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Gut microbiota of provisioned and wild rhesus macaques (*Macaca mulatta*) living in a limestone forest in southwest Guangxi, China

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Abstract

The gut microbiota plays an important role in animal health and is strongly affected by the environment. Captivity and human source food have been shown to influence drastically the gut microbiota composition and function of wild animals. Therefore, in the present study, the gut microbiota of provisioned and wild populations of limestone-living rhesus macaques (*Macaca mulatta*) were compared using high-throughput 16S rRNA sequencing and bioinformatic analyses. The results indicated that provisioned macaques had a higher microbial richness than wild macaques, but there was no significant difference in the evenness of the gut microbiota between the two populations. Provisioned macaques also showed a higher abundance of Firmicutes and a lower abundance of Bacteroidetes than wild macaques. Functional analysis revealed that wild macaques had enriched microbial pathways involved in glycan biosynthesis and metabolism, transport and catabolism, and the digestive and endocrine systems, while provisioned macaques were richer in pathways associated with signaling molecules and interaction, neurodegenerative diseases. These differences were likely due to modification of the gut microbiota of the provisioned macaques to enable the digestion of new foods.

KEYWORDS
food provisioning, gut microbiota, limestone forest, rhesus macaque

1 | INTRODUCTION

Animal intestines feature complex microbial ecosystems that play important roles in host digestive function, metabolism, immune regulation, and disease resistance (Cao et al., 2008; Marino, 2016; Murphy et al., 2010; Saxena et al., 2012; Wallace et al., 2011)—indeed, the gut microbiome is even considered the second genome in animals (Zhu, Wang, & Li, 2010). The gut microbiota is affected by multiple intrinsic and extrinsic factors. Previous studies have identified host genetics as a crucial determinant of the gut microbiota (Bonder et al., 2016; Kurilshikov, Wijmenga, Fu, & Zhernakova, 2017), which varies not only between species due to differences in the digestive tract characteristics and functions (Ley et al., 2008), but also within species, with genetically similar individuals having greater gut microbial similarities than genetically different individuals (Goodrich et al., 2016, 2014). However, recent studies have indicated that environmental factors play more crucial roles in shaping the gut microbiota than host genetics (Barelli et al., 2015; David et al., 2014; Nelson, Rogers, Carlini, & Brown, 2013; Rothschild et al., 2018; Vangay et al., 2018), with genetically unrelated individuals who live together in the

long term having similar gut microbiota and relatives who live apart exhibiting significant differences in their gut microbiota (Rothschild et al., 2018).

The effects of the environment on the gut microbiota are strongly associated with diet (Amato et al., 2016; Barelli et al., 2015; Gomez et al., 2015; Scott, Gratz, Sheridan, Flint, & Duncan, 2013), with the gut microbiome of mammals exhibiting varied responses to altered dietary patterns (Angelakis et al., 2016; David et al., 2014; Muegge et al., 2011). For example, the gut microbiome of US immigrants from non-Western countries is characterized by reduced diversity and functional losses compared with those of preimmigration and newly arrived individuals (Vangay et al., 2018). Furthermore, a study on the effects of the 1975 Japanese diet (a more diverse and healthy dietary pattern than the modern Japanese diet) on the gut microbiota revealed that after 28 days, the proportions of unclassified Lachnospiraceae, *Parabacteroides* spp., and unclassified Rikenellaceae had significantly decreased and the proportion of *Sutterella* spp. had markedly increased in the gastrointestinal tracts of 10 young adults compared with people consuming the modern Japanese diet (Kushida et al., 2018). In addition, the gut microbiota of black howler monkeys (*Alouatta pigra*) has been shown to vary within habitats in terms of microbial richness, diversity, and composition, most likely due to seasonal variations in diet (Amato et al., 2015).

Captivity has an important effect on the gut microbiota of mammals, with captive animals having a lower gut microbial diversity than wild animals. Captive environments differ from wild environments in terms of diet, lifestyle, and contact with other individuals, all of which could alter the structure of the gut microbiota for most mammals (McKenzie et al., 2017). In general, wild animals have a more varied diet than captive animals, which explains the difference in gut microbial diversity (McKenzie et al., 2017; Nelson et al., 2013; Uenishi et al., 2007). In addition, wild animals must adapt to seasonal variations in ecological factors, such as the availability of food resources and climate, which typically involves a change in feeding strategies (Hansen et al., 2010; Huang, Wu, Zhou, Li, & Cai, 2008; Zhou, Huang, Wei, & Huang, 2018). Therefore, diet is likely to be the main factor that causes changes in the gut microbiota in captive animals. Consequently, investigation of the gut microbiota may provide an insight into the effects of food provisioning on captive populations to help improve their management and conservation.

To explore the effect of food provisioning on the gut microbiota of rhesus macaques (*Macaca mulatta*), we compared the gut microbiota of food-provisioned rhesus macaques from Guangxi Longhu Mountain Natural Reserve (hereafter "Longhu Mountain") with that of completely wild rhesus macaques from Guangxi Chongzuo White-headed Langur National Natural Reserve (hereafter "Chongzuo"). Limestone-living rhesus macaques preferably feed on young leaves, which are supplemented with mature leaves when supplies of their preferred foods are sparse (Tang et al., 2016), whereas the food-provisioned macaques in Longhu Mountain live in a natural environment but also receive a portion of their diet from reserve staff and tourists, such as corn and peanut. This food provisioning is likely leads to a high-fat diet, which probably reduce the gut microbial diversity (Jami, White, & Mizrahi, 2014; Ley, Turnbaugh, Klein, & Gordon, 2006; Murphy et al., 2010). To date, there has been much research on the gut microbiota of rhesus macaques, including as laboratory animals for human gut microbiota research (Ardeshir et al., 2014; Martin et al., 2013; O'Sullivan et al., 2013), and the relationship between the gut microbiota and ecology of these monkeys (Cui, Wang, Yu, Ye, & Yang, 2019; Yasuda et al., 2015; Zhao et al., 2018), but the effect of partial food provisioning on the gut microbiota of rhesus macaques has not been investigated. Therefore, this research may provide advice for the management and protection of provisioned macaques.

We compared the gut microbial composition and diversity in rhesus macaques inhabiting Longhu Mountain and Chongzuo through the collection of fecal samples, as it has been shown that the composition of the gut microbiota in the large intestine is highly correlated with the composition in the feces (Yasuda et al., 2015). The gut microbiota in the fecal samples were then assessed using high-throughput 16S rRNA sequencing. We predicted that (a) the gut microbial diversity would be lower in provisioned rhesus macaques than in wild rhesus macaques, reflecting the findings for captive versus wild mammals (McKenzie et al., 2017; Nelson et al., 2013; Uenishi et al., 2007); (b) food provisioning would cause the gut microbiota of the provisioned macaques to be richer in high-fat diet bacteria; and (c) the gut microbiota of wild rhesus macaques would be richer in bacteria that contribute to cellulose degradation due to leaves being taken as a staple food (Tang et al., 2016).

2 | MATERIALS AND METHODS

2.1 | Study sites and fecal sample collection

Longhu Mountain is located in Long'an County in Guangxi Province (22°56′–23°00′N, 107°27′–107°41′E), and Chongzuo is located approximately 140 km away in Jiangzhou District and Fusui County in Guangxi Province (22°15′–22°17′N, 107°29′–107°32′E). Both reserves have limestone landscapes and vegetation that mainly comprises tropical and subtropical evergreen and deciduous forests (Yao et al., 2012; Zhang, Huang, & Huang, 2007). The habitat in Chongzuo has been fragmented by human activities, but the rhesus macaques that inhabit the reserve do not range close to or interact directly with humans. By contrast, animals in Longhu Mountain commonly range close to humans, and the reserve staff regularly feed them corn to attract tourists and also sell peanuts to tourists to feed these monkeys. Furthermore, the monkeys in Longhu Mountain also consume other foods provided by the tourists, such as bread, fruits, and drinks.

At Chongzuo, fecal samples were collected from a group of rhesus macaques comprising approximately 20 individuals at a stationary point to ensure that the fecal samples were collected from this group. At Longhu Mountain, fecal samples were collected at a stationary provisioned point at which a group of approximately 400 rhesus macaques resided. Within 20 min of defecation, samples of

the fecal interiors, which do not contact the air or soil, were collected into sterile collection tubes using bamboo sticks while wearing polyethylene gloves. The samples were frozen in dry ice immediately after collection, transported to an ultralow-temperature refrigerator in the laboratory, and stored at −80°C until DNA extraction.

In total, 35 fecal samples were collected from provisioned rhesus macaques from Longhu Mountain in October and November 2018, and 23 fecal samples were collected from wild rhesus macaques from Chongzuo in September, November, and December 2018. The age–sex classes of the sampled individuals were not known due to limitations of the current condition.

