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Microecosystem of yak rumen on the Qinghai-Tibetan Plateau is stable and is unaffected by soil or grass microbiota

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Abstract

The rumen of livestock grazing on the Qinghai-Tibetan Plateau (QTP) acts as a transfer station for the circulation of soil, grass, faecal mineral elements and nutrients. Whether the microorganisms from the soil and grass could circulate through livestock rumen and excreted faeces. We studied the structural composition and interactive networks of microbiomes (bacteria and fungi) in soil, grass, and grazing yaks (rumen and faeces) on the QTP by using 16S rRNA gene and internally transcribed spacer (ITS) sequencing technology and to calculate the contribution rate of microorganisms from one habitat to another habitat using SourceTracker analysis. The meta-co-occurrence network revealed that soil, grass, rumen, and faeces comprise four independent habitats. The bacterial and fungal composition was significantly different in these four habitats. Soil microbiota showed the highest alpha diversity and microbial network complexity. Rumen microbiota demonstrated the highest microbial network stability and synergy, while grass endophytes showed the lowest microbial network complexity, stability, and synergy. According to the SourceTracker model, grass contributes 0.02% to the rumen microbes of yaks, while soil microorganisms do not circulate in the rumen. The soil and grass microbiota originating from faeces were 4.5% and 1.2%, respectively. The contribution of soil to grass was found to be 1.1%. Overall, the rumen microbiota of yaks is relatively stable and is only minimally influenced by the microbiota inhabiting the environment under natural grazing conditions. However, the contribution of yaks to soil and grass microbiota is relatively high when compared with the contribution of soil and grass to yaks microbiota.

INTRODUCTION

The rumen of ruminants is a complex habitat that comprises abundant bacteria, fungi, and protozoa, which perform important functions, such as feed fermentation (Zhou et al., 2017), immunity regulation (Martin et al., 2010), disease prevention (Tlaskalova-Hogenova et al., 2011), energy balance (Shabat et al., 2016), and host adaptation. However, neither the source of the microbiome nor its composition is well understood. The colonization of the gut and rumens of new borns are well studied. Bi et al. (2021) reported an active microbiome in the intestines of lambs before birth and Zhu et al. (2021) reported that the intestinal microbes of dairy calves originate from the mother. In addition, Henderson et al. (2015), Huws et al. (2016), and Malmuthuge et al. (2019) studied on how to modify existing gut and rumen microbiomes.

The Qinghai-Tibetan Plateau (QTP) is the highest altitude grassland and is the largest continuous grazing area in the world, accounting for 44% of the 40 million hectares of grassland in China. The plateau is home to

approximately 20 million yaks (*Bos grunniens*). Yaks graze on natural pastures of traditional ranches that lie at least 3000 m above sea level without the need for supplementary feeding, which is of great significance to the livelihoods of Tibetan herdsmen and the management of the local ecosystem (Liu et al., 2021). Livestock ingest forage and large amounts of soil when grazing, and ruminants absorb trace elements predominantly through soil ingestion (Rodrigues et al., 2012). The QTP is a primitive closed environment, and previous studies have reported that the mineral elements and nutrients that are required by yaks come from the soil and herbage and are excreted in the yak faeces before being recycled back into the soil to be re-absorbed by the herbage (Allison et al., 2010, 2013; Zhou et al., 2019). According to Attwood et al. (2019), a large number of microorganisms inhabit soil, grass, and yak faeces. However, it remains unknown whether microorganisms circulate through the soil, grass, and yaks under the grazing conditions of the QTP.

The objective of this study was to investigate the bacterial and fungal community composition and the interaction networks of the soil, grass, rumen, and yak faeces on the QTP and to calculate the contribution rate of microorganisms to the four habitats using SourceTracker. Considering the differences in growth and living conditions generally outside versus inside the rumen, we assumed that a small amount of the microorganisms in the rumens of yaks are originally sourced in the soil and pasture and are returned as manure.

EXPERIMENTAL PROCEDURES

Study sites and animals

All trial procedures of this study have been approved by the Animal Ethics Committee of Lanzhou University (File No: 2010-1 and 2010-2). For the purpose of reducing error, we selected three study sites (Xiahe County, Maqu County, and Naqu County) that had similar geographic conditions, soil type, and vegetation type and had been grazing for decades (Figure S1). The vegetation type in the study area is typical alpine meadow, and the soil type is subalpine meadow. The major species of edible forage are presented in Table S5. Yaks commonly graze in a full-grazing system at the study sites, with forage as the only feed. We selected 12 healthy yaks aged 5 years (284.38 $\pm$ 8.36 kg) at each sampling site for the collection of rumen fluid and faecal samples. A 10-day sampling interval was set to ensure that the forage was at the same phenological stage at all three sampling points. Soil, grass, rumen fluid, and faecal samples were collected from Xiahe County (July 15), followed by Maqu County (July 25), and finally Naqu County (August 5).

Collection and analysis of soil, grass, rumen, and faeces samples

A total of six quadrats (0.5 m $\times$ 0.5 m) were randomly placed at each experimental site. The quadrats were located >50 m apart in order to surpass the space pertinence of the microbial variables; thus, each quadrat could be considered independent of the others (Zhou et al., 2019). Grass samples were cut 5 cm above the ground with scissors, transported to the laboratory in an icebox, and processed as described by Beckers et al. (2017). The samples were cleared of epiphytic bacteria by sequential washing (surface sterilization) with (a) 70% ethanol (40 s), (b) a 2.5% sodium hypochlorite solution (0.1% Tween 80) (10 min), and (c) 70% ethanol (30 s), before rinsing two to three times with sterile water. The grass samples were then stored at -80°C for molecular and chemical composition analyses. After the plant samples were collected, three soil samples were randomly collected from the upper 15 cm of each quadrat using a soil auger with a diameter of 10 cm and were mixed, homogenized, sieved (<2 mm) to remove roots and other plant materials, and stored in an ice box. Thus, six composite soil samples (regarded as sub-samples) were obtained from each of the three experimental sites (forming a total of 18 sub-samples). The soil samples were brought to the laboratory immediately after collection and stored at -80°C for molecular and physical and chemical properties analyses.

Twelve yaks with similar average body weights were selected at each site to collect rumen contents, which were sampled in the morning. A total of 36 samples were collected using an oral stomach tube (Shen et al., 2012). The equipment was cleaned thoroughly with fresh water between each sample collection, and the first 50 ml was discarded to ensure that no contamination occurred (Zhou et al., 2017). Subsequently, the samples (approximately 20 ml) were transported to the laboratory in an ice box and stored at -80°C for DNA extraction and analysis of ruminal fermentation parameters. Faecal samples ($n = 36$) were collected under natural grazing conditions with little disturbance from humans or livestock. Samples were collected immediately after defecation with sterile gloves, and samples for bacterial and fungal DNA preparation were collected from inside the stool under aseptic conditions. The samples were then transported to the laboratory in an ice box and stored at -80°C for DNA extraction and analysis of mineral elements and chemical compositions.

