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# Environment and host species identity shape gut microbiota diversity in sympatric herbivorous mammals

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## Summary

**The previous studies have reported that the mammalian gut microbiota is a physiological consequence; nonetheless, the factors influencing its composition and function remain unclear. In this study, to evaluate the contributions of the host and environment to the gut microbiota, we conducted a sequencing analysis of 16S rDNA and shotgun metagenomic DNA from plateau pikas and yaks, two sympatric herbivorous mammals, and further compared the sequences in summer and winter. The results revealed that both pikas and yaks harboured considerably more distinct communities between summer and winter. We detected the over-representation of Verrucomicrobia and Proteobacteria in pikas, and Archaea and Bacteroidetes in yaks. Firmicutes and Actinobacteria, associated with energy-efficient acquisition, significantly enriched in winter. The diversity of the microbial community was determined by the interactive effects between the host and season. Metagenomic analysis revealed that methane-metabolism-related pathway of yaks was significantly enriched in summer, while some pathogenic pathways were more abundant in pikas. Both pikas and yaks had a higher capacity for lipid degradation in winter. Pika and yak shared more OTUs when food shortage occurred in winter, and this caused a convergence in gut microbial composition and function. From winter to summer, the network module number increased from one to five in pikas, which was different in yaks. Our study demonstrates that the host is a dominant factor in shaping the microbial communities and that seasonality promotes divergence or convergence based on dietary quality across host species identity.**

## Introduction

The profile of mammalian gut microbial communities often displays extensive changes owing to numerous factors including host species and the external environment (Eichmiller *et al*., 2016; Yang *et al*., 2016; Amato *et al*., 2018; Kohl *et al*., 2018; Perofsky *et al*., 2018). The most prevalent factors are host species identity (Kropackova *et al*., 2017; Amato *et al*., 2018), the environment (Maurice *et al*., 2015; Ren *et al*., 2017) and behaviour (Moeller *et al*., 2016; Zhang *et al*., 2018). Some studies have shown that the influence of host species identity on the structure and function is much stronger than that of the dietary niche in primates (Amato *et al*., 2018; Perofsky *et al*., 2018), and the cladogram of microbial community similarity is determined by host species identity in the American pika (Galbreath *et al*., 2009). The giant pandas harboured a more similar gut microbial community with their carnivorous relatives (e.g. bears), though they have evolved an herbivorous adaption (Wei *et al*., 2015; Huang *et al*., 2020). Baleen whales host a gut microbiome with similarities to their terrestrial relatives (e.g. hippopotamuses and cows), though they lived in the ocean (Sanders *et al*., 2015). Nevertheless, UniFrac distances have revealed significant clustering in specialized myrmecophagous mammals, which include armadillos, anteaters, aardvarks, pangolins and aardwolves, even though they belong to

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phylogenetically distant lineages representing different orders (Delsuc *et al*., 2014). The bat and birds evolved a similar gut microbiome to adapt the behaviour of flying (Song *et al*., 2020). This finding confirmed that dietary and behavioural adaptation is a major driving factor of convergence in gut microbiota composition over host species identity (Delsuc *et al*., 2014). Furthermore, a comparison of the gut microbiomes of common, silver and bighead carps from wild and laboratory environments revealed that captive populations of the species harbour more similar gut microbiomes than wild populations, suggesting that the environment is a dominant factor structuring the gut microbiome of invasive carps living in artificial habitats (Eichmiller *et al*., 2016). However, these studies have often focused on multiple species within a season (Muegge *et al*., 2011), or one species in different environments (Rothschild *et al*., 2018). Therefore, limited information is available regarding the gut microbiota of multiple species living in sympatry across different seasons, and the coaction between host species identity and season or other environmental factors.

The yak (*Bos mutus*), which is an Artiodactyla ruminant, is the most typical herbivorous species on the Qinghai–Tibetan Plateau (QTP). The species arose in north-eastern Eurasia (northern China, Inner Mongolia, eastern Siberia and northern mid-Asia) 2.5 million years ago (Ma). They possess excellent tolerance to cold and hypoxic conditions (Krishnan *et al*., 2018; Liu *et al*., 2019). The yak was domesticated approximately 6000–12 000 years ago, and it was a mainstay of Tibetan pastoral societies, with more than 14 million domestic yaks grazing freely on the QTP (Guo *et al*., 2006; Qiu *et al*., 2015). The plateau pika (*Ochotona curzoniae*) is an indigenous member of Lagomorpha on the QTP that also prefers cold and hypoxic environments (Liu *et al*., 2012; Qu *et al*., 2013; Solari and Hadly, 2018) and has been widely distributed in the QTP since 3.4 Ma (Yu *et al*., 2000; Dahal *et al*., 2017). The two species have coexisted for approximately 2.4 million years, since the divergence between lagomorphs and artiodactyls approximately 115 million years ago (Cooper and Fortey, 1998; Archibald, 2003). The traditional view is that they are competitors owing to their consumption of similar plants and their competition for space (Liu *et al*., 2012; Zhang *et al*., 2014; Harris *et al*., 2015). However, these traditional concepts contradict the current factual data, because during the last few decades, with the rapid growth of the yak population, the density of plateau pika markedly increased (Dong *et al*., 2013; Qu *et al*., 2016; Qu *et al.,* 2011). Moreover, overgrazing altered the plant community structure by increasing the population of toxic plants (Lai and Smith, 2003; Smith *et al.,* 2019), these changes further contributed to the population expansion of plateau pika, because the plateau pika, as a small mammal, is well tolerated to the toxic plants (Kohl *et al*., 2014; Li *et al*., 2019). Furthermore, toxic plants increase the gut microbial diversity in small mammals and expand the scope of dietary niche, which may be beneficial to the survival of pika (Kohl *et al*., 2014; Li *et al*., 2019). Thus, this strange phenomenon may imply that they are not just competitors, but some mutually beneficial interactions may have occurred.

In this study, we employed a $2 \times 2$ experimental design to quantify the effects of host species identity and environmental factors on the gut microbiota of sympatric herbivorous mammals on the Tibetan Plateau and investigate the gut microbial diversity of sympatric populations of plateau pikas and yaks in winter and summer. We also aimed to determine the factors related to the genetic background and the environment that shaped the gut microbiota. We performed a cross-factorial comparison based on 16S rDNA sequencing and shotgun sequencing of the metagenome of the gut microbiota in faecal samples. We aimed to test whether host species identity or the seasonality dominates the determination of gut microbiota composition and function.

