupled with effect size.

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lignocellulose digestion in GP. The results revealed that diet conversion, habitat environment and lifestyle could remarkably influence the GM of GP. In addition, results suggested that increasing the ability of lignin degradation with GM may aid to change the GM of reintroduced pandas to resemble those of wild pandas.

1. Introduction

Gut microbiota (GM) play beneficial roles in the production of enzymes, food digestion, homeostasis and immune systems of hosts (Claesson et al., 2012; Round and Mazmanian, 2009; Sommer and Backhed, 2013). Consequently, changes in the composition, diversity, or abundance of GM are frequently associated with diseases and immune system problems (Evans et al., 2013; Zhernakova et al., 2016). In addition, a considerable body of studies over the past decade have revealed that host diet, stressors, and biogeography are major factors that affect GM dynamics (Knight and Girling, 2003; Versalovic and Relman, 2006).

Giant pandas (*Ailuropoda melanoleuca*, GPs) are solitary animals endemic to China, and their wildlife population sizes are small (Yang et al., 2018; Zhang et al., 2018; Schaller et al., 1985). GPs belong to the order Carnivora and possess the typical carnivorous digestive system, yet they are well known for their unique diet comprising bamboo (Wei et al., 2015; Zhu et al., 2011). Interestingly, the GP has not evolved any genome encoding enzymes specific for cellulose digestion, despite their unique dietary adaptation (Hu et al., 2017). Therefore, it is not known how GPs can rely on high fiber diets characterized by low nutritional value components. It has consequently been hypothesized that GPs rely on symbiotic gut microbial populations to degrade nutritional components of their highly fibrous diets including cellulose, hemicelluloses, and lignin, which are all key components of their bamboo diet (Hu et al., 2017). Bamboo fermentation tests using GP gut microbiomes showed that they can degrade lignocellulose (Scoma et al., 2020). However, whether this activity contributes to the nutrition of GPs is open to debate. An in vitro enzyme activity assay for cellulose and hemicellulose-degradation in GP feces showed that GP had the lowest enzyme activity among major herbivores (Guo et al., 2018). Whether this in vitro result could represent the situation in vivo is still an open question (Joaan et al., 2014). Zhu et al. reported that the gut microbes of GP had genes coding cellulose and hemicellulose-digesting enzymes (Zhu et al., 2011). Zhang et al. found that the abundance of the genes encoding hemicellulose-digesting enzymes was significantly higher in bamboo-eating GPs than in milk-eating GPs, and suggested that hemicelluloses are the main energy source in the diet of GPs (Zhang et al., 2018). Despite the investigation of this hypothesis by multiple studies, the dynamic expression and regulation of the genes encoding were still unclear (Wei et al., 2018).

GP cubs exhibit unique dietary conversion phases, changing from milk to bamboo diets during development. Significant shifts in the composition of GM concomitantly occur in GP infants during the transition to more solid and varied diets (Sghir et al., 2000). Thus, investigating GM variation within GPs during dietary shifts may reveal the mechanisms underlying the dietary specialization of GPs. Studies on the GM of milk-fed and bamboo-fed GPs are quite rare (Guo et al., 2018; Zhang et al., 2018). Due to low number of subjects and differences in experimental design, including diet period, diet amount and start time, it has been difficult to infer the influences of diet conversion on the GM of GPs.

Habitat environment conversion resulted in changes in the composition of GM in deer mice and primate (Schmidt et al., 2019; Clayton et al., 2016). GPs undergo a habitat environment conversion in their wild-training process (Tang et al., 2019). Studies on the wild-training process of GPs have compared the GM composition between the captive, semi-wild, reintroduced and wild GPs, whereas the influence of habitat environment on GM has received little attention (Yao et al., 2019a,b; Tang et al., 2019). Moreover, the differences in microbiota between individuals are a significant confounding factor when comparing community structures (Xue et al., 2015). The GM of one-year old GPs has reached a stable state, and its functions are mostly close to those of the adult microbiotata (Zhang et al., 2018). Based on a previous observation, bamboo is the main part in the diet of reintroduced GPs diet structure after the age of 14 months. Thus, approximately 14-month old and older GPs can be chosen for a wild-training process habitat conversion experiment (Yao et al., 2019a,b; Tang et al., 2019).

The reintroduction of captive GPs is effective at increasing their wild population sizes and mitigating population declines. The reintroduction of extirpated or threatened species is a remedial measure that can generally prevent species extinctions, and has been used in conservation efforts for wolves (Smith et al., 2000) and giant tortoises (Gibbs et al., 2010). Remarkable achievements have been made in the GP conservation breeding program (e.g., through mating, artificial insemination, and parental care behaviors), contributing to the sustainment and increase in captive GP populations that can be used in reintroduction efforts to supplement wild populations (D. Li et al., 2017; Y. Li et al., 2017; Wei et al., 2015; Zhang et al., 2004). Human-associated microbial communities may change quickly and profoundly due to the actions and experiences of the host (David et al., 2014). Likewise, accumulating evidence has emphasized that gastrointestinal disease is a primary cause of GP death (Tun et al., 2014), indicating that their gut microbial communities play crucial roles in improving reintroduction success rates. A recent study compared the GM of the reintroduced and wild GPs, yet robust conclusions on the influence of lifestyle on GM could not be drawn since only one reintroduced GP was available (Yao et al., 2019a,b; Tang et al., 2019).

Consequently, the aims of this study were to evaluate the influence of dietary change, habitat environment conversion and different lifestyle on the diversity and composition of GP gut microbial communities to understand the interactions between the host and GM, which will further support the successful reintroduction of GPs and future conservation efforts.

2. Materials and methods

2.1. Experimental design

2.1.1. Diet conversion experiment

Five one-month-old captive GPs from the Shenshuping Base of the China Conservation and Research Center for the Giant Panda (CCRCGP) that had been fed by breast milk were chosen for the diet conversion experiment. The diet conversion experiment was separated three stages: OMD (only formula milk as their diet), MBD (formula milk and bamboo diet), and OBD (only bamboo as their dominant diet). In the OMD stage, GPs that received 200–600 mL formula milk per day, and their feces were collected at 2, 3, 4, 5, 6, and 7 months of age. In the MBD stage, the GPs received 1 L of formula milk per day and bamboo was introduced to their daily diets with weights based on age. At 8 months of age, 3.0 kg of bamboo; at 9 months of age, 3.0 kg bamboo; at 10 months of age, 5.0 kg bamboo; at 11 months of age, 5.0 kg bamboo; at 12 months of age, 8.0 kg bamboo; and at 13 months of age, 8.0 kg bamboo. In addition, 200–500 g bamboo shoots per day were also given for each GP. In the OBD stage, the GPs were fed with 15 kg of bamboo per day and feces were collected at 14, 15, 16, 17, 18, and 19 months of age. In addition, 0.5–2 kg bamboo shoots, 200–500 g steamed bread and 300 g carrots per day were given for each GP. The health status of GPs was monitored daily by veterinarians. The GPs received no dietary supplements and

antibiotics during the experiment. The nutritional details of formula milk are in Supplementary Table S1.

2.1.2. Habitat environment conversion and lifestyle experiment

Two14-month-old and one 16-month-old captive GPs at the Hetaoping Base of CCRCGP were chosen for the habitat environment conversion experiment. The experiment was separated into three stages: wild training I, wild training II, and reintroduction groups. The GPs were maintained in wild training I until 20–22 months old, and in wild training II for 6 more months until they were 26–28 months old. Finally, the GPs were reintroduced to the Liziping National Nature Reserve in Ya'an, Sichuan (29°2′ N, 102°46′ E), a natural forest environment without any human disturbance. In the wild-training, the GPs were living together with their mothers, and bamboos formed the staple food for the GPs. After learning skills to survive in the wild during wild-training I and II, the GPs were separated from their mothers 2–3 months before being reintroduced to the natural forest environment. The feces were collected from wild GPs as controls of the reintroduced samples for the different lifestyle experiment. GPs were fitted with GPS collars during the wild-training II and reintroduction stages after approval from the State Forestry Administration of China. Captive GPs lived in man-made limited space and tourists could visit. Wild training I and wild training II GPs lived in a large area and natural habitat or primeval forest without any disturbance by humans (the environment space of wild training II was larger than wild training I). Reintroduced GPs lived in primeval forest, where the wild GPs live.

2.2. Captivity conditions

The captivity facilities were located at the Shenshuping Base of CCRCGP in Wolong, Sichuan (31°1′N, 103°18′E) at an altitude of approximately 1500 to 1700 m. The base was open for tourists. GPs were housed alone in a room with a 580 cm × 230 cm × 270 cm animal house and a 580 × 1300 cm playground. The room was constructed by a rail network with playground equipment (e.g., a sliding board) and a pool that provided water. Trees and bamboo were planted around the room and adjacent rooms were separated by concrete walls.

2.3. Wild-training I conditions

The wild-training I area was located at the Hetaopinge Base of CCRCGP, Wolong, Sichuan (31°4′N, 103°13′E), at an altitude of approximately 2840 m. Tourists were not allowed to visit the area. The wild-training GPs lived with their mothers and learned skills to survive in the wild from their mothers (e.g., tree climbing skills, foraging and avoiding the danger). Wild-training GPs were housed in a room similar that in captivity, surrounded by natural forest area of approximately 2300–3200 m^2. Each GP was trained in a separate wild training area. Bamboos formed the staple food for GP.

2.4. Wild-training II conditions

The larger wild-training II facilities were located at the Tiantaishan Base, Wolong, Sichuan (31°1′N, 103°34′E). The base is a temperate deciduous forest at an altitude of approximately 2070–2140 m. Tourists were not allowed to visit the approximately 1.4 km^2 area. The wild training II area was constructed with steel plates and a barbed-wire fence, with a stream flowing through the area. At the Tiantaishan Base, the bamboo species *Fargesia robusta*, and *Bashania faberi* are the dominant food sources of GPs, comprising 72.2% and 20.4% of the vegetation, respectively. *Yushania brevipaniculata* were interspersed in the *Fargesia robusta* bamboo forest. The wild-training II area is of high biodiversity, and similar to those occupied by wild GPs in the Wolong National Natural Reserve. Each GP was trained in a separate wild-training II area.

