The rumen microbiome of yak co-evolves with its host probably adding the adaptation to its harsh environments

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Research

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Abstract

Background

Rumen microbes play an important role in ruminant energy supply and animal performance. Previous studies showed that yak (Bos grunniens) rumen microbiome and fermentation differ from other ruminants. However, little is understood on the features of the rumen microbiome that make yak adapted to its unique environmental and dietary conditions. Here we investigated the rumen microbiome and metabolome to understand how yak adapts to the coarse forage and harsh environment in the high Qinghai-Tibetan plateau.

Result

Metataxonomic analysis of the rumen microbiota revealed that yak (Bos grunniens), domesticated cattle (Bos taurus), and dzo (a hybrid between the yak and domestic cattle) have distinct rumen microbiota. Metagenomic analysis displayed a larger gene pool encoding a richer repertoire of carbohydrate-active enzymes (CAZymes) in the rumen microbiome of yak and dzo than cattle. Some of the genes encoding glycoside hydrolases (GH) that mediate the digestion of cellulose and hemicellulose were significantly enriched in the rumen of yak than cattle, but the cattle rumen microbiome had more genes assigned to GH57 that primarily includes amylases. The rumen fermentation profile differed also, with cattle having a higher molar proportion of acetate but a lower molar proportion of propionate than dzo and yak. Metabolomic analysis showed differences in both rumen microbial metabolic pathways and metabolites, mainly amino acids, carboxylic acids, sugars, and bile acids. Notably, styrene degradation, primary bile acid biosynthesis, glyoxylate, and dicarboxylate metabolism significantly differed between cattle and dzo; streptomycin biosynthesis was significantly different between cattle and yak; and the pathways for biotin metabolism and styrene degradation significantly differed between dzo and yak. Correlation analysis revealed certain microbial species correlated with differential rumen metabolites. Nine differential metabolites showed a positive correlation with seven species belonging to Bacteroides and Alistipes but a negative correlation with ten species belonging to Prevotella and Ruminococcus.

Conclusion

The present study showed that the rumen microbiome of yak and its host had probably co-evolved aiding in the adaptation of yak to the harsh dietary environment of the Qinghai-Tibetan plateau. In particular, the yak rumen microbiome has more enzymes involved in the degradation of rough forage than that of cattle, providing sufficient energy for its host.

Background

The rumen microbiota enables the digestion of feed and production of both the energy source (about 70% of the energy required by ruminants) in the form of volatile fatty acids (VFA, mainly acetate, propionate, and butyrate) and the protein source (about 70% of the total protein reaching the small intestines) in the form of microbial protein that the ruminants can directly utilize. The rumen microbiota metabolism significantly differed between cattle and dzo; streptomycin biosynthesis was significantly different between cattle and yak; and the pathways for biotin metabolism and styrene degradation significantly differed between dzo and yak. Correlation analysis revealed certain microbial species correlated with differential rumen metabolites. Nine differential metabolites showed a positive correlation with seven species belonging to Bacteroides and Alistipes but a negative correlation with ten species belonging to Prevotella and Ruminococcus.
alteration of rumen microbiota can impact rumen function and energy utilization in the host body, rumen microbiota may also contribute to host adaptation. We hypothesized that besides the adaptative evolution of the respiratory and the circulatory systems, the digestive system, especially the rumen microbiota, of yak had probably also undergone adaptative evolution to ingest and digest the available local feed. However, it remains to be determined if and how the yak rumen microbiota helps its host to adapt to its harsh environment.