## Results

### *Gut microbial composition of plateau pikas and yaks*

We collected 124 faecal samples from plateau pikas and yaks (Table S1) and sequenced their 16S rDNA to characterize the gut microbiota from 2015 to 2018. In total, we obtained 8 487 518 sequences. We evaluated the contributions of the host and seasonality to the gut microbiota and found that plateau pikas and yaks could be differentiated in both summer and winter based on weighted UniFrac and unweighted UniFrac distances (Fig. 1A and B) (unweighted UniFrac metric, PERMANOVA, permutations = 999, season: $F_{1,123} = 6.3$, $R^2 = 0.03$, $P = 0.001$; species: $F_{1,123} = 69.7$, $R^2 = 0.35$, $P = 0.001$; season × species: $F_{1,123} = 69.7$, $R^2 = 0.02$, $P = 0.003$; and weighted UniFrac metric, permutations = 999, PERMANOVA, season: $F_{1,123} = 7.8$, $R^2 = 0.03$, $P = 0.001$; species: $F_{1,123} = 95.4$, $R^2 = 0.41$, $P = 0.001$; season × species: $F_{1,123} = 69.7$, $R^2 = 0.03$, $P = 0.001$). We found that season explained 3.1% and 3.4% of bacterial variance based on the unweighted UniFrac metric and weighted UniFrac metric, respectively, while species explained 34.8% and 41.3% of bacterial variance based on the unweighted UniFrac metric and weighted UniFrac metric respectively (Fig. 1A and B). We measured the effects of species, season and interannual variation and displayed the results using mean pairwise Bray–Curtis dissimilarities to explore the factors shaping the gut microbiota. The distances between the two species were significantly greater than those within

 

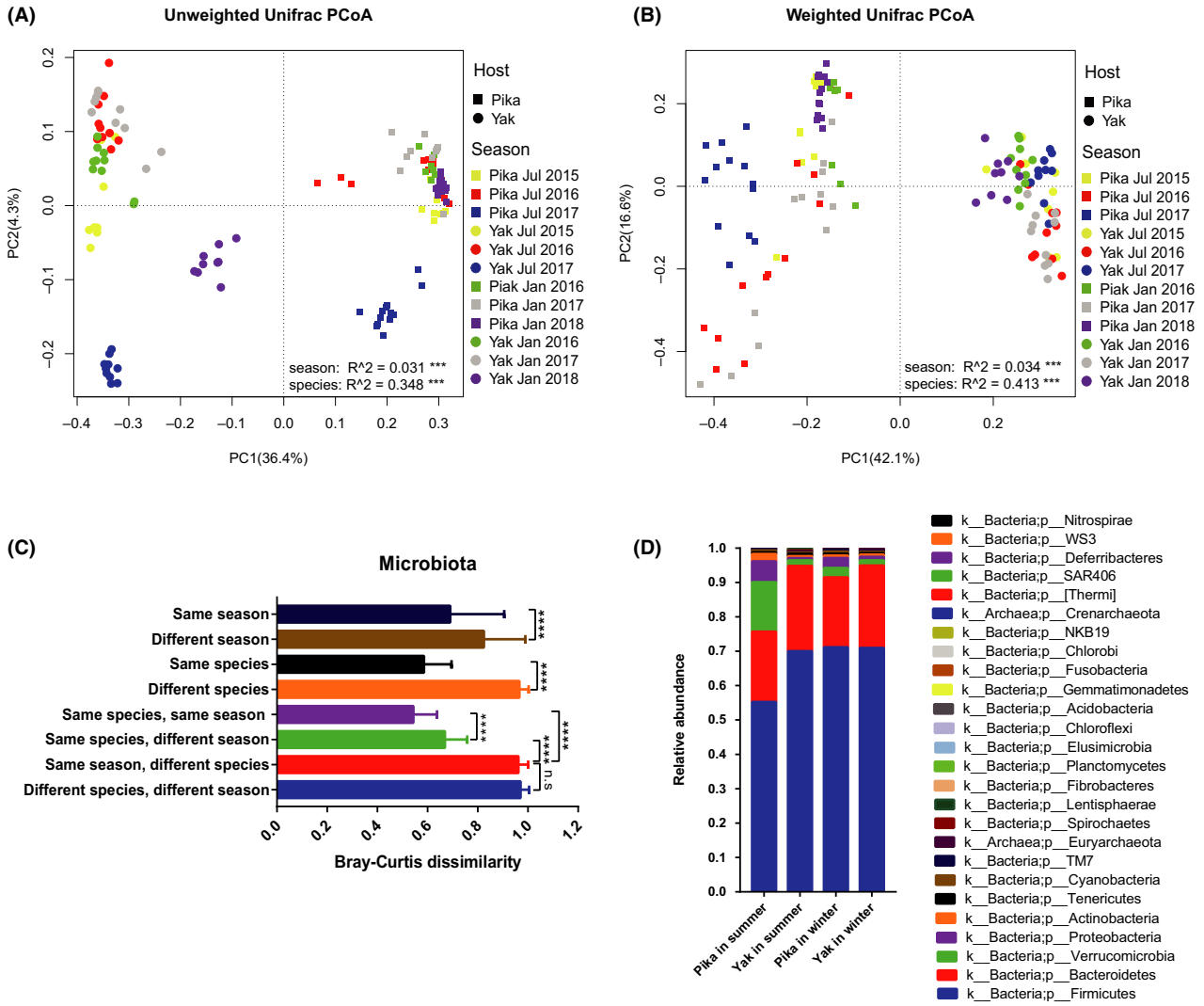


**Fig. 1.** The structure of the gut microbiota within plateau pikas and yaks between seasons.
A. PCoA based on unweighted UniFrac distances.
B. PCoA based on weighted UniFrac distances.
C. Bray–Curtis dissimilarities between host phylogenies and seasons.
D. Average relative abundance of gut microbiota at the phylum level.

the same species (Mann–Whitney *U*-test, $Z = 15.3$, $P < 0.0001$) (Fig. 1C), and the communities from the same season were more similar than those from different seasons (Mann–Whitney *U*-test, $Z = 6.3$, $P < 0.0001$).

At the phylum level, the gut microbiota of plateau pikas and yaks was dominated by Firmicutes, Bacteroidetes, Verrucomicrobia and Proteobacteria (Fig. 1D; Fig. S1A). Firmicutes, the most abundant phylum (Fig. 1D; Table S2), significantly enriched in January than in July in plateau pikas (Mann–Whitney *U*-test, $Z = 4.7$, *FDR* $P < 0.0001$) (Fig. S1B), and also significantly enriched in yaks than in plateau pikas in July (Mann–Whitney *U*-test, $Z = -5.0$, *FDR* $P < 0.0001$) (Fig. S1B). Bacteroidetes, as the second most abundant phylum (Fig. 1D; Table S2), displayed no significant difference between July and January in both plateau pikas and yaks (plateau pika: Mann–Whitney *U*-test, $Z = -0.2$, *FDR* $P = 0.851$; yak: Mann–Whitney *U*-test, $Z = -0.2$, *FDR* $P = 0.811$) (Fig. S1C), but a significantly higher abundance was found in yaks than in plateau pikas in both July and January (July: Mann–Whitney *U*-test, $Z = -2.6$, *FDR* $P < 0.01$; January: Mann–Whitney *U*-test, $Z = -2.6$, *FDR* $P < 0.05$) (Fig. S1C). A significantly greater abundance of Verrucomicrobia was found in July compared with that in January in plateau pikas (Mann–Whitney *U*-test, $Z = -3.5$, *FDR* $P < 0.001$) (Fig. S1D), and also, it was significantly enriched in plateau pikas compared with that in yaks both in July and in January

 