2.5. Reintroduced and wild conditions

According to the learning status in wild-training II, individuals G1 and G2 were chosen for reintroduction. The approximately 479.4 km^2 reintroduction area is a nature forest, at the Liziping National Nature Reserve, Ya'an, Sichuan (28°51′-29°08′N, 102°10′-102°29′E). Approximately 60 mammal species, e.g. GPs, takin, leopard, tufted deer and porcupine live in this reserve. GPs live at the altitude of approximately 2400–3400 m. In this area, *Bashania spanostachya* and *Fargesia dulcicula* bamboos constitute the dominant food for reintroduced and wild GPs.

2.6. Sample collection

2.6.1. Diet conversion experiment

Feces from each individual were collected every month during the diet conversion experiment. Fecal samples were collected immediately after defecation in sterilized plastic bags, and kept cold with dry ice during transport to the laboratory. A total of 180 fecal samples were taken for microbial community compositional analysis via high-throughput 16S rRNA gene sequencing from the five captive GP cubs. Following quality assessment (Bokulich et al., 2013), a total of 168 fecal samples were subjected to bacterial 16S rRNA sequencing and 108 of those samples were subjected to fungal ITS sequencing. The diet conversion experiment comprised three different periods, including the only milk diet period from 2 to 7 months old (OMD, 16S rRNA sequencing $n = 56$; ITS sequencing $n = 36$), the milk and bamboo mixed diet period from 8 to 13 months old (MBD, 16S rRNA sequencing n = 56; ITS sequencing n = 36), and the bamboo only diet period from 14 to 19 months old (OBD, 16S rRNA sequencing n = 56; ITS sequencing n = 36; metagenomic binning $n = 60$) (Supplementary Table S2).

2.6.2. Habitat environment conversions and lifestyle experiments

To evaluate the effects of habitat environment and lifestyle conversions, the fecal samples of GPs only bamboo fed were collected during two months before wild-training II and reintroduction, and finally one year after reintroduction to the wild. The wild-training II and reintroduced GPs were tracked with GPS collar to collect the feces. Feces of wild GPs in the natural forest environment were also collected to be compared with the feces of the reintroduced GPs. Samples were aseptically collected in sterilized plastic bags and immediately frozen in dry ice. A total of 61 fecal samples from wild-training II and reintroduced GPs were collected. Following quality control, 48 fecal samples were successfully subjected to 16S rRNA gene sequencing and 33 samples to fungal ITS sequencing. The habitat environment conversions comprised three stages including the wild-training I (16S rRNA sequencing $n = 17$; ITS sequencing $n = 10$), wild-training II (16S rRNA sequencing $n = 16$; ITS sequencing $n = 12$), and reintroduced (16S rRNA sequencing n = 16; ITS sequencing $n = 13$) individuals. An additional 15 and 12 fecal samples were collected from wild GPs for 16S rRNA gene and ITS high-throughput sequencing, respectively. The reintroduced and wild are two different lifestyles, with different foraging habit and activity histories. According to the learning status of wild-training II GP, G1 and G2 were chosen for reintroduction. Detailed information on samples is in Supplementary Table S3.

2.7. DNA extraction, amplicon sequencing, and metagenomics analysis

Microbial genomic DNA was isolated from fecal samples with the MoBio PowerFecal DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) following the manufacturer's instructions. Fungal DNA was extracted using the E.Z.N.A.™ Fungal DNA Mini Kit (OMEGA Bio-tek, Inc., Norcross, GA, USA) according to manufacturer's instructions. The quality of extracted DNA was assessed by electrophoresis in 1% agar gel. Bacterial and fungal amplicon libraries were prepared by amplifying the V4 hypervariable region of 16S rRNA genes and the ITS1 region, respectively, as described previously (Huang et al., 2015). Amplicon

sequencing was performed on the Illumina Hiseq 2500 (diet conversion experiment) and Illumina Miseq 2500 (habitat environment conversion and lifestyle shift experiment) platforms to generate 150 bp paired-end reads.

Metagenomic binning ($n = 60$, fecal samples from OBD) was conducted using metabat2 to bin samples from the single-sample assemblies and the co-assembly as described previously (Stewart et al., 2018).

2.8. Data analysis

Raw paired-end sequences were preprocessed using the HiSeq Control Software (diet conversion experiment) and the MiSeq Control Software (habitat environment conversion and lifestyle shift experiment) programs. Briefly, paired-end reads were merged using FLASH (V1.2.7, http://ccb.jhu.edu/software/FLASH/), with overlap greater than or equal to10 bp and the mismatch rate less than0.1. After filtering low-quality reads, clean amplicon reads were imported into the QIIME 1.9.1 (http://qiime.org/scripts/split_libraries_fastq.html), with q less than or equal to 19 (Caporaso et al., 2010). The 16S rRNA gene and ITS sequences were clustered at the 97% nucleotide sequence similarity level to generate operational taxonomic units (OTUs). OTUs were assigned to taxa using the SILVA 132 (Quast et al., 2013) and UNITE 7.2 (Koljalg et al., 2013) reference databases for the bacterial and fungal libraries, respectively, using mothur (threshold values 0.8–1.0) and blast (e-value ≤ 1e-05) methods. Chao1 and Shannon index richness/diversity metrics were calculated in QIIME 1.9.1 (Identity = 97%) and visualized in Prism7. Principal Coordinates Analysis (PCoA) was conducted on the OTU compositional matrices using the Bray-Curtis (BC) distance, as implemented in QIIME 1.9.1 with the default settings and visualized in R3.5.0 (WGCNA, stats and ggplot2 packages). Linear discriminant analysis coupled with effect sizes (LEfSe) was conducted in the galaxy platform (http://huttenhower.sph.harvard.edu/galaxy/root). A cladogram with circular representations of taxonomic compositions and phylogenetic trees were produced using GraPhlAn (Truong et al., 2015).

Whole genome shotgun libraries composed of approximately 400 bp clone inserts were generated for associated samples. Metagenomic sequencing of the library was performed on the Illumina HiSeq4000 platform (Illumina, Inc., San Diego, CA, USA) using 2 × 150 bp paired-end sequencing mode at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). Filtering low quality reads were conducted in Seqprep (https://github.com/jstjohn/SeqPrep) and Sickle (https://github.com/najoshi/sickle) software, with a length <50 bp and those reads with an average quality score < 20. Meanwhile, reads that matched the genomes of the host (*Ailuropoda melanoleuca*) and the plants *Malus domestica*, *Daucus carota*, *Zea mays*, *Oryza sativa* and *Glycine* max (https://www.ncbi.nlm.nih.gov/genome/) were removed using the bwa aligner (http://bio-bwa.sourceforge.net/). The filtered and microbiota-enriched reads were subjected to contig assembly using IDBA-UD (http://i.cs.hku.hk/~alse/hkubrg/projects/idba_ud/) (Peng et al., 2012). RefineM (https://github.com/dparks1134/RefineM) was used to compare the annotation of contigs with the annotation results of bins and 16S rRNA genes, and to remove inconsistent contigs. The Metabat2 genome binning program was used to bin the contigs of the sample assemblies (Stewart et al., 2018) into metagenome assembled genomes (MAGs). A total of 449 draft MAGs were recovered and dRep (https://github.com/MrOlm/drep) was used to replicate MAGs (Olm et al., 2017). Dereplication resulted in a total of 22 high quality MAGs with completeness values ≥ 70% and contamination ≤ 10%, as assessed by CheckM (https://github.com/Ecogenomics/CheckM/wiki) (Parks et al., 2015). The high-quality MAGs were retained for further analyses. Prodigal (http://compbio.ornl.gov/prodigal/) was used to predict genes within the high quality MAGs (Hyatt et al., 2010). Functional annotation of the predicted genes was conducted via BLASTP (BLAST 2.2.31+, http://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis against the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/) (e-value ≤ 10^{-5}) and Carbohydrate-active enzymes 5.0 (CAZys, http://www.cazy.org/) databases (e-value ≤ 10^{-5}). Housekeeping in the 22 MAGs were identified using amphora2 (https://github.com/martinwu/AMPHORA2).

One-way analysis of variance (ANOVA) analyses (experiment design: each row represents matched) were conducted in SPSS 22, used to identify significant differences in the alpha diversity (including Chao-1 richness estimate and Shannon diversity index) among communities, as based on amplicon sequencing. All tests for significance were two-sided and used a p value < 0.05 to determine statistical significance. Bray-Curtis dissimilarity differences in GM community composition among different groups were tested using permutational MANOVA (PERMANOVA) with 9999 random permutations in the vegan package of R 3.5.0, and used to identify significant differences in GM structure among different groups. The Bonferroni correction was applied for multiple comparisons. Differences were taken as statistically significant at p value < 0.05. Linear discriminant analysis coupled with effect sizes (LEfSe) was conducted in the galaxy platform (http://huttenhower.sph.harvard.edu/galaxy/root). Non-parametric factorial Kruskal-Wallis sum-rank tests were used to test differences at phylum and genus level taxonomic compositions between groups in the LEfSe. LDA (Linear Discriminant Analysis) was used to estimate the effect sizes of each feature using a normalized relative abundance matrix. An LDA value > 4.0 was considered statistically significant.

