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# Seasonal shift in gut microbiome diversity in wild Sichuan takin (*Budorcas tibetanus*) and environmental adaptation

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ABSTRACT

In this study, we investigated the change in microbiome composition of wild Sichuan takin (*Budorcas tibetanus*) during winter and spring and analyzed the physiological implications for such changes. Diversity analyses of the microbiome (average 15,091 high-quality reads per sample) in 24 fecal samples (15 from winter, 9 from spring) revealed that spring samples had higher species diversity and were compositionally different from winter samples ($P < 0.05$). Taxonomic composition analysis showed that the relative abundance increased in spring for *Patescibacteria* (2.7% vs. 0.9% in winter, $P < 0.001$) and *Tenericutes* (1.9% vs. 1% in winter, $P < 0.05$). Substantial increases in relative abundance of *Ruminococcaceae* and *Micrococcaceae* were identified in spring and winter, respectively. Mann-Whitney U and ANCOM identified seven differentially abundant genera: *Enterococcus, Acetitomaculum*, *Blautia*, *Coprococcus* 1, *Lachnospiraceae* UCG 008, *Ruminococcus* 2 and *Ralstonia*. All seven genera were significantly more abundant in spring (average 0.016–1.2%) than winter (average 0–0.16%), with the largest difference found in *Ruminococcus* (1.21% in spring vs. 0.16% in winter). The other six genera were undetectable in winter. Functional prediction and pathway analysis revealed that biosynthesis of cofactors (ko01240) had the highest gene count ratios in the winter, followed by the two-component system (ko02020). Seasonal variation affects the gut microbiomes in wild Sichuan takins, with winter associated with lower species diversity and spring with enrichment of cellulose-degrading genera and phytopathogens. Such changes were crucial in their adaptation to the environment, particularly the difference in food abundance.



## 1. Introduction

Gut microbiomes are crucial to animal physiology and are highly associated with individual health [1]. Understanding the relationships between gut microbial community composition and host adaptations is a major goal of wildlife research [2,3]. Recent advances in sequencing techniques and bioinformatics capability have greatly facilitated the understanding of host-microbiome interaction and allowed rapid microbial characterization. Environmental change is known to affect variations in the gut microbiomes in a way that reflects host fitness, and seasonal variations are one of the environmental factors that alter the microbial compositions in the gut environment [4–6]. The effect of seasonal changes on gut

*Abbreviations:* ANCOM, Analysis of the composition of microbiomes; ASVs, Amplicon sequence variants; KO, KEGG Orthology; PCoA, Principal coordinate analysis; PERMANOVA, permutational multivariate analysis of variance; SRA, Sequence Read Archive; UCGs, uncultured genus-level groups

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microbiomes has been demonstrated in various animals, including marine invertebrates, birds, mammals, non-human primates, and humans [4–7], suggesting that these seasonal associated variations in gut microbiome composition are ubiquitous.

Sichuan takin (*Budorcas tibetanus*) is an endangered large ungulate that mainly inhabits the mountainous terrain of Sichuan and Gansu in China [8]. Their unique habitat poses challenges to survival and necessitates seasonal migration to support nutritional requirements, particularly because of the decreased food availability in winter [9]. As ruminants, the specialized digestive system of Sichuan takins is colonized by rumen microbes capable of breaking down and using cellulose as an energy source [10]. Thus, the condition of rumen microorganisms could reflect the takin physiological status and indirectly provide information on nutrient availability. Despite recognition of the increasing importance of gastrointestinal microbiomes, there are only two studies on takin gut microbiomes so far [11,12]. In golden takins, microbial species richness and rumen microbial abundance were higher in feces collected during spring [12]. The only study on Sichuan takins was performed in captivity [11], but captive animals usually harbor very different microbial communities from wild animals, as also seen in several other species [13,14]. To the best of our knowledge, no study has investigated the relationship between seasonal change and gut microbiomes in wild Sichuan takins. Characterization of microbial composition across seasons is critical to understanding the effects on host physiology and adaptation to the different seasonal environmental conditions. Therefore, this study aimed to characterize the intestinal microbiomes of wild Sichuan takins and determine whether seasonal variation alters microbial community composition. The findings could provide important insight into the physiological response to environmental stress and enhance our current knowledge on the conservation of this endangered species.

## 2. Materials and methods

### 2.1. Sample collection

Fecal samples in this study were collected in December 2018 and April 2019, respectively, in the Tangjiahe national nature reserve, Sichuan province, China. All were fresh fecal samples collected with sterile disposable gloves. The freshness of feces was confirmed from its phenotypic properties, including a smooth, wet, and light green appearance and a special urine smell. Each sample was preserved in a separate sterile container and stored in thermal bags at − 14 °C. The samples were immediately processed after being transported to the laboratory.

### 2.2. DNA extraction and PCR amplification

Microbial community genomic DNA was extracted from 24 fecal samples using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.) according to manufacturer's instructions. The DNA extract was checked on 1% agarose gel, and DNA concentration and purity were determined with NanoDrop 2000 UV–vis spectrophotometer (Thermo Scientific, Wilmington, USA). The hypervariable region V3-V4 of the bacterial 16S rRNA gene was amplified with primer pairs 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) by an ABI GeneAmp® 9700 PCR thermocycler (ABI, CA, USA). The PCR amplification of 16S rRNA gene was performed as follows: initial denaturation at 95ºC for 3 min, followed by 27 cycles of denaturing at 95 ºC for 30 s, annealing at 55ºC for 30 s and extension at 72ºC for 45 s, and single extension at 72 ºC for 10 min, and end at 4 ºC. The PCR mixtures contain 5 × *TransStart* FastPfu buffer 4 μL, 2.5 mM dNTPs 2 μL, forward primer (5 μM) 0.8 μL, reverse primer (5 μM) 0.8 μL, *TransStart* FastPfu DNA Polymerase 0.4 μL, template DNA 10 ng, and finally $ddH_2O$ up to 20 μL. PCR reactions were performed in triplicate. The PCR product was extracted from 2% agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to manufacturer's instructions and quantified using Quantus™ Fluorometer (Promega, USA).

### 2.3. Illumina MiSeq sequencing

Purified amplicons were pooled in equimolar and paired-end sequenced on an Illumina MiSeq PE300 platform (Illumina, San Diego, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (Project ID: PRJNA918737).

