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Dynamic changes in the gastrointestinal microbial communities of Gangba sheep and analysis of their functions in plant biomass degradation at high altitude

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Abstract

Background While Gangba sheep being well known for their unique flavour and nutritional value, harsh environmental factors negatively affect their growth and development, leading to poor productivity. The gastrointestinal tract microbiota plays an important role in host nutrient absorption and metabolism. The identification of dynamic changes in the gastrointestinal microbial communities and their functions is an important step towards improving animal production performance and health.

Results A comprehensive multi-omics survey of the microbial communities of the Gangba sheep gastrointestinal tract was performed under three distinct feeding strategies: natural grazing, semi-grazing with supplementation, and barn feeding. The dynamic changes, cross-kingdom partnerships and functional potential profiles were analysed and the results revealed that the feeding strategies had a greater impact on the microbial communities than the site of the gastrointestinal tract. The different microbial associations among the groups were revealed by co-occurrence networks based on the amplicon sequence variants (ASVs). Moreover, a Gangba sheep gastrointestinal microbial genomic catalogue was constructed for the first time, including 1146 metagenome-assembled genomes (MAGs) with completeness > 50% and contamination < 10%, among which, 504 bacterial and 15 archaeal MAGs were of high quality with completeness > 80% and contamination < 10%. About 40% of the high-quality MAGs displaying enzyme activity were related to the microbial species that contribute to plant biomass degradation. Most of these enzymes were expressed in rumen metatranscriptome datasets, especially in *Prevotella* spp. and *Ruminococcus* spp., suggesting that gastrointestinal microbial communities in ruminants play major roles in the digestion of plant biomass to provide nutrition and energy for the host.

Conclusions These findings suggest that feeding strategies are the primary cause of changes in the gastrointestinal microbiome. Diversification of livestock feed might be an effective strategy to maintain the diversity and ecological multifunctionality of microbial communities in the gastrointestinal tract. Additionally, the catalogue of microbial genomes and the encoded biomass-degrading enzymes identified here provide insights into the potential microbial functions of the gastrointestinal tract of Gangba sheep at high altitudes. This paves the way for microbial interventions to improve the growth performance, productivity and product quality of ruminant livestock.

Keywords High altitude, Gangba sheep, Feeding strategies, Gastrointestinal tract, Biomass-degrading enzymes

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Background

Organisms living in high-altitude regions are constantly exposed to severe physiological constraints and challenges due to the extreme environment [1–3]. The East African Plateau (average altitude, 2400–3700 m), Andes (average altitude, 4000 m) and Himalayas (average altitude, 4500 m) are among the high-altitude regions of the world [4, 5]. Hypobaric hypoxia, dehydration and low temperatures are the primary environmental challenges faced by animals living in these high-altitude regions [1]. As an indigenous breed in Tibet, Gangba sheep can not only survive in harsh high-altitude environments over the long term but are also famous for their unique flavour and high nutritional value [6]. However, the number and individual productivity of sheep cannot be fully developed by traditional grazing. An increasing number of studies have demonstrated that semi-grazing and barn feeding, as alternative strategies to traditional grazing, can improve the growth performance of local domestic animals [7, 8]. Therefore, there is an urgent need to develop an appropriate feeding strategy that is suitable for high-altitude contexts while also developing sustainable grassland ecosystems in these regions.

Microbial life thrives in various environments [9–13] and performs basic functions, such as driving global nutrient cycling [14] and biogeochemical cycles [15], affecting the health of plants and animals [12, 13, 16]. Gastrointestinal microorganisms have co-evolved with their hosts to form complex ecosystems in which microbial communities participate in the digestion of nutrients, maintain the integrity of the digestive tract, stimulate the immune system, and modulate the host metabolism [17–20]. Increasing attention is being paid to the gastrointestinal microbiome signatures of high-altitude livestock, with findings suggesting that high-altitude environments shape the unique gastrointestinal microbiomes and functional signatures of livestock [21, 22]. However, the diversity and functional landscape of the gastrointestinal microbiome of Gangba sheep remains underexplored.

Previous studies of ruminant livestock had mainly focused on the characterization and composition of rumen microbial communities, as well as the degradation and fermentation of lignocellulose by microbial strains [23, 24]. Recent studies have shown that the integrated catalogues of gastrointestinal microbial genomes or genes can contribute to the digestion of overall food and the absorption of nutrients [25, 26]. Thus, an understanding of how these microbes within the community interact with one another to contribute to the host's gastrointestinal functions is essential for constructing beneficial microbial communities. In fact, it is well established that the sites of the gastrointestinal tract (GIT) and the feeding (FD) strategy have significant effects on the composition and function of the gastrointestinal microbiome [7, 27, 28]. Thus, it is necessary to consider these factors when examining the dynamic changes in microbial communities and how the microbiome impacts the gastrointestinal functions of the host at high altitudes.

In this study, it was hypothesised that different FD strategies can lead to variation in interkingdom interactions between bacteria and fungi along the GIT, which alters the gastrointestinal microbiome and affects its metabolism and potential functions, thereby affecting the growth performance of Gangba sheep. Specifically, cross-kingdom partnerships within the microbial communities were investigated, the comprehensive associations between specific microbial taxa and metabolites were evaluated, and the potential functions that enable the breakdown of plant biomass by the Gangba sheep gastrointestinal microbiome were assessed. These findings fill the gap in our understanding of the functional significance of the microbial genomic catalogue of the Gangba sheep digestive tract at high altitudes. This work will facilitate further studies of plant fibre decomposition in the digestive tracts of ruminants.

Materials and methods

Ethics statement

All animal experimental procedures were performed following the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of the People's Republic of China. All animal experiments were approved by the Committee on Experimental Animal Management of the Chinese Academy of Agricultural Sciences (Beijing).

Study design, sample collection and processing

The study was carried out in a local village-owned farm (88°08' - 88°56' E, 27°56' -28°45' N, average altitude, 4,700 m) located in Kamba County in the east of Tibet Autonomous Region, China. During the study period, the average maximum temperature was 23°C, the average minimum temperature was 9°C, the highest maximum temperature was 26°C, the lowest minimum temperature was 5°C, and the monthly rainfall ranged from 27.6 to 334.7 mm. Eighteen Gangba rams of the same age (one year old) and weight (14.48 ± 0.26 kg) were randomly divided into three groups: natural grazing (NG, $n = 6$), semi-grazing with supplementation (SG, $n = 6$) and barn feeding (BF, $n = 6$). The animal experiments lasted for 80 days, following a seven-day adaptation period. The sheep in the NG group were kept on pasture without any supplementation. The sheep in the SG group were grazed on the same pasture from 10:00 to 16:00 h and kept in

individual pens at night, with a pellet diet of 0.55–0.90 kg (including concentrates and oat hay) provided when they were off the pasture. The sheep in the BF group were kept in individual pens and were provided a pellet diet of 0.85–1.35 kg (Table S1). The pellet diet was adjusted daily based on the previous day's intake, allowing for refusal of 10%. All Gangba sheep were provided water ad libitum throughout the experimental period. For calculation of the average daily weight gain, all sheep were weighed every two weeks. At the end of the feeding experiment, all sheep were stunned, slaughtered and dissected, after the prevention of food and water intake for 12 h overnight. The rumen, duodenum, jejunum and ileum of each animal were separated, and the lumen contents were homogenized separately. The homogenized contents from each region were collected in triplicate and immediately stored in liquid nitrogen for subsequent analysis.