The pH, total phosphorus (TP), organic matter (OM), ammonia nitrogen (NO_3-N), nitrate nitrogen (NH_4-N), moisture, microbial carbon (MBC), and microbial nitrogen (MBN) contents of the soil samples were measured according to Zhou et al. (2019). The dry matter (DM), organic matter (OM), crude protein (CP), ether extract (EE), neutral detergent fibre (NDF), and acid detergent fibre (ADF) contents of the forage samples were

measured using standard methods (Fan et al., 2019). The pH, NH_4-N, and volatile fatty acids concentration of rumen fluid of yaks were analysed using the methods outlined by Shen et al. (2012). The TP, total organic carbon (TOC), total nitrogen (TN), total potassium (TK), NDF, Mg, Ash, and ADF contents of the faecal samples were measured according to Zhou et al. (2019).

DNA extraction, sequencing, sequence processing, and analysis

Soil, forage, rumen, and faecal total DNA were extracted using a PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) and the extracted product was detected using 1% agarose gel electrophoresis. The V3–V4 hypervariable region of the bacterial 16S rRNA gene was amplified with the primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′) (Dennis et al., 2013). PCR was run at 94°C for 5 min, followed by 28 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s, with a final extension at 72°C for 7 min. The fungal internally transcribed spacer (ITS) region was amplified using an Eppendorf Mastercycler Gradient Thermocycler (Germany) with the primers ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2 (5′-TGCGTTCTTCATCGATGC-3′) (Miao et al., 2016). Cycling parameters were 95°C for 5 min, followed by 28 cycles at 95°C for 45 s, 55°C for 50 s, and 72°C for 45 s, with a final extension at 72°C for 10 min.

The bacterial and fungal PCR products were confirmed using 1% agarose gel electrophoresis, and the purified amplicons were pooled in equimolar amounts, paired-end sequenced (2 × 300 bp) on an Illumina MiSeq PE300 platform, and sequenced according to standard protocols by the Allwegene Company (Beijing, China). Paired-end reads of the original DNA fragments were merged using FLASH software (version 1.2.11) and quality-filtered using QIIME software (version 1.9.0; Caporaso et al., 2010). Sequences from mitochondria and chloroplasts were eliminated via gel purification. Effective sequences were retained and reads that could not be assembled were discarded. UPARSE software (version 7.0) was used to cluster unique sequences with 97% or more similarity into operational taxonomic units (OTUs) (Edgar, 2013). Each OTU was annotated using the SILVA database (SSU123) in MOTHUR (Tiedje & Cole, 2007). Samples with the least amount of data were used as standards for normalization. Soil, grass, rumen, and faecal bacterial and fungal community diversities were calculated using QIIME software (version 1.9.0; Caporaso et al., 2010).

Statistical analysis

Statistical analyses were performed using SAS (SAS Institute Inc., version 9.2, USA). Normal distributions were checked using the Shapiro–Wilk test, and the homoscedasticity of the variances was analysed using Levene's test. Significant differences in the variances of the parameters were evaluated, depending on the distribution of the estimated parameters, using the Kruskal–Wallis rank sum test. Post hoc comparisons were conducted using Tukey's honest significant difference tests. The relative abundances of bacteria and fungi in the soil, grass, rumen, and faeces were analysed using analysis of variance (ANOVA). The differences in the microbial communities were analysed by principal coordinate analysis (PCoA) (Lozupone & Knight, 2005). LEfSe was used to identify significantly different bacterial and fungal communities in different habitats (Segata et al., 2011). Four habitats sensitive OTUs were identified according to the method described by Hartman et al. (2018). First, we used correlation based indicator species analysis with the indicspecies package in R 3.2.5 (De Caceres et al., 2010) to calculate the point-biserial correlation coefficient of an OTU's positive association to one or a combination of habitat types. The analysis was conducted with 10^4 permutations and considered significant at $p < 0.05$. Additionally, the likelihood ratio tests (LRT) was used to evaluate the differential OTU abundance among habitat types with the edgeR package (Robinson et al., 2010). Finally, sensitive OTUs were defined as these confirmed by both indicator species analysis and LRT at $p < 0.05$. The co-occurrence networks were constructed using the WGCNA package based on Spearman's correlation matrices, according to the methods reported by Qiu et al. (2021). The Mantel test and Procrustes analysis were performed to summarize the correlations (synergy) between the bacteria and fungi in the soil, grass, rumen, and faeces using the vegan package. M^2 is an important index of Procrustes analysis. The smaller its value is, the stronger the synergy between two groups of data is, and the smaller the distance between two paired sample points is in the visualized figure (Vogl et al., 2021). Random forest model was performed using the randomForest package. Bayesian source tracking was performed to identify the proportion of microbial communities in a target microbial community that were derived from other sources using the SourceTracker algorithm (https://github.com/uo-green-lab/dust-2015) in MacQIIME (v1.9.1) (Knights et al., 2011).

RESULTS

Alpha and beta diversity in the microbial communities of soil, grass, rumen, and faeces

The bacterial and fungal diversities in the soil, grass, rumen, and faeces are shown in Figure 1A,B.

