Temporal and spatial variations in body mass and thermogenic capacity associated with alterations in the gut microbiota and host transcriptome in mammalian herbivores

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HIGHLIGHTS

- Pika body mass increased with altitude.
- Pika thermogenesis increased and decreased with summer and winter altitude.
- Metabolites of amino acids were enriched in summer.
- *Prevotella*, *Bacteroides*, etc. may contribute to thermogenesis in pikas.

GRAPHICAL ABSTRACT
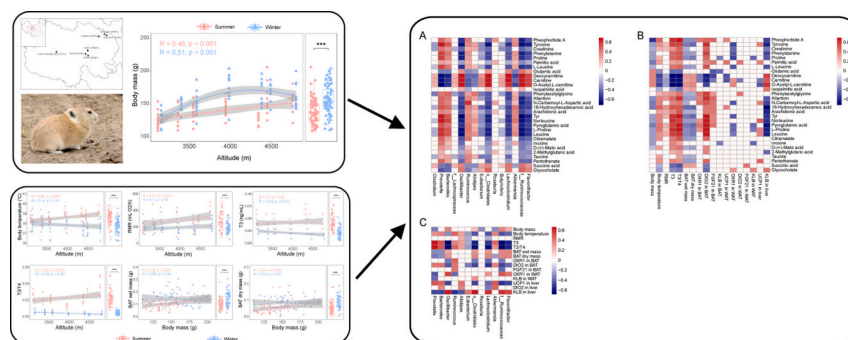
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ABSTRACT

Most wild animals follow Bergmann's rule and grow in body size as cold stress increases. However, the underlying thermogenic strategies and their relationship with the gut microbiota have not been comprehensively elucidated. Herein, we used the plateau pikas as a model to investigate body mass, thermogenic capacity, host transcriptome, gut microbiota and metabolites collected from seven sites ranging from 3100 to 4700 m on the Qinghai–Tibetan Plateau (QTP) in summer and winter to test the seasonal thermogenesis strategy in small herbivorous mammals. The results showed that the increase in pika body mass with altitude followed Bergmann's rule in summer and an inverted parabolic shape was observed in winter. However, physiological parameters and transcriptome profiles indicated that the thermogenic capacity of pikas increased with altitude in summer and decreased with altitude in winter. The abundance of Firmicutes declined, whereas that of

Bacteroidetes significantly increased with altitude in summer. Phenylalanine, tyrosine, and proline were enriched in summer, whereas carnitine and succinate were enriched in winter. Spearman's correlation analysis revealed significant positive correlations between *Prevotella*, *Bacteroides*, *Ruminococcus*, *Alistipes* and *Akkermansia* and metabolites of amino acids, pika physiological parameters, and transcriptome profiles. Moreover, metabolites of amino acids further showed significant positive correlations with pika physiological parameters and transcriptome profiles. Our study highlights that the changes in body mass and thermogenic capacity with altitude distinctly differentiate small herbivorous mammals between summer and winter on the QTP, and that the gut microbiota may regulate host thermogenesis through its metabolites.

1. Introduction

Bergmann's rule states that endothermic vertebrate species from relatively cool climates are larger than their conspecifics from relatively warm climates (Bergmann, 1848). Global surveys have discovered that >50 % of mammalian species, particularly herbivores, follow Bergmann's rule (Ashton et al., 2000; Meiri and Dayan, 2003). Studies have shown that the Swedish moose (*Alces alces*) (Sand et al., 1995), wild house mice (*Mus musculus*) (Suzuki et al., 2020), and plateau pikas (*Ochotona curzoniae*) (Lin et al., 2008) exhibit latitudinal or altitude body weight gain trends in relatively cold climates; this is thought to be adaptation to cold environments by reducing body heat loss (Blackburn et al., 1999). According to Bergmann's rule, heat produced in endothermic animals is related to volume, whereas heat loss is related to body surface area (Bergmann, 1848). Because relatively large animals have a low area-to-volume ratio on their body surface, resulting in relatively low heat loss, a relatively large body conserves energy in cold environments (Bergmann, 1848; Mayr, 1963). However, this perception has been questioned because large animals generate and dissipate more heat than smaller ones owing to their relatively large volume and body surface area, which may consume more energy (McNab, 1971). Therefore, explaining thermogenesis in animals with body weight changes that conform to Bergmann's rule is a hotspot in evolutionary ecology. However, previous studies have only focused on the geographical variation in population traits on spatial scales and rarely considered shifts in species life history strategies under the integration of temporal and spatial scales. Therefore, integrative studies are required to explore thermogenesis in herbivorous mammals on a spatiotemporal scale.

The diet of herbivorous mammals contains a high proportion of cellulose, hemicellulose, and polysaccharides, which are non-self-degradable and can only be converted into short-chain fatty acids (SCFAs) as a form of energy to the host by relying on carbohydrate enzymes produced by the gut microbiota (Flint et al., 2014). Furthermore, substantial evidence suggests that gut microbiota is critical for energy extraction and body weight maintenance of the host (Backhed et al., 2004; Fang et al., 2019; Li et al., 2015b). Recently, ecologists proposed that gut microbiota may contribute to Bergmann's rule as they found that the overall differences in the gut microbiota are associated with changes in the body weight of wild house mice at varying latitudes (Suzuki et al., 2020). Moreover, studies on human gut microbiota have shown that the proportions of Firmicutes and Bacteroidetes are positively and negatively correlated with latitude, respectively, in large-scale geography (Suzuki and Worobey, 2014). A high ratio of Firmicutes to Bacteroidetes contributes to energy harvesting from diets (Jumpertz et al., 2011) and is associated with increased body mass (Ley et al., 2006). Additionally, the gut microbiota significantly influences endothermic thermoregulation (Bo et al., 2019; Khakisahneh et al., 2021; Zhang et al., 2018). Transplantation of gut microbiota from cold-exposed mice to germ-free mice increases host insulin sensitivity and promotes the browning of white adipose tissue (WAT), thereby enhancing host thermogenesis (Chevalier et al., 2015). Conversely, gut microbiota depletion via antibiotic treatment impairs thermogenic capacity in mice (Li et al., 2019a). This suggests that gut microbiota contributes to host energy demands. Metabolites, small molecule products of metabolism, provide insight into the metabolic processes of the

organism (Holmes et al., 2008). Metabolites are the end products of cellular regulatory processes, and their levels can be considered as the ultimate reaction of biological systems to genetic or environmental fluctuations (Fiehn, 2002). Moreover, metabolites contribute more to the host phenotype than taxonomic composition (Xue et al., 2020). Therefore, multi-omics analysis is urgently needed to reveal the molecular mechanisms underlying host thermogenic strategies.

Plateau pikas (*Ochotona curzoniae*) are herbivorous small mammals living on the Qinghai–Tibetan Plateau (QTP) (Smith and Foggin, 1999). They are diurnal and non-hibernating, and interact with the surrounding environment through excavation activities (Arthur et al., 2008; Smith et al., 2019). Plateau pikas are widely distributed 3100–5200 m above sea level (ASL) in alpine meadows, and variations in their body size during summer follow Bergmann's rule (Lin et al., 2008). Wang found that the basal metabolic capacity of plateau pikas might be enhanced by increasing altitude in summer (Wang, 2006); however, Speakman et al. identified that pikas suppress energy demand in winter (Speakman et al., 2021). Elucidating the thermogenic capacity of plateau pikas at varying altitudes in different seasons will elucidate their life history strategies that change with climate. Hence, it is essential to explore the thermogenic strategies of pikas and their relationship with gut microbiota at large spatial and temporal scales.

In this study, we used plateau pika as an animal model and collected data on body mass, physiological parameters, host transcriptome, metagenomics, and metabolomics of cecum contents from seven altitude gradients in summer and winter. We aimed to investigate (i) whether the body mass variation of plateau pikas with altitude conforms to Bergmann's rule in summer and winter and whether the variation in physiological parameters with altitude is distinct between summer and winter; (ii) whether host transcriptome profiles are upregulated with altitude in summer and winter; (iii) whether the changes in gut microbial composition and function, and metabolites of pikas with altitude are consistent between summer and winter, and (iv) whether changes in the gut microbiota and metabolites are related to the host thermogenic capacity. This study provides insight into the thermogenic strategies of herbivorous mammals in adapting to cold stress and the contribution of the gut microbiota and metabolites to host thermogenesis.

2. Materials and methods

2.1. Sample collection

In total, 158 wild adult plateau pikas were captured using rope-wrap traps from seven different altitudes in summer (July, $n = 84$) and winter (January, $n = 74$) on the QTP (Fig. 1A, B, and Table S1). The seven altitudes were Menyuan 1 (3118 m ASL), Menyuan 2 (3363 m ASL), Gangcha (3550 m ASL), Dulan 1 (3945 m ASL), Dulan 2 (4343 m ASL), Maduo (4450 m ASL), and Kunlun Mountain pass (4761 m ASL). Subsequently, body mass, body temperature (T_b), and resting metabolic rate (RMR) were measured, and each pika was euthanized and dissected on a sterile dissecting table. Afterward, we collected blood from the heart chambers to measure thyroid hormone levels. Additionally, the cecal contents, liver, and WAT were collected and cryopreserved in a portable liquid nitrogen tank. Subsequently, the samples were transported to the laboratory and stored at −80 °C. Afterwards, brown adipose tissue

(BAT) was collected from the largest interscapular region and weighed on an electronic balance (± 0.0001 g; FA2104, Liang Ping, China). Finally, the BAT samples were dried at 60 °C to a constant weight and reweighed. The animal procedures were approved by the Animal Care and Ethics Committee of the Northwest Institute of Plateau Biology, Chinese Academy of Sciences (Approval no: nwipb2019110801).