DNA extraction and amplicon sequencing

Microbial genomic DNA extraction was performed on the gastrointestinal contents using a Mag-Bind® Soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.). The qualified DNA was used for amplification of the V3–V4 region of the bacterial 16S rRNA gene using the following barcoded primer pair (338F: 5'-ACTCCTACGGGAGGC AGCAG-3'; 806R: 5'-GGACTACHVGGGTWTCTAAT-3') and the ITS region of the fungal rRNA gene was amplified using the following primer set (ITS1F: 5'-CTT GGTCATTTAGAGGAAGTAA-3'; ITS2R: 5'-GCTGCG TTCTTCATCGATGC-3'). Paired-end libraries were generated using a NEXTFLEX ® Rapid DNA-Seq Kit and amplicon sequencing was performed on an Illumina PE300 platform (Illumina, San Diego, USA) at Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). Raw Illumina sequencing reads were filtered via fastp (v0.19.6) [29] to remove low-quality (quality scores < 20), short (< 50 bp) and ambiguous bases of reads, and merged via FLASH (v1.2.11) [30]. Then the high-quality sequences were denoised according to the DADA2 procedure [31] using the QIIME2 plugin (version 2020.2) [32] to obtain amplicon sequence variants (ASVs). Chloroplast and mitochondrial sequences were also removed, and only sequences from bacteria and fungi were kept. To minimize the effects of sequencing depth on alpha and beta diversity, all 16S rRNA sequences from each sample were rarefied to 21,446 and all ITS sequences from each sample were rarefied to 38,847. ASVs were classified against the SILVA bacterial 16S rRNA database (v138) [33] and UNITE fungal ITS database v8.0 [34] using classify-sklearn (Naive Bayes).

Co-occurrence networks

To examine cross-kingdom interactions among microbial taxa, co-occurrence networks were constructed based on the Spearman correlations ($|r| > 0.6$, $P < 0.001$) among the ASV relative abundances within the community using the "igraph" v.1.5.1 package [35]. ASVs with a mean relative abundance > 0.0001 were included in these networks. Co-occurrence network modules were inferred via weighted correlation network analysis with the WGCNA package (v.1.72–1) in R [36]. The networks were calculated and visualized using the "Gephi" interactive platform [37].

Analysis of metabolome

The 72 samples of gastrointestinal contents from the three FD strategies (NG, SG and BF) and four locations of the GIT (rumen, duodenum, jejunum and ileum) with six replicates were added to a solution (acetonitrile: methanol = 1:1(v:v)) for metabolite extraction. The supernatant was removed and blown dry under nitrogen after low-temperature ultrasonication. The samples were then re-dissolved in solution (acetonitrile: water = 1:1 (v/v)) and the supernatant was obtained following low-temperature ultrasonication. Liquid Chromatography-Mass Spectrometry (LC–MS/MS) analysis was performed on a Thermo UHPLC-Q Exactive HF-X system at Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The metabolome analysis was performed using the free online Majorbio Cloud platform (cloud.majorbio.com) [38].

Metagenome assembly and analysis

The qualified DNA extracted from the 36 rumen and ileum samples was used for paired-end library construction via NEXTFLEX ® Rapid DNA-Seq (Bioo Scientific, Austin, TX, USA). Metagenome shotgun sequencing was performed on an Illumina NovaSeq 6000 platform (Illumina Inc., San Diego, CA, USA) with 150 bp paired-end reads at a sequencing depth of 10 GB per sample. The raw reads were processed using Trimmomatic v0.39 [39] to trim adaptors and low-quality reads and then the possible eukaryotic genome sequences were removed by mapping against the host reference genome. The remaining reads were assembled into contigs for each sample using SPAdes v3.13.1 with k-mers 21, 33, 55 and 77 [40]. Contigs with a length > 500 bp were mapped back to the reads by BWA-MEM (v. 0.7.17) [41]. The metagenome-assembled genomes (MAGs) were co-assembled via metabat2 v2.12.1 [42], maxbin v2.2.6 [43] and concoct v1.0.0 [44]. All MAGs were pooled and dereplicated using dRep v3.4.5 with the parameters as follows: -sa 0.95 -nc 0.60

-comp 50 -con 10 -p 50' [45, 46]. This resulted in 1146 MAGs, including 1107 bacterial and 39 archaeal MAGs with completeness > 50% and contamination < 10%, among which, 519 MAGs were high quality with completeness > 80% and contamination < 10%.

Annotation and functional characterization of MAG-encoded proteins

Ruminant gastrointestinal microbial MAGs were investigated in this study, including 519 assembled as described above, and 410 [47], 538 [48], 913 [49] and 4934 [50] MAGs previously reported were re-annotated via prokka v1.14.6 [51]. All re-annotated genes were mapped to dbCAN HMMdb (family level) and dbCAN-sub HMMdb (subfamily level) using dbCAN3 web server with HMMER tool (E-Value < 1e-15, coverage > 0.35), and also mapped to CAZyDB by dbCAN3 with DIAMOND tool (E-Value < 1e-102) [52] to identify three functional categories: cellulase (GH5, GH6, GH7, GH8, GH9, GH12, GH44, GH45, GH48, GH51, GH74 and GH124), hemicellulase (GH5, GH8, GH10, GH11, GH16, GH26, GH30, GH43, GH44, GH51, GH62, GH98 and GH141) and pectinase/esterase (GH28, CE1, CE2, CE3, CE4, CE5, CE6, CE7, CE8, CE12, CE13, CE15, PL1, PL2, PL3, PL9, and PL10) [53]. Only when all three tools reported the same match was a sequence considered to be a valid hit.

Taxonomic assignments of MAGs

In order to normalize the taxonomic annotations of all genomes in the collection, all MAGs were pooled and re-identified using GTDB-Tk v2.3.2 [54]. Amino acid alignment of the GH5, GH8, GH9, GH51, GH10, GH16, GH26, GH30, GH43, GH28, CE6, CE8, CE12 and PL1-encoding proteins (> 100 amino acid sequences) was performed using MUSCLE v3.8, respectively. All phylogenomic trees were constructed with Fasttree v2.1.11 [55] and visualized with iTol [56].

Assessment of gene copy numbers

To validate the activity of enzyme identified by bioinformatics analysis, quantitative PCR (qPCR) was performed on rumen samples to determine the absolute abundance of ten randomly selected genes. The PCR amplification products were ligated into the pMD19-T cloning vector (Takara, Cat#6013) and then transformed into *Escherichia coli* TG1-competent cells. Calibration curves were constructed with target genes in a pMD19-T vector at tenfold dilution. The copy numbers of the target genes were calculated according to the relative calibration curves of the plasmid copy numbers. All data analyses were conducted using GraphPad Prism 9.1.2 (GraphPad Software, The North Parker, USA).

Gene expression within rumen microbial metatranscriptomes

Rumen microbial metatranscriptomic data sets were obtained from the National Centre for Biotechnology Information [57] to assess the expression of cellulase, hemicellulase and pectinase/esterase in other rumen. The raw data were trimmed using Trimmomatic v0.39 [39] to filter out both low-quality reads and adapters after quality determination with fastqc v0.11.8 [58] and multiqc v1.5 [59]. The data were then aligned to the gastrointestinal microbial MAGs of Gangba sheep using STAR v2.7.10a [60] with the default settings. The expression levels of the genes were calculated using RSEM v1.3. [61] with the rsem-calculate-expression parameter. The FPKM values of the genes served as a measure of the gene expression levels.

Data analyses

Permutational multivariate analysis of variance (PERMANOVA) was performed on the Bray–Curtis dissimilarities to determine the factors influencing the GIT microbial communities based on the relative abundance profiles of the ASVs using the R package "vegan" (v.2.6–4). Non-metric multidimensional scaling (NMDS) plots were constructed to assess and visualize differences among the three FD strategies via the "Adonis" function of the vegan package (v.2.6–4) in R. Bacterial and fungal alpha diversity were examined based on the Shannon and Chao1 indices calculated by Mothur v1.30.1 [62]. The differences in alpha diversity across the FD strategies were tested using a nonparametric Kruskal–Wallis rank sum test and were validated by the False Discovery Rate (FDR) followed by a pairwise Wilcoxon test.