2.2 | DNA extraction, 16S rRNA amplification, and sequencing

Total bacterial genomic DNA was extracted from all fecal samples using an E.Z.N.A.® soil DNA Kit (Omega Bio-tek) according to the manufacturer's instructions. The DNA concentration and purity were determined using a NanoDrop 2000 UV–Vis spectrophotometer (Thermo Scientific), and DNA quality was assessed by 1% agarose gel electrophoresis. The V3–V4 hypervariable region of the 16S rRNA gene was amplified by polymerase chain reaction (PCR) (GeneAmp 9700; ABI) using the universal bacterial primers (338F, 5′-ACTCCTACGGGAGGCAGCAG-3′; 806R, 5′-GGACTACHVGGGTWTCTAAT-3′) (Mori et al., 2014). The initial PCR was conducted using TransGen AP221-02: TransStart® Fastpfu DNA Polymerase with 20 μl of reaction mixture containing 10 ng of template DNA, 4 μl of 5 × FastPfu Buffer, 2 μl of 2.5 mM deoxyribonucleotide triphosphates (dNTPs), 0.8 μl of each primer (5 μM), and 0.2 μl of bovine serum albumin. The PCR conditions included initial denaturation at 95°C for 3 min, followed by 28 cycles of denaturation at 95°C for 30 s, annealing at 53°C for 30 s, and extension at 72°C for 45 s, and final extension at 72°C for 10 min, following which the samples were incubated at 10°C until the reaction stopped. The PCR products were eluted from a 2% agarose gel, purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences), and quantified using QuantiFluor™-ST (Promega) according to the manufacturer's protocol. The purified PCR fragments were pooled at equimolar concentrations, and paired-end sequencing (2 × 300) was undertaken on an Illumina MiSeq platform (Illumina) by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai).

2.3 | Data analysis

Raw FASTQ files were demultiplexed and filtered using Trimmomatic and merged using FLASH. Reads at any site that had an average quality score of < 20 over a 50-bp sliding window were truncated. The primers' barcodes were matched, allowing exactly two nucleotide mismatches, and any reads that contained ambiguous bases were removed. Sequences with overlaps of > 10 bp were merged according to their overlap sequence. Operational taxonomic units (OTUs) were clustered with a 97% similarity cutoff using UPARSE (USEARCH version 7.1, http://drive5.com/uparse/), and chimeric sequences were identified and removed using UCHIME. Taxonomic classification of each 16S rRNA gene sequence was performed through comparison against the 16S rRNA Greengenes 135 bacteria database (Release 13.5 http://greengenes.secondgenome.com/) using the RDP Classifier algorithm (http://rdp.cme.msu.edu/), with a confidence threshold of 70%.

The evenness and richness of the gut microbiota were assessed by calculating alpha diversity indices (Shannon index, Simpson index, abundance-based coverage estimator [ACE], and Chao estimator) using the Mothur program (version v.1.30.1; http://www.mothur.org/wiki/Schloss_SOP#Alpha_diversity). Rank abundance, rarefaction, and diversity curves were also plotted to reflect the sequencing depth. For beta diversity analysis, weighted and unweighted UniFrac distance matrices were calculated and visualized using principal coordinate analysis (PCoA). Permutational multivariate analysis of variance (PERMANOVA) was used to further identify the differences in gut microbiota between the two populations, and a histogram of bacterial composition was plotted according to the results of the taxonomic analysis. The Wilcoxon rank-sum test was used to identify differences in alpha diversity indices and community structure of the gut microbiota between the two populations, using false discovery rate (FDR)-adjusted *p*-values. These analyses were run with the R statistical software (version 3.2.2), using the plot function to plot the curves and histogram and the vegan package to undertake the PERMANOVA analysis. Differences in the structure of the gut microbial communities were further analyzed using the linear discriminant analysis effect size (LEfSe) (http://huttenhower.sph.harvard.edu/galaxy/root?tool_xml:id=lefse_upload). Phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) (Langille et al., 2013) was then applied to predict the functional profiles of the gut microbial communities, and the Wilcoxon rank-sum test was used to test for differences in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of the gut microbiota of the two populations, using FDR-adjusted *p*-values. Differences in the KEGG pathways were analyzed using the IBM SPSS statistical software (version 23.0). All data were analyzed using the Majorbio I-Sanger Cloud Platform (http://www.i-sanger.com).

3 | RESULTS

3.1 | Sequencing quality evaluation

In total, 3,108,909 sequences of the hypervariable V3–V4 region of the 16S rRNA gene were obtained from the 58 fecal samples, 2,291,877 of which were valid. These corresponded to an average of 53,601.9 ± 6,038.6 reads per sample, which were then subsampled to an equal sequencing depth (31,200 reads per sample). A total of 1,183 OTUs were clustered using a sequence similarity of 97%. The rank abundance, rarefaction, and alpha diversity curves that were constructed based on these OTUs revealed that the sequencing

depth was sufficient (Figures A1, A2), while Good's coverage estimations revealed that approximately 99.5%–99.8% of the species were obtained for all of the samples (Table A1).

3.2 | Alpha and beta diversity analyses

Alpha diversity analyses based on the 1,183 OTUs (Table A1) revealed that there were no significant differences in the Shannon or Simpson indices between the wild and provisioned populations (Figure 1a,b), indicating a similar evenness. However, the provisioned population had significantly higher ACE and Chao1 estimator values than the wild population (Figure 1c,d), indicating that there were differences in bacterial richness. PCoA based on unweighted and weighted UniFrac distances demonstrated that the gut microbes were strongly clustered by population, as indicated by the beta diversity (Figure 2, Figure A3), and the PERMANOVA based on unweighted and weighted UniFrac distances revealed significant differences between the two populations (R^2 = 0.153, adjusted p < .001 for both analyses).

3.3 | Gut microbial community structure

Taxonomic analysis revealed that the 1,183 OTUs obtained from the fecal samples consisted of 14 classified bacterial phyla, 1 unclassified phylum, and 153 microbial genera. The most dominant phyla were Firmicutes (65.02% ± 20.63%) and Bacteroidetes (23.98% ± 17.49%), followed by Actinobacteria (3.46% ± 4.37%), Spirochetes (3.39% ± 8.32%), Proteobacteria (1.29% ± 1.97%), and Tenericutes (1.20% ± 0.96%) (Figure 3a). The dominant genus was *Prevotella* (18.28% ± 15.68%), followed by no-rank Ruminococcaceae (9.99% ± 6.85%), and no-rank Clostridiaceae (7.82% ± 11.41%) (Figure 3b). The proportions of other bacterial phyla and genera are shown in Tables A2 and A3.

3.4 | Differences in gut microbial composition

The proportions of gut bacteria significantly differed between the two populations according to Wilcoxon rank-sum tests. At the phylum level, Bacteroidetes, Spirochetes, WPS-2, and Fibrobacteres were more enriched in the wild population than in the provisioned population, whereas Firmicutes and Verrucomicrobia were more enriched in the provisioned population (Figure 4a; Table A2). At the genus level, *Prevotella*, *Treponema*, no-rank S24–7, and *Coprococcus* were more enriched in the wild population, whereas no-rank Clostridiaceae, *Enterococcus*, *Lactobacillus*, no-rank Peptostreptococcaceae, *Sarcina*, *Kurthia*, and *Lactococcus* were more enriched in the provisioned population (Figure 4b). Other significant differences between the two populations at the genus level are presented in Table A3.

To further identify shifts in the gut microbial composition between the two populations, we used LEfSe to detect differences in the relative abundances of the bacterial taxa at the phylum, class, order, family, and genus levels. Differences in the abundances of bacterial phyla were caused by differences in the bacterial genera (Figure A3a), with similar results being observed to those described above for the Wilcoxon rank-sum test only with more different genera being found. Thus, the wild population showed higher abundances of *Prevotella*, *Treponema*, *Ruminococcus*, *Coprococcus*, no-rank S24–7, no-rank Ruminococcaceae, and *Oscillospira*, whereas the provisioned population showed higher abundances of no-rank Clostridiacese, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Sarcina*, and *Kurthia* (Figure A3b).

3.5 | Differences in the functional profiles of the gut microbiota

To further explore the functions of the gut microbiota, we predicted the functional profiles of the gut microbial communities from the two populations of rhesus macaques using PICRUSt (mean nearest sequenced taxon index [NSTI]: overall = 0.13 ± 0.03; wild = 0.15 ± 0.03; and provisioned = 0.12 ± 0.03). We also investigated the effects of food provisioning on the functional profiles of the gut microbiota by examining differences in the KEGG pathways using Wilcoxon rank-sum tests. There was no significant difference between the populations at KEGG pathway level 1 (Table A4). However, at KEGG pathway level 2, pathways associated with glycan biosynthesis and metabolism, transport and catabolism, and digestive and endocrine systems were significantly richer in the wild population, whereas pathways related to signaling molecules and their interaction and neurodegenerative diseases were richer in the provisioned population (Figure 5; Table A5).