Zhang et al. [19] compared the rumen metagenome and rumen epithelial metatranscriptome of cattle and yak living at different altitudes. Their results showed that compared with cattle living at low altitudes, the rumen microbiome of the yak is enriched with VFA fermentation pathways and the yak rumen wall is more effective in absorbing VFA. That study suggests the contributions of the rumen microbiome to the adaptative evolution of ruminants living at high altitudes. However, their VFA results were obtained in vitro, and they only focused on VFA production and methanogenesis. Because diet, environment, and feeding can profoundly affect the rumen microbiome [23, 24], these confounding factors should be eliminated in comparative studies of different species or breeds of ruminants. The objective of this study was to elucidate the potential mechanism by which the rumen microbiota contributes to yak adaption. To achieve this goal, we compared the rumen microbiota (composition and structure), fermentation, and function (both the metagenome and metabolome) among yak, cattle, and dzo (designated as a species), all of which were kept at the same high altitude and fed the same diet. This study provided new knowledge of the yak rumen microbiome that might help understand its adaptation to high-altitude environments.

**Results**

**Yak has the highest rumen propionate molar proportion and cellulase activity.**

We determined the rumen fermentation characteristics and cellulase activities of fresh rumen samples to compare the feed fermentation and cellulolytic capacities among yak, Qaidam yellow cattle, and dzo. No significant difference was observed in rumen pH among the three ruminant species (Table 1), but the rumen concentration of total VFA in dzo was lower ($P < 0.05$) than in cattle. The molar proportion of propionate and butyrate in yak was higher than in cattle ($P < 0.05$), while the molar proportion of acetate and acetate : propionate (A:P) ratio showed the opposite trend. The rumen microbiome of yak had the highest ($P < 0.05$) activity of carboxymethyl cellulase and avicelase (Table 1).
Table 1

Rumen fermentation parameters and fibrolytic enzyme activities among cattle, dzo and yak.

| Item                        | Cattle | Dzo    | Yak    | SEM    | P value |
|-----------------------------|--------|--------|--------|--------|---------|
| pH                          | 7.10   | 7.37   | 7.29   | 0.05   | 0.077   |
| Total VFA (mM)              | 74.00a | 58.07b | 70.08ab | 2.65   | 0.032   |
| VFA proportion, mol/100mol  |        |        |        |        |         |
| Acetate                     | 77.92a | 76.48b | 75.10c | 0.26   | <0.001  |
| Propionate                  | 13.23b | 14.10a | 14.38a | 0.17   | 0.008   |
| Butyrate                    | 7.11b  | 7.61b  | 8.56a  | 0.18   | 0.001   |
| Isobutyrate                 | 0.58   | 0.70   | 0.70   | 0.03   | 0.092   |
| Isovalerate                 | 0.62   | 0.70   | 0.81   | 0.05   | 0.263   |
| Valerate                    | 0.53a  | 0.42b  | 0.45b  | 0.02   | 0.040   |
| A:P ratio                   | 5.90a  | 5.44b  | 5.24b  | 0.08   | <0.001  |
| Fibrolytic enzyme activities (mU) |        |        |        |        |         |
| Xylanase                    | 485.80 | 648.25 | 789.27 | 109.25 | 0.542   |
| Carboxymethyl cellulase     | 158.02b| 182.63ab| 220.69| 9.58   | 0.021   |
| Avicelase                   | 187.76b| 204.60b| 248.14a| 6.74   | <0.001  |
| Acetylesterase              | 149.76 | 154.75 | 146.54 | 3.57   | 0.065   |

P-values were calculated using ANOVA (n = 9 per ruminant species).

The rumen microbiota structure and composition are different among the three species.

Using metataxonomic analysis, we compared the rumen microbiota among the three ruminant species. The sequencing depth coverage was >98% for bacteria and 99% for fungi for all the samples. The three species had different bacterial and fungal rumen microbiota. Yak had a lower (P<0.05) ACE richness estimate of rumen bacteria than cattle and dzo, but no significant difference was observed in Simpson diversity index among the three ruminant species. Simpson evenness was higher (P<0.05) for yak than for cattle and dzo (Additional file 1: Table S1). With respect to fungi, cattle had a lower (P<0.05) observed species richness (both ACE and Chao1 estimates) than dzo and yak, but Simpson evenness for cattle was higher (P<0.05) compared with yak and dzo (Additional file 2: Table S2). The β-diversity of the bacterial and fungal microbiotas was compared using PCoA based on Bray-Curtis dissimilarity, and ANOSIM analysis showed a difference (P=0.001) in the overall rumen microbiota of both bacteria and fungi among the three ruminant species (Fig. 1a, b). Cattle and dzo had more similar rumen bacterial microbiota compared to yak, while yak and dzo had a more similar fungal microbiota compared to cattle. Overall, the rumen microbiota of bacteria and fungi of cattle clustered separately from that of yak, while that of dzo fell in between.