Results

Dynamic changes in the microbial communities along the Gangba sheep GIT under different FD strategies

The effects of the FD strategies on the growth and slaughter performance of Gangba sheep were investigated. The results revealed that the performance indices were significantly higher in the BF and SG groups than in the NG group (Table S2). These results suggest that supplemental feeding or barn feeding at the right time are more valuable strategies than the traditional natural grazing strategy for combating extreme environments. Both the FD strategy [7] and the location of the GIT [26, 63, 64] play

a role in the assembly of the microbial community. Here, the factors influencing the GIT microbial communities were compared via PERMANOVA (Distance = "Bray–Curtis", Permutations = 9999, p-value < 0.001). Compared with the location of the GIT, the FD strategy had a stronger effect on the bacterial and fungal communities at the ASV level. The beta and alpha diversity of the bacteria and fungi under the three FD strategies were examined according to the abundance of ASVs. Clear separation (bacteria: Stress 0.188, $R^2 = 0.2537$, $P < 0.0001$; fungi: Stress 0.092, $R^2 = 0.4747$, $P < 0.0001$) was observed among the FD strategies via NMDS analysis (Fig. 1A). Analysis of the alpha diversity showed significant differences between the NG group and BF group in terms of community diversity (i.e., Shannon index) and community richness (i.e., Chao1 index). Although there was no significant difference between the SG and BF groups in terms of bacterial community diversity and community richness, nor between the NG and SG groups in terms of fungal community diversity (Fig. 1B), these findings nonetheless suggest that the FD strategy strongly influences the diversity of the GIT microbiota. Furthermore, the microbial composition differed markedly across the FD strategies, with most of the gastrointestinal microbial

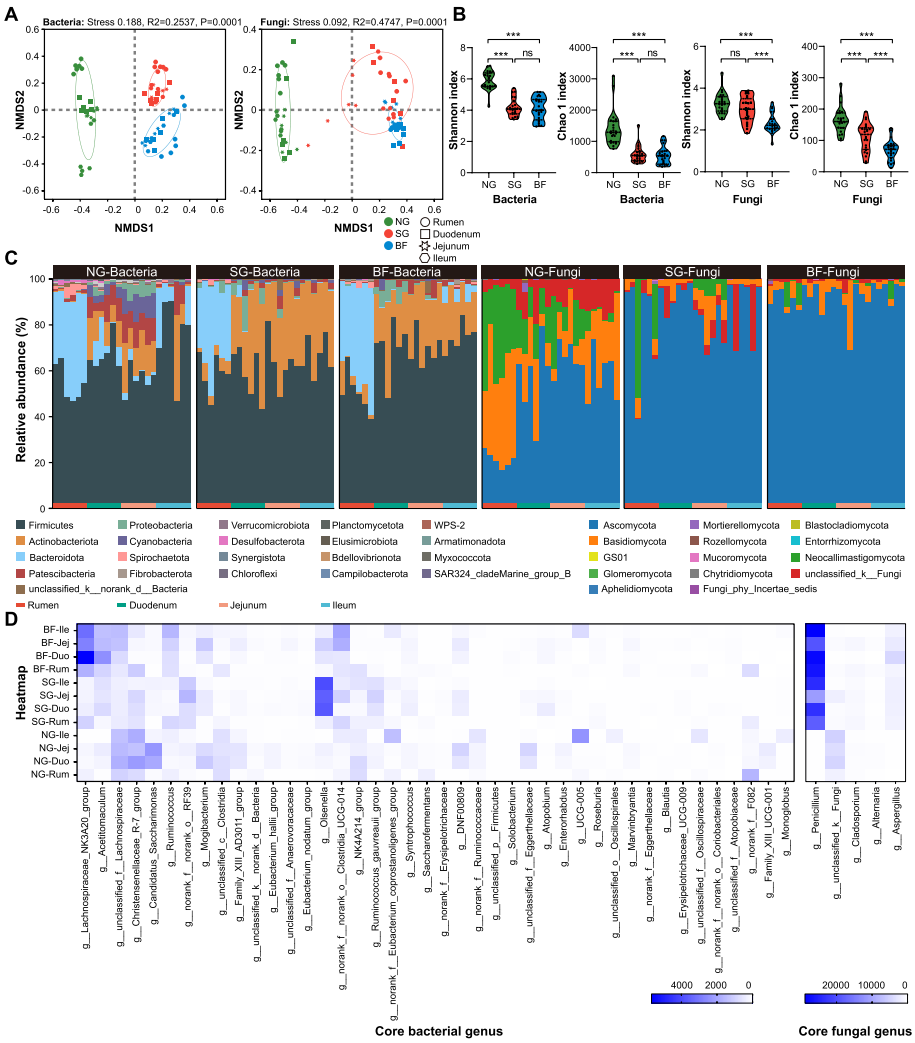

Fig. 1 Dynamic changes in the microbial communities along the Gangba sheep gastrointestinal tract (GIT) and the influence of feeding (FD) strategies. **A** Non-metric multidimensional scaling (NMDS) of bacterial and fungal communities based on amplicon sequence variant (ASV) abundance. Significant differences among the FD strategies were examined using the ADONIS test (Bacteria: Stress 0.188, $R^2 = 0.2537$, $P < 0.0001$; Fungi: Stress 0.092, $R^2 = 0.4747$, $P < 0.0001$). NMDS of all samples: colours indicate distinct FD strategies and shapes indicate the locations of the GIT. **B** The alpha diversity among the NG, SG and BF groups, including community diversity (i.e., Shannon index) and community richness (i.e., Chao1 indices). **C** Comparisons of the microbial communities in the different groups at the phylum level. **D** Core microbial genera at four sites of the GIT under different FD strategies. NG, natural grazing; SG, semi-grazing with supplementation; BF, barn feeding

taxa co-enriched in the NG group. In contrast to the bacterial communities in the SG and BF groups, the NG group was predominantly composed of Fimicutes, Bacteroidota and Spirochaetota in the rumen, while Fimicutes, Bacteroidota, Actinobacteriota, Patescibacteria, Cyanbacreria and Proteobacteria accounted for larger proportions in the duodenum and jejunum. Interestingly, the number of members of the phylum Actinobacteriota was significantly higher in the SG and BF groups than in the NG group. The fungal community of the NG group was predominantly composed of Ascomycota, Basidiomycota and Neocallimastigomycota across the GIT, while the SG and BF groups were mainly composed of Ascomycota (Fig. 1C). These results indicate that the FD strategies changed the characteristic spectrum of the microbial community composition, which may alter the robustness of the microbial community. All Gangba sheep shared a core set of microbial taxa that were defined as genera present in 80% of the samples [65], including 42 bacterial and 5 fungal genera, belonging to five bacterial and two fungal phyla, respectively. These were mainly composed of members of the Lachnospiraceae, Anaerovoracaceae, Eggerthellaceae, Atopobiaceae, Erysipelotrichaceae, Oscillospiraceae, Aspergillaceae and Ruminococcaceae. Compared with the NG group, the *Olsenella*, norank_f__norank_o__Clostridia_UCG-014, *Penicillium* and *Aspergillus* genera were enriched in both the SG and BF groups, while Lachnospiraceae_NK3A20_group and *Acetitomaculum*, *Ruminococcus* were enriched in the BF group at the genus level. Further detailed analyses revealed that large members of Prevotellaceae served as core bacteria in the rumen and ileum (Fig. 1D and Figure S1). Overall, these findings reveal notable variations in symbiotic bacteria and fungi under different FD strategies despite sharing major bacterial and fungal taxa. The latter may sustain the basic functionality of the host at high altitudes.