3. Results

3.1. Variation in gut bacterial composition during giant panda diet conversion

After quality filtering, a total of 13,650,999 bacterial 16S rRNA gene sequences were obtained from 168 fecal samples from the diet conversion experiment. The sequences were clustered into 3027 OTUs at the 97% sequence identity threshold. Both community richness (Chao1 index) and diversity (Shannon index) varied with host diet, and significant differences in these values were observed between the three experimental groups ($p < 0.05$, ANOVA test) (Fig. 1a, b). Specifically, gut bacterial diversity increased when transitioning from OMD to MBD and OBD diets, while richness conversely declined. Principal Coordinates Analysis (PCoA) also indicated that samples from the same diet group clustered together and were separated from the other groups (Fig. 1c). Significant differences in community structure were identified among the three groups based on BC distances (Bray Curtis: $F = 47.89$, $R^2 = 0.31$, $p < 0.001$).

Proteobacteria and Firmicutes comprised >90.0% of the total relative abundance in all groups (Fig. 1d). Proteobacteria was the most dominant phylum in OMD (comprising 85.5% of the total relative abundance) and MBD (57.7%) communities (Fig. 1e). Firmicutes was the most dominant phylum in OBD (58.3%) communities, and its relative abundance increased during diet conversion (Non-parametric factorial Kruskal-Wallis sum-rank test, LDA > 4) (Fig. 1d, e).

The distribution of the 10 most abundant genera in each group (comprising >80.0% of the total relative abundance in each group) were further investigated (Fig. 1f). The three most abundant genera in the OMD samples were *Escherichia-Shigella* (80.1%), *Streptococcus* (7.9%), and *Lactobacillus* (1.9%). The abundance of *Escherichia-Shigella* sharply decreased in the OBD communities relative to the OMD communities (Non-parametric factorial Kruskal-Wallis sum-rank test, LDA > 4) (Fig. 1e, f). The three most abundant genera in the MBD communities were *Escherichia-Shigella* (43.8%), *Streptococcus* (16.8%), and *Lactobacillus* (10.1%). The relative abundance of *Streptococcus* was significantly higher in the MBD group than in the other groups (7.9% and 11.4% in OMD and OBD) (Non-parametric factorial Kruskal-Wallis sum-rank test, LDA > 4) (Fig. 1e, f). *Pseudomonas* (13.4%), *Lactobacillus* (12.7%), and *Clostridium* (12.2%) were the three most abundant genera in the OBD communities. In addition, the relative abundance of

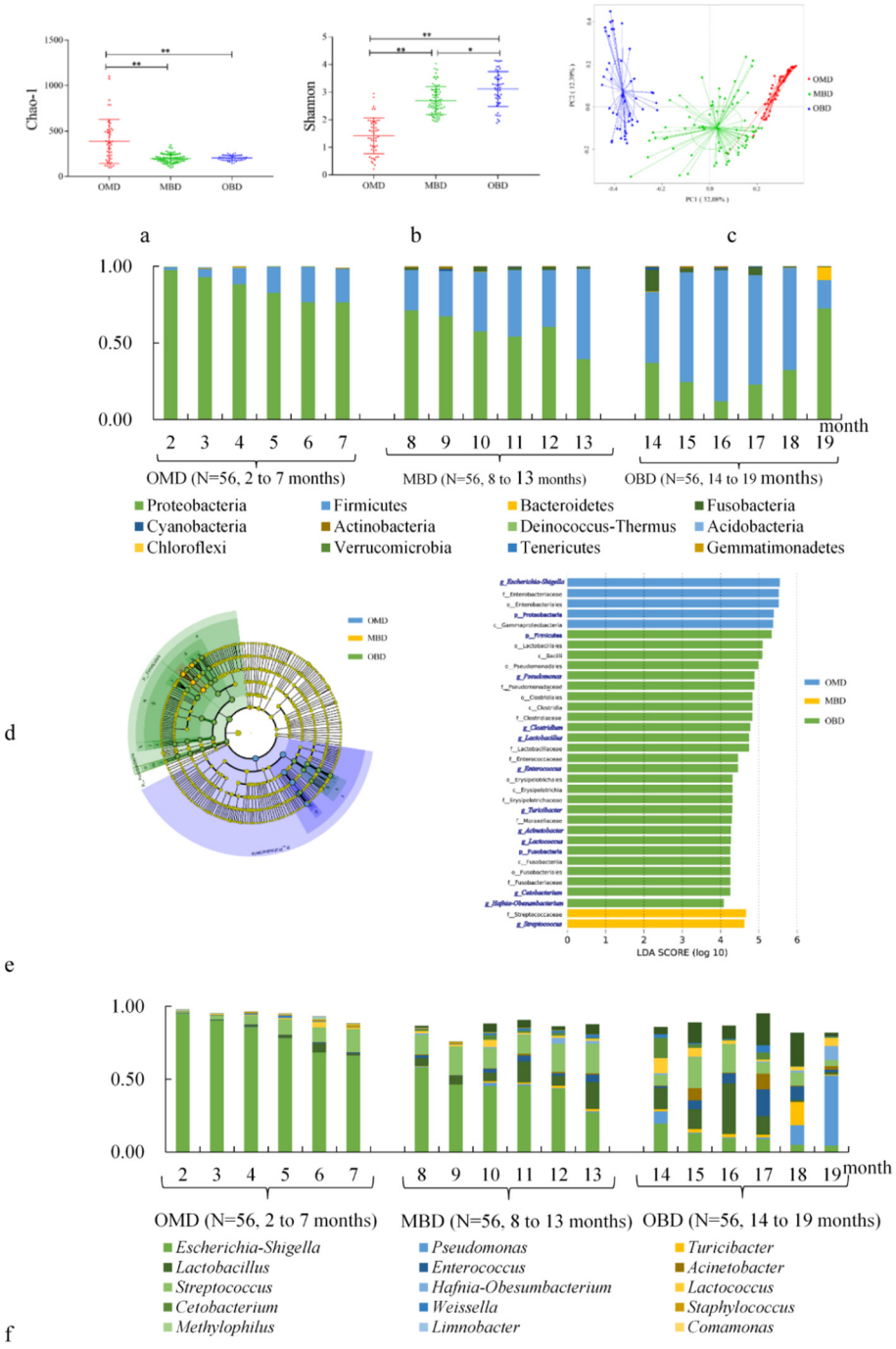

Fig. 1. Gut microbiome variation due to dietary change. (a) Variation in gut microbiome richness during dietary shifts. Each circle represents a sample, wherein OMD group treatment communities: red, MBD: green, and OBD: blue. (b) Variation in gut microbiome diversity during dietary shifts. Circles represent samples and are colored as indicated in panel a. (c) Principal Coordinates Analysis (PCoA) of gut microbiome structures from the OMD, MBD, and OBD experimental groups. PC1 and PC2 are shown on the x and y axes along with the percent variation explained by each. Circles represent samples and are colored as indicated in panel a. (d) Differences in overall bacterial phylum-level compositions of the communities from the OMD, MBD, and OBD experimental groups. (e) Significantly different bacterial taxa among the OMD, MBD, and OBD experimental groups, as identified by linear discriminant analysis coupled with effect size (LEfSe) using the default parameters. Blue symbols: OMD, orange: MBD, green: OBD. Blue text shows phyla and genera. (f) Differences in overall bacterial genus-level of the communities from the OMD, MBD and OBD experimental groups. *: $0.01 < p < 0.05$; **: $p < 0.01$.

Pseudomonas, *Lactobacillus*, *Clostridium*, *Enterococcus*, *Lactococcus*, *Turicibacter*, *Acinetobacter*, *Cetobacterium*, and *Hafnia-Obesumbacterium* abundances also significantly increased when transitioning from the OMD (0.2%, 1.9%, 0.1%, 0.4%, 1.0%, 0.0%, 0.2%, 0.0%, and 0.0%, respectively) to the OBD (13.4%, 12.7%, 12.2%, 7.0%, 4.8%, 3.9%, 3.9%, 3.6% and 2.4%, respectively) communities (Non-parametric factorial Kruskal-Wallis sum-rank test, LDA > 4) (Fig. 1e, f).

3.2. Metagenomic analysis of gut bacterial metabolic functions in giant pandas

A total of 22 high quality MAGs were obtained from the metagenomics analysis. Thirteen MAGs were classified to the genus level (Table 1). We focused on the KEGG database-mapped metabolic pathways associated with *Streptococcus*, *Pseudomonas*, *Lactobacillus*,

Table 1
Genome statistics for the 22 drafts metagenome-assembled-genomes that were reconstructed.

Draft genome	Total bases (Mbp)	Number of contigs	GC content (%)	Completeness (%)	Contamination (%)	Taxonomic identification
CB1	1892	168	0.42	92.1	6.21	*Streptococcus*
CB2	1131	197	0.39	70.6	0.27	*Lactobacillus reuteri*
CB3	2549	257	0.4	79.4	3.9	*Acinetobacter* sp.
CB4	2601	197	0.41	80.2	2.51	*Acinetobacter* sp.
CB5	2100	214	0.35	84.5	0.57	*Lactococcus lactis*
CB6	1826	20	0.41	99.2	0.26	*Lactobacillus* sp.
CB7	1768	64	0.38	88.3	0.51	*Lactococcus* sp.
CB8	2463	131	0.38	98.5	0.47	*Enterococcus* sp.
CB9	3262	8	0.64	71.7	0.14	*Pseudomonas*, sp.
CB10	1800	50	0.53	98.6	0.00	*Lactobacillus fermentum*
CB11	5103	299	0.59	97.9	1.86	*Agrobacterium* sp.
CB12	6299	221	0.67	99.0	0.77	*Delftia acidovorans*
CB13	2715	398	0.37	82.3	1.35	*Fluviicola taffensis*
CB14	3775	583	0.6	78.4	2.89	Enterobacteriaceae
CB15	3105	435	0.62	82.3	2.17	Sphingomonadaceae
CB16	4364	20	0.67	99.5	1.99	Xanthomonadaceae
CB17	2250	148	0.31	98.9	0.00	Fusobacteriaceae
CB18	1509	279	0.47	78.2	3.77	Gammaproteobacteria
CB19	5325	456	0.71	98.2	7.82	Burkholderiales
CB20	2310	104	0.34	87.2	0.60	Unidentified bacteria
CB21	4284	781	0.37	77.4	2.13	Unidentified bacteria
CB22	4301	565	0.38	85.4	1.21	Unidentified bacteria

Enterococcus, *Lactococcus*, and *Acinetobacter* in the diet conversion experiment due to their differential abundances based on 16S rRNA gene analyses. Catalase-peroxidase (EC 1.11.1.21, *katG*), catechol 2,3-dioxygenase (EC 1.13.11.2, *dmpB*), NADPH: quinone reductase (EC 1.6.5.5, *qor*) and triacylglycerol lipase (EC 3.1.1.3, *TGL2*) that are associated with lignin degradation were observed in all of these genomes (Fig. 2a). The *katG* and *TGL2* were mostly found in *Pseudomonas*. The *dmpB* was enriched in *Enterococcus*, and the *qor* was abundant in *Lactococcus*.

