



RESEARCH ARTICLE

Association of Intestinal Fungal Dysbiosis Related to *blastocystis* sp. in Yaks on Plateau

Danqu Lamu^{1,2*}, Xialing Zhao^{1,2}, Chenyang Xia^{1,2}, Wujin Cuomu^{1,2}, Yundan Ciren³ and Rongsheng Mi⁴

¹Institute of Animal Husbandry and Veterinary Medicine, Xizang Academy of Agricultural and Animal Husbandry Sciences, Lhasa 850009, China; ²Key Laboratory of Animal Genetics and Breeding on Tibetan Plateau, Ministry of Agriculture and Rural Affairs, Lhasa 850009, China; ³Municipal Agricultural and Animal Husbandry Science Research and Extension Center of Shigatse, Shigatse 857000, China; ⁴Key Laboratory of Animal Parasitology of Ministry of Agriculture, Laboratory of Quality and Safety Risk Assessment for Animal Products on Biohazards (Shanghai) of Ministry of Agriculture, Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Shanghai 200241, China

*Corresponding author: Yyxf521369@163.com

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ABSTRACT

Blastocystis sp. is a zoonotic protozoan that infects various animals, including plateau yaks. We conducted this study to compare the microbiota of *Blastocystis* sp.-positive and -negative yaks on the cold plateau. Fecal samples of *Blastocystis* sp. positive and negative yaks were assigned to BSP and CON groups and subjected to amplicon sequencing. A total of 2021402 (BSP) and 2702263 (CON) raw reads, and 1973301 (BSP) and 2625224 (CON) filtered reads, were obtained. Alpha diversity evaluation showed that Pielou ($P < 0.01$), Shannon ($P < 0.01$), and Simpson ($P < 0.05$) in BSP yaks were significantly lower than in CON animals. Beta diversity analysis revealed significant differences in PCA ($P < 0.01$), PCoA ($P < 0.01$), NMDS ($P < 0.05$), UPGMA, and Anosim ($P < 0.01$) between the two yak groups. Microbiota biomarker analysis identified thirty-six distinguished genera including *Aspergillus*, *Wallemia*, *CoPrinellus*, *Mucor*, *Sarocladium*, *Coniochaeta*, and *Anaeromyces* between *Blastocystis* sp. positive and negative yaks. Our results unveil potential avenues for the development of effective therapies against *Blastocystis* sp.

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INTRODUCTION

The *Blastocystis* sp. belongs to class of Blastocystea, a well-known parasitic pathogen which is mainly detected in the intestines and other digestive organs of humans and various animals (Zanetti *et al.*, 2020). The prevalence of *Blastocystis* sp. in people ranges from 4% to 42% in underdeveloped countries with more than one billion infected individuals (Abedi *et al.*, 2022). These food-borne protozoa are mainly transmitted by ingesting contaminated water and foods (Sun *et al.*, 2024) and cause severe diarrhea, abdominal pain, and inflammatory bowel disease in animals and humans (Zhang *et al.*, 2023). However, infections have been confirmed in health hosts without visible symptoms (Asghari *et al.*, 2021). Individuals with immunodeficiencies tend to have increased occurrences of *Blastocystis* sp. infections (Asghari *et al.*, 2021).

Long-haired yaks are culturally and economically important for highland ethnic groups that live on the

alpine-cold region with altitude over 3000 m (Dong *et al.*, 2023; Ji *et al.*, 2025). There are more than 18 million yaks in China, accounting for about 90% of the world yak population (Fan *et al.*, 2023). Yaks provide highly nutritious meat and milk, bone marrow extracts used in traditional medicine, low-cost fuels (dung), and fur products, while serving as pack animals and playing an important cultural role (Dong *et al.*, 2023; Lu *et al.*, 2023). However, parasitic diseases are serious health threats for yaks, leading to reduced productivity and economic losses (Ji *et al.*, 2025). *Blastocystis* sp. is a common pathogen common in plateau yaks, with a prevalence rate of 27.07% to 87.9% (Ren *et al.*, 2019; Ji *et al.*, 2025).

Trillions of indigenous microbes form gut microflora including bacteria, fungi, and protozoa, and are highly associated with host functions including metabolism, immunity and development of the nervous system (Zmora *et al.*, 2019). Gut microbiota imbalance, commonly called dysbiosis, can increase inflammation, disrupt intestinal

barrier, and cause immune dysfunction (Parhizkar *et al.*, 2025). Previous studies have reported dysbacteriosis in *Cryptosporidium parvum* infected yaks and mice (Lu *et al.*, 2023; Xu *et al.*, 2025), *Toxoplasma gondii* infected mice (Yan *et al.*, 2022), and *Giardia* spp. infected mice (Fekete *et al.*, 2024). Available data suggest that yaks infected with *Blastocystis* sp. May have different gut microflora when compared to non-infected ones. Therefore, we conducted this study to comparatively analyze the fungal microbiota of *Blastocystis* sp. positive and negative yaks on the cold plateau.

