

# The Gut microbiota in Sheep of Different Breeds in Qinghai Province

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## Research article

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# Abstract

Background: Gut microbiota play important roles in their co-evolution with mammals and may influence their emissions into numerous habitats. Mammal lifestyle is related to immune and metabolic system, driven by difference in their gut microbiota composition.

Results: Using 16S rDNA genes Illumina sequencing, we dissect the specificity of gut microbiota among four breeds of sheep and find the difference between TSs and other three types of sheep (DsSs, STHSs and DrSs). The results also indicate that gut microbes effect on the adaptation of high-elevation animals, which can contribute to the survival of their hosts in high-altitude conditions. Besides, the analysis of function abundance profile shows that commensal bacteria are entirely possible to coevolved with their host genomes by gene lineages contribute to energy metabolism. And the study of low methane metabolism in mammals at high-elevation environment may give theoretical basis for the biologic supervision of greenhouse gas emissions, which is the byproduct of high-methane-producing animals.

Conclusions: In conclusion, our results reveal the value of characterizing mammalian gut microbiomes to fully understand mammal's lifestyle among different breeds.

## Background

The significance of gut microbiome is well known, it is an extremely complicated and diverse population and has been explored extensively [1, 2]. Recently, a new term 'superorganism' is applied to describe the strong tie between the gut commensal and its reliable host [3, 4]. The intestinal microbiota is linked to a diverse range of conditions, including gathering energy, promoting intestinal epithelial cell proliferation and enhancing the immune system [5, 6], and can be deservedly regarded as an 'organ' playing a significant part in the metabolic process [3, 7, 8]. It has been found that more than 3.3 million commensal genes are resided in humans, which is equivalent to 150 times of human genes [9]. In ruminants, the composition of the gastrointestinal commensals, their effect on host immunity and welfare have been explored for several years. A previous study suggested that it was a great achievement of using gut microbiota by ruminant animals [10]. And it's true that combining high-throughput 'omics' technologies with ruminant's genomes, the unprecedented well-being of achievement can be harvested [3].

Initially, the researches about gut microorganisms were dependent on the sequence alignment from genomic libraries to screen the functional genes, or through PCR amplification [3]. In *Escherichia coli*, heterologous gene expression was determined by Sanger Chain Termination Method and obtained the autoradiographic map [11]. However, it could only obtain the genes expressed in *E. coli*, and certainly leads to a large missing of available genes. Through this method, the first gene was acquired from *F. succinogenes* and encoding cellulases [12]. In the following years, seven *F. succinogenes* genes were detected through these traditional genetic methods, which encoded fiber-degrading enzymes [13]. This was certainly an extraordinary progress, but after *F. succinogenes* S85 genomic sequencing was finished, it was found that there were 104 open reading frames to participate in the disruption of plant cell wall

[14]. Accordingly, the great advantage of genomic sequencing is intuitively clear, more enzymes were detected than that of previous researches in the *F. succinogenes* genome.

Metagenomics is performed to analyze the genome of microbial communities in an environmental sample, including the genomic sequence-based analysis and functional prediction. It is applied to screening the specific functions and detecting new bioactives in diverse ecosystems [15]. Moreover, it is a vital step to model and connect the microbial structure and function to that of the host [16, 17]. Some researchers found that the gut microbiota of mammals have a large identical part of their functions, suggesting that the understanding of human researches can provide many common views for ruminants [18]. In addition, research has shown that there is a relationship between microbial lineages and their specific environment [19]. However, those gut microbes are not frequently emerged in other environments [20]. Some study also showed that gut microbes were extensively shared among various mammals [21], indicating that some views are commonly applied to both humans and domestic animals.

In the present study, to better analyze and clarify the relationship between microbial lineages and the host species from a perspective of gut microbiota, amplification of V3 + V4 region of the 16s rDNA was performed, which was followed by, the most credible techniques, Illumina MiSeq Reagent Kit PE250 sequencing. Apart from the shared characteristics are presented among all sheep, typical microbial population features corresponding to different species of sheep are also mentioned in our research.

## Results

### Description of the sequencing data

The samples we obtained were from the feces of a great variety of sheep, which were comprised 40 subjects, namely 10 Dorset sheep (DrS), 15 Small Tail Han sheep (STHS), 5 Tibetan sheep (TS) and 10 Dorper sheep (DrS). We received 1, 694, 264 raw bases on a PE250 instrument, as mentioned in the experiment method. Behind quality-filtering (also as described in the methods), 1, 359, 405 total sequences with an average of 433 bp in length were obtained for the following analysis. In order to normalize the total count for further alpha and beta diversity analysis, the high-quality sequences in all samples were optimized to the minimum number.

