Dietary changes and lifestyle shifts affect the gut microbiomes of giant pandas

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Abstract

Gut microbiota (GM) are important for the health of giant pandas (Ailuropoda melanoleuca, GP), in addition to the utilization of bamboo in their diets. However, it's not fully understood how diet conversions and environmental factors contribute to the compositions of giant panda GM. Consequently, we evaluated how dietary changes and lifestyle shifts influence the GM of giant pandas using high-throughput sequencing and genome-resolved metagenomics. The gut microbial communities of giant pandas were more similar when their hosts exhibited the same diets or lifestyles. High fiber diets significantly increased the diversity (Shannon index) and decreased the richness (Chao1 index) of gut bacterial communities ($p < 0.05$). In addition, the abundances of Streptococcus, Pseudomonas, Enterococcus, Lactococcus, Acinetobacter, and Clostridium significantly increased with bamboo consumption (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. Further, the biodiversity of GM in wild or reintroduced pandas were higher than those of wild-training pandas, especially fungal communities. The GM structure in reintroduced giant pandas notably converged to that of wild pandas. These results revealed Streptococcus, Pseudomonas, Enterococcus, Lactococcus, Acinetobacter, and Clostridium may contribute to lignocellulose digestion in GP. Captivity generally led to decreased biodiversity of GM in giant pandas. Adaptations to increased environmental threats or stressors may aid the conversion of reintroduced giant panda GM to those like wild pandas. In summary, we indicated that diet and lifestyle could influence GM remarkably in GP.

INTRODUCTION

Gut microbiota (GM) play beneficial roles in the homeostasis and immune systems of hosts in addition to improving their general health and nutritional status (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 research over the past decade has 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*) are endemic to China, but exhibit rare wild populations due to decreasing population sizes (Shengzhi et al 2018, Zhang et al 2018). They are well known for their unique diet comprising bamboo, despite that they belong to the order Carnivora and possesses a typical carnivorous digestive system (Wei et al 2015, Zhu et al 2011). Interestingly, the giant panda has not evolved any enzymes specific for cellulose digestion, despite their unique dietary adaptation (Hu et al 2017). Therefore,

it is not known how giant pandas rely on high fiber diets characterized by low-nutritional components. It has consequently been hypothesized that giant pandas 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 diets (Hu et al 2017). Despite the investigation of this hypothesis by multiple studies (Zhu et al 2011), it has remained unresolved (Wei et al 2018). Nevertheless, it is clear that the GM of giant pandas play roles in their dietary metabolisms, although the extent of these roles may be unclear.

Giant panda cubs also exhibit unique dietary conversion phases, changing from milk to bamboo diets during development. Significant shifts in the compositions of GM concomitantly occur in giant panda infants during the transition to more solid and varied diets (Sghir et al 2000). Accordingly, investigating GM variation within giant pandas during dietary shifts may provide evidence for the mechanisms underlying the dietary specialization of giant pandas. Indeed, several studies have compared the GM of milk- and bamboo-fed giant pandas (Guo et al 2018, Zhang et al 2018). However, diet was not the only unique variable in the comparison groups of these studies, and it is thus difficult to infer the influences of diet on the GM of giant pandas from these studies.

The reintroduction of captive giant pandas 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 giant panda conservation breeding program (e.g., through mating, artificial insemination, and parental care behaviors), contributing to the sustainment and increase of giant panda populations that can then be used in reintroduction efforts to supplement wild populations (Li et al 2017, Wei et al 2015, Zhang et al 2004). As indicated above, human-associated microbial communities can be quickly and profoundly altered by typical human activities and ecological backgrounds (David et al 2014). Likewise, accumulating evidence has emphasized that gastrointestinal disease is a primary cause of giant panda deaths (Tun et al 2014), indicating that their gut microbial communities play crucial roles in improving reintroduction success rates. However, studies of giant pandas have only compared GM compositions between captive and wild giant pandas, while few have evaluated the GM characteristics of pandas with different lifestyles (Wei et al 2015, Wu et al 2017, Zhu et al 2011). Moreover, these studies have investigated samples of captive and wild giant pandas from different individuals, although individual microbiota differences are a significant confounding factor when comparing community structures (Xue et al 2015). Thus, little is known regarding the impact of lifestyle variation on the GM of giant pandas. Consequently, the aims of this study were to evaluate the influence of dietary and lifestyle changes on the diversity and composition of giant panda gut microbial communities in order to understand the interactions among the host and their GM and inform future conservation efforts.

MATERIALS AND METHODS

Experimental design

Diet conversion experiment

Five six-month-old captive giant pandas from the Shenshuping Base of China Conservation and Research Center for the Giant Panda (CCRCGP) that were only receiving milk in their diets were chosen for the diet conversion experiment. Three experimental groups were established in these individuals at different developmental times: OMD (only milk as their diet), MBD (milk and bamboo diet), and OBD (only bamboo as their diet). Giant pandas that only received milk as their diet received 700-800 mL of milk per feeding, and their corresponding feces were collected at 2, 3, 4, 5, 6, and 7 months of age. Bamboo was subsequently introduced to the their daily diets with different weights based on age for 8 (3.0 kg of bamboo and 1 L of milk), 9 (3.0 kg bamboo and 1 L milk), 10 (5.0 kg bamboo and 1 L milk), 11 (5.0 kg bamboo and 1 L milk), 12 (8.0 kg bamboo and 1 L milk), and 13 (8.0 kg bamboo and 1 L milk) month old pandas. Subsequently, only bamboo was fed to these individuals with 15 kg of bamboo fed every day and feces collected at 14, 15, 16, 17, 18, and 19 months of age. The milk nutritional details are shown in Supplementary Table S1.

Lifestyle shift experiment

Three newborn to 4-month-old giant pandas at the Hetaoping Base of CCRCGP were chosen for the lifestyle shift experiment. Three primary experimental groups were established at different times for the same individual cohort: wild training I, wild training II, and reintroduced groups. The cub giant pandas were maintained in wild training I until 11-15 months old. Then the three wild-trained I giant pandas were subsequently moved to wild training II environment for 7-15 more months until they were 27-39 months old. Finally, two well trained giant pandas after wild trained II were reintroduced to the Liziping National Nature Reserve in Ya'an of Sichuan (29°2' N, 102deg46' E), a natural forest environment without any human disturbances. The wild-training giant pandas were living together with their mothers separately. After learning skills to survive in the wild during wild-training I and II, they left their mothers 2-3 months before reintroducing to the wild. Feces were also collected from wild giant pandas as controls. Giant pandas were fitted with GPS collars during the wild-training II and reintroduction stages after approval from the State Forestry Administration of China.

Captivityconditions

Giant pandas were housed in a room comprising a 580 cm x 230 cm x 270 cm animal house and a 580 x 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. The captivity facilities were located at the Shenshuping Base of CCRCGP in Wolong, Sichuan (31deg1'N, 103deg18'E) at an altitude of about 1500 to 1,700 m.

Wild-training I conditions

The wild-training I area was located at the Hetaopinge Base of CCRCGP, Wolong, Sichuan (31deg4'N, 103deg13'E), and lacked visitation by tourists. They wild-training giant pandas live with their mothers and learn skills to survive in the wild from their mothers (e.g., tree climbing skills, foraging and avoiding the danger). Wild-training giant pandas were housed in a room similar to the captives, and an area around 2300-3200 m^2 natural forest. The altitude is around 2,840 m.

Wild-training II conditions

The larger wild-training II facilities were located at the Tiantaishan Base, Wolong, Sichuan (31deg1'N, 103deg34'E), were about 1.4 km^2 wide and lacked visitation by tourists. The wild training II area was constructed with steel plates and a barbed-wire fence, with a stream flowing throughout the areas. At the Tiantaishan Base, the bamboo species *Fargesia robusta* , and *Bashania faberi* are the dominant food sources of giant pandas, with the former comprising 72.2% and 20.4% of the vegetation respectively. Some of *Yushania brevipaniculata*interspersed among *Fargesia robusta* bamboo forest. The base is a temperate deciduous forest at an altitude of about 2,070–2,140 m. The wild-training II area exhibits high biodiversity, and is a similar habitat to those occupied by wild giant pandas in the Wolong National Natural Reserve. Each giant panda was housed in a separate wild-training II area.

Sample collection

Diet conversion experiment

Feces from each individual were collected in each room of the captive group facilities every month during the diet conversion experiment. A total of 180 fecal samples were taken for microbial community compositional analysis via high-throughput 16S rRNA gene sequencing from the five captive giant panda cubs. Following quality assessment, a total of 168 fecal samples were subjected to 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=49; ITS sequencing n=29), the milk and bamboo mixed diet period from 8 to 13 months old (MBD, 16S rRNA sequencing n=74; ITS sequencing n=50), and the bamboo only diet period from 14 to 19 months old (OBD, 16S rRNA sequencing n=45; ITS sequencing n=29; metagenomic binning n=60) (Supplementary Table S2).

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Lifestyle-shift experiments

To evaluate the effects of lifestyle shifts, the fecal samples of giant pandas only bamboo fed were collected during two months before wild-training II and reintroduction, and finally one year after reintroduction to the wild. Fecal samples from wild-training II and reintroduced individuals were collected from their corresponding giant pandas with GPS collar tracking. Feces were also collected from wild giant pandas as controls. A total of 61 fecal samples were recovered from the experimental individuals for sequence analysis. Following quality control, 49 fecal samples were successfully subjected to 16S rRNA gene sequencing and 35 samples to fungal ITS sequencing. The lifestyle-shifts comprised three stages including the wild-training I (16S rRNA sequencing n=17; ITS sequencing n=12), wild-training II (16S rRNA sequencing n=16; ITS sequencing n=13), and reintroduced (16S rRNA sequencing n=16; ITS sequencing n=10) individuals. An additional 15 and 12 fecal samples were collected from wild giant pandas for 16S rRNA gene and ITS high-throughput sequencing, respectively. According to the learning status of wild-training II giant panda, G1 and G2 were chosen for reintroduction. Detailed information for samples is provided in Supplementary Table S3.

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) following the manufacturer's protocols. Fungal DNA was extracted using the E.Z.N.A.™ Fungal DNA Mini Kit (OMEGA Bio-tek, Inc., Norcross, GA) according to manufacturer's instructions. Successful DNA isolation was confirmed by 1% agar gel electrophoresis. Bacterial amplicon libraries were prepared by amplifying the V4 hypervariable region of 16S rRNA genes, while fungal amplicon libraries were prepared by amplifying the ITS1 region, as described previously (Huang et al 2015). Amplicon sequencing was performed on the Illumina Hiseq 2500 (diet conversion experiment) and Illumina Miseq 2500 (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 single-sample assemblies and the co-assembly, as described previously (Stewart et al 2018) and in further detail below.