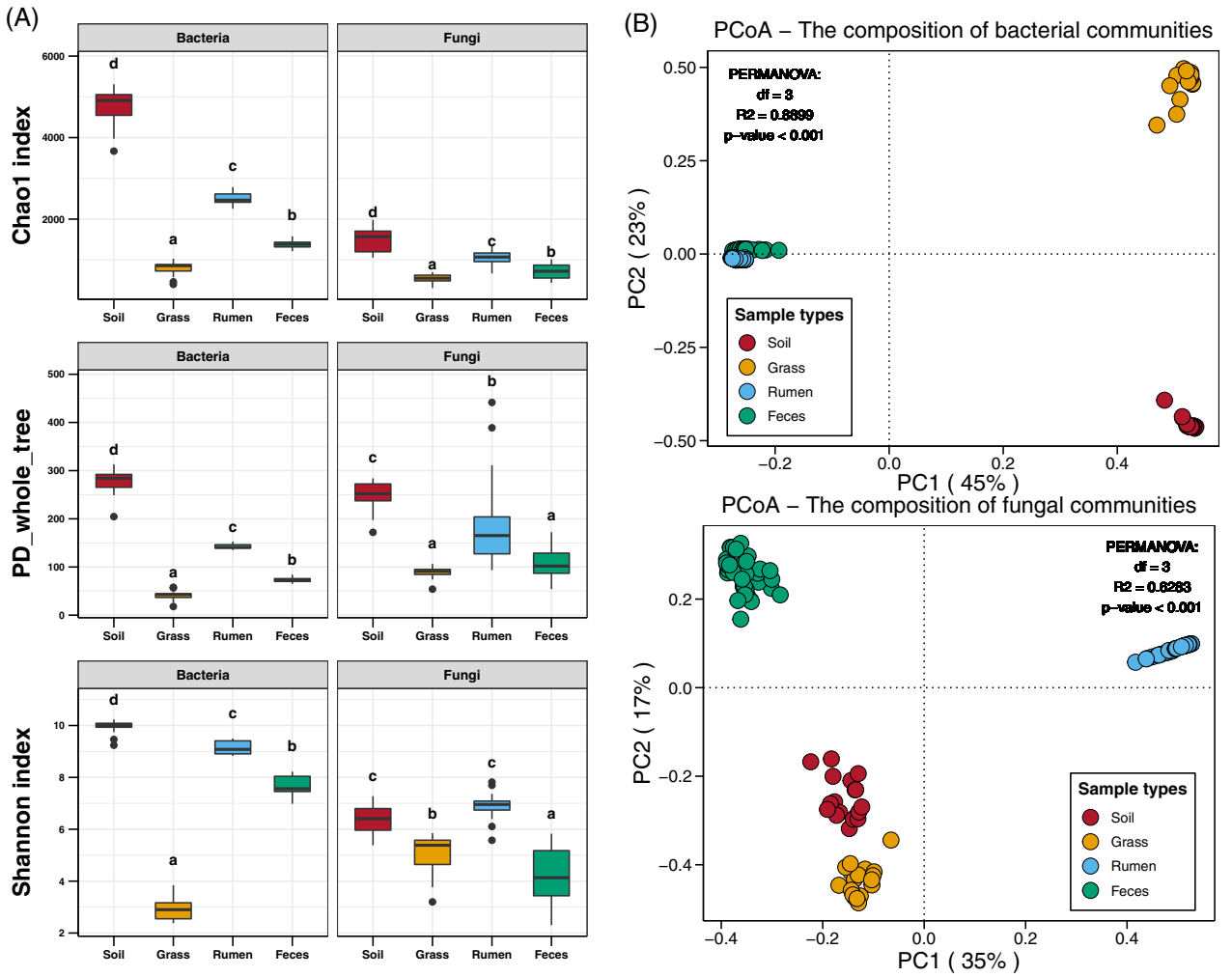

FIGURE 1 Bacterial and fungal diversity indices of the soil, grass, rumen, and faeces. (A) Variations in community alpha-diversities. (B) Community dissimilarities in the soil, grass, rumen, and faeces, calculated using principal coordinates analysis (PCoA). The letters (a, b, c, d) indicates boxes with different superscripts differ significantly ($p < 0.05$).

In general, the highest alpha diversity (Chao 1 and Shannon index) for bacteria was found in the soil, followed by rumen and faeces, with the lowest diversity observed in the grass. In terms of fungal diversity, the highest Chao 1 index was found in the soil, while the lowest was observed in grass; and the lowest Shannon index was observed in faeces (Figure 1A). PCoA indicated significant differences in the bacterial and fungal communities (Figure 1B) in the four habitats. PC1 clustered the bacterial communities of the rumen and faeces together. In addition, PC2 indicated that the bacterial community inhabiting the grass was separate from that in the soil. The fungal communities in the soil and grass were clustered together but were clearly distinguishable by PC2. Additionally, the fungal community in the rumen was clearly separated from that of the faeces by PC1.

Bacterial and fungal community compositions in soil, grass, rumen, and faeces

The bacterial and fungal community compositions at the phylum level are shown in Figure 2A,C, while that at the genus level are shown in Figure 2B,D, respectively. At the phylum level, the compositions in the rumen and the faeces were mainly composed of Firmicutes and Bacteroidetes. Proteobacteria was the dominant bacterial phylum in both soil and grass. At the genus level, *Christensenellaceae* R-7 group was the dominant bacterial genus in both rumen and faeces. *Bromus tectorum* was the dominant bacterial genus in grass. Ascomycota and Basidiomycota dominated the fungal phyla in all four habitats. The highest relative abundance of Ascomycota was observed in the faeces, accounting for 84.3% of the total, while the highest

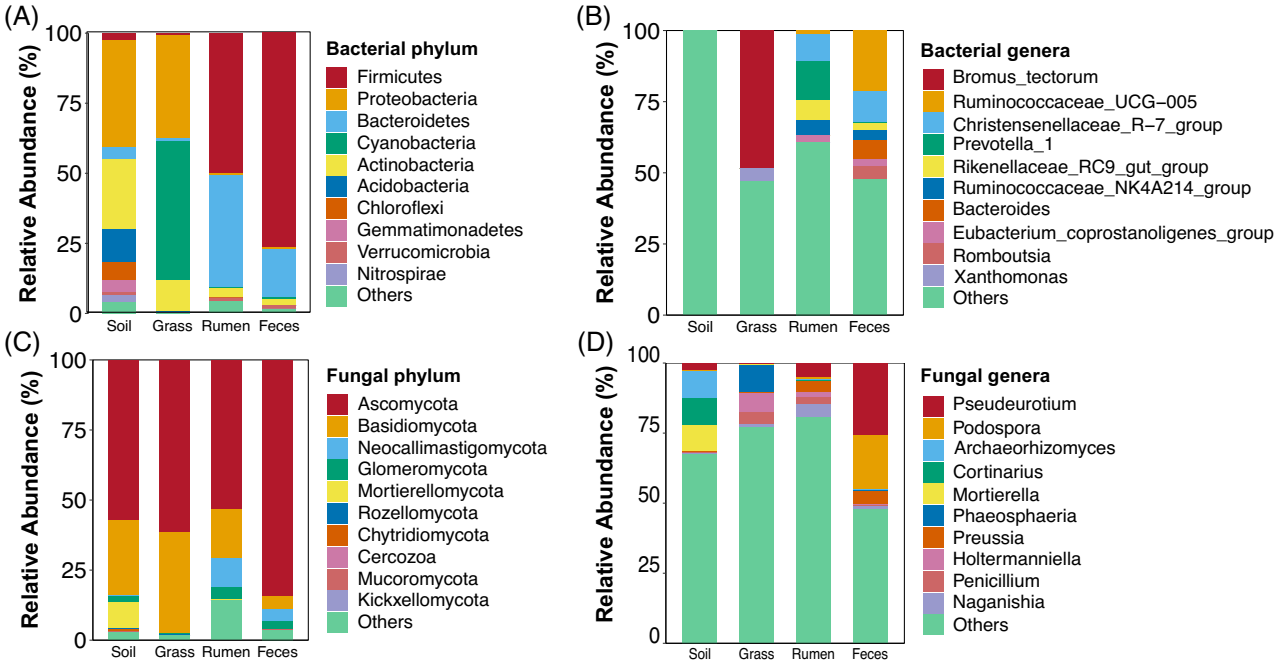

FIGURE 2 The relative abundance of dominant bacterial (A, B) and fungal (C, D) communities at the phyla and genus level in the soil, grass, rumen, and faeces

relative abundance of Basidiomycota was observed in the grass, accounting for 36.0%. *Pseudeurotium* was the dominant fungal genus in the rumen and faeces. *Cortinarius*, *Archaeorhizomyces*, and *Mortierella* were the dominant fungal genus in the soil. *Phaeosphaeria* was the dominant fungal genus in the grass.