### Gut microbiota is associated with sheep species

After simplifying the original data, 1, 359, 405 high-quality available sequences were acquired. According to 97% species similarity, 7039, 6887, 4112, and 8257 OTUs were separately acquired from samples at groups DrS, DsS, TS, and STHS (Table 1), respectively. A total of 9682 OTUs were detected from all samples, of which 2448 obtained in all groups mentioned as core OTUs (Figure 1(A)). The core OTUs contained nearly 25.28 % of the entire OTUs. Furthermore, 225, 203 OTUs were uniquely detected in DrS and DsS groups, and 168, 654 unique OTUs were found in group TS and STHS, respectively. The number of observed OTUs in the TS samples was fewer than that of the other three.

To confirm the quality of our sequencing data, we examined alpha and beta diversities of bacterial fraction of the sheep microbiota. Several alpha diversity indices diverged significantly between the four types of sheep (Fig.1 (B) and (C)). In terms of the Chao1 estimator, 3160, 2598, 1585 and 2207 average OTUs were noted in samples at groups DsS, STHS, TS and DrS, respectively, and the ACE estimator indicated 3263, 2646, 1627 and 2267 (Table S2), which was consistent with the results shown in Chao1, suggested that lower OTU richness was got in TS samples than the other threes (Fig.1 (B) and (C)). The Shannon–Wiener index may straightly expose the heterogeneity of a community according to the amount of species present and their related abundance [22]. The Shannon–Wiener estimator of groups DsS, STHS, TS and DrS were 9.92, 9.33, 8.09 and 9.06, respectively. Within the groups, Chao1 and Shannon index visually reflected that the abundance and diversity of intestinal microbial population in the TS group were lower than those in groups DsS, STHS and DrS, and the difference between these four groups was significant ( $P < 0.05$ ; Figure 1 (B) and (C)). Collectively, these data pointed towards a more diverse bacterial population in TS compared to the other threes, and indicated differences in intestinal microbial composition in association with sheep species.

The bacterial microbiome composition is significantly altered in different types of sheep

Using the unweighted Unifrac similarity and Bray-Curtis, distances between each sample were calculated for the purpose of examining the overall differences in microbial composition among the four sheep species.

To expose relationships based on bacterial abundance and their phylogenetic relatedness, the principal coordinates analysis (PCoA) was presented according to the phylogenetic-tree-based Unifrac metric. As shown in Fig. 2 (A), all samples were assembled into three clusters, scattered points in the principal component denoted different types and their relationship between each other. There was significant differences between types in relation to microbiome composition (ANOVA,  $p < 0.01$ ). TSs were mainly aggregated in cluster B, whereas DsSs were mostly converged in cluster A. Moreover, DrSs and STHSs were more scattered and found between cluster A and cluster B. Both principal components accounted for 28.1% (PC1) and 8.5% (PC2) of the explained variance. Interestingly, two lambs in STHSs were clustered separately as shown in Fig. 2 (A), suggesting that there was a general difference in gut microbiome between adult sheep and lambs.

Nonmetric multidimensional scaling (NMDS) was used to further clarify the difference among all the types in bacterial population composition, which was performed using the Bray-Curtis similarity for all the samples at OUT level[1]. Be regarded as a dominant ordination method that could exhibit the non-linear relationship between samples, NMDS has been widely applied in the study of gut microbiome. As shown in Fig. 2 (B), there was distinguishing clustering of TSs samples, meanwhile samples from DsSs were very close to DrSs. However, the samples from STHS were more dispersed (Fig. 2 (B)).

Additionally, hierarchical clustering analysis of all samples was used to exhibit the similarity between samples, which was performed with Unweighted pair-group method with arithmetic means (UPGMA) and used the Bray-Curtis similarity. Two primary groups were perceived in this analysis. One cluster contains

the whole samples of TS and the other cluster contains the all samples from DrS (Fig. 3). It was similar with the results above, that TS samples were distinctive compared with other types. And the gut bacterial composition in sheep is largely influenced by the type of samples.

#### Comparisons of gut microbiota at different levels

In order to clarify the diversity of gut bacterial composition in different types of sheep, we estimated the gut microbiota in different taxonomical levels. The overall bacterial composition of different groups at the phylum level is identified in Fig. 4 (A) which shows that *Firmicutes* was the most predominant phylum in the 40 samples, followed by *Bacteroidetes*. The higher abundance of phylum *Spirochaetes*, *Proteobacteria* and *Verrucomicrobia* were found in TSs than those in the other three types, but the *Deferribacteres* was lacked in TSs (Fig. 4 (A)).

When analyzed on the family level from all samples, as shown in Fig. 4 (B), no significant differences were detected between these four groups. *Ruminococcaceae* and *WCHB1-25* were the most abundant families in DsS, DrS and STHS groups, whereas *Alcaligenaceae*, *Desulfovibrionaceae* and *Barnesiellaceae* were almost absent. As for TS groups, the most abundant families were *Spirochaetaceae*, *S24-7*, *Prevotellaceae*, *Barnesiellaceae* and *Succinivibrionaceae*, while BS11 and *WCHB1-25* were almost absent in the TS samples (Fig. 4 (B)).