2.2. Measurement of body mass, T_b, and RMR

The body mass of the plateau pika and T_b were measured using a spring scale (± 2 g, PESO-40300/7, PESOLA, Switzerland) and a rectal thermometer (± 0.1 °C, TP677, MITIR, China), respectively.

RMR was determined through oxygen consumption readings at local temperatures using a Field Metabolic System (FMS, Sable Systems International, Las Vegas, NV, USA). Seven metabolic chambers (each containing a pika) were used for measurements, and one empty metabolic chamber was used as a reference. The airflow rate was 500–600 mL/min, and the FMS was run for 2 h after 0.5 h of acclimation. RMR was calculated by taking the average of at least three consecutive readings of oxygen consumption.

2.3. Serum thyroid hormone assay

We quantified serum triiodothyronine (T3) and thyroxine (T4) concentrations using liquid chromatography-tandem mass spectrometry (LC-MS/MS) as previously described (Kunisue et al., 2011). First, we added 200 μL methanol to each 100 μL serum sample, homogenized for 5 min, precipitated the protein at 4 °C for 1 h, and centrifuged at 12,000 $\times g$ for 5 min. Subsequently, the supernatant was discarded, and 200 μL of deionized water was added. After centrifugation, 5 μL of the sample was directly injected for analysis at a flow rate of 0.6 mL/min.

2.4. Gene expression analysis

Real-time quantitative polymerase chain reaction (RT-qPCR) was used to determine the expression levels of oxidation resistance 1 (OXR1), iodothyronine deiodinase 2 (DIO2), uncoupling protein 1 (UCP1), fibroblast growth factor 21 (FGF21), and beta-Klotho (KLB). Total RNA was extracted from BAT, WAT, and liver using TRIzol reagent, according to the manufacturer's instructions. The final reaction volume of 12 μL contained 6 μL of 2× SYBR Premix EX Tag II (Vazyme Biotech Co.,Ltd., Nanjing, Jiangsu, China), 4 μL diluted cDNA template, and 1 μL of forward primer and reverse primer. RT-qPCR was performed using a Tianlong Real-Time PCR System (Tianlong Technology Co., Ltd., Xi'an, China) under the following conditions: 95 °C for 30 s, 40 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. We used the $2^{-\Delta\Delta CT}$ method to calculate relative gene expression. Primer sequences used in this study are listed in Table S2.

2.5. DNA extraction and sequencing

Total DNA was extracted from the cecal contents of the pika using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). Subsequently, the DNA concentration was determined using a NanoDrop ND-1000 (Thermo Fisher, Waltham, MA, USA). Afterward, the extracted DNA was fragmented to a length of approximately 400 bp using Covaris M220 (Covaris, Woburn, MA, USA). The DNA library was constructed via end repair, 3′ A tail addition, adapter ligation, and purification. Then library quality was assessed using a Qubit 2.0 (Thermo Fisher, Waltham, MA, USA) and an Agilent Bioanalyzer 2100. Qualified libraries were pooled for paired-end sequencing on an Illumina HiSeq platform.

2.6. Metagenomic data processing

Raw data quality control was performed using fastp software (https://github.com/OpenGene/fastp) to trim the 3′- and 5′-ends of reads and cut low-quality reads (length < 50 bp, quality scores <20, or having N bases). Subsequently, the filtered reads were assembled de novo using Megahit v1.1.2 (Li et al., 2015a). Afterward, the assembled contigs (> 300 bp) were employed for open reading frames prediction using MetaGene v0.3.38 (Noguchi et al., 2006). Non-redundant contigs were identified with 95 % sequence identity and 90 % coverage using CD-HIT (Fu et al., 2012). The original sequences were then mapped to the predicted genes to determine their abundance using SOAPaligner v2.21 (Li et al., 2009). Finally, the contigs were annotated using DIAMOND (Buchfink et al., 2015) against the RefSeq database (Pruitt et al., 2007) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) (https://www.kegg.jp/) database was used to obtain taxonomy and
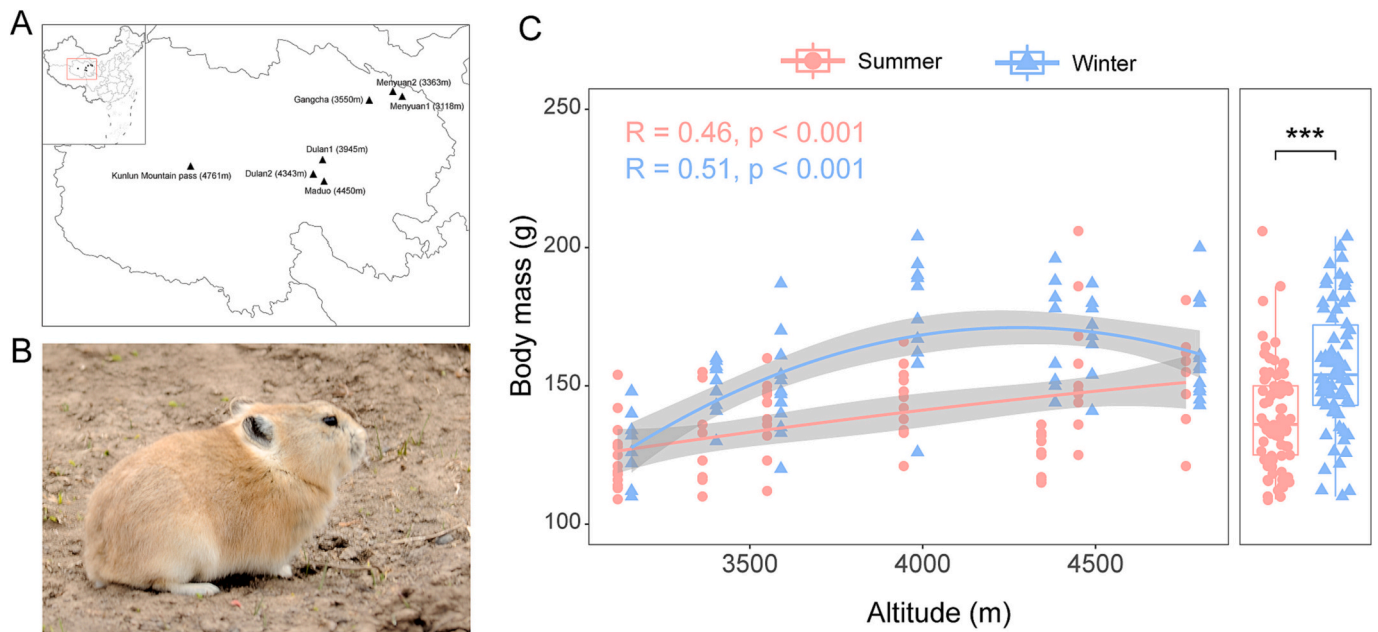

Fig. 1. Sampling sites and body mass variation of plateau pikas. (A) Sampling sites, (B) photo of plateau pikas, and (C) variation in pika body mass with altitude and its differences between summer and winter. *** p < 0.001.

potential functional profiles for downstream analysis.

2.7. Untargeted metabolomics study

A 100 mg sample was ground in liquid nitrogen and mixed with 200 μL pre-cooled water and 800 μL pre-cooled methanol/acetonitrile (1:1, *v*/v). Ultrasound was performed in an ice bath for 60 min, followed by incubation at $-20\,°C$ for 1 h to precipitate proteins and centrifugation at 16,000 *g* for 20 min at 4 °C. The supernatant was evaporated in a high-speed vacuum centrifuge. For mass spectrometry, 100 μL of acetonitrile-water solution (1:1, v/v) was added and centrifuged at 14,000 *g* for 15 min at 4 °C. The supernatant sample was taken for analysis.

Chromatographic analysis was performed on an Agilent 1290 Infinity LC ultra-high performance liquid chromatography system (UHPLC). The injection volume was 5 μL, column temperature was 25 °C, and flow rate was 0.3 mL/min. The chromatographic mobile phases were A: water +25 mM ammonium acetate +25 mM ammonia and B: acetonitrile. The chromatographic gradient elution procedure was as follows: 0–0.5 min, 95 % B; 0.5–7 min, 95 % to 65 % B; 7–9 min, 65 % to 40 % B; 9–10 min, 40 % B; 10–11.1 min, 40 % to 95 % B; 11.1–16 min, 95 % B.

Positive and negative ion modes were detected by electrospray ionization. The mass spectrometry analysis was performed with a Triple-TOF 5600 mass spectrometer (AB SCIEX). The raw data were converted to .mzXML format by ProteoWizard and then the XCMS program was used for peak alignment, retention time correction, and peak area extraction. For the XCMS extracted data, ion peaks with missing values >50 % within the group were removed, and positive and negative ion peaks were identified by the software SIMCA-P 14.1 (Umetrics, Umea, Sweden) respectively. Orthogonal partial least squares discriminant analysis (OPLS-DA) was performed after Pareto-scaling pre-processing of the data. Metabolites with variable importance for the projection (VIP) > 2 and p < 0.05 were considered as differential metabolites. The online platform MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/MetaboAnalyst/) was used to conduct the metabolic pathway enrichment analysis (Pang et al., 2021).