MATERIALS AND METHODS

Blastocystis sP. Examination: In Damxung county, Xizang, fecal samples of 49 diarrheic yaks were collected and examined for *Blastocystis* sp. via 18S SSU rRNA amplification and sequencing as our previous study reported (Ji *et al.*, 2025).

Microbiota sequencing of yaks: *Blastocystis* sp. positive fecal samples (n=6,) and negative samples (n=6) were assigned to BSP (BSP1-6) and CON (CON1-6) groups, respectively, and total DNA extraction from the samples using the MP FastDNA Extraction Kit (MP Biomedicals, USA). The quality of DNA samples was evaluated by agarose gel (1.5%) electrophoresis and NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) as previously reported (Lu *et al.*, 2023; Xu *et al.*, 2025). After that, those DNA products from BSP and CON yaks were piloted for ITS1 amplification by targeting the ITS1F/ITS2R gene fragment (Li *et al.*, 2020). Later, all the amplicons from yaks were subjected to Illumina sequence using an MiSeq sequencer at Bioyi Biotechnology Co., Ltd (Wuhan, China).

Bioinformatics study of yaks: All the raw sequencing data was analyzed using QIIME2 (2023) and R packages (v4.2.2). Briefly, sequenced demultiplexed ITS1 reads were processed in QIIME2. Primer sequences were removed using Cutadapt with a primer mismatch error of 0.2. Raw sequences were quality-filtered with a minimum Phred score of 20, a quality window size of 3 bp, and a minimum retained length fraction of 0.5. Denoising was performed via DADA2 without fixed truncation length (trunc-len = 0), setting the maximum expected error to 2 and truncating reads at a Phred quality threshold of 20. Low-abundance ASVs were filtered by removing features with a total frequency below 10 and those present in fewer

than 2 samples. First, data quality control was performed via DADA2 plugin for raw data demultiplex and merging, followed by Non-singleton amplicon sequence variants (ASVs) generation through aligning yak sequences with mafft (Kato and Standley, 2013). Secondly, taxonomy was assigned to yak ASVs by employing classify-sklearn method in QIIME2 targeting the UNITE database link (<https://unite.ut.ee/>) (Nilsson *et al.*, 2019). Thirdly, α -diversity metrics containing Chao1, Shannon, Simpson index, and etc. of BSP and CON yaks were met via QIIME2 (Chen *et al.*, 2024). Beta diversity analysis of yaks' sequence data in both groups was performed to compare microbiome structure variation by using metrics of Jaccard and Bray-Curtis, and later visualized through analysis of nonmetric multidimensional scaling (Chen *et al.*, 2024), principal coordinate (Xu *et al.*, 2025), principal component (Lu *et al.*, 2023), and unweighted pair-group method with arithmetic means hierarchical clustering (Deng *et al.*, 2021). The statistical differences among gut microflora structure between BSP and CON yak groups were examined using ANOSIM. Shared and unique ASVs between the BSP and CON groups were determined using a Venn diagram using the R package, and the compositions and abundances of different microbiota taxa were visualized by employing GraPhlAn (Aya *et al.*, 2024). The biomarker ASVs and fungi between BSP and CON yaks were examined via analysis of ZicoSeq and T-test (Xu *et al.*, 2025; Zeyneb *et al.*, 2025). The gut microbial functions of yaks were predicted and functionally compared between BSP and CON groups using PICRUSt2 by aligning databases of MetaCyc and KEGG (Kanehisa *et al.*, 2017; Caspi *et al.*, 2020; Douglas *et al.*, 2020).

Statistical Analysis: Statistical analysis between BSP and CON groups was performed using SPSS (28.0). All results of yaks were depicted as means \pm standard deviation whereas, $P < 0.05$ was considered as statistically significant.

RESULTS

Blastocystis sp. positive samples from yaks: Twenty-six yak samples were positive for *Blastocystis* sp., and among them six genomes were successfully sequenced. Those sequences have high homology (99.68%-100.00%) with available references (PP439446.1, OR117671.1) and were deposited into NCBI database with accession numbers PX934862-PX934867.