In contrast to the family level, there are significant differences between TS group and the other three groups on the genus level. The main genera in TS group included *Treponema*, *Succinivibrio*, *Akkermansia* and *Prevotella*, while *Bifidobacterium*, *Sharpea* and *YRC22* were absent (Fig. 4 (C)). Moreover, in the groups of DsS, STHS and DrS, *Treponema* remained the predominant population, and *Coprococcus* and *Roseburia* were relatively less abundant. However, it's worth noting that a large number of microbes in TS samples were relatively abundant, when compared to other three groups.

#### Function abundance profile of gut microbiota from all species

To estimate the function of gut microbiota of all four groups of sheep, we established the function abundance profile based on OTUs in the Kyoto Encyclopedia of Genes and Genomes (KEGG) Database (Fig. 5). Predominant functions performed by gut microbiota of different groups were membrane transport; carbohydrate metabolism; amino acid metabolism; replication and repair; translation; energy metabolism and so on (Table S3). Genes in respect of digestive system, excretory system and transport and catabolism were significantly different in TS group (Table S3, t-test,  $p < 0.05$ ), compared with DsS and STHS groups. It was noteworthy that all these genes in TS were found to be significantly increased. Remarkably, methane metabolism is significantly decreased in TS group (Fig. 5, t-test,  $p < 0.05$ ), compared with DsS, DrS and STHS groups, which is consistent with previous reports [23, 24]. In contrast, DsS, DrS and STHS samples, which is related to high methane production [25], shows higher relative abundance than in TSs (Fig. 5, t-test,  $p < 0.05$ ). These results suggest that commensal bacteria are entirely possible to coevolved with their host genomes by gene lineages contribute to energy metabolism [24, 26, 27].

## Discussion

Tibetan civilization has always been concerned by scientists all over the world and speculated as the most critical factors to influence the human and animals. The Qinghai-Tibetan Plateau (QTP) provides the most extreme survival circumstance for all creatures [24]. TSs have adapted to surviving in this harsh plateau environment, where Tibetans raise these animals for food and sustenance [24, 28]. Several studies revealed that the gut microbes of ruminants help them survival at high altitude, which involve in the energy metabolism pathway [24, 26, 27]. However, although the insights into mammalian gut microflora have extended to various fields, such as metabolism, immunology and phylogeny [19, 29, 30], few researches have been declared the relationship between the sheep breeds and the gut microbiota.

In our research, the intestinal microflora of fecal inclusion has been examined by bacterial diversity and abundance in different breeds of sheep. These results indicate that there are significant differences of the gut microbiota between TSs and the other three breeds of sheep (DrSs, DsSs and STHSs), besides, the bacterial diversity and composition in TSs are lower. However, the bacterial abundance in TSs are higher than those in the other three breeds of sheep. The microbial diversity of TSs altered significantly compared with the other three breeds, which is similar with the earlier reports in high-altitude mammals [24, 27]. PCoA clustering analysis reveals that the microbial structure is distinct between the TSs and the other three breeds (Figure 2A). Besides, hierarchical clustering analysis shows that TS samples clearly cluster together, indicating that the intestinal microbial population of TSs are highly conserved for the comparison between interspecies. From different taxonomical levels, the abundance of gut bacterial composition is also distinct among different types of sheep. This phenomenon is probably due to the fact that Tibetan sheep have adapted to the high-altitude environment, while the other three breeds, as introduced later, are convergent to the commensal composition of Tibetan sheep. In addition, our study shows that the gut bacterial composition of lambs is quite different from that in adult sheep, suggesting that the gut microbial composition in lambs will develop towards adult sheep under their survival environment.

It is also demonstrated that adaptive evolution exists in commensal bacteria of high-altitude organisms, particularly in ruminants, which may alter host metabolic repertoires [9, 18, 21, 31–33]. For example, methane can contribute to energy loss, which is a residual product of ruminal fermentation by methanogens [24, 34]. Our result indicates that methane metabolism in TSs is significantly lower than that in the other three breeds of sheep (Fig. 5, t-test,  $p < 0.05$ ), demonstrating that a low methane metabolism has been occurred in TSs after they are independent from the shared ancestor of sheep. The result of low methane metabolism in high-altitude mammals suggests that the gut microbiomes may impact their adaptation in high-elevation environment and the conversions in gut microbiome structure and composition might cause the low methane metabolism.

## Conclusion

On the whole, our 16s rDNA analyses provide essential views into the gut microbiome of different breeds of sheep and highlight the difference between TSs, which are the high-altitude mammals, and the other three types of sheep (DsSs, STHSs and DrSs). In addition, our data show that the gut microbes play an important role in the adaptation of high-elevation organisms. Future understanding might be got through analyses of systems of gut microbiome in high-elevation animals, which can contribute to the adaptation of their hosts in high-altitude conditions. Furthermore, the investigation of low methane metabolism in mammals at high-elevation environment and their distinct microbial structures may provide theoretical basis for the biologic supervision of greenhouse gas emissions, which is the byproduct of high-methane-producing animals.

