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ORIGINAL ARTICLE

Metagenomics reveals contrasting energy utilization efficiencies of captive and wild camels (*Camelus ferus*)

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Abstract

Captive conditions can affect the symbiotic microbiome of animals. In this study, we compared the structural and functional differences of the gastrointestinal microbiomes of wild Bactrian camels (*Camelus ferus*) between wild and captive populations, as well as their different host energy utilization performances through metagenomics. The results showed that wild-living camels harbored more microbial taxa related to the production of volatile fatty acids, fewer methanogens, and fewer genes encoding enzymes involved in methanogenesis, leading to higher energy utilization efficiency compared to that of captive-living camels. These findings suggest that the wild-living camel fecal microbiome demonstrates a series of adaptive characteristics that enable the host to adjust to a relatively barren field environment. Our study provides novel insights into the mechanisms of wildlife adaptations to habitats from the perspective of the microbiome.

Key words: adaptability, energy utilization, gastrointestinal microbiome, metagenomics, wild and captive, wild Bactrian camel

INTRODUCTION

In recent years, numerous studies have confirmed that symbiotic microorganisms in the gastrointestinal tract (GIT) of humans and animals play a vital role in the health, nutrition, physiology, and behavior of the host (Hanning & Diaz-Sanchez 2015; Sharon *et al*. 2016; Gilbert *et al*. 2018; Davidson *et al*. 2020; Peixoto *et al*. 2021). An obvious example is that ruminants have the ability to convert carbohydrates, such as plant fibers that are not digestible by the animals themselves, into energy substances that can be digested and absorbed by the host (Ravachol *et al*. 2015). This ability is mainly attributed to the symbiotic microbiota in the rumen at the front of the digestive tract of ruminants (Allen & Mertens 1988). These microbes, mainly different bacteria, convert plant material into metabolic end products, such as volatile fatty acids (VFAs, primarily acetic, propionic, and butyric acids) and methane (Koh *et al*. 2016; Tapio *et al*. 2017). VFAs can be absorbed by the rumen wall and enter the blood circulation as usable energy material (Russell & Rychlik 2001), whereas methane is emitted into the

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atmosphere as an unused carbon source, resulting in major energy losses for ruminants (Mizrahi 2012). Changes in the abundance of specific microbial taxa can affect the energy acquisition of ruminants, resulting in changes in the feed efficiency phenotype of animals (Shabat *et al*. 2016).

Since the impact of human activities on the environment has increased, captive breeding conservation programs are being launched to save endangered species (IUCN/SSC 2013; Attard *et al*. 2016; Harding *et al*. 2016). Changes in lifestyle (in captivity vs in the wild) have resulted in alterations to energy requirements. Exploring how captivity affects the microbiome in response to various energy requirements will lead to a deeper understanding of wildlife adaptation mechanisms in different habitats. Several recent studies have identified differences in symbiotic microbes between wild and captive populations (Clayton *et al*. 2016; McKenzie *et al*. 2017; Gibson *et al*. 2019; Schmidt *et al*. 2019; Trevelline *et al*. 2019; Tang *et al*. 2020), indicating that the confined environment has a significant impact on the GIT microflora of wild animals.

However, most current studies on the differences between captive and wild animals have only considered variations in community composition and structure. Little attention has been paid to the effects of these microbial divergences on host physiological functions, such as energy utilization. This is mainly due to the fact that most current research on GIT microbiota has focused on the targeted amplicon sequencing of a single gene (e.g. 16S rRNA) to identify the GIT microbiome, as a metataxonomic approach. While less cost-effective, the application of a shotgun metagenomic approach to characterize the GIT microbiome offers multiple advantages (Hilton *et al*. 2016). First, the shotgun metagenomic approach does not depend on PCR. Therefore, it is not affected by PCR artifacts (Soergel *et al*. 2012). Furthermore, metagenomic sequencing can also provide functional knowledge of the microbiome (Dinsdale *et al*. 2008), which is crucial to understand how environmental change-induced variations in the composition of GIT microbes affect the host biological processes.

To further explore how captive conditions can alter the GIT microbiome of wildlife and its effect on host metabolic functions in energy utilization, we utilized the metagenomics to compare differences in the composition structure and metabolic function of GIT microbes between wild and captive populations of wild Bactrian camel (WBC; *Camelus ferus*), which is the only wild camel remaining in the world (Mohandesan *et al*. 2017) that can live in the extreme barren desert. This will provide new insights in understanding the mechanisms of adaptations of wild populations to their living environments.

MATERIALS AND METHODS

Sample collection

Fecal samples were collected from 7 captive and 6 wild WBCs (see Table S1, Supporting Information). The feces of captive WBCs (C1-C2 aged 6–7 years old; C3-C7 aged 10–20 years old) were sampled from September 26, 2020, to September 27, 2020, at Gansu Endangered Animals Protection Center (GEAPC), located in Wuwei City, Gansu Province, China (37°52′45″N, 102°52′54″E). Captive WBCs were reared in the breeding center in 2 large enclosures and were fed alfalfa (*Medicago sativa*) hay daily. They have been treated with ivermectin twice a year for 4 years, but none of them were administered with drugs within 3 months prior to sampling. Fecal samples of wild WBCs (aged 10–20 years old) were collected on September 30, 2020, at Dunhuang Xihu National Nature Reserve (DXNNR), located in Dunhuang City, Gansu Province, China (40°20′39″N, 093°41′51″E), which is about 830 km away from GEAPC. DXNNR covers an area of 660 000 hectares and is adjacent to the Kumtag Desert and Lop Nur, where the vegetation is mainly xerophytic shrubs. Fresh fecal samples were collected into sterile centrifuge tubes, labeled, and stored in a mobile refrigerator before being sent to the laboratory; they were then stored at −20 °C until DNA extraction.

This study was approved by the Ethics and Animal Welfare Committee of Beijing Forestry University and carried out in accordance with the approved protocol.