2.8. Statistical analysis

Statistical analyses were performed using SPSS v21.0 (IBM Corp., Armonk, NY, USA). Differences in body mass, T_b, T3, T3/T4 ratio, gene expression, and KEGG enzyme in summer and winter were compared using Student's *t*-test. Furthermore, RMR and BAT wet/dry mass for summer and winter were compared using ANCOVA with body mass as a covariate. The differences in alpha and beta diversities and the relative abundance of microbiota and KEGG categories between summer and winter were analyzed using the Mann–Whitney *U* test. Moreover, the relationship among body mass, T_b, RMR, T3, T3/T4, alpha diversity, beta diversity, functional diversity, microbes, KEGG categories, and altitude, and that between BAT wet/dry mass and body mass were explored using linear regression. Principal coordinate analysis (PCoA) was performed based on Bray–Curtis dissimilarity using the "vegan" package in R (https://www.r-project.org/). We used the "pheatmap" package to assess the strength and significance of associations between dominant microbes and altitude and cecal metabolites, as well as cecal metabolites and physiological phenotypes, by spearman's correlation. Lastly, Spearman's correlation was calculated among major bacteria genera, cecal metabolites, and physiological phenotypes/host transcriptome and visualized as a heatmap using the "pheatmap" package in R.

3. Results

3.1. Body mass, physiological parameters, and thermogenic capacity in different seasons

The body mass of plateau pikas was significantly higher in winter than in summer (p < 0.001), and the variation in body mass significantly increased with increasing altitude in summer ($R = 0.46$, p < 0.001) and winter ($R = 0.51$, p < 0.001) (Fig. 1C). Furthermore, T_b, RMR, T3, and T3/T4 ratios were significantly higher in summer, and BAT wet and dry masses in winter (p < 0.001; Fig. 2). Pearson's correlation analysis was used to examine the correlations between physiological parameters and altitude or body mass. In summer, T_b ($R = 0.36$, p = 0.001), RMR ($R = 0.37$, p = 0.011), and T3/T4 ratio ($R = 0.29$, p = 0.033) were significantly positively correlated with altitude, and BAT wet mass ($R = 0.28$, p = 0.027) was significantly positively associated with body mass (Fig. 2A, B, D, and E). However, T3 ($R = -0.29$, p = 0.016) and T3/T4 ratio ($R = -0.49$, p < 0.001) were significantly negatively associated with altitude in winter (Fig. 2C and D).

3.2. Host transcriptome profiles

The host transcriptome was identified as the relative expression of genes associated with thermogenesis in BAT, WAT, and liver. The expressions of thermogenic-related genes differed between summer and winter (Fig. 3). In BAT, the expression levels of OXR1 and FGF21 increased with altitude in summer, whereas those of UCP1, OXR1, DIO2, FGF21, and KLB decreased with altitude in winter (Fig. 3A–E). Moreover, the expression levels of OXR1, DIO2 and FGF21 were significantly higher in summer than in winter (p < 0.01; Fig. 3P). In WAT, the expression levels of UCP1, DIO2, FGF21, and KLB exhibited an increasing trend with altitude in summer and a downward trend with altitude in winter (Fig. 3F, H–J). In addition, the expression levels of UCP1 and DIO2 were significantly higher in summer than in winter, whereas that of OXR1 exhibited the opposite trend (p < 0.05; Fig. 3Q). In the liver, the expression levels of OXR1, DIO2, FGF21, and KLB increased and decreased with altitude in summer and winter, respectively (Fig. 3L–O). Moreover, the expression levels of UCP1 and DIO2 were significantly higher in summer than in winter, whereas that of KLB exhibited the opposite trend (p < 0.05; Fig. 3R).

3.3. Gut microbial diversity and composition

Metagenomic sequencing generated 13,547,820,070 reads, with 85,745,697 ± 419,679 reads (mean ± standard error of the mean) per sample (Table S3). After quality control and removal of host DNA sequences, 13,261,164,988 reads were retained, with 83,931,424 ± 417,893 reads per sample. Furthermore, after de novo assembly, 285,860,981 contigs were generated, with 1,809,247 ± 14,351 contigs per sample (N50 length of 736 ± 14 bp).

We calculated the Shannon and richness indices of the gut microbiota in plateau pikas based on the genus profile of bacteria and fungi; they were significantly higher in winter than in summer (p < 0.001; Fig. 4A, B, F, G). Moreover, bacterial Shannon index was significantly positively correlated with altitude in summer ($R = 0.23$, p = 0.033) and winter ($R = 0.32$, p = 0.005); however, the richness index showed a positive association with altitude only in summer ($R = 0.023$, p = 0.035; Fig. 4A, B). Among the fungal diversity indices, only the Shannon index increased significantly with altitude in summer ($R = 0.22$, p = 0.045; Fig. 4F). Furthermore, PCoA revealed that bacteria and fungi in summer and winter differed significantly on the PCo1 and PCo2 axes (p < 0.001; Fig. 4C–E, H–J). Additionally, variations in bacterial beta diversity on the PCo1 axis were significantly associated with altitude in summer ($R = -0.29$, p = 0.008), and variations on the PCo2 axis were significantly associated with altitude in summer ($R = 0.52$, p < 0.001) and winter ($R = 0.47$, p < 0.001; Fig. 4D and E). Changes in fungal beta diversity on
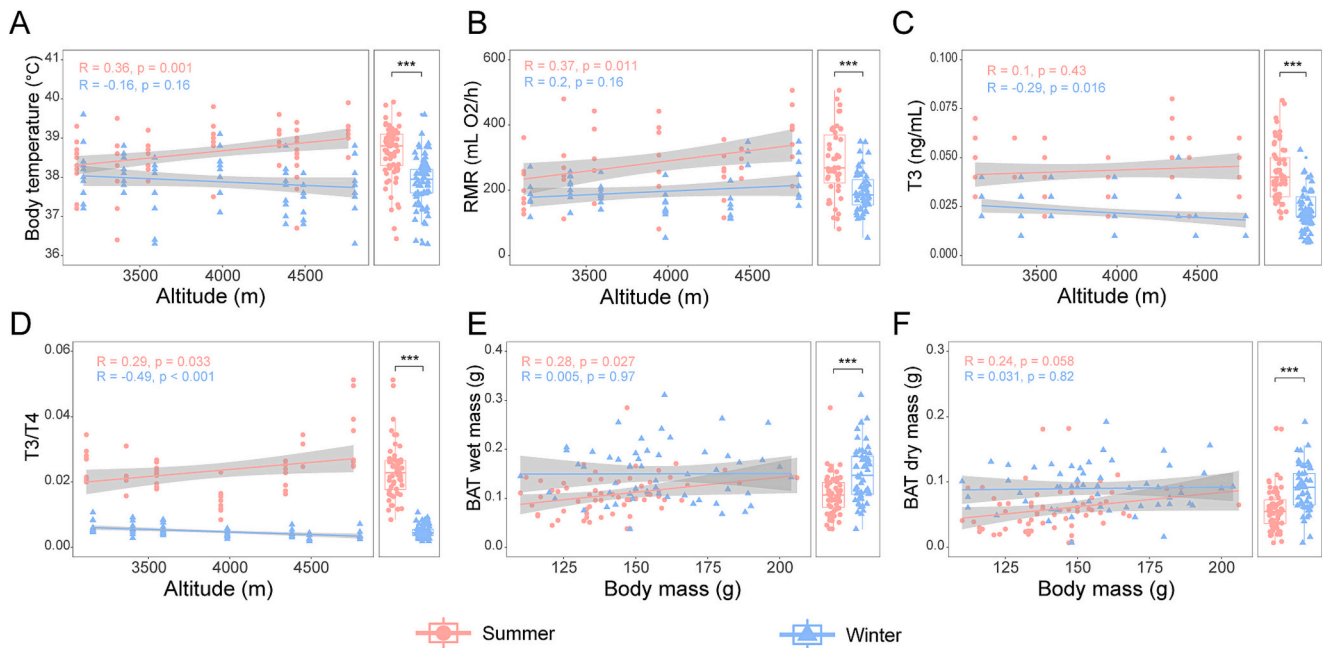
Fig. 2. Plasticity of physiological phenotypes in plateau pikas during summer and winter. (A) T_b, (B) RMR, (C) T3, (D) T3/T4, (E) BAT wet mass, and (F) BAT dry mass. *** p < 0.001.

the PCo2 axis were significantly associated with altitude in winter (R = 0.47, p < 0.001; Fig. 4J).