Data analysis

Raw paired-end sequences were preprocessed using the HiSeq Control Software (diet conversion) and the MiSeq Control Software (lifestyle shift) programs. After filtering low-quality reads, clean amplicon reads were imported into the QIIME software package and analyzed as previously described (Caporaso et al 2010). Briefly, the 16S rRNA gene and ITS sequences were clustered at the 97% nucleotide sequence similarity level to generate representative operational taxonomic unit (OTU) sequences using the SILVA (Quast et al 2013) and UNITE (Koljalg et al 2013) reference databases for the bacterial and fungal libraries, respectively. Chao1 and Shannon index richness/diversity metrics were calculated in QIIME (V1.9.1) and visualized in R (V3.5.0). Principal coordinates analysis (PCoA) was conducted on the OTU compositional matrices using the Bray-Curtis (BC) distance, as implemented in QIIME with the default settings. 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).

A whole genome shotgun (WGS) library composed of around 400 bp clone inserts was generated for associated samples. Metagenomic sequencing of the library was performed on the Illumina HiSeq4000 platform (Illumina, Inc., San Diego, CA, USA) using 2x150 bp paired-end sequencing mode at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). The Seqprep (*https://github.com/jstjohn/SeqPrep*) and Sickle (*https://github.com/najoshi/sickle*) were then used to filter low quality reads with a length less than 50 bp and those reads with an average quality score < 20. The bwa aligner (*http://bio-bwa.sourceforge.net/*) was also used to remove reads that matched to the host (*Ailuropoda melanoleuca*) genome sequence as well as to genome sequences from the common plants *Malus domestica* , *Daucus carota* , *Zea mays* , *Oryza Sativa* and *Glycine max*(*https://www.ncbi.nlm.nih.gov/genome/*). The filtered

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and microbiota-enriched reads for each sample were then subjected to contig assembly using IDBA-UD (*http://i.cs.hku.hk/˜alse/hkubrg/projects/idba_ud/*) (Peng et al 2012). 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 was used to de-duplicate them (Olm et al 2017). Dereplication resulted in a total of 22 high quality bins, as assessed by CheckM (Parks et al 2017) and completeness values [?] 70% and contamination [?] 10%. The high-quality bins were retained for further analyses. Prodigal (*http://compbio.ornl.gov/prodigal/*) was used to predict genes within the high quality bins (Hyatt et al 2010). Functional annotation of the predicted genes was conducted via BLASTx analysis against the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/) and Carbohydrate-active enzymes (CAZy, http://www.cazy.org/) databases. Housekeeping phylogenetic marker genes were also identified in the 22 reconstructed genomes using amphora2 (https://github.com/martinwu/AMPHORA2).

One-way analysis of variance (ANOVA) analyses were used to identify significant differences in the alpha diversity 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. Analysis of molecular variance (AMOVA) tests were used to identify significant differences in GM structure among different treatment groups based on BC distances, with a p value < 0.05 used to identify statistical significance. Non-parametric factorial Kruskal-Wallis sum-rank tests were also used to detect significant differences in the phylum- or genus-level taxonomic compositions between groups in the LEfSe analyses. Further, LDA 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.

RESULTS

3.1 Variation of gut bacterial composition due to 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 3,027 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) (Figure 1a, b). Specifically, gut bacterial diversity increased when transitioning from OMD to MBD and OBD diets, while richness conversely declined. PCoA analysis also indicated that samples from the same diet group clustered together and separately from those of different groups (Figure 1c). Indeed, significant differences in community structure were identified among the three groups based on BC distances ($p < 0.05$, AMOVA).

Proteobacteria and Firmicutes were the dominant phyla among communities sampled during diet conversion, comprising more than 90.0% of the total sequences (Figure 1d). However, Proteobacteria was the most dominant phylum in OMD (comprising 85.5% of the total sequences) and MBD (57.7%) communities, while Firmicutes was the most dominant phylum in OBD (58.3%) communities. Further, the abundance of Proteobacteria was significantly highest in OMD (85.5%) communities than in other groups and was considerably lower in the OBD (35.1%) communities (non-parametric factorial Kruskal-Wallis sum-rank test, LDA > 4) (Figure 1e). Conversely, Firmicutes abundances increased markedly from the OMD (13.5%) to the OBD (58.3%) communities (Non-parametric factorial Kruskal-Wallis sum-rank test, LDA>4), and were significantly higher in the OBD samples (Figure 1d, e).

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

1e, f). Lastly, *Pseudomonas*(13.4%)*, Lactobacillus* (12.7%), and *Clostridium* (12.2%) were the three most abundant genera in the OBD communities. In addition,*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) (Figure 1e, f).

3.2 Metagenomic analysis of gut bacterial metabolic functions in giant pandas

A total of 22 draft MAGs were obtained from metagenomics analysis, in which 13 were classified to the genus level (Table 1). We focused on the KEGG database-mapped metabolic pathways associated with*Streptococcus, Pseudomonas, Lactobacillus, Enterococcus, Lactococcus,* and *Acinetobacter* in the diet conversion experiment due to their significantly different abundances among groups 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 (Figure 2a). Further, several genes involved in 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*) were observed in the genomes. In addition, genes involved in 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*) were also observed. The enzymes involved in cellulose digestion pathways are shown in a schematic in Figure 2b.

To better evaluate the capacity for lignocellulose degradation in the communities, specific genes identified in the metagenomic binning analyses were annotated using the CAZys database (Figure 2c). The auxiliary activities (AAs) family including AA3, AA4, AA6, and AA7 representatives that are associated with lignin degradation were abundant among communities. These AA families were mostly found in*Streptococcus* (accounting for 30.7% of their total genes), in addition to *Pseudomonas* (5.6%), *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 beta-1,4-beta-glucanases (EC 3.2.1.74), 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, respectively. Conversely, GH2 was mostly observed in *Enterococcus* (3.1%) MAGs, while GH8 were mostly observed in *Pseudomonas* (0.2%) and*Acinetobacter* (0.1%) MAGs.

3.3 Gut fungal community variation in giant pandas after 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. Although richness and diversity varied with diet conversions, significant differences in fungal community richness and diversity were not observed among communities from the three experimental groups ($p > 0.05$, ANOVA) (Figure 1a, b). However, PCoA indicated that the communities from hosts with the same diet clustered together and separately from others (Figure 1c). Likewise, significant differences in community structure based on BC distances were identified among the three groups ($p < 0.05$, AMOVA).

Ascomycota was the most dominant phyla among communities in the diet conversion experiment, followed by Basidiomycota (Figure 1d). Basidiomycota notably increased markedly in the OBD (41.9%) communities relative to the OMD (7.6%) communities (Non-parametric factorial Kruskal-Wallis sum-rank test, LDA>4) (Figure 1e). 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 abun-

dant genera among communities of the OMD treatment. *Candida* (3.0%), *Microidium* (2.7%), and *Gibberella* (1.0%) were the three most abundant genera in the MBD communities. Lastly, *Cystofilobasidium* (9.0%), *Guehomyces* (8.1%), and *Microidium* (5.2%) were the three most abundant genera in the OBD communities (Figure 1f). *Candida* abundances were significantly higher in the OMD (37.0%) communities and were significantly lower in the OMD (37.0%) to OBD (0.1%) communities (Non-parametric factorial Kruskal-Wallis sum-rank test, LDA>4) (Figure 1e, f). In addition, *Cystofilobasidium* , *Guehomyces,* and *Gibberella* were significantly more 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 different lifestyles

A total of 4,747,957 bacterial 16S rRNA gene sequencing genes were obtained from samples in the lifestyle change experiments. After removal of mitochondria and chloroplasts sequences, 4,644,322 16S rRNA gene sequences were clustered into 3,720 OTUs at the 97% sequence identity threshold. The richness and diversity of gut bacterial communities varied with lifestyle shifts and significant differences were observed among the wild-training I, wild-training II, reintroduced and wild groups (Figure 3a, b). The richness and diversity of gut microbial communities from wild pandas were higher than those of wild-training I or wild-training II groups. Specifically, gut bacterial community richness in the wild pandas was significantly higher than those of wild-training I pandas ($p < 0.05$, ANOVA test). Moreover, community diversity in the wild pandas was significantly higher than those of the wild-training II and reintroduced pandas ($p < 0.05$, ANOVA test). PCoA of bacterial community composition indicated that samples from hosts with the same lifestyle clustered together and distinctly from others (Figure 3c).

Proteobacteria and Firmicutes were the dominant phyla among lifestyle shift samples regardless of lifestyle, comprising more than 98.0% of the sequences (Figure 3d). In particular, Proteobacteria was the dominant phylum in wild-training I panda gut communities (61.0%) and was significantly higher than in those of other groups (6.4% in wild-training II and 15.2% in reintroduced pandas) (Non-parametric factorial Kruskal-Wallis sum-rank test, LDA>4) (Figure 3d, e). Conversely, Firmicutes was the dominant phylum in the wild-training II (92.5%) and reintroduced (84.2%) pandas, and was 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 (Figure 3e, f). *Escherichia* (30.6%), *Acinetobacter* (22.4%), and *Streptococcus* (20.1%) were the most abundant genera in the communities of wild-training I pandas (Figure 3d). Among these, *Escherichia* abundances were significantly enriched in the communities of the wild-training I group, and significantly lower in the reintroduced group (Non-parametric factorial Kruskal-Wallis sum-rank test, LDA>4) (Figure 3e). In addition, the abundances of *Acinetobacter* in the communities of wild-training I pandas (22.4%) were significantly higher than in those of wild-training II (0.4%) and reintroduced (3.2%) pandas (Non-parametric factorial Kruskal-Wallis sum-rank tests, LDA>4). *Streptococcus* (64.2%) was the dominant genus in wild-training II panda communities and were significantly more abundant than in those of wild-training I (20.1%) and reintroduced (5.2%) pandas, followed by *Leuconostoc* (13.0%) and *Clostridium* (10.9%). *Clostridium* (40.2%), *Leuconostoc* (22.8%), and *Turicibacter* (8.0%) were the most abundant genera in the reintroduced pandas, and were significantly more abundant than in wild-training I (5.5%, 3.8%, and 0.4%, respectively) and wild-training II (10.9%, 13.0%, and 1.7%, respectively) panda gut communities. *Clostridium* and *Turicibacter* were notably significantly higher in the reintroduced panda gut communities (40.2% and 8.0%, respectively) compared to those of the wild-training I pandas (10.9% and 1.7%, respectively) to (Non-parametric factorial Kruskal-Wallis sum-rank test, LDA>4).

The GM composition of reintroduced pandas was more similar to those of wild-training I and wild-training II pandas (Figure 3c). However, 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 Figure 1). Specifically, Firmicutes (84.2%), Acidobacteria (0.02%), and Cyanobacteria (0.01%) were more abundant in the reintroduced panda communities ($p < 0.05$, Wilcoxon test) (Supplementary Figure 1a). In

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contrast, Proteobacteria (51.4%), Bacteroidetes (24.9%), Verrucomicrobia (1.5%), and Actinobacteria (0.8%) were more abundant in wild pandas ($p < 0.05$, 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 pandas ($p < 0.05$, Wilcoxon test) (Supplementary Figure 1b). Conversely, *Pseudomonas* (28.3%), *Sphingobacterium* (8.8%), *Flavobacterium* (6.0%), and *Pedobacter* (5.4%) were more abundant in the wild pandas ($p < 0.05$, Wilcoxon test).

