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ORIGINAL ARTICLE

Comparison of the gut microbiota composition between the wild and captive Tibetan wild ass (*Equus kiang*)

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Keywords
16S rRNA sequencing, captive, gut microbiota, The Qinghai-Tibet Plateau, Tibetan wild ass (*Equus kiang*).

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Abstract

Aims: The gut microbiota has a great effect on the health and nutrition of the host. Manipulation of the intestinal microbiota may improve animal health and growth performance. The objectives of our study were to characterize the faecal microbiota between wild and captive Tibetan wild asses and discuss the differences and their reasons.

Methods and Results: Through high-throughput sequencing of the 16S rRNA V4-V5 region, we studied the gut microbiota composition and structure of Tibetan wild asses in winter, and analysed the differences between wild and captive groups. The results showed that the most common bacterial phylum in Tibetan wild ass faeces samples was Bacteroidetes, while the phylum Firmicutes was dominant in captive Tibetan wild ass faecal samples. The relative abundance of Firmicutes, Tenericutes and Spirochaetes were significantly higher ($P < 0{\cdot}01$) than in the wild groups.

Conclusions: Captivity reduces intestinal microbial diversity, evenness and operational taxonomic unit number due to the consumption of industrial food, therefore, increasing the risk of disease prevalence and affecting the health of wildlife.

Significance and Impact of the Study: We studied the effect of the captive environment on intestinal micro-organisms. This article provides a theoretical basis for the ex-situ conservation of wild animals in the future.

Introduction

The Qinghai-Tibetan plateau provides one of the most extreme environments for the survival of humans and other mammals (Zhang *et al.* 2016). The Tibetan wild ass (*Equus kiang*) is a unique species on the Qinghai-Tibetan plateau and is widely distributed in Qinghai, Gansu, Xinjiang, Sichuan and Tibet (Wu and Yi 2000; Moehlman 2002). It is a key protected species in China and is listed in the International Union for Conservation of Nature Red List 2012 of threatened species. Intensive research has been performed regarding the conservation of this species (Joseph and Bard-Jorgen 2005; Yifan and Jianping 2006; Yin *et al.* 2007; St-Louis and Côté 2009; Kefena *et al.* 2012; Dong *et al.* 2015; Guo *et al.* 2018). With the development of wildlife protection plans, the change in environment during ex-situ conservation comes with a change in animal health.

The microbial community of the gastrointestinal tract remains balanced in terms of species, quantity and location in healthy organisms. Animal intestines have large, diverse and dynamically changing bacterial communities that play important roles in host immunity, nutrient metabolism and energy acquisition (Yun *et al.* 2017). The composition of the mammalian gut microflora is associated with many environmental factors, among which living conditions are a major part (Guan *et al.* 2016).

Captive environments affect the composition of gut microbe in wild animals (Xenoulis *et al.* 2010; Guan *et al.* 2016, 2017). Changes in the intestinal microbe composition are associated with host health and disease (Quigley 2010; Costa *et al.* 2012; Morgan *et al.* 2012; Qin *et al.* 2012). Diet is a key factor affecting microbial diversity in the host gut (Ley *et al.* 2008; Yin *et al.* 2017; Qin *et al.* 2018). As industrial food consumption increases in humans and wildlife, each dietary change is accompanied by an adjustment of intestinal microbes, resulting in the loss or extinction of certain intestinal microbes (Zhang *et al.* 2018). Recent studies have shown that diet-induced loss of microbial diversity can be amplified over generations, resulting in reduced intestinal microbial diversity and increased risk of population extinction (Sonnenburg *et al.* 2016).

Therefore, the objectives of our study were (i) to characterize the faecal microbiota between wild and captive Tibetan wild asses; (ii) to analyse the differences between faecal samples from different environments; (iii) discuss the causes for the differences, and finally, (iv) to explore the relationship between diet, gut flora and host health. The study of intestinal microbial diversity, which can be used to assess host health and related diseases, provide a theoretical basis for the future breeding or release of wild animals.

Materials and methods

Faecal samples from Tibetan wild asses living in the wild were collected from different regions of the headwaters of the Yellow River, Maduo County on Qinghai-Tibet Plateau in January 2018. A total of 140 wild Tibetan wild ass faecal samples were collected. All samples were collected after natural defecation. Animals were not scared, nor driven, and drugs were not used to promote defecation. Captive Tibetan wild ass faecal samples were collected from the Qinghai-Tibet Plateau wild animal park in January 2018. In total 28 captive Tibetan wild ass samples were collected. None of the animals had received anti-inflammatory drugs or antimicrobials within the last 3 months.

All sample collection processes were performed in accordance with the requirements of the authorizing ethics committee.