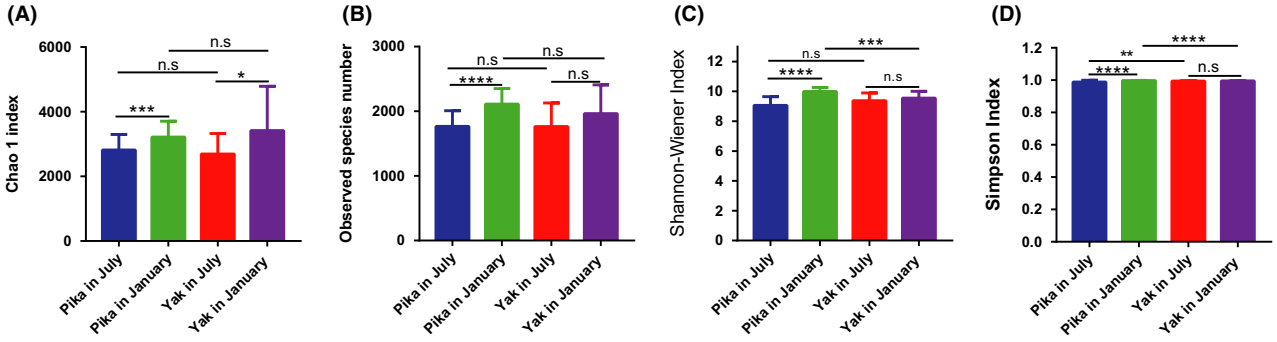


**Fig. 2.** Alpha diversity of the gut microbiota across all samples. Asterisks show significant differences between groups; significance is listed in Table S4.
A. Chao1 index.
B. Observed species number.
C. Shannon–Wiener index.
D. Comparison of the Simpson index among groups.

(July: Mann–Whitney *U*-test, $Z = 4.7$, *FDR* $P < 0.0001$; July: Mann–Whitney *U*-test, $Z = 2.8$, *FDR* $P = 0.05$) (Fig. S1D). We further identified the variations among different groups at the genus level (Fig. S1E; Table S3), and found that the relative abundance of *Akkermansia* was significantly greater in July than in January in plateau pikas (Mann–Whitney *U*-test, $Z = -3.5$, $P < 0.001$) (Fig. S1E and F; Table S3).

We found that the alpha diversity showed significant differences between seasons and different host species identity (Fig. 2A–D; Table S4). Interestingly, the differences in Chao1 index and the observed species number among different groups were similar, while the response of the Shannon–Weiner and Simpson index to seasons and host species identity were similar. We are not sure these similar responses among different diversity indexes are stochastic or associated with their own definition. Because the Chao1 index and the observed species number were often used to measure the richness, the Shannon–Weiner index and Simpson index were used to measure the community heterogeneity. To clarify how the host species identity and season affected microbial alpha diversity, we conducted a general linear mixed-model analysis. Our results showed that season was the dominant variables explaining the variation in the Chao1 index and the observed species number (Tables 1 and 2). However, the complex multiplicative effect, as well as the additive effect, was the most important variables explaining the variation in the Shannon–Weiner and Simpson index (Tables 1 and 2). Our results showed that season and host species played different roles on different alpha indexes of the same gut microbial communities.

The weighted UniFrac distances, which mirror the similarity between gut microbiota communities in terms of the overlap between phylogenetic trees, suggested that the microbiota communities between the two species were significantly more similar in January than in July (Mann–Whitney *U*-test, $Z = 5.5$, $P < 0.0001$). Distances between July and January for plateau pikas were significantly greater than those for yaks (Mann–Whitney *U*-test, $Z = 6.4$, $P < 0.0001$) (Fig. 3A–C).

**Table 1.** Results of the general linear mixed-effect model analysis examining the effects of host (plateau pika/yak) and season (July/January) on the diversity of the gut microbiome, including the Chao1 index, Shannon–Wiener index, observed species number and Simpson index.

| Alpha diversity indices | Model | Adjusted $R^2$ | d.f. | *F*-value | *P*-value | *N* | AICc | $\Delta_i$AICc | $\omega_i$AICc |
|---|---|---|---|---|---|---|---|---|---|
| Chao1 index | Season | 0.097 | 1,122 | 14.250 | < 0.001 | 5 | 2020.40 | 0.00 | 0.65 |
| | Host + Season | 0.090 | 2,121 | 7.098 | < 0.001 | 6 | 2022.55 | 2.15 | 0.22 |
| | Host × Season + Host + Season | 0.092 | 3,120 | 5.149 | 0.002 | 7 | 2023.54 | 3.14 | 0.13 |
| Shannon–Wiener index | Host × Season + Host + Season | 0.342 | 3,120 | 22.340 | < 0.001 | 7 | 177.90 | 0.00 | 1.00 |
| | Host + Season | 0.242 | 2,121 | 20.600 | < 0.001 | 6 | 194.34 | 16.44 | 0.00 |
| Observed species number | Season | 0.144 | 1,122 | 21.630 | < 0.001 | 5 | 1798.34 | 0.00 | 0.44 |
| | Host + Season | 0.148 | 2,121 | 11.680 | < 0.001 | 6 | 1798.90 | 0.56 | 0.33 |
| | Host × Season + Host + Season | 0.152 | 3,120 | 8.328 | < 0.001 | 7 | 1799.58 | 1.24 | 0.23 |
| Simpson index | Host × Season + Host + Season | 0.231 | 3,120 | 13.320 | < 0.001 | 7 | -912.61 | 0.00 | 1.00 |
| | Host + Season | 0.144 | 2,122 | 11.380 | < 0.001 | 6 | -900.58 | 12.03 | 0.00 |

Selected models are ranked according to the corrected Akaike information criterion weight ($\omega_i$AICc). $\omega_i$AICc indicates the relative likelihood that model i is the best model for the observed data among a given set of models. $\Delta_i$AICc is the difference between the AICc from a considered model i and that for the model with the lowest AICc value (best model).

 

**Table 2.** Parameter estimation based on the minimum adequate model (selected based on the AIC) for the effects of host and season on community structure, including the Chao1 index, Shannon–Wiener index, observed species number and Simpson index, in the 124 samples from the Tibetan Plateau.

| Fixed effects | Estimate | SE | d.f. | *t* Value | *P*-value |
|---|---|---|---|---|---|
| Chao1 index | | | | | |
| Intercept | 3311.560 | 103.352 | 110 | 32.042 | < 0.001 |
| Season | −547.344 | 144.997 | 110 | −3.775 | < 0.001 |
| Shannon–Wiener index | | | | | |
| Intercept | 9.994 | 0.084 | 108 | 119.150 | < 0.001 |
| Host | −0.443 | 0.122 | 108 | −3.643 | < 0.001 |
| Season | −0.934 | 0.116 | 108 | −8.048 | < 0.001 |
| Host × Season | 0.757 | 0.171 | 108 | 4.418 | < 0.001 |
| Observed species number | | | | | |
| Intercept | 2041.261 | 42.215 | 110 | 48.354 | < 0.001 |
| Season | −275.413 | 59.225 | 110 | −4.650 | < 0.001 |
| Simpson index | | | | | |
| Intercept | 0.998 | 0.001 | 108 | 966.108 | 0.021 |
| Host | −0.003 | 0.001 | 108 | −1.674 | 0.097 |
| Season | −0.009 | 0.001 | 108 | −6.092 | < 0.001 |
| Host × Season | 0.008 | 0.002 | 108 | 3.827 | 0.002 |

The model is a mixed-effect model where the dependent variable is explained by the host (plateau pika and yak), season (July and January) and their interaction.

Consistent with the results of weighted UniFrac distances, plateau pikas possessed less unique OTUs in January (985, 6.79%) than in July (2812, 21.28%) (Fig. 3D and E; Fig. S3).