Table 1: Sequencing information of yaks in different groups

| Samples | Input | Filtered | Denoised | Merged | Non-chimeric | Non-singleton |
|---------|--------|----------|----------|--------|--------------|---------------|
| BSP1 | 336596 | 329053 | 328604 | 311662 | 301328 | 301328 |
| BSP2 | 319762 | 311916 | 310808 | 293729 | 278206 | 278206 |
| BSP3 | 304970 | 298190 | 297552 | 287775 | 279905 | 279905 |
| BSP4 | 355368 | 345978 | 345070 | 319979 | 312885 | 312884 |
| BSP5 | 417729 | 407826 | 407091 | 394647 | 383548 | 383548 |
| BSP6 | 286977 | 280338 | 279815 | 259576 | 251409 | 251409 |
| CON1 | 421131 | 409429 | 408700 | 390301 | 380619 | 380618 |
| CON2 | 441677 | 429889 | 429390 | 385758 | 376308 | 376308 |
| CON3 | 499879 | 484603 | 483259 | 465167 | 449024 | 449024 |
| CON4 | 354226 | 345126 | 344254 | 324173 | 309642 | 309642 |
| CON5 | 518106 | 502922 | 502049 | 470390 | 455897 | 455897 |
| CON6 | 467244 | 453255 | 452743 | 411496 | 401024 | 401023 |

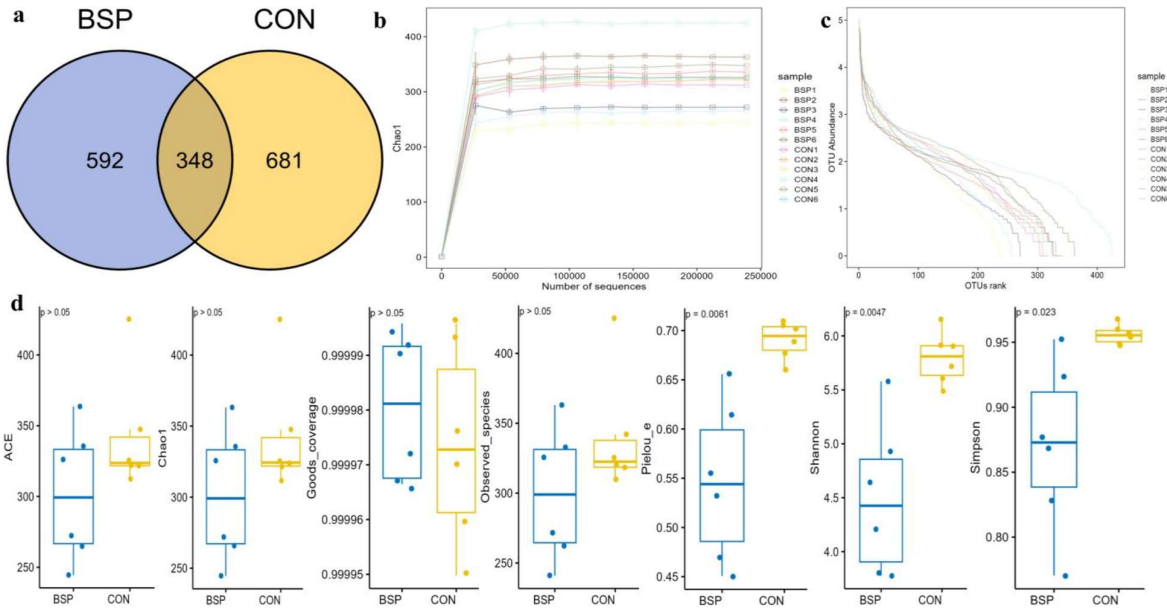


Fig. 1: Venn diagram and α -diversity analysis of yaks among various groups. (a) Venn diagram, (b) Rarefaction curve, (c) Rank abundance curve, (d) Index.

Table 2: α -diversity analysis of yaks among various groups

| Samples | Chao1 | ACE | Good's Coverage | No. of species Observed | Pielou | Shannon | Simpson |
|---------|------------------|------------------|-------------------|-------------------------|-------------------|------------------|-------------------|
| BSP1 | 244.5 | 244.509279988514 | 0.999966504492585 | 241 | 0.531670997722883 | 4.20705376826415 | 0.868104310233324 |
| BSP2 | 363.090909090909 | 363.562327172741 | 0.999991626123146 | 363 | 0.655895235683528 | 5.57761878663437 | 0.952240615950947 |
| BSP3 | 272.25 | 272.404730330951 | 0.999991626123146 | 272 | 0.470513980442498 | 3.80526433311749 | 0.828685590086471 |
| BSP4 | 265.5 | 264.816305320644 | 0.999970691431012 | 262 | 0.613340785335457 | 4.92722597269491 | 0.922985294102135 |
| BSP5 | 335.8 | 335.598978723731 | 0.999966504492585 | 333 | 0.450755629821152 | 3.77705197335894 | 0.770450984168597 |
| BSP6 | 326 | 326.228829978086 | 0.999995813061573 | 326 | 0.556354352132052 | 4.64485124337385 | 0.877565508304684 |
| CON1 | 311.75 | 312.498349781112 | 0.999970691431012 | 310 | 0.677492590208911 | 5.60701296022043 | 0.949195644145052 |
| CON2 | 323.625 | 321.682636753635 | 0.999958130615731 | 318 | 0.687583760239441 | 5.71580332082478 | 0.95678549587332 |
| CON3 | 321.25 | 321.871700469657 | 0.999974878369439 | 320 | 0.659228533390967 | 5.48605245297768 | 0.947051085019711 |
| CON4 | 425 | 425.245063150942 | 0.999995813061573 | 425 | 0.704705687036249 | 6.1530101764912 | 0.959690035459473 |
| CON5 | 347.5 | 347.418396638516 | 0.999949756738877 | 342 | 0.701439911679963 | 5.90461772457652 | 0.953956230160736 |
| CON6 | 325.066666666667 | 325.525064440523 | 0.999991626123146 | 325 | 0.70836002640234 | 5.91076566964018 | 0.96716443239305 |