### 2.4. Bioinformatics analysis

Amplicon Sequence Variants (ASVs) were identified using QIIME2 version 2019.7 [15], and chimeric sequences were removed by DADA2. Taxonomic classification was conducted by the QIIME2 plugin feature classifier, and each of the ASVs was analyzed by RDP Classifier version 2.2 against the Silva v138 database [16]. The phylogenetic tree was generated using qiime phylogeny align-to-tree-mafft-fasttree command for calculating phylogeny-dependent diversity measurements. All sequence data were rarefied to 8, 117 sequences per sample for the diversity analysis. Total sum scaling was applied to the ASV table to calculate the relative abundance in each sample, and the compositions of ASVs relative abundance with taxonomic classification were shown with a stacked bar chart.

Several alpha diversity indices, including Chao1, observed ASVs, Pielou's evenness, Shannon index, and Simpson index, were calculated to address species abundance, evenness, and diversity (Table 1). Stool samples were grouped by season, and the significant differences in microbial diversity and compositions were tested by the Mann-Whitney U test. Two dissimilarity matrices, weighted UniFrac distance and unweighted UniFrac distance, were included as beta diversity measures. Principal coordinate analysis (PCoA) was applied to distance matrices to visualize the results of beta diversity. A permutational multivariate analysis of variance (PERMANOVA) test was used to compare the microbial community structures in QIIME2 to evaluate the statistical significance of beta diversity. Analysis of the composition of microbiomes (ANCOM) was used to compare the compositional difference [17].

PICRUST2 was adopted to conduct the KEGG Orthology (KO) prediction based on microbial community structures [18]. All the predicted KOs were subject to analysis of ALDEx2 (BH adjusted p-value < 0.1) to identify distinct KOs between season groups [19]. In addition, statistically significant KOs were loaded into the R package *clusterprofiler* for over-representation test with the default setting to identify pathways with high number counts of KOs [20]. Subsequently, the identified pathways were further visualized by *Pathview* Web, and changes in KOs were further investigated [21].

**Table 1**
Alpha-diversity of the gut microbiomes in Sichuan takins in this study.

| Diversity indices | Spring | Winter | P values |
|---|---|---|---|
| Chao1 | 515.7 (427.8–573.1) | 450.2 (193.9–475.6) | < 0.05 |
| Faith's phylogenetic diversity | 30.7 (26.4–32.9) | 26.6 (15.6–30.1) | < 0.05 |
| Observed ASVs | 504 (403–552) | 446 (193–462.5) | < 0.05 |
| Pielou's evenness | 0.9 (0.9–0.9) | 0.9 (0.8–0.9) | 0.38 |
| Shannon | 8.5 (8–8.7) | 8.4 (6.1–8.4) | < 0.05 |
| Simpson | 1 (1–1) | 1 (1–1) | < 0.05 |

Median (interquartile range)

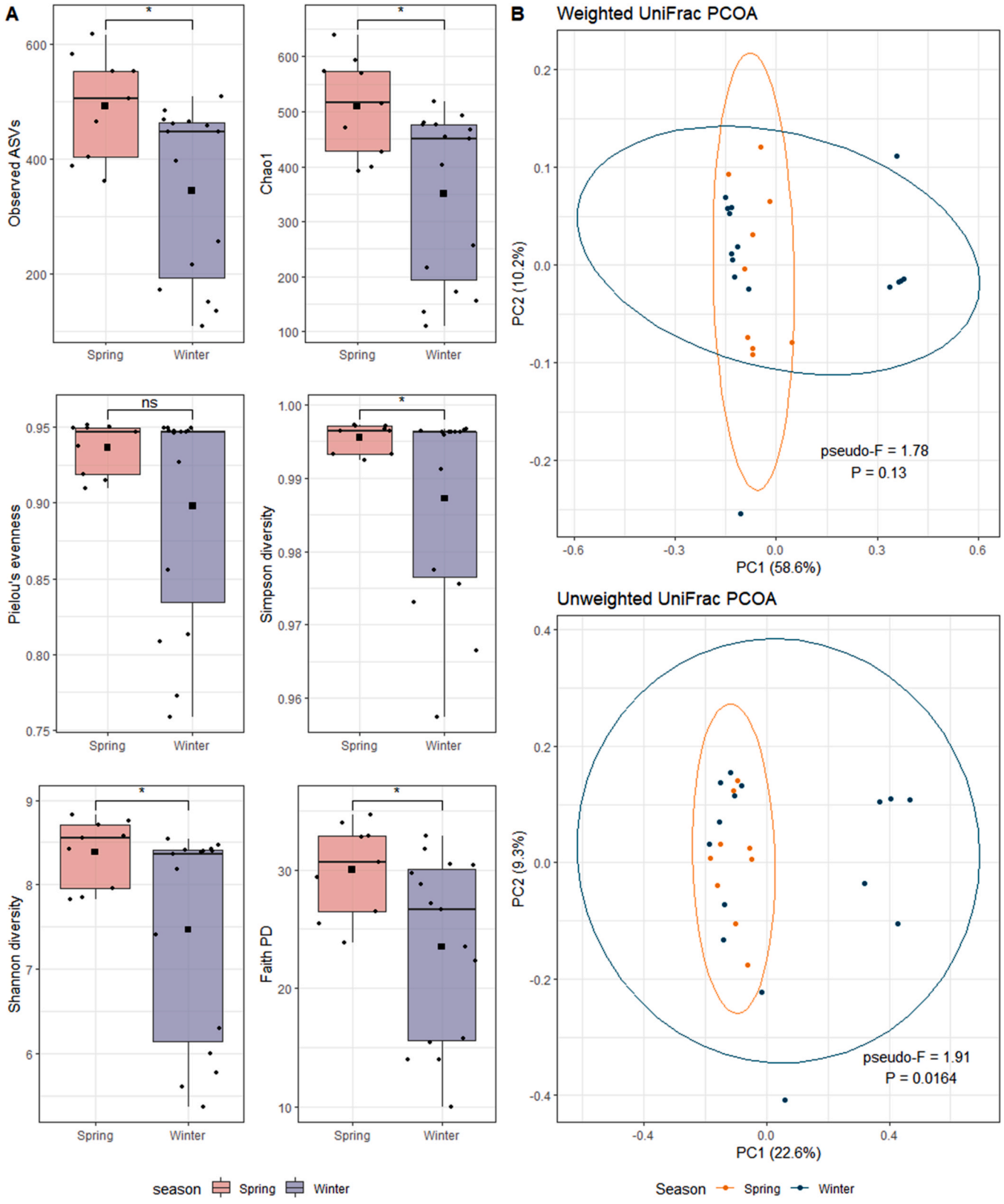


**Fig. 1.** Diversity comparisons of Sichuan takin gut microbial community between winter and spring. (**A**) Alpha diversity. Species richness was estimated using observed ASVs and Chao1. Species evenness and diversity were determined using Pielou's evenness, Simpson's diversity, Shannon diversity, and Faith's phylogenetic diversity. Samples from different seasons are represented with purple (winter) and pink (spring). Boxes indicate interquartile range, lines indicate medians, squares indicate means, and whiskers represent the range. (**B**) PCoA plots of Beta diversity. Sample distances were calculated with weighted UniFrac and unweighted UniFrac. The ellipses represent 95% confidence ellipses. Samples from different seasons are represented with blue (winter) and yellow (spring). Pairwise comparisons: ns, not significant ($P > 0.05$); *: $P \leq 0.05$.