The patterns of cross-kingdom interactions within the microbial communities

To probe the potential mechanism underlying the cross-kingdom interactions within the microbial communities under the different FD strategies and in the different locations of the GIT, Spearman correlations between the microbial taxa at the ASV level were employed to construct co-occurrence networks from an ecological perspective, and the network topology characteristics were evaluated (Fig. 2A, B and C). This analysis focused on the significant correlations ($|r| > 0.6$, $P < 0.001$) between the ASVs and the mean relative abundance > 0.0001, where significant ASV-level shifts in connectivity and complexity within the co-occurrence networks were observed. This implies that the microbial communities exhibited distinct roles among the locations of the GIT and under the distinct FD strategies. The co-occurrence networks in the NG group were more complex (2156 ASVs/nodes and 20,771 edges) than those in the SG group (989 ASVs/nodes and 5674 edges) and BF group (800 ASVs/nodes and 4350 edges) (Fig. 2D, E and Figure S2). Surprisingly, although there were more nodes and edges in these networks in the NG group as compared to the SG and BF groups, 99% of positive corrections for each co-occurrence network were detected (Fig. 2E). This indicates that the gastrointestinal-resident microbial communities may function as a consortium at high altitudes. Furthermore, a more complex community and higher modularity were observed in the NG group compared to the SG and BF groups, where microbes interacted with one another in a more intimate way, with higher network connectivity (average degree) and modularity indices (≥ 0.647) (Fig. 2F and G). These results indicate that the SG and BF groups not only had reduced microbial community diversity but also reduced the richness of species involved in symbiotic microbial interactions, probably due to the composition of the feed. This finding suggests that the enrichment of the feed materials under the SG and BF conditions helped maintain the diversity and function of the microbial communities. Thus, future studies should investigate the dynamic change in the microbial communities and the effects of key microorganisms on the host under the influence of diverse combinations of feed materials.

Functional profile of the gastrointestinal microbiome

To further explore the potential functions regulated by the microbiome, untargeted metabolomics was conducted on 72 gastrointestinal content samples. In total, 927 of the 6456 metabolites in the gastrointestinal metabolome were annotated to the KEGG database and mapped to the 231 KEGG pathways; these were mainly classified into amino acid metabolism, lipid metabolism, metabolism of cofactors and vitamins, carbohydrate metabolism, and digestive system (Fig. 3A and Table S3). The four sites of the GIT under the different FD strategies exhibited different metabolic profiles. The dominant pathways enriched were tryptophan metabolism (map00380), arginine and proline metabolism (map00330), tyrosine metabolism (map00350), and lysine degradation (map00310) within amino acid metabolism; glycerophospholipid metabolism (map00564), arachidonic acid metabolism (map00590), linoleic acid metabolism (map00591), and steroid hormone biosynthesis (map00140) within lipid metabolism; nicotinate and nicotinamide metabolism (map00760) and porphyrin metabolism (map00860) within metabolism of cofactors and vitamins; ascorbate and aldarate metabolism (map00053),

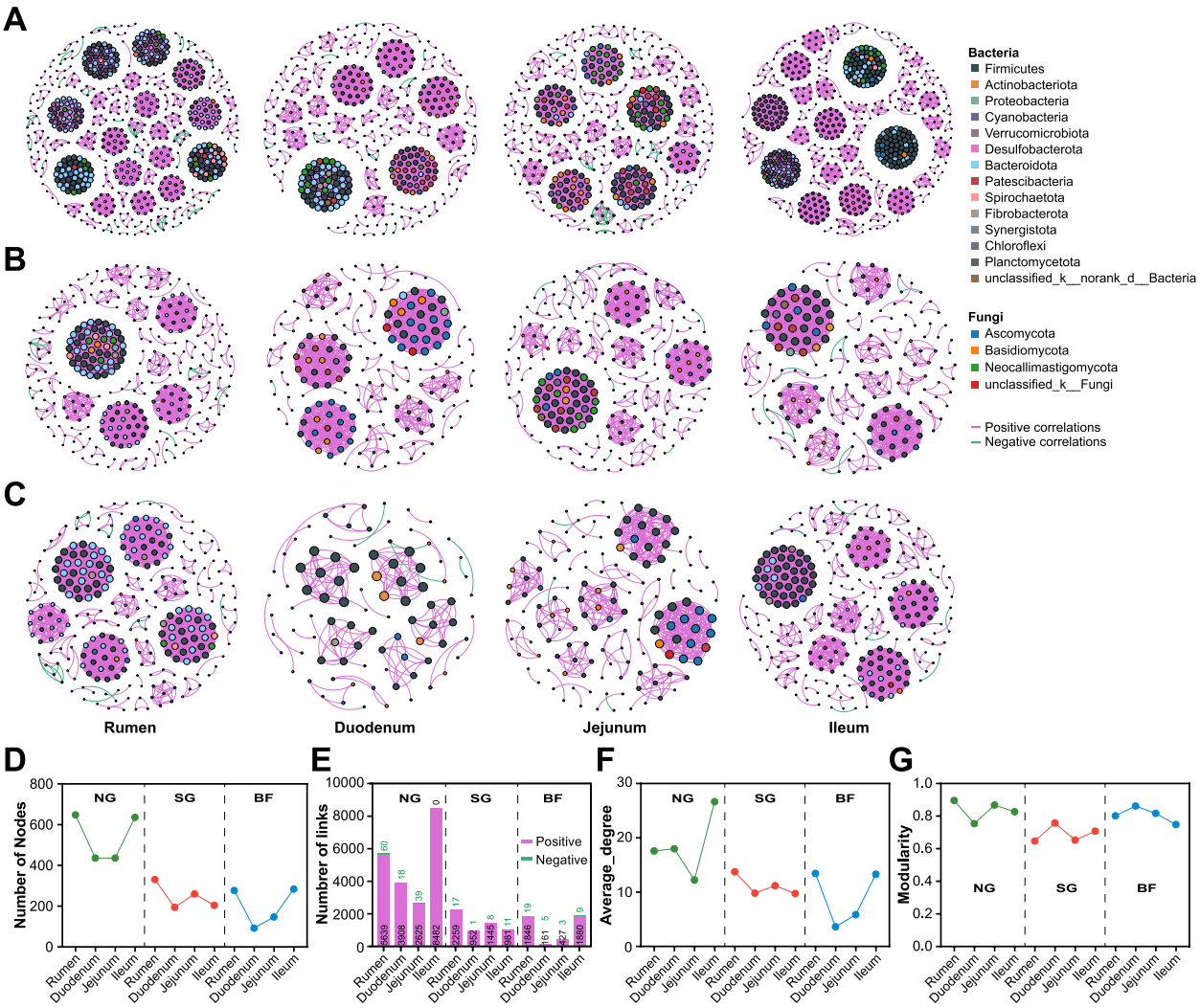

Fig. 2 Co-occurrence networks of microbial taxa in the distinct communities. Co-occurrence networks of the microbiome along the gastrointestinal tract (GIT) in the NG **A**, SG **B**, and BF **C**. Network properties including the number of nodes **D**, number of linkers **E**, average degree **F**, and network modularity **G**. The nodes serve as amplicon sequence variants (ASVs) and are coloured according to the phyla in which the ASV belongs. The size of each node represents the degree. The edges reflect strong and significant correlations based on Spearman correlations ($|r| > 0.6$, $P < 0.001$) among the ASVs. The colours of the edges reflect positive and negative correlations between ASVs. NG, natural grazing; SG, semi-grazing with supplementation; BF, barn feeding

galactose metabolism (map00052), and amino sugar and nucleotide sugar metabolism (map00520) within carbohydrate metabolism; and bile secretion (map04976), protein digestion and absorption (map04974), and fat digestion and absorption (map04975) within digestive system (Figure S3).