The LEfSe results showed that the plateau pikas and yaks harboured 78 significantly different taxa (Fig. 4A, LDA score > 3, $P < 0.05$). However, the seasonality created only 14 significantly different taxa (Fig. 4B, LDA score > 3, $P < 0.05$). Verrucomicrobia, Proteobacteria and Actinobacteria were markedly more abundant in the plateau pika gut microbiota, and Archaea and Bacteroidetes were the most abundant taxa in the yak gut microbiota (Fig. 4A). Verrucomicrobia and Corynebacteria (Actinobacteria) were significantly enriched in July, and Firmicutes were significantly enriched in January (Fig. 4B).

### *Functional variation in microbial communities caused by the host and seasonality*

The metagenomic sequencing data were used to examine the gut microbiota functional similarities among hosts and seasons by PCoA of the Bray–Curtis dissimilarities and the relative abundances of gene enrichment in KEGG orthology (KO) pathways. The results suggested that the gut microbial function between plateau pikas and yaks showed significant differences both in summer and in winter (Fig. 5A) (PERMANOVA, permutations = 999, Bray–Curtis metric: season: $F_{1,8} = 3.6$, $R^2 = 0.191$, $P = 0.002$; species: $F_{1,8} = 5.3$, $R^2 = 0.280$, $P = 0.001$; season × species, $F_{1,8} = 2.1$, $R^2 = 0.109$, $P = 0.068$). For the variation of gut microbial functions, season explained 19.1%, while host species explained 28.0% (Fig. 5A). The Bray–Curtis dissimilarities showed that the distances between plateau pikas and yaks in summer were significantly greater than those in winter (Fig. 5B) (Mann–Whitney *U*-test, $Z = 3.6$, $P = 0.0003$). This result indicated that the microbiota of plateau pikas was more similar to that of yaks in winter than in summer. We performed Welch *t*-tests for KEGG pathways and found that Vibrio cholerae *pathogenic cycle* (ko05111), *amoebiasis* (ko05146) and *lipopolysaccharide biosynthesis* (ko00540) were more abundant in plateau pikas than in yaks (Fig. S4A; Table S5). For yaks, *methane metabolism* (ko00680) was significantly enriched in the summer (Fig. S4A; Table S5). In winter, *adipocytokine signalling pathway* (ko04920) and *fatty acid degradation* (ko00071) were significantly upregulated in both plateau pikas and yaks (Fig. S4C and D; Table S5). Conversely, *adipocytokine signalling pathway* (ko04920), *fatty acid degradation* (ko00071), *amyotrophic lateral sclerosis* (ALS) and *synthesis and degradation of ketone bodies* were more abundant in yaks than in plateau pikas in winter (Fig. S4B; Table S5).

### *Phylogenetic molecular ecological network analysis*

We examined networks of microbial communities using pMENA, which is a powerful tool for comparing the species interactions (links) between different microbial taxa (nodes) (Zhou *et al.*, 2011). For plateau pikas in July, 76 nodes and 642 links were assigned to five modules, which was the largest module number among the four groups (Pika in July and January respectively; Yak in July and January respectively) (Fig. S5A–D; Table S6). However, 46 nodes and 512 links formed only one module in yaks in July (Fig. S5B; Table S6). Both plateau pikas and yaks in January formed only one module (Fig. S5C and D). Moreover, the parameter of modularity (fast_greedy) was the same (0.310) in January for plateau pikas and yaks, while it was 0.750 and 0.380 for plateau pikas and yaks in July respectively (Table S6). Thus, the networks were more similar between plateau pikas and yaks in January than in July in terms of modularity properties. Not only the modularity properties but also other network properties displayed more similarities between plateau pikas and yaks in January than in July, such as the total nodes, average path distance (GD), geodesic efficiency (E), harmonic geodesic distance (HD), centralization of degree (CD), centralization of betweenness (CB), centralization of stress centrality (CS), centralization of eigenvector centrality (CE), density (D), transitivity (Trans), connectedness (Con) and efficiency (Table S6).

 

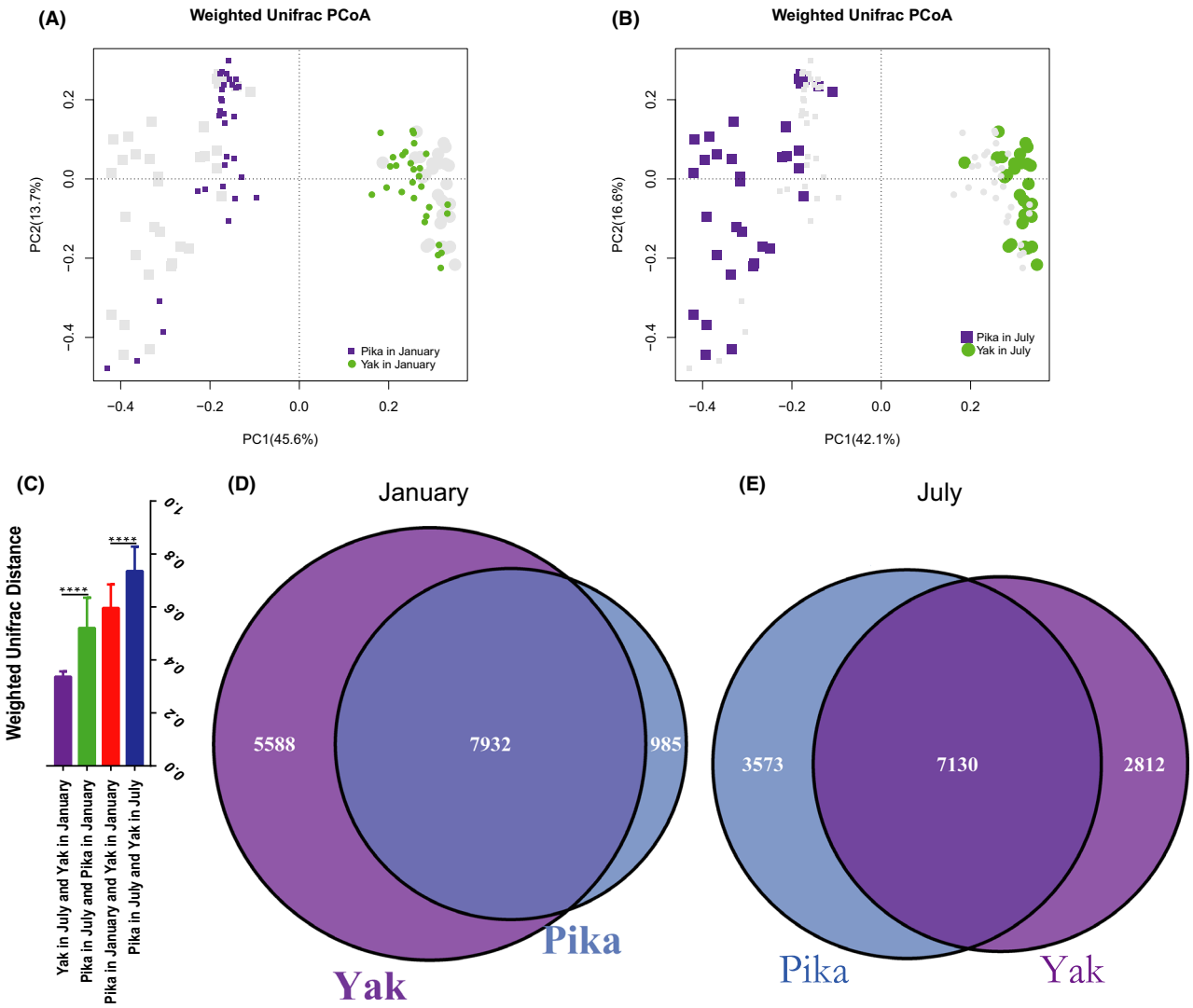


**Fig. 3.** The response of the gut microbiota to season.
A. PCoA of weighted UniFrac distances in January (winter).
B. PCoA of weighted UniFrac distances in July (summer).
C. Comparison of microbial community similarities among groups.
D. Shared OTU number for plateau pikas and yaks in January.
E. Shared OTU number for plateau pikas and yaks in July.