DNA extraction and metagenomic sequencing

Complete genomic DNA was extracted from fresh camel fecal samples using the Qiagen Fast Stool Mini Kit (QIAGEN Sciences, USA). A mechanical disruption step of bacterial cells was added by bead-beating with disruptor tubes (Omega Bio-tech, USA) using the TissueLyser (Onebio tech, China) at a frequency of 45 HZ for 250 s. The concentration and purity of the extracted DNA were examined using a TBS-380 and NanoDrop2000, respectively. The quality of the extracted DNA was verified using 1% agarose gel electrophoresis. DNA was purified using a Qiagen DNA Purification Kit (QIAGEN Sciences, USA).

Covaris M220 (Gene Company Limited, China) was used for DNA fragmentation and approximately 400 bp

fragments were filtered using a NEXTFLEX Rapid DNA-Seq kit (Bioo Scientific, Austin, TX, USA) to construct a paired-end library. Paired-end sequencing was conducted using the NovaSeq Reagent Kits on the Illumina Novaseq 6000 platform (Illumina Inc., San Diego, CA, USA). Sequenced data associated with this project have been deposited in the NCBI Sequence Read Archive (Accession Number: PRJNA690892).

Bioinformatics

Paired-end reads were trimmed to exclude Illumina sequencing adaptors, and low-quality reads (length <50 bp, with a quality value <20, or having *N* bases) were eliminated using fastp (version 0.20.0) (Chen *et al*. 2018). The genome of the wild Bactrian camel was aligned and omitted from the reads using BWA (version 0.7.9a) (Li & Durbin 2009). Metagenomic data were assembled using MEGAHIT (version 1.1.2) (Li *et al*. 2015). Contigs with a length ≥300 bp were selected as the final assembly results. The open reading frames (ORFs) of each contig assembled were predicted using MetaGene (Noguchi *et al*. 2006). Predicted ORFs with a length ≥100 bp were retrieved and translated into amino acid sequences using the NCBI translation table.

A non-redundant gene catalog was constructed using CD-HIT (version 4.6.1) (Fu *et al*. 2012) with 90% sequence identity and 90% coverage. The longest sequence of each cluster was taken as the representative sequence. Bacterial and archaeal genes were selected from the non-redundant gene catalog to construct a prokaryotic gene catalog. The gene abundance profiles of each sample were calculated by a Blast search of high-quality reads based on the prokaryotic gene catalog with 95% identity using SOAPaligner (version 2.21) (Li *et al*. 2008). Representative sequences were aligned to the NCBI NR database and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database using Diamond (version 0.8.35) (Buchfink *et al*. 2014) for taxonomic and functional annotations with an e-value cutoff of 1e-5. The relative abundance of sequences annotated to the same taxon was then added together to generate the taxonomic profile. It was suggested that assembly- and reads-based methods would likely generate different results (Tamames *et al*. 2019), therefore, apart from assembly-based tools, we also performed reads-based analysis. Kraken2 (Wood *et al*. 2019) was used against the pre-built 8 GB MiniKraken database with abundance estimates from Bracken (Lu *et al*. 2017) to test the performance of reads-based taxonomic profiling, and then Pavian package (Breitwieser & Salzberg 2020) was used for the visualization of the results.

Statistical analyses

All analyses were performed on gene abundance normalized to reads per kilobase per million reads. The Bray-Curtis algorithm was used in QIIME (Caporaso *et al*. 2010) to calculate the distance between pairs of samples, and a beta diversity distance matrix was obtained to conduct hierarchical clustering analysis, which was constructed using the unweighted pair group method with arithmetic mean (UPGMA) algorithm. The "stats" package of R and Python's "SciPy" package were used for two-group comparisons. Statistical testing was performed using the two-tailed Wilcoxon rank-sum test ($P < 0.05$) with the false discovery rate for multiple comparison correction. All given confidence intervals are calculated for a confidence level 0.95 with the bootstrap method. Furthermore, linear discriminant analysis (LDA) effect size (LEfSe) analyses were performed using LEfSe software (http://huttenhower.sph.harvard.edu/lefse/) (Segata *et al*. 2011) with an LDA score threshold of 4.0. KEGG pathway analysis was performed using the KEGG mapper tool, whereas correlation network analysis was conducted using Python's NetworkX package (Hagberg *et al*. 2008).

RESULTS

Sequence statistics

One sample collected from captive WBCs showed 80 to 340-fold higher proportion of *Acinetobacter* compared to the other samples in the analysis of the microflora structure, and therefore was excluded from the dataset before further analysis. Of all the 12 WBC fecal samples, a total of 868 632 920 (72 386 077 ± 2 558 463) raw reads were obtained, and 851 151 000 (70 929 250 ± 2 514 606) reads remained after quality control, representing 98% of the raw data. In total, 624 128 922 (52 010 744 ± 2 619 153) optimized reads were obtained after the elimination of host sequences (*Camelus ferus*), accounting for 72% of the raw reads. A total of 10 328 850 contigs from the gene assembly were collected and 13 770 155 ORFs were identified after gene prediction. In total, 4 311 327 catalog genes were constructed with an average sequence length of 552 bp in the non-redundant prokaryotic gene catalog.