The dominant bacterial phyla were Firmicutes (66.26 %), Bacteroidetes (24.03 %), and Proteobacteria (3.79 %); the dominant bacterial genera were *Clostridium* (8.94 %), *Prevotella* (8.57 %), *Bacteroides* (7.42 %), and *unclassified_f_Lachnospiraceae* (6.87 %); and the dominant bacterial species included *Firmicutes_bacterium_ASF500* (3.66 %), *Oscillibacter_sp._1–3* (2.99 %), *Prevotella_sp._CAG:485* (1.73 %), *Firmicutes_bacterium_CAG:65* (1.59 %), and *Ruminococcus_flavefaciens* (1.52 %) (Fig. S1). Furthermore, a comparative analysis of the bacterial abundance at the phylum level showed that the abundances of Firmicutes, Spirochaetes, Actinobacteria, Synergistetes, and Fusobacteria were significantly higher in the pika gut in winter (p < 0.001), similar to those of Bacteroidetes, Proteobacteria, Verrucomicrobia, and Chlamydiae in summer (p < 0.05) (Fig. 5A; Table S4). At the genus level, *Clostridium*, *unclassified_f_Lachnospiraceae*, *Oscillibacter*, *Eubacterium*, and *unclassified_o_Clostridiales* were significantly higher in the pika gut in winter (p < 0.001), similar to *Prevotella, Bacteroides, Ruminococcus*, and *Alistipes* in summer (p < 0.001) (Fig. 5B; Table S4). At the species level, 25 species were significantly relatively abundant in the pika gut in winter (p < 0.05), whereas 12 were significantly relatively abundant in summer (p < 0.05) (Fig. S3A; Table S4). The fitted linear regression showed that Firmicutes and Spirochaetes were negatively associated with altitude in summer, whereas Bacteroidetes was positively associated with this altitude. Furthermore, Proteobacteria, Chlamydiae, Synergistetes and Cyanobacteria were positively associated with altitude in winter, whereas Spirochaetes showed a negative association (Fig. 5E and Fig. S2A). Moreover, the fitted linear regression and heatmap showed that the bacterial genera and species were significantly correlated with altitude in summer and winter (Fig. 5F, Fig. S2B and Fig. S3C).

The composition of the dominant fungal phyla, genera, and species is shown in Fig. S4. Regarding the differential abundance of the most abundant fungal phyla, Ascomycota was significantly higher in the pika gut in summer, similar to Glomeromycota, Microsporidia, Neocallimastigomycota, and Chytridiomycota in winter (Fig. 5C; Table S4). At the genus level, *Penicillium, Debaryomyces, Pseudogymnoascus,* and *Puccinia* were significantly more abundant in the pika gut in summer, similar to *Rhizophagus, Aspergillus, and Ophiocordyceps* in winter (Fig. 5D; Table S4). Lastly, at the species level, nine species exhibited significantly

higher abundance in the pika gut in summer, and six exhibited a similar trend in winter (Fig. S3B; Table S4). The fitted linear regression and heatmap showed that fungal phyla, genera, and species were significantly associated with altitude in summer and winter (Fig. 5G, H; Fig. S3D).

3.4. Functional profiles of pika gut microbiota

The functional diversity of the pika gut microbiota was determined based on the KEGG level 3 profile. Shannon index was significantly higher in the pika gut in summer than in winter, whereas the richness of the KEGG profile showed the opposite trend (Fig. 6A and B). Only the richness index was significantly correlated with altitude in winter (Fig. 6B). Additionally, PCoA analysis based on Bray–Curtis dissimilarity showed clear distinctions in PCo1 and PCo2 of KEGG categories between summer and winter (Fig. 6C). Variations in functional diversity on the PCo1 and PCo2 axes were significantly negatively associated with altitude in summer (Fig. 6D, E).

Statistical analysis revealed that the top 10 most abundant metabolic categories at KEGG level 2 differed significantly between summer and winter and were significantly associated with altitude, except for replication and repair (Fig. 7; Table S5). Moreover, 25 third-level pathways—including three amino acid and four carbohydrate metabolism pathways, among others—were significantly higher in the pika gut in summer. Additionally, 22 pathways—including two amino acid, seven carbohydrate, and four energy metabolism pathways, among others—were significantly upregulated in the pika gut in winter (Fig. S5A; Table S5). The heatmap also displayed the association between KEGG level 3 and altitude in summer and winter (Fig. S5B).

We focused on the key enzymes involved in starch and sucrose metabolism and SCFAs production (Fig. S6). They were all significantly higher in the pika gut in winter; two were involved in starch and sucrose metabolism, two in acetate production, two in butyrate production, and eight in propanoate formation.

3.5. Cecal metabolome

Comparative analysis revealed 24 significant differential metabolites in the positive ion mode, 13 of which were enriched in summer and 11 in
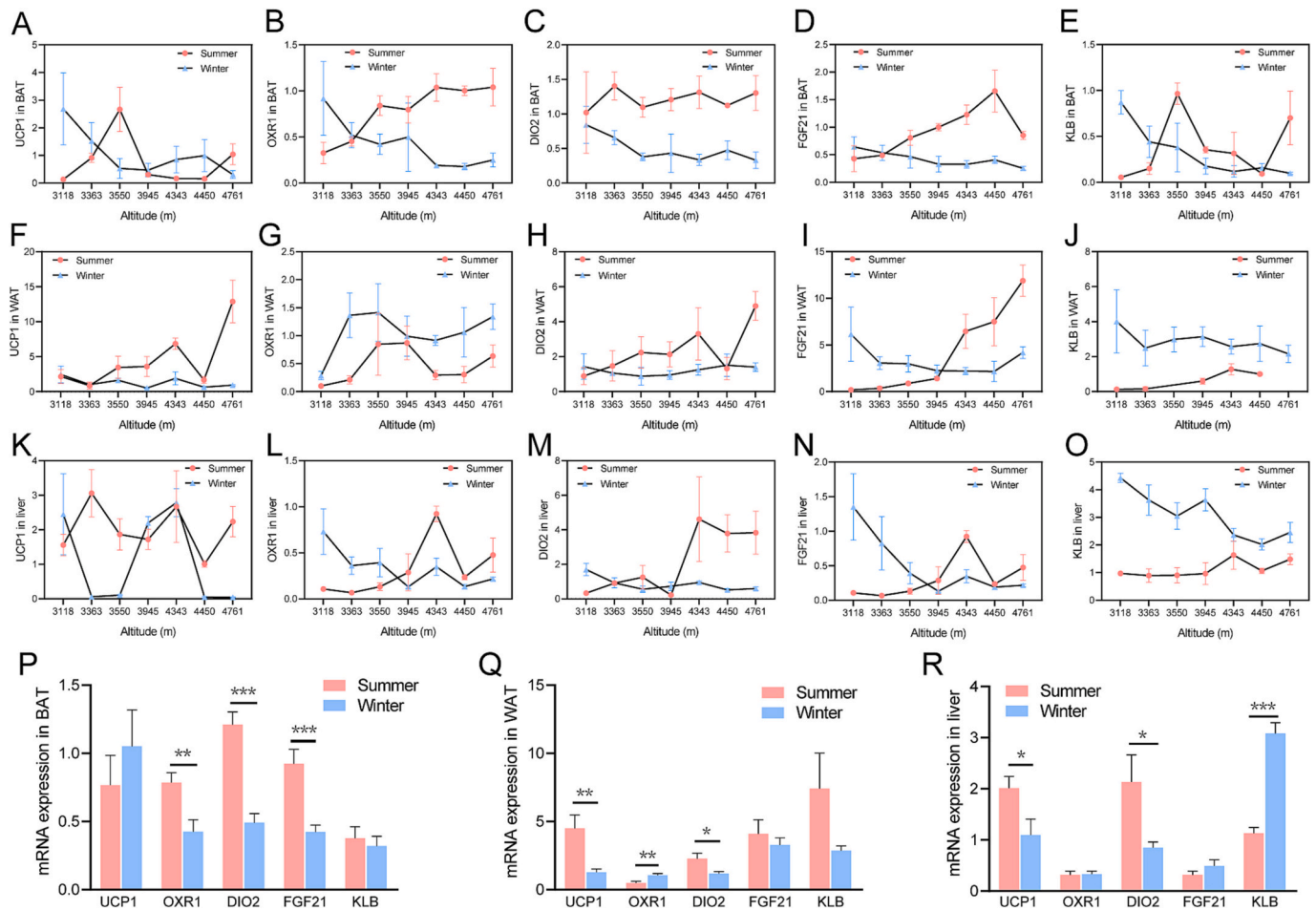
Fig. 3. Gene expression in different tissues of plateau pikas during summer and winter. The expression of (A) UCP1, (B) OXR1, (C) DIO2, (D) FGF21, and (E) KLB in BAT with altitude and (P) their differences between summer and winter. The expression of (F) UCP1, (G) OXR1, (H) DIO2, (I) FGF21, and (J) KLB in WAT with altitude and (Q) their differences between summer and winter. The expression of (K) UCP1, (L) OXR1, (M) DIO2, (N) FGF21, and (O) KLB in liver with altitude and (R) their differences between summer and winter. * p < 0.05, ** p < 0.01, *** p < 0.001.

winter (Fig. 8A). There were 41 significantly different metabolites in the negative ion mode, 35 were enriched in summer and 6 were enriched in winter (Fig. 8B). Significantly different cecal metabolites revealed 10 enriched metabolic pathways in the positive ion mode, mainly including phenylalanine, tyrosine and tryptophan biosynthesis, D-Glutamine and D-glutamate metabolism, and phenylalanine metabolism (Fig. 8C). Twelve enriched metabolic pathways were identified in the negative ion mode, and primarily consisted of phenylalanine, tyrosine and tryptophan biosynthesis, taurine and hypotaurine metabolism, and arachidonic acid metabolism (Fig. 8D).