## Discussion

In previous studies, host species identity has been reported as the most important factor shaping the gut microbiota (Knowles *et al.*, 2019), while in other studies, environmental factors, such as diet and season, have been reported as the dominant factors (Delsuc *et al.*, 2014; Eichmiller *et al.*, 2016; Smits *et al.*, 2017). However, these studies could not discriminate the effects of the environment from those of host species identity (Delsuc *et al.*, 2014; Eichmiller *et al.*, 2016; Knowles *et al.*, 2019). Some studies have focused on a single species in different environments (Rothschild *et al.*, 2018) or multiple species in varied habitats (Muegge *et al.*, 2011; Knowles *et al.*, 2019) to elucidate the respective influences of the environment and host on the gut microbiota. Nevertheless, the above-mentioned studies were conducted in a single season. Although they employed a scheme with multiple species in varied environments, host preferences for specific dietary items and contents may have weakened the environmental effects because the animals living in different environments may have foraged for similar diets or contents by selecting similar diet items (Delsuc *et al.*, 2014; Knowles *et al.*, 2019). This would lead to a greater similarity between the gut microbiota of species living in different environments,

 


**Fig. 4.** LEfSe identification of several microbial families with significant differences ($LDA > 3$, $P < 0.05$).
A. Differences between plateau pikas and yaks.
B. Differences between July and January.

mistakenly attributing the similarity to host species identity, and mask environmental effects (Mikaelyan *et al.*, 2015). However, foraging differences caused by the seasonality are ubiquitous for animals living in the field. Thus, assessing the environmental influence of the seasonality may be more credible than assessing different environments in the same season. Thus, we explored the influence of sympatric hosts and different seasons.

Recently, some studies have investigated the relative importance of host diet and host species identity in shaping the gut microbiome diversity using multivariate analyses of gut microbiomes from sympatric mammals and a robust analytical framework. The studies have revealed that microbial community assembly in the mammalian gut is regulated by multiple factors, including host genetics, diet and social behaviour (Mikaelyan *et al.*, 2015; Kim *et al.*, 2018; Knowles *et al.*, 2019; Lutz *et al.*, 2019; Song *et al.*, 2020). Our study explored the variation in the gut microbiota within sympatric, unrelated hosts between two seasons using a $2 \times 2$ factorial analysis. The results in this study indicated that yaks and plateau pikas could be distinguished from each other in both summer and winter based on unweighted and weighted UniFrac distances, which suggested that strong effect of host species identity was the dominant factor shaping the gut microbiota (Fig. 1A and B). However, the bacterial communities varied with season, especially in plateau pikas, which displayed a greater seasonality in microbial composition than yaks (Fig. 1A and B). Furthermore, the dominant taxa, such as Firmicutes, Bacteroidetes, Verrucomicrobia, Ruminococcaceae and *Akkermansia*, exhibited a significant difference between July and January for plateau pikas and yaks (Fig. 1D; Fig. S1A–F). Seasonality potently changed the vegetation form and nutrition, the height and coverage were significantly higher in July than in January, and the crude

 

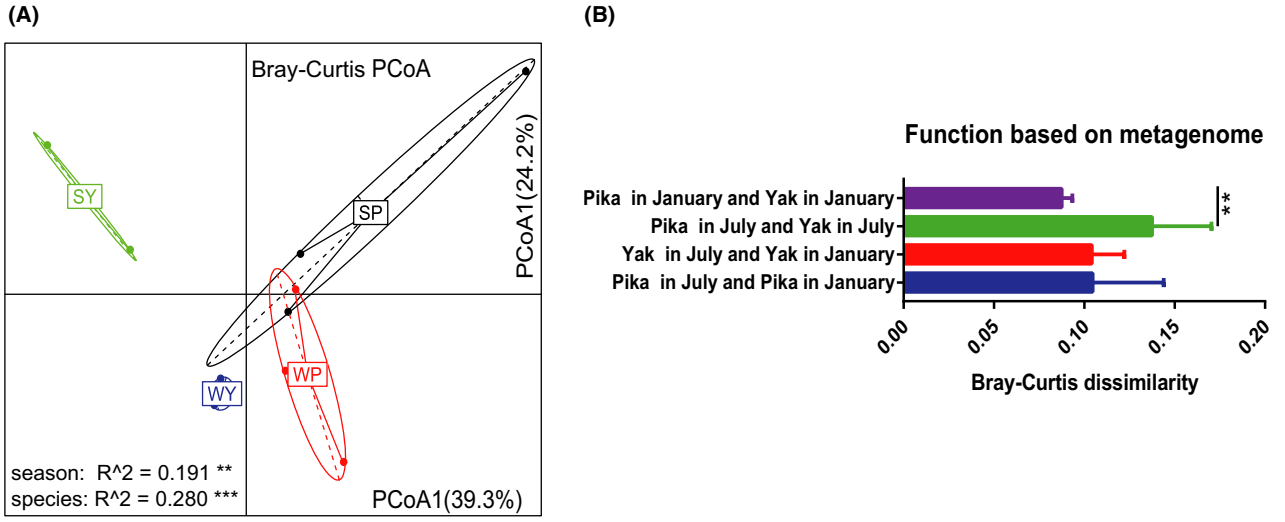


**Fig. 5.** Functions based on shotgun metagenomic sequencing. SP and SY represent plateau pikas and yaks in July respectively; WP and WY represent plateau pikas and yaks in January respectively.
A. Bray–Curtis dissimilarity PCoA based on the relative abundances of KO pathways.
B. Bray–Curtis dissimilarities of KO pathways showing that the distance in January is significantly shorter than that in July. KO, KEGG orthology.

protein, crude fat and total sugars were significantly higher in July than in January (Table S7). Conversely, the crude fibre and polysaccharides were significantly higher in January than in July (Table S7). These results implied that the accessibility and nutrition value of the diet were significantly higher in July than in January, and indigestible substances such as cellulose may be more abundant in January (Table S7). These differences may further influence the host gut microbiota, potentially resulting in the growth of more cellulose-degrading bacteria. This result was consistent with that from a previous report in which host species identity and environmental factors influenced the gut microbiome (Youngblut *et al.*, 2019).

Youngblut *et al.* (2019) also suggested that host species identity and diet modulate different aspects of gut microbiome diversity, showing a stronger cophylogenetic signal for microbial taxa associated with host species identity or diet. Environmental filtering and microbe–microbe interactions also differ among host clades.