Sequence information and alpha diversity analysis of yaks:

A total of 2021402 (BSP) and 2702263 (CON) raw reads, and 1973301 (BSP) and 2625224 (CON) filtered sequences were obtained (Table 1). Those sequences were assigned to 1621 ASVs, with 348 that were shared between the two yak groups (Fig. 1a). The rarefaction curves of BSP and CON yaks peaked rapidly and maintained horizontal lines, which illustrated that the sequencing depth of yak samples were sufficient (Fig. 1b). The rank abundances of yaks decreased gradually, which indicated the high evenness of animals (Fig. 1c). Alpha diversity evaluation showed that Pielou, Shannon, and Simpson with P-values less than 0.01 in BSP yaks were significantly lower than those in CON animals (Table 2, Fig. 1d).

Comparing analysis of the microbiota of yaks in different groups:

At the phylum level, Ascomycota (BSP=48.80%, CON=45.53%), Basidiomycota (BSP=38.86%, CON=37.69%), and Neocallimastigomycota (BSP=12.23%, CON=16.44%) were the dominant phyla in both yaks' groups (Fig. 2a). At the class level, Tremellomycetes (35.76%), Leotiomycetes (31.07%), and Dothideomycetes (13.43%) were the main classes in BSP yaks, while Dothideomycetes (27.30%), Tremellomycetes

(19.53%), and Neocallimastigomycetes (16.53%) were the dominate classes in CON yaks (Fig. 2b). At the order level, Filobasidiales (35.45%), Thelebolales (31.03%), and Neocallimastigales (12.24%) were the dominant orders in BSP yaks, while Pleosporales (26.82%), Filobasidiales (19.51%), and Neocallimastigales (16.58%) were the foremost orders in CON yaks (Fig. 2c). At taxonomic level, the families including Filobasidiaceae (35.54%), Thelebolaceae (31.10%), and Neocallimastigaceae (12.27%) were the dominant families in BSP groups, while Sporormiaceae (23.56%), Filobasidiaceae (19.60%), and Neocallimastigaceae (16.70%) were the foremost families in CON group (Fig. 2d). At the genera level, *Naganishia* (51.46%), *Cladosporium* (11.94%), and *Caecomyces* (7.35%) were the dominant genera in BSP yaks, while *Naganishia* (21.28%), *Preussia* (18.30%), and *Caecomyces* (8.49%) were the primary genera in CON yaks (Fig. 2e).

Biomarker fungi in yaks between groups of BSP and CON:

The β -diversity analysis indicated clear differences between yaks in BSP and CON groups by employing analysis of PCA ($P < 0.01$), PCoA ($P < 0.01$), NMDS ($P < 0.05$), UPGMA, and ANOSIM ($P < 0.01$) (Fig. 3). T-test analysis showed that ASV735 ($P < 0.01$),