Several genes related to the digestion of hemicellulose including alpha-glucuronidase (EC 3.2.1.139, *aguA*), xylan 1,4-beta-xylosidase (EC 3.2.1.37, *XYL4*) and endo-1,4-beta-xylanase (EC 3.2.1.8, *xynA*) genes, were observed in the genomes. In addition, genes related to the cellulose digestion including cellulase (EC 3.2.1.4, *aguA*), beta-glucosidase (EC 3.2.1.21, *bglB*), 6-phospho-beta-glucosidase (EC 3.2.1.86, *celF*), and protein-Npi-phosphohistidine—cellobiose phosphotransferase (EC 2.7.1.205, *celB*) genes, were observed. The corresponding enzymes involved in cellulose digestion pathways are shown in Fig. 2b.

To further evaluate the capacity for lignocellulose degradation in the communities, specific genes identified in the metagenomic binning analyses were annotated using the CAZys database (Fig. 2c). The auxiliary activities (AAs) family including AA2, AA3, AA4, AA6, and AA7 representatives that are associated with lignin degradation were abundant in the communities. The AA families were mostly found in *Streptococcus* (accounting for 30.7% of their total genes, especially AA3, AA4, AA6 and AA7), followed by *Pseudomonas* (5.6%, especially AA2, AA3, AA4, AA5 and AA7), *Lactococcus* (5.6%), *Lactobacillus* (4.7%), *Enterococcus* (2.6%), and *Acinetobacter* (0.9%).

A total of 26 CAZy families representing the glycoside hydrolases (GHs) and carbohydrate esterases (CEs) classes that are involved in hemicellulose digestion were observed, but mostly in the *Acinetobacter* MAGs (20.4%). Nevertheless, the genes were also observed in the *Pseudomonas*, *Streptococcus*, *Enterococcus*, and *Lactobacillus* MAGs, comprising 14.6%, 11.7%, 7.2%, and 7.0% of their total genes, respectively. Several families involved in cellulose digestion including GH1, GH2, GH3, GH5, and GH8 were identified that represented 1,4-beta-cellobiosidases (EC 3.2.1.91), and beta-1,4-beta-glucanases (EC 3.2.1.74). GH1, GH3, and GH5 genes were mostly observed in the *Acinetobacter* MAGs, accounting for 4.4%, 2.1%, and 0.3% of their total genes. GH2 was mostly observed in *Enterococcus* (3.1%) MAGs, and GH8 was mostly observed in *Pseudomonas* (0.2%) and *Acinetobacter* (0.1%) MAGs.

3.3. Gut fungal community variation in giant pandas during diet conversion

After quality filtering and assembly, 7,172,982 fungal ITS sequences were obtained from 108 fecal samples from the diet conversion experiments. The sequences were clustered into 15,547 OTUs at the 97% sequence identity threshold. The richness and diversity of fungi were approximately similar in three experimental groups ($p > 0.05$, ANOVA) (Fig. 3a, b). However, PCoA indicated that the communities from hosts with the same diet clustered together and were separated from the other groups (Fig. 3c). Likewise, significant differences in community structure based on BC distances were identified among the three groups (Bray Curtis: $F = 8.54$, $R^2 = 0.12$, $p < 0.001$).

Ascomycota was the most dominant phylum among communities in the diet conversion experiment, followed by Basidiomycota (Fig. 3d). The relative abundance of Basidiomycota was higher in the OBD (33.2%) communities than in the OMD (7.6%) communities (Non-parametric factorial Kruskal-Wallis sum-rank test, LDA > 4) (Fig. 3e). The 10 most abundant fungal genera in each group were further investigated, excluding unidentified genera. *Candida* (37.0%), *Saccharomyces* (6.2%), and *Microidium* (6.1%) were the three most abundant genera in the OMD. *Candida* (3.0%), *Microidium* (2.7%), and *Gibberella* (1.0%) were the three most abundant genera in the MBD communities. *Cystofilobasidium* (9.0%), *Guehomyces* (8.1%), and *Microidium* (5.2%) were the three most abundant genera in the OBD (Fig. 1f). The relative abundance of *Candida* was significantly higher in the OMD (37.0%) communities and lower in the OMD to OBD (0.1%) (Non-parametric factorial Kruskal-Wallis sum-rank test, LDA > 4) (Fig. 3e, f). The relative abundance of *Cystofilobasidium*, *Guehomyces*, and *Gibberella* were significantly higher abundant in the OBD communities (9.0%, 8.1%, and 2.8%, respectively) than in the OMD communities (0.03%, 0.0%, and 0.01%, respectively) (Non-parametric factorial Kruskal-Wallis sum-rank test, LDA > 4).

3.4. Variation in gut bacterial communities of giant pandas with habitat environment conversion

After removal of mitochondria and chloroplasts sequences, the remaining 7,950,647 sequences were clustered into 3720 OTUs at the 97% sequence identity threshold. The bacterial community composition of reintroduced pandas was closer to those of wild GPs than to those of wild-training I and wild-training II GPs (Fig. 4a). We compared the gut bacterial community composition of wild-training I, wild-training II

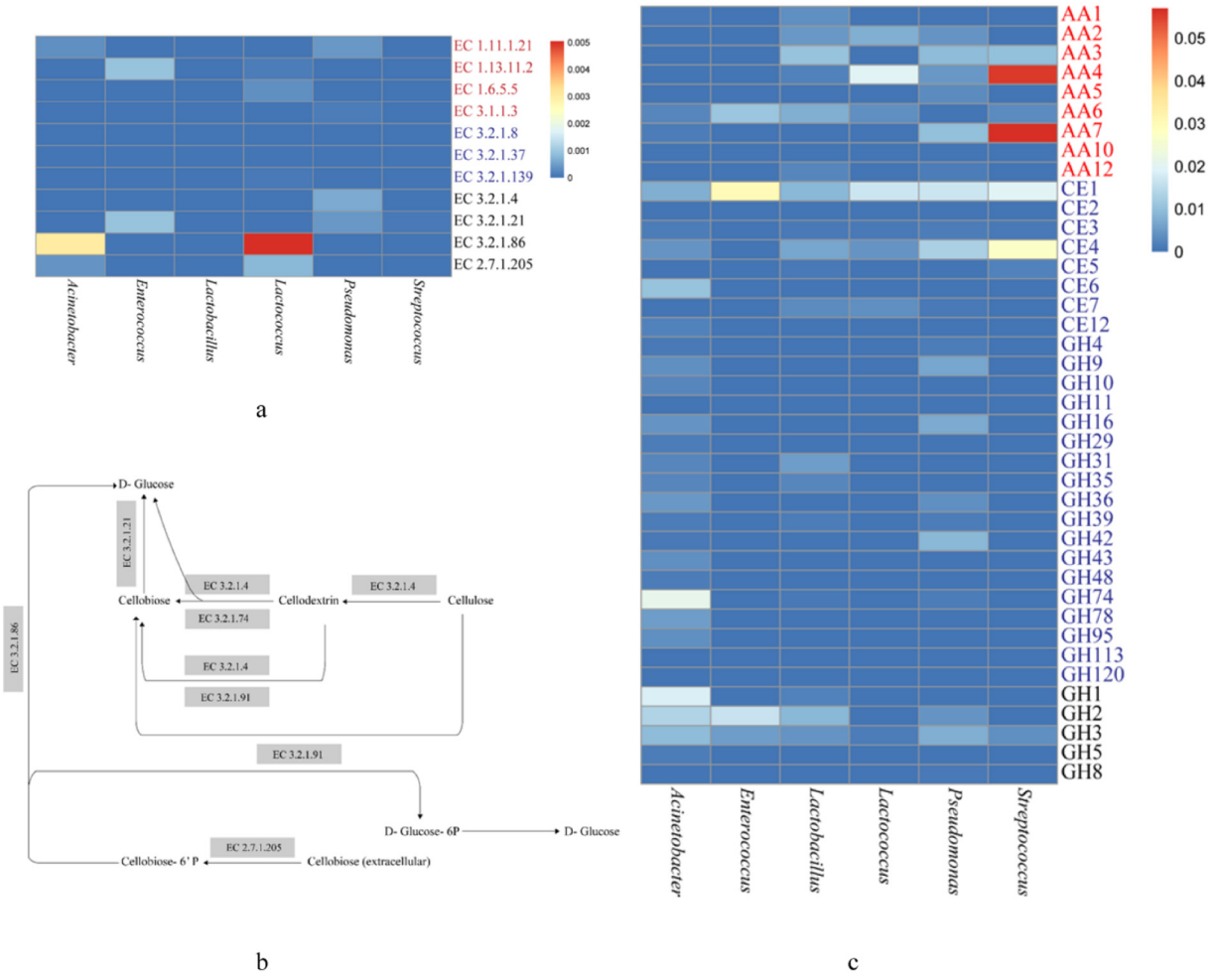

Fig. 2. Distribution of cellulose, hemicellulose, and lignin degradation associated genes across selected genomes. (a) Distribution of enzyme-encoding genes involved in cellulose, hemicellulose, and lignin degradation pathways, as determined by KEGG database mapping. (b) Cellulose degradation pathways showing cellulose degradation genes encoding key enzymes. EC 3.2.1.74 and EC 3.2.1.91 were identified by GH3, GH5, and GH8 in the CAZy database. (c) Carbohydrate-active enzyme families involved in cellulose, hemicellulose, and lignin degradation pathways determined via the CAZy database (GH: glycoside hydrolase, CE: carbohydrate esterases, AA: auxiliary activities). Red text indicates enzymes involved in lignin degradation, blue text indicates enzymes involved in hemicellulose degradation, and black text indicates enzymes involved in cellulose degradation.

and reintroduced pandas, to analyze the habitat environment influence on the gut bacterial communities of giant pandas.