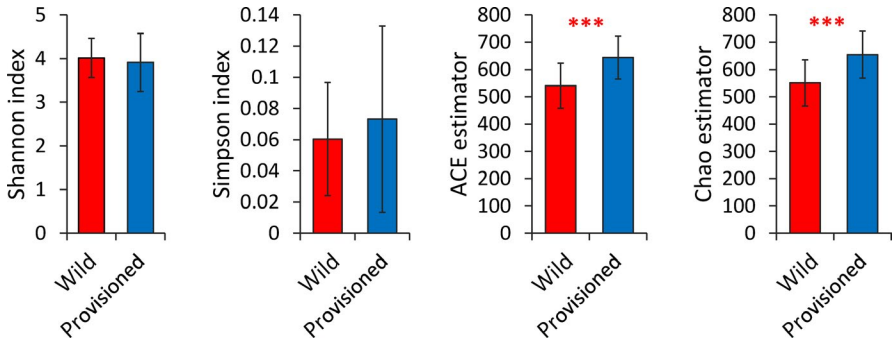

FIGURE 1 Alpha diversity of the gut microbiota of rhesus macaques from Chongzuo (wild) and Longhu Mountain (provisioned). The p-value is represented by "*"; significant difference p < .001 is marked as "***"

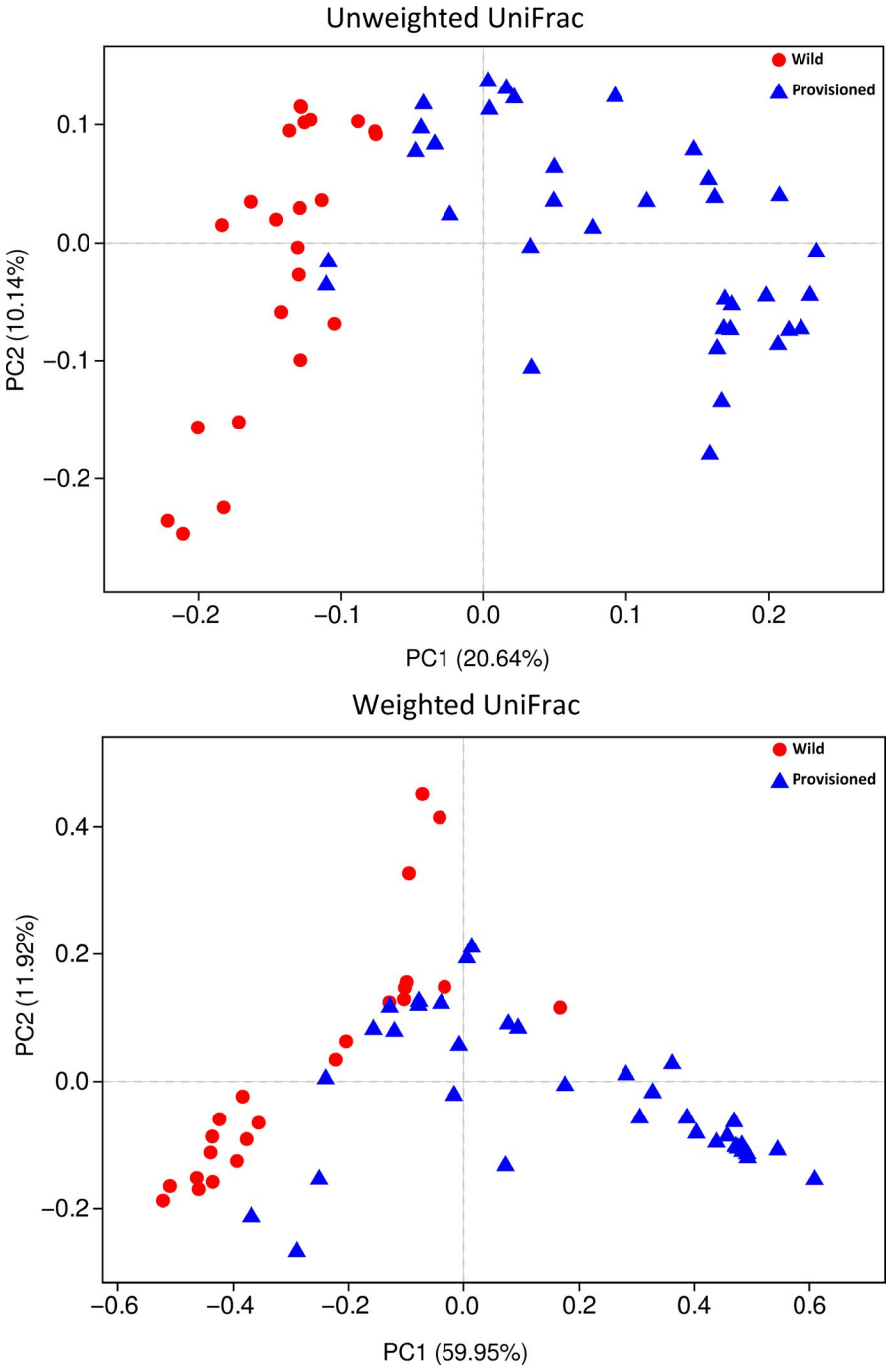

FIGURE 2 PCoA of structure differentiation and interindividual similarity on gut microbiota of rhesus macaques from Chongzuo (wild) and Longhu Mountain (provisioned)

4 | DISCUSSION

Changes in the gut microbiota as a result of environmental variations are often strongly associated with diet (Amato et al., 2016; Barelli et al., 2015). In the present study, the gut microbiota of rhesus macaques inhabiting Longhu Mountain and Chongzuo formed two distinct clusters by population, which matches previous findings for rhesus macaques, humans, and other primates species inhabiting different environments (Amato et al., 2013; Gomez et al., 2015; Kohl, Varner, Wilkening, & Dearing, 2018; Rothschild et al., 2018; Vangay et al., 2018; Zhao et al., 2018). These differences may be explained by differences in dietary composition, as macaques in Chongzuo exclusively depend on natural foods, whereas those in Longhu Mountain are provided with additional foods. In general, wild animals have a higher gut microbial diversity than captive animals due to their more complex dietary composition (Amato et al., 2013; McKenzie et al., 2017; Nelson et al., 2013; Uenishi et al., 2007). However, our results did not support these findings or our first prediction that provisioned macaques would have a significantly lower gut microbial diversity. This may be because although rhesus macaques inhabiting Longhu Mountain are regularly provided with food, they also heavily depend on natural foods, such as leaves, flowers, and fruits (Wang, Jiang, Liu, & Feng, 1994), which may cause them to have similar digestion requirements as their wild

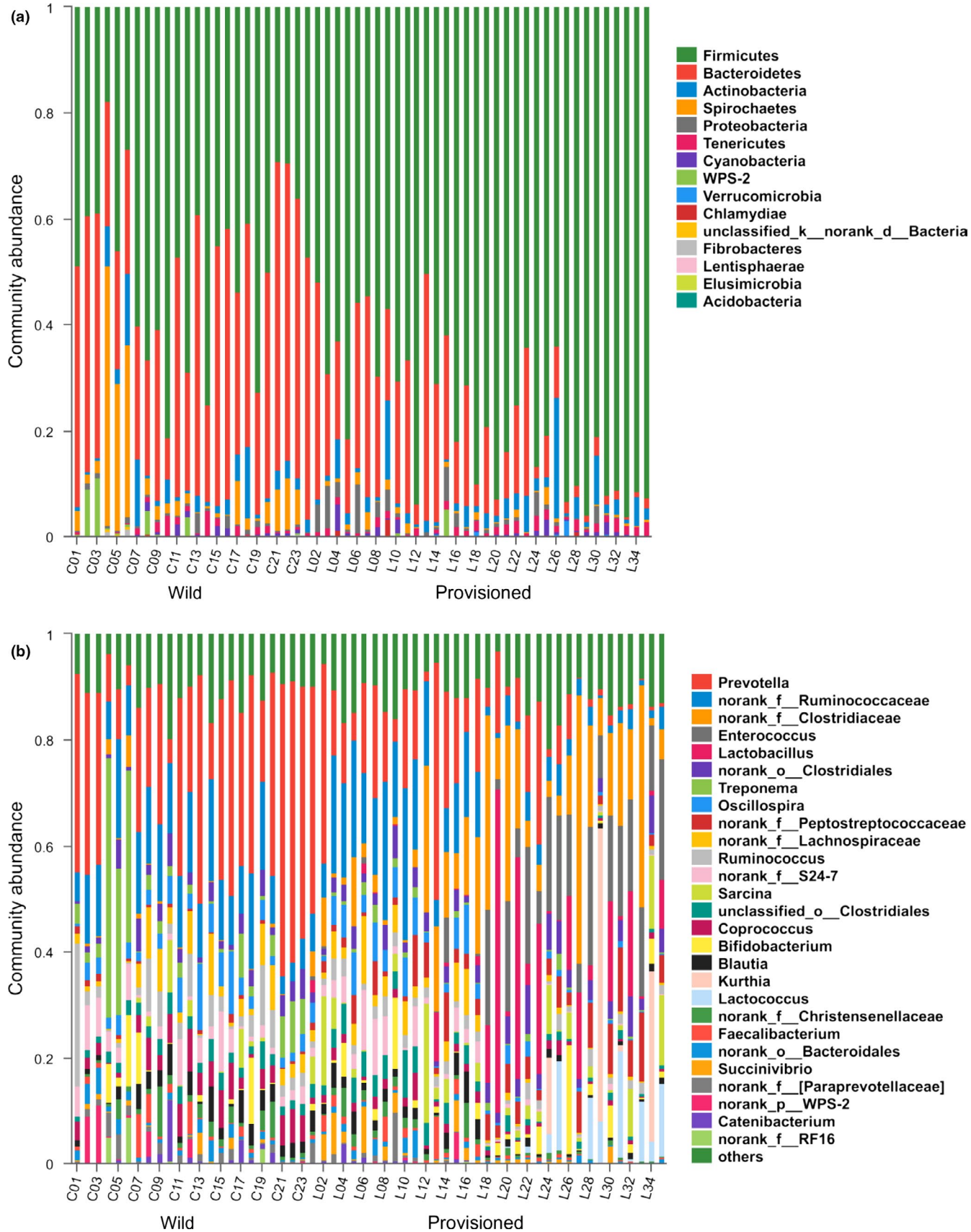

FIGURE 3 Stacked bar graphs illustrate the abundances of phyla and genus on gut microbiota from Chongzuo (wild) and Longhu Mountain (provisioned). (a) At phylum level; (b) At genus level