## Methods

### Description of samples

Sheep were obtained from specialized farms (Qinghai Province, China) and were analyzed faecal 16s rDNA sequences from 40 individuals including 10 Dorset sheep (DrS), 15 Small Tail Han sheep (STHS), 5 Tibetan sheep (TS) and 10 Dorper sheep(DrS). Each types of sheep we selected were self-propagated and fed by the profit-making sheep farm and had similar genetic context. All screened animals were healthy and no other diseases appeared prior to the sample selection. Table S1 provides the detailed information of each sheep we sampled.

### DNA Extraction

Following the manufacturer's instructions of Omega Bio-tek, microbial genomic DNA was extracted from 500 mg of each fecal sample using the fecal DNA kit. Meanwhile, we measured the DNA quality with 1% agarose gel electrophoresis, and examined the concentration through the NanoDrop Spectrophotometer, storing DNA samples at  $-20^{\circ}\text{C}$ .

### PCR Amplification of 16s rDNA

The V3-V4 region, which was 468bp within the 16s rDNA gene, was used to build the illumine sequencing library and amplified with the broadly conserved primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3'). Different identifier codes were added at each primer for the further illumina sequencing. Polymerase chain reaction (PCR) was applied in a 50  $\mu\text{l}$  reaction system including 2x Phanta Max Master Mix (Vazyme, China), 10 mM each primer, 16  $\mu\text{l}$  each ddH<sub>2</sub>O and 5  $\mu\text{l}$  DNA template. The PCR program was initial denaturation at  $95^{\circ}\text{C}$ , with 8 cycles of denaturation at  $95^{\circ}\text{C}$  for 30 s, and followed annealing at  $55^{\circ}\text{C}$  for 30 s, extension at  $72^{\circ}\text{C}$  for 45 s, with a final elongation phase at  $72^{\circ}\text{C}$  for 5 min. The PCR products were performed by Quant-It Pico Green kit (Invitrogen, United States) and put them together for further library preparation. Barcoded samples were combined equal concentrations according to volume of sequencing. By Agilent 2100 Bioanalyzer (Agilent Technologies, United States), we performed the library concentration and eluted with Tris\_HCl (pH 8.5). After

denaturation, barcoded samples were combined following the volume of sequencing and sequenced on a PE250 v3 instrument using 600 cycles MiSeq Reagent Kit on a MiSeq Platform (Illumina; United States).

## Bioinformatics and Statistical Analysis

In our research, the generating sequences have been uploaded to the National Center for Biotechnology Information (NCBI) under accession number AR180907. The QIIME (Quantitative Insights Into Microbial Ecology, v1.8.0) was performed to process the raw reads, and then the paired reads were assembled by FLASH v1.2.7 [35, 36]. Subsequently, QIIME was used to filter and analyze the joined sequences. By UPARSE 7.0, operational taxonomic units (OTUs) were obtained with based on a 97% identity threshold. Eventually, the whole OTUs were categorized to distinct taxonomic levels by Ribosomal Database Project (RDP) classifier 2.2 [37]. Based on the OTUs information, R package VennDiagram was performed to complete the venn diagram. In addition, the phylogenetic tree was obtained by MAGA 5.2 after sequences alignment. Alpha diversity was measured by MOTHUR, which was referred to the microbial community diversity. Bray-Curtis distance and unweighted Unifrac was evaluated the similarities of different samples with R package vegan. The Bray-Curtis distance is founded on common OTUs among samples to provide equal weight to differences in each taxa [1, 38]. The Unifrac is used to create the phylogenetic tree for samples and the taxa which are phylogenetically related will give less divergent Unifrac values, while the unrelated taxa will generate larger differences [39]. OIIME was performed to generate phylogenetic beta diversity, and further to do principal coordinate analysis (PCoA) and hierarchical clustering analysis by R program based on Bray-Curtis distance and unweighted Unifrac.

Function abundance profiles of gut microbiome were constructed based on OUT abundance of different types of sheep standardized by PICRUS<sub>t</sub> (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) and the functional information of microbial community can be searched against the KEGG PATHWAY Database (<http://www.genome.jp/kegg/pathway.html>). ANOVA and student's t-test were performed to exam significant differences between various groups.

## Abbreviations

DrS: Dorset sheep; STHS: Small Tail Han sheep; TS: Tibetan sheep; DrS: Dorper sheep; NCBI: National Center for Biotechnology Information; QIIME: Quantitative Insights Into Microbial Ecology; OTU: operational taxonomic units; RDP: Ribosomal Database Project; PCoA: principal coordinate analysis; PICRUS<sub>t</sub>: Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; NMDS: Nonmetric multidimensional scaling; UPGMA: Unweighted pair-group method with arithmetic means; KEGG: Kyoto Encyclopedia of Genes and Genomes; QTP: Qinghai-Tibetan Plateau.