The richness of gut microbial communities from wild-training II and reintroduced groups were significantly higher than that of wild-training I groups ($p < 0.05$, Kruskal-Wallis test) (Fig. 4b). However, the diversities of gut bacterial communities were approximately similar in the three experimental groups ($p > 0.05$, Kruskal-Wallis test) (Fig. 4c).

Proteobacteria and Firmicutes were the dominant phyla in all groups, comprising >98.0% of the total abundance (Fig. 4d). Proteobacteria was the dominant phylum in wild-training I panda gut communities (61.0%) and its relative abundance was higher in the wild-training I groups than in the other groups (6.4% in wild-training II and 15.2% in reintroduced GPs) (Non-parametric factorial Kruskal-Wallis sum-rank test, LDA > 4) (Fig. 4d, e). Firmicutes was the dominant phylum in the wild-training II (92.5%) and reintroduced (84.2%) pandas, and significantly higher than in the communities of wild-training I pandas (37.8%) (Non-parametric factorial Kruskal-Wallis sum-rank tests, LDA > 4).

The distributions of the 10 most abundant bacterial genera in each group were further investigated, excluding unidentified genera (Fig. 4e, f). *Escherichia* (30.6%), *Acinetobacter* (22.4%), and *Streptococcus* (20.1%) were the most abundant genera in the communities of wild-training I GPs (Fig. 4d). *Escherichia* was significantly enriched in the wild-training I group, and significantly depleted in the reintroduced group (Non-parametric factorial Kruskal-Wallis sum-rank test, LDA > 4) (Fig. 3e). In addition, the relative abundance of *Acinetobacter* in the wild-training I GPs (22.4%) was significantly higher than in the wild-training II (0.4%) and reintroduced (3.2%) GPs (Non-parametric factorial Kruskal-Wallis sum-rank tests, LDA > 4). *Streptococcus* was more abundant (64.2%) the dominant genus in wild-training II GPs and significantly more abundant than in the wild-training I (20.1%) and reintroduced (5.2%) GPs. *Leuconostoc* (13.0%) and *Clostridium* (10.9%) were abundant the wild-training II GPs. *Clostridium* (40.2%), *Leuconostoc* (22.8%), and *Turicibacter* (8.0%) were the most abundant genera in the reintroduced GPs. The relative abundance of *Clostridium* and *Turicibacter* were significantly higher in the reintroduced GPs (40.2% and 8.0%, respectively) than in the wild-training I (5.5% and 0.4%) and wild-training II (10.9% and 1.7%) GPs (Non-parametric factorial Kruskal-Wallis sum-rank test, LDA > 4).

3.5. Variation in gut bacterial communities of giant pandas with different lifestyle

We compared the gut bacterial community composition of reintroduced and wild GPs, to analyze the influence of lifestyle on the gut bacterial communities. The bacterial diversity of wild GPs was significantly higher than that of the reintroduced GPs ($p < 0.05$, Kruskal-Wallis test), and no significant differences of bacterial richness were

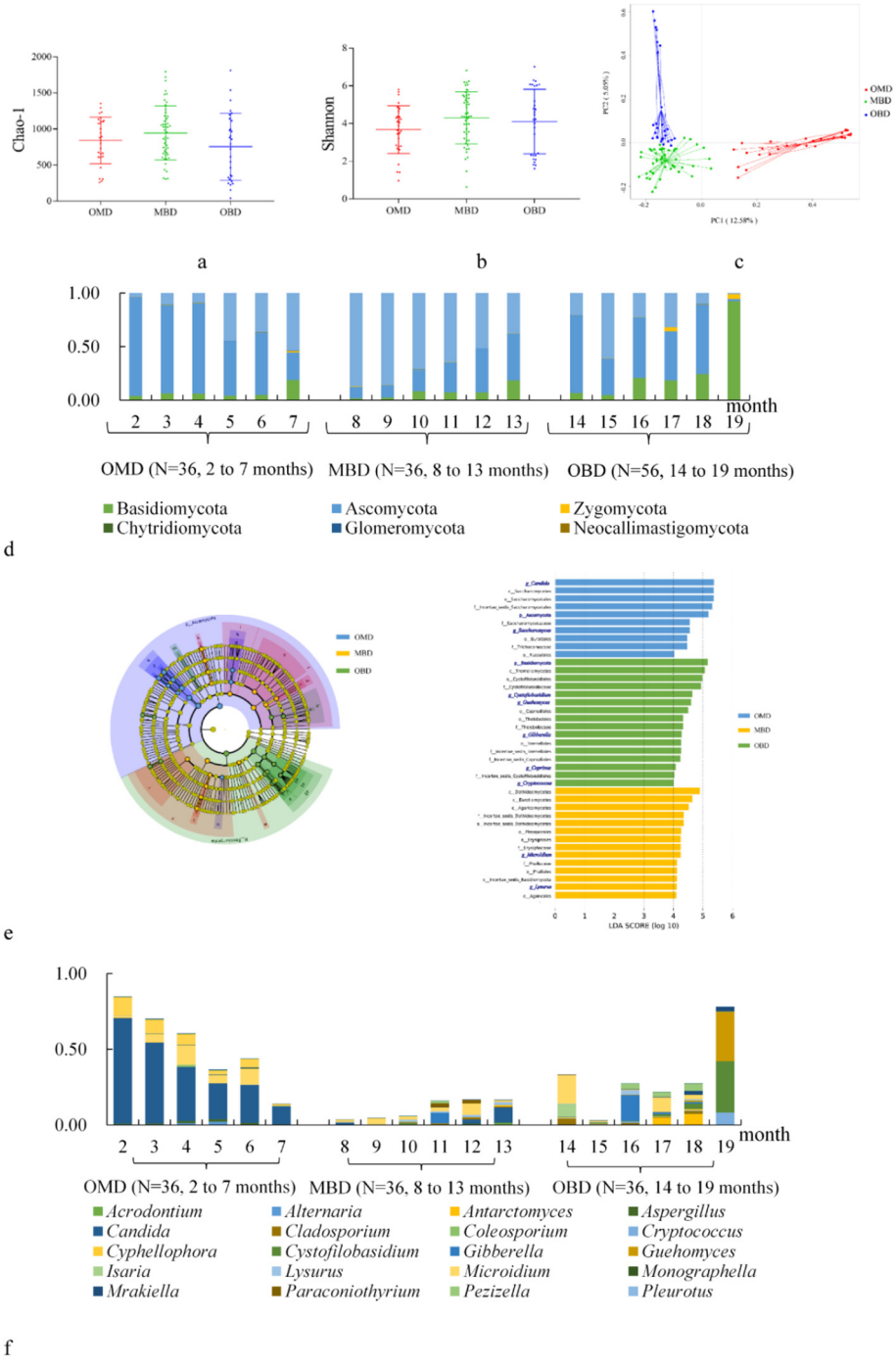

Fig. 3. Gut fungal community variation due to dietary change. (a) Variation in gut fungal richness during dietary shifts. Each circle represents a sample, wherein OMD group treatment communities: red, MBD: green, and OBD: blue. (b) Variation in gut fungal diversity during dietary shifts. Circles represent samples and are colored as indicated in panel a. (c) Principal Coordinates Analysis (PCoA) of gut microbiome structures from the OMD, MBD, and OBD experimental groups. PC1 and PC2 are shown on the x and y axes along with the percent variation explained by each. Circles represent samples and are colored as indicated in panel a. (d) Differences in overall fungal phylum-level compositions of the communities from the OMD, MBD, and OBD experimental groups. (e) Significantly different fungal taxa among the OMD, MBD, and OBD experimental groups, as identified by linear discriminant analysis coupled with effect size (LEfSe) using the default parameters. Blue symbols: OMD, orange: MBD, green: OBD. Blue text shows phyla and genera. (f) Differences in overall fungal genus-level of the communities from the OMD, MBD and OBD experimental groups. *: $0.01 < p < 0.05$; **: $p < 0.01$.

observed between the reintroduced and wild GPs ($p > 0.05$, Kruskal-Wallis test) (Supplementary Fig. 1).

Significant differences were also observed between the communities of the reintroduced and wild pandas at the phylum and genus levels ($p < 0.05$, Wilcoxon test) (Supplementary Fig. 2). At the phylum level, Firmicutes was the predominant phylum in the reintroduced panda (84.2%), and its relative abundance was significantly higher than it in the wild GPs ($p < 0.05$, Wilcoxon test) (Supplementary Fig. 2a). The relative abundances of Proteobacteria (51.4%), Bacteroidetes (24.9%) and Verrucomicrobia (1.5%) in the wild GPs were significantly higher than those in the reintroduced GPs ($p < 0.05$, Wilcoxon test).