Then, the changes in the metabolites between the NG and BF groups were examined. The results indicated that the metabolites differentially responded to the FD strategies along the GIT (Figure S4A). When comparing the first 10 KEGG pathways with a significant difference between the NG and BF groups, it was observed that the majority of KEGG pathways in the rumen and ileum were present in the metabolism and organismal systems, respectively. Tryptophan metabolism and tyrosine metabolism associated with amino acid metabolism were significantly different between the NG and BF groups across the four sites of the GIT. Lipid metabolism, including linoleic acid metabolism, steroid hormone biosynthesis, arachidonic acid metabolism and glycerophospholipid metabolism, was significantly altered between the NG and BF groups in the rumen. Digestive system, nervous system, endocrine system and immune system, associated with organismal systems, were significantly different between the NG and BF groups in the ileum (Fig. 3B and Figure S4B). The microbe–metabolite

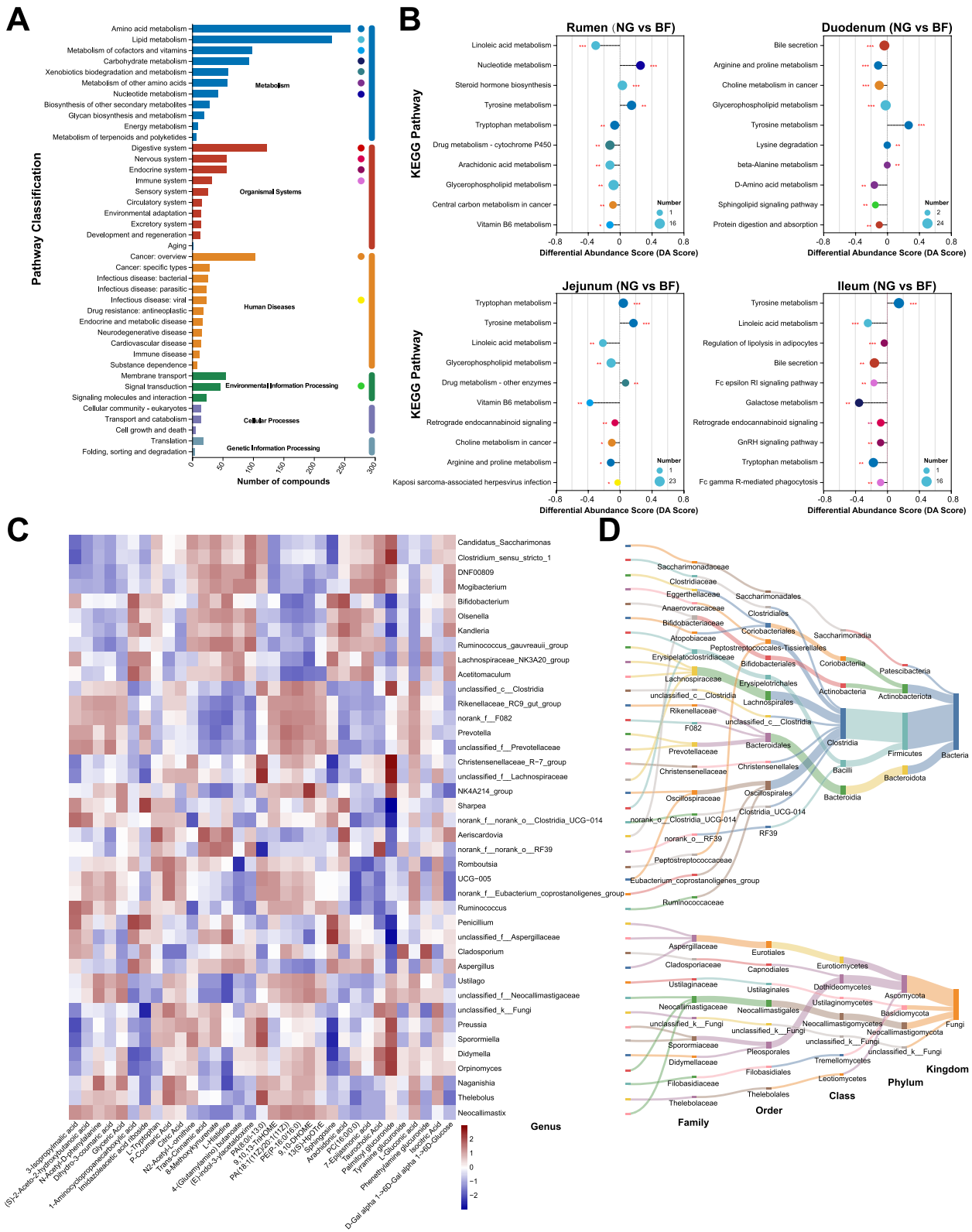

Fig. 3 Metabolome analysis of the gastrointestinal tract (GIT) of Gangba sheep. **A** Pathway classification of all identified metabolites. **B** Comparison of significantly enriched KEGG pathways between the NG and BF groups. **C** Pearson correlations between the top 40 microbial genera and a total of 33 of the top 300 metabolites involved in amino acid metabolism, lipid metabolism, carbohydrate metabolism and digestive system pathways. **D** Sankey diagram showing the microbial distribution. NG, natural grazing; BF, barn feeding

correlation heatmap showed associations between the top 40 microbial genera and a total of 33 of the top 300 metabolites that participate in amino acid metabolism, lipid metabolism, carbohydrate metabolism and digestive system pathways. These microbial genera were mainly distributed in Fimicutes, Actinobacteriota, Bacteroidota, Ascomycota and Neocallimastigomycota, among which, Lachnospiraceae and Oscillospiraceae belong to Fimicutes, Bifidobacteriaceae to Actinobacteriota, Prevotellaceae to Bacteroidota, Aspergillaceae and Sporormiaceae to Ascomycota, and Neocallimastigaceae to Neocallimastigomycota (Fig. 3C and D). These data suggest that these microbial taxa may be implicated in amino acid metabolism, lipid metabolism, carbohydrate metabolism and digestive system pathways. These findings provide a starting point for understanding the functions potentially represented within the microbial community.

Profiling of the sheep metagenome

A total of 420,271,747,204 bp of metagenome sequencing data generated a total of 1,922,043,104 optimized reads, with 28,654,345 ± 1,172,567 optimized reads and 4,235,488,928 ± 173,491,624 bases (Mean ± standard error of mean [SEM]) per sample after removing low-quality bases, adapters and the host genomes. The clean reads were assembled using metaSPAdes [40] and then grouped into a total of 13,598,464 reads (> 500 bp). The resulting 8,674,037 contigs were grouped into 12,452 bins via metabat2 v2.12.1 [42], maxbin v2.2.6 [43] and concoct v1.0.0 [44], using the default parameters. The MAGs were aggregated and dereplicated using dRep v3.4.5 [45, 46]. Finally, 1107 bacterial and 39 archaeal MAGs were obtained based on completeness > 50% and contamination < 10%. A total of 519 high-quality MAGs with completeness > 80% and contamination < 10% were classified into 13 bacterial phyla and one archaea phylum, among which, 98.84% of MAGs, including 98.81% of bacterial and 100% of archaeal MAGs, were assigned to known genera, and 62.04% of MAGs, including 62.70% of bacterial and 40% of archaeal MAGs, were assigned to known species (Fig. 4A). Further, 49.21% of 504 bacterial MAGs were assigned to Bacillota_A, 29.76% to Bacteroidota, 6.75% to Bacillota, 4.76% to Bacillota_C and 4.76% to Actinomycetota at the phylum level. At the family level, 18.06% of 504 MAGs were assigned to Lachnospiraceae, 12.30% to Bacteroidaceae, 7.14% to Acutaibacteraceae, 4.96% to Osillospiraceae and 4.17% to Ruminococcaceae (Fig. 4B, C, D and Table S4). A comparative analysis of the 7314 MAGs, including 519 assembled in this study and 6795 collected from previous studies (see the method) [47–50], was performed. The MAGs identified in the Gangba sheep GIT were widely distributed across the GIT microbial genomes of ruminants (Figure S5).