3.6. Associations between gut microbiota, metabolites, and physiological phenotypes/host transcriptome

We further analyzed the correlation between gut microbiota and cecal metabolites using Spearman's correlation analysis; the results revealed that genera *Prevotella*, *Bacteroides*, *Ruminococcus*, *Alistipes*, and *Akkermansia* were significantly positively correlated with 23, 22, 22, 13, and 17 metabolites, respectively. *Oscillibacter*, o_*Clostridiales*, *Lachnoclostridium*, f_*Ruminococcaceae*, and *Flavonifractor* were all obviously negatively associated with 23 metabolites (Fig. 9A). Spearman's correlation analysis was performed to identify potential metabolite-physiological phenotype interactions. We found that 19, 10, 22, and 23 metabolites were significantly positively correlated with T_b, RMR, T3, and T3/T4 ratio, respectively. and 7, 21, 10, and 7 metabolites were markedly positively associated with OXR1 in BAT, DIO2 in BAT, FGF21

in BAT, and UCP1 in liver, respectively (Fig. 9B). Furthermore, we found that *Prevotella*, *Bacteroides*, *Ruminococcus*, *Alistipes*, and *Akkermansia* directly exhibited significant positive correlations with pika physiological phenotypes and transcriptome profiles (Fig. 9C).

4. Discussion

In this study, the shift of body mass with altitude of plateau pikas followed Bergmann's rule in summer (Fig. 1C), consistent with previous findings (Lin et al., 2008). Similar results were obtained in wild house mice (Suzuki et al., 2020), sifakas (*Propithecus diadema*) (Lehman et al., 2005), cerulean warblers (*Dendroica cerulea*) (Jones et al., 2005), and house sparrows (*Passer domesticus*) (Brommer et al., 2015). However, body mass in winter exhibited an inverted parabolic shape with altitude. Geist (1987) found that the body weights of deer and wolf increased with latitude at low and middle latitudes, but decreased with latitude at high latitudes. Thus, Geist (1987) argues that Bergmann's rule is flawed and proposes the hypothesis that body size is primarily affected by the availability of food resources. Moreover, the increasing body size of white-tailed deer with latitude in accordance with Bergmann's rule can be explained by food resource availability (Wolverton et al., 2009). Thus, the body weight loss of pikas at high altitudes in winter may be related to the scarcity of food resources in extreme cold environments.

The T_b and RMR of pikas increased with altitude in summer (Fig. 2A, B). Consistent with our results, the T_b of yellow-necked mice (*Apodemus flavicollis*) and bank voles (*Myodes glareolus*) increased with altitude in
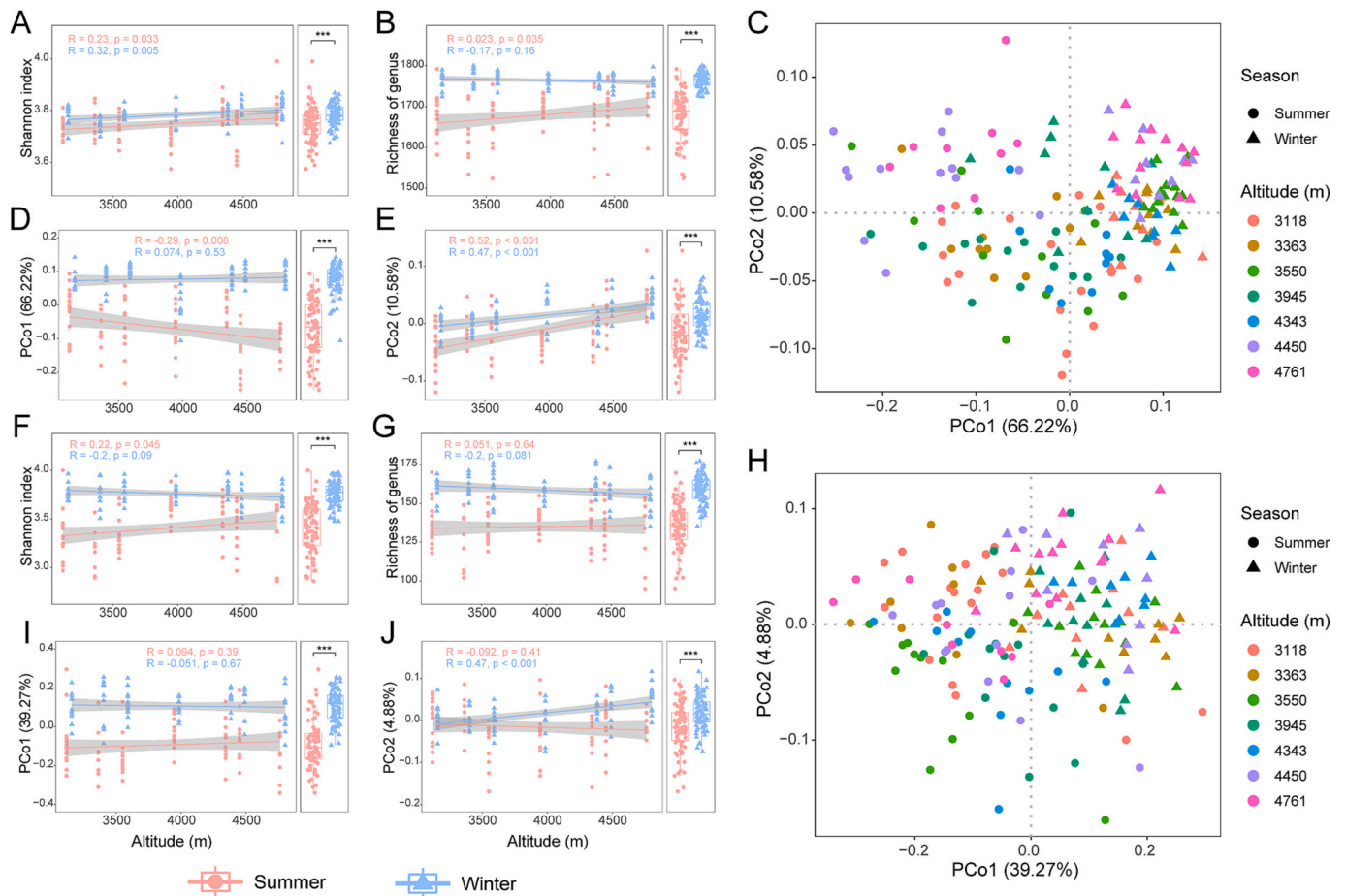
Fig. 4. Gut microbial diversity in plateau pikas during summer and winter. Bacterial diversity of (A) Shannon index and (B) richness of genus. (C) PCoA based on Bray–Curtis distance and sample distribution on (D) PCo1 and (E) PCo2 axes of bacteria. Fungal diversity of (F) Shannon index and (G) richness of genus. (H) PCoA based on Bray–Curtis distance and sample distribution on (I) PCo1 and (J) PCo2 axes of fungi. *** p < 0.001.

summer (Melcore et al., 2022). This phenomenon can be understood that endotherms compensate for the increased heat dissipation caused by the reduction in ambient temperature by increasing thermal production (Hassi et al., 2005; Khakisahneh et al., 2020; Zhang et al., 2018). Similar research showed that deer mice (*Peromyscus maniculatus*) (Hammond et al., 1999), Lesotho mole-rats (*Cryptomys hottentotus mahali*) (Broekman et al., 2006) and Cape White-eyes (*Zosterops virens*) (Thompson and Downs, 2017) living at high altitudes have higher metabolic rates than those living at lower altitudes. Additionally, the metabolic rate of rats was significantly increased when the ambient temperature was reduced from 25 °C to 5 °C (Gautier, 2000). Summer records the highest temperature and most abundant food resources in the QTP; the maximum biomass is approximately 420 g/m^2 at 3300 m and the minimum biomass is 132 g/m^2 at 4400 m in summer (Jing et al., 2004; Li et al., 2004). Although thermoregulation in elevated altitudes requires high energy, pikas may compensate for increased energy expenditure by increasing food intake (Zhu et al., 2012). Similar to other rodents that enhance, energy intake and expenditure to adapt to cold environments by increasing food intake (Khakisahneh et al., 2020; Zhang and Wang, 2006; Zhao et al., 2022). In contrast, the lowest winter temperature on the QTP is approximately −20 °C (Wang et al., 2011), and endotherms may reduce energy demands when the ambient temperature is below 0 °C (Sheriff et al., 2009). Similar studies found that the T$_b$ of yaks (*Bos grunniens*) decreases with altitude in winter (Han et al., 2002), and the energy expenditure of North American red squirrels (*Tamiasciurus hudsonicus*) decreases with the reduction of ambient temperatures in winter (Fletcher et al., 2012; Humphries et al., 2005). Furthermore, Speakman et al. (2021) identified that the lower energy

demands of pikas in winter were primarily due to food deprivation. The RMR of African striped mice (*Rhabdomys pumilio*) was positively correlated with food availability and decreased during periods of food resource scarcity (Rimbach et al., 2018). Additionally, snowshoe hares (*Lepus americanus*) living at high latitudes conserve energy by suppressing field metabolic rate during food shortages in winter (Sheriff et al., 2009). Lower energy expenditure contributes to overwintering survival (Desforges et al., 2021), so pikas must suppress energy demands to adapt to extremely cold environments when food supply is insufficient in winter.