## Declarations

Ethics approval and consent to participate

All experiments were approved by the Ministry of Health in China for the care and use of laboratory animals and supervised by the Research Ethics Committee of Northwest A&F University. Informed consent was obtained from the animal owners in advance.

Consent for publication

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this published article, and also available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no conflict of interest.

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Authors' contributions

WTM and DKC conceived and designed experiments; XTY and JJC performed all experiments. XTY, CXZ and YXQ collected and analyzed the data. XTY and JJC drafted the manuscript. All authors read and approved the final manuscript.

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Not applicable.

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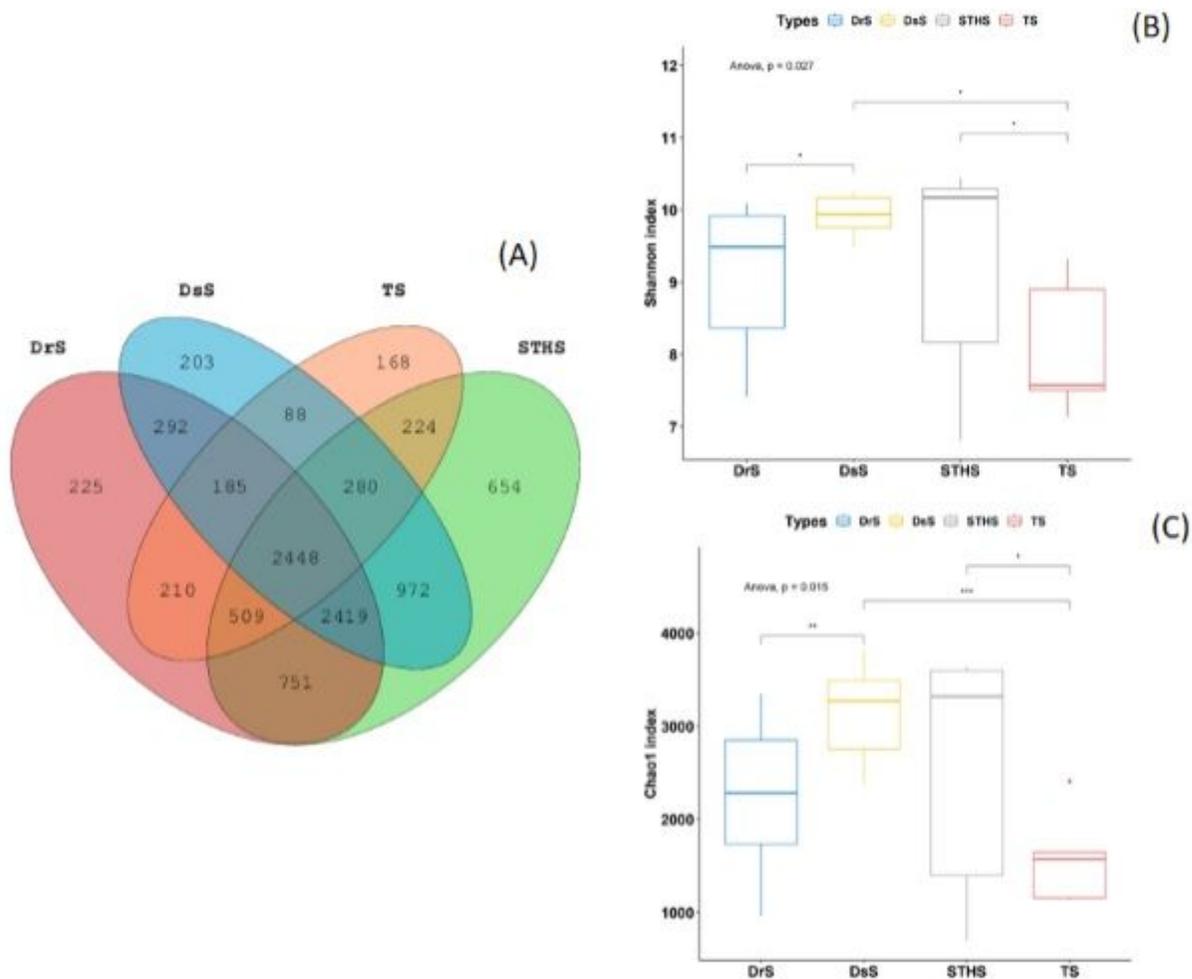
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## Tables