For prokaryotic MAGs, a total of 1,070,836 protein-coding sequences were predicted and annotated using Prokka. Enzymes such as cellulases, hemicellulases and pectinase/esterase are important in the degradation of plant cell walls [66]. This study analysed carbohydrate-active enzymes (CAZymes) against the CAZy database to explore the potential functional categories of cellulases, hemicellulases and pectinases/esterases. CAZymes were present extensively in the ruminant gastrointestinal microbial taxa, including CAG-74 and Ruminococcaceae from Bacillota_A (Fig. 4E and Figure S6A), and Bacteroidaceae and UBA932 from Bacteroidota (Fig. 4E and Figure S6B). These taxa generally coded several cellulases (aver 4.67—8.76), hemicellulases (aver 12.73—24.68) and pectinases/esterases (aver 3.86—13.35) per MAG and were primarily enriched in the GIT of ruminants. Upon further analysis, it was found that the members of Bacteroidaceae harboured the most cellulases, hemicellulases and pectinase/esterase (aver 7.45, 24.68 and 13.35) (Figure S6A and Figure S6B). These findings suggest that these taxa probably possess the dual capability of breaking down plant cell walls and fermenting basic sugars at a combined trophic level. The numerous cellulase, hemicellulase and pectinase/esterase-coding genes in the MAGs may account for the widespread distribution of the microbiome in cellulose-rich habitats, such as the rumen.

Expression of key enzymes for plant cell wall breakdown

The livestock gut microbiome encodes enzymes such as cellulases, hemicellulases and pectinases/esterases, which help to degrade plant polysaccharides, including cellulose, hemicellulose and pectin, and play a key role in the digestive function of livestock [21]. To probe the actual functions of these enzymes within the Gangba sheep GIT microbiome in more detail, qPCR assays for GH5, GH10, GH43, GH48, GH51, PL1, CE2 and CE15 were performed. High gene copy numbers were observed for GH5 (*Prevotella*, *Ruminococcus* and *UBA3839*), GH10 (*Prevotella*), GH43 (*Prevotella*), GH48 (*Ruminococcus*), GH51 (*Prevotella*), PL1 (*Ruminococcus*), CE2 (*Ruminococcus*) and CE15 (*Fibrobacter*) originally detected within the rumen of Gangba sheep (Fig. 5A). This implies that the degradation of plant polysaccharides is the primary responsibility of microorganisms in this habitat. Meanwhile, the importance of cellulases, hemicellulases and pectinases/esterases in other rumen was evaluated by integrating both the microbial metagenome of the Gangba sheep GIT and metatranscriptome data from previous studies. About 40% of the annotated MAGs exhibited the activity of cellulases, hemicellulases or pectinases/esterases (Fig. 5B). These enzymes were mainly distributed

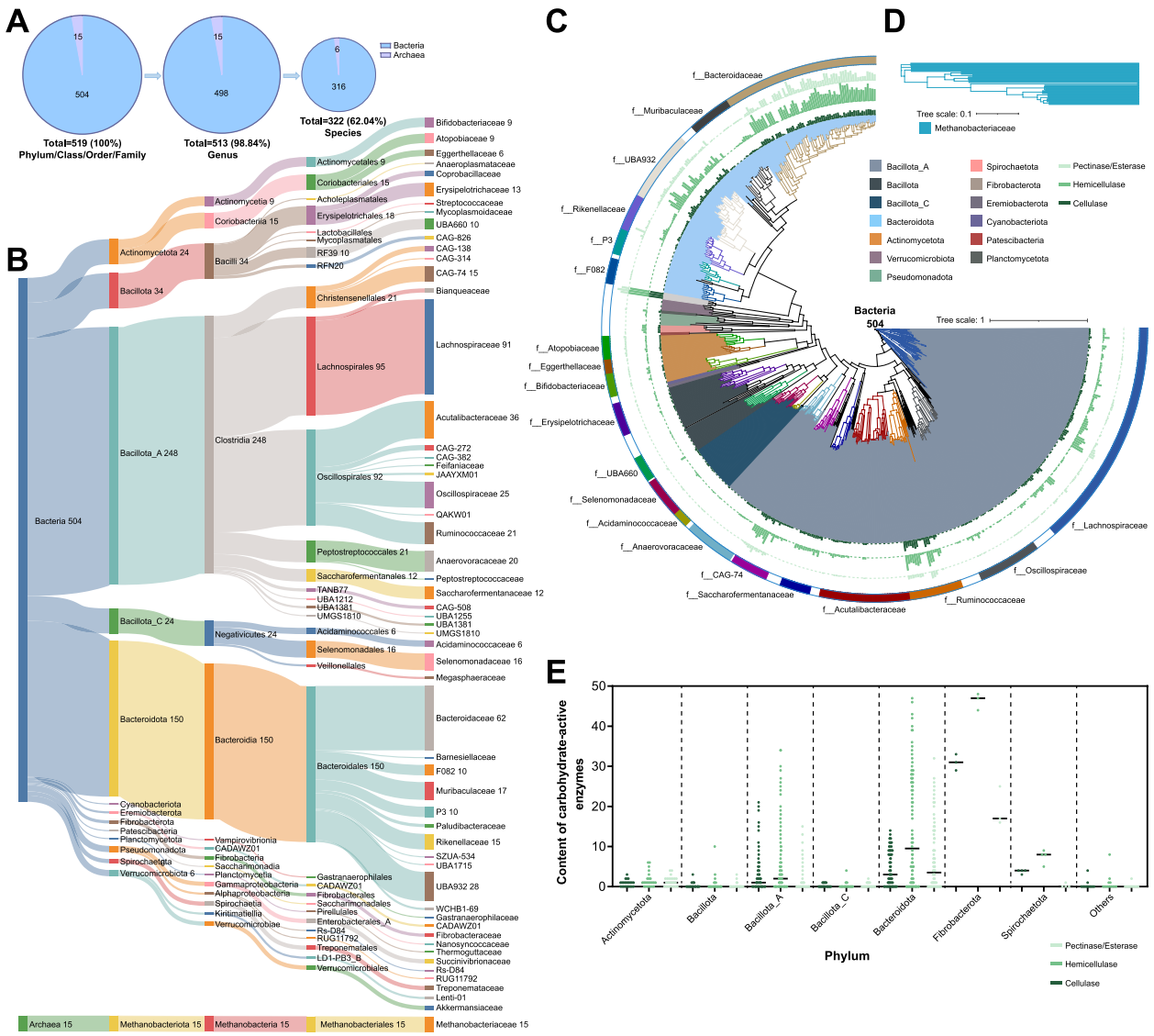

Fig. 4 Taxonomic characteristics of 504 bacterial and 15 archaeal metagenome-assembled genomes (MAGs) assembled in this study. **A** Classification of 519 MAGs at different taxonomic levels. The numbers represent the number of MAGs that could be annotated at the respective levels. **B** Sankey diagram showing the microbial distribution of MAGs. Phylogenomic tree of bacteria **C** and archaea **D**, as well as the distribution of carbohydrate-active enzymes in the Gangba sheep metagenome. Coloured bars represent the amount of cellulases, hemicellulases and pectinases/esterases in each MAG, respectively. **E** Contents of cellulases, hemicellulases and pectinases/esterases in each bacterial or archaeal MAG at the phylum level

in Bacteroidaceae and Lachnospiraceae, followed by UBA932, Ruminococcaceae, CAG-74, Oscillospiraceae, Muribaculaceae, and so on, belonging to the phyla Bacteroidota and Bacillota_all (including Bacillota_A, Bacillota, Bacillota_C), and their respective phyla (Fig. 5C). A large number of enzymes were expressed across the microbial taxa while a small portion of enzymes were expressed in certain microbial taxa. For example, CE6 only existed in Bacteroidota and Fibrobacterota. Moreover, the level of gene expression was classified into low expression (FPKM < 1), medium expression (FPKM 1 – 10) and high expression (FPKM > 10). High expression levels of these enzymes were found in members of Bacteroidaceae and Ruminococcaceae. Among these taxa, *Prevotella* (various *Prevotella* spp.) and *Ruminococcus*_all (including *Ruminococcus* and *Ruminococcus*_D) with high expression levels possessed large sets of enzymes, including CE8,