Differences in fecal microbial composition between captive and wild WBCs

There were significant differences in the microbial community structure between the captive and wild

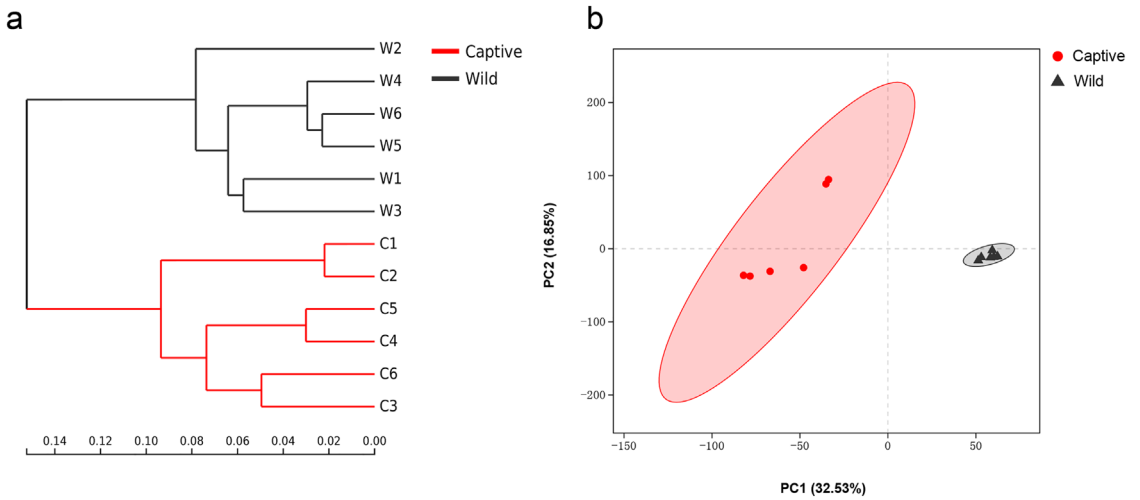

Figure 1 (a) Hierarchical cluster tree and (b) principal component analysis (PCA) plot of the gastrointestinal microbiome in all camel samples at the species level.

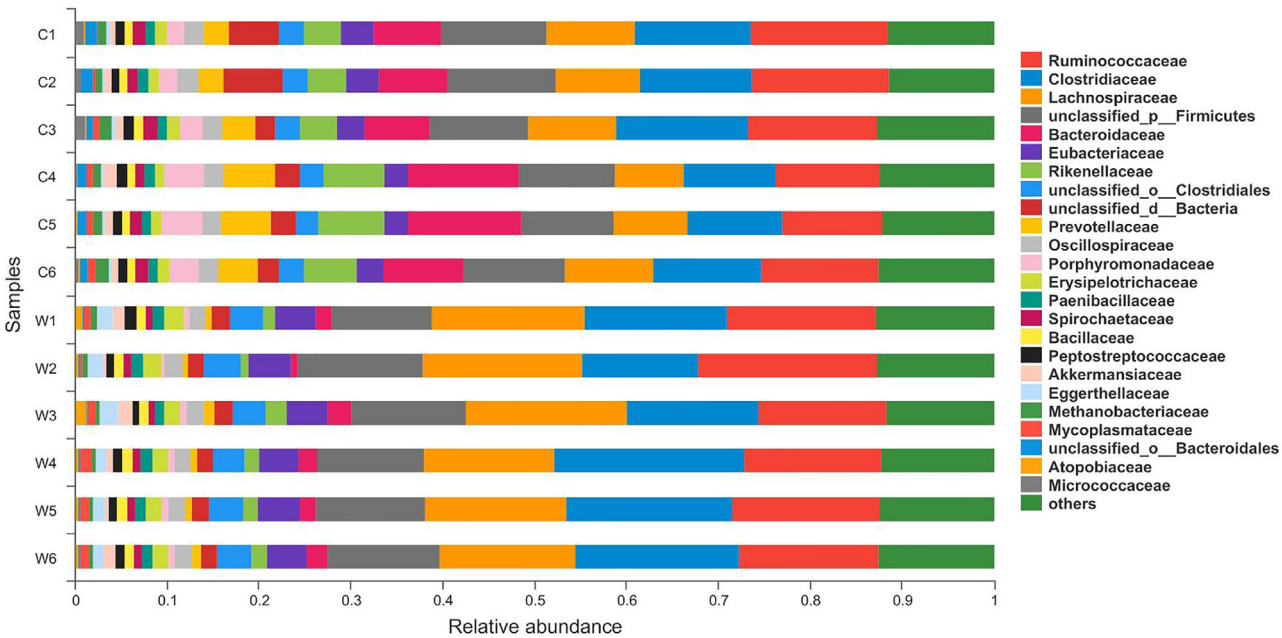

Figure 2 Fecal microbiome community composition at the family level in wild Bactrian camels (WBCs) in the wild and in captivity.

WBCs. Species hierarchical clustering analysis and principal component analysis of the microbiome showed that the fecal microbial communities of captive and wild WBCs were markedly distinct and formed separate groups (Fig. 1). At the family level, Ruminococcaceae, Clostridiaceae, Lachnospiraceae, and unclassified Firmicutes, which are dominant in both captive and wild WBCs (Fig. 2), all showed higher relative abundances in wild WBCs, and this was especially apparent for Lachnospiraceae (relative abundance was approximately 16%, almost twice that of captive WBCs), whereas Bacteroidaceae, Rikenellaceae, and Prevotellaceae were significantly more abundant in the captive group than in the wild group (Fig. 3a). At the genus level, the abundance of gastrointestinal microbiota in wild WBCs was significantly higher than that in captive populations for