3.6 Variation in gut fungal communities of giant pandas with different lifestyles

A total of 4,362,547 fungal ITS sequences were obtained from samples in the lifestyle shift experiments. After removal of mitochondria and chloroplast sequences, 4,218,492 ITS sequences were clustered into 7,438 OTUs at the 97% sequence identity threshold. Fungal community richness was significantly higher in the GM of reintroduced and wild pandas compared to those of wild-training I and wild-training II ($p < 0.05$, ANOVA test) (Figure 3a). The fungal diversity of wild-training I panda communities was significantly lower than in those of other three groups ($p < 0.05$, ANOVA test) (Figure 3b). PCoA indicated that fungal communities from hosts with the same lifestyle clustered together and separately from others (Figure 3c).

Ascomycota and Basidiomycota were the dominant phyla of fungal communities in the lifestyle shift experiments regardless of lifestyle, although their abundances varied by lifestyle (Non-parametric factorial Kruskal-Wallis sum-rank test, LDA>4) (Figure 3d, e). Ascomycota had notably higher relative abundances in wild-training I panda gut communities (92.4%), while Basidiomycota were more abundant in wild-training II (33.4%) pandas (Non-parametric factorial Kruskal-Wallis sum-rank test, LDA>4) (Figure 3e). At the genus level, *Candida* (83.1%) was the most dominant genus in wild-training I panda communities and were significantly more abundant than in wild-training II (3.2%) and reintroduced (2.8%) pandas, followed by *Williopsis* (2.7%) and *Cryptococcus* (0.7%) (Non-parametric factorial Kruskal-Wallis sum-rank test, LDA>4) (Figure 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. Further, the abundances of *Cryptococcus* (12.3%), *Cystofilobasidium* (8.1%), *Purpureocillium* (3.8%), and *Penicillium* (2.5%) were significantly higher in wild-training II panda communities than in those of 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) pandas (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 abundances of *Mrakiella* (9.2%) were significantly higher in the reintroduced panda communities relative to the wild-training I (0.1%) and wild-training II (0.7%) pandas (Non-parametric factorial Kruskal-Wallis sum-rank test, LDA>4).

Although the gut fungal community compositions in the reintroduced and wild pandas were more similar to each other than they were to those of the wild-training I and wild-training II pandas, significant genus level differences were observed between the reintroduced and wild panda communities ($p < 0.05$, Wilcoxon test). Specifically, 11 genera were significantly different between reintroduced and wild panda communities (excluding unidentified genera), including *Candida* and *Calycina* which were among the 10 most abundant genera (Supplementary Figure 2). *Candida* (2.8%) were more abundant in reintroduced pandas, while *Calycina* (13.2%) was more abundant in wild pandas ($p < 0.05$, Wilcoxon test).

DISCUSSION

To understand whether giant panda GM could contribute to their host's ability to digest bamboo and inform the successful reintroduction of giant pandas, we assessed the variation in their GM during diet conversions and lifestyle shifts. The richness, diversity, and composition of giant panda gut microbial communities varied with diets and were influenced by lifestyle shifts. Diet and surrounding environments play important roles in shaping the GM of animals, including of humans (Koren et al 2012), snub-nosed monkeys (Miriam et al 2013), black honeybees (Zhao et al 2018) and mice (Miriam et al 2013). Similarly, our results suggest that diet conversion and lifestyle shifts are critical factors influencing the GM of giant pandas.

Gut microbiome dynamics associated with dietary shifts

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Gut bacterial community diversity increased when transitioning from the OMD to OBD diets of pandas, while richness exhibited an opposite trend. Gut bacterial community richness has been observed as higher in pandas with lower fiber diets compared to those with higher fiber diets (Guo et al 2018, 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). However, high fiber diets can increase the diversity of GM in humans, while high fat diets are associated with lower diversity (Carlotta et al 2010, Tap et al 2016, Zhernakova et al 2016). Competitive interactions among bacteria are ubiquitous in natural systems, although 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 et al2016). Indeed, lignocellulose is a cross-linked structure that is difficult to degrade. Thus, bacteria may need to form consortia to synergistically achieve lignocellulose degradation (Deng et al 2016, Perez et al 2002). Thus, fiber content could be an important factor underlying variation in richness and diversity of gut microbial populations among the pandas in the OMD, MBD, and OBD groups. Consequently, our results support that high fiber diets could increase the diversity, but decrease the richness of gut bacterial communities in giant pandas.

Among all sampled gut communities, the Proteobacteria and Firmicutes dominated, which is consistent with previous studies of giant panda GMs (Yang et al 2018, Zhang et al 2018). Proteobacteria may be more dominant in the guts of herbivores with low metabolic rates (Dill-McFarland et al 2016), which is consistent with the low expenditure and physical activity of giant pandas (Yonggang et al 2015). 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). Interestingly, Firmicutes abundances exhibited a gradient in the transition from OMD to OBD group communities. Firmicutes abundances have also been positively associated with fiber content in human guts (Carlotta et al 2010), in addition to significantly associated with supplemented dietary fiber in dogs (Costa et al 2012). The positive association of Firmicutes abundances with lignocellulose ingestion in giant pandas could suggest that they are important for digesting high fiber bamboo foods into more labile nutritional components.

At the genus level, *Streptococcus* (Firmicutes phylum) abundances have been shown to significantly increase upon introduction of a bamboo diet (Ouwehand et al 2010). Moreover,*Streptococcus* are associated with giant panda gut mucus (Williams et al 2016) that is critical for dietary conversions of giant pandas from low diet to high fiber diets. 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 observed the presence of the gene encoding Protein-Npi-phosphohistidine-cellobiose phosphotransferase (EC 2.7.1.205, *celB*) in the *Streptococcus* MAG, which is important for cellulose digestion (Lai et al 1997), thus indicating the potential for cellobiose utilization by the *Streptococcus* in these panda gut communities. In addition, genes encoding 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 , Rytioja et al 2014). In particular, GHs are often associated with digestion of cellulose and hemicellulose (Stewart et al 2018), and were accordingly identified in the *Streptococcus* MAG via comparison to the CAZy databases. It should be noted that 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). Accordingly, several enzyme-encoding genes involved in hemicellulose and lignin degradation were present in the *Streptococcus* MAG including CE1, CE3, CE4, CE5, AA3, AA4, AA6, and AA7 group genes (Zhang et al 2018, Zhen and Jr 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 (Lifeng et al 2011). Consistent with these genomic predictions, *Pseudomonas, Clostridium,Lactobacillus, Enterococcus, Lactococcus* and *Acinetobacter*abundances exhibited a gradient of increase when transitioning from the OMD to OBD group pandas. *Clostridium* and *Enterococcus* have been

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positively correlated with crude fiber digestibility, while *Lactobacillus* , *Enterococcus,* and *Pseudomonas* have been positively associated with acid detergent fiber digestibility (Niu et al 2015). Moreover, the involvement of *Pseudomonas* and *Acinetobacter* in the degradation of lignin has been previously demonstrated (Jimenez et al 2015). Thus, our results indicate that *Streptococcus* , *Pseudomonas* ,*Enterococcus, Lactococcus, Acineto-bacter,* and *Clostridium* may contribute to the utilization of cellulose and hemicellulose from bamboo, thereby providing energy and nutrients for their giant panda hosts.

Interestingly, gut bacterial communities of giant pandas 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 giant pandas were more similar to those of bears and entirely distinct to those of herbivores via comparison among 57 mammalian species including giant pandas, its close relatives, typical carnivores, and distantly related herbivores (Xue et al 2015). We therefore hypothesize that the gut bacterial communities of bears and even carnivores have the potential to metabolize fiber or otherwise that these bacterial communities have evolved in concert with giant panda evolution. Nevertheless, additional research is needed to evaluate the above hypothesis.

No significant differences were observed in the richness and diversity of fungal communities among the three dietary groups. Interestingly, Basidiomycota abundances significantly increased in the transition from the OMD to OBD diets, suggested that they may play a role in the utilization of bamboo by giant pandas. Ascomycota and Basidiomycota have been previously shown to dominate the fungal gut communities of giant pandas (Tun et al 2014, Zhang et al 2018b), which coincides with their dominance in soil (Xu et al 2012) and bamboo (Zhou et al 2017) fungal communities. These observations have led to the hypothesis that giant panda gut microbiomes may originate from their food sources or even from soils (Hannula et al 2019, Nina et al 2013). *Candida* was the dominant fungal genus in the OMD communities and significantly declined in abundance in the transition from the OMD to OBD diets. *Candida* abundances in gut fungal communities have 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), suggesting that *Candida* may be involved in milk metabolism in the guts of newborn giant pandas. *Cystofilobasidium* ,*Guehomyces,* and *Gibberella* abundances increased markedly in the transition from the OMD to OBD diets (Non-parametric factorial Kruskal-Wallis sum-rank test, LDA>4). Therefore, we also hypothesized that *Cystofilobasidium* , *Guehomyces,* and *Gibberella* may contribute to the ability of giant pandas to digest bamboo.

Gut microbiome dynamics associated with lifestyle shifts

Wild giant pandas exhibited significantly higher gut bacterial community richness and diversity than did wild-training I/II pandas, consistent with previous studies of primates (Clayton et al 2016) and bears (Borbon-Garcia et al 2017). The increased richness and diversity could be due to the exposure to more diverse microbial meta-communities via habitats of wild giant pandas compared to those of wild-training I pandas (Burns et al 2016, Schmidt et al 2019, Wu et al 2017).

Proteobacteria and Firmicutes dominated the bacterial communities among all hosts with different lifestyles, which is consistent with previous studies (Zhang et al 2018, Zhu et al 2011). However, Proteobacteria was the most dominant phylum in the gut of wild-training I giant pandas, while Firmicutes was the most abundant phylum in wild-training II and reintroduced pandas. Proteobacteria have also been observed as the dominant phylum in the gut communities of wild-training I giant pandas (Wei et al 2015), while Firmicutes have been observed as the dominant phylum in the guts of wild pandas (Zhu et al 2011). Similarly, Firmicutes were the dominant phylum in wild deer mice and musk deer (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). Non-captive giant pandas require maximal energy from their diets due to environmental stressors that wild-training I giant pandas do not experience (Schmidt et al 2019). Consequently, we also hypothesized that environmental stressors/threats (including pathogens, intraspecific competition, and interspecific competition, among others) may be important factors that drive the composition of panda intestinal bacterial communities during lifestyle shifts. In addition to the above, Bacteroidetes, Verrucomicrobia, and Actinobacteria abundances were higher in

the wild giant panda communities compared to those of the reintroduced pandas (Non-parametric factorial Kruskal-Wallis sum-rank test, LDA>4). Bacteroidetes have been positively associated with the digestion of carbohydrates and proteins, and may help facilitate the development of gut immune systems (Ley et al 2006, Li et al 2017). In addition, Actinobacteria have been positively associated with fat digestion (Wu et al 2011). Thus, these results suggest that wild giant pandas had more complex environmental habitats and food choices that likely affected their GM.