## 3. Results

### 3.1. Sequencing metadata

Illumina MiSeq sequencing of 24 fecal samples (15 from winter, 9 from spring) yielded 1, 379, 797 (mean: 57, 491; range: 42, 854–73, 797) raw 16S rDNA reads. After quality trimming, pair-end joining, and chimeric filtering, 362, 200 high-quality reads were obtained for downstream analyses. Individual samples that passed quality checks generated an average of 15, 091 reads per sample. Rarefaction curves based on observed amplicon sequence variants (ASVs) indicated sufficient sequencing depth (Fig. S1). The analysis identified 6, 725 unique features, assigned into 2 domains, 17 phyla, 28 classes, 51 orders, 92 families, and 233 genera.

### 3.2. Comparison of Sichuan takin gut microbiome diversity between spring and winter

We determined alpha and beta diversity indices for measuring gut microbial species abundance, diversity, and structural differences in takin samples collected during spring and winter. In general, spring samples had higher alpha diversity than winter samples (Fig. 1 A, Table 1). Both Chao1 estimator and observed ASVs (Fig. 1 A) indicated that spring samples had a greater number of microbial species, as shown in the boxplot distribution (median 504 vs. 446 for spring and winter samples, $P < 0.05$ [observed ASVs]; median 515.7 vs. 450.2, $P < 0.05$ [Chao1]). However, species evenness did not exhibit seasonal differences (median 0.9 vs. 0.9 in spring and winter samples, $P = 0.38$ [Pielou's evenness]). Shannon, Simpson, and Faith phylogenetic diversity measurements revealed that spring samples had significantly more microbial diversity than winter samples (median 8.5 vs. 8.4 in spring and winter samples, $P < 0.05$ [Shannon]; median 1 vs. 1, $P < 0.05$, [Simpson]; median 30.7 vs. 26.6, $P < 0.05$ [Faith]; Table 1).

We measured phylogenetic distance (microbial community compositional differences) using two beta diversity indices: unweighted UniFrac distance and weighted UniFrac distance. Distances between samples were visualized with PCoA (Fig. 1B). The first two principal components of weighted UniFrac explained more data variation than those of unweighted UniFrac (Axis 1 = 58.6%, Axis 2 = 10.2% [weighted UniFrac]; Axis 1 = 22.6%, Axis 2 = 9.3% [unweighted UniFrac]). However, PERMANOVA of weighted UniFrac did not find a significant seasonal difference (PERMANOVA pseudo-F = 1.78, $P = 0.13$). In contrast, unweighted UniFrac distances revealed distinct microbial compositions between spring and winter (PERMANOVA pseudo-F = 1.91, $P < 0.05$), suggesting that observed seasonal differences were related to low-abundance, phylogenetically similar taxa.

### 3.3. Taxonomic composition of Sichuan takin gut microbial communities

We also investigated taxonomic compositions at the phylum, family, and genus levels to characterize dominant groups in the gut of wild Sichuan takins. Our analysis revealed that the gut microbial community comprised 17 bacterial phyla, with seven at > 1% relative abundance (Fig. 2): *Firmicutes* (average ± SD: 59.7% ± 22.9), *Actinobacteria* (13.9% ± 20.7), *Bacteroidetes* (13.8% ± 6.5), *Proteobacteria* (6.9% ± 9.7), *Patescibacteria* (1.6% ± 1.3), *Tenericutes* (1.3% ± 1), and *Verrucomicrobia* (1.2% ± 3.2). We also identified 92 families, with 17 being dominant (relative abundance > 1%). Among these 17 families (accounting for 90.5% of the total microbial composition), *Ruminococcaceae* had the highest relative abundance (35.9% ± 18.2), followed by *Micrococcaceae* (10.5% ± 19.6), *Lachnospiraceae* (6.9% ± 4.6), *Christensenellaceae* (5.9% ± 3.8), *Pseudomonadaceae* (3.9% ± 8.7), *Rikenellaceae* (3.8% ± 2.8), *Prevotellaceae* (3.2% ± 1.9), *Clostridiales* Family XIII (2.9% ± 2), *Eggerthellaceae* (2.1% ± 1.5), *Bacillaceae* (2.1% ± 4.1), *Bacteroidaceae* (2.0% ± 1.5), *Muribaculaceae* (2.0% ± 1.4), *Planococcaceae* (1.6% ± 3.1), *Peptostreptococcaceae* (1.7% ± 1.2), *Saccharimonadaceae* (1.6% ± 1.3), *Enterobacteriaceae* (1.3% ± 3.9), and *Akkermansiaceae* (1.2% ± 3.2). Finally, we identified 233 genera, but only 22 genera had a relative abundance of > 1%. The top 10 most abundant genera were *Arthrobacter* (10.5% ± 19.5), *Christensenellaceae* R7 group (5.8% ± 3.7), *Ruminococcaceae* UCG 010 (5.6% ± 3.8), *Ruminococcaceae* UCG 005 (5.2% ± 2.4), *Ruminococcaceae* UCG 013 (5.1% ± 3.5), *Ruminococcaceae* UCG 014 (4.5% ± 2.9), *Eubacterium coprostanoligenes* (4.0% ± 2.6), *Pseudomonas* (3.9% ± 8.7), *Rikenellaceae* RC9 (2.1% ± 1.7), and *Bacillus* (2.1% ± 4.1).