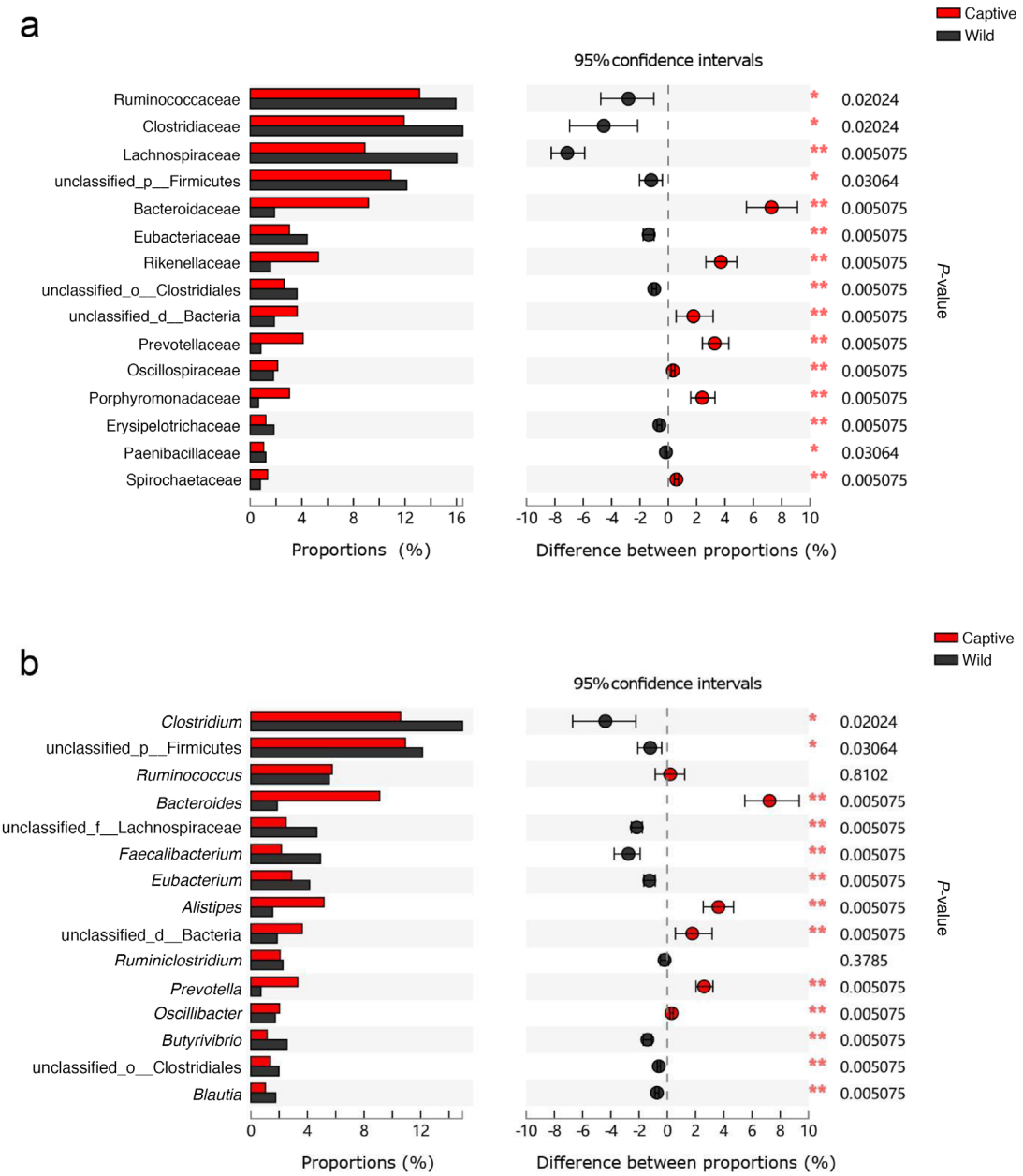

Figure 3 Two-tailed Wilcoxon rank-sum test on (a) fecal microbial families and (b) genera for the 2 groups of camels (relative abundance of top 15 families and genera are shown respectively). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

many taxa, such as *Clostridium* spp., *Faecalibacterium* spp., *Eubacterium* spp., *Butyrivibrio* spp., and *Blautia* spp., which all presented a high degree of contribution to energy metabolism (Fig. 4), whereas *Bacteroides* spp., *Alistipes* spp., and *Prevotella* spp. were more abundant in the captive population (Fig. 3b). The LEfSe analyses showed that *Methanobrevibacter* was significantly more prevalent in captive WBCs than in wild WBCs and was the biomarker for archaeal communities in captive WBCs with the greatest impact on the significant difference between groups (LDA = 5.28; Fig. 5).

We used additional reads-based metagenomic sequence classification software, Kraken2, to verify if the results were validated across different bioinformatic

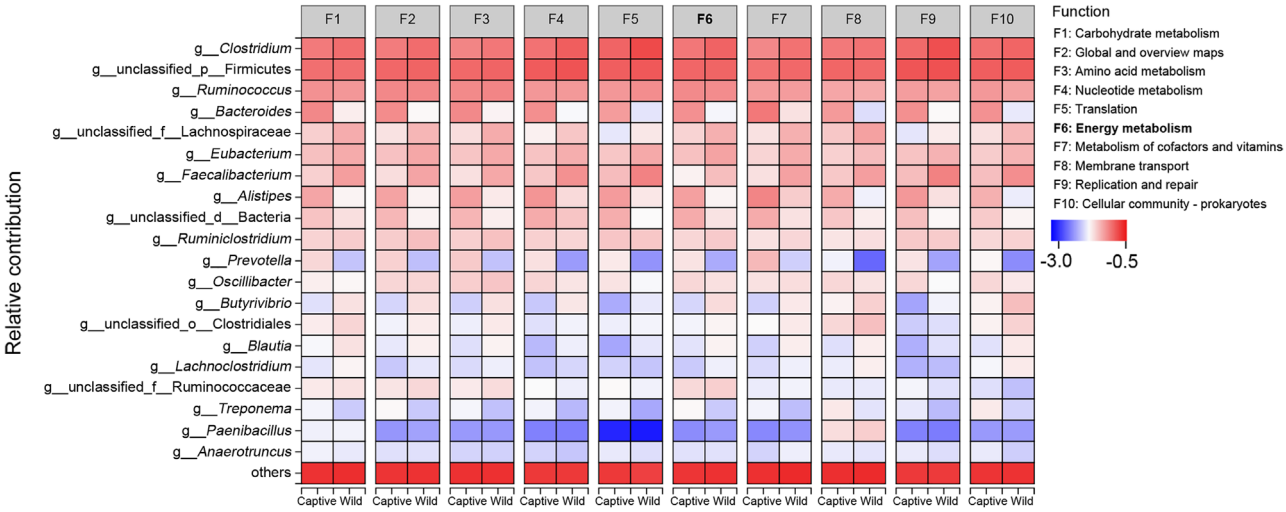

Figure 4 Heatmap showing the relative contribution of the top 20 dominant genera in functions of KEGG pathway level 2 in wild Bactrian camels (WBCs) in captivity and in the wild.