We further investigated the composition and function of rumen microbiota of the three ruminant species using metagenomics. We selected six samples from each ruminant species based on the metataxonomic results to maximize depth coverage with the budget we had. We obtained a total of >1.36 billion raw reads (>75.8 million reads per sample) totaling >206 Gb of sequence data (>11.4 Gb per sample). After the removal of host DNA and quality filtering, we obtained >1.35 billion reads in total and >75.1 million reads per sample. The average length of the Open reading frames (ORFs) was about 494 bp. PCoA analysis of the metagenomic data also revealed difference (P = 0.001) in the overall bacterial microbiota among the three ruminant species (Fig. 1c). Bacteroidetes, Firmicutes, Proteobacteria, Euryarchaeota were the predominant phyla in all samples, with Bacteroidetes (53.71%) and Firmicutes (24.74%) being the most predominant. The relative abundance of Proteobacteria, Verrucomicrobia, Planctomycetes, Synergistetes, Cyanobacteria, Chloroflexi, and Acidobacteria was higher (P<0.05) in yak than in cattle, while Chlamydiae was more predominant (P<0.05) in cattle and Actinobacteria more predominant (P<0.05) in dzo (Additional file 3: Figure S1). At the genus level, Bacteroides, Alistipes, Butyrivibrio, Faecalibacterium, and Pseudobutyrivibrio were more predominant in cattle than in yak (P<0.05), while the opposite is true (P<0.05) for Ruminococcus, Selenomonas, Oscillabacter, Treponema, and Fibrobacter (Additional file 4: Figure S2). At the species level, nine species, most of which
belong to *Bacteroides* and *Alistipes*, were more predominant (*P*<0.05) in cattle than in yak or dzo; four species were more predominant (*P*<0.05) in dzo; and 18 species (most belonging to *Prevotella*) were more predominant (*P*<0.05) in yak (Fig. 2).

Because the yak rumen had a higher propionate molar proportion (Table 1), we compared the relative abundance of bacterial species that either produce or utilize succinate, a fermentation intermediate of propionate production via the succinate pathway [25]. Of the 12 succinate-producing bacterial species, *Prevotella brevis*, *P. bryantii*, *P. albensis*, *Fibrobacter succinogenes*, *Selenomonas ruminantium*, and *Mitsuokella jalaludini* were enriched (*P*<0.05) in the yak rumen compared to the rumen microbiota of cattle or dzo (Fig. 3a), so was the succinate-utilizing species identified, *Selenomonas ruminantium* (Fig. 3b). The bacterial genera likely using the acrylate pathway for propionate production did not differ (*P*>0.05) in relative abundance among the three ruminant species (Additional file 5: Figure S3). The relative abundance of *Ruminococcus flavefaciens*, *R. albus*, and *F. succinogenes*, the best-known species of cellulolytic bacteria in the rumen, were enriched (*P*<0.05) in yak compared to cattle (Additional file 6: Figure S4).