Verrucomicrobia have been notably not previously observed in the guts of giant pandas although they were relatively abundant in the gut communities of wild pandas in our study. Verrucomicrobia have been observed in the gut communities of primates and termites in addition to various other environments (Dedysh et al 2006, He et al 2010, Lee et al 2009, Manjula et al 2016, Su et al 2016). The more microbial species that 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). These observations support our suggestion that wild giant pandas were more adapted to natural environments and interacted with more diverse microbial species than did wild-training I giant pandas.

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

The composition of gut bacterial communities in the reintroduced giant panda were closer to those of the wild pandas, although significant differences were observed between the reintroduced and wild panda communities at the phylum and genus levels (Non-parametric factorial Kruskal-Wallis sum-rank test, LDA>4). These observations suggest that the reintroduced giant pandas still maintained differences in their GM relative to wild pandas despite living in the same environment. For example, *Clostridium* , *Leuconostoc* ,*Turicibacter,* and *Acinetobacter* were more abundant in reintroduced panda communities. Conversely, *Pseudomonas* ,*Sphingobacterium,* and *Flavobacterium* were more abundant in the communities of wild pandas. All of these genera are associated with cellulose, hemicellulose, and lignin degradation (Dahal and Kim 2016, Jimenez et al 2015, Williams et al 2016, Zhu et al 2011), which is an important characteristic for the complete convergence of reintroduced giant pandas to wild pandas. Different habitats can influence the composition of GM through contact with different habitats, foods, and other materials (Borbon-Garcia et al 2017, Li et al 2017, Chave 2010). Thus, these results suggest that additional time is needed for the complete conversion of reintroduced giant panda gut communities to those of wild pandas.

As with the bacterial communities, the richness and diversity of reintroduced and wild giant panda fungal communities were significantly higher than in those of wild-training I pandas ($p < 0.05$, ANOVA). Thus, captivity could lead to decreased richness and diversity of gut fungal communities of giant pandas. The underlying mechanism behind these differences are likely the same as for the bacterial communities, wherein food, space, and interactions with human keepers are limited for captive giant pandas (Schaller et al 1985), and thereby limit potential interactions with meta-communities relative to wild pandas. Ascomycota and Basidiomycota were the dominant phyla in the communities of wild-training I, wild-training II, and reintroduced pandas, although their relative abundances varied among groups. Ascomycota and Basidiomycota have been observed as dominant in the vaginas of giant pandas (Chen et al 2017), the guts of humans (Christian et al 2013), 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). Indeed, Ascomycota and Basidiomycota are ubiquitous and abundant among most environments. Given that gut microbiome composition is driven by the frequency of contact with microbial species by hosts (Schmidt et al 2019, Smits et al 2017, Wheeler et al 2012), these

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results suggest that environmental microbiota may be one of the most important lifestyle factors that affect giant panda gut fungal communities.

Considerable variation was observed among the fungal genera associated with lifestyles. *Candida* was the dominant genus in the gut communities of captive pandas, which may help in the digestion and absorption of carbohydrates (Christian et al 2013, Iannotti et al 1973). Captive giant pandas are ensured a fixed amount of carbohydrates (e.g. shoots and panda cakes) compared to semi-captive and reintroduced pandas (Schaller et al 1985). In contrast, *Cryptococcus* was enriched in the gut communities of wild-training II giant pandas, while *Mrakiella* was abundant in those of the reintroduced pandas. *Cryptococcus* is common in natural environments and can remain in non-infective states in bodies while later reactivating and spreading to other body areas, causing serious diseases in hosts with weakened immune systems (Hagen et al 2017, Litvintseva and Mitchell 2009). *Mrakiella* are found in soils and waters, especially in low-temperature environments within various regions (Thomas-Hall et al 2010). These results consequently suggested that diet and the microbial species within specific environments may be important factors that shape the composition of intestinal fungal communities during lifestyle shifts.

A total of 31 fungal genera exhibited significantly different abundances in the gut communities of reintroduced and wild giant pandas (Non-parametric factorial Kruskal-Wallis sum-rank test, LDA>4), albeit with low relative abundances except *Calycina*. *Calycina* was enriched in the communities of the wild pandas and belongs to the Helotiales order (Zhang and Zhuang 2004) that is associated with root endophytes (Tedersoo et al 2010). These results support that wild giant pandas may have more comprehensive dietary structures or more contact with microbial meta-communities within environments compared to reintroduced pandas, which would then enhance the dietary diversity of reintroduced pandas and contribute to the recovery of natural gut fungal community compositions as seen in wild pandas.

CONCLUSIONS

Overall, these data strongly indicate that diets and lifestyles are associated with the richness, diversity, and compositions of the GM of giant pandas. Specifically, the GM of giant pandas was more similar when the hosts exhibited the same diet or lifestyle. High fiber diets significantly increased the diversity, while decreasing the richness of gut bacterial populations of giant pandas ($p < 0.05$). In addition, the abundances of *Streptococcus* , *Pseudomonas* ,*Enterococcus, Lactococcus, Acinetobacter,* and *Clostridium* significantly increased with bamboo consumption. Reconstruction of 22 metagenome-assembled-genomes (MAGs) indicated that gut bacterial populations were potentially responsible for bamboo digestion via degradation of cellulose, hemicellulose, and lignin. Captivity resulted in decreased GM diversity, especially of fungi, in the pandas. Specifically, gut bacterial community richness and diversity in wild giant pandas were significantly higher than in those of wild-training I or wild-training II pandas ($p < 0.05$). Likewise, fungal community richness and diversity of reintroduced and wild giant pandas were significantly higher than in those of wild-training I and wild-training II pandas ($p < 0.05$). Notably, the composition of GM in reintroduced giant pandas converged to those of wild pandas. Food choices and environmental meta-communities could therefore drive the structure of giant panda gut microbiomes. Further, we suggest that adaptation to increasing environmental threats or stressors could help converge the GM compositions of giant pandas to those of wild pandas.

Authors' contributions

LZ, HZ and YH conceived the idea. LJ, ZG and DW performed the experiments. LJ, SY, CL and WD

performed the statistical analyses. GZ, YX, RW, GL and HW collected the samples. AZ, YH, HZ, LZ and TY wrote the first draft of the manuscript. LJ, SY, CL, BL and L.Z. contributed substantially to revisions. LJ, SY and CL equally contributed to this work. All authors have read and approved the final manuscript.

Data Accessibility Statement

Final DNA sequence assembly uploaded as online.

Ethics approval and consent to participate

All sample collection protocols in this study were approved by the China Conservation and Research Center for the Giant Panda. The experimental procedures were fully in compliance with the current laws on animal welfare and research in China.

Consent for publication

Not applicable.

Conflict of interest

The authors declare that they have no conflict of interest.