Genomic DNA from the samples were extracted by the CTAB method. DNA purity and concentration were monitored on a 1% agarose gel. DNA samples were diluted to 1 ng μl^{-1} using sterile water. Universal 16S PCR primers (515F, 5′-GTGCCAGCMGCCGCGGTAA-3′ and 907R, 5′-CCGTCAATTCCTTTGAGTTT-3′) were used to amplify the V4 and V5 regions of the 16S rRNA. All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, MA). The polymerase chain reaction was carried out using the following mixture in a final volume of 30 μl: 10 μl of template DNA, 3 μl of each primer (6 $\mu mol\ l^{-1}$), 15 μl of Phusion Master Mix (2×) and 2 μl of ddH_2O. Next, DNA was amplified using the following conditions: denaturation for 1 min at 98°C, followed by 30 cycles of 10 s at 98°C for denaturation, 30 s at 50°C for annealing and 30 s at 72°C for extension, as well as a final extension step at 72°C for 5 min. The yield

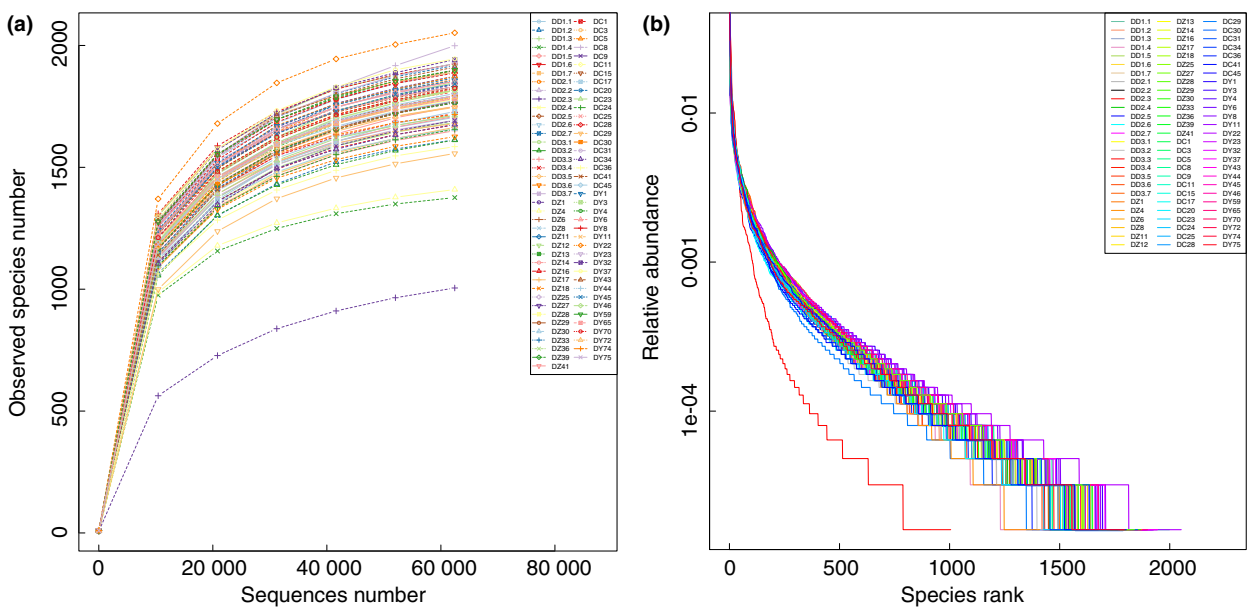

Figure 1 Tibetan wild ass rarefaction curves (a) and rank abundance curve (b). [Colour figure can be viewed at wileyonlinelibrary.com]

of PCR products was estimated using 2% agarose gel electrophoresis. PCR products were then purified with the GeneJETTM Gel Extraction Kit (Thermo Scientific, Waltham, MA).

The library was sequenced on an Ion S5™ XL platform and 400 bp single-end reads were generated. The single-end method was used to construct a small fragment library for single-end sequencing. By cutting and filtering reads, OTUs (operational taxonomic units) were clustered and species annotation and abundance analysis were performed to reveal sample species composition.

Novogene was commissioned to complete all experiments (DNA extraction, PCR amplification, library preparation and sequencing) and data analysis.

All diversity indices in our samples were calculated with QIIME (ver. 1.9.1) and displayed with R software (ver. 2.15.3). In R, NMDS analysis was displayed using the vegan package, principal coordinates analysis (PCoA) was displayed using the WGCNA package, stat package and ggplot2 package. Cluster analysis was preceded by principal component analysis (PCA), which was applied to reduce the dimensionality of the original variables using the factor Mine R package and ggplot2 package. Cross-group and intra-group differences were tested using the MRPP function in the vegan package.