We then stratified these data by season to observe potential differences in dominant taxa (Table 2). At the phylum level, similar phyla were dominant across both seasons, such as *Bacteroidetes* (13.8% vs. 13.8% in winter, $P = 0.95$), *Proteobacteria* (6.0% vs. 7.5% in winter, $P = 0.35$), and *Cyanobacteria* (1.1% vs. 0.6% in winter, $P = 0.26$). However, relative abundance increased in spring for *Patescibacteria* (2.7% vs. 0.9% in winter, $P < 0.001$) and *Tenericutes* (1.9% vs. 1% in winter, $P < 0.05$). We also observed substantial changes in the average composition of *Firmicutes* and *Actinobacteria* during spring (68.9% vs. 54.2% in winter, $P = 0.48$, [*Firmicutes*]; 4.2% vs. 19.7% in winter, $P = 0.56$ [*Actinobacteria*]), although the difference was not significant (Table 2). Analysis at the family level revealed that differences between *Firmicutes* and *Actinobacteria* were mainly driven by fluctuations in the abundance of *Ruminococcaceae* and *Micrococcaceae*. *Micrococcaceae* abundance declined during the transition from winter to spring (16% vs. 1.3% in spring, $P = 0.53$), whereas *Ruminococcaceae* increased over the same period (32.7% vs. 41.1% in spring, $P = 0.68$). Notably, the latter is a well-known family of fibrinolytic bacteria responsible for the degradation of plant cellulose. At the genus level, *Arthrobacter* (15.9% vs. 1.3% in spring, $P = 0.53$) and uncultured genus-level groups (UCGs) of *Ruminococcaceae* were the primary genera associated with fluctuations in *Micrococcaceae* and *Ruminococcaceae*.

### 3.4. Differential abundance analyses

Since over 200 genera, including low-abundance taxa, were present in takin gut microbiomes, statistical analyses were performed to identify differentially abundant features. Results from Mann-Whitney U tests revealed 28 differentially abundant genera (Table S1). More than half belong to *Firmicutes* (18/28, 64%), followed by *Proteobacteria* (3/28, 10.7%), *Bacteroidetes* (2/28, 7.1%), *Cyanobacteria* (2/28, 7.1%), *Tenericutes* (1/28, 3.6%) and *Patescibacteria* (1/28, 3.6%). Next, we applied ANCOM (analysis of composition of microbiomes) to determine seasonal differences in composition (Fig. 3, Table S2). The results indicated a similar profile of differently abundant genera ($n = 20$) as the Mann-Whitney U test. These genera belong to four phyla (Table S2), with *Firmicutes* remaining the dominant phylum (10/20, 50%), followed by *Actinobacteria* (6/20, 30%), *Proteobacteria* (3/20, 15%), and *Bacteroidetes* (1/20, 5%).

We then compared differentially abundant ASVs from the two statistical tests using a Venn diagram (Fig. 4 A). Mann-Whitney U and ANCOM identified 41 unique taxa, representing 17.6% (41/233) bacterial genera. At the intersection of the Venn diagram, we noted seven ASVs that were identified as differentially abundant by both tests. These shared seven genera were *Enterococcus, Acetitomaculum, Blautia, Coprococcus* 1, *Lachnospiraceae* UCG 008, *Ruminococcus* 2, and *Ralstonia*. Besides *Ralstonia* (from *Proteobacteria*), the remaining six belong to *Firmicutes*. All seven genera were significantly more abundant in spring (Fig. 4B; average 0.016–1.2% [spring] vs. average 0–0.16% [winter]), with the largest difference found in *Ruminococcus* (Average 1.21% vs 0.16% in winter). The other six genera were undetectable in winter but presented in spring (average 0.02–0.16%), suggesting that they are important indicators of seasonal change.