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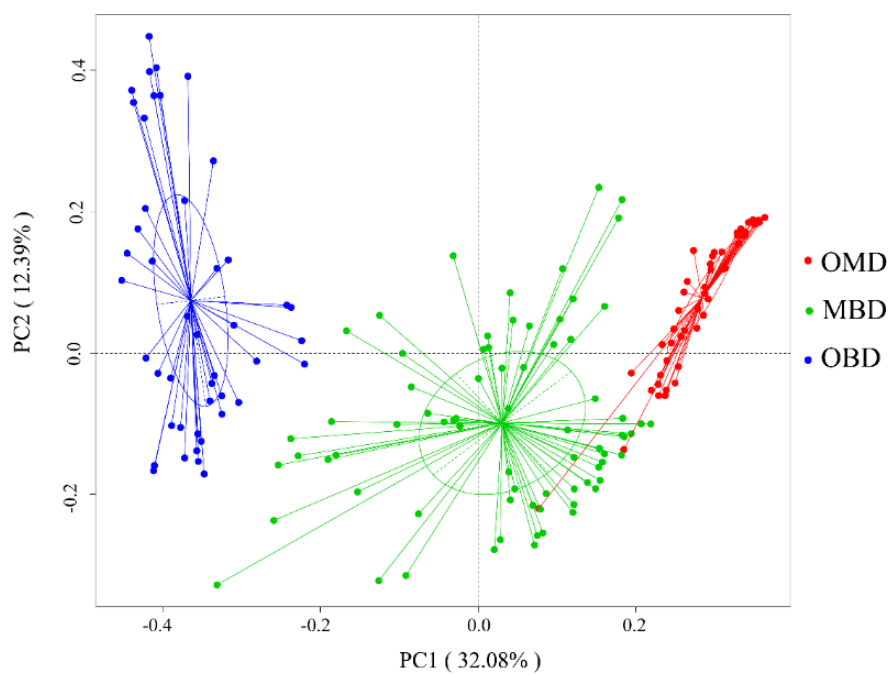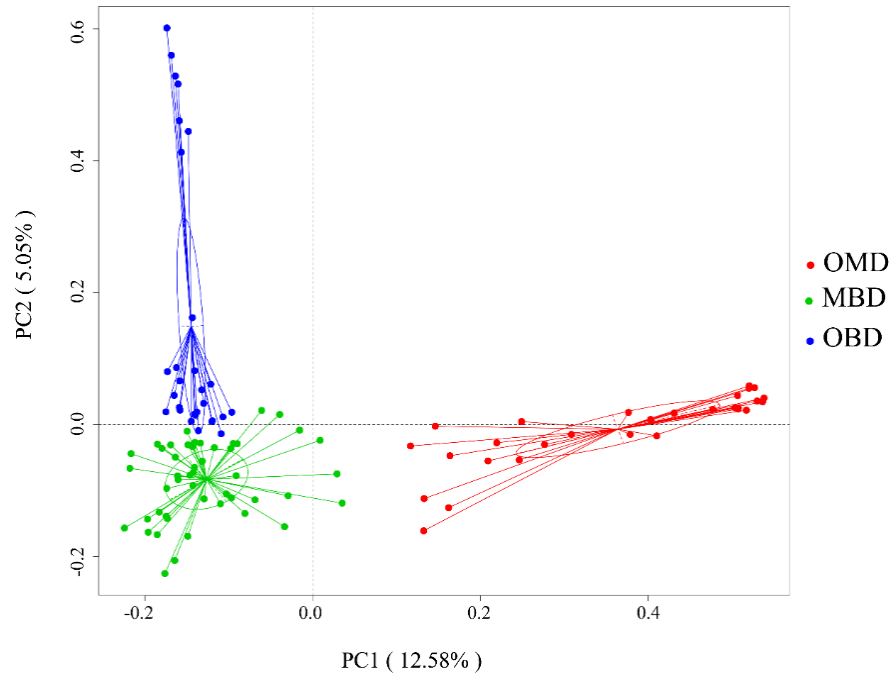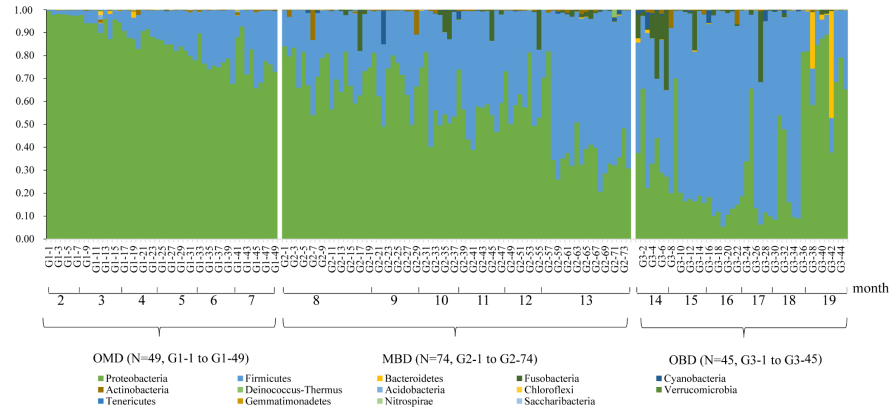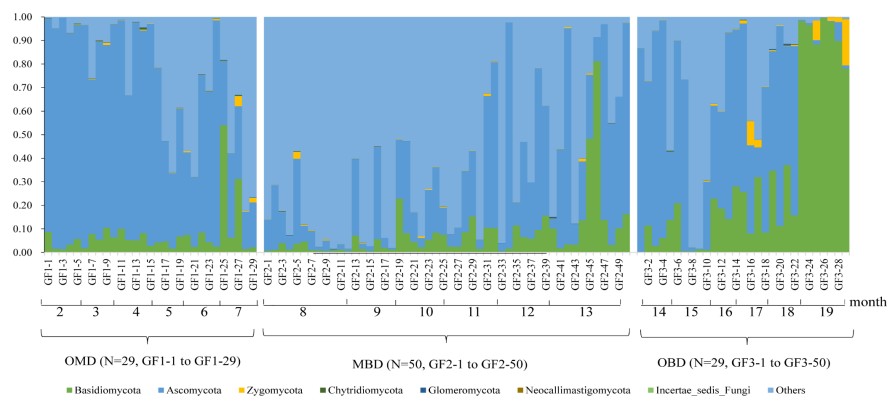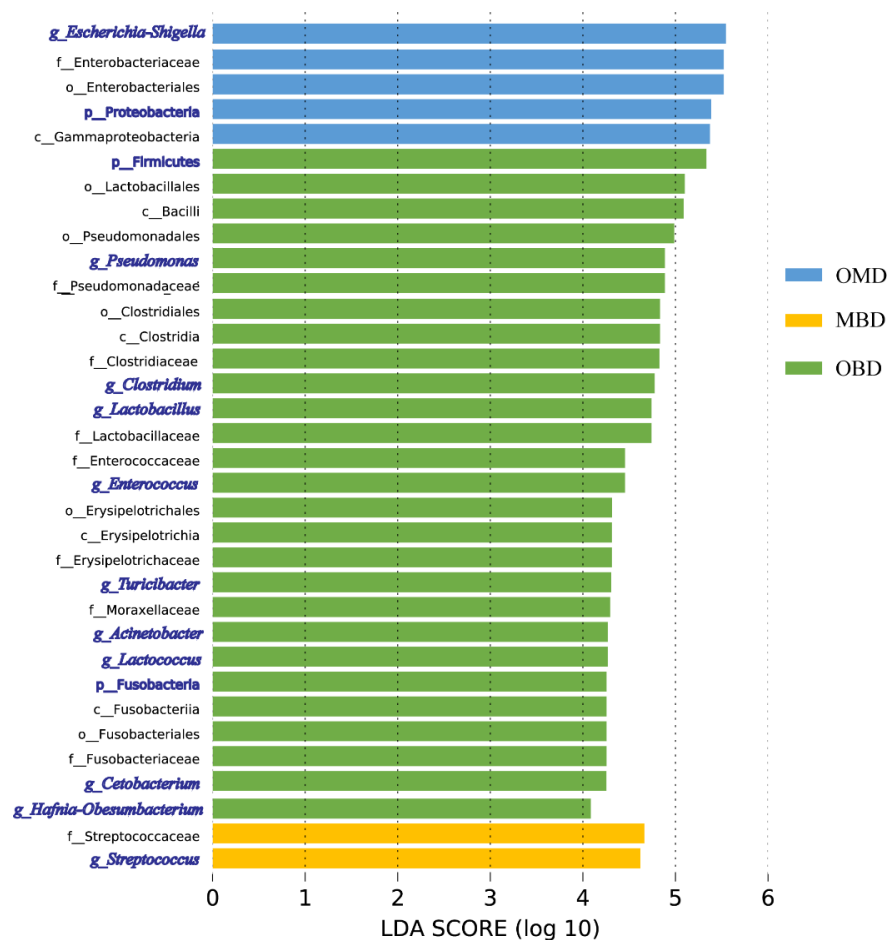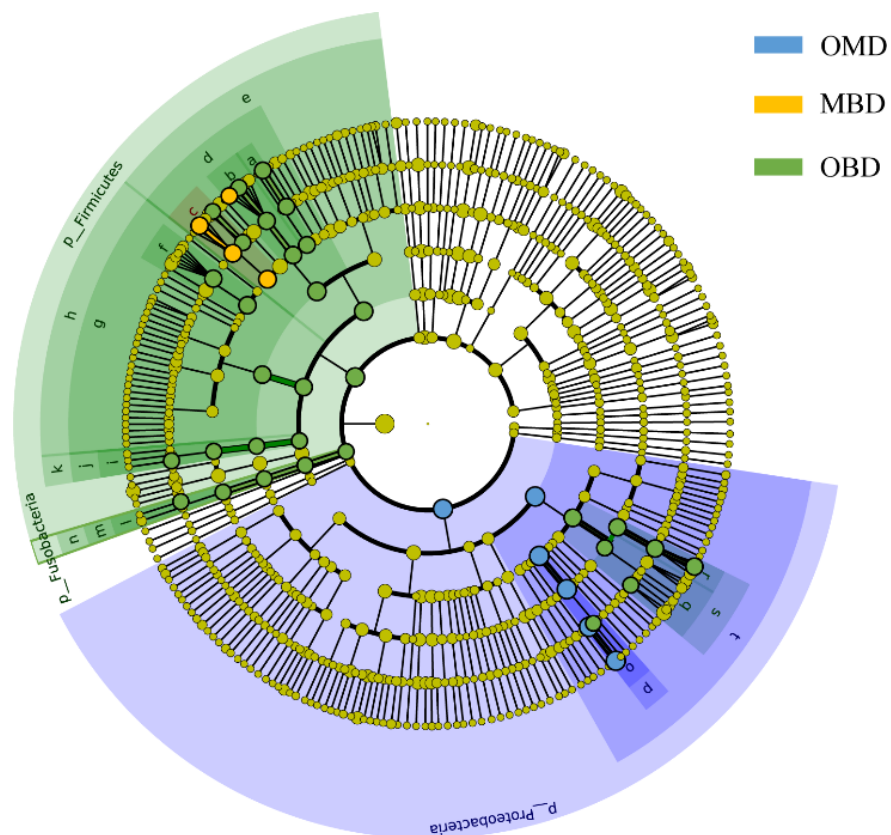
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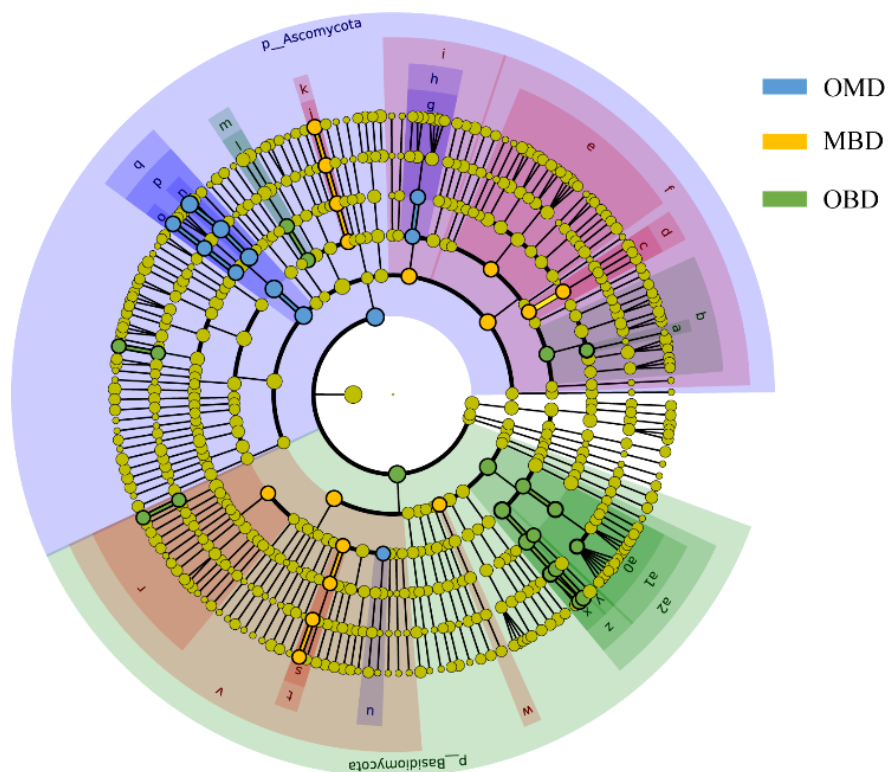
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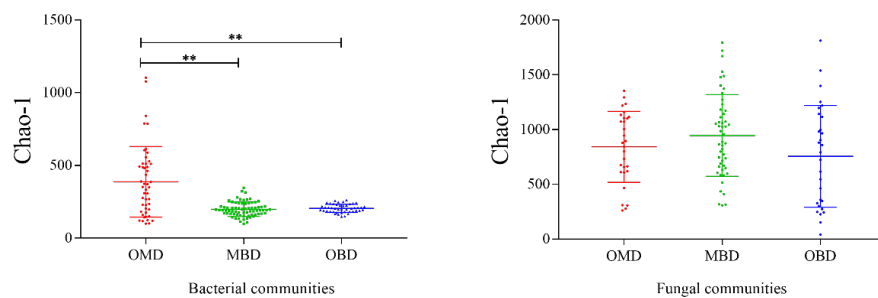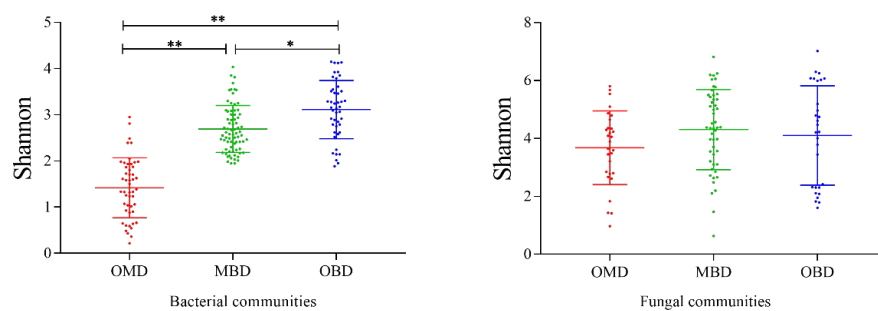
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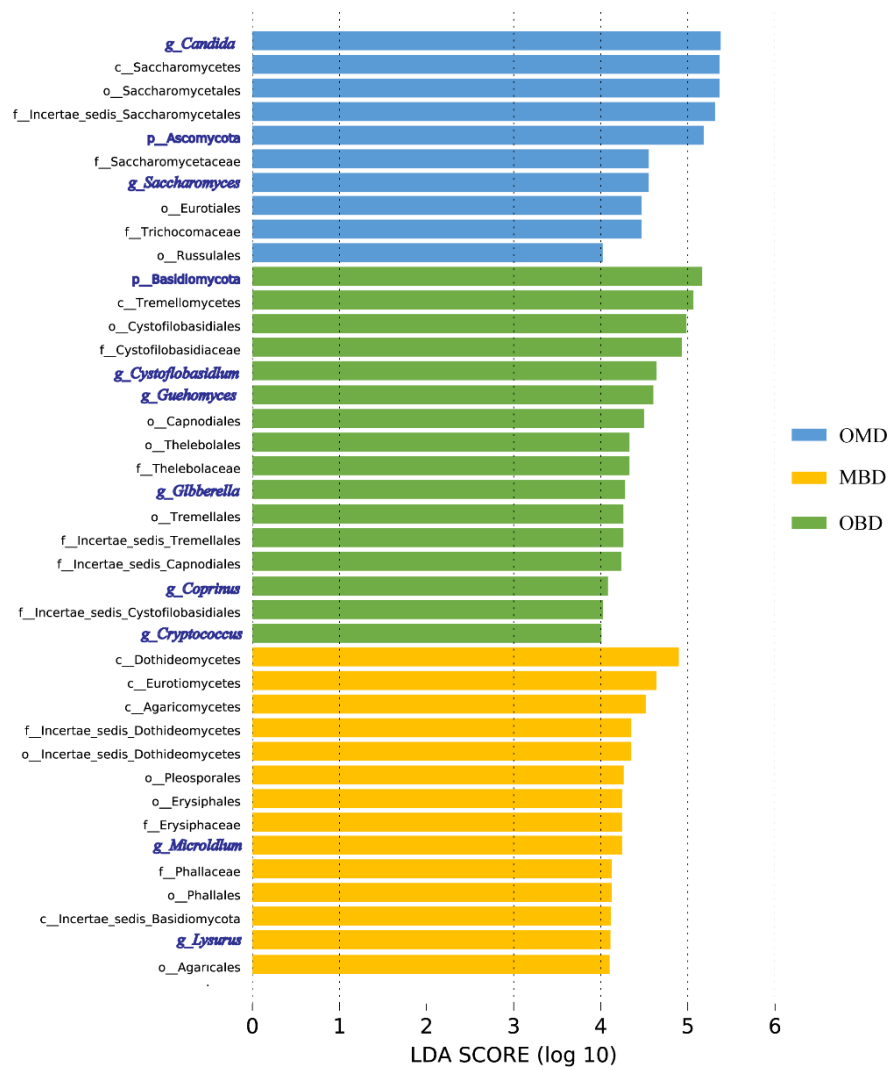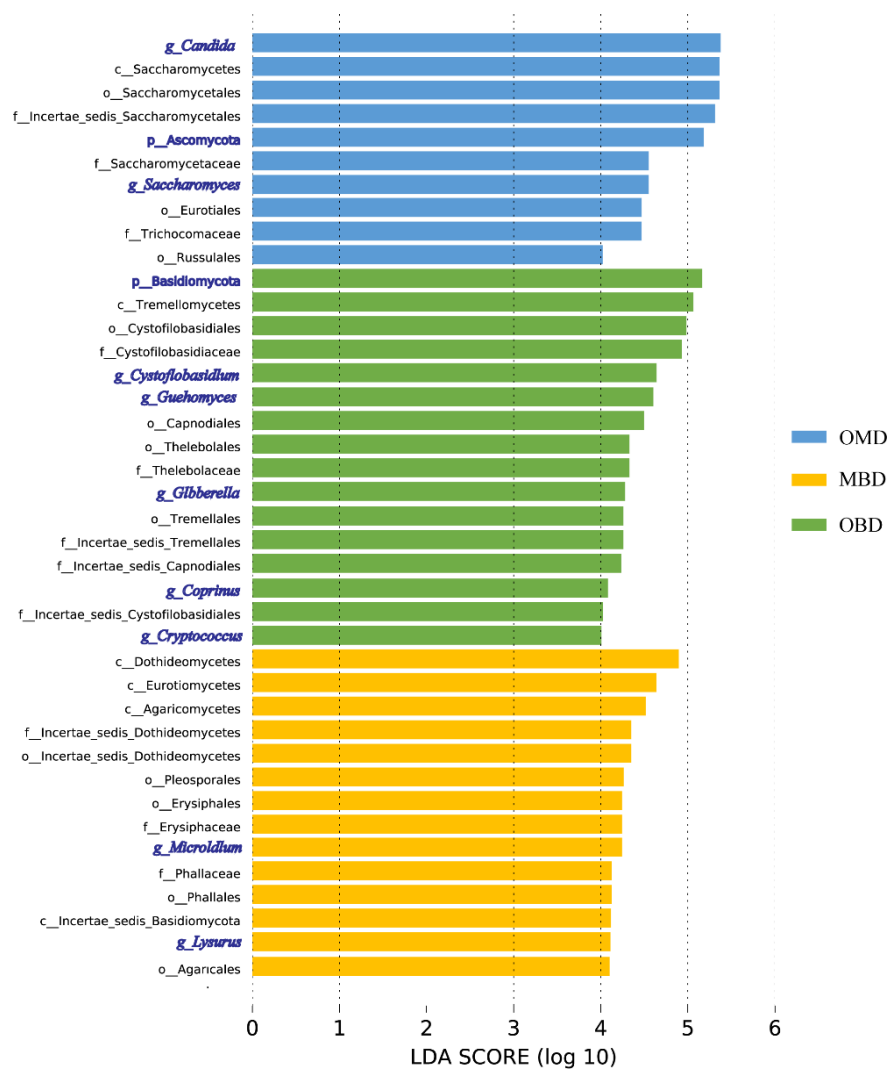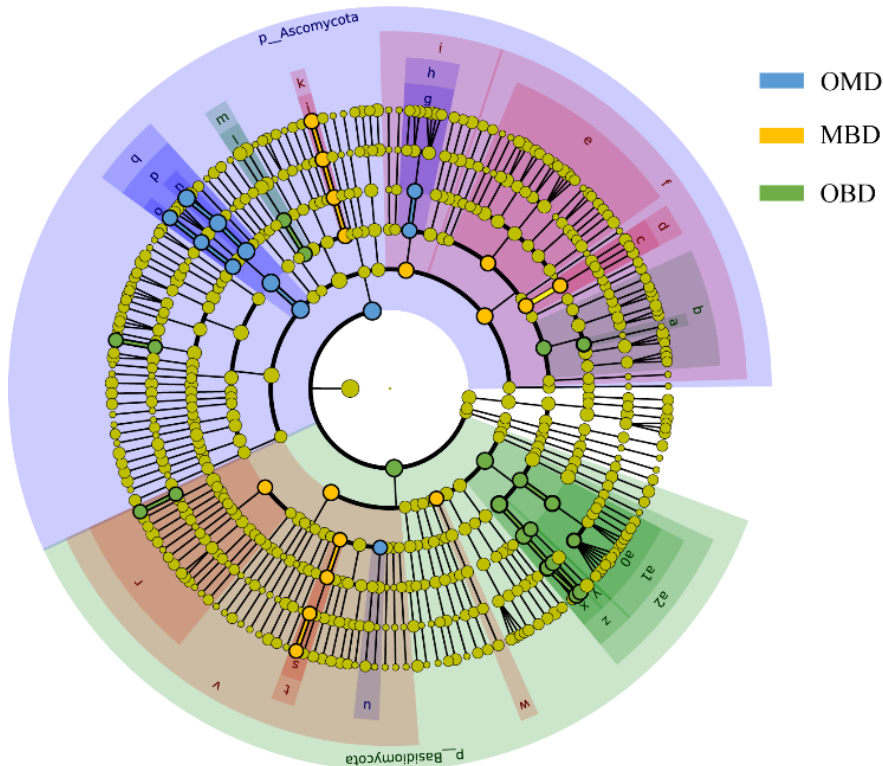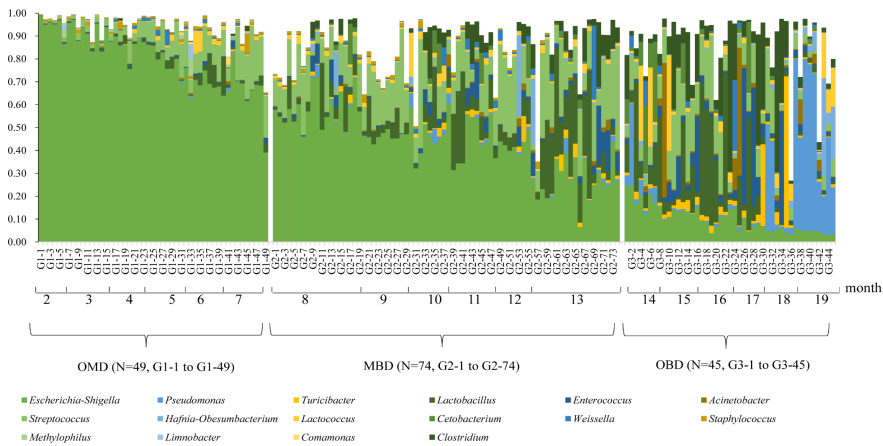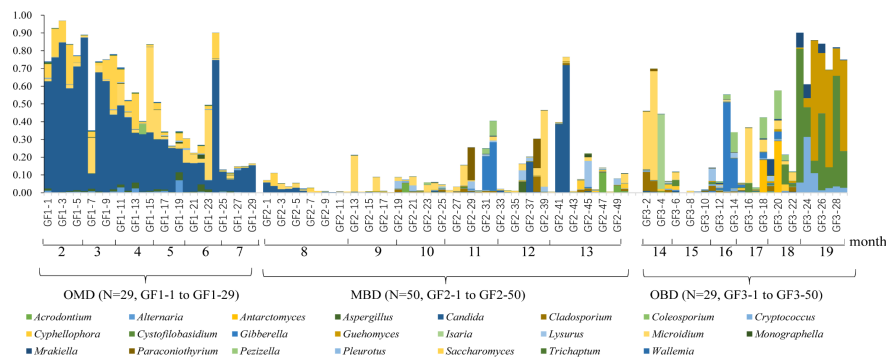Bacterial communities