**Table 1:** Sequence data of samples.

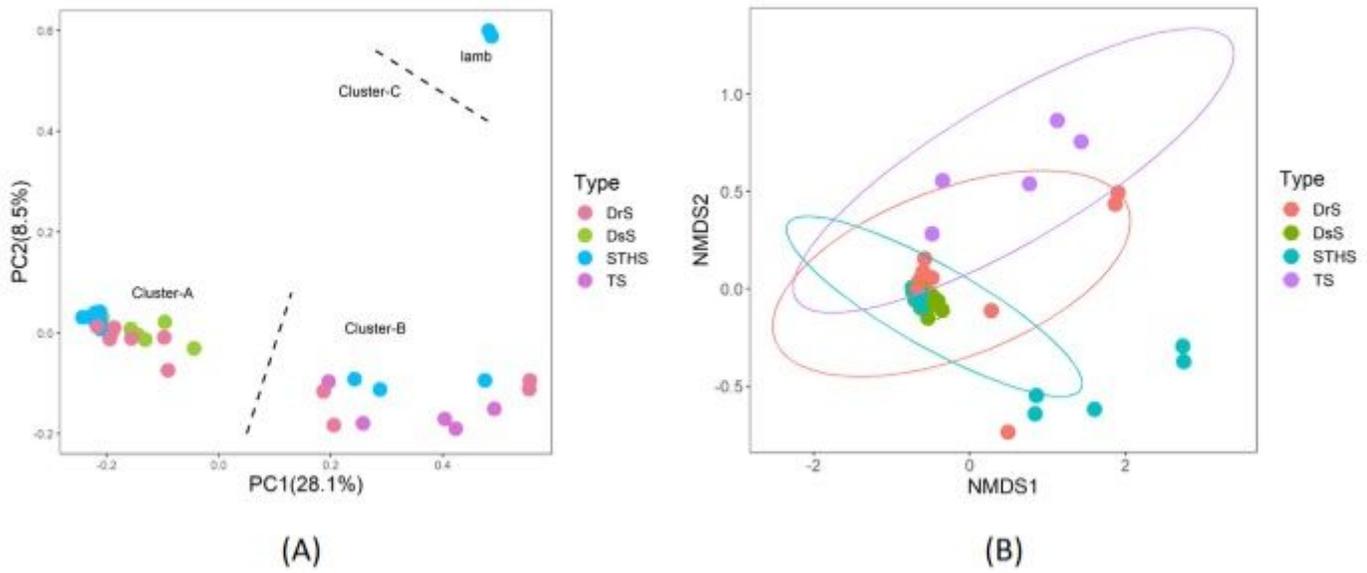
Group	Raw sequences	High quality valid sequences	OTUs	Average valid sequences of sample
DrS	444002	355132	7039	35514
DsS	641786	507753	6887	33851
TS	203332	167035	4112	33407
STHS	405144	329485	8257	32949