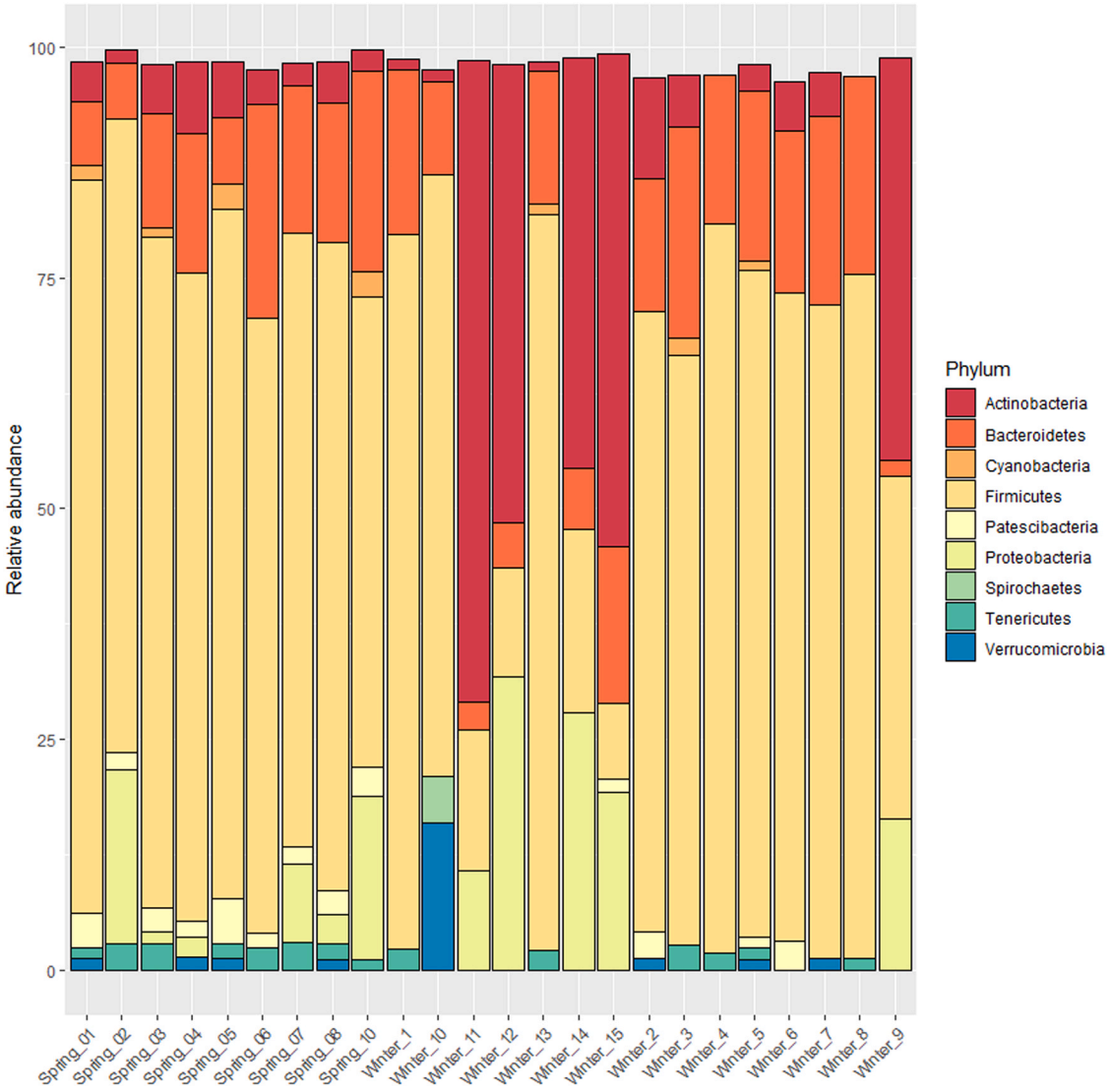


**Fig. 2.** Comparison of phyla composition per sample. The relative abundance of the bar represents 100% of microbial composition, and the size of colored regions represents the relative abundance (proportional contributions to total composition) of each phylum shown. For clarity, only major phyla (> 1% relative abundance) are listed in the color key.

### *3.5. Metabolic pathway prediction*

We hypothesized that seasonal microbial variations would influence the underlying microbial metabolism. Thus, we examined the metabolic potential of microbial communities through predicting functional orthologs in PICRUST2. We identified 306 significantly different KEGG Orthology functions (KOs) and visualized them with MA plots in *ALDEx2* (Fig. 5). These significant KOs were subjected to an over-representation test, resulting in 156 unique KOs mapped to KEGG pathways. Of these KOs, 100 mapped significantly to 20 pathways (adjusted $P < 0.05$, Fig. 6). Gene count ratios revealed that mapped KOs were not equally distributed across the 20 pathways but highly aggregated within a few pathways. Fifteen of the pathways were winter-specific and three were spring-specific (Fig. 6). Two pathways were shared between seasons: two-component system (ko02020) and galactose metabolism (ko00052). In terms of the most active pathways for each season, biosynthesis of cofactors (ko01240) had the highest gene count ratios in the winter, followed by the two-component system. During spring, the two-component system had the highest gene count ratios.

We then used *Pathview* to visualize pathway IDs with the highest gene count ratios: biosynthesis of cofactors and two-component system. For biosynthesis of cofactors (active only in winter), 21 KOs were involved, with the primary pathways being ubiquinone biosynthesis, porphyrin metabolism, and folate biosynthesis (Fig. S2 & S3). For two-component system, winter-associated pathways were phosphate assimilation, multidrug efflux, copper efflux, potassium limitation associated transport, tricarboxylate transport, citrate fermentation, and oxidative phosphorylation (Fig. S4). Spring-associated pathways were Mn2 + starvation, anaerobic fumarate respiratory system, and formate dehydrogenase (Fig. S4).

## 4. Discussion

In springtime, everything grows, seeds germinate and flowers blossom. In the Inner Canon of the Chinese Emperor, the oldest textbook of Chinese medicine, it was mentioned that in order to lead a healthy life, human being and animals should behave in a way so that is in the same pace with nature. In this study, we successfully characterized the wild Sichuan takin gut microbiome using high-throughput sequencing and demonstrated that the microbial community exhibits clear seasonal variation. Specifically, we observed that the gut microbiomes of wild Sichuan takins had greater species abundance and diversity in spring compared with winter (Fig. 1,

**Table 2**
Seasonal comparisons for ASVs relative abundance at Phylum level, Family and Genus taxonomic level. Top 10 taxa with more than 1% average relative abundance were shown.

| Taxonomic Level | Taxa | Winter | Spring | P values |
|---|---|---|---|---|
| Phylum | *Firmicutes* | 54.2 ± 27.2 | 68.9 ± 7.9 | 0.48 |
| | *Actinobacteria* | 19.7 ± 24.6 | 4.2 ± 2.0 | 0.56 |
| | *Bacteroidetes* | 13.8 ± 6.9 | 13.8 ± 6.2 | 0.95 |
| | *Proteobacteria* | 7.5 ± 11.0 | 6 ± 7.4 | 0.35 |
| | *Verrucomicrobia* | 1.5 ± 4.0 | 0.8 ± 0.5 | 0.2 |
| | *Tenericutes* | 1 ± 0.9 | 1.9 ± 0.9 | < 0.05 |
| | *Patescibacteria* | 0.9 ± 0.9 | 2.7 ± 1.1 | < 0.001 |
| | *Cyanobacteria* | 0.6 ± 0.5 | 1.1 ± 1.0 | 0.26 |
| Family | *Ruminococcaceae* | 32.7 ± 21.8 | 41.1 ± 8.4 | 0.68 |
| | *Micrococcaceae* | 16 ± 23.3 | 1.3 ± 2.0 | 0.53 |
| | *Lachnospiraceae* | 6.8 ± 5.5 | 7.1 ± 2.7 | 0.82 |
| | *Christensenellaceae* | 5.3 ± 3.6 | 7 ± 4.0 | 0.35 |
| | *Rikenellaceae* | 4 ± 3.0 | 3.7 ± 2.5 | 0.95 |
| | *Prevotellaceae* | 2.9 ± 1.9 | 3.7 ± 1.9 | 0.29 |
| | Clostridiales Family XIII | 2.4 ± 2.0 | 3.7 ± 1.7 | 0.14 |
| | *Muribaculaceae* | 2.2 ± 1.6 | 1.5 ± 0.9 | 0.45 |
| | *Eggerthellaceae* | 2.1 ± 1.6 | 1.9 ± 1.5 | 0.68 |
| | *Bacteroidaceae* | 1.9 ± 1.7 | 2.1 ± 1.3 | 0.63 |
| Genus | *Arthrobacter* | 15.9 ± 23.1 | 1.3 ± 2.0 | 0.53 |
| | *Christensenellaceae* R 7 group | 5.2 ± 3.6 | 6.8 ± 3.9 | 0.35 |
| | *Ruminococcaceae* UCG 010 | 5.2 ± 4.3 | 6.2 ± 2.7 | 0.6 |
| | *Ruminococcaceae* UCG 005 | 4.7 ± 2.8 | 6.1 ± 1.6 | 0.14 |
| | *Ruminococcaceae* UCG 013 | 4.6 ± 3.6 | 5.8 ± 3.3 | 0.56 |
| | *Ruminococcaceae* UCG 014 | 4.3 ± 3.6 | 4.8 ± 1.0 | 0.41 |
| | *Eubacterium coprostanoligenes* | 4 ± 3.1 | 4 ± 1.9 | 0.91 |
| | *Pseudomonas* | 6.1 ± 10.5 | 0 ± 0.6 | 0.89 |
| | *Rikenellaceae* RC9 gut group | 2.2 ± 1.8 | 2 ± 1.5 | 0.82 |
| | *Bacteroides* | 1.9 ± 1.7 | 2.1 ± 1.3 | 0.63 |