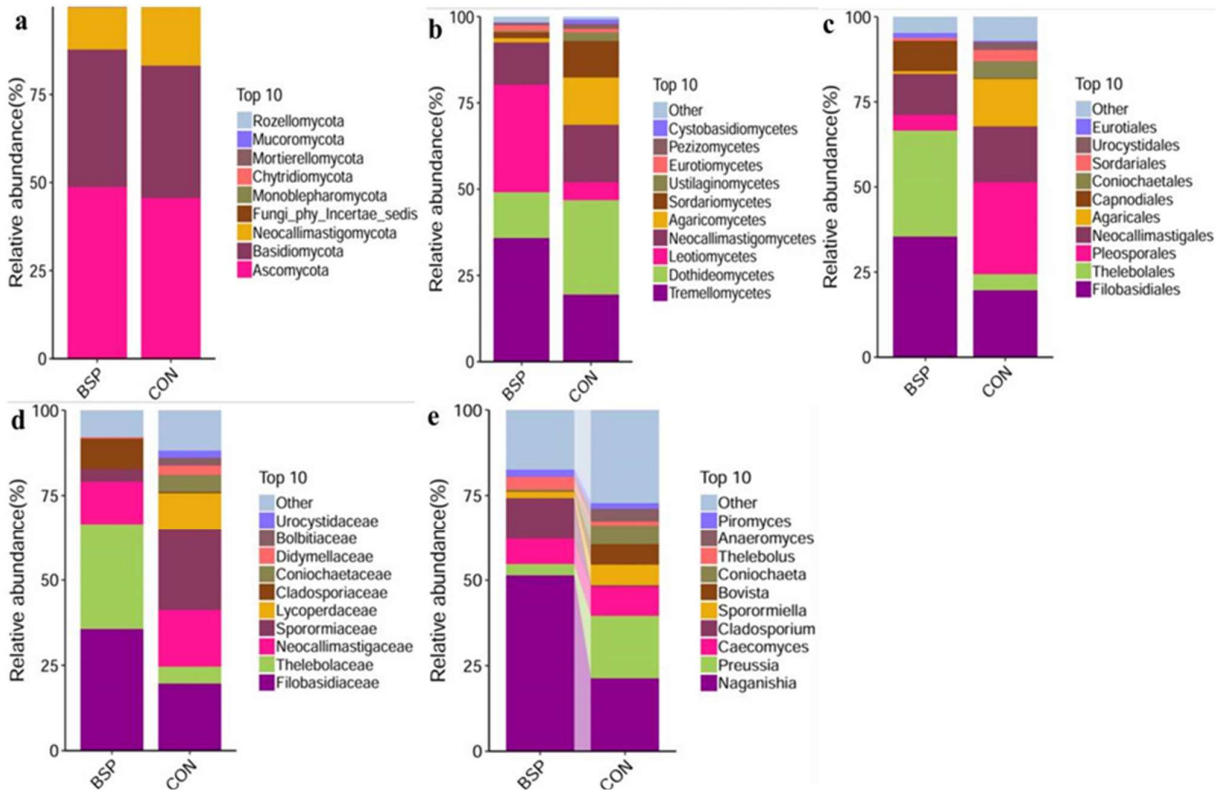


Fig. 2: Microbiota comparative analysis of yaks among various groups. (a) Phylum, (b) Class, (c) Order, (d) Family, (e) Genera.