The thermogenic capacity of pikas increased and decreased with altitude in summer and winter, respectively. Previous studies in pikas showed that the T3/T4 ratio increased with altitude in summer (Wang, 2006). In humans, decreased serum T3 levels were observed at lower ambient winter temperatures (Hassi et al., 2001). The T3/T4 ratio increases as ambient temperature decreases in mice (Zhao et al., 2022). Another study discovered a positive relationship between thyroid hormone levels and basal metabolic rate (Li et al., 2010). Thyroid hormone supplementation in Mongolian gerbils (Khakisahneh et al., 2021) and Brandt's voles (Liu et al., 1997) resulted in a marked increase in RMR. These results indicate that the thermogenic capacity of pikas corresponds to their energy demands. Moreover, BAT wet and dry masses increased with body weight in summer, and a similar phenomenon was observed in deer mice in the laboratory (Coulson et al., 2021). The thermogenic properties of BAT are essential for T$_b$ maintenance in mammals during cold periods (Townsend and Tseng, 2014; Wen et al., 2019). Therefore, high body weight and BAT content help pikas maintain T$_b$ at high altitudes in both summer and winter. Additionally, we
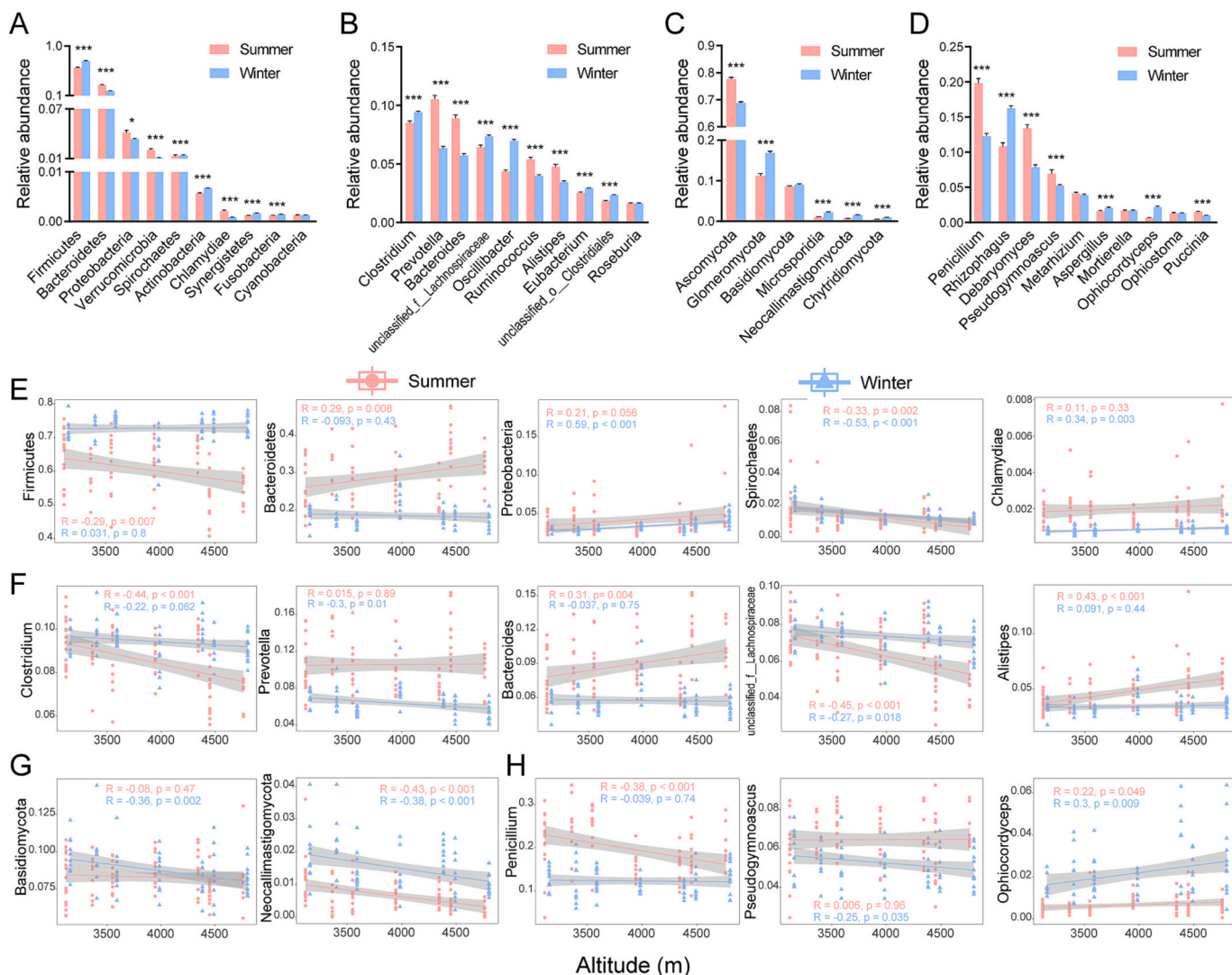
Fig. 5. Changes in gut microbial taxa in plateau pikas during summer and winter. Differences in the most abundant (A) bacterial phyla, (B) bacterial genera, (C) fungal phyla, and (D) fungal genera between summer and winter. Dominant (E) bacterial phyla, (F) bacterial genera, (G) fungal phyla, and (H) fungal genera were significantly correlated with altitude in summer or winter. * p < 0.05, *** p < 0.001.
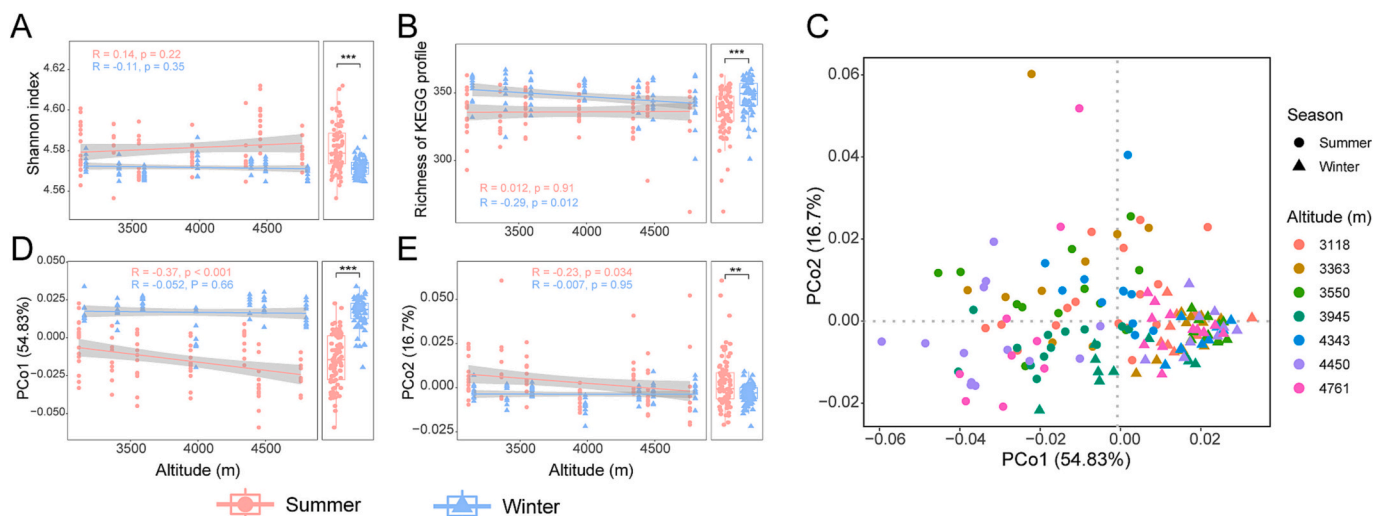

Fig. 6. Microbial functional diversity in plateau pikas during summer and winter. Functional diversity of (A) Shannon index and (B) richness of KEGG profile. (C) PCoA based on Bray–Curtis distance and sample distribution on (D) PCo1 and (E) PCo2 axes. ** p < 0.01, *** p < 0.001.
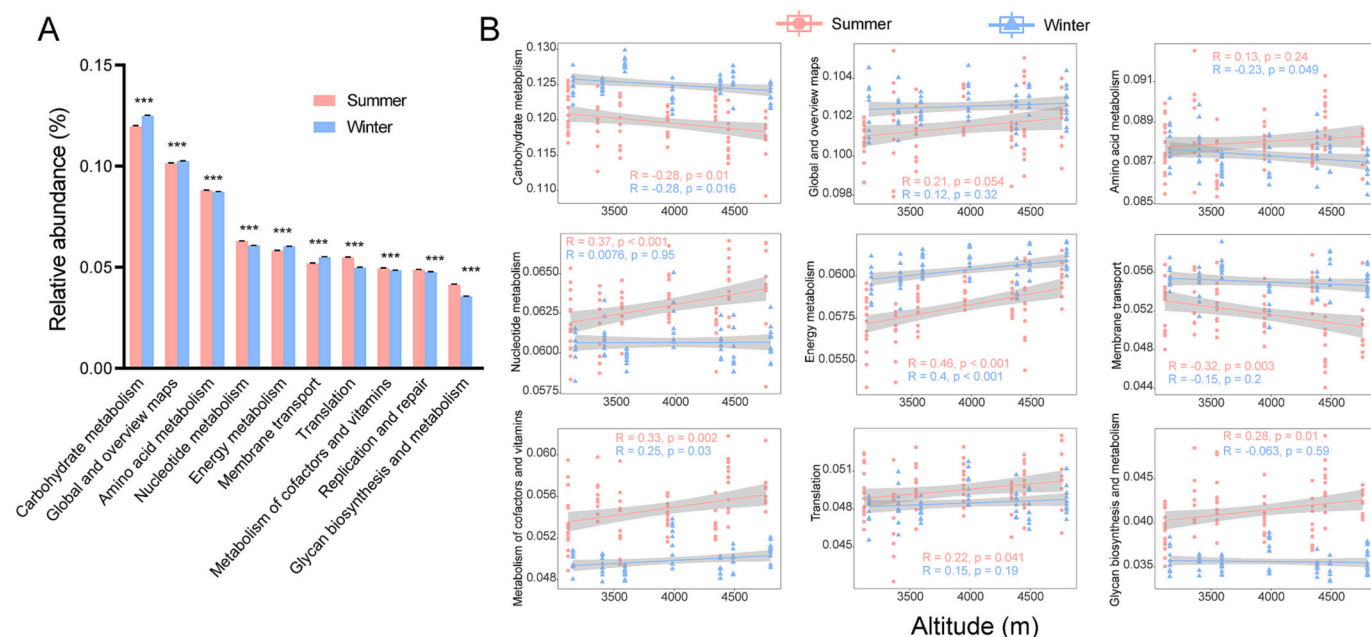
Fig. 7. Changes in gut microbiota function in plateau pikas during summer and winter. (A) Comparison of the gut microbial KEGG level 2 functions of pikas between summer and winter. (B) KEGG level 2 functions were significantly correlated with altitude in summer or winter. *** p < 0.001.

observed that BAT wet and dry masses were higher in winter, implying that pikas have a high potential for thermogenesis during cold winters.