Mean ± standard deviation

Table 1), in line with a previous study on golden takins [12]. Our data also agrees with previous research across diverse wild animal species, demonstrating that the increase in gut microbial diversity during spring is common [12,22,23]. In the present study, we observed that the dominant bacterial phyla in the guts of wild Sichuan takins were *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, and *Proteobacteria* (Fig. 2), corroborating previous work that found these phyla to be similarly dominant in takin gut microbial composition [11,12]. Among these phyla, it was noted that *Actinobacteria* and *Firmicutes* showed particularly strong seasonal variation.

The seasonal change in the gut microbiome in wild Sichuan takin is important to its adaptation to the food availability in the environment. At the family level, *Micrococcaceae* and *Ruminococcaceae* in the phyla *Actinobacteria* and *Firmicutes*, respectively, showed the strongest seasonal variation. The *Micrococcaceae* genus *Arthrobacter* is a well-known psychrotolerant bacteria that unsurprisingly displays a sharp decline during the winter-spring transition [24]. In contrast, the cellulose-degrading *Ruminococcaceae* accounts for the most significant increase in spring and dominated (> 30% relative abundance) in the wild Sichuan takin gut microbial composition (Table 2). Members of *Ruminococcaceae* are a major constituent of rumen bacteria, and their number is often increased in high-fiber or high-fruit diets [25]. Here, no single genus was responsible for the increase in *Ruminococcaceae*, with various genera being enriched during spring (Table 2). Thus, spring appears to facilitate the growth of multiple cellulose-degrading *Ruminococcaceae*. In fact, further analysis at the genus level revealed over 40 differentially abundant genera associated with seasonal changes. Seven of these (*Enterococcus, Acetitomaculum, Blautia, Coprococcus* 1, *Lachnospiraceae* UCG 008, *Ruminococcus* 2 and *Ralstonia*) were identified by both Mann-Whitney U test and ANCOM (Fig. 4). All these seven genera were undetectable from the samples collected in winter and were only present from those collected in spring. The increase in plant materials and their consumption by wild Sichuan takins likely explain the enrichment of these taxa, as *Blautia*, *Acetitomaculum, Coprococcus,* and *Ruminococcus* all belong to either *Ruminococcaceae* or *Lachnospiraceae* [26,27]. Similar to *Ruminococcaceae*, *Lachnospiraceae*

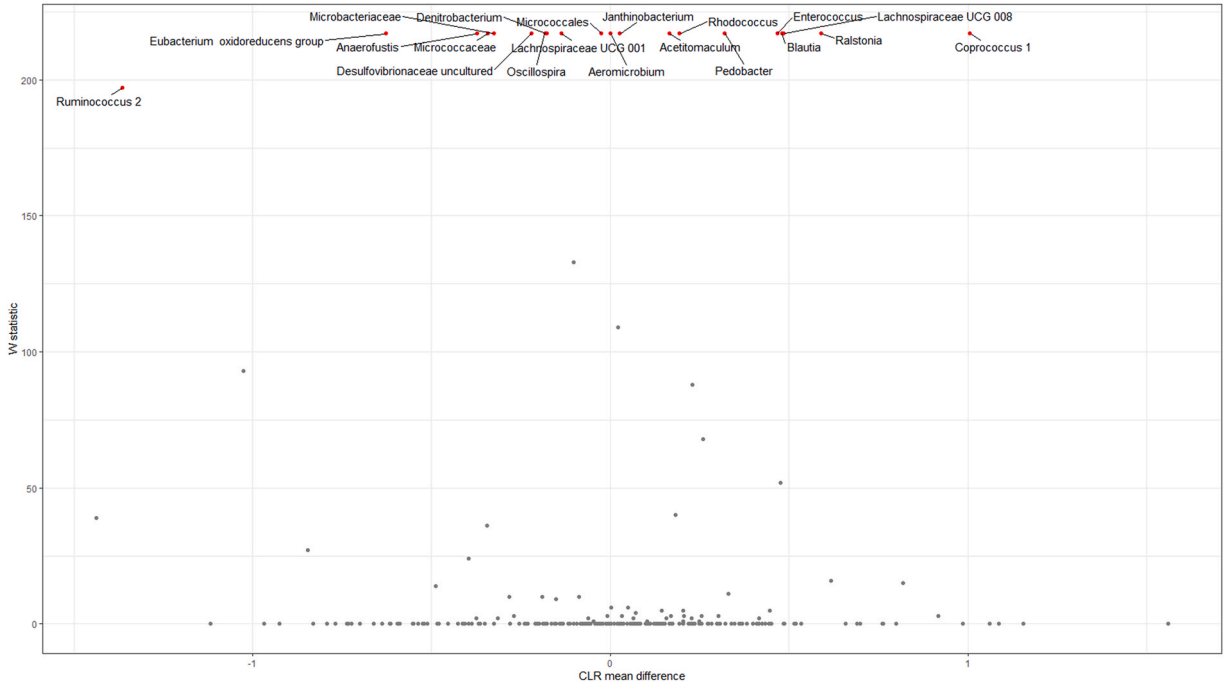


**Fig. 3.** ANCOM volcano plot of differential abundance. A positive x-axis represents an ASV that is abundant in spring samples, and a negative x-axis indicates higher abundance in winter samples. Only significantly different ASVs were labeled.