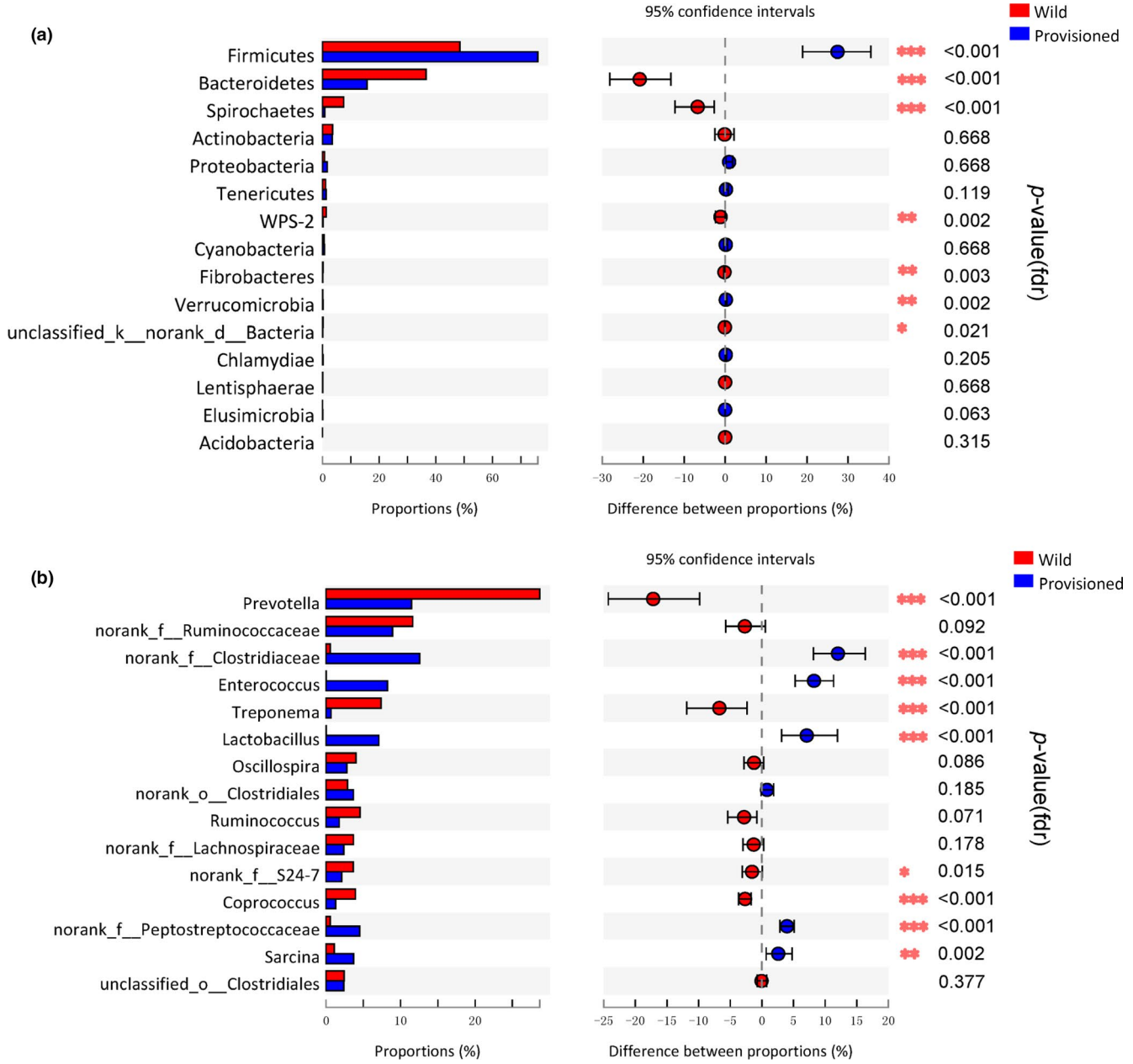

FIGURE 4 Abundance differences analysis (Wilcoxon rank-sum test) of gut microbiota community on rhesus macaques from Chongzuo (wild) and Longhu Mountain (provisioned). (a) At phylum level. (b) At genus level, only the first 15 bacterial species with significant differences were showed

counterparts. These results imply that the provisioned macaques in Longhu Mountain have maintained their ability to digest natural foods, which has undoubtedly improved their survival in this provisioned environment. However, more detailed comparisons are required in the future.

The gut microbiota of the rhesus macaques sampled in the present study was dominated by Firmicutes and Bacteroidetes, which is similar to the findings of previous studies on rhesus macaques and other primates species (Gomez et al., 2015; Su et al., 2016; Szekely et al., 2010; Trosvik, Rueness, Muinck, Moges, & Mekonnen, 2018; Zhao et al., 2018). In general, Firmicutes species can decompose various substances to help their host digest and absorb nutrients via digestive enzymes (Kaakoush, 2015), whereas Bacteroidetes species assist the host in degrading carbohydrates and proteins in foods (Fernando et al., 2010; Jami et al., 2014). However, the abundances of these bacteria varied greatly between the two populations, with rhesus macaques in Longhu Mountain having a higher ratio of Firmicutes/Bacteroidetes. Since an increased prevalence of Firmicutes and decreased prevalence of Bacteroidetes can improve the digestion and absorption of food energy (Bird & Conlon, 2015; Jami et al., 2014; Ley et al., 2006; Murphy et al., 2010), the ratio of Firmicutes/Bacteroidetes increases in response to the consumption of a high-fat diet (Jami et al., 2014; Ley et al., 2006; Murphy et al., 2010) and has been linked to obesity (Ley et al., 2005; Turnbaugh et al., 2006; Vebo, Karlsson, Avershina,

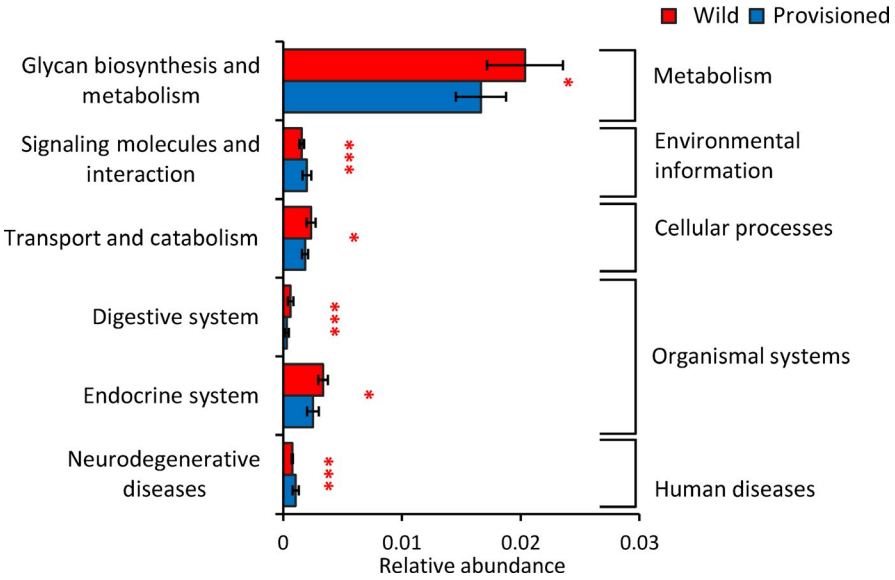

FIGURE 5 Predicted functional metagenomic on gut microbiota of rhesus macaques from Chongzuo (wild) and Longhu Mountain (provisioned). The *p*-value is represented by "*". Significant difference 0.01 < *p*< 0.05 is marked as "*" and *p* < .001 is marked as "***

Finnby, & Rudi, 2016). Therefore, this result supports our second prediction that the gut microbial community structure of food-provisioned rhesus macaques would be richer in high-fat-diet bacteria. This is likely due to rhesus macaques in Longhu Mountain being provided with corn and peanuts, both of which are rich in starch and fats, as well as rhesus macaques from Chongzuo spending more time foraging for food than those from Longhu Mountain, which may decrease the ratio of Firmicutes/Bacteroidetes in the gut (Denou, Marcinko, Surette, Steinberg, & Schertzer, 2016).