Results

Eighty-one faecal samples from wild and captive Tibetan wild asses were selected for sequencing, of which 60 samples were from wild animals (DY, DC and DZ), classified as the wild group (DYW), and 21 samples were from captive animals (DD1, DD2, DD3), classified as the captive group (DDD). A total of 4 809 901 high-quality reads were obtained from wild group and classified into 3542 OTUs, while 1 693 293 high-quality reads were obtained from the captive group and classified into 3155 OTUs. The number of OTUs present in both the wild and captive groups was 2928, with 614 unique OTUs in the wild group, and 227 unique OTUs in the captive group.

The rarefaction curves and rank abundance curves of the wild and the captive Tibetan wild ass faecal samples (Fig. 1) show the richness and evenness of the species in the samples. As the sample size increased, the number of observed species gradually stabilized and there were no further significant growth or fluctuations. The results show that the curve had reached a plateau and the sequencing data were reasonable. The number of samples in this study was sufficient to study the intestinal microbial diversity of Tibetan wild asses in the field and in captivity.

We detected a total of 27 phyla, 47 classes, 81 orders, 134 families and 241 genera from 81 Tibetan wild ass faecal samples. In the wild group, we detected 26 phyla, 44 classes, 74 orders, 117 families and 199 genera, while in the captive group, 26 phyla, 43 classes, 71 orders, 121 families and 204 genera were detected.

In the wild group, Bacteroidetes (42·59%) was the predominant phylum, and Anaerovorax (2·29%) was the predominant genus. In the captive group, Firmicutes (49·74%) was the predominant phylum, and *Streptococcus* (4·39%) was the predominant genus. In order to show the relative abundance of bacterial communities more intuitively, we have chosen the top 10 species for each sample or group and generated a percentage stacked histogram of relative abundance at the phylum and genus levels in Fig. 2.

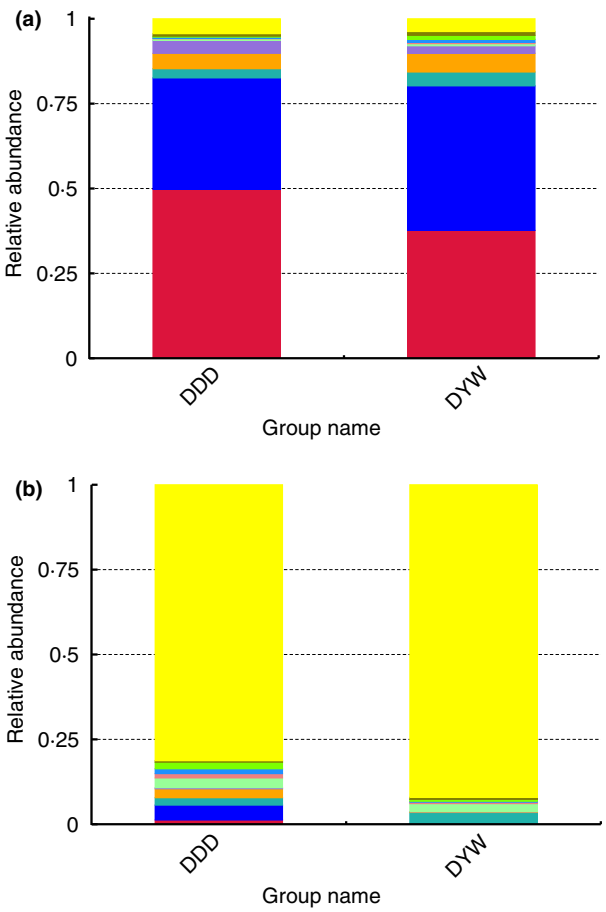

Figure 2 Relative abundance histogram. A histogram of the relative abundance of gut microbiota among groups in wild and captive Tibetan wild asses at the phylum level others; Proteobacteria; Melainabacteria; Euryarchaeota; Fibronacteres; Verrucomicrobia; Spirochaetes; Kiritimatiellaeota; Tenericutes; Bacteroidetes; Firmicutes (a) and genus level (b) others; Akkermansia; unidentified_Spirochaetaceae; Oribacterium; unidentified_Prevotellaceae; unidentified_Ruminococcaceae; Gillisia; unidentified_Clostridiales; unidentified_Bacteroidales; Streptococcus; Bacteroides. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 1 Alpha-diversity of gut microbiota in faeces samples from wild and captive Tibetan wild asses