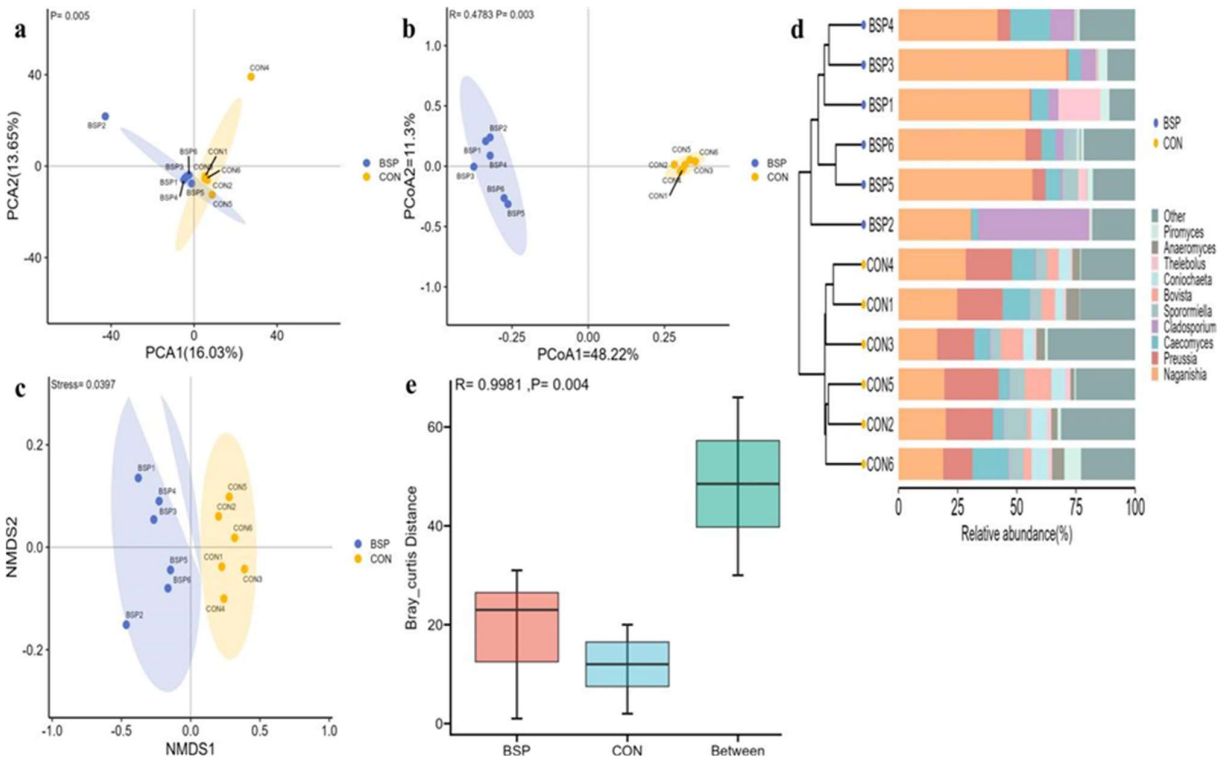


Fig. 3: β-diversity analysis among various groups. (a) PCA, (b) PCoA, (c) NMDS, (d) UPGMA, (e) ANOSIM.

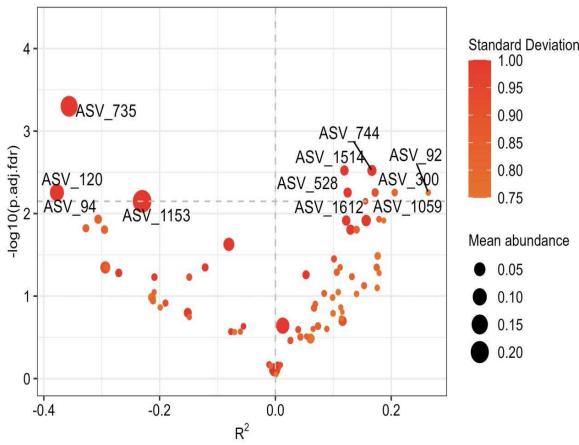


Fig. 4: Exploring distinguished ASVs between BSP and CON yaks by piloting ZicoSeq analysis.

ASV94 ($P < 0.05$), ASV120 ($P < 0.05$), and ASV1153 ($P < 0.05$) were markedly higher in BSP yaks, while ASV744 ($P < 0.01$), ASV1514 ($P < 0.01$), ASV528 ($P < 0.05$), ASV1059 ($P < 0.05$), ASV300 ($P < 0.05$), ASV92 ($P < 0.05$), and ASV1612 ($P < 0.05$) were significantly higher in CON yaks (Fig. 4). T-test showed that *Naganishia* ($P < 0.001$), *AsPergillus* ($P < 0.05$), *Neocallimastix* ($P < 0.05$), *Walleimia* ($P < 0.01$), *CoPrinellus* ($P < 0.05$), *Candida* ($P < 0.01$), *Vishniacozyma* ($P < 0.05$), *Meyerozyma* ($P < 0.05$), *Geosmithia* ($P < 0.05$), *Mucor* ($P < 0.05$), and *Sarocladium* ($P < 0.05$) were obviously higher in BSP animals, while *Preussia* ($P < 0.0001$), *SPorormiella* ($P < 0.05$), *Bovista* ($P < 0.01$), *Coniochaeta*

($P < 0.0001$), *Anaeromyces* ($P < 0.001$), *Disciseda* ($P < 0.001$), *Panaeolus* ($P < 0.01$), *Urocystis* ($P < 0.01$), *Neosascochyta* ($P < 0.0001$), *Cystobasidium* ($P < 0.05$), *Lasiobolus* ($P < 0.05$), *Trichocladium* ($P < 0.001$), *SPorormiaceae gen Incertae sedis* ($P < 0.05$), *Gelasinospora* ($P < 0.05$), *Deconica* ($P < 0.05$), *Doratomyces* ($P < 0.05$), *NothoPhoma* ($P < 0.01$), *Periconia* ($P < 0.001$), *Microascus* ($P < 0.05$), *Gamsia* ($P < 0.05$), *Xenodidymella* ($P < 0.01$), *Neostagonospora* ($P < 0.05$), *Plenodomus* ($P < 0.05$), *Paraconiothyrium* ($P < 0.05$), and *Aureobasidium* ($P < 0.01$) were significantly higher in CON yaks (Fig. 5).