At the genus level, *Clostridium* (40.2%), *Leuconostoc* (22.8%), *Turicibacter* (8.0%), *Acinetobacter* (3.2%), and *Yersinia* (2.6%) were more prevalent in the reintroduced GPs ($p < 0.05$, Wilcoxon test)

Fig. 4. Gut bacterial community variation due to habitat environment conversions. (a) Variation in gut microbiome richness during habitat environment shifts. Circles represent samples, with Wild-training I panda communities shown in blue, wild-training II in red and reintroduced in green, and wild in purple. (b) Variation in gut microbiome diversity during habitat environment. Circles represent samples, as indicated in panel a. (c) Principal Coordinates Analysis (PCoA) of bacterial community structures from the wild-training I, wild-training II, reintroduced, and wild groups. PC1 and PC2 are shown on the x and y axes along with the percent variation explained by each. Circles represent samples and are colored as indicated in panel. (d) Differences in overall bacterial phylum-level compositions of the communities from the wild-training I, wild-training II, and reintroduced pandas. (e) Significantly different bacterial taxa among the wild-training I, wild-training II, and reintroduced groups, as identified by linear discriminant analysis coupled with effect size (LEfSe) using the default parameters. Blue symbols show wild-training I samples, orange: wild-training II, and green: reintroduced. Blue text shows phyla and genera. (f) Differences in overall bacterial genus-level composition of the communities from the wild-training I, wild-training II, and reintroduced experimental groups. *: $0.01 < p < 0.05$, **: $p < 0.01$.

(Supplementary Fig. 2b). *Pseudomonas* (28.3%), *Sphingobacterium* (8.8%), *Flavobacterium* (6.0%), and *Pedobacter* (5.4%) were more abundant in the wild GPs ($p < 0.05$, Wilcoxon test).

3.6. Variation in gut fungal communities of giant pandas with habitat environment conversion

After removal of mitochondria and chloroplast sequences, 8,142,570 ITS sequences were clustered into 7438 OTUs at the 97% sequence identity threshold. The fungal community composition of reintroduced GPs was closer to those of wild GPs than to those of wild-training I and wild-training II GPs (Fig. 5a).

Fungal community richness was significantly higher in the GM of reintroduced pandas compared to those of wild-training ($p < 0.05$, ANOVA test) (Fig. 5b). The fungal diversity of wild-training I GPs was significantly lower than those of wild-training II and reintroduced GPs ($p < 0.05$, ANOVA test) (Fig. 5c).

The relative abundance of Ascomycota was higher in wild-training I GPs (92.4%) than in the other groups, and that of Basidiomycota was higher in the wild-training II (33.4%) and reintroduced GPs (21.8%) than in wild-training I GPs (Non-parametric factorial Kruskal-Wallis

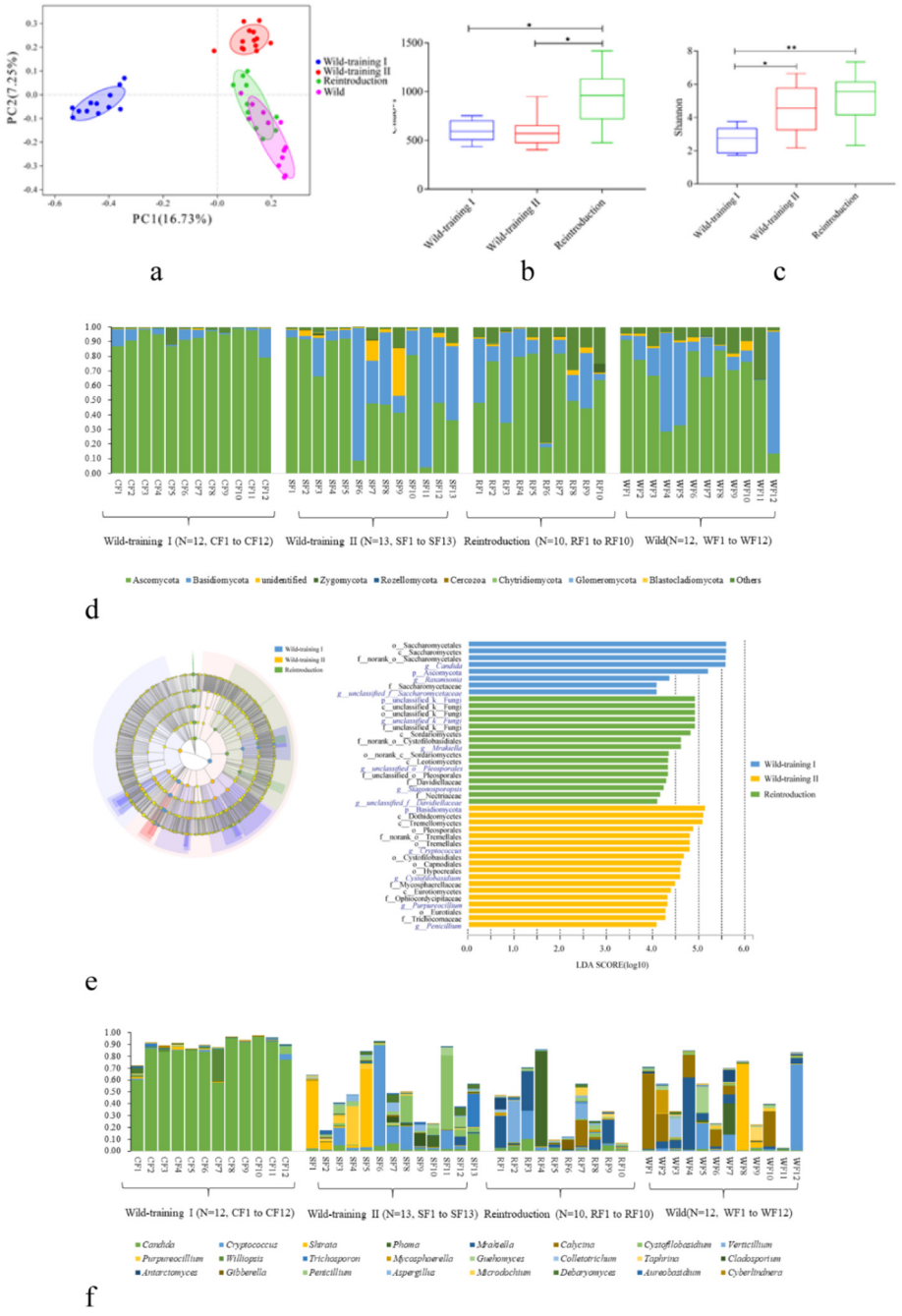

Fig. 5. Gut fungal community variation due to habitat environment conversions. (a) Variation in gut microbiome richness during habitat environment shifts. Circles represent samples, with Wild-training I panda communities shown in blue, wild-training II in red and reintroduced in green, and wild in purple. (b) Variation in gut microbiome diversity during habitat environment. Circles represent samples, as indicated in panel a. (c) Principal Coordinates Analysis (PCoA) of fungal community structures from the wild-training I, wild-training II, reintroduced, and wild groups. PC1 and PC2 are shown on the x and y axes along with the percent variation explained by each. Circles represent samples and are colored as indicated in panel. (d) Differences in overall fungal phylum-level compositions of the communities from the wild-training I, wild-training II, and reintroduced pandas. (e) Significantly different fungal taxa among the wild-training I, wild-training II, and reintroduced groups, as identified by linear discriminant analysis coupled with effect size (LEfSe) using the default parameters. Blue symbols show wild-training I samples, orange: wild-training II, and green: reintroduced. Blue text shows phyla and genera. (f) Differences in overall fungal genus-level composition of the communities from the wild-training I, wild-training II, and reintroduced experimental groups. *: $0.01 < p < 0.05$, **: $p < 0.01$.

sum-rank test, LDA > 4) (Fig. 5d, e). At the genus level, *Candida* (83.1%) was the most dominant genus in the wild-training I GPs followed by *Williopsis* (2.7%) and *Cryptococcus* (0.7%), and significantly more abundant than in the wild-training II (3.2%) and reintroduced (2.8%) GPs (Non-parametric factorial Kruskal-Wallis sum-rank test, LDA > 4) (Fig. 3e, f). *Cryptococcus* (12.3%), *Shiraia* (10.3%), and *Cystofilobasidium* (8.1%) were the most abundant genera in the wild-training II panda gut communities. The relative abundances of *Cryptococcus* (12.3%), *Cystofilobasidium* (8.1%), *Purpureocillium* (3.8%), and *Penicillium* (2.5%) were significantly higher in the wild-training II GPs than in the wild-training I (0.7%, 0.7%, 0.06%, and 0.09%, respectively) and reintroduced (3.2%, 0.6%, 0.1%, and 1.0%, respectively) GPs (Non-parametric factorial Kruskal-Wallis sum-rank test, LDA > 4). *Mrakiella* (9.2%), *Phoma* (8.3%), and *Verticillium* (4.9%) were the most abundant genera in the reintroduced pandas. The relative abundance of *Mrakiella* (9.2%) was significantly higher in the reintroduced GPs than in the wild-training I (0.1%) and wild-training II (0.7%) GPs (Non-parametric factorial Kruskal-Wallis sum-rank test, LDA > 4).

3.7. Variation in gut fungal communities of giant pandas with different lifestyle

Fungal richness and diversity were approximately similar in both the wild and reintroduced GPs. Significant differences at phylum and genus level between the reintroduced and wild GPs were observed ($p < 0.05$, Wilcoxon test) (Supplementary Fig. 1). The relative abundance of eleven genera were significantly different between the reintroduced and wild GPs (Supplementary Fig. 3). For example, *Candida* (2.8%) was more abundant in the reintroduced GPs, while *Calycina* (13.2%) was more abundant in the wild GPs ($p < 0.05$, Wilcoxon test).