We found that both host species identity and season modulated gut microbiome diversity and showed that they regulated different aspects of diversity (Fig. 2A–D; Table S4; Table 1). These results corresponded to those of a study across multiple species, in which each factor modulates a different aspect of diversity and host species identity is moderately correlated with diet (Youngblut *et al.*, 2019). The Chao1 index and observed species number were used to determine the species richness, rather than the species abundance and evenness, and they are sensitive to rare taxa (Chao, 1984). Seasonality causes a shift in the diet content, providing different niches for different microbial species. Thus, seasonality may yield greater effects on the richness of the Chao1 index and observed species number in GLM analysis (Tables 1 and 2). However, the Shannon–Wiener index and Simpson index are used to assess community heterogeneity, and they depend on not only the species number, but also abundance and evenness (Shannon, 1948; Simpson, 1949). The host species and season displayed a multiplicative effect on the Shannon–Wiener index and Simpson index (Tables 1 and 2). These results indicate that the host species and season affect not only the microbial species number but also the abundance and evenness.

There is a broad debate on the shaping factor of host gut microbial community (Spor *et al.*, 2011), because the gut microbes are always in dynamic development (Sonnenburg *et al.*, 2016; Nishida and Ochman, 2018), this makes it difficult to access the relative influencing factors on the gut microbial community (Perofsky *et al.*, 2018). The gut microbes form a complex ecosystem depending on the internal environment of the host, and interact actively with their host (Perofsky *et al.*, 2018). Thus, it automatically suggests the ecologists to assume that the host species is the dominant factor shaping the gut microbiota (Amato *et al.*, 2018; Nishida and Ochman, 2018). However, this hypothesis often fails to explain the convergence of the gut microbial communities when the host species share diet or habitat, though the host species are distant in evolutionary phylogeny (Groussin *et al.*, 2017; Perofsky *et al.*, 2018; Lutz *et al.*, 2019). Thus, it is difficult to assess the influencing factors on the gut microbial community. The results of our study

 

indicated that the host played a dominant role in shaping the bacterial communities of plateau pikas and yaks, although we still found trends of convergence in January and divergence in July in the community structure (Fig. 3A–C). This phenomenon also appeared in the composition of OTUs; plateau pikas and yaks shared more OTUs in January than in July, and the influence of the season tended to overcome the influence of host species identity (Fig. S3; Fig. 3D and E). In summer, plateau pikas prefer to eat forbs with plant secondary metabolites (PSMs) (Jiang and Xia, 1985; Wang *et al*., 1992), and yaks prefer *Gramineae* and *Cyperus rotundus* plants (Dong *et al*., 2006). Dietary niche separation may lead to divergence in the composition and structure of gut microbial communities (Li *et al*., 2018). However, the diets of the two animals become more deficient and uniform in winter, when both plateau pikas and yaks face similar harsh environments and share withered aboveground parts of plants (Liu *et al*., 2012; Li *et al*., 2018). This may have led to the observed convergence in the composition and structure of their gut microbial communities.

The responses of some bacterial taxa to host or season were different (Fig. 4A and B). *Akkermansia* is a vital probiotic that degrades intestinal mucosal protein and resists the stimulation of harmful substances by advancing the immunity of the host (Everard *et al*., 2013). It is also associated with a slim and healthy body (Barton *et al*., 2017). Plateau pikas forage for plants rich in PSMs in July (choline, tannins and terpenoids), such as *Taraxacum sp., Plantago asiatica, Potentilla nivea* and *Oxytropis sp*. (Lai and Smith, 2003; Dai *et al*., 2012, 2014). Thus, higher abundance of *Akkermansia* may improve adaptability to the toxic compounds by advancing the immunity and ability to withstand toxic compounds in the host in July (Fig. 4A and B). Methanogens (Archaea) can improve cellulose digestibility and energy utilization by decreasing the accumulation of $H_2$ and $CO_2$, which causes the interruption of fermentation (Williams *et al*., 1994). The enrichment of methanogens (Archaea) in yaks was beneficial for the efficient utilization of diet resources (Fig. 4A). In addition, a higher abundance of Firmicutes is closely associated with efficient energy harvest and degradation of cellulose (Chevalier *et al*., 2015). The Firmicutes enriched in both plateau pikas and yaks may help the hosts harvest more energy from the diet to resist the cold temperatures in winter (Fig. 4B). The host preferences of bacteria were associated with their characteristics, and the seasonal preferences of bacteria were linked to the content of the diet.

The function of organisms often depends on their structure, and similar structures yield similar functions (Hildebrandt *et al*., 2009). Strong effects of seasonality on the functions of the gut microbiota have been reported in wild animals (Maurice *et al*., 2015). In our study, a significant difference between plateau pikas and yaks suggested that the function of the gut microbiota was closely associated with the host species identity and characteristics of the host (Fig. 5A and B). However, seasonal gut microbial community divergence in July and convergence in January (Fig. 5A and B) suggested that the function of gut bacterial communities was shaped by the host and season. In summer, some pathogenic and induced inflammatory response pathways were more abundant in plateau pikas than in yaks, such as Vibrio cholerae *pathogenic cycle* (ko05111), *amoebiasis* (ko05146) and *lipopolysaccharide biosynthesis* (ko00540) (Fig. S4A). This may be associated with the previous reports that plateau pikas had extremely high mortality caused by density dependence and higher infection rate of intestinal parasites in summer (Wang, 2003; Pech *et al*., 2007; Qu *et al*., 2013). These results were also consistent with the higher abundance of probiotics in *Akkermansia* (Fig. 4b), which advance the immunity of the host (Everard *et al*., 2013). For yaks in summer, *methane metabolism* was significantly enriched (ko00680) (Fig. S4A; Table S5). This may have enhanced the persistence of rumen fermentation and corresponded to the higher abundance of methanogens in the rumen (Williams *et al*., 1994) (Fig. 4A). Furthermore, flavonoids are widely contained in fresh plants (Yang *et al*., 2008), and *flavonoid-related metabolism* (ko00941 and ko00944) showed significant differences between plateau pika and yak in summer, and no significant difference in winter (Fig. S4A and B). This also implied that seasonal dietary feature might induce a difference in gut microbial function. In winter, *adipocytokine signalling pathway* (ko04920) and *fatty acid degradation* (ko00071) were significantly upregulated in both plateau pikas and yaks (Fig. S4C and D; Table S5), suggesting that the functions of the gut microbiota converged when the hosts were confronted with the same challenge of diet shortage (Wang *et al*., 2006b). Moreover, *lysine biosynthesis* (ko00300), which can accelerate fat degradation (Arslan, 2006), was more abundant in plateau pikas than in yaks (Fig. S4B; Table S5). Conversely, *adipocytokine signalling pathway* (ko04920), *fatty acid degradation* (ko00071), *ALS* and *synthesis and degradation of ketone bodies* were more abundant in yaks than in plateau pikas in winter (Fig. S4B; Table S5). These results suggested that the adaptive strategies of plateau pikas and yaks were markedly different. The plateau pika, with a relatively stable body mass throughout the year, accumulates brown adipose tissues in summer and primarily relies on enhancing non-shivering thermogenesis rather than decreasing body weight and white fat deposits to withstand cold in winter (Wang *et al*., 2006a,

 

b). Conversely, yaks convert the abundant energy and nutrition from their diets into white adipose tissues, exhibiting marked weight gain in summer and resisting the cold in winter by burning the white adipose tissues (Long *et al*., 2005; Xue *et al*., 2005). Thus, discrepancies in life-history characteristics and response patterns between hosts primarily drove the divergence and convergence in gut microbial function. Our results were consistent with those of previous studies, which reported that gut microbiota function is closely associated with the lifestyle of the host and diet content (Perofsky *et al*., 2018; Trosvik *et al*., 2018). Overall, the microbial function tended to form respective features with the dietary niche expenditure in summer, and this mechanism converged with a limited dietary niche in winter. Furthermore, these regulatory mechanisms could beneficially contribute towards the coexistence of two sympatric herbivorous mammals.