Rhesus macaques in the Chongzuo population were also found to have a higher proportion of cellulose-degrading Fibrobacteres species in their gut microbiota, which may be explained by their greater consumption of cellulose-rich food (Ransom-Jones, Jones, McCarthy, & McDonald, 2012), supporting our third prediction. A previous study indicated that rhesus macaques inhabiting limestone forests tend to be folivorous and prefer young leaves as staple foods, supplementing their diet with mature leaves when required (Tang et al., 2016), which are rich in cellulose (Richard, 1985). Therefore, since the current study was conducted during the dry season when young leaves and fruits are scarce, macaques in Chongzuo may have had a more fiber-rich diet than the provisioned macaques in Longhu Mountain, explaining why they hosted greater proportions of Fibrobacteres in their guts. Some bacterial genera were also present at significantly higher proportions in the Chongzuo population than in the Longhu Mountain population, likely due to differences in the dietary compositions of the two populations. These included the genera *Prevotella* and *Treponema*, which enable calories to be extracted from indigestible polysaccharides such as xylan and cellulose (De Filippo et al., 2010); *Ruminococcus*, which is important for cellulose and hemicellulose fermentation in ruminants (Ntaikou, Gavala, Kornaros, & Lyberatos, 2008; Pettipher & Latham, 1979); and *Coprococcus* and genera in the family Ruminococcaceae, which are commonly found in the guts of ruminants and other mammals and occur as fibrolytic communities that are likely associated with cellulose degradation (Biddle, Stewart, Blanchard, & Leschine, 2013; Henderson et al., 2015).

Another phylum that was detected in the gut of the rhesus macaques was WPS-2, which has rarely been detected in the primate gut microbiota previously. This lesser-known bacterial phylum was first detected in polychlorinated biphenyl-polluted soil from Wittenberg, Germany (Nogales et al., 2001), and was single cloned from the canine oral microbiome (Dewhirst et al., 2012). However, the functional roles of members of this phylum in the host remain unclear. The occurrence of WPS-2 in the gut microbiota of rhesus macaques in the present study may be attributed to geophagy, which is a common behavior in most primates. Primates obtain minerals by licking rocks or eating soil (Hsu, Agoramoorthy, & Lin, 2001; Li et al., 2014; Pebsworth, Bardi, & Huffman, 2012), which may result in bacteria from the soil colonizing the gut. However, the specific reasons for the presence of WPS-2 in the guts of these rhesus macaques warrants further research.

The gut microbiota is closely associated with host health, and certain metabolites of the gut microbiota play important roles in host metabolism, digestion, and immunity (Castellazzi et al., 2017; Miani et al., 2018; Million et al., 2018; Rooks & Garrett, 2016; Tamburini & Clemente, 2017). In the present study, we used PICRUSt to predict the functional profiles of the gut microbiota of the two populations of rhesus macaques. However, it should be noted that this tool has some limitations in its capacity to predict functions, as only 16S marker gene sequences corresponding to bacterial and archaeal genomes are currently included and the accuracy is not high in the case of the reference genome pool (Langille et al., 2013). NSTI was used to quantify the availability of nearby genome representatives for each microbiome sample, and the accuracy of PICRUSt decreases as NSTI increases (Langille et al., 2013). However, the mean NSTI value was 0.13 ± 0.03, which is within the range of that previously reported for mammals (NSTI = 0.14 ± 0.06; (Langille et al., 2013)), indicating that our results are interpretable. The enriched functional profiles of the digestive and endocrine systems in rhesus macaques from Chongzuo may be attributable to their completely natural diet. Individuals in the wild population spend more time and energy on foraging than

those in the provisioned population, and natural food items, such as leaves, contain greater levels of relatively indigestible fibers and toxic compounds (Richard, 1985). Thus, the enrichment of functional flora for digestion may facilitate the adaptation of animals to environmental variations. Furthermore, the gut microbiota of rhesus macaques from Chongzuo was enriched with Fibrobacteres, *Prevotella*, *Treponema*, *Coprococcus*, and *Ruminococcus*, which facilitate cellulose digestion (De Filippo et al., 2010; Ntaikou et al., 2008; Pettipher & Latham, 1979; Ransom-Jones et al., 2012) and so may explain the enrichment of the digestive system.

In conclusion, although there was no significant difference in the diversity of gut microbes between provisioned and wild rhesus macaques, there was great variation in the richness and bacterial community structure of the two populations, indicating that food provisioning alters the gut microbiota of this species. In particular, food provisioning increased the ratio of Firmicutes/Bacteroidetes, which helped the rhesus macaques from Longhu Mountain to digest high-fat foods more easily while maintaining a similar gut microbiota diversity. This suggests that an excessive reliance on provisioned feeding may increase the risk of obesity and gradually reduce the ability of these monkeys to survive in the wild. Therefore, a balance of provisioned and wild feeding is crucial to sustain the ability of rhesus macaques to digest a range of foods, which will allow them to effectively adapt to environmental variations.

ETHICS STATEMENT

We were permitted to enter the study site and collect samples under the Guangxi Chongzuo White-Headed Langur National Nature Reserve ticket code 145141911048–00879801 and Guangxi Longhu Mountain Nature Reserve ticket code 1450119M0030-0000986. This study did not involve any animal tissues. All fecal samples were collected after the animals left to avoid a stress reaction.

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CONFLICT OF INTERESTS

None declared.

AUTHORS' CONTRIBUTIONS

Ting Chen: Formal analysis-Equal, Writing-original draft-Equal; Yuhui Li: Investigation-Equal; Jipeng Liang:Investigation-Equal; Youbang Li:Writing-review & editing-Equal; Zhonghao Huang: Conceptualization-Equal, Writing-review & editing-Equal.

DATA AVAILABILITY STATEMENT

The raw sequencing data from the current study are available in the NCBI repository at https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA590350, and the SRA accession number is PRJNA590350.

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APPENDIX

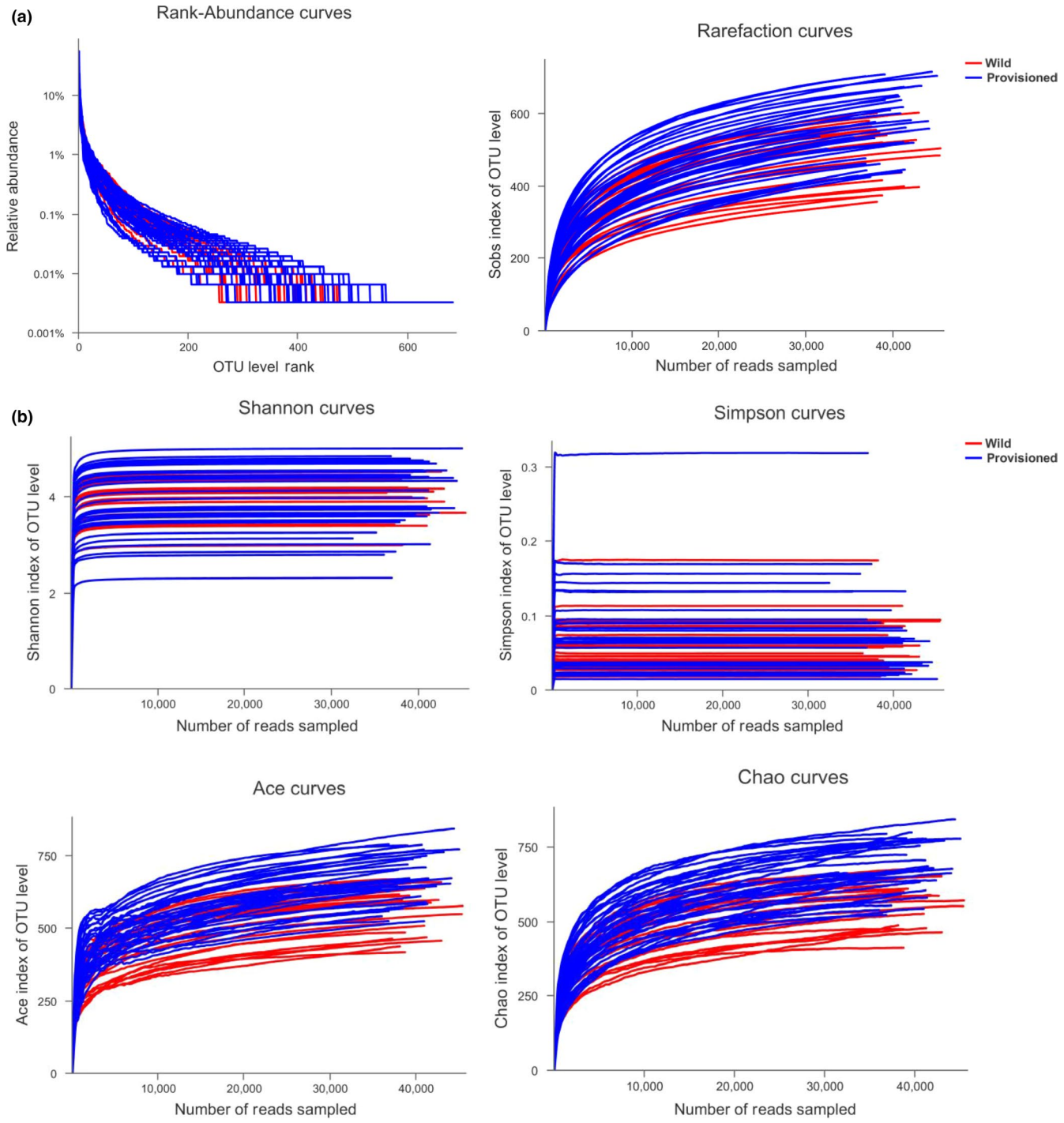

FIGURE A1 (a) Rank abundance distribution curves and rarefaction curves; (b) Alpha diversity curves.