4. Discussion

To understand the influence of diet, habitat environment and lifestyle on the gut microbiota (GM) of giant panda (GP), we assessed the variation in their GM during diet changes, habitat environment conversions and with the different lifestyle. The richness, diversity and composition of GP gut microbial communities varied with diet conversion and were influenced by habitat environment and lifestyle. Diet, surrounding environments and lifestyle play important roles in shaping the GM of humans (Koren et al., 2012) as well as animals including snub-nosed monkeys (Miriam et al., 2013), black honeybees (Zhao et al., 2018) and mice (Miriam et al., 2013). Similarly, our results suggest that they are also critical factors in influencing the GM of GPs.

4.1. Gut microbiome changes associated with dietary changes

The diversity of gut bacterial communities of GPs increased when transitioning from the milk diet (OMD) to bamboo diet (OBD), and richness exhibited an opposite trend. Guo et al. reported that the richness and diversity of gut microbiome increased until the GPs were 6 months old and then declined (Guo et al., 2018). The different results may have been due to differences in diet, which are known to affect GM (Carlotta et al., 2010). The gut bacterial community richness of GPs has been observed to be higher with low fiber diet than with high fiber diet (Wu et al., 2017). These results may be explained by the variable structural complexity of fiber and the relatively low richness of bacteria that can use fiber as a growth substrate (Lynd et al., 2002). High fiber diets can increase the diversity of GM in humans, (Carlotta et al., 2010; Tap et al., 2016; Zhernakova et al., 2016; Heisel et al., 2017), and based on our results, the GM of GPs responds similarly. Competitive interactions among bacteria are ubiquitous in natural systems, many studies have shown that lignocellulose is a complex substrate that promotes positive interactions and synergistic growth of bacterial populations compared to labile substrates like glucose and fat (Haruta et al., 2002; Sarunyou et al., 2012; Deng and Wang, 2016). Since lignocellulose is a cross-linked structure that is difficult to degrade, bacteria may need to form consortia to degrade lignocellulose (Deng and Wang, 2016; Perez et al., 2002). Overall, fiber content could be an important factor in affecting the richness and diversity of gut microbial populations of the GPs. Consequently, we speculated that high fiber diets could increase the diversity, but decrease the richness of gut bacterial communities in GPs.

Similar with previous studies (Yang et al., 2018; Zhang et al., 2018), Proteobacteria and Firmicutes dominated the GM of GPs. The low expenditure and physical activity of GPs (Nie et al., 2015) may favor Proteobacteria that are known to be dominant in the guts of herbivores with low metabolic rates (Dill-McFarland et al., 2016). Firmicutes are typically dominant in the guts of mammalian herbivores and play critical roles in fiber digestion (Dill-McFarland et al., 2016; Nelson et al., 2010). The relative abundance of Firmicutes has also been positively associated with fiber content in human guts (Carlotta et al., 2010) and with supplemented dietary fiber in dogs (Costa et al., 2012). In our study, the relative abundance of Firmicutes increased with increasing bamboo content in the diet, suggesting that Firmicutes were important for digesting the high fiber bamboo foods into more labile nutritional components.

At the genus level, the abundance of *Streptococcus* (in the phylum Firmicutes) has been shown to significantly increase upon introduction of a bamboo diet (Ouwehand et al., 2010). Moreover, *Streptococcus* is associated with GP gut mucus (Williams et al., 2016) that is critical in dietary changes of GPs from low to high fiber diet. Mucus helps protect guts from injuries due to high fiber contents and aids the movement of high fiber components through the gut (Montagne et al., 2003). We detected the genes encoding Protein-Npi-phosphohistidine-cellobiose phosphotransferase (EC 2.7.1.205, *celB*) in the *Streptococcus* MAG, which are important for cellulose digestion (Lai et al., 1997). In addition, beta-glucosidase (EC 3.2.1.21, *bglB*) and 6-phospho-beta-glucosidase (EC 3.2.1.86 *celF*) were identified in the *Streptococcus* MAG, as inferred from comparison to the KEGG databases. Beta-glucosidase (EC 3.2.1.21) and 6-phospho-beta-glucosidase (EC 3.2.1.86) are both involved in cellulose digestion (Ghorai et al., 2010; Rytioja et al., 2014). In particular, GHs that are often associated with digestion of cellulose and hemicellulose (Stewart et al., 2018) were accordingly identified in the *Streptococcus* MAG indicating that *Streptococcus* in the GP gut have potential for cellulose utilization. Cellulose and hemicellulose are cross-linked with lignin, and the removal of lignin is the first step in digesting cellulose and hemicellulose (Rytioja et al., 2014). Several genes encoding enzymes involved in hemicellulose and lignin degradation were detected in the *Streptococcus* MAG including CE1, CE3, CE4, CE5, AA3, AA4, AA6, and AA7 group genes (Zhang et al., 2018; Zhen and Smith, 2016). Moreover, several other cellulose, hemicellulose, and lignin degradation associated genes were also observed in the *Pseudomonas*, *Enterococcus*, *Lactococcus*, and *Acinetobacter* MAGs including cellulase (EC 3.2.1.4) and 1,4-beta-cellobiosidase (EC 3.2.1.91). The combined activities of cellulase and 1,4-beta-cellobiosidase can convert cellulose into cellobiose, and cellobiose is a key intermediate in the conversion of cellulose to D-glucose (Zhu et al., 2011). The relative abundance of *Pseudomonas*, *Clostridium*, *Lactobacillus*, *Enterococcus*, *Lactococcus* and *Acinetobacter* that carried the cellulose degradation linked genes increased with increasing bamboo content diet. *Clostridium* and *Enterococcus* correlated positively with crude fiber digestibility, while *Lactobacillus*, *Enterococcus*, and *Pseudomonas* were positively associated with acid detergent fiber digestibility (Niu et al., 2015). Moreover, the *Pseudomonas* and *Acinetobacter* were involved in in the degradation of lignin (Jiménez et al., 2015). Thus, our results indicated that *Streptococcus*, *Pseudomonas*, *Enterococcus*, *Lactococcus*, *Acinetobacter*, and *Clostridium* may contribute to lignocellulose digestion and the utilization of cellulose and hemicellulose from bamboo, thereby providing energy and nutrients for their GP hosts.

Interestingly, gut bacterial communities of GPs were more similar to those of carnivores than herbivores in a previous comparison of human GM and 59 other mammalian species (Ley et al., 2008). Likewise, Xue et al. observed that the composition of gut bacterial communities in GPs were similar to those of bears and entirely distinct from those of herbivores (Xue et al., 2015).The gut microbiota of humans (Filippo et al., 2010), roosters (Coon et al., 1989), dogs (Silvio et al., 2000; Moore et al., 1980) and African lions (Jia, 2017) are capable to digest fiber. We hypothesized that the gut bacterial communities of bears and even others have the potential to metabolize fiber or otherwise that these bacterial communities have been evolved in concert with GP evolution. Nevertheless, additional study is needed to evaluate the above hypothesis.

Consistent with Zhang et al. (2018) who reported that gut fungal communities are less important than gut bacterial communities for the development of GPs, the changes in the fungal communities across the diet groups were minor. Similar to previous studies (Tun et al., 2014; Zhang et al., 2018), Ascomycota and Basidiomycota dominated the fungal communities of GPs. Ascomycota and Basidiomycota were prevalent in the fungal communities in soil (Xu et al., 2012) and bamboo (Zhou et al., 2017). Bamboo could influence the GM composition of GPs

through nutrition and microbiome, and over fifty fungal genera were shared by bamboo and GPs (Jin et al., 2020). Soil microbiome was a main source of the GM of foliar-feeding insects GM (Hannula et al., 2019; Nina et al., 2013). These observations have led to the hypothesis that the gut microbiomes of GPs may originate from their food sources or even soil. *Candida* was the dominant fungal genus in the OMD and significantly decreased in the transition from the OMD to OBD diet. The relative abundance of *Candida* in gut fungal communities has been strongly associated with the consumption of carbohydrates (Christian et al., 2013; Iannotti et al., 1973). Milk has higher carbohydrate contents than bamboo (Mainka et al., 1989). Possibly *Candida* was involved in metabolizing milk in the guts of GPs on containing diets. The relative abundances of *Cystofilobasidium*, *Guehomyces*, and *Gibberella* increased markedly in the transition from the OMD to OBD diet (Non-parametric factorial Kruskal-Wallis sum-rank test, LDA > 4). Although little is known about the function of these three genera (Chen et al., 2017), we hypothesized that they may contribute to the ability of GPs to digest bamboo.

4.2. Gut microbiome dynamics associated with habitat environment conversion

Consistent with the differences in GM of wild and captive primates (Clayton et al., 2016) and bears (Borbón-García et al., 2017), the richness of the gut bacteria and the richness and diversity of gut fungi of reintroduced GPs were higher than those of GPs in the beginning of wild-training. The more microbial species a host comes into contact with, the more likely it is that those species will persist in the host's gut microbiome (Schmidt et al., 2019; Smits et al., 2017; Chave, 2010). Previous studies have shown that the richness of GM of wild GPs was higher than that of captive GPs, and the higher richness could be due to exposure to more diverse microbial meta-communities in the natural environment (Burns et al., 2016; Schmidt et al., 2019; Wu et al., 2017). Possibly the increase in space from the beginning of the wild training to reintroduction lead to the increased resources (e.g. food, water, social interactions) (Schaller et al., 1985) and interactions with meta-communities (Schmidt et al., 2019) that contributed to the diversity and richness increase.