Sample	Observed_species	Shannon	Simpson	Chao1	ACE	Goods_coverage
DD1·1	1705	8·560	0·993	1812·014	1822·969	0·997
DD1·2	1613	8·244	0·990	1704·377	1723·025	0·997
DD1·3	1684	8·354	0·990	1772·946	1786·140	0·997
DD1·4	1376	8·472	0·994	1452·703	1455·215	0·998
DD1·5	1750	8·344	0·986	1880·569	1889·458	0·996
DD1·6	1720	8·667	0·993	1810·725	1819·446	0·997
DD1·7	1750	8·688	0·994	1850·665	1869·866	0·997
DD2·1	1818	8·868	0·995	1920·097	1933·872	0·997
DD2·2	1770	8·708	0·994	1885·545	1904·017	0·996
DD2·3	1005	5·957	0·930	1152·396	1171·362	0·997
DD2·4	1660	8·634	0·993	1773·305	1775·951	0·997
DD2·5	1764	7·811	0·953	1875·000	1888·276	0·996
DD2·6	1715	7·687	0·946	1810·174	1820·362	0·997
DD2·7	1769	8·072	0·972	1900·790	1904·063	0·996
DD3·1	1693	8·503	0·992	1847·962	1837·006	0·996
DD3·2	1612	8·198	0·989	1738·196	1738·003	0·997
DD3·3	1650	8·370	0·991	1785·631	1772·260	0·996
DD3·4	1674	8·503	0·990	1774·000	1784·444	0·997
DD3·5	1664	8·609	0·993	1774·571	1789·636	0·997
DD3·6	1626	8·596	0·994	1744·719	1737·675	0·997
DD3·7	1693	8·727	0·994	1788·050	1797·103	0·997
DZ1	1874	8·582	0·990	1986·267	2013·325	0·996
DZ4	1409	8·124	0·988	1500·838	1500·093	0·997
DZ6	1793	8·523	0·991	1900·505	1905·494	0·997
DZ8	1891	8·675	0·992	2035·361	2022·190	0·996
DZ11	1831	8·748	0·993	1930·100	1937·509	0·997
DZ12	1817	8·514	0·989	1921·659	1936·995	0·997
DZ13	1788	8·657	0·993	1928·041	1924·393	0·996
DZ14	1709	8·687	0·994	1812·000	1824·012	0·997
DZ16	1810	8·649	0·993	1896·900	1915·408	0·997
DZ17	1680	8·896	0·995	1765·562	1762·498	0·997
DZ18	1861	8·869	0·995	2008·877	2011·590	0·996
DZ25	1842	8·886	0·994	1928·671	1941·348	0·997
DZ27	1840	8·688	0·990	1975·591	1978·662	0·996
DZ28	1814	8·378	0·988	1982·125	1980·656	0·996
DZ29	1771	8·452	0·990	1910·183	1914·916	0·996
DZ30	1658	8·552	0·992	1752·031	1765·289	0·997
DZ33	1867	8·720	0·993	1973·260	1997·880	0·996
DZ36	1848	8·604	0·993	1997·638	2007·443	0·996
DZ39	1765	8·444	0·989	1923·888	1899·486	0·996
DZ41	1794	8·762	0·994	1933·087	1925·171	0·996
DC1	1899	8·776	0·994	2054·793	2040·100	0·996
DC3	1746	8·847	0·995	1834·033	1838·680	0·997
DC5	1873	8·763	0·994	2035·515	2031·729	0·996
DC8	1999	8·948	0·995	3207·036	2373·265	0·993
DC9	1692	8·452	0·989	1811·929	1828·708	0·996
DC11	1778	8·700	0·993	1882·046	1899·458	0·997
DC15	1826	8·661	0·992	1918·130	1925·591	0·997
DC17	1729	8·295	0·990	1898·375	1910·953	0·996
DC20	1918	8·855	0·994	2045·401	2053·585	0·996
DC23	1713	8·414	0·988	1827·895	1824·986	0·997
DC24	1656	8·216	0·987	1791·264	1795·118	0·996
DC25	1831	8·935	0·995	1960·242	1944·414	0·997
DC28	1887	8·952	0·995	2027·000	2008·004	0·996

(Continued)

Table 1 (*Continued*)

Sample	Observed_species	Shannon	Simpson	Chao1	ACE	Goods_coverage
DC29	1557	8·048	0·989	1668·467	1674·144	0·997
DC30	1788	8·417	0·990	1896·005	1906·001	0·997
DC31	1854	8·710	0·992	1963·000	1974·194	0·996
DC34	1676	8·236	0·988	1812·573	1800·127	0·996
DC36	1585	8·368	0·990	1706·731	1707·016	0·997
DC41	1773	8·245	0·988	1921·479	1922·046	0·996
DC45	1803	8·494	0·989	1949·250	1934·658	0·996
DY1	1856	8·715	0·993	1935·377	1952·197	0·997
DY3	1926	9·032	0·995	2054·211	2053·668	0·996
DY4	1897	8·907	0·994	2026·814	2030·692	0·996
DY6	1853	8·789	0·994	1955·511	1975·373	0·996
DY8	1924	9·043	0·995	2036·718	2040·398	0·997
DY11	1891	9·170	0·996	2002·132	1998·883	0·997
DY22	2052	8·998	0·994	2177·845	2177·968	0·996
DY23	1926	8·818	0·994	2084·049	2082·460	0·996
DY32	1942	8·865	0·994	2116·527	2100·596	0·996
DY37	1945	8·984	0·995	2072·467	2079·782	0·996
DY43	1911	8·924	0·995	2040·868	2042·800	0·996
DY44	1773	8·669	0·992	1865·205	1890·189	0·997
DY45	1842	8·775	0·994	1951·120	1970·065	0·996
DY46	1816	8·641	0·992	1979·125	1982·641	0·996
DY59	1899	9·036	0·995	2006·622	2022·777	0·996
DY65	1783	8·839	0·994	1902·691	1903·654	0·997
DY70	1824	8·782	0·994	1947·142	1956·814	0·996
DY72	1789	8·574	0·992	1900·500	1910·883	0·996
DY74	1717	8·665	0·993	1816·569	1805·623	0·997
DY75	1794	8·374	0·987	1927·526	1932·330	0·996