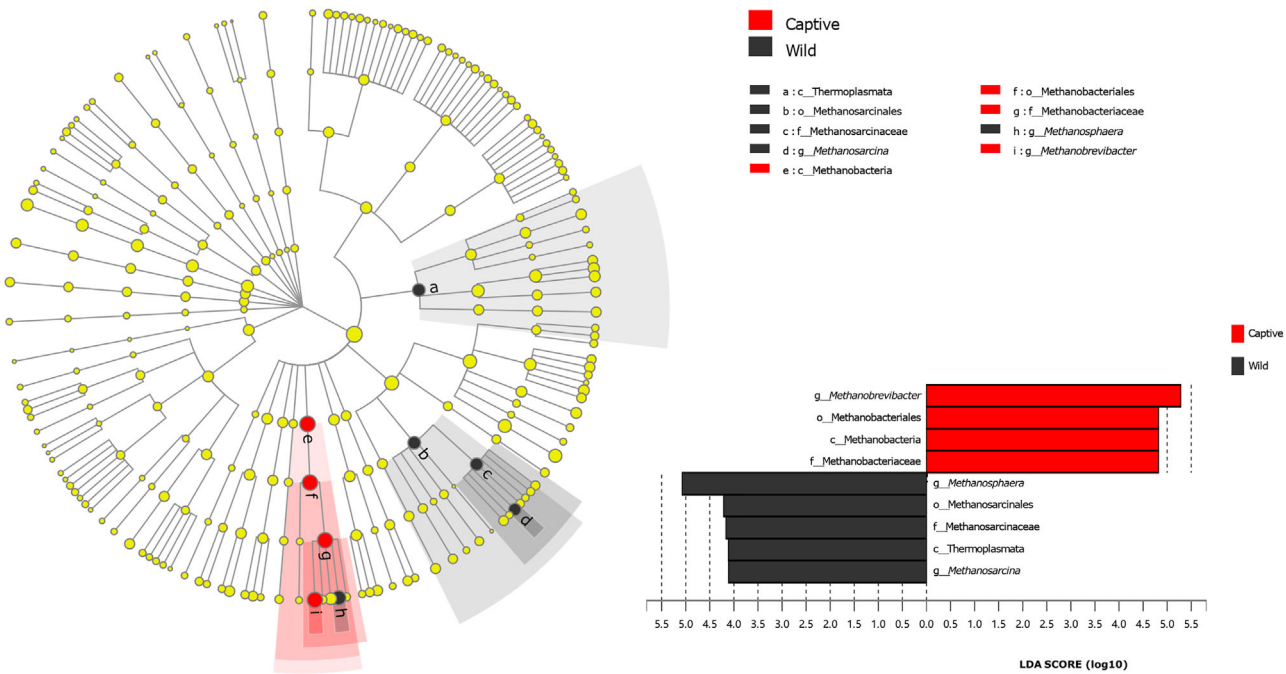

Figure 5 Linear discriminant analysis (LDA) effect size (LEfSe) analyses of fecal archaea communities in wild Bactrian camels (WBCs) in captivity and in the wild. The cladogram shows the biomarkers of archaea in captive and wild WBCs, the sizes of dots are proportional to the relative abundances of taxa. The bar plot of the LDA score shows taxa with scores greater than 4.

pipeline. Similar to the assembly-based method, Kraken2 identified Ruminococcaceae, Clostridiaceae, and Lachnospiraceae as most abundant families (see Tables S2 and S3 and Fig. S1, Supporting Information). They all had higher relative abundances in wild WBCs than captive groups, whereas Bacteroidaceae, Rikenellaceae, and Prevotellaceae showed the opposite trends (see Table S3 and Fig. S2, Supporting Information). Although the results concerning the difference between captive and wild individuals stay valid across different pipeline, it should