Consistent with previous studies (Zhang et al., 2018; Zhu et al., 2011), Proteobacteria and Firmicutes dominated the bacterial communities during habitat environment conversion. However, Proteobacteria was the most dominant phylum in the gut of wild-training I GPs, while Firmicutes was the most abundant phylum in wild-training II and reintroduced pandas. Proteobacteria was the dominant phylum in the gut communities of wild-training I GPs (Wei et al., 2015), and Firmicutes in the guts of wild GPs (Zhu et al., 2011). Similarly, Firmicutes was the dominant phylum in wild deer mice and musk deer (D. Li et al., 2017; Y. Li et al., 2017; Schmidt et al., 2019) that primarily ingest insoluble fibers that are degraded by cellulose and hemicellulose-digesting enzymes including cellulase, beta-glucosidase, and xylan 1,4-b-xylosidase (Costa et al., 2012; Zhu et al., 2011). The results indicated that the proportion of cellulolytic bacteria increased after GPs were relocated to larger and more complex environment.

At the genus level, *Escherichia* was significantly enriched in the wild-training I panda gut bacterial communities (Non-parametric factorial Kruskal-Wallis sum-rank test, LDA > 4). *Escherichia* has also been observed as the major bacterial taxa in the guts of captive GPs (Xue et al., 2015), humans (Lagier et al., 2012) and pigs (Niu et al., 2015). *Streptococcus* and *Leuconostoc* were more abundant in the wild-training II GP gut communities, while *Clostridium*, *Leuconostoc*, and *Turicibacter* were enriched in the reintroduced GPs (Non-parametric factorial Kruskal-Wallis sum-rank test, LDA > 4). These genera may be involved in the digestion of bamboo in wild GPs (Oyeleke and Okusanmi, 2008; Zhu et al., 2011), which could help the GPs to gain adequate energy from limited nutritional sources.

Similar to the diet conversion experiment, Ascomycota and Basidiomycota were the dominant fungal phyla in the GM of wild-training I, wild-training II, and reintroduced pandas, although their relative abundances varied between groups. Ascomycota and Basidiomycota have been observed as dominant in the vaginas of GPs (Chen et al., 2017), the guts of humans (Christian et al., 2013), the guts of dogs (Handl et al., 2011), bamboo (Zhou et al., 2017), soils (Xu et al., 2012), and in the near-surface atmosphere (Bowers et al., 2013). Considerable variation was observed among the fungal genera associated with habitat environment conversion. *Candida* was the dominant genus in the gut communities of the captive GPs. Since captive GPs receive a fixed amount of carbohydrates (e.g. shoots and panda cakes) compared to semi-captive and reintroduced GPs (Schaller et al., 1985), *Candida* may help in the digestion and absorption of carbohydrates (Christian et al., 2013; Iannotti et al., 1973). *Cryptococcus* was enriched in the gut communities of wild-training II GPs, and *Mrakiella* was abundant in those of the reintroduced GPs. *Cryptococcus* is common in natural environments and can remain in non-infective states in bodies while later reactivating and spreading to other body areas (Hagen et al., 2017; Litvintseva and Mitchell, 2009). *Mrakiella* is found in soils and waters, especially in low-temperature environments within various regions (Thomas-Hall et al., 2010). As we know, bamboo microbiome could influence giant panda GM, and vagina microbiome could influence infant microbiome (Jin et al., 2020; Hannula et al., 2019). The results suggest that similar to the bacterial communities of the GPs, the changes in the gut fungal communities were possibly due to exposure to more diverse environmental microbiota.

4.3. Gut microbiome dynamics associated with different lifestyle

The bacterial diversity of the GM of wild GPs was higher than those of reintroduced GPs. Yao et al., reported that the semi-wild GP had less stable gut microbial community than wild GP (Yao et al., 2019a,b). The relative abundance of Firmicutes, including *Clostridium*, *Leuconostoc* and *Turicibacter*, was higher in the GM of the reintroduced GPs than in the GM of the wild GPs. Similar with the wild GPs in Xiaoxiangling and Minshan mountains (Yao et al., 2019a,b), Proteobacteria, including *Pseudomonas*, were dominant in the GM of the wild GPs. *Clostridium* in the GM of GPs may focus on cellulose and hemicellulose digestion (Yao et al., 2019a,b; Dahal and Kim, 2016; Jiménez et al., 2015; Zhu et al., 2011). AA2 including lignin peroxidase and manganese peroxidase related genes that are linked to the degradation of polycyclic aromatic hydrocarbons (Wang et al., 2003) were abundant in *Pseudomonas*. *Pseudomonas* involved in degradation of lignin had been demonstrated in wheat (Jiménez et al., 2015). The ability of *Pseudomonas* to degrade secondary compounds (e.g., cyanide compounds and aromatic compounds) may be related to the high proportion of *Pseudomonas* in the wild GPs (Yao et al., 2019a,b; Zhu et al., 2018). In addition, the abundance of Bacteroidetes, including *Sphingobacterium*, was higher in the wild than in the reintroduced GPs. Bacteroidetes has been positively associated with the digestion of carbohydrates and proteins, and may help facilitate the development of gut immune systems (Ley et al., 2006; D. Li et al., 2017; Y. Li et al., 2017). Interestingly, *Sphingobacterium* isolated from soils had been demonstrated could degrade polycyclic aromatic hydrocarbons (Nam et al., 2015; Son et al., 2011). Polycyclic aromatic hydrocarbon may be formed from lignin (Zhou et al., 2014). Thus, we speculate that the wild GPs could be adapted to higher lignin content food than the reintroduced GPs. Contact with different habitats, foods, and other materials can influence the composition of GM (Borbón-García et al., 2017; D. Li et al., 2017; Y. Li et al., 2017; Chave, 2010). Thus, the results suggested that additional time is needed for the complete conversion of reintroduced GP gut communities to resemble those of wild GPs. Moreover, improving the lignin degradation ability of the GM of reintroduced GPs may help its GM to become similar with that of wild GPs.

The relative abundances of Verrucomicrobia and Actinobacteria were higher in the wild GP communities compared to those of the reintroduced GPs (Non-parametric factorial Kruskal-Wallis sum-rank test, LDA > 4). Verrucomicrobia has been observed in the gut communities of primates and termites (Dedysh et al., 2006; He et al., 2010; Lee et al., 2009; Manjula et al., 2016; Su et al., 2016). However, Verrucomicrobia has not been previously detected in the guts of GPs, possibly because the focus has mostly been on captive GPs and lower number of wild GPs were included (Xue et al., 2015; Zhang et al., 2018; Wu et al., 2017; Schmidt et al., 2019; Smits et al., 2017; Chave, 2010). Actinobacteria has been positively associated with fat digestion (Wu et al., 2011). The food choices of wild and reintroduced GPs were different (Schaller et al., 1985), which may explain the differences in GM between reintroduced and wild GPs.

There were no significant differences in the richness and diversity of fungal GM between reintroduced and wild GPs, and no significantly different phyla were observed between the reintroduced and wild GPs. Up to now, more studies focus on bacterial GM, while the composition and diversity of fungal gut microbiome was not clearly understood (Kamada et al., 2013; Vemuri et al., 2020). The structure of fungal communities could be more complex than bacteria in spite of the technical limitation of fungal species and function annotation (Chen et al., 2017; Tun et al., 2014; Zhang et al., 2018). Overall, we speculated that the fungi of GM response to lifestyle shift less than bacteria due to the incomplete annotation of fungi.

A total of 31 fungal genera were differentially abundant in the gut communities of reintroduced and wild GPs (Non-parametric factorial Kruskal-Wallis sum-rank test, LDA > 4), albeit only *Calycina* was not of low relative abundance. *Calycina* that was enriched in the GM of the wild GPs belongs to the order Helotiales (Zhang and Zhuang, 2004) that is associated with root endophytes (Tedersoo et al., 2010). The results suggest that wild GPs may have a more comprehensive dietary structure or more contact with microbial meta-communities compared to reintroduced GPs, which would then enhance the dietary diversity of reintroduced GPs and contribute to the recovery of natural gut fungal community compositions as seen in wild GPs. Moreover, the composition of GM varied in different individuals due to host genetic influence, this phenomenon had been demonstrated in GPs and human (Zhang et al., 2018; Spor et al., 2011). The composition of the fungal GM of reintroduced GPs was closer to that of wild GPs than to those of wild-training I or wild-training II GPs, indicating that habitat environment may play an important role in shaping the GM of GPs.

5. Conclusions

Overall, our results indicated that diet conversion, habitat environment and lifestyle are associated with the richness, diversity, and composition of the GM of GPs. High fiber diets increased the diversity of gut bacterial populations of GP. Possibly the enriched gut bacteria contributed to lignocellulose digestion. Both habitat environment and lifestyle played an important role in shaping the GM of GP. Taking into account the differences in the GM of the reintroduced and wild GPs may be crucial for improving the success of GP reintroduction. Due to the limited population of GPs, five GPs were involved in the diet conversion experiment and three in the environment conversion experiment, calling for caution in generalizing the conclusions over the whole GP population. More GPs and GPs from different areas are required to explore the GM composition and diversity during diet, habitat environment and lifestyle conversion in the future.

Ethics approval and consent to participate

All sample collection protocols in this study were approved by the CCRCGP. The experimental procedures were fully in compliance with the current laws on animal welfare and research in China.

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CRediT authorship contribution statement

Lei Jin: Performed the experiments, Statistical analyses, Writing - review & editing. **Yan Huang:** Conceived the idea, Review & editing. **Shengzhi Yang:** Statistical analyses. **Caiwu Li:** Statistical analyses, Collected the samples. **Daifu Wu:** Performed the experiments, Collected the samples. **Ke Zhao:** Review & editing. **Yongguo He:** Collected the samples. **Bei Li:** Review & editing. **Guiquan Zhang:** Performed the experiments. **Yaowu Xiong:** Collected the samples. **Rongping Wei:** Performed the experiments. **Guo Li:** Collected the samples. **Hongning Wu:** Collected the samples. **Wenwen Deng:** Statistical analyses. **Hemin Zhang:** Review & editing. **Likou Zou:** Conceived the idea, Methodology, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no competing interests.

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Availability of data and materials

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

Consent for publication

Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.145316.

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