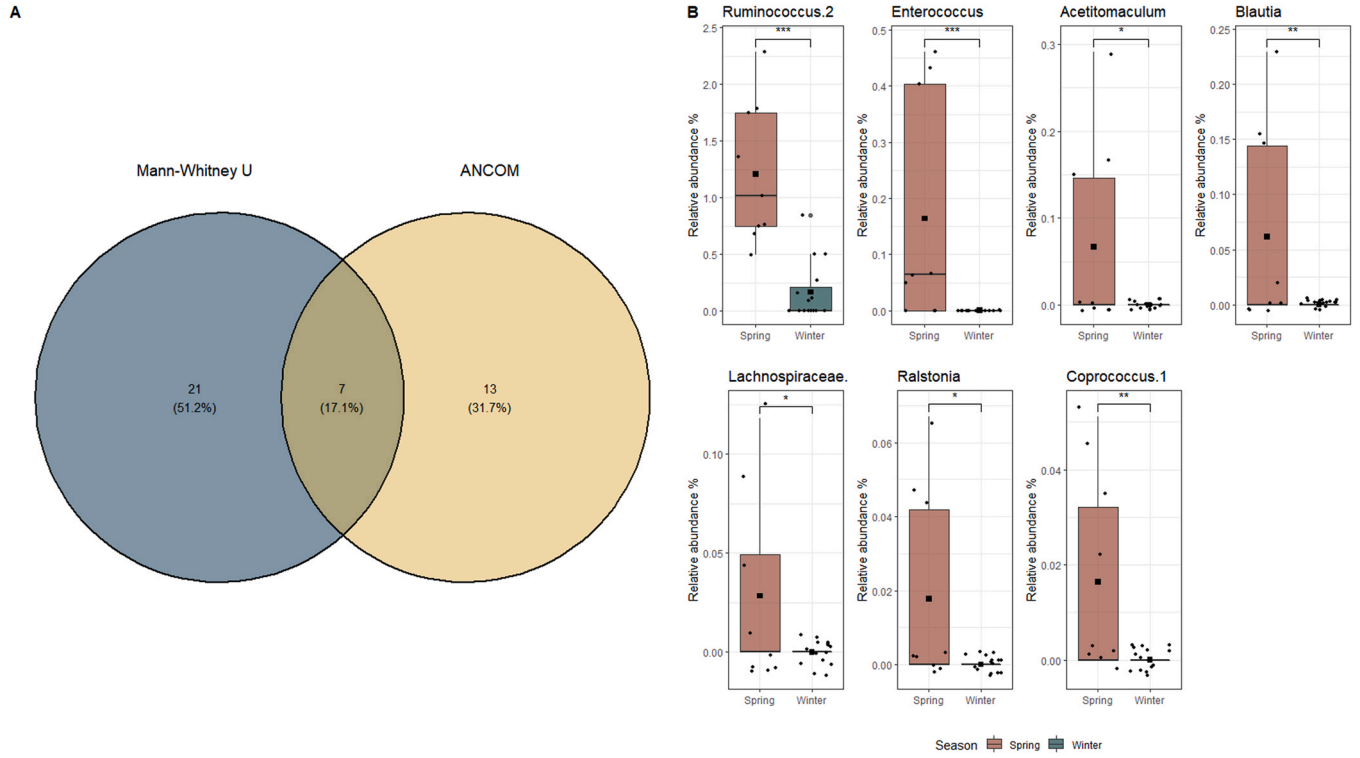


**Fig. 4.** Differentially abundant genera across winter and spring. (A) Venn diagram showing unique and shared differentially abundant genera between Mann-Whitney U test and ANCOM. (B) Comparison of seven differentially abundant genera between spring and winter was identified using both statistical tests. Boxes indicate interquartile range, lines indicate medians, squares indicate means, and whiskers represent range. Pairwise comparisons: ns, not significant ($P > 0.05$); *: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$.

is a family of plant degraders, and their genomes contain numerous carbohydrate-active enzymes and sugar transport genes [28,29]. In addition, members of *Ralstonia* (from *Proteobacteria*) are soil-borne phytopathogens that colonize the plant vascular system [30]. These bacteria probably entered the gut microbial community when their host plants were ingested by the takins. In fact, these seasonal changes in the gut microbiome of wild Sichuan takins are in line with results in our previous study on wild herbivorous and omnivorous cattle, which also showed that the changes in their microbiomes were closely related to the diet of the cattle [31].

Both known and unknown metabolic pathways of bacteria in the gut microbiome in the wild Sichuan takins have been activated and

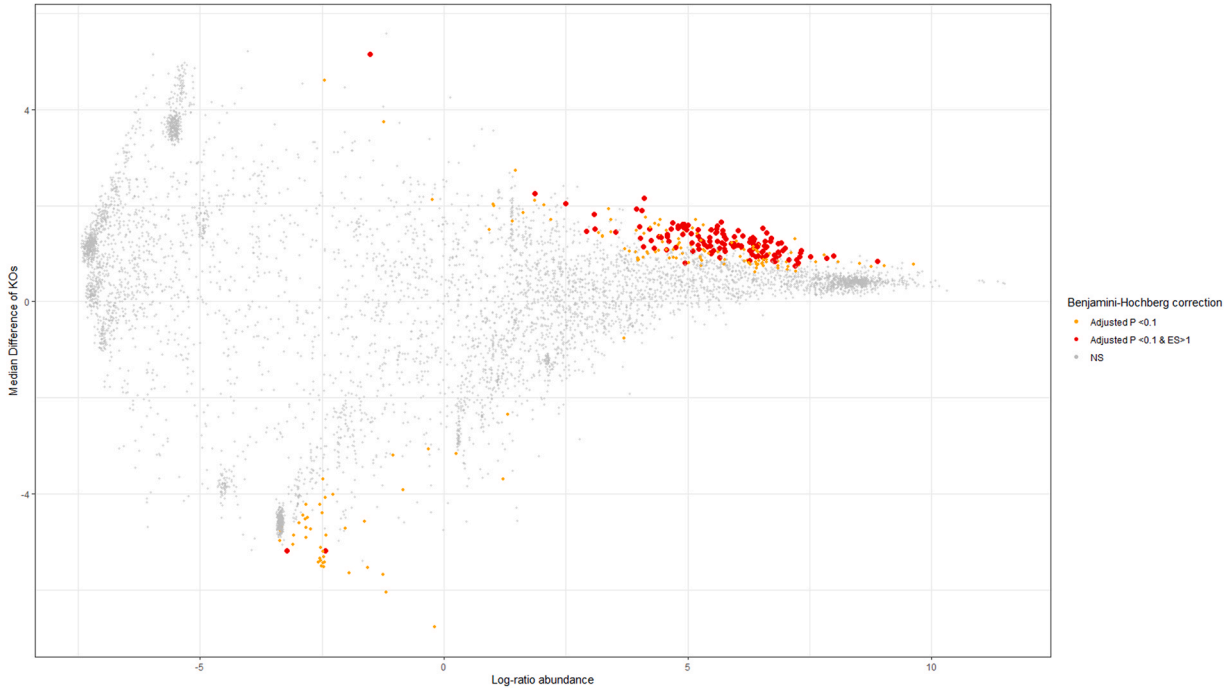


**Fig. 5.** MA plot generated with ALDEx2. The plot shows significantly distinct KOs between spring and winter samples, determined by PICRUST2. KOs with BH adjusted $p < 0.1$ and effect size > 1 were considered to be significantly distinct.