The alpha diversity indices (including Shannon, Simpson, Chao1, ACE, Goods_coverage) are shown in Table 1 (cut-off = 62 431). The Goods coverage index was above 99%, indicating a high level of diversity was found in the samples. The Shannon, Chao1 and ACE indices in the wild group were higher than in the captive group ($P_{Shannon} = 0{\cdot}01627 < 0{\cdot}05$, $P_{Chao1} = 0{\cdot}000381 < 0{\cdot}01$, $P_{ACE} = 0{\cdot}000838 < 0{\cdot}01$), but the Goods coverage index in the wild group was significantly lower than that in the captive group ($P = 0{\cdot}009368 < 0{\cdot}01$).

The PCA plot (Fig. 3a) and the PCoA plots (Fig. 3b) showed that the wild and the captive group formed two distinct areas on the graph. The similarity of the community structure was higher and the composition was more similar. The similarity between the two groups was obviously smaller than within the samples. In the PCA plot, the wild the captive groups were obviously separated, meaning that the similarity between the groups was small.

We also used a nonmetric multidimensional scaling (NMDS) plot to analyse discrepancies between the groups. Weighted and nonweighted methods were used for NMDS analysis, resulting in stress values of 0·088 and 0·102, respectively, which are both <0·2 indicating that NMDS can accurately differentiate the samples. NMDS is a nonlinear model, whether it is weighted analysis or nonweighted analysis, and the wild and captive groups were clearly separated. For individuals, different groups of individuals will also be clustered into the corresponding group, indicating that the difference between the two groups was quite remarkable (Fig. 3c). MRPP testing between the wild and captive groups was $A = 0{\cdot}1136 > 0$. The difference between the groups was greater than the difference within the groups, indicating that the study groups were reasonable. The significance of $0{\cdot}001 < 0{\cdot}01$, showed that the wild group and the captive group were significantly different.

The Metastat method was used to test the microbial species abundance data for wild and captive faecal samples. According to the q value at the phylum level and genus there was a significant difference between the species ($P < 0{\cdot}01$), and a plot of the difference between the species can be seen in the abundance distribution box map (Figs 4 and 5).

Discussion

In the analysis of alpha diversity, the Shannon, Chao1 and ACE indexes of the wild group were larger than