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Figure 1. Gut microbiome variation due to dietary conversion. (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. *: $0.01 < p < 0.05$; **: $p < 0.01$.
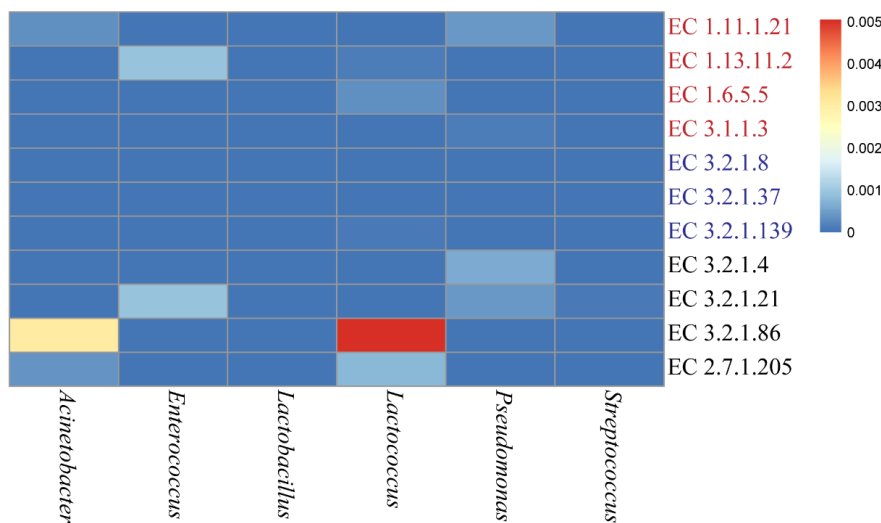