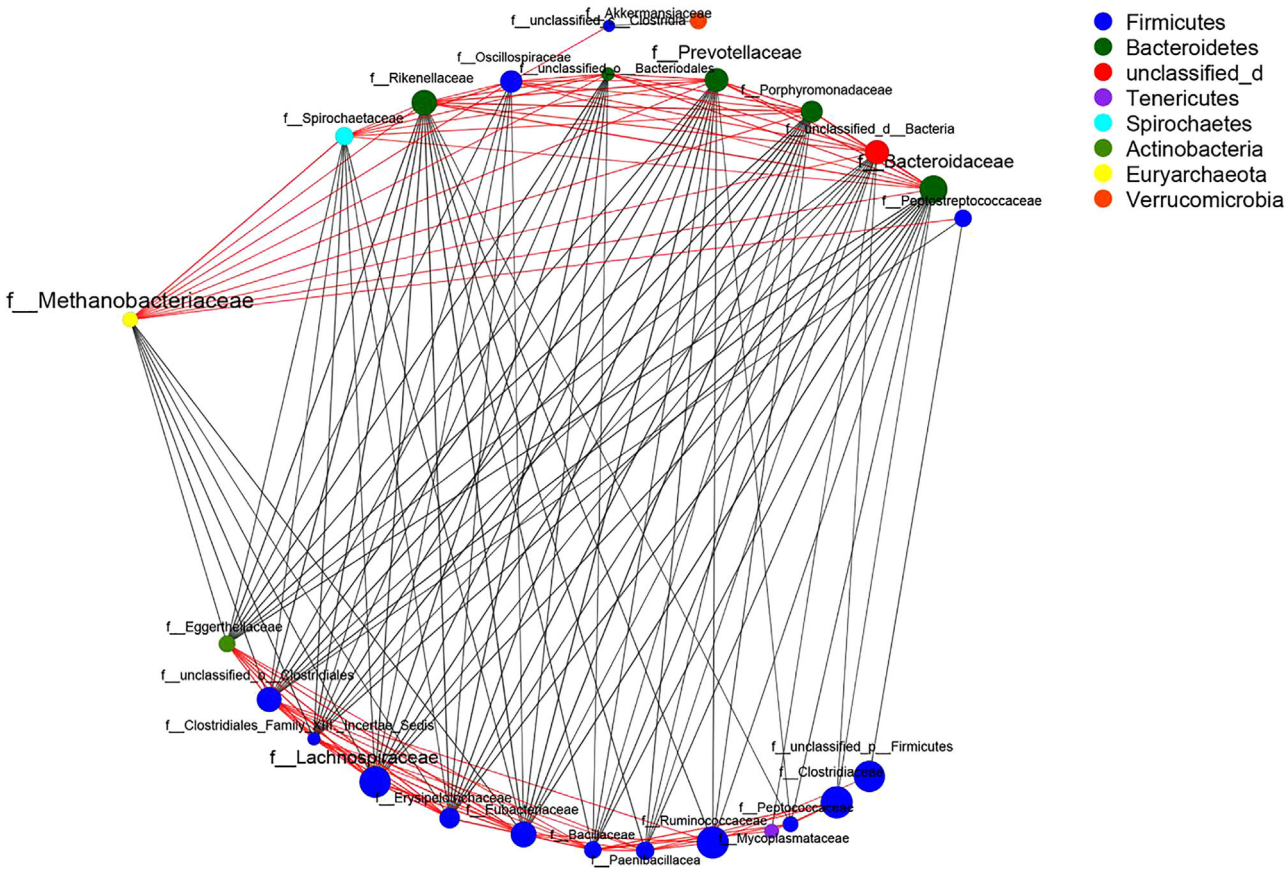

Figure 6 Correlation network analysis of fecal microbial communities in wild Bactrian camels (WBCs) in captivity and in the wild. The red lines represent positive correlations, and the black lines represent negative correlations; the sizes of dots are proportional to the relative abundances of taxa.

be noted that there are significant variations among some taxa using the 2 methods. For example, the relative abundance of Burkholderiaceae identified through Kraken2 (4.27%) was higher than through assembly-based method (0.29%), while Eubacteriaceae had lower abundance in the profile of reads-based method (0.52%) than in assembly-based method (3.72%) (see Table S3 and Fig. S1, Supporting Information). In addition, a higher number of reads were reported as "unclassified" by the assembly-based method.

Correlation analysis of fecal microbial community composition in WBCs

The correlation network analysis of the top 25 microbial families indicated that, of the 2 dominant phyla, most families of Firmicutes were negatively correlated with Methanobacteriaceae, whereas families of Bacteroidetes were positively correlated with Methanobacteriaceae (Fig. 6). In addition, most internal correlation within Bacteroidetes and Firmicutes was positive.

Function analysis of fecal microbiomes based on energy harvesting

A considerable distinction in gastrointestinal microbiota function was observed between captive and wild WBCs. Based on the principal component analysis of the KEGG pathway function (level 2), it was apparent that the microbiome functions of the captive and wild WBCs were aggregated within the group but were distinctly separated from each other (Fig. 7a). The prominent functions of the gastrointestinal microbiome in WBCs are carbohydrate metabolism, amino acid metabolism, nucleotide metabolism, replication and repair, and energy metabolism. Notably, the abundance of

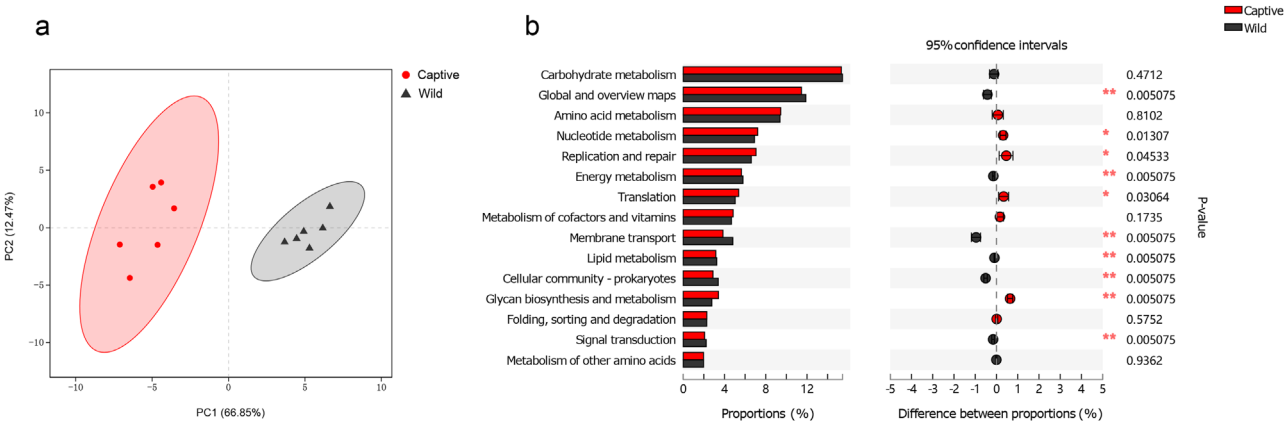

Figure 7 (a) Principal component analysis (PCA) plot and (b) two-tailed Wilcoxon rank-sum test based on KEGG pathway level 2 at the genus level in wild Bactrian camels (WBCs) in captivity and in the wild. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

genes associated with global and overview maps, energy metabolism, membrane transport, lipid metabolism, cellular community-prokaryotes, and signal transduction, were significantly higher ($P < 0.01$) in wild WBCs than in captive WBCs (Fig. 7b).