The transcriptome profiles at the molecular level indicated that the thermogenic capacity of pikas increased and decreased with altitude in summer and winter, respectively (Fig. 3). Bai et al. (2015) revealed elevated expression of UCP1 and DIO2 in pikas exposed to low temperatures in a laboratory hypobaric chamber reproducing a living altitude of 4100 m. The thermogenic capacity of BAT primarily depends on the uncoupling activity of UCP1 (Ricquier, 2011). DIO2 mediates the intracellular conversion of T4 into bioactive T3 to enhance thermogenesis (de Jesus et al., 2001). OXR1 can scavenge reactive oxygen species and toxic compounds produced by the normal metabolism of aerobic organisms and prevent oxidative DNA damage (Volkert et al., 2000). FGF21 promotes glucose utilization and increases energy expenditure by enhancing insulin sensitivity of adipose tissue and thermogenesis of BAT (Szczepanska and Gietka-Czernel, 2022). Xue et al. (2018) revealed that the browning of WAT under cold exposure varies between mice and bats. The expression patterns of thermogenic-related genes diverge between seasons, indicating that pikas harbor distinct thermogenic strategies between summer and winter, although their body weight increased with altitude in both seasons.

The alpha diversity of pika gut bacteria increased with altitude, and PCoA displayed a distinct separation of bacterial communities between summer and winter and significant variations with altitude in both seasons. Previous studies have shown that low temperatures lead to increased diversity of gut microbiota in Mongolian gerbils (Khakisahneh et al., 2020). Higher gut microbial diversity contributes to the fermentation efficiency of dietary fiber (Tap et al., 2015), providing functional redundancy to cope with environmental fluctuations (Louca et al., 2018). This facilitates the adaptation of pikas to the cold environment of high altitude where food resources are scarce.

The diversity of Firmicutes decreased with altitude, whereas that of Bacteroidetes increased with altitude in wild house mice (Suzuki et al., 2019). Firmicutes specialize in complex polysaccharide degradation (Cann et al., 2016), and Bacteroidetes assist in protein catabolism, producing food nutrients (Wu et al., 2011). Therefore, our results indicated that the changes in Firmicutes and Bacteroidetes with altitude corresponded to the decrease in crude fiber content with altitude and the increase in crude protein content with altitude in vegetation on the QTP

region, respectively (He et al., 2020). Furthermore, the abundance of Firmicutes was higher in winter, whereas that of Bacteroidetes was higher in summer. Consistent with our previous report, crude cellulose was enriched in winter, and crude protein was relatively high in the vegetation nutrition around the pika habitat in summer (Fu et al., 2021). Firmicutes were primarily attributed to the genera *Clostridium*, *unclassified_f_Lachnospiraceae*, *Oscillibacter*, and *Eubacterium*, and their abundance was significantly higher in winter than in summer. These bacteria degrade cellulose and hemicellulose and convert them into SCFAs, which can be absorbed and used for energy by the host (Biddle et al., 2013; Iino et al., 2007; Lee et al., 2012; Rodriguez-Castano et al., 2019; Yoda et al., 2005; Zhu et al., 2011). Meanwhile, the genera *Prevotella*, *Bacteroides*, and *Alistipes* are the major members of Bacteroidetes and were significantly more abundant in summer than in winter. *Bacteroides* is associated with the high-protein and high-fat modern diet typical of Western countries (De Filippo et al., 2010) and has been observed to increase considerably in caloric restriction experiments (Santacruz et al., 2009). The abundance of *Alistipes* is increased in mice fed a high-fat diet (Daniel et al., 2014). Furthermore, *Prevotella*, *Bacteroides*, and *Alistipes* are all beneficial in promoting the production of SCFAs (Shah and Collins, 1990; Yin et al., 2018). Similarly, the higher summer abundance of *Ruminococcus* facilitates the breakdown of carbohydrates to produce SCFAs (Ezaki, 2015). Evidently, the gut microbiota of pikas degrades high protein and high fat diets to produce energy material and thus support the host's energy production strategy in summer, while digesting low quality food rich in fiber and hemicellulose to produce energy and thus supply the host's energy saving strategy in winter.