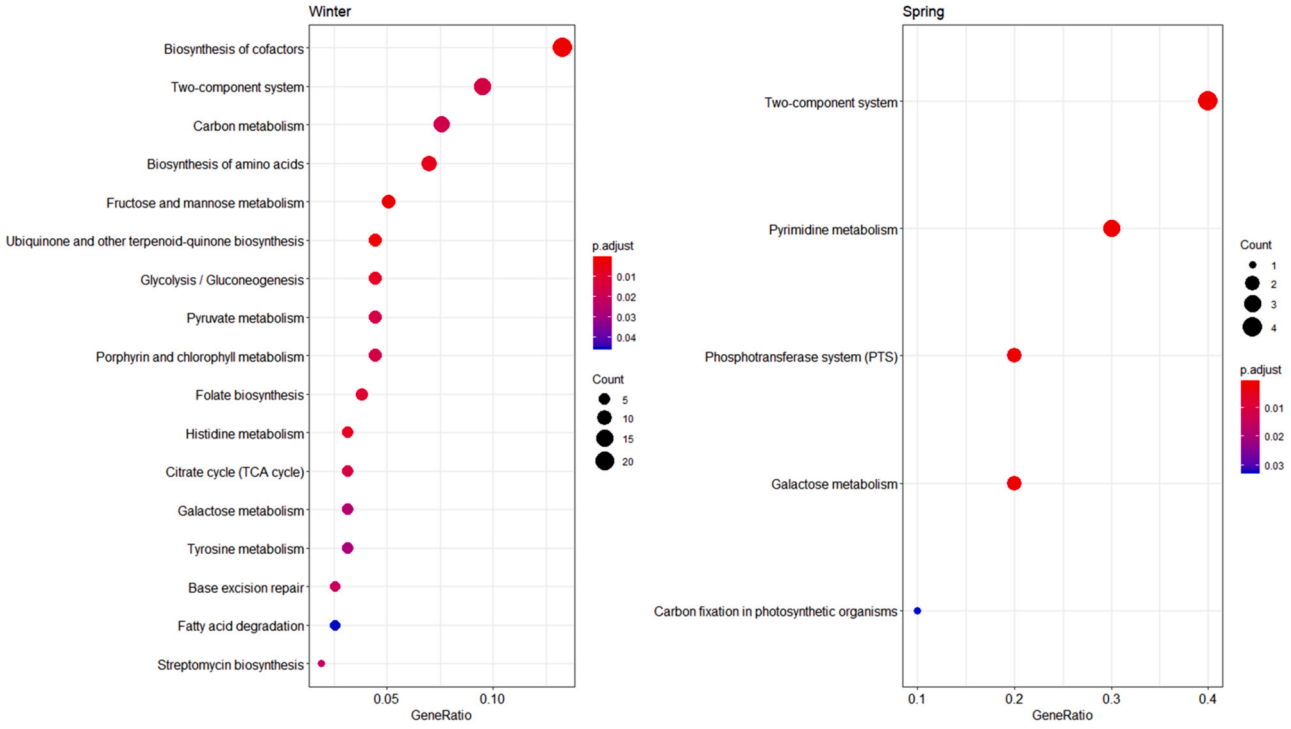


**Fig. 6.** Over-representation analysis of significant KOs (P < 0.1).

deactivated during seasonal change. The gut microbiome of animals produces a variety of metabolites that act as signaling molecules to influence host physiology. Therefore, the microbial composition could reflect underlying metabolic functions. In this study, our *in silico* functional analysis found over 300 seasonally associated KOs, but only half could be mapped to 20 potential KEGG pathways, indicating that a significant amount of metabolic function is still unexplored. Among these 20 pathways, biosynthesis of cofactors and two-component system pathways had relatively high gene count ratios, implying that they may play an important role in seasonal change (Fig. 6). Interestingly, two-component system pathways appear to exhibit season-specific profiles (Fig. S4). Therefore, we speculate that specific pathways must have been activated/deactivated during seasonal change, leading to up- or down-regulation in gene transcription and hence protein levels, ultimately leading to physiological changes in the animals that help them adapt to the environment. To follow up on this association between metabolic pathways and seasonal change, future transcriptomic and metabolomic studies focusing on specific mRNA, proteins and other small molecules as a more robust evaluation of metabolic function for verifying the predicted pathways are warranted [32–34].

## 5. Conclusions

In conclusion, our data suggest that seasonal variation affects the gut microbiomes in wild Sichuan takins. Winter is associated with lower species diversity, probably due to lower nutrient intake. In spring, cellulose-degrading genera and phytopathogens were enriched, indicating frequent ingestion of plant materials, and potentially reflecting the physiological status of wild Sichuan takins. Functional analysis revealed diverse differences in pathways of two-component regulatory systems, suggesting that seasonal change triggers different responses in the gut microbiomes. Overall, the present findings have improved the current understanding of the co-evolution and seasonal adaptations of wild Sichuan takins and their gut microbiomes.

## CRediT authorship contribution statement

**Tian-Pei Guan:** Conceptualization, Methodology, Resources, Funding acquisition, Writing - Review & Editing. **Jade LL Teng:** Supervision, Resources, Validation, Formal analysis, Project administration, Writing - Original Draft, Writing - Review & Editing. **Jordan YH Fong:** Validation, Formal analysis, Data Curation, Visualization, Project administration, Writing - Original Draft, Writing - Review & Editing. **Patrick CY Woo:** Supervision, Resources, Formal analysis, Writing - Original Draft, Writing - Review & Editing. **Susanna KP Lau:** Writing - Review & Editing.

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## Ethical statement

Collection of fecal samples from the wild Sichuan takins was approved by the administration of Tangjiahe Nature Reserve.

## Declaration of competing interests

The authors declare that they have no competing interests.

## Author Contributions

TPG conceived and designed the study and collected samples. TPG, JLLT, and PCYW contributed reagents, materials, and analytical tools. JLLT and JYHF analyzed the data. JYHF performed the laboratory work. TPG, JLLT, JYHF, and PCYW wrote the manuscript. SKPL

revised the manuscript. All authors read and approved the manuscript.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.csbj.2022.12.035.

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