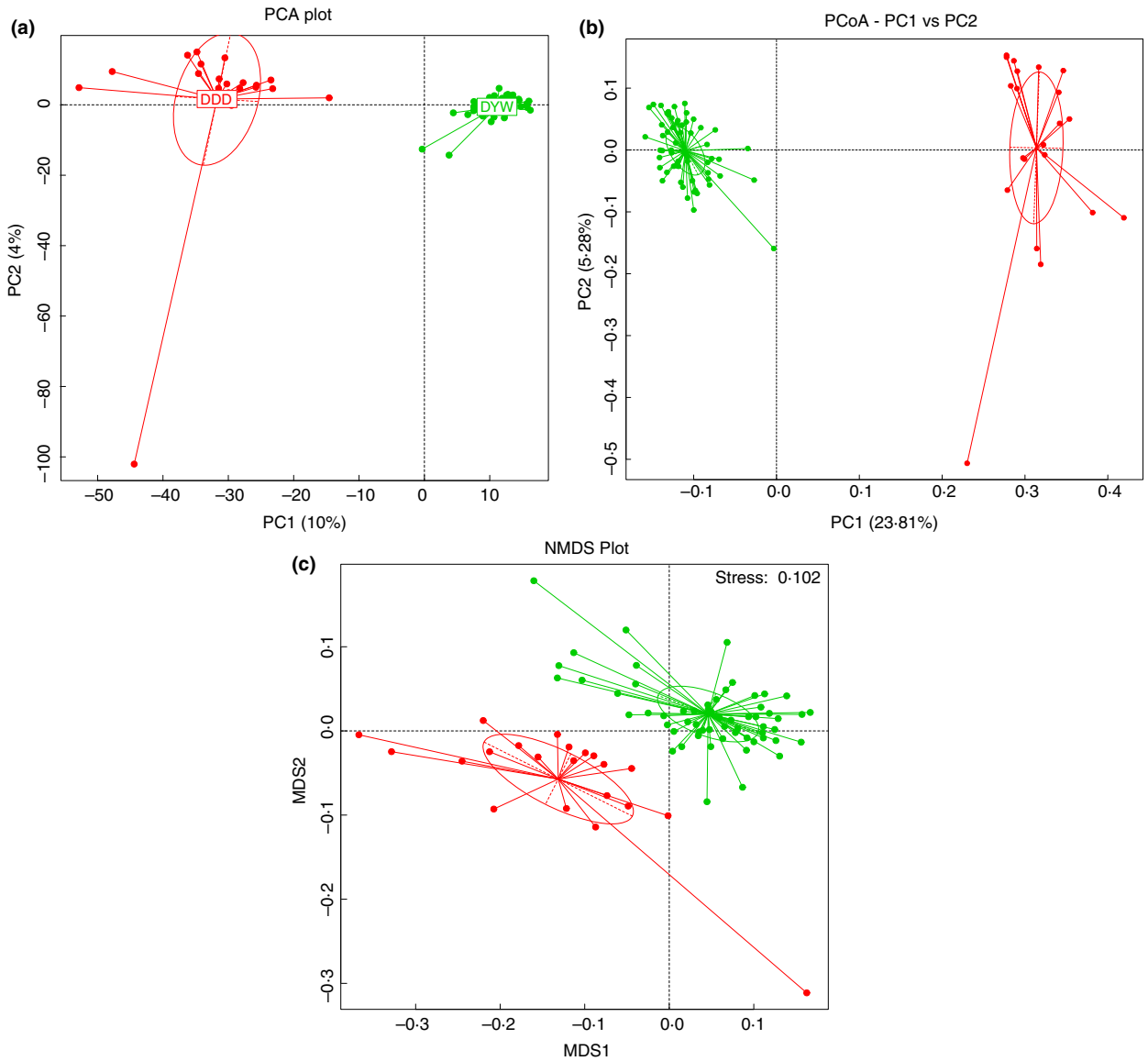

Figure 3 The principal component analysis (PCA) of the gut microbiota of Tibetan wild asses in wild and captive groups (a). Principal coordinate analysis (PCoA) of the gut microbiota of Tibetan wild asses in wild and captive groups (b). NMDS analysis of gut microbiota of Tibetan wild asses from wild and captive collections (c) (● DDD; ● DYW). [Colour figure can be viewed at wileyonlinelibrary.com]

those of the captive group, which suggests that the bacterial diversity of gut microbes in the wild Tibetan wild ass population is significantly higher than for those individuals in captivity. Although the intestinal microbial diversity of the wild Tibetan wild ass was higher, fewer microbes were identified, and the exploration of wild animal intestinal flora has a broader prospect.

The Bacteroides and Firmicutes phyla made up more than 80% of the total bacterial content. This is consistent with previous studies of intestinal microbial diversity in mammals (Eckburg *et al.* 2005; Mariat *et al.* 2009; Middelbos *et al.* 2010; Qin *et al.* 2010; Van den Abbeele *et al.* 2010; Zhu *et al.* 2011; Guan *et al.* 2016) and these organisms facilitate the digestion of cellulose and hemicellulose in food (Wu *et al.* 2016). However, the numbers of bacteria from these two phyla were significantly different in the different host groups ($P < 0{\cdot}01$). Bacteroidetes was the dominant phylum in the wild group, while Firmicutes was the dominant phylum in the captive group.

In winter, captive Tibetan wild asses are fed semi-dry oat grass (fiber content 353·1 g kg^{-1}), feed (protein 17·5%, fat 2%) and carrots (proportional to 8 : 2 : 1), and