High network complexity often implies the occurrence of extensive mutualistic interactions among bacteria (Shi *et al*., 2016). Multiple mechanisms may be responsible for increasing network complexity. Bacterial abundance varied with interactions among the members or in response to environmental factors. Previous studies have shown that dietary availability is an important driver of network structure (Henzi *et al*., 2009; Foster *et al*., 2012). Additionally, PSMs significantly increase the diversity of the gut microbiota and enhance network complexity (Kohl *et al*., 2014; Li *et al*., 2019). The greater network complexity of plateau pikas than that of yaks in July may have been associated with the preferences of plateau pikas for toxic plants (Fig. S5A; Table S6), such as *Taraxacum sp., Plantago asiatica, Potentilla nivea* and *Oxytropis sp*., and the fact that they harboured gut microbes that degrade PSMs (Dai *et al*., 2012, 2014). A similar pattern also occurred in voles (*Lasiopodomys brandtii*); they developed more diverse communities and complex networks to resist the toxicity of *Cleistogenes squarrosa* (Li *et al*., 2019), because more complex networks may provide more flexible metabolic networks, allowing adaptation to a diverse diet, which improves the fitness of the host (Li *et al*., 2019). In winter, the diets of plateau pikas and yaks became more deficient and uniform, which may have driven the bacterial network simplification and convergence observed for these animals (Fig. S5C and D; Table S6).

In summary, our results demonstrated that both host species identity and environment combine to shape the composition and function of gut microbiota in the herbivorous mammals. However, the patterns of host and seasonal influence are different. The gut microbiomes of mammals appear to mirror their host species identity, and overlapping or separated dietary niches can drive convergence or divergence in compositions and functions of the gut microbiota across mammalian host species identity. The effects of host and season were not completely isolated, as they often exhibited complex and varied interactions under specific conditions. Our study provides deep insights into the factors affecting the gut microbial diversity, and the sampling scheme might be useful in future studies. However, our study is still limited as far as showing a more in-depth understanding of the determinants of the gut microbiota. Thus, future research should explore how gut microbes respond to diet niche changes, and how the response pattern influences the population dynamics.

## Experimental procedures

### *Sample collection*

The faecal samples from plateau pikas and yaks were collected from 2015 to 2018 in Reshui village, Gangcha County, Qinghai Province (Table S1; altitude: 3650 m, N: 37°9′39″, E: 100°28′40″), where the average annual temperature was 2°C (Zhao *et al*., 2012). We followed behind the yaks, and the faecal samples were immediately picked when the animals defecated. The faeces in the core of excrement were picked to avoid contamination. Only regular excretion was collected for the study. Plateau pikas were trapped using a live-trapping method (Wang *et al*., 2006b) and then locked in cages (cages had been sterilized using 75% alcohol). Each trapped pika remained in single cage with number. The faeces were collected within 1 min of when they defecated. The collected samples were placed in liquid nitrogen in the field and then stored at −80°C freezer upon return to the laboratory in Xining. There are only two typical seasons (warm in July and cold in January) on the QTP (Dong *et al*., 2013), and we collected samples in these 2 months (Table S1). A total of 124 fresh samples were collected (Table S1). All captured plateau pikas were transported to Xining for further breeding studies in the laboratory. Moreover, we randomly selected 10 samples of $1.0 \times 1.0$ $m^2$ in July 2015 and January 2016 respectively. We surveyed the height and coverage of vegetation in each quadrat and then harvested the aboveground biomass for nutrient determinations. We measured crude protein and crude fibre using the Kjeldahl determination and Weende analysis (Casal *et al.,* 2000; Pepkowitz and Shive, 1942; Gidenne and Thierry, 2014). Moreover, crude fat and polysaccharide were determined by the Soxhlet extractor method and phenol–sulfuric acid method respectively (Sheng *et al*., 2010; Jones *et al*., 2017). The monosaccharide was determined using Fehling's agent (International Organization for Standardization). The total sugar content is equal to the sum of monosaccharide and polysaccharide. All of the results are listed in Table S7. This project was

 

approved by the Animal Ethics Committee of Northwest Plateau Institute of Biology, Chinese Academy of Sciences (nwipb2015110801).

### *DNA extraction and sequencing*

We extracted microbial DNA using the TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, California, USA). The V3 and V4 regions of 16S rDNA were amplified using the primers 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′) (Marcus *et al*., 2010; Claudia and Qunfeng, 2012). The quantification and purification of polymerase chain reaction (PCR) products were performed using a QuantiFluorTM fluorometer (Promega Biotech, Madison, WI, USA). Negative controls included template controls, which were not used for DNA extraction and PCR amplification. PCR products were mixed in ratios of equal densities. Then, the purification of mixed PCR products was conducted using a Qiagen Gel Extraction Kit (Qiagen, Dusseldorf, North Rhine-Westphalia, Germany). Subsequently, sequencing libraries were produced using the TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, CA, USA) according to the manufacturer's recommendations, and index codes were added. The assessment of library quality was carried out on the QUBIT 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and Agilent Bioanalyzer 2100 system (Agilent Technologies, Palo Alto, CA, USA). Finally, the library was sequenced on an Illumina HiSeq 2500 platform (Illumina, San Diego, CA, USA) with a strategy of 250 bp paired-end reads.

Total genomic DNA was extracted from 12 faecal samples (three for each species in July and January) using a QIAamp DNA Stool Mini Kit (Qiagen, Dusseldorf, Germany) according to the manufacturer's instructions. The DNA concentration was measured using a NanoDrop ND-1000 (Thermo Scientific, Waltham, MA, USA).

### *Shotgun metagenomic sequencing*

The qualifying DNA samples were randomly interrupted using a Covaris ultrasonic crusher with a length of approximately 150 bp. We prepared the libraries following the steps of end repair, added 3'A tail, ligate adapters and purification. Then, the assessment of library quality was conducted using the Qubit 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 System. Next, the qualifying libraries were sequenced on an Illumina HiSeq platform with a method of paired-end reads (Tringe and Rubin, 2005; Law *et al*., 2013). Finally, the sequencing produced an average of 540 million metagenome jointed reads (12 Gb) per sample.

### *16S rDNA data analysis*

The reads were trimmed based on a cut-off *Q*-value $> 20$, missing bases $> 10\%$ and relative abundance $< 40\%$. The high-quality paired reads were merged into a single tag based on the overlapping region using FLASH v1.2.11, with a minimum match length of 10 bp and 2% mismatch allowed in the overlapping region. Filtering was performed according to the protocols provided by the QIIME pipeline (version 1.9.1) (Caporaso *et al*., 2010). After processing, we aligned the clean tags against the Gold database (r20110519) based on the UCHIME algorithm to identify and discard chimeras and obtain effective tags. The effective tags were searched in the Greengenes 13_8 reference database and clustered into operational taxonomic units (OTUs) based on a 97% identity based on the UCLUST algorithm (DeSantis *et al*., 2006). The representative OTUs were classified using PYNAST, and the taxonomy of OTUs was assigned using the UCLUST algorithm. After that, the mitochondrion, chloroplast and the OTUs that are unclassified at the Kingdom level were removed before downstream analysis. To balance the differences in sequencing depth among different samples, we conducted a normalization based on the minimum value of the sequence counts among all samples before the calculation of alpha and beta diversity, and the composition.