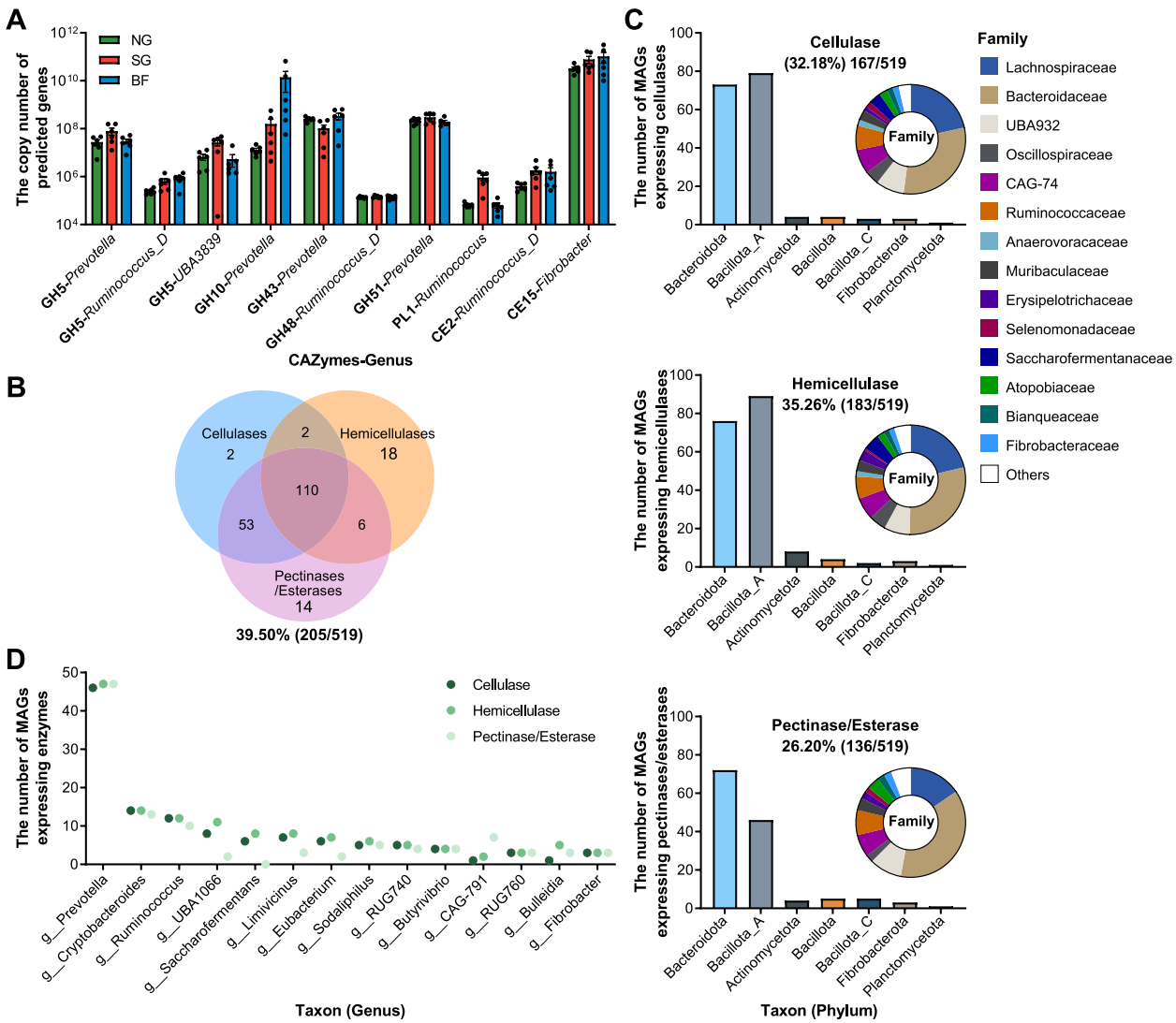

Fig. 5 The profiles of predicted carbohydrate-active enzymes at the transcript level. **A** The copy number of predicted carbohydrate-active enzymes in the gastrointestinal tract (GIT) of Gangba sheep. **B** The number of metagenome-assembled genomes (MAGs) that expressed cellulases, hemicelluloses and pectinases/esterases. Classification of MAGs that expressed cellulases, hemicelluloses and pectinases/esterases at the respective levels **C** and genus level **D**

GH48, GH9, GH10, GH51, GH5, GH43, GH26, GH28, GH141, CE7, CE12 and PL1 (Fig. 5D, Figure S7 and Table S5), acting on celluloses, hemicelluloses and pectins. This indicates that these taxa may play important roles in plant biomass degradation. The presence and potential functions of these enzymes in the GIT enable them to improve the digestibility and utilization of plant-based feed and maintain the stability of the GIT.

Discussion

As an important indigenous livestock breed, Gangba sheep contribute to human culture and survival under extreme environments, such as prolonged hypobaric hypoxia, cold and food scarcity. While studies have demonstrated that gastrointestinal microbiomes of animals co-evolve and co-develop with the host [67, 68] and are critical for digestion and absorption, a comprehensive survey of the gastrointestinal microbiomes of Gangba sheep covering the locations of the GIT under distinct FD strategies has, to date, not been performed. Thus, this study describes the dynamic changes in microbial communities and the potential functions of specific taxa in response to the sites of the GIT and distinct FD strategies. Metagenomic and qPCR analyses revealed the microbial genomic catalogue of the Gangba sheep GIT and the microbial species related to the cellulases,

hemicellulases and pectinases/esterases contribute to plant biomass degradation at high altitudes. In addition, the MAGs assembled in this study and the metatranscriptome data collected from previous studies revealed the expression levels of biomass-degrading enzymes in other rumen. These findings offer guidance for the potential application of microbial strains as probiotic sources in animal feed.

An improved understanding of microbial community dynamics in the Gangba sheep GIT may assist with the exploitation of microbial functions in domestic animals. The current study revealed, for the first time, that the dynamic pattern of microbial communities along the GIT of Gangba sheep is driven by distinct FD strategies. Generally, both the FD strategy [7] and the location of the GIT [26, 63, 64] may affect the assembly of the microbial community. However, in the present study, the FD strategy had a stronger effect on the gastrointestinal microbial communities than the location of the GIT. To some extent, this is probably a reflection of the rather extreme shortage of food resources in high-altitude habitats. It is widely acknowledged that dietary alterations [69] and physical exercise [70] influence gut microbiome diversity and composition, which, in turn, influence intestinal metabolomes. In Gangba sheep, shifts in gastrointestinal symbiotic microbial diversity and composition are driven by FD strategies, resulting in a decreased number of microbial species in the GIT of the SG and BF groups compared with the NG group. However, relatively stable environmental factors such as temperature and humidity may accelerate the growth and reproduction of Actinobacteriota, resulting in a higher number of members of the phylum Actinobacteriota in the SG and BF groups as compared to the NG group. The identification of interaction patterns in microbial communities is essential to further our understanding of the functions of microbial communities as a whole. Previous studies have shown that the modularity of microbial co-occurrence networks varies with the environment, such as soil properties [71], and may represent different niches [72]. Here, although the number of nodes and links in the SG and BF group networks were reduced compared to the NG group, especially in the duodenum and jejunum, the modularity of all co-occurrence networks was high (> 0.6), indicating a certain structural and functional stability. But the loss of several key symbiotic partners may eventually influence the biotic niches of microbes and the physiological outcomes of the host.