Differences in the bacterial and fungal microbial composition of soil, grass, rumen, and faeces

The number of OTUs that were uniquely identified in each specific habitat (soil, grass, rumen, and faeces) and those between the habitats were calculated from the Venn diagram (Figure 3A,B). The proportion of bacterial and fungal OTUs that was shared by all four ecosystems was 0.6% and 3.8%, respectively. A higher overlap was clearly observed in the bacterial OTUs of the soil and grass (13.8%) than that observed for the soil and rumen (1.6%) or the soil and faecal samples (1.6%). A higher overlap was clearly observed in the bacterial OTUs of the soil, grass, and rumen (1.3%) than that observed for the soil, grass, and faecal samples (1.2%). Similarly, a higher overlap was observed between fungal OTUs in the soil and faeces samples (12.5%) than the soil and grass (6.1%) or the soil and rumen (4.7%). A higher overlap was clearly observed in the fungal OTUs of the soil, grass, and rumen (4.7%) than that observed for the soil, grass, and faecal samples (4.1%). Approximately, 43% of all bacterial OTUs were exclusively found in the soil, with lower proportions found in the grass (5.8%), rumen (18.4%), and faecal (9.2%) samples. The 26.7% of all fungal OTUs were exclusively found in the soil, which is much higher than that observed in the grass (6.5%), rumen (21.2%), and faecal (10.2%) samples.

We also performed LEfSe analysis to detect groups or species that were responsible for the significant differences observed among the soil, grass, rumen, and faeces samples. The LEfSe analysis indicated that the composition of the bacterial and fungal communities differed significantly in the soil, grass, rumen, and faeces (Figure 3C,D). A total of 99 bacterial and 134 fungal clades exhibited statistically significant differences in the soil, grass, rumen, and faeces with a linear discriminate analysis (LDA) threshold of 4.0 (Figure S2). The phyla Proteobacteria, Actinobacteria, Acidobacteria, Chloroflexi, Gemmatimonadetes, and Mortierellomycota; and the genera *Inocybe*, *Mortierella*, *Cortinarius*, and *Archaeorhizomyces* were significantly enriched in the soil; while the phyla Cyanobacteria and Basidiomycota; and the genera *Bromus tectorum*, *Holtermanniella*, *Penicillium*, and *Phaeosphaeria* were enriched in the grass. The phyla Bacteroidetes, Neocallimastigomycota, and Glomeromycota; and the genera *Prevotella* 1, *Rikenellaceae* RC9 gut group, *Ruminococcaceae* NK4A214 group, *Naganishia*, *Gigaspora*, and *Pilidium* were enriched in the rumen; while the phyla Firmicutes and Ascomycota; and the genera *Pseudeurotium*, *Podospora*,

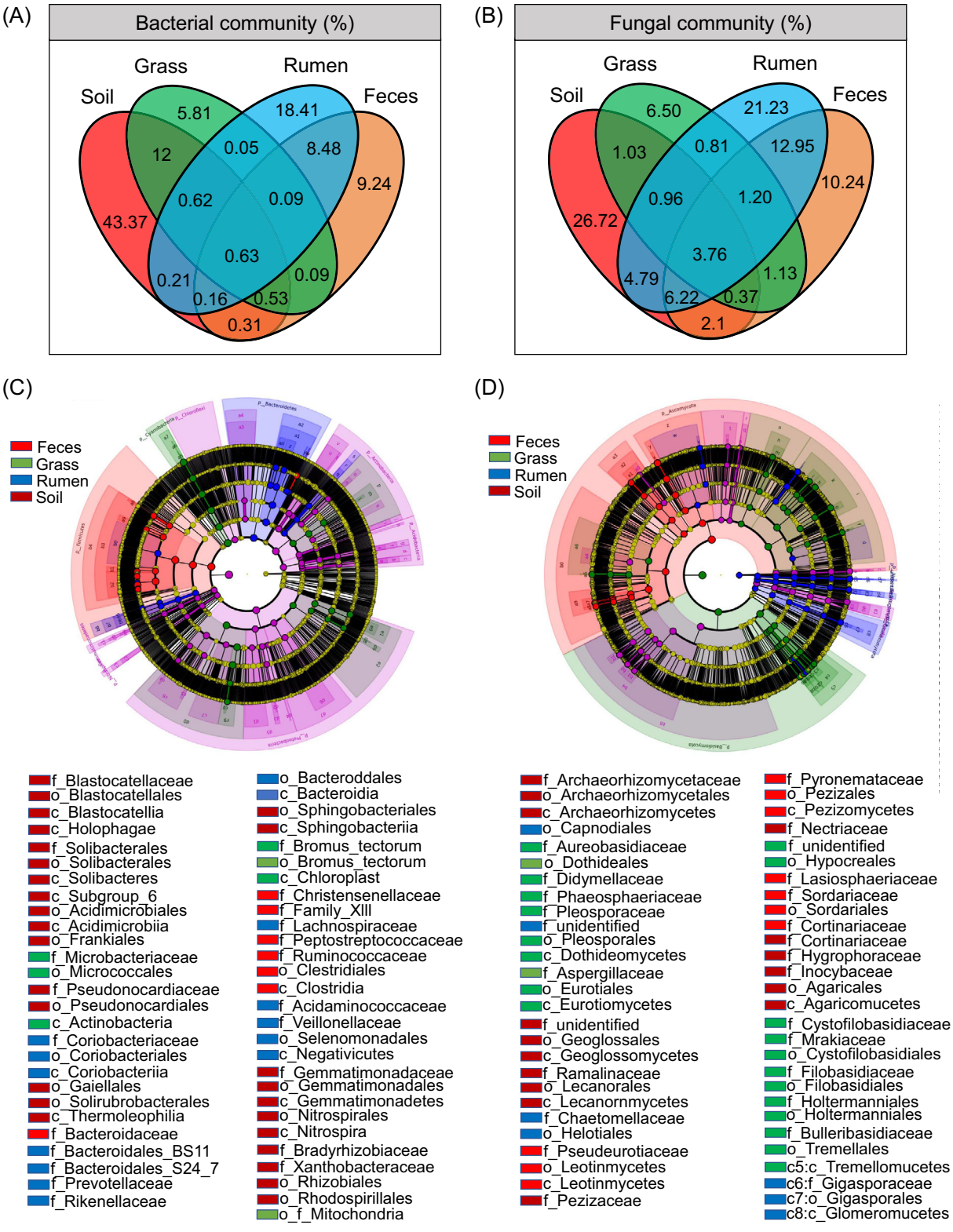

FIGURE 3 Difference in the bacterial and fungal microbial composition of the soil, grass, rumen, and faeces. (A) Venn diagram showing the different and similar operational taxonomic units (OTUs) of the bacterial (A) and fungal (B) communities in the soil, grass, rumen, and faeces. Cladogram of the phylogenetic distribution of bacterial (C) and fungal (D) lineages in soil, grass, rumen, and faeces