### *Metagenomic data analysis*

The raw data were processed to acquire clean data for subsequent analysis, producing metagenomic joined reads. The DIAMOND software (V0.9.9) was used to blast Unigenes to a functional database based on the parameter setting of blastp (Buchfink *et al*., 20155; Li *et al*., 2014). The functional database was the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa *et al*., 2006) database (Version 2018-01-01, http://www.kegg.jp/kegg/). For the blast result of each sequence, the best blast hit was used for subsequent analysis (Wang *et al*., 2007; Edgar, 2010; Karlsson *et al*., 2013). We analysed the relative abundances of different functional hierarchies in KEGG pathways, which equalled the sum of relative abundances annotated at each functional level. The samples were plotted according to principal coordinate analysis (PCoA) of the Bray–Curtis dissimilarities based on level-3 functional profiles using the R package 'ade4'. These dissimilarities were also used to determine the distances between groups by performing a Mann–Whitney *U*-test. Functional differences in microbial communities were detected and analysed mainly using STAMP v2.1.3 with White's non-parametric *t*-test.

 

### *Phylogenetic molecular ecological network analysis*

Phylogenetic molecular ecological network analysis (pMENA) was conducted using the online software Molecular Ecological Network Analysis Pipeline with random matrix theory (RMT)-based methods (http://ieg4.rccc.ou.edu/MENA/login.cgi) (Deng *et al*., 2012). In total, only the OTUs that were present in more than 50% of all samples in each group were used for network construction (groups including pikas in July and January, respectively, and yaks in July and January respectively; Fig. S5; Table S6). The parameters of the networks, such as co-occurrences (positive or negative), average degree (avgK), average clustering coefficient (avgCC), density (D), average path distance (GD), betweenness centrality (BC), and modularity, were calculated using the 'Global Network properties' and 'Module Separation and modularity calculation' on the website, which was following the methods of Deng *et al*. (2012). Finally, the results were visualized using CYTOSCAPE v3.3.0.

### *Statistical analyses*

Statistical analysis of the composition and diversity indices (including alpha and beta diversity) of gut microbial communities were calculated using the QIIME pipeline and visualized in GRAPHPAD PRISM v7.00 using a Mann–Whitney *U*-test. PCoA graphs and OTU Venn diagrams were plotted using R 3.2.2. Permutational analysis of variance (PERMANOVA) tests was run in R, using the function *Adonis2* (by = 'terms') implemented in *vegan* based on 999 permutations.

We conducted general linear mixed-model analysis to measure the effects of host species identity and seasonality on the diversity profile of the gut microbiota. We used the Akaike information criterion (AIC) corrected for the specimens to select the most parsimonious model (Burnham and Anderson, 2002), the year was considered as a random effect in GLMs. The tests were considered significant at $P < 0.05$, and the data were analysed using R 3.2.2. The linear discriminant analysis effect size (LEfSe) was obtained using Galaxy (LDA scores $> 3$, $P < 0.05$) (Segata *et al*., 2011) (http://huttenhower.sph.harvard.edu/galaxy/).

### *Data accessibility*

The 16S rDNA and the shotgun metagenomic data in this study can be freely retrieved from the NCBI Sequence Read Archive with Project Accession Nos. PRJNA590457 and PRJNA590585 respectively.

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**Conflict of interest**

The authors declare that they have no conflict of interest.

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## Supporting information

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

**Fig. S1.** The relative abundance of gut microbiota members across all samples. Significance differences were measured using a Mann-Whitney *U*-test. (A) The relative abundance of gut microbiota taxa at the phylum level, (B) Firmicutes with significant differences among groups, (C) Bacteroidetes with significant differences among groups, (D) Verrucomicrobia with significant differences among groups, (E) Genus level, (F) *Akkermansia* with significant differences among groups.

**Fig. S2.** Alpha diversity in three years, significance was tested with Mann-Whitney *U*-test. (A) Chao1 index and Shannon-Wiener index, (B) observed species number, (C) Shannon-Wiener index, (D) Simpson index.

**Fig. S3.** Venn diagrams for plateau pikas and yaks in July and January.

**Fig. S4.** Functions based on shotgun metagenomic sequencing. (A) KO pathways with a significant difference between plateau pikas and yaks in summer, (B) KO pathways with a significant difference between plateau pikas and yaks in winter, (C) KO pathways of yaks with a significant difference between July and January, (D) KO pathways of plateau pikas with a significant difference between July and January. KO, KEGG orthology.

**Fig. S5.** Networks built based on random matrix theory cooccurrence models using the 16S rDNA data. Nodes represent OTUs, and links between the nodes indicate interactions. Red and green lines represent positive and negative correlations respectively. Nodes with the top ten links are annotated with taxonomic names. (A) Network for plateau pikas in July, (B) network for yaks in July, (C) network for plateau pikas in January, (D) network for yaks in January.

**Table S1.** Sample information of 16s rDNA sequencing.

**Table S2.** The average relative abundance of gut microbiota members at the phylum level. The gut Firmicutes, Bacteroidetes, Verrucomicrobia and Proteobacteria are the most abundant taxon. The relative abundance of Firmicutes is the

 

most abundant in yaks, with percentages of 70.02% and 70.91% in July and January, respectively, whereas the percentages are only 55.22% and 71.12% in plateau pikas in July and January respectively. Bacteroidetes is the second most abundant phylum, with relative abundances of 20.47% (plateau pikas in July), 20.33% (plateau pikas in January), 24.75% (yaks in July) and 20.33% (yaks in January). Verrucomicrobia is the third abundant taxon, with relative abundances of 14.39% (plateau pikas in July), 2.74% (plateau pikas in January), 1.65% (yaks in July), and 1.51% (yaks in January).

**Table S3.** The average relative abundance of gut microbiota members at the genus level. The most abundant taxa are the unclassified Ruminococcaceae, with relative abundances of 14.69% (plateau pikas in July) and 19.51% (plateau pikas in January), with the relative abundances of 46.77% (yaks in July) and 43.85% (yaks in January). In plateau pikas, the relative abundances of *Akkermansia* are 14.36% and 2.72% in July and January respectively. In yaks, the relative abundances of *Akkermansia* is 1.05% and 1.34% in July and January respectively.

**Table S4.** Results of Mann-Whitney U-test comparing alpha diversity.

**Table S5.** KO pathways in level 3 based on the shotgun-sequenced metagenome. KO, KEGG orthology.

**Table S6.** Network indices for plateau pikas and yaks in July and January. SP and SY represent plateau pikas and yaks in July, respectively, and WP and WY represent plateau pikas and yaks in January respectively.

**Table S7.** Average temperature and precipitation of July and January in Gangcha County (Jia, 2012). Vegetation height, coverage, and nutrition profile presented as the mean ± SD in Reshui village, Gangcha County. The significance of each pair of vegetation data was tested with Welch's *t*-tests, standard deviation.

 