Network and fungi function examination of yaks among various groups:

Network investigation showed that *Arcuadendron* was positively correlated with *Mycosylva*, *Mortierella*, *Bradymyces*, and *Bolbitius*. *Coniochaeta* was positively correlated to *Panaeolus*, and *Periconia*. On the other hand, *Neostagonospora* was negatively associated with *Xenodidymella* (Fig. 6). Function comparison analysis of MetaCyc pathways showed that the abundance of PWY-7118 ($P < 0.05$), PWY-6317 ($P < 0.05$), PWY-7385 ($P < 0.05$), TYRFUMCAT-PWY ($P < 0.05$), NONOXIPENT-PWY ($P < 0.05$), and PWY-5659 ($P < 0.05$) were significantly more abundant in BSP yaks, while PWY-5189 ($P < 0.05$), THRESYN-PWY ($P < 0.05$), PWY-7282 ($P < 0.05$), PWY-5920 ($P < 0.05$), PWY-5690 ($P < 0.05$), GLYOXYLATE-BYPASS ($P < 0.05$), SO4ASSIM-PWY ($P < 0.05$), PWY-7279 ($P < 0.05$), PWY-3781 ($P < 0.05$), and PWY-6609 ($P < 0.05$) were significantly greater in CON yaks (Fig. 7).

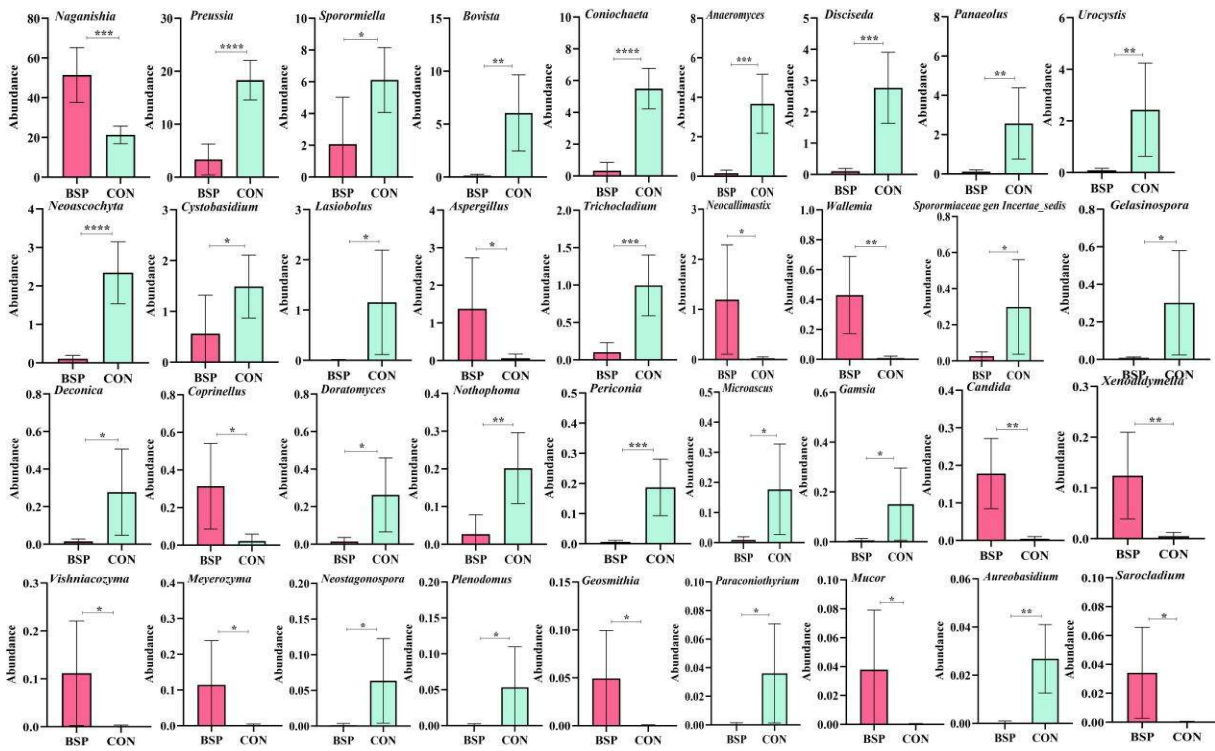


Fig. 5: Exploring fungi difference between BSP and CON yaks by using T-test. Significance is presented as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$; data were depicted as the mean \pm SEM ($n = 6$).

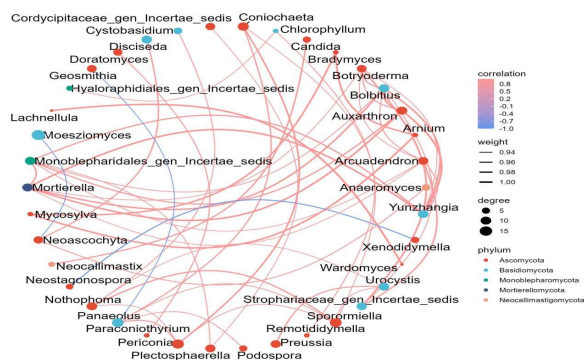


Fig. 6: Network analysis of yaks in different groups.