There were also major variations in the methane metabolism pathway between captive and wild WBCs (Fig. 8). Analyses of differences in metabolic pathways of methanogenesis showed that genes encoding almost all enzymes (9 of 11 reactions) involved in the metabolism of carbon dioxide (CO_2) to methane (CH_4) were significantly lower in wild WBCs than in captive animals in terms of relative abundance ($P < 0.05$). F420 is an essential hydride carrier in the methanogenesis pathway using H_2 and CO_2 (Mills *et al*. 2013), and genes encoding all enzymes involved in the metabolism of F420 biosynthesis were found to be significantly lower in wild WBCs ($P < 0.05$).

DISCUSSION

Microorganisms living in the rumens of ruminants, such as camels, can convert indigestible plant materials such as lignocellulose into energy substances that can be absorbed and utilized by the host, therefore provide energy to the host (Russell & Rychlik 2001; Koike & Kobayashi 2009). Meanwhile, microbial-led methane metabolism is the primary source of energy loss in ruminants (Morgavi *et al*. 2010). The results showed that there were significant differences in fecal microbial composition between captive and wild WBCs. Ruminococcaceae, Clostridiaceae, and Lachnospiraceae, which are highly abundant in wild WBCs, play an important role in converting plant fibers into VFAs to provide energy for hosts (Boutard *et al*. 2014; Molina-Guerrero *et al*. 2021). Most notably, Lachnospiraceae, one of the richest taxa in the rumen microbial composition of ruminants (Seshadri *et al*. 2018), was considerably enriched in wild WBCs, including *Butyrivibrio* spp. and *Blautia* spp., which exhibited a high relative abundance in wild WBCs and can ferment a variety of plant polysaccharides into VFAs, mainly acetate and butyrate (Boutard *et al*. 2014). The formation of propionate is predominantly restricted to *Faecalibacterium* spp. and *Eubacteria* spp. (Flint *et al*. 2012; Engels *et al*. 2016), both of which were significantly more prevalent in wild WBCs than in captive WBCs. *Clostridium* spp., which are abundant in wild WBCs, also play an important role in the fermentation process of plant polysaccharides (Boutard *et al*. 2014), and this is one of the most abundant and important microbial taxa in the rumen (Lopetuso *et al*. 2013).

The higher relative abundance of *Bacteroides* spp. and *Prevotella* spp. in captive WBCs might be due to the difference in diet between captive and wild WBCs. The main food of captive WBCs in the GEAPC is alfalfa hay, whereas the food of camels in the wild is mainly woody shrubs, which are widely distributed in the DXNNR. As a leguminous herb with symbiotic nitrogen-fixing rhizobia, alfalfa is often used worldwide as high-quality livestock forage (Bouton 2013), with more protein and digestible carbohydrates than shrubs (Hoiechek 1984; Scholtz *et al*. 2009). *Bacteroides* were usually associated with protein-rich diet, whereas *Prevotella* has been linked with high-fiber diet (Wu *et al*. 2011). Increase of *Bacteroides* spp. and *Prevotella* spp. in captive animals than in wild counterparts has also been reported in other species, such as musk deer (Li *et al*. 2017) and loris (Cabana *et al*. 2019). The comparatively higher abundance of these 2 genera