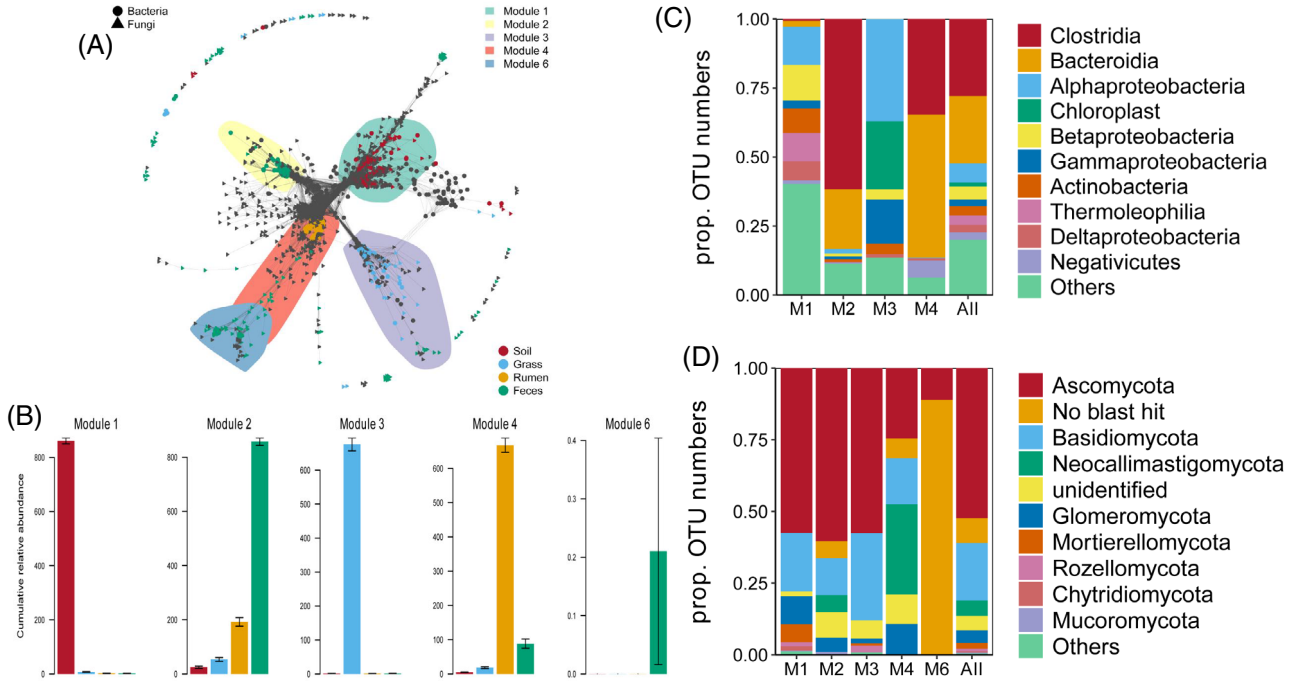

FIGURE 4 Co-occurrence patterns of the soil, grass, rumen, and faeces sensitive operational taxonomic units (OTUs). (A) Co-occurrence networks visualizing significant correlations ($p < 0.001$; indicated with grey lines) between bacterial and fungal OTUs in the soil, grass, rumen, and faeces communities. Circles indicate bacteria, triangles fungi. OTUs are coloured by their association with the different habitats, and grey OTUs are insensitive to treatment. Shaded areas represent the network modules containing OTUs that are sensitive to the four microbial systems. (B) Cumulative relative abundance (as counts per million; *y* axis in ×1000) of all bacteria and fungi of the habitat types sensitive modules. The cumulative relative abundance in samples of soil (red), faeces (green), rumen (yellow), and grass (blue) indicates the overall response of habitat types sensitive modules. (C) Qualitative taxonomic composition of soil, grass, rumen, and faeces sensitive modules is reported as the proportion of OTUs per bacterial species. (D) Qualitative taxonomic composition of soil, grass, rumen, and faeces sensitive modules is reported as the proportion of OTUs per fungal species.

Ruminococcaceae UCG-005, *Bacteroides*, and *Romboutsia* were enriched in the faeces (Table S1–S4).

Bacterial and fungal co-occurrence patterns, network complexity, and the stability and synergy of microbiota in soil, grass, rumen, and faeces

The distribution patterns of sensitive OTUs in meta-co-occurrence patterns of bacterial and fungal communities in the soil, grass, rumen, and faeces are shown in Figure 4A–D. High proportions of OTUs that are sensitive to different habitats were clustered in different modules (Modules 1–4 and 6) (Figure 4A). Sensitive OTUs that are specific to the soil, grass, and rumen were clustered together in Modules 1, 3, and 4, respectively. Modules 2 and 6 primarily consisted of sensitive OTUs that are specific to faeces (Figure 4B). The microecosystem-responsive modules are comprised of different sets of bacteria and fungi. Module 1 mainly comprised *Betaproteobacteria* and *Alphaproteobacteria*, which were sensitive to the soil habitat. Module 3 was predominantly *Alphaproteobacteria*, *Chloroplast*, and *Gammaproteobacteria*, which are sensitive to the grass habitat. *Clostridia* and *Bacteroidia* were the major bacteria observed in Modules 2 and 4, whereas *Clostridia* and *Bacteroidia* were observed in higher abundances in the faeces and rumen, respectively (Figure 4C). The major bacteria in Module 4 was Neocallimastigomycota, which is sensitive to the rumen habitat (Figure 4D). Ascomycota and Basidiomycota accounted for a large proportion of the fungi in Modules 1–3. Module 1 contained *Glomeromycota* and *Mortierellomycota*, which are sensitive to the soil habitat.

The networks of bacterial and fungal communities in different habitats demonstrated distinct co-occurrence patterns (Figure 5). Different interaction networks of bacteria and fungi were observed in different habitats. The network topological parameters, node and edge numbers, and the degree of betweenness and assortativity, were used to assess the complexity of the bacterial and fungal network, with higher node and edge numbers and smaller betweenness and assortativity representing greater network complexity. The ratio of negative and positive correlations (neg/pos) was used to assess the bacterial network stability, with a higher neg/pos ratio representing greater network stability. The results showed that the highest complexity of bacterial and fungal interactions was observed in the soil