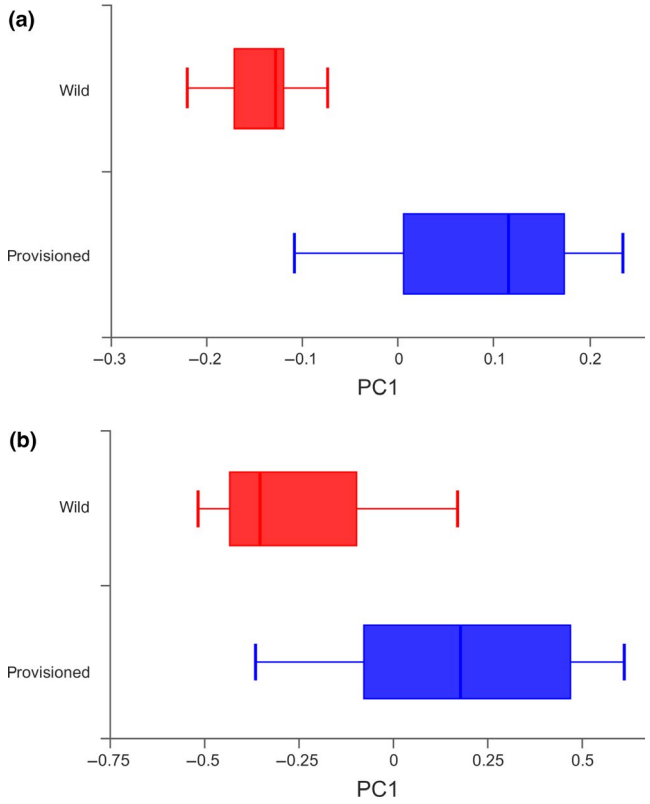

FIGURE A2 The dispersion degree box plot of PCoA of structure differentiation and inter-individual similarity on gut microbiota of wild and provisioned rhesus macaques; (a) based on unweighted UniFrac distance; (b) based on weighted UniFrac distance.

TABLE A1 Alpha diversity index of rhesus macaques gut microbiota

	Wild	Provisioned	*p*-value (fdr)
Shannon	4.02 ± 0.45	3.91 ± 0.66	.763
Simpson	0.06 ± 0.04	0.07 ± 0.06	.763
Ace	540.64 ± 82.81	644.14 ± 78.65	<.001
Chao	551.29 ± 84.30	654.73 ± 86.12	<.001
Coverage	0.997 ± 0.000	0.996 ± 0.001	<.001

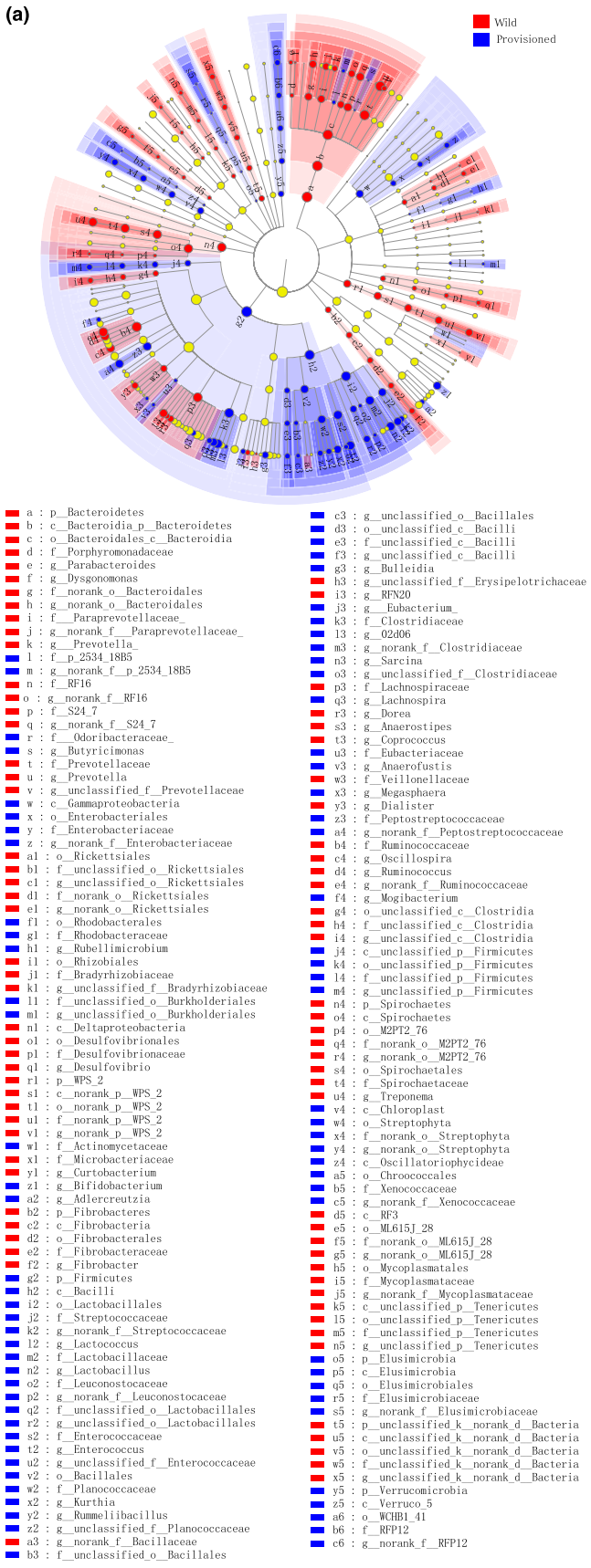

FIGURE A3 Linear discriminant analysis effect size (LEfSe) analysis on gut microbiota composition of wild and provisioned rhesus macaques (LDA > 2, $p < .05$).

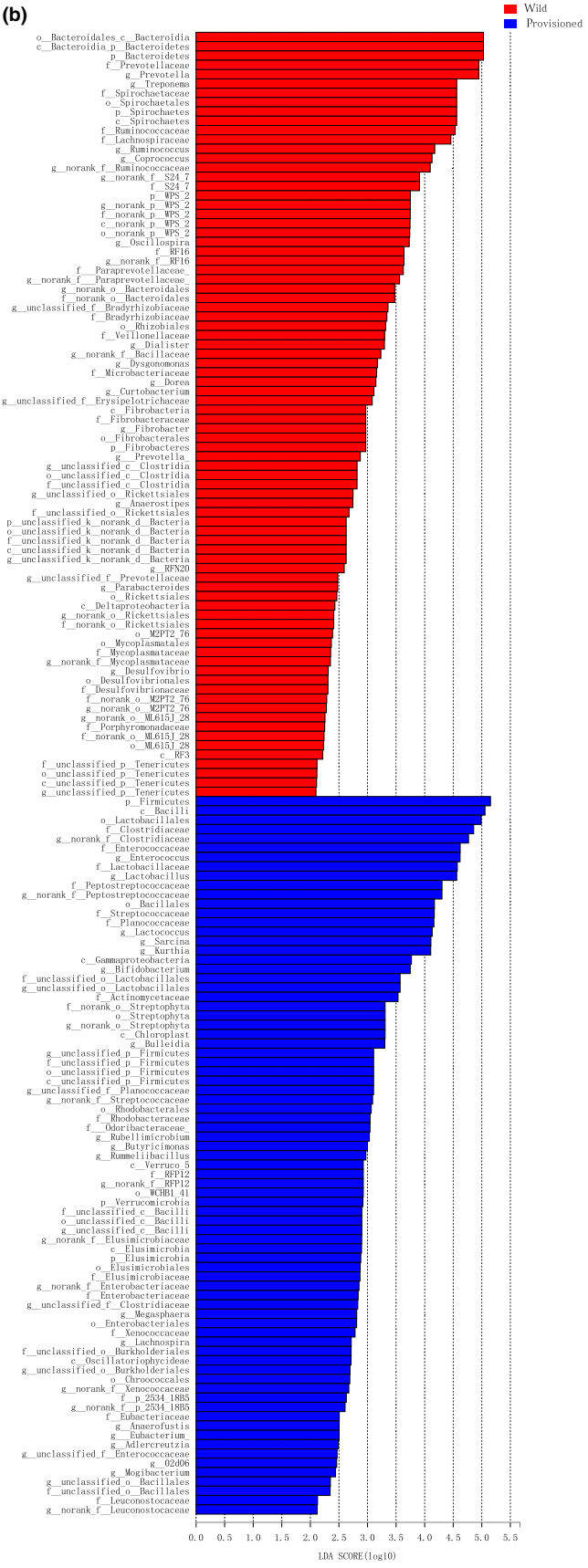

FIGURE A3 (Continued)