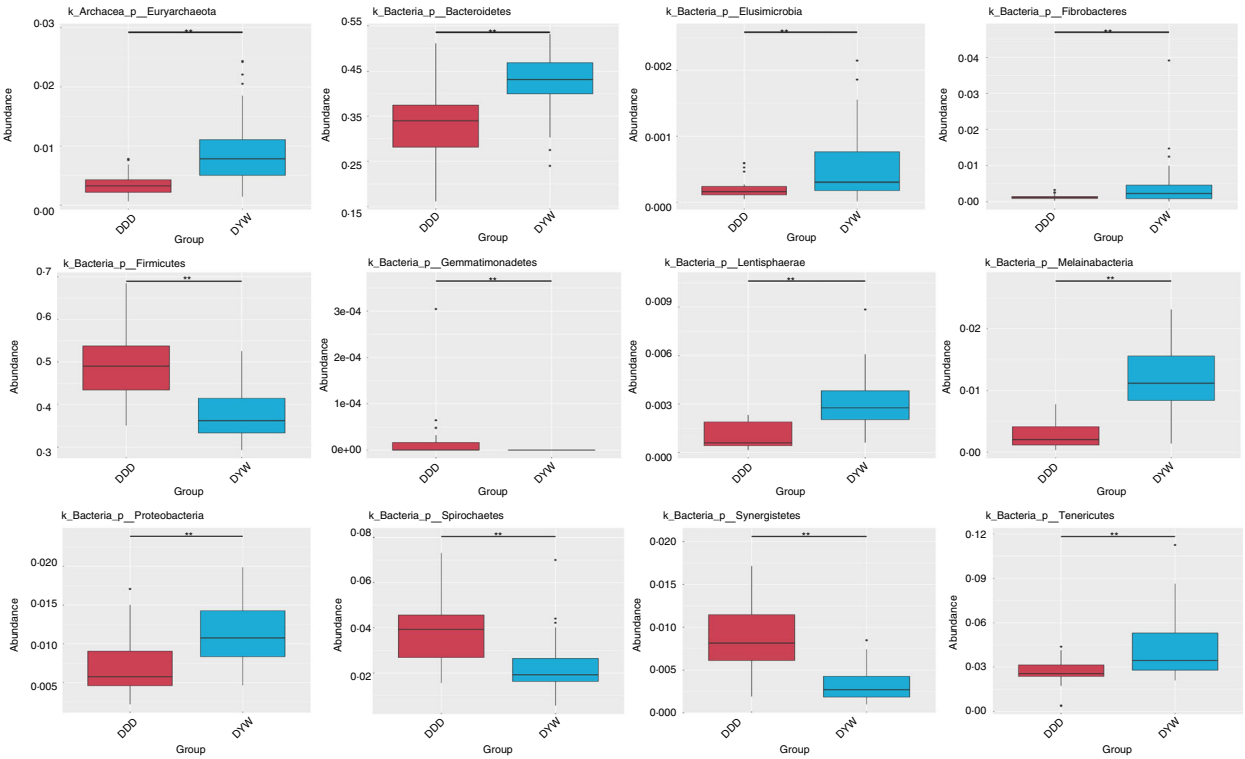

Figure 4 Box diagram of species differences between wild and captive Tibetan wild asses at the phylum level. [Colour figure can be viewed at wileyonlinelibrary.com]

more fat and protein may reduce microbial diversity and lead to an increase in the number of Firmicutes and Actinobacteria (Zhang *et al.* 2012; He *et al.* 2013; Cani 2018). Thus the diversity of the gut microbiota was significantly lower in the captive group than in the wild group, with higher numbers of Actinobacteria and Firmicutes (Middelbos *et al.* 2010), and lower numbers of Bacteroidetes. The wild Tibetan wild asses feed mostly on Gramineae, Leguminosae and Cyperaceae plants, including *pedicularis*, *Stipa purpurea*, *Brylkinia caudate*, *Poa annua*, *Carex myosuroides* and *Potentilla chinensis* (Yin *et al.* 2007; Dong *et al.* 2015). In the wild, due to food shortage, protein and fat intake decreased, and the Bacteroidetes content increased to help host to increase their nutrition.

A disruption of the symbiosis between the microbiota and host is known as dysbiosis and is described in multiple chronic diseases, such as obesity and malnutrition (Castaner *et al.* 2018; Zhang *et al.* 2018; Jeong *et al.* 2019), neurological disorders (Kurokawa *et al.* 2018; Quagliariello *et al.* 2018; Sun and Shen 2018), inflammatory bowel disease (IBD) (Costa *et al.* 2012; Roche-Lima *et al.* 2018), metabolic syndrome (Zhao *et al.* 2018), cancer and other diseases (Katsimichas *et al.* 2018; Lu *et al.* 2018; Panebianco *et al.* 2018; Pulikkan *et al.* 2018; Zitvogel *et al.* 2018). We presume that the health of the wild group of Tibetan wild asses was better than the captive group. On the one hand, in the case of captivity, the feeding density is high and there is long-term contact with human beings, with a higher probability of zoonosis among animals in captivity, and generally poorer health than animals in the wild. On the other hand, the intestinal microbial composition and content of the captive group was greatly altered, which can present as qualitative changes, such as increased proportions of harmful bacteria and reduced levels of beneficial bacteria. The captive Tibetan wild asses had more Spirochaetes, Proteobacteria and Campylobacter; groups of bacteria that contain pathogens (Ludwig *et al.* 2010), Proteobacteria is closely related to IBD and *Clostridium difficile* infection. *Campylobacter* is the most frequent cause of foodborne disease. At same time, the captive group samples had a lower content of Bacteroidetes, the basal microbiota, which is one of the richest phyla in a healthy human body and its levels can be a predictor of an animal's health.