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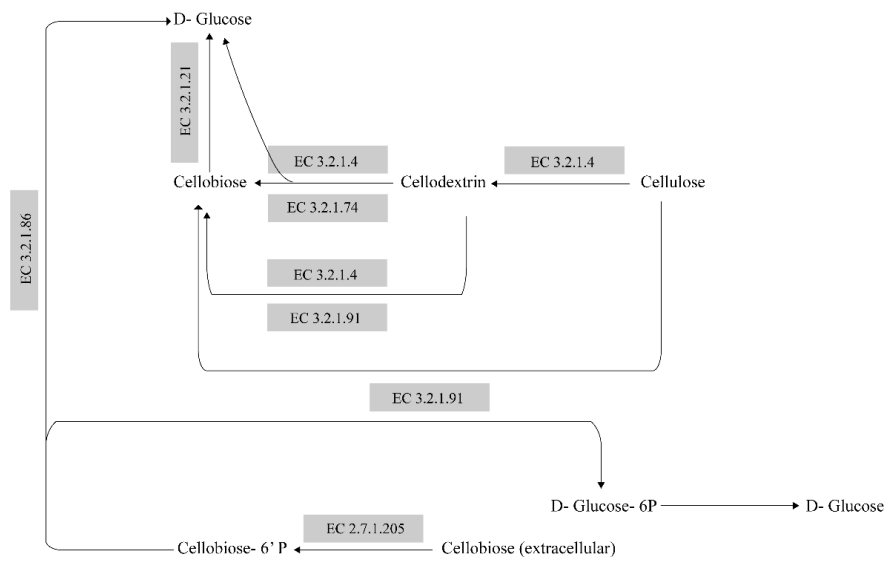
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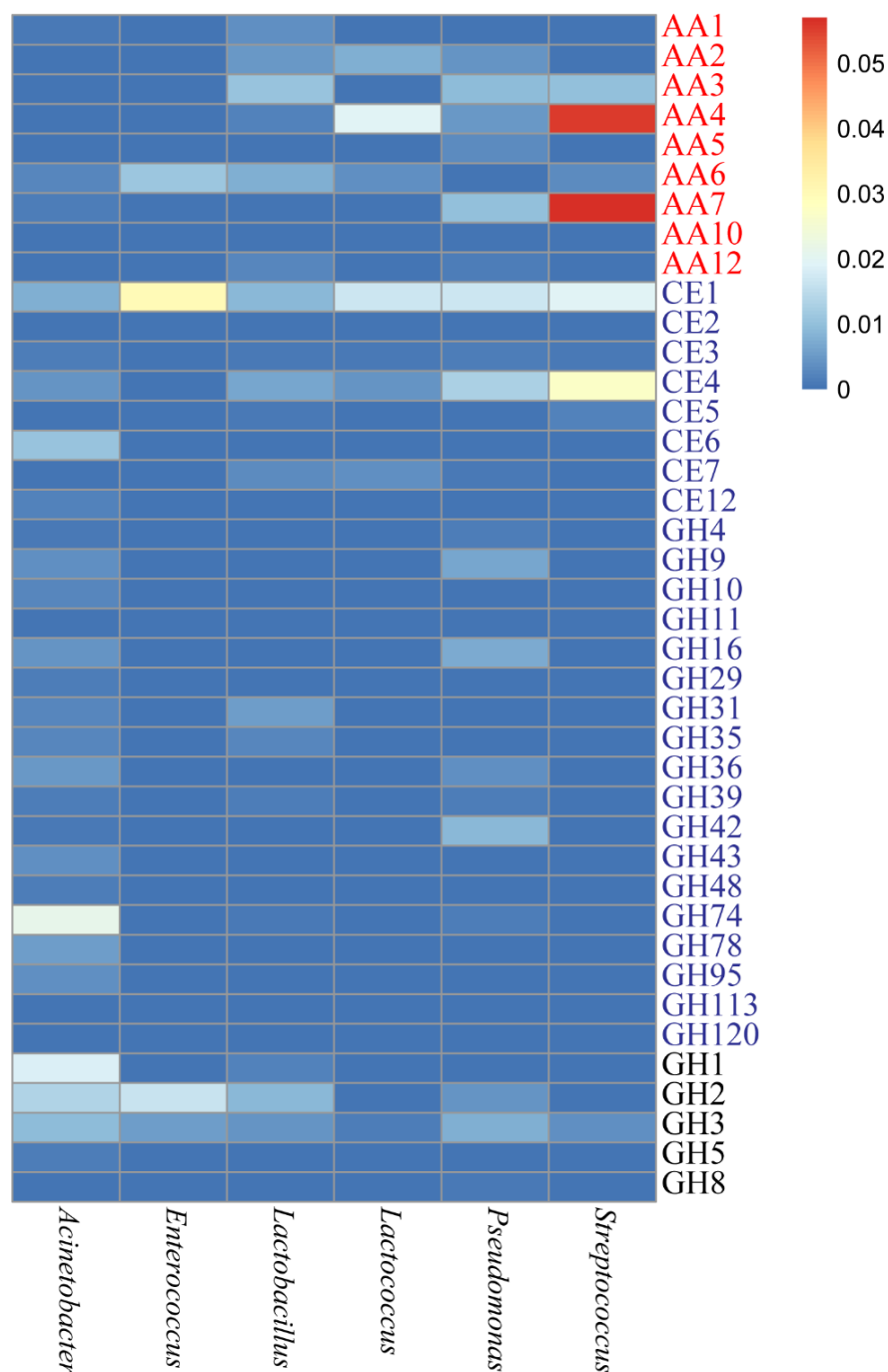
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Figure 2. Distribution of cellulose, hemicellulose, and lignin degradation associated genes across selected genomes. (a) Distribution of enzyme-encoding genes involved in cellulose, hemicellulose, and lignin degradation pathways, as determined by KEGG 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: glyco-
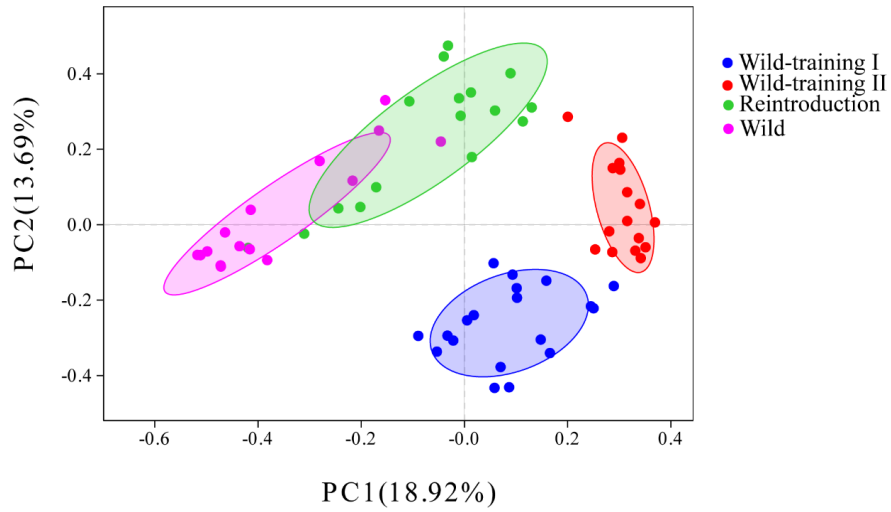
side hydrolase, CE: carbohydrate esterases, AA: auxiliary activities). Red text indicates enzymes involved in lignin degradation, blue text indicates enzymes involved in hemicellulose degradation, and black text indicates enzymes involved in cellulose degradation.
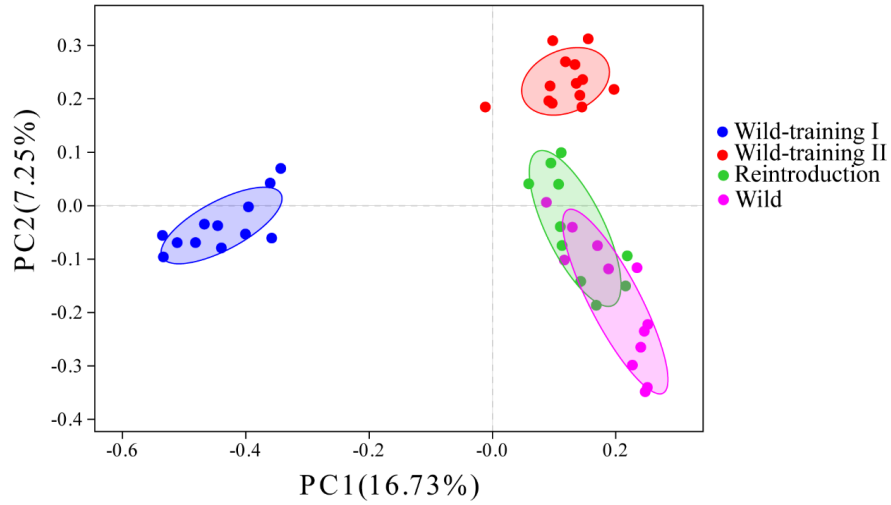
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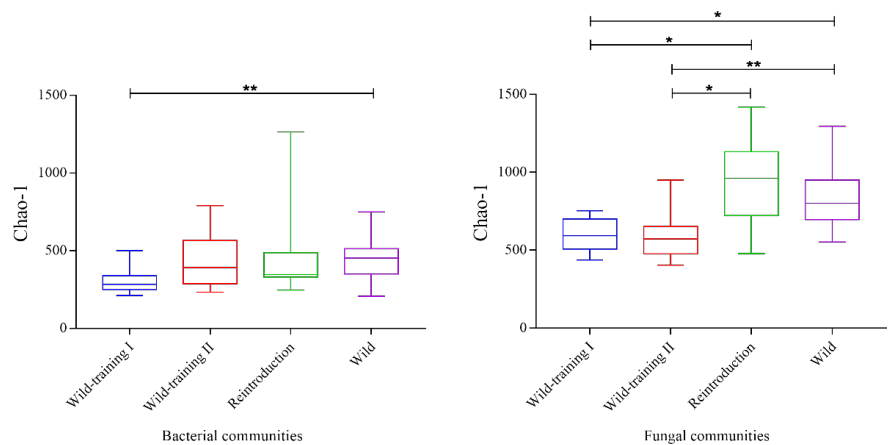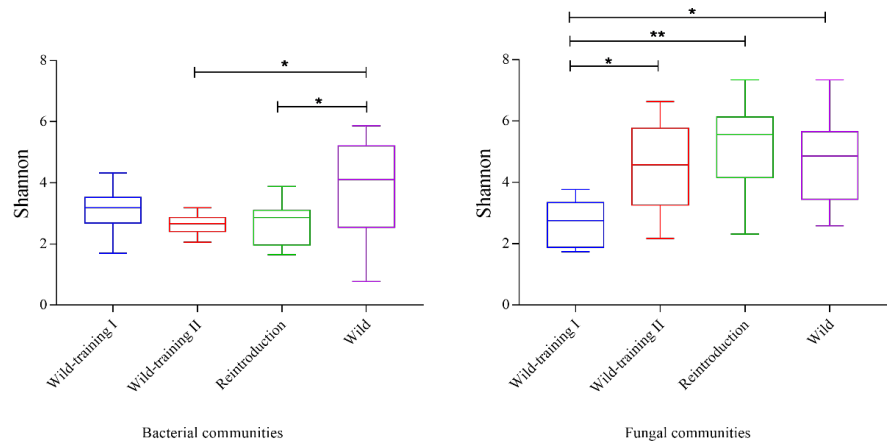
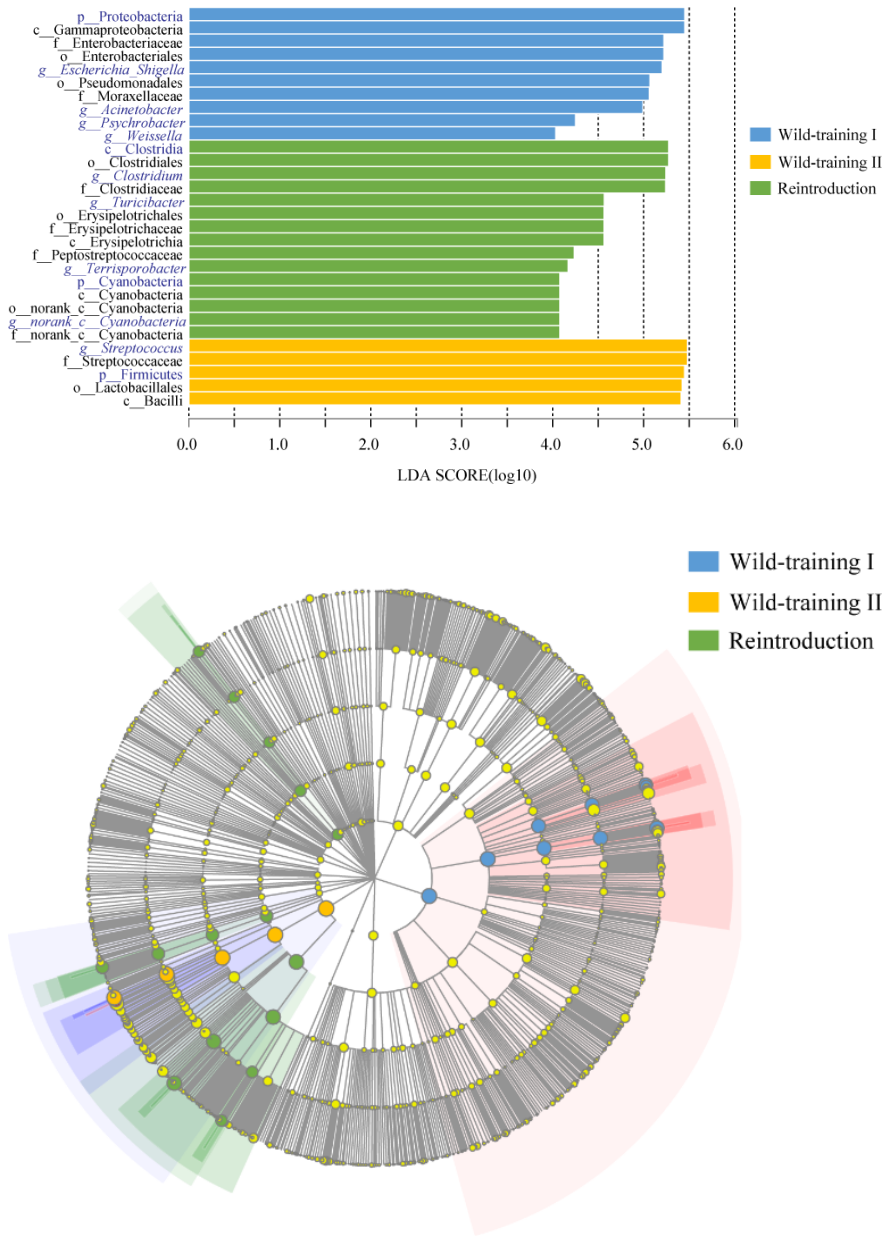Bacterial communities