TABLE A2 The proportion differences of gut microbiota community of rhesus macaques from two different environments (at phylum level)

Species name	Wild (%)	Provisioned (%)	*p*-value (fdr)
Firmicutes	48.49 ± 16.71	75.93 ± 15	<.001
Bacteroidetes	36.58 ± 14.67	15.73 ± 14.05	<.001
Spirochaetes	7.45 ± 12.25	0.73 ± 0.89	<.001
Actinobacteria	3.54 ± 4.21	3.4 ± 4.46	.668
Proteobacteria	0.7 ± 0.86	1.68 ± 2.37	.668
Tenericutes	1.07 ± 1.21	1.28 ± 0.75	.119
WPS-2	1.3 ± 3	0.17 ± 0.84	.002
Cyanobacteria	0.55 ± 0.65	0.69 ± 1.08	.668
Fibrobacteres	0.18 ± 0.33	0.01 ± 0.02	.003
Verrucomicrobia	0.01 ± 0.01	0.18 ± 0.54	.002
Unclassified Bacteria	0.13 ± 0.14	0.05 ± 0.04	.021
Chlamydiae	<0.01	<0.01	.205
Lentisphaerae	<0.01	<0.01	.668
Elusimicrobia	<0.01	<0.01	.063
Acidobacteria	<0.01	0	.315

TABLE A3 The proportion differences of gut microbiota community of rhesus macaques from two different environments (at genus level)

Species name	Wild (%)	Provisioned (%)	*p*-value (fdr)
Prevotella	28.66 ± 15.12	11.47 ± 12.01	<.001
No-rank Ruminococcaceae	11.64 ± 4.68	8.94 ± 7.87	.092
No-rank Clostridiaceae	0.56 ± 1.29	12.58 ± 12.58	<.001
Enterococcus	<0.01	8.24 ± 9.14	<.001
Treponema	7.4 ± 12.25	0.68 ± 0.86	<.001
Lactobacillus	<0.01	7.09 ± 13.37	<.001
Oscillospira	4.03 ± 2.99	2.79 ± 2.78	.086
No-rank Clostridiales	2.87 ± 1.88	3.68 ± 1.95	.185
Ruminococcus	4.58 ± 5.79	1.77 ± 1.01	.071
No-rank Lachnospiraceae	3.68 ± 3.47	2.38 ± 2.88	.178
No-rank S24-7	3.66 ± 3.07	2.11 ± 2.99	.015
Coprococcus	3.95 ± 2.35	1.29 ± 0.99	<.001
No-rank Peptostreptococcaceae	0.56 ± 0.47	4.53 ± 3.34	<.001
Sarcina	1.12 ± 2.52	3.7 ± 5.42	.002
Unclassified Clostridiales	2.42 ± 1.03	2.37 ± 1.99	.377
Blautia	2.74 ± 1.99	1.78 ± 1.49	.14
Bifidobacterium	2.08 ± 4.27	2.26 ± 4.18	.113
No-rank Christensenellaceae	1.96 ± 2.23	1.13 ± 1.4	.126
Kurthia	0	2.95 ± 10.87	.029
Lactococcus	<0.01	2.59 ± 5.41	.016
Unclassified Ruminococcaceae	1.4 ± 0.81	1.11 ± 0.99	.178
Faecalibacterium	1.33 ± 2.19	1.04 ± 1.63	.739
No-rank Bacteroidales	1.52 ± 1.37	0.83 ± 1.05	.042
No-rank RF39	1.02 ± 1.17	1.26 ± 0.75	.116

(Continues)

TABLE A3 (Continued)

Species name	Wild (%)	Provisioned (%)	*p*-value (fdr)
No-rank Coriobacteriaceae	1.25 ± 1.29	0.89 ± 0.58	.859
Succinivibrio	0.49 ± 0.9	1.4 ± 2.29	.212
No-rank [Paraprevotellaceae]	1.23 ± 1.3	0.57 ± 1.63	<.001
No-rank WPS-2	1.3 ± 3	0.17 ± 0.84	.003
Unclassified Lachnospiraceae	0.78 ± 0.71	0.64 ± 0.55	.8
Catenibacterium	1.1 ± 2.53	0.28 ± 0.64	.167
Butyrivibrio	0.73 ± 1.04	0.35 ± 0.33	1
Bulleidia	0.26 ± 0.24	0.66 ± 0.53	<.001
No-rank RF16	0.85 ± 2.1	0.06 ± 0.07	<.001
No-rank [Mogibacteriaceae]	0.33 ± 0.25	0.44 ± 0.35	.422
Unclassified Lactobacillales	0	0.73 ± 0.97	<.001
No-rank Streptophyta	0.14 ± 0.21	0.5 ± 0.98	.091
Dorea	0.46 ± 0.5	0.15 ± 0.14	.006
No-rank YS2	0.41 ± 0.58	0.18 ± 0.24	.6
Dialister	0.47 ± 1.18	0.09 ± 0.17	.06
[*Ruminococcus*]	0.33 ± 0.39	0.21 ± 0.19	.398
Clostridium	0.26 ± 0.26	0.25 ± 0.37	.179
Unclassified Bacteroidales Bacteroidia	0.15 ± 0.17	0.31 ± 0.66	.859
No-rank Rikenellaceae	0.15 ± 0.41	0.16 ± 0.41	.364
[*Prevotella*]	0.19 ± 0.32	0.06 ± 0.14	.023
Unclassified Firmicutes	<0.01	0.25 ± 0.38	<.001
Unclassified Planococcaceae	0	0.24 ± 0.76	.0633
No-rank Streptococcaceae	0	0.23 ± 0.42	<.001
Roseburia	0.15 ± 0.22	0.08 ± 0.1	.645
Rummeliibacillus	0	0.22 ± 0.71	.063
Collinsella	0.08 ± 0.1	0.11 ± 0.11	.314
Fibrobacter	0.18 ± 0.33	0.01 ± 0.02	.005
No-rank Erysipelotrichaceae	0.12 ± 0.15	0.06 ± 0.07	.232
No-rank RFP12	<0.01	0.18 ± 0.54	<.001
Unclassified no-rank Bacteria	0.13 ± 0.14	0.05 ± 0.04	.029
Lachnospira	0.03 ± 0.04	0.13 ± 0.11	<.001
Pediococcus	<0.01	0.16 ± 0.76	.173
Unclassified Bacilli	<0.01	0.16 ± 0.2	<.001
Unclassified Clostridia	0.14 ± 0.3	0.01 ± 0.02	.003
Candidatus Rhabdochlamydia	<0.01	0.14 ± 0.54	.232
Slackia	0.09 ± 0.07	0.06 ± 0.04	.276
No-rank Enterobacteriaceae	0	0.14 ± 0.67	<.001
Unclassified Clostridiaceae	0	0.14 ± 0.2	<.001
Unclassified Prevotellaceae	0.09 ± 0.07	0.04 ± 0.06	.003
RFN20	0.07 ± 0.1	0.03 ± 0.08	.009
Anaerostipes	0.1 ± 0.22	<0.01	.006
YRC22	0.05 ± 0.1	0.05 ± 0.08	.536
[*Eubacterium*]	0.02 ± 0.04	0.08 ± 0.06	<.001
No-rank Dehalobacteriaceae	0.08 ± 0.24	0.01 ± 0.02	.655

(Continues)

TABLE A3 (Continued)