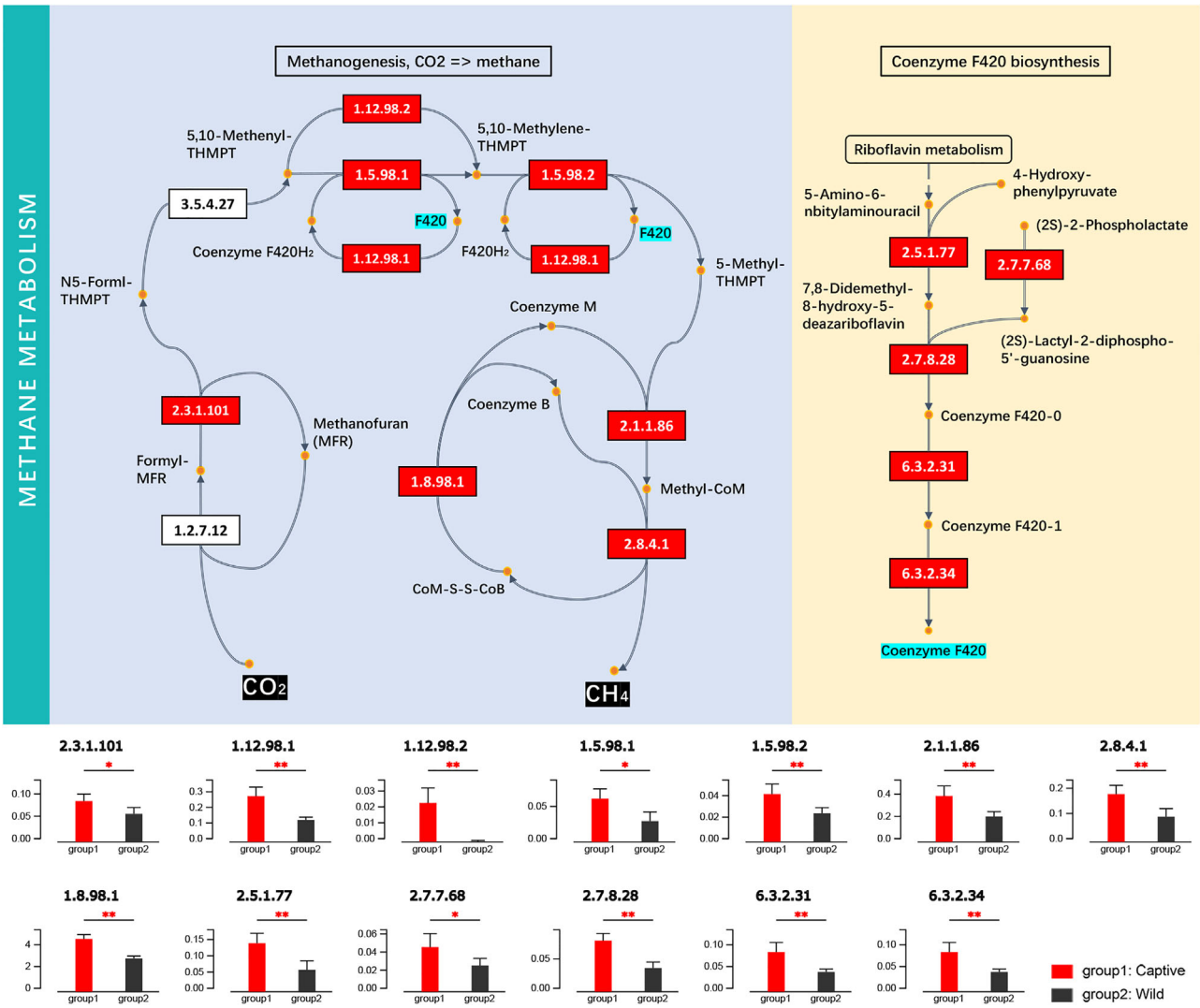

Figure 8 Methane metabolism pathway analysis of fecal microbiomes of wild Bactrian camels (WBCs) in captivity and in the wild. Each box represents an enzyme, with red denoting that the relative abundance of which was significantly higher in captive WBCs; the differences in relative abundances are shown in the bar plots.

and their greater contribution to energy metabolism in captive WBCs could be attributable to the high protein and carbohydrate diet composition.

Methanobrevibacter belonging to Methanobacteriaceae is the dominant genus of the archaea community, accounting for more than 70% of the total archaea and is the main contributor to methane production in the ruminant rumen (Henderson *et al*. 2015). In this study, *Methanobrevibacter* spp. also displayed the highest relative abundance in the gut archaea community of WBCs and was also a biomarker which could distinguish captive WBCs from wild WBCs. The relatively less methanogenic archaea and more bacteria associated with VFA production in wild WBCs than captive WBCs might constitute the structural basis of their adaptation to the harsh environment in the wild (Zhang *et al*. 2016).

Microbiota cohabit ecological niches, and interactions between microbial species might be present (Faust & Raes 2012). This study showed that Methanobacteriaceae, as the most important methanogenic archaea, was negatively correlated, in terms of relative abundance, with energy-metabolizing bacteria, such as Lachnospiraceae and Ruminococcaceae, which are all related to the production of VFAs, indicating that methanogenic archaea

and VFA-producing bacteria might have a competitive relationship in resource utilization (Hibbing *et al*. 2010). The increased generation of VFAs inhibits methanogenesis through competition for hydrogen during methane metabolism (McAllister *et al*. 1996). Methanobacteriaceae were positively correlated with Bacteroidaceae and Prevotellaceae, indicating that the declining abundance of the 2 families may accompany with a decreased abundance of Methanobacteriaceae synchronously. The Methanobacteriaceae produce almost all enzymes involved in methane metabolism in wild WBCs and were significantly lower compared to levels in captive WBCs, thus might suggest inhibited methane production in wild WBCs and a reduction in energy waste caused by methane release.

Wild animals are likely to rely more on efficient energy conversion than captive animals because they require more vigor and strength to cope with more challenging and hazardous environments (Liukkonen-Anttila *et al*. 2000; O'Regan & Kitchener 2005). Compared to that of captive WBCs, the higher energy metabolism associated genes of wild WBCs suggests that their GIT microbiome may be more capable of transforming limited food resources into energy that can be utilized by the host. Higher levels of membrane transport function also indicate higher metabolic levels in wild WBCs (Kleinzeller 1981).

Although there have been efforts to use the fecal microbiome as a proxy for monitoring the microbial communities in the rumen (Noel *et al*. 2019; Andrade *et al*. 2020), it is uncertain to which extent the fecal microbiome may represent the rumen microbiome of wild WBCs. Previous research showed that the relative abundance of bacteria differed between rumen and feces of domestic Bactrian camel (He *et al*. 2018). In addition, there is evidence of a correlation between microbiome and methane emission, but that link was weaker in feces than in the rumen (Noel *et al*. 2019). The current study may therefore underestimate the influence of captivity on the gut microbiome as only the fecal microbiome was included.

Overall, the fecal microbiome of WBCs in the wild showed a series of adaptive characteristics that may enable them to acclimate to the harsher wild environment, as compared to that in animals living in captivity, which need to be tested through future physiological or functional studies. More VFA-related microbiota, fewer methanogens, and fewer genes encoding enzymes involved in the methanogenic pathway may enable wild WBCs to convert foodstuffs into energy substances more efficiently than captive WBCs, with fewer energy losses resulting from methane emissions. This could allow wild WBCs to sustain a higher level of energy harvesting with less food acquisition to maintain a higher metabolic function. Greater energy efficiency is a way for wildlife to adapt to harsh environments, and this adaptation can be achieved through the symbiotic microbiome of the host. The huge differences in methanogenic archaea shown in wild and captive ruminants also provide new insights into ways to address the global warming effect exacerbated by massive volumes of methane emitted by livestock.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SUPPLEMENTARY MATERIALS

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Comparison of reads-based and assembly-based methods on the relative abundance of the top 30 bacteria at the family level.

Figure S2 Relative abundance of the specific taxon across samples using Kraken2.

Table S1 Metadata for each individual wild Bactrian camel

Table S2 Taxnomic abundance (%) profile estimated by kreken2 at the diffferenct taxonomy level

Table S3 Comparison of reads-based and assembly-based methods on the relative abundance of the top 30 bacteria at the family level

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