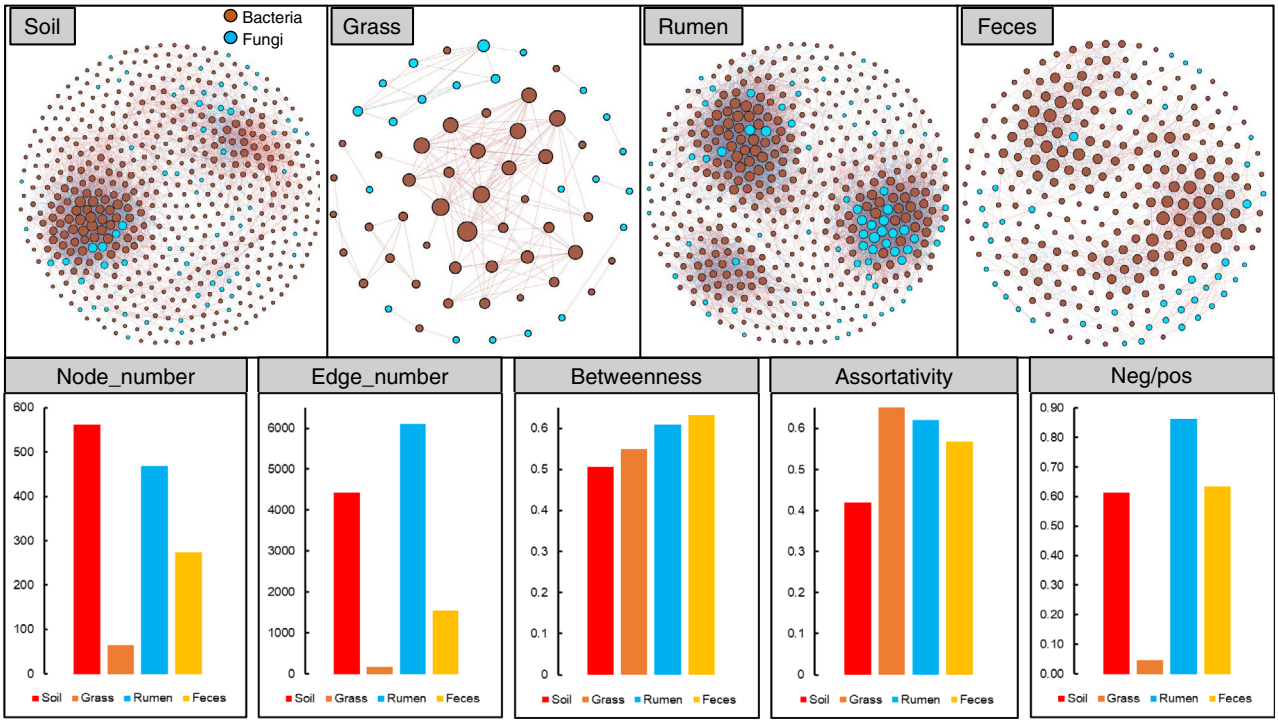

FIGURE 5 Co-occurrence network of bacteria and fungi in the soil, grass, rumen, and faeces. The number of nodes and edges and the degree of betweenness and assortativity of bacteria and fungi in the soil, grass, rumen, and faeces co-occurrence patterns. Neg/pos, the ratio of negative correlation to positive correlation

habitat, followed by rumen and faeces. The interaction between bacteria and fungi is the simplest in the grass habitat. The grass ecosystem also has the lowest OTU number, the lowest diversity, and so on. We found that the highest bacterial and fungal community stability was observed in the rumen habitat, with the lowest stability observed in the grass habitat. No differences were observed between faeces and soil.

The bacterial and fungal community synergy in the soil, grass, rumen, and faeces was described using the Mantel test and Procrustes analysis, which were used to compare the correlation between bacterial and fungal communities in different types of samples and assess the synergy between changes in the environmental pressure, and bacterial and fungal communities. The results indicate that the bacterial and fungal communities in the four types of samples show synergistic adaptability to environmental changes. Of these, bacteria and fungi in the rumen showed the strongest synergy, followed by soil and faeces, and grass had the weakest synergy (Figure 6).

The relationship between environmental factors and bacterial and fungal community in soil, grass, rumen, and faeces

The relationship between environmental factors and bacterial and fungal community in soil, grass, rumen, and faeces was analysed using random forest models (Figure 7). We observed that OM, NH_4-N, and MBC displayed significant correlations with bacterial community, whereas moisture and MBC were significantly correlated with fungal community in the soil samples; DM and ADF exhibited significant correlations with bacterial community, whereas ADF and NDF were significantly correlated with fungal community in the grass samples; Propionate, butyrate, and isobutyrate displayed significant correlations with bacterial community, whereas butyrate and isobutyrate were significantly correlated with fungal community in the rumen samples; TP exhibited significant correlations with bacterial community, whereas ADF, Ash, and TP were significantly correlated with fungal community in the faecal samples.

The transmission of microorganisms in the four habitats

SourceTracker was used to explore the contribution rate of microbes in the soil, grass, rumen, and faeces habitat (Figure 8). The proportions of sequences in the rumen that originated from the soil and grass microbiota were 0 and 0.02, respectively. The proportion of sequences in the faecal microbiota originating from the rumen microbiota was 3.55. The proportion of sequences in the soil and grass microbiota that originated from faecal microbiota was 4.48 and 1.18, respectively, and the proportion of sequences in the grass microbiota that originated from the soil was 1.06.

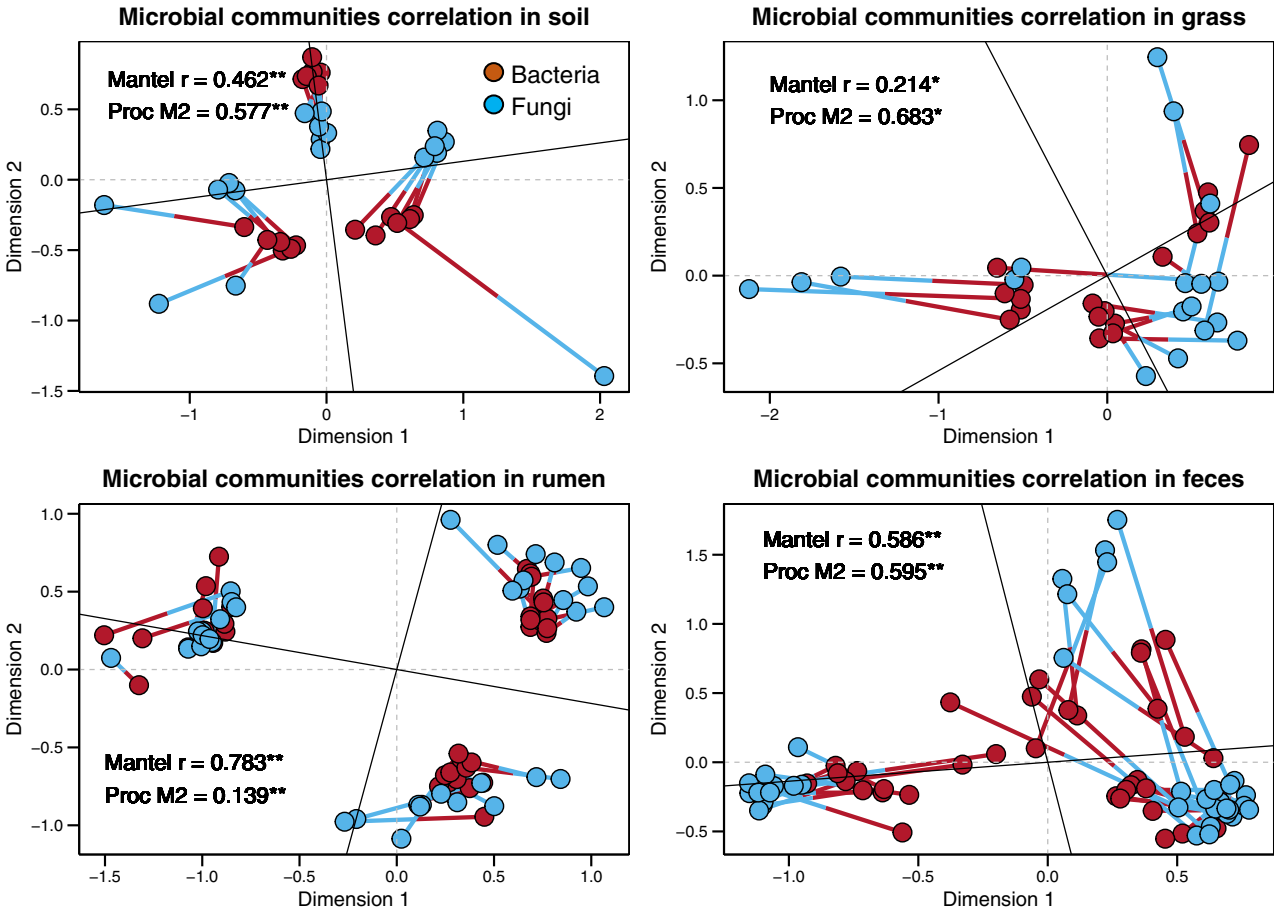

FIGURE 6 Mantel test and Procrustes analysis revealed the correlations between the bacteria and fungi in the soil, grass, rumen, and faeces. The red colour represents the bacteria and the blue colour represents the fungi. The same line represents bacterial and fungal communities from the same sample.