Species name	Wild (%)	Provisioned (%)	*p*-value (fdr)
Adlercreutzia	0.03 ± 0.03	0.07 ± 0.05	<.001
Sphaerochaeta	0.04 ± 0.04	0.05 ± 0.05	.461
No-rank GMD14H09	0.05 ± 0.12	0.03 ± 0.09	.614
Flexispira	0.05 ± 0.05	0.03 ± 0.06	.221
L7A E11	0.03 ± 0.07	0.04 ± 0.08	.487
No-rank Rickettsiales	0.06 ± 0.07	0.02 ± 0.02	.002
p-75-a5	0.03 ± 0.03	0.03 ± 0.03	.607
Mogibacterium	0.02 ± 0.02	0.04 ± 0.04	.009
Lachnobacterium	0.04 ± 0.06	<0.01	.44
Phascolarctobacterium	0.03 ± 0.06	0.01 ± 0.02	.31
Desulfovibrio	0.04 ± 0.05	<0.01	.003
Unclassified Enterococcaceae	0	0.04 ± 0.05	<.001
Unclassified Bacillales	0	0.04 ± 0.15	.063
CF231	0.01 ± 0.01	0.03 ± 0.05	.652
Streptococcus	0.01 ± 0.02	0.02 ± 0.07	.533
Parabacteroides	0.02 ± 0.06	0.01 ± 0.02	.001
Unclassified Tenericutes	0.02 ± 0.05	<0.01	.022
Unclassified Coriobacteriaceae	<0.01	0.02 ± 0.02	.487
No-rank p-2534-18B5	0	0.02 ± 0.05	<.001
02d06	<0.01	0.02 ± 0.02	<.001
No-rank Lactobacillaceae	<0.01	0.02 ± 0.1	.967
No-rank ML615J-28	0.02 ± 0.02	<0.01	.063
No-rank Leuconostocaceae	0	0.02 ± 0.04	<.001
Anaerofustis	<0.01	0.01 ± 0.01	<.001
Unclassified Alphaproteobacteria	<0.01	0.01 ± 0.03	.14
Dehalobacterium	<0.01	<0.01	.801
No-rank mitochondria	<0.01	<0.01	.271
Megasphaera	<0.01	0.01 ± 0.05	.015
Coprobacillus	<0.01	<0.01	.138
Brachyspira	<0.01	<0.01	.377
No-rank Mycoplasmataceae	<0.01	0	.001
Anaeroplasma	<0.01	<0.01	.615
No-rank M2PT2-76	<0.01	<0.01	.001
Anaerovibrio	<0.01	<0.01	.479
No-rank Veillonellaceae	<0.01	<0.01	.888
No-rank RF32	<0.01	<0.01	.314
Actinobacillus	<0.01	<0.01	.406
Oxalobacter	<0.01	<0.01	.536
Unclassified Burkholderiales	0	<0.01	.091
Solibacillus	0	<0.01	.372
Unclassified Spirochaetes	<0.01	<0.01	.698
Bacillus	<0.01	<0.01	.232
No-rank Prevotellaceae	<0.01	<0.01	.254

(Continues)

TABLE A3 (Continued)

Species name	Wild (%)	Provisioned (%)	*p*-value (fdr)
Akkermansia	<0.01	<0.01	.194
No-rank Alphaproteobacteria	<0.01	<0.01	1
Acinetobacter	<0.01	<0.01	.126
No-rank Victivallaceae	<0.01	<0.01	.652
No-rank Anaeroplasmataceae	<0.01	<0.01	.828
Veillonella	<0.01	<0.01	.795
Rickettsiella	0	<0.01	.262
No-rank Xenococcaceae	0	<0.01	.043
Unclassified Erysipelotrichaceae	<0.01	0	.003
Unclassified Rickettsiales	<0.01	0	.006
No-rank Elusimicrobiaceae	<0.01	<0.01	.081
Butyricimonas	<0.01	<0.01	.022
No-rank Caulobacteraceae	<0.01	<0.01	.406
Unclassified Bradyrhizobiaceae	<0.01	<0.01	.003
Rubellimicrobium	0	<0.01	.063
Kocuria	<0.01	<0.01	.859
Sphingomonas	<0.01	<0.01	.336
Alloscardovia	<0.01	<0.01	.958
Unclassified Micrococcaceae	0	<0.01	.184
Dysgonomonas	<0.01	0	.077
No-rank Peptococcaceae	<0.01	<0.01	.917
No-rank Planococcaceae	0	<0.01	.536
Curtobacterium	<0.01	<0.01	.025
No-rank Bifidobacteriaceae	0	<0.01	.536
No-rank Pseudonocardiaceae	<0.01	<0.01	.822
Candidatus Phytoplasma	0	<0.01	.536
No-rank Bacillaceae	<0.01	0	.035
Anaerococcus	<0.01	<0.01	.346
rc4-4	<0.01	<0.01	.129
Sutterella	<0.01	<0.01	.614
Ruminobacter	0	<0.01	.262
Unclassified Erythrobacteraceae	0	<0.01	.184
No-rank R4-45B	<0.01	<0.01	1
Unclassified Proteobacteria	<0.01	<0.01	.629
Brevundimonas	<0.01	<0.01	.751
Epulopiscium	0	<0.01	.372
Actinomyces	0	<0.01	.184
No-rank 32-20	<0.01	0	.346
Arcanobacterium	0	<0.01	.372
Bacteroides Bacteroidaceae	<0.01	0	.346
Unclassified Acholeplasmatales	0	<0.01	.536
Aerococcus	0	<0.01	.536

TABLE A4 The differences of KEGG Pathways Level 1

Pathway in level 1	Relative abundance (%)		*p*-value (fdr)
	Wild	Provisioned	
Cellular processes	3.55 ± 0.52	3.02 ± 0.55	.6
Environmental information processing	13.82 ± 1	15.09 ± 0.82	.578
Genetic information processing	20.96 ± 0.46	20.59 ± 0.8	.945
Human diseases	0.7 ± 0.03	0.74 ± 0.05	.656
Metabolism	46.22 ± 0.92	45.64 ± 0.6	.81
None	0.21 ± 0.02	0.19 ± 0.03	.824
Organismal systems	0.78 ± 0.07	0.63 ± 0.08	.072
Unclassified	13.77 ± 0.13	14.1 ± 0.29	.781

TABLE A5 The differences of KEGG Pathways Level 2

Pathway in level 2	Relative abundance (%)		*p*-value (fdr)
	Wild	Provisioned	
Amino acid metabolism	9.4 ± 0.19	9.12 ± 0.38	.938
Biosynthesis of other secondary metabolites	0.86 ± 0.04	0.79 ± 0.06	.543
Carbohydrate metabolism	10.2 ± 0.37	10.3 ± 0.35	1
Energy metabolism	5.82 ± 0.22	5.47 ± 0.29	.658
Enzyme families	2.23 ± 0.06	2.22 ± 0.09	1
Glycan biosynthesis and metabolism	2.04 ± 0.32	1.67 ± 0.21	.031
Lipid metabolism	2.73 ± 0.17	2.82 ± 0.17	1
Metabolism	2.37 ± 0.07	2.6 ± 0.18	.543
Metabolism of cofactors and vitamins	4.27 ± 0.21	4.21 ± 0.2	1
Metabolism of other amino acids	1.34 ± 0.07	1.45 ± 0.08	.567
Metabolism of terpenoids and polyketides	1.64 ± 0.1	1.61 ± 0.08	.89
Nucleotide metabolism	4.18 ± 0.19	4.26 ± 0.27	1
Xenobiotics biodegradation and metabolism	1.6 ± 0.1	1.8 ± 0.21	.456
Folding, sorting and degradation	2.46 ± 0.1	2.33 ± 0.1	.656
Genetic information processing	2.77 ± 0.06	2.79 ± 0.11	1
Replication and repair	9.39 ± 0.29	9.17 ± 0.46	.824
Transcription	2.95 ± 0.13	3.15 ± 0.12	.683
Translation	6.2 ± 0.19	5.98 ± 0.32	.677
Membrane transport	12.24 ± 0.94	13.39 ± 0.75	.656
Signal transduction	1.45 ± 0.1	1.53 ± 0.14	.839
Signaling molecules and interaction	0.16 ± 0.02	0.2 ± 0.04	0

(Continues)

TABLE A5 (Continued)

Pathway in level 2	Relative abundance (%)		*p*-value (fdr)
	Wild	Provisioned	
Cell communication	0	0	1
Cell growth and death	0.56 ± 0.03	0.51 ± 0.03	.533
Cell motility	2.76 ± 0.51	2.33 ± 0.55	.539
Cellular processes and signaling	3.81 ± 0.15	3.85 ± 0.19	1
Transport and catabolism	0.23 ± 0.04	0.18 ± 0.03	.031
Circulatory system	<0.01	<0.01	.444
Digestive system	0.06 ± 0.02	0.03 ± 0.02	0
Endocrine system	0.34 ± 0.04	0.25 ± 0.05	.031
Environmental adaptation	0.17 ± 0.01	0.15 ± 0.01	.617
Excretory system	0.02 ± 0.01	0.02 ± 0.01	.399
Immune system	0.1 ± 0.01	0.08 ± 0.02	.1
Nervous system	0.11 ± 0	0.1 ± 0.01	.658
Sensory system	0	0	1
Cancers	0.1 ± 0.01	0.08 ± 0.01	.092
Cardiovascular diseases	<0.01	<0.01	.631
Immune system diseases	0.04 ± 0.01	0.05 ± 0.01	.549
Infectious diseases	0.37 ± 0.02	0.4 ± 0.03	.456
Metabolic diseases	0.11 ± 0.01	0.1 ± 0.01	.695
Neurodegenerative diseases	0.08 ± 0.01	0.1 ± 0.03	0
Poorly characterized	4.84 ± 0.08	4.88 ± 0.11	1