DISCUSSION

Yaks are important food animals; therefore, effective breeding and disease prevention control programs are pivotal for development of the yak industry. In this study, six *Blastocystis* sp. positive yaks from 49 animals were screened and ITS amplicon sequencing of protozoa-positive and negative yaks was performed. There were 4 723 665 raw and 4 598 525 filtered sequences obtained from yaks (Table 1). Alpha diversity indexes of Pielou ($P < 0.01$), Shannon ($P < 0.01$), and Simpson ($P < 0.05$) were differed significantly between the two yak groups (Fig. 1d), which was consistent with *C. Parvum* infected pigs (Chen *et al.*, 2023), *Blastocystis* sp. infected people (Even *et al.*, 2021), and *Eimeria* spp. infected chickens (Liu *et al.*, 2024), but not in line with *C. Parvum* infected yaks (Lu *et al.*, 2023). These results suggest that parasitic infection may reduce the diversity and evenness of the host microbiota.

Beta diversity analysis revealed significant differences between yaks in BSP and CON groups (Fig. 3), which demonstrates structural difference of microbiota in different animals. Our results are in agreement with *C. Parvum* infected animals and *Blastocystis* sp. infected humans (Even *et al.*, 2021; Dong *et al.*, 2023). Furthermore, microbiota difference was confirmed by taxa analysis (Fig. 2). The intestine microbiota shifts also affected their functions in yaks, with nineteen distinguished pathways (Fig. 7). Among them, *Blastocystis* sp. infection enriched six pathways in animals, but decreased thirteen MetaCyc pathways in infected animals. Thirty-six genera were markedly different between BSP and CON yaks. Among them was *AsPergillus*, an opportunist pathogenic genus infecting people and animals (Barac *et al.*, 2024). Higher abundance of *Walleimia*, *CoPrinellus*, *Mucor* and *Sarocladium* was previously reported in rheumatoid arthritis patients (Sun *et al.*, 2022), *C. Parvum* infected yaks (Lu *et al.*, 2023), adults with non-alcoholic steatohepatitis (Demir *et al.*, 2022) and people with chronic pulmonary disease (Martinsen *et al.*, 2021). *Candida* is a pathogenic genus positively associated with cancer (Dohman *et al.* 2022). There are higher levels of incidence of that genus in BSP yaks, which many contribute to the infection status in yaks. *Coniochaeta* is related to degraded cellulose (Jiménez *et al.*, 2020), and *Anaeromyces* is associated with amino acid and

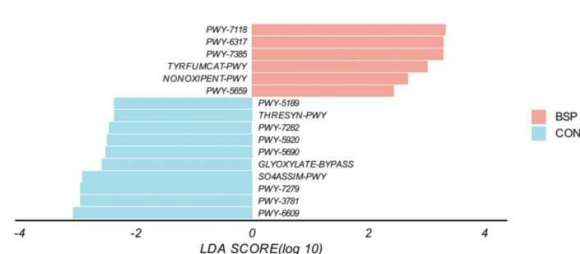


Fig. 7: Functional comparative analysis of MetaCyc pathways of yaks in different groups.

carbohydrate metabolism (Yang *et al.*, 2024). The lower abundance of these two genera in BSP yaks may indicate that *Blastocystis* sp. decreased substance metabolism in yaks. Lower abundance of *Urocystis* in *Blastocystis* sp. positive yaks was in line with *C. Parvum* infected yaks (Lu *et al.*, 2023), while yaks with this genus inversely correlated with parasite infection. Higher abundance of *Neoscochyta*, *Aureobasidium*, and *Lasiobolus* were examined in healthy hosts (Lu *et al.*, 2023; Li *et al.*, 2024; Xu *et al.*, 2024) while the lower abundance of those genera may be associated with the infection of *Blastocystis* sp. in yaks.

Conclusions: In conclusion, *Blastocystis* sp. was associated with intestinal fungal dysbiosis in yaks, with thirty-six biomarker genera including *AsPergillus*, *Walleimia*, *CoPrinellus*, *Mucor*, *Sarocladium*, *Coniochaeta*, and *Anaeromyces*. As yaks are native reservoirs of *Blastocystis* sp., appropriate measures should be taken to mitigate the threat posed by this parasite on local herdsmen and plateau animals. Our results provide baseline potential avenues for future studies on *Blastocystis* sp.

Data available statement: All of the available raw data of yaks was placed in the NCBI Sequence Read Archive under consent number: PRJNA1417247.

Animal ethics approval: All the experiment procedures were instructed and approved by Xizang Academy of Agricultural and Animal Husbandry Sciences (XAAA202500275).

Authors contribution: DL: research topic and methodology. DL, XZ, XC, WC, YC and MR: solutions, reagents, lab materials and analytical tools. DL: writing martial, drafting and preparation procedures. DL: paper writing, review and rewriting. DL: supervision and visualization. All authors reviewed and approved the final manuscript.

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