## Figures



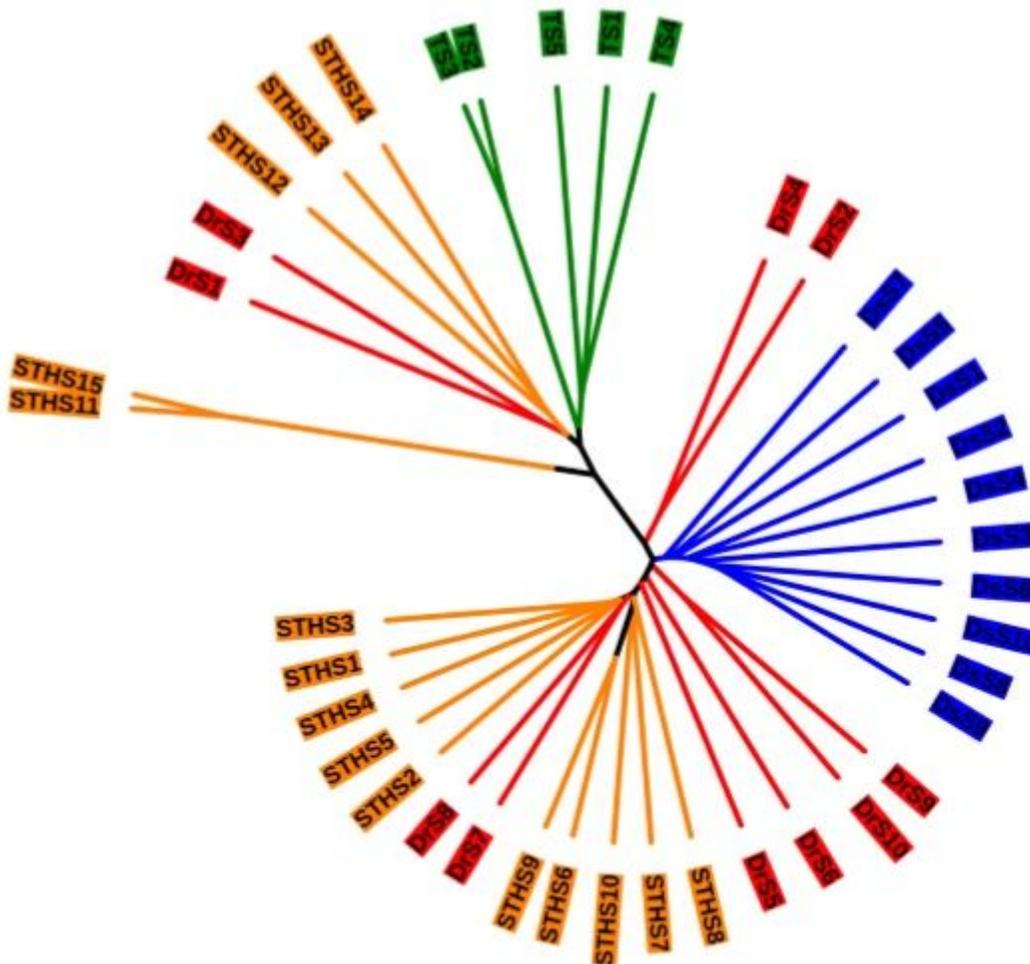
**Figure 1**

The diversity of community composition and microbial diversity index analysis. (A) Venn diagram showing overlap in OTUs of differential abundance in DrS, DsS, TS and STHS. (B) Shannon index. (C) Chao1 index. Different asterisks represent statistical significance (\* $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).



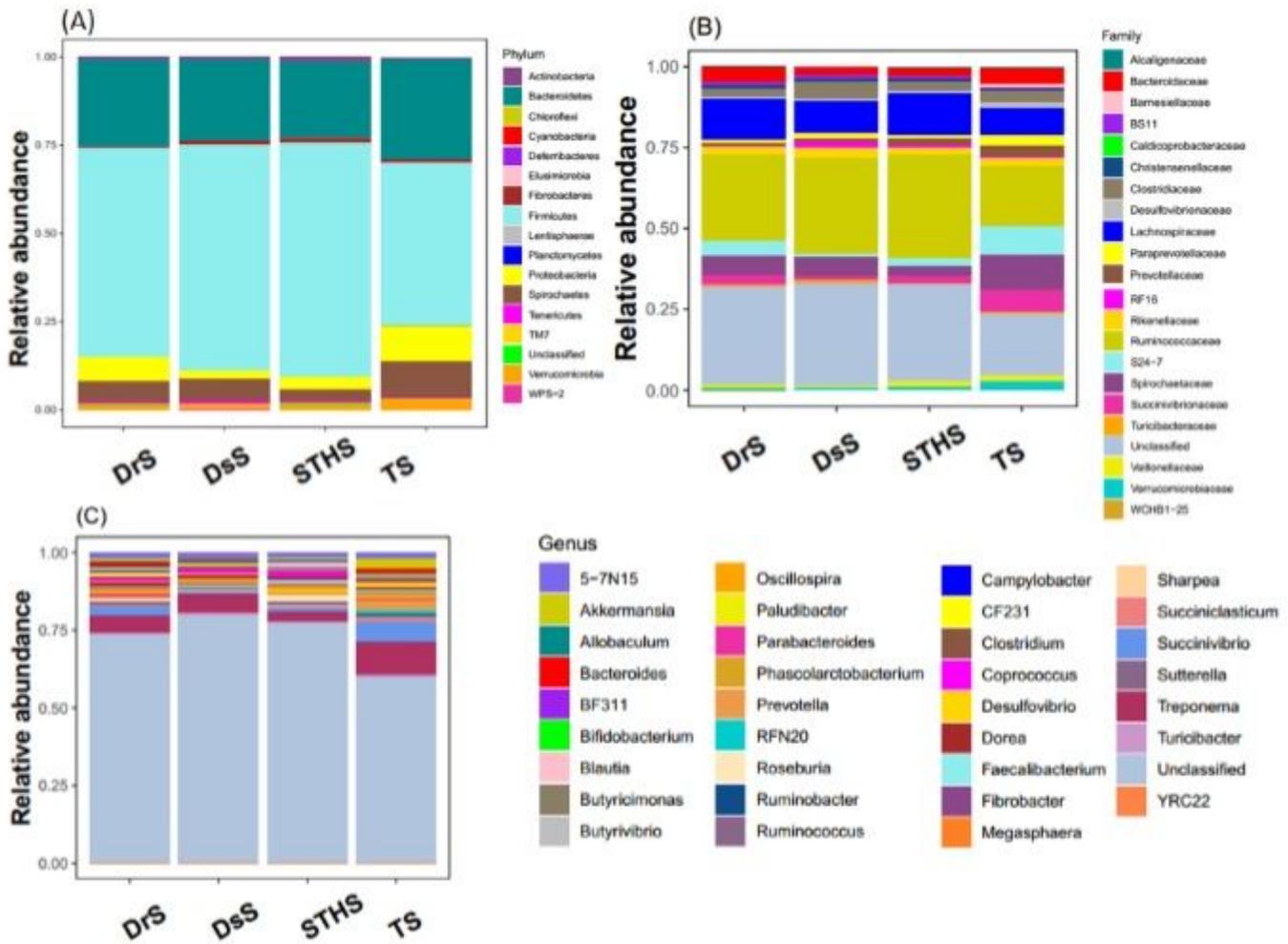
**Figure 2**

The intestinal microbial altered in different types of sheep. (A) PCoA plot of similarities between the different groups. Principal component (PC) 1 and 2 accounted for 28.1% and 8.5% of the variance, respectively. (B) NMDS showing the alteration of bacterial population based on Bray-Curtis distance.