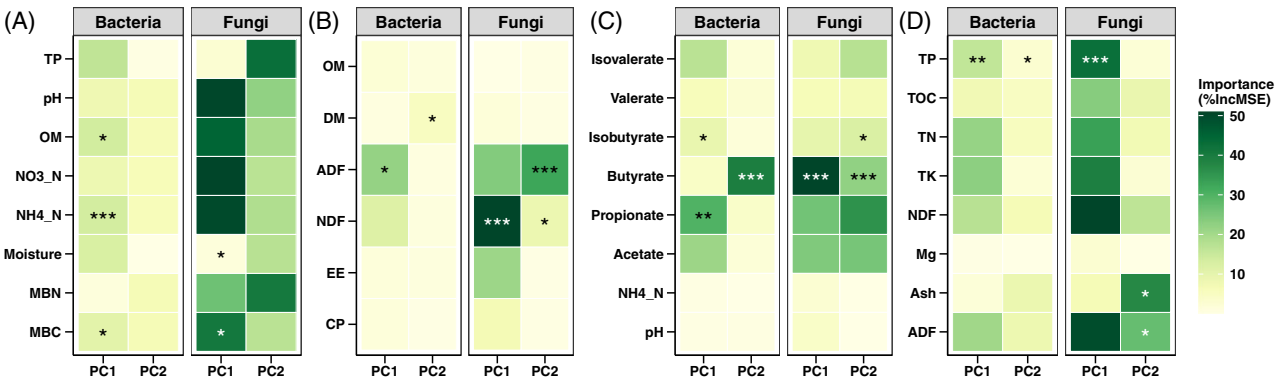

FIGURE 7 Random forest model identifying the significantly environmental factors influencing the community of bacteria and fungi in soil, grass, rumen, and faeces.

DISCUSSION

The same DNA extraction kit was used for all experiments in order to ensure the same lysis efficiency (Beckers et al., 2017). The sequencing error (and potential creation of erroneous sequences) is therefore considered to be similar for the soil, grass, rumen, and faecal samples. To control for differences in the sampling effort across soil, grass, rumen, and faeces, normalization analysis was performed based on the lowest number of sequences within a sample (Beckers et al., 2017). Initially, the alpha diversity was estimated by focusing on Chao1, PD_whole_tree, and Shannon, and soil showed the highest alpha diversity. Gans et al.

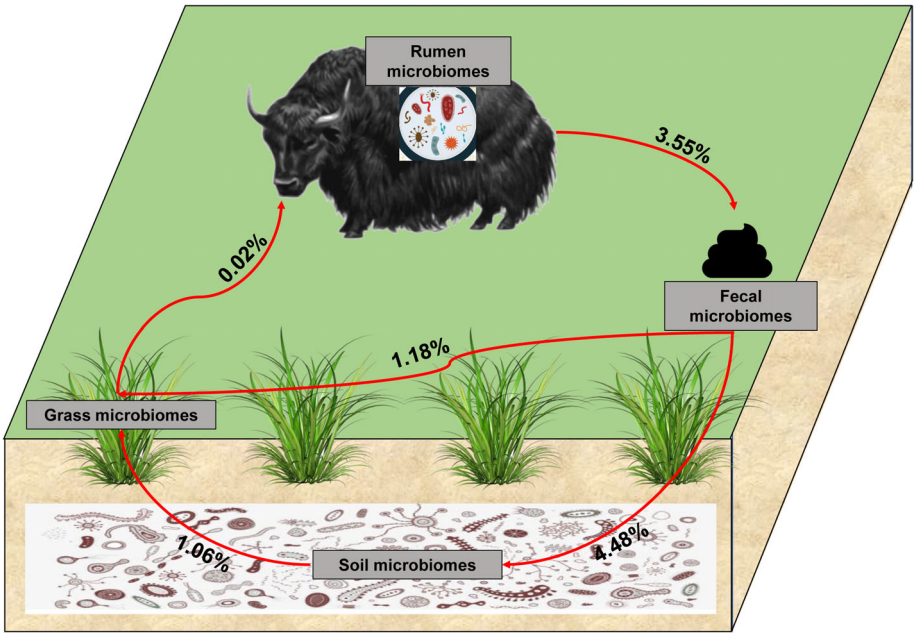

FIGURE 8 Microbial source tracking estimations of microbial source contributions in the soil, grass, rumen, and faeces

(2005) reported that soil, a reservoir of microbial species, is renowned for its vast microbial diversity and is considered the most genetically diverse ecosystem on earth. With the development of sequencing technology and the development of specific pipelines for bioinformatics, researchers have a better understanding of forage endophytes (Cernava & Cernava, 2022; Basile & Lepek, 2020). Cernava and Cernava (2022) pointed that endophytes engage in more intimate interaction with their hosts although they generally occur at substantially lower numbers than rhizosphere microbes. In this study, the lowest alpha diversity was also obtained for the forage endophytes when compared with other habitats. Durso et al. (2017) reported that microbial diversity is higher in the rumen than the faeces, which was confirmed by the results of this study.

In the current study, we found that the microbial communities in the four systems differed significantly. The dominant phyla in the bacterial communities inhabiting the soil were Proteobacteria and Actinobacteria. This finding was consistent with previous research conducted in a similar region (Chen et al., 2017; Sun et al., 2021; Zhou et al., 2019). Proteobacteria play important roles in energy metabolism and phylogenetic value (Mukhopadhya et al., 2012). Actinobacteria have strong DNA repair mechanisms and the ability to survive in low temperatures (Yergeau et al., 2010). These phenomena indicate that these bacteria, which are dominant in the soil, are well adapted to the low-temperature and hypoxic conditions of the QTP. The dominant phyla observed in the bacterial community within the grass samples, Cyanobacteria and Proteobacteria, is consistent with previous findings (Hassani et al., 2018; Lugtenberg et al., 2016) and is to be expected because Cyanobacteria and Proteobacteria play important roles in carbon (MacCready et al., 2021) and nitrogen fixation (Gutiérrez-García et al., 2019). Durso et al. (2017) reported that Firmicutes and Bacteroidetes are dominant in the rumen and faeces; however, the relative abundance of the dominant phyla was found to vary greatly. This finding is consistent with the results of our study. However, we also found that Ascomycota and Basidiomycota dominated the fungal communities of the soil, grass, rumen, and faecal samples from the QTP. This is consistent with previous studies conducted on alpine meadows, which showed that most fungi inhabiting these environments belong to the phyla Ascomycota and Basidiomycota and are parasitic in soil, plants, humans, poultry, and insects (Chen et al., 2017; Sun et al., 2021; Treseder et al., 2014; Wu et al., 2021).