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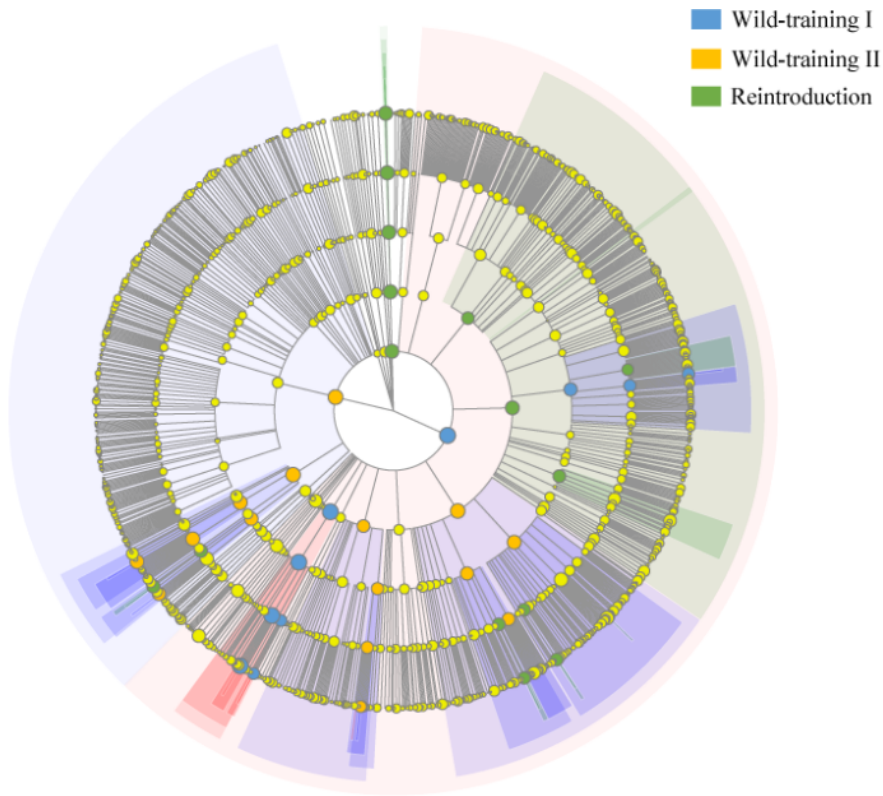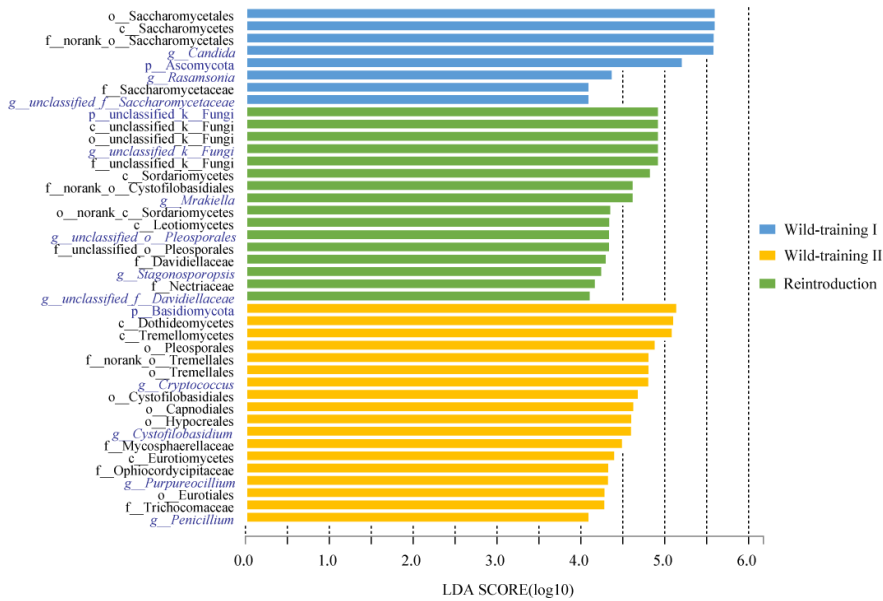
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Figure 3. Gut bacterial community variation due to lifestyle changes. (a) Variation in gut microbiome richness during lifestyle shifts. Circles represent samples, with Wild-training I panda communities shown in blue, wild-training II in red, reintroduced in green, and wild in purple. (b) Variation in gut microbiome diversity during lifestyle shifts. 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 lifestyle 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 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, red: 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.

Tables

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,*

Draft genome	Total bases (Mbp)	Number of contigs	GC content (%)	Completeness (%)	Contamination (%)	Taxonomic identification
CB13	2715	398	0.37	82.3	1.35	*Fluviicola taffensis*
CB14	3775	583	0.6	78.4	2.89	Enterobacteriace
CB15	3105	435	0.62	82.3	2.17	Sphingomonadace
CB16	4364	20	0.67	99.5	1.99	Xanthomonadace
CB17	2250	148	0.31	98.9	0.00	Fusobacteriaceae
CB18	1509	279	0.47	78.2	3.77	Gammaproteoba
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

Supplementary Materials

Supplementary Figure 1. Significant differences in bacterial phyla and genera between reintroduced and wild pandas.

Supplementary Figure 2. Significant differences in fungal genera (with relative abundances > 0.05%) between reintroduced and wild pandas.

Supplementary Table 1. Nutrition formula for the milk diet.

Supplementary Table 2. Information about the giant pandas included in the diet conversion experiment.

Supplementary Table 3. Information about the giant pandas included in the lifestyle shift experiment.