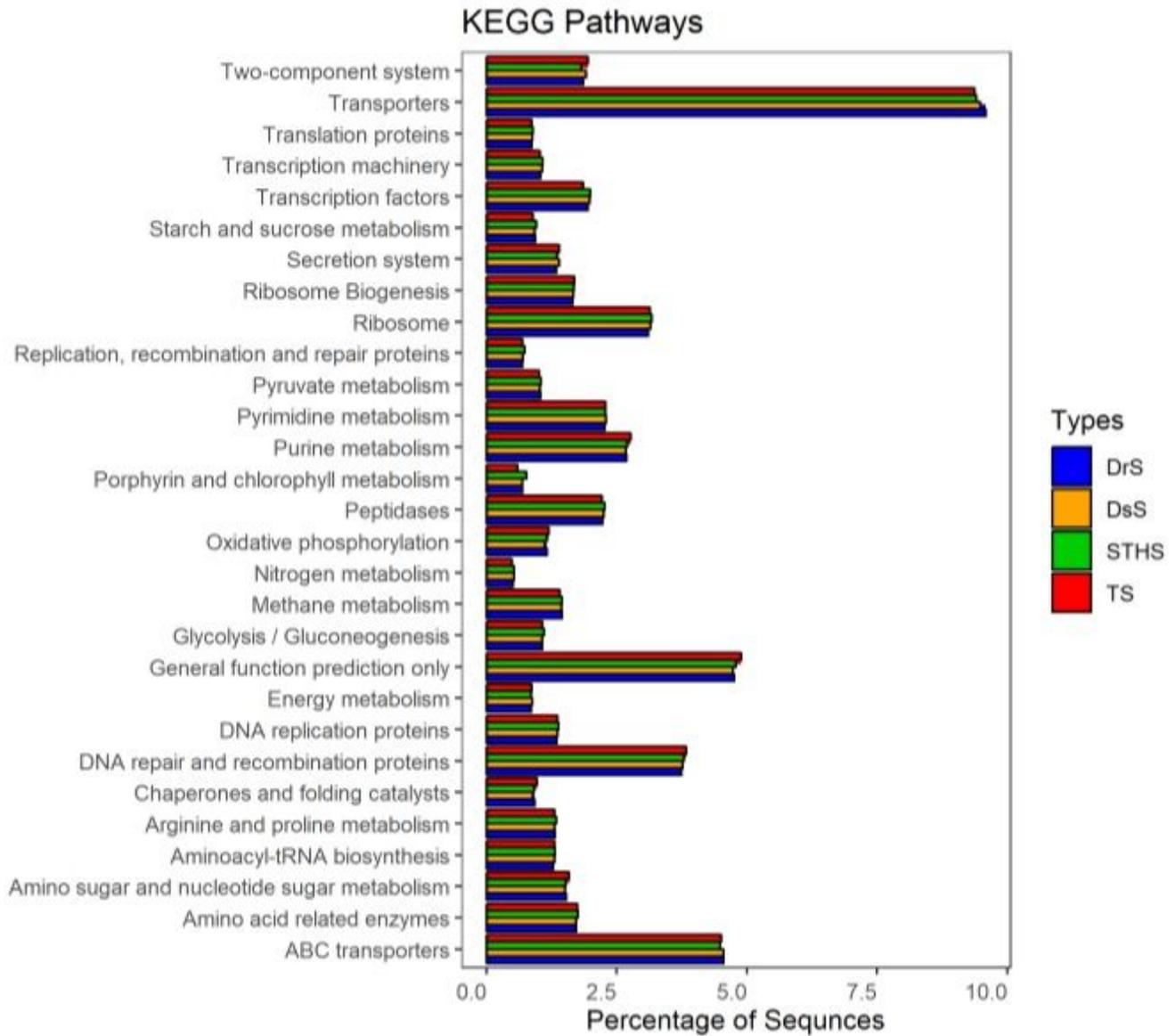
**Figure 3**

Hierarchical clustering of bacterial communities according to Bray-Curtis distance.



**Figure 4**

Microbial composition of different samples. Each bar represents the average relative abundance of each bacterial taxon within a group. (A) Taxa assignments at Phylum level. (B) Taxa assignments at Family level. (C) Taxa assignments at Genus level.



**Figure 5**

Function abundance profile of gut microbiota of different groups. KEGG, Kyoto Encyclopedia of Genes and Genomes.