The sensitive OTUs in the meta-network of bacteria and fungi in the four habitats are grouped into distinct modules that reflect the microorganisms inhabiting different habitats, which are greatly affected by environmental factors. Previous studies also reported that the four different micro-ecological systems have unique micro-ecological environments (Attwood et al., 2019; Malmuthuge et al., 2019; Semenov et al., 2010). This was consistent with our results. The complexity and stability of the bacterial and fungal interaction networks in the four habitats were further explored separately. The results demonstrated that the complexity of the bacterial and fungal communities in the different habitats was strongest in the soil, followed by the rumen and faeces, and weakest in the grass. Interestingly, we

found that the network complexity of the interactions between bacteria and fungi in different habitats was the same as the change in the community diversity index. The reason was that soil habitats have the highest abundance and OTU number, and grass endophytes have the lowest abundance and OTU number. Fan et al. (2018) reported that negative interactions might weaken competitive relationships in the bacterial community, whereas positive interactions are likely to strengthen such relationships. Therefore, the larger the neg/pos ratio, the weaker the competition between microbial communities, and the more stable the microbial network structure. In the current study, the highest bacterial and fungal network stability was observed in the rumen, followed by the faeces and soil microbial systems, and the weakest was in the grass. Previous studies have found that a mature rumen habitat is particularly stable, and the fact that many nutritional interventions cannot be effective over long periods and that the microbiota immediately return to their original state once such interventions cease, indicates that the mature rumen microbial system is relatively stable and unaffected by the external environment (Griffith et al., 2017). The bacterial and fungal network stability is lower in soil and is affected by vegetation, temperature, and rainfall (Li et al., 2021; Sun et al., 2021; Zhou et al., 2019). The low bacterial and fungal network stability observed in grass is affected by soil flora, temperature, humidity, and several other environmental factors (Beckers et al., 2017; Massoni et al., 2021). The bacteria and fungi in the rumen also have the strongest synergy, followed by faeces and soil, while forage has the weakest synergy. The network stability and synergistic changes in the interactions between bacteria and fungi were found to be similar in the different habitats.

Livestock grazing on the natural grasslands of QTP occurs throughout the year without any supplementary feeding (Zhou et al., 2017). Grazing livestock feed on plants, and ferment, and digest plants in the rumen, indicating the possibility of a clear connection between plant and animal microbiota. It is well known that as soon as plant material enters the rumen, it is colonized by a succession of different microbes that initiate digestion (Huws et al., 2016). Previous results from the global rumen microbial survey found that the microbes in the rumen may be derived from plant feed, water, or soil microbes, which account for an average of 3% of the sequences found in the rumen microbiome (Henderson et al., 2015). In our study, the proportion of sequences in the rumen microbiota originating from the grass was 0.02. This is possibly because most of the plant endophytes that are ingested by grazing livestock are degraded in the rumen, including the DNA fragments and proteins, and only a very small proportion remain within the rumen (Kingston-Smith et al., 2008). This may also explain the particularly strong stability of the rumen microbes, which were observed to be unaffected by the environment.

Although the interaction between grass and ruminant microbial communities is clear, ruminants ingest significant quantities of soil. Previous research has shown that livestock intentionally eat soil during the grazing process (Rodrigues et al., 2012), with the daily average soil intake of grazing cattle at 1000 g (Mayland et al., 1975). Considering that each gram of soil can contain 1×10^9 to 1×10^{10} microorganisms (Gans et al., 2005), grazing livestock are ingesting 1×10^{12} to 1×10^{13} soil microorganisms per day. The potential impact of this is estimated to account for 2.6% of the rumen microbiota in cattle (Attwood et al., 2019). However, the rumen microbiota were not found to originate from soil microorganisms in the present study. This inconsistency may be because the soil and rumen environments have different physical and chemical conditions such as temperature and pH, and therefore, that microorganisms entering the rumen environment from the soil will not survive long enough to metabolize (Attwood et al., 2019).

After grass enters the rumen, the nutrients are decomposed under the action of microorganisms, the plant material is further fermented in the hindgut and is finally excreted from the body as faeces (Liu et al., 2021). The excrement of grazing livestock affects both soil microorganisms and plant endophytes (Semenov et al., 2010; Zhang et al., 2021). Semenov et al. (2010) reported that intestinal pathogens that were labelled with green fluorescent protein (GFP) before entering the gastrointestinal tract of animals via feed were finally excreted in faeces, from where they entered the soil. Meanwhile, Bardgett et al. (1998) reported the impact of faecal deposition on dryland pastures, indicating that it provides an additional substrate for microbial growth and metabolism and changes the nutrient availability. This is consistent with our results: the proportion of sequences in the grass microbiota that originated from faeces was 1.18, while that in the soil microbiota that originated from faeces was 4.48.

Soil microbes have long been considered central to managing the productive capacity of ecosystems (Zhang et al., 2021). Soil microbes provide the primary reservoir of microbiota that colonize the rhizosphere, root rhizoplane, and ultimately the wider endophytic microbiota within plants (Beckers et al., 2017). Previous studies have shown that the subset of bacteria that inhabit the soil does not require dispersal factors such as wind, insects, or water to reach the leaves and flowers of *Arabidopsis thaliana* (Massoni et al., 2021). In addition, the soil microbiome has been found to affect microbial communities in the stems and leaves of poplar trees (Beckers et al., 2017). The interface between rhizosphere, soil, and roots plays a key role in creating a selective barrier, and the ratio of endophytic competence to colonization is limited to specific

microbial species (Beckers et al., 2017). In our study, the great loss of diversity from the soil to the grass-endophytic compartment supports this opinion and demonstrates that only a limited number of bacteria and fungi can adapt to an endophytic lifestyle. The proportion of sequences in the grass microbiota that originated from the soil was only 1.06.

CONCLUSIONS

According to the meta-co-occurrence network, the four habitats associated with the soil, grass, rumen, and faeces are relatively independent. The bacterial sensitivities of the rumen and faecal habitats are similar and differ from those in the soil and grass. *Negativicutes* and *Neocallimastigomycota* are sensitive microbiota that exist only in the rumen. In addition, significant differences were observed in 99 bacteria and 134 fungal clades inhabiting the soil, grass, rumen, and faeces using LefSe analysis. The highest alpha diversity and microbial network complexity were observed in the soil, and the highest microbial network stability and synergy were observed in the rumen. Grass endophytes had the lowest microbial network complexity, stability, and synergy. SourceTracker showed that soil and grass have little effect on the microorganisms inhabiting yak, whereas the contribution of yak microorganisms to the soil and grass is relatively high. The results of this study indicate that after long-term evolution and natural selection, the rumen microbiota of grazing yaks has reached a relatively stable state that has allowed them to adapt to the harsh environment of the QTP.

AUTHOR CONTRIBUTIONS

Fujiang Hou planned and designed the experiments. Qingshan Fan performed the animal experiments and sample collection. Qingshan Fan and Fujiang Hou analysed the data and wrote the manuscript. Kaili Xie, Xiongxiong Cui, Guangyun Zhang, Haozhe Zheng and Shenghua Chang helped in sample collection. All authors have read and approved the final manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The raw reads were deposited at NCBI (under BioProject accession ID: PRJNA793078).

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