PCoA distinguished the functional diversity of the pika gut microbiota between summer and winter and showed obvious alterations with increasing altitude during summer (Fig. 6). Previous studies have shown that the beta diversity of the gut microbial function differs significantly between seasons (Fan et al., 2022) and correlates significantly with altitude (Li et al., 2019b). This suggests that seasonality and spatiality impact gut microbial function. Specifically, carbohydrate and energy metabolism was higher in winter, which plays a crucial role in the host's altitude adaptation (Bai et al., 2022; Fan et al., 2020; Su et al., 2021). The enrichment of glycan biosynthesis and metabolism in summer favors the efficiency of food energy conversion (Xu et al., 2016), and the pathway is also enriched in Tibetan chickens (*Gallus gallus*) (Du et al.,
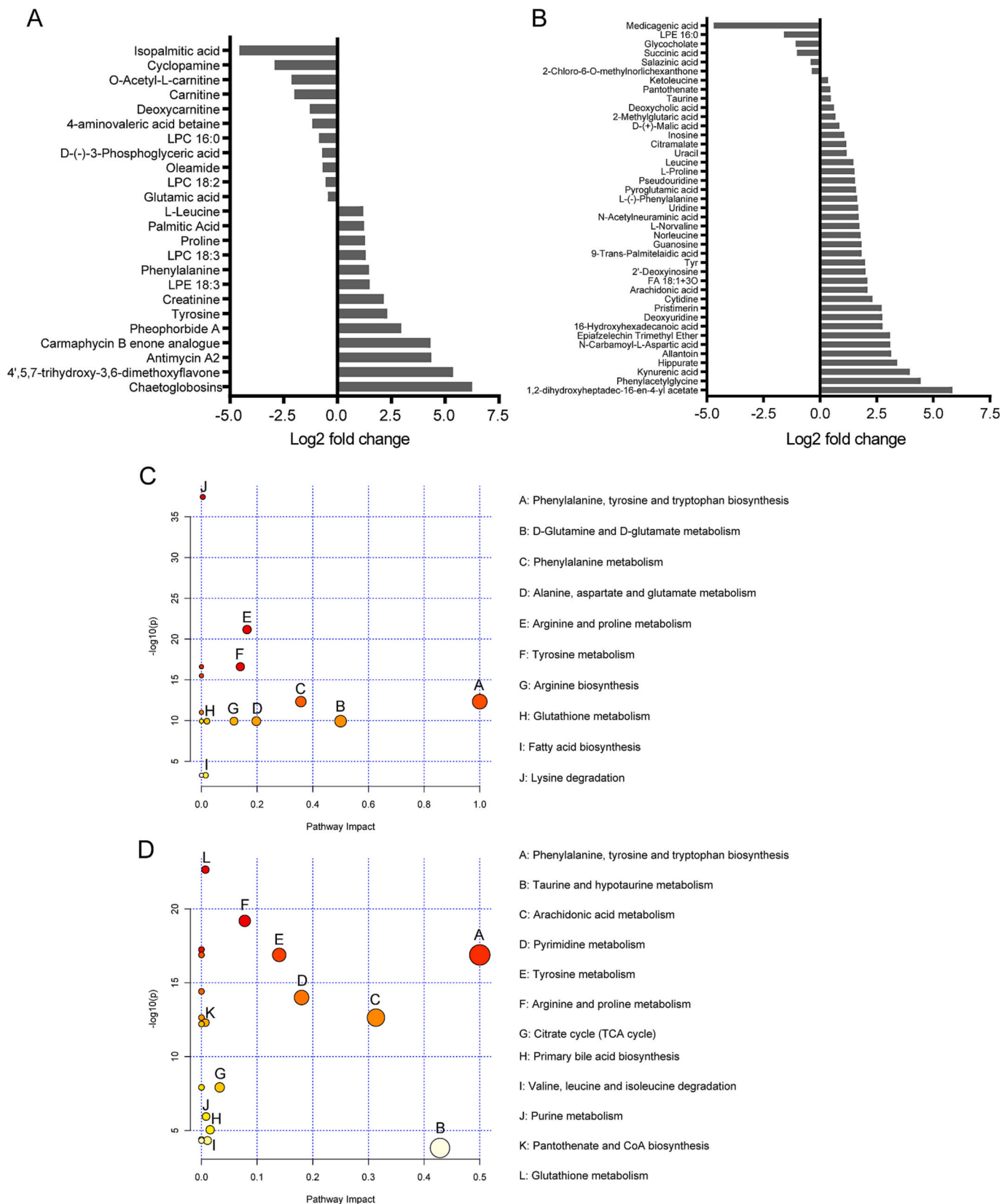
Fig. 8. Metabolites differences between summer and winter. The summer/winter fold change in (A) positive and (B) negative ion patterns of significantly different cecal metabolites in pikas between summer and winter. Pathway enrichment analysis was performed using the significantly different cecal metabolites in (C) positive and (D) negative ion patterns of pikas between summer and winter.
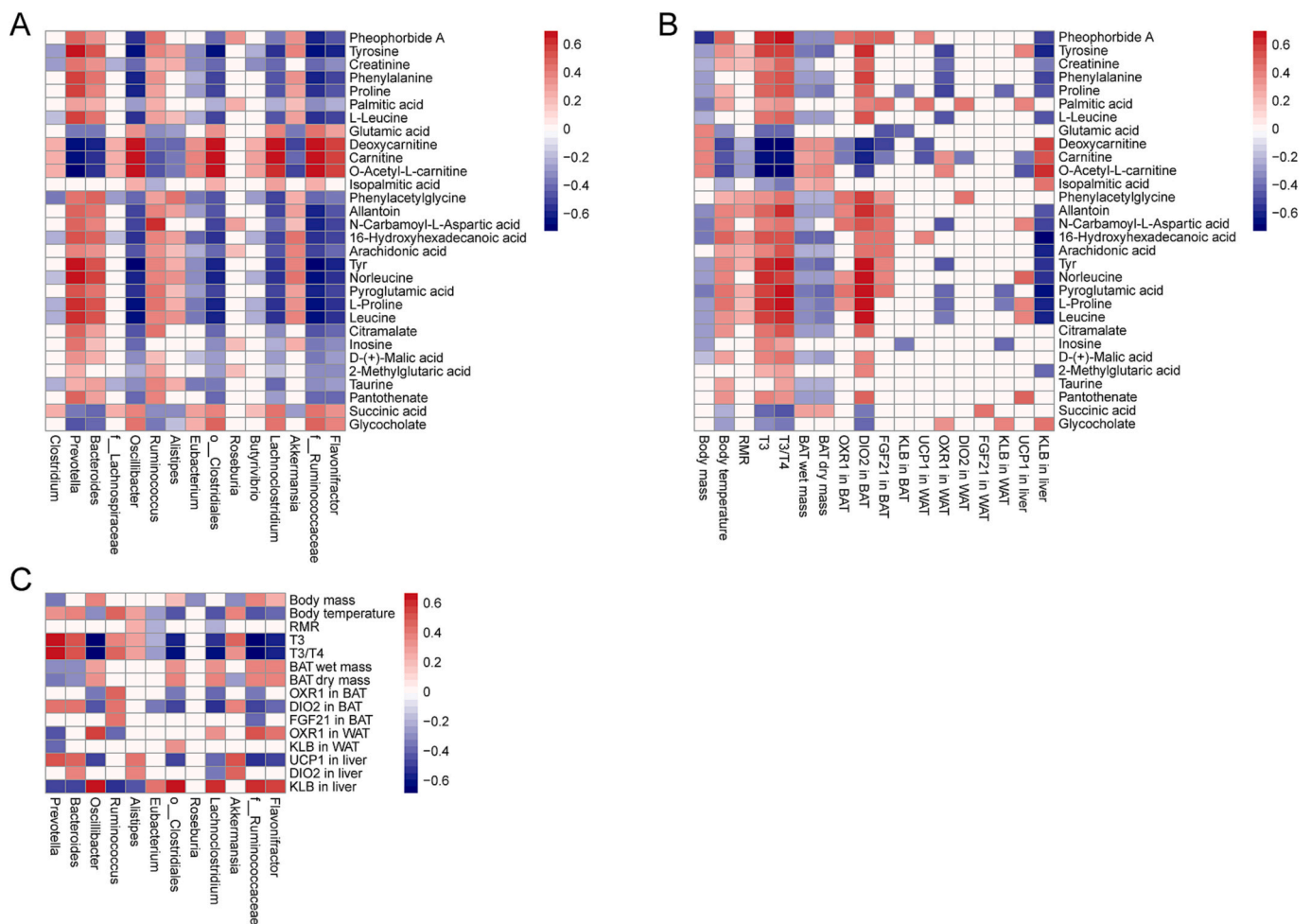
Fig. 9. Interactions among gut microbiota, metabolites, and physiological phenotypes/host transcriptome. (A) Spearman's rank correlations between gut microbiota and metabolites. (B) Spearman's correlations between metabolites and physiological phenotypes/host transcriptome. (C) Spearman's correlations between gut microbiota and physiological phenotypes/host transcriptome. Only false discovery rate (FDR)-corrected $p < 0.05$ is displayed.

2022) and *Gynaephora* (Cao et al., 2021) at high altitudes. The TCA cycle, which is enriched in summer, is involved in the catabolism of carbohydrates, proteins, and fats, and is the primary pathway for adenosine triphosphate (ATP) production (Akram, 2014); therefore, its increased abundance can meet the increased energy requirements of the host. Gut microbial functions of pikas in summer are associated with diverse substance utilization, such as proteins, fats, and carbohydrates, while they specialize in carbohydrate utilization in winter.

The metabolites enriched in summer were mainly amino acids, such as phenylalanine, tyrosine, and proline. Phenylalanine is converted to tyrosine by the reaction of phenylalanine hydroxylase (Davis and Kaufman, 1993). Tyrosine is involved in the tyrosine metabolic pathway to produce thyroxine, which facilitates the host thermogenesis (Bowden and Goldis, 2023). Moreover, proline is tightly associated with energy metabolism (Bursell, 1981). Carnitine, which plays a key role in energy production, is enriched in winter. It conveys fatty acids to the mitochondria where they are oxidized to produce ATP (Carillo et al., 2020). Succinate is an important component of the TCA cycle and its enrichment in winter may be in response to low quality foods (Amato et al., 2015).

Prevotella, Bacteroides, Ruminococcus, Alistipes and *Akkermansia* are probiotics that favor the production of the energy substance SCFAs (Ezaki, 2015; Houtman et al., 2022; Shah and Collins, 1990; Yin et al., 2018). Our results suggest that these probiotics are positively associated with metabolites of amino acid, thereby contributing to the thermogenic capacity of pikas. A study on dairy cows indicates that *Prevotella* may be

a key contributor to microbial metabolites (Xue et al., 2020). Studies on Mongolian gerbils and Brandt's voles have shown that *Prevotella* and *Ruminococcus* contribute to host thermogenesis (Bo et al., 2019; Khakisahneh et al., 2021), and *Bacteroides* was positively associated with host RMR (Khakisahneh et al., 2020). Furthermore, the gut microbiota may communicate with host organs through its metabolites to regulate systemic homeostasis (Cani and Knauf, 2016; Rastelli et al., 2018; Williams et al., 2014). Thus, our results imply that the gut microbiota may contribute to host thermogenesis through the metabolites of amino acids.

5. Conclusions

In this study, the variation in body mass of plateau pikas with altitude followed Bergmann's rule in summer and an inverted parabolic shape was observed in winter. T_b, RMR, and T3/T4 ratio increased with altitude in summer, whereas T3 and T3/T4 ratio decreased with altitude in winter. The expression of thermogenic-related genes was upregulated with altitude in summer, but downregulated with altitude in winter. These results suggest that the thermogenic capacity of pikas is enhanced with altitude in summer and attenuated with altitude in winter, which indicates that pikas adopt an energy expenditure strategy in summer and energy conservation strategy in winter.

The alpha diversity of pika gut microbiota was significantly higher in winter than in summer. The abundance of Firmicutes declined, whereas that of Bacteroidetes significantly increased with altitude in summer.

The gut microbial function associated with energy-related glycan biosynthesis and metabolism and the TCA cycle was also positively correlated with altitude in summer. Phenylalanine, tyrosine, and proline were enriched in summer, whereas carnitine was enriched in winter. Furthermore, *Prevotella*, *Bacteroides*, *Ruminococcus*, *Alistipes*, and *Akkermansia* were positively correlated with the thermogenic capacity of pikas. Overall, our findings emphasize that the changes in body mass and thermogenic capacity with altitude distinctly differentiate small herbivorous mammals on the QTP between summer and winter, and that specific gut microbes may regulate host thermogenesis through their metabolites.

Ethics

The animal procedures were approved by the Animal Care and Ethics Committee of the Northwest Institute of Plateau Biology, Chinese Academy of Sciences (Approval no: nwipb2019110801).

CRediT authorship contribution statement

Shien Ren: Investigation, Formal analysis, Writing – original draft, Visualization. **Liangzhi Zhang:** Investigation, Data curation. **Xianjiang Tang:** Investigation, Data curation. **Yaqi Zhao:** Investigation. **Qi Cheng:** Investigation. **John R. Speakman:** Writing – review & editing. **Yanming Zhang:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.167776.

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