An understanding of the potential functions of the gastrointestinal microbiome is critical if we are to identify the mechanisms that shape host nutrition and fitness. Therefore, the current study further focused on the roles of altered microbes in the functional profiles of the GIT of Ganba sheep under distinct FD strategies. The findings not only revealed associations between the microbial species and their metabolism pathways but also demonstrated the enzymes related to the microbial species that contribute to the degradation of plant biomass in the Gangba sheep GIT. It has been shown that lipid metabolism, amino acid metabolism, carbohydrate metabolism, and metabolism of cofactors and vitamins contribute to the host [73, 74]. These metabolism pathways were predominant in the Gangba sheep GIT and may be required to maintain normal physiological functions. The current results indicated that the microbial communities had distinct functions in different nutrient environments. Compared with the BF group, most of the metabolic pathways enriched in the rumen and organismal systems enriched in the ileum were significantly different in the NG group. Lipid metabolism serves as an important physiological function of the human body, playing a role in the digestion and absorption of lipid products from food [75]. Lipid metabolism, including "linoleic acid metabolism", "steroid hormone biosynthesis", "arachidonic acid metabolism" and "glycerophospholipid metabolism" was significantly altered between the NG and BF groups in the rumen. This suggests that FD strategies have major effects on the digestion and absorption of lipid products from food. The digestive system, nervous system, endocrine system and immune system, associated with organismal systems, were significantly different between the NG and BF groups in this ileum. The ileum is the last part of the small intestine and is responsible for digestion and absorption, secretion, and immune function [76]. Further, several metabolism pathways were significantly different across the distinct FD strategies, e.g., tyrosine metabolism, meanwhile, while some were conserved, suggesting that they are not sensitive to the FD strategy. This is reasonable given that these metabolism pathways are required to maintain primary physiologic functions for host survival and health. Lachnospiraceae, Prevotellaceae, Oscillospiraceae, Aspergillaceae and Neocallimastigaceae were found to be closely related to the amino acid metabolism, lipid metabolism, carbohydrate metabolism and digestive system pathways. Analysis of the microbiome and metabolome can provide a detailed characterization of both microbial taxa and their potential functions and can reveal the important biological processes of microorganisms in complex ecosystems.

Recent studies have shown that biomass-degrading enzymes enriched in the goat gut microbiome degrade lignocellulose by hydrolytic strategies [53] to affect

overall food digestion and nutrient absorption [77]. The current results suggested that the microbiome of the Gangba sheep GIT may play similar roles. Specifically, most members of the microbial communities contained cellulases, hemicellulases and pectinases/esterases. *Fibrobacter*, which is the only member of the phylum Fibrobacterota, is a major microbial taxon responsible for cellulose digestion [64]. In the current study, while the number of MAGs of *Fibrobacter* assembled was modest, the results showed that they possessed the largest amounts of cellulases, hemicelluloses and pectinases/esterases. This may suggest that *Fibrobacter* spp. efficiently converts plant biomass to energy by hydrolysis in the GIT of ruminants [78]. Moreover, a number of biomass-degrading enzymes were enriched in the members of the phyla Bacteroidota and Bacillota, and expressed in the Gangba sheep GIT. Further investigation revealed that most of these enzymes within *Prevotella* spp. and *Ruminococcus* spp. were expressed at high levels (FPKM > 10) in other rumen metatranscriptome data of ruminant livestock from previous studies. These results might be explained by the potential functions of *Prevotella* spp. and *ruminococcus* spp. in the plant-rich diets of the host [79]. Recently, improvement in the feed efficiency of domestic animals has been encouraged [80], and a thorough understanding of the expression levels of biomass-degrading enzymes in the GIT is critical for improving food digestion and nutrient absorption. The current findings not only provide novel insight into the plant biomass degradation mechanisms in the complex GIT of ruminant livestock but also provide evidence supporting the bio-utilization of plant biomass by specific microbes in the future.

Conclusion

The present study comprehensively compared the microbial communities of variations locations of the GIT under different FD strategies and described the symbiotic relationships between the gastrointestinal microbes and their predicted functions in Gangba sheep. Further, this study provided a detailed characterization of the Gangba sheep gastrointestinal microbial catalogue, including bacterial and archaeal MAGs, and identified the enzymes responsible for the degradation of plant biomass, and their degradation mechanisms, in the GIT of Gangba sheep. Further studies should aim to provide a more detailed description of the regulation effects of FD strategies on gastrointestinal microbial communities in order to achieve precision nutrition and health outcomes in livestock in high-altitude regions.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40168-024-02022-5.

Additional file 1: Table S1. Dietary composition and nutrient content.

Additional file 2: Table S2. Growth and slaughter performance of Gangba sheep.

Additional file 3: Figure S1. Core microbial genera along the gastrointestinal tract (GIT) of Gangba sheep. NG, natural grazing; SG, semi-grazing with supplementation; BF, barn feeding.

Additional file 4: Figure S2. The changes in microbial taxa within the co-occurrence networks along the gastrointestinal tract (GIT) and the influence of the feeding (FD) strategies: NG (A), SG (B) and BF (C). NG, natural grazing; SG, semi-grazing with supplementation; BF, barn feeding.

Additional file 5: Table S3. Metabolite information of the gastrointestinal tract (GIT) of Gangba sheep.

Additional file 6: Figure S3. Classification of the metabolic pathways in the gastrointestinal tract (GIT) of Gangba sheep.

Additional file 7: Figure S4. Differential predicted functions between the NG and BF groups. (A) Volcano plots illustrating the up- and down-regulation of the metabolites. (B) Comparison of significantly enriched KEGG pathways between the NG and BF groups. NG, natural grazing; BF, barn feeding.

Additional file 8: Table S4. Information on metagenome-assembled genomes (MAGs) assembled in this study.

Additional file 9: Figure S5. Phylogenomic tree of bacteria (A) and archaea (B) in the gastrointestinal tract (GIT) of ruminant livestock.

Additional file 10: Figure S6. The number of metagenome-assembled genomes (MAGs) predicted containing carbohydrate-active enzymes in Bacillota_A (A) and Bacteroidota (B).

Additional file 11: Figure S7. Phylogenomic tree of predicted carbohydrate-active enzymes within the rumen microbial metatranscriptome. The number of amino acid sequences is larger than 100.

Additional file 12: Table S5. Expression of genes encoding cellulases, hemicelluloses and pectinases/esterases in metagenome-assembled genomes (MAGs).

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Not applicable.

Authors' contributions
Xiaoqing Zhang conceived the ideas. XL designed this study. Xiaoqing Zhang performed the animal experiment. XL, HD, NT, JZ, QZ, HL and MS collected the gastrointestinal content samples. XL and Xiaoxue Zhang performed the computational analysis of transcriptome. Xiaoxue Zhang performed quantitative PCR (qPCR). Unless otherwise specified, XL performed all of the computational analyses and drafted of the manuscript. XL and Xiaoqing Zhang made revision and provided funding for this research. All authors read and approved the final manuscript.

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Data availability
The raw sequencing reads of amplicon sequencing and metagenome sequencing were deposited into CNCB with BioProject accession number PRJCA026346. The microbial metagenome assemblies used in this study are available at the JGI (https://genome.jgi.doe.gov/portal/HungateCollection/HungateCollection.info.html), Edinburgh DataShare (DOI:https://doi.

org/10.7488/ds/2296), NCBI BioProject under accession PRJNA631951, ENA under accession PRJEB31266, respectively. The available meta-transcriptomic datasets were obtained from the NCBI SRA database using accession number SRA075938.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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