In summary, there were significant differences in gut microbial composition and structure between wild and captive Tibetan wild asses. We believe that food, bacterial content and animal health are connected and changes in the numbers of different bacteria play an important role for the host.

With the intake of large amounts of industrial food, the intestinal microbial diversity of captive Tibetan wild asses decreased, increasing the risk of disease. Other methods of

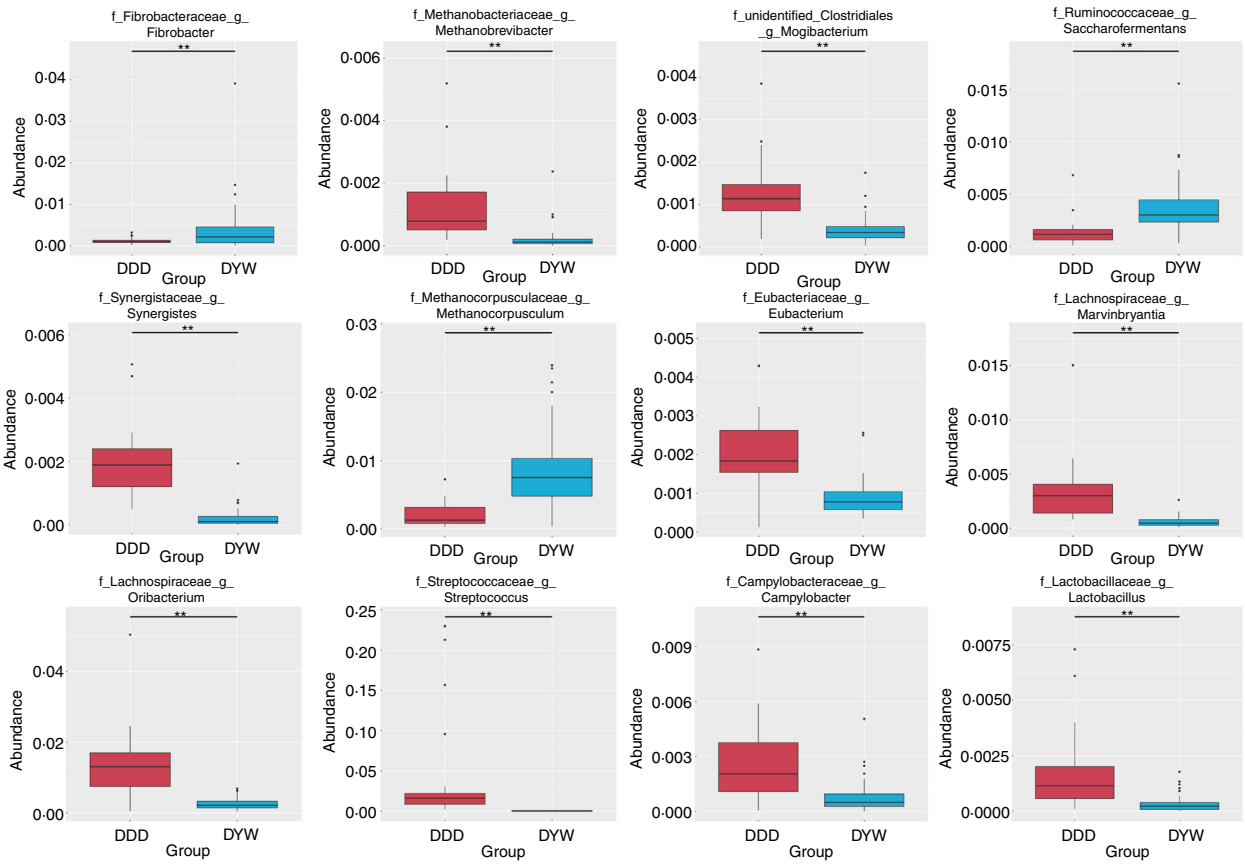

Figure 5 Box diagram of species differences between wild and captive Tibetan wild asses at the genus level. [Colour figure can be viewed at wileyonlinelibrary.com]

feeding that better approximate nature should be chosen to protect rare and endangered wildlife in a captive environment. The gut microbiota of the Tibetan wild ass is complex and this study of its composition and function is of great significance to the protection of the Tibetan wild ass. In addition, it is important to conduct more research to understand how environmental differences directly affect the diversity of bacteria in stool samples.

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Statement on the welfare of animals

All procedures performed in studies involving animals were approved by the Ethics and Welfare of Experiment Animals Committee affiliated to Northwest Institute of Plateau Biology.

Conflict of Interest

The authors declare that they have no conflict of interest.

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