**The yak rumen microbiota has a larger fibrolytic capacity but a smaller amylolytic capacity.**

The CAZymes encoded by the rumen microbiome are of critical importance to feed digestion in ruminants [26, 27]. To explore the microbial potential to degrade fiber in the rumen of cattle, dzo, and yak, we sequenced and comparatively analyzed their rumen metagenomes. The cattle showed a lower (*P*<0.05) relative abundance of CAZymes than dzo or yak (Table 2). The abundance of six types of CAZymes was quite different among the three ruminant species. Dzo had the highest abundance of GHs, whereas yak owned the highest abundance of GTs (Table 2). We further compared the GH and CE families that are involved in polysaccharide degradation. Our results revealed that GH48 (primarily exoglucanases [27]) and GH45 (mostly endoglucanases [27]) were more abundant (*P*<0.05) in yak than in cattle (Table 3). Among the GHs encoding hemicellulases, GH5 and GH44 (both mostly xyloglucanases [27]), GH16 and GH17 (both mostly β-glucanases [27]), and GH11 (primarily endoxylanases [28]) were more (*P*<0.05) abundant in yak than in cattle (Table 3). Similarly, PL11 (rhamnogalacturonan endolyase or exolyase) and PL4-4 (rhamnogalacturonan endolyase) were more abundant (*P*<0.05) in yak than in cattle (Additional file 7: Table S3). On the other hand, yak had the lowest (either significantly or numerically) abundance of the GH families encoding amylases (Table 3). Of the detected carbohydrate esterases, CE12 (mostly acetyl esterase acting on xylan, pectin, or rhamnogalacturonan) was more abundant (*P*<0.05) in yak than in cattle, whereas CE13 (pectin acetyl esterase) was more abundant (*P*<0.05) in cattle than in dzo or yak (Table 3). Of the 15 most abundant GT, GT2, GT4, and GT9 were more abundant (*P*<0.05) in yak, while GT1 was more abundant (*P*<0.05) in cattle (Additional file 8: Table S4). Among the top 15 differential CBMs detected (Additional file 9: Table S5), CBM48, CBM72, CBM78, CBM12, CBM58, CBM5, and CBM41 were more abundant (*P*<0.05) in cattle, whereas CBM67, CBM61, CBM40, CBM51, CBM57, CBM77, and CBM70 were enriched in yak compared to the other two ruminant species.

| Item     | Cattle   | Dzo      | Yak      | SEM    | P value |
|----------|----------|----------|----------|--------|---------|
| Sum      | 44,914.81\(^b\) | 48,529.87\(^a\) | 48,156.05\(^a\) | 528.91 | 0.002   |
| GH       | 21,449.70\(^b\) | 23,392.58\(^a\) | 22,277.84\(^ab\) | 290.21 | 0.012   |
| GT       | 9,081.16\(^b\) | 9,665.29\(^b\) | 10,837.93\(^a\) | 216.86 | <0.001 |
| CE       | 7,909.66 | 8,349.70 | 7,812.54 | 102.82 | 0.066   |
| CBM      | 4,810.67 | 4,998.95 | 5,095.12 | 53.21  | 0.077   |
| PL       | 826.66\(^b\) | 1,068.62\(^a\) | 1,084.87\(^a\) | 32.48  | <0.001 |
| AA       | 836.96\(^b\) | 1,054.73\(^a\) | 1,047.74\(^a\) | 26.26  | <0.002 |

GH: Glycoside Hydrolases; GT: Glycosyl Transferases; CE: Carbohydrate Esterases; CBM: Carbohydrate-Binding Modules; PL: Polysaccharide Lyases; AA: Auxiliary Activities

\(P\) values were calculated using ANOVA (n = 9 per ruminant species).

Different superscripts in a row designate significant difference (*P*<0.05).
Table 3
Abundance (in TPM) of cellulase, hemicellulose, acetyesterase and amylase genes in the rumen metagenome of cattle, dzo, and yak.

| CAZymes       | CAZymes family | Cattle   | Dzo      | Yak      | SEM | P-value |
|---------------|----------------|----------|----------|----------|-----|---------|
| Cellulases    |                |          |          |          |     |         |
| GH48          | GH48           | 0.75b    | 1.85ab   | 1.99a    | 0.22| 0.030   |
| GH6           |                | 0.00     | 0.16     | 0.00     | 0.04| 0.137   |
| GH5           |                | 111.47b  | 156.61b  | 212.09a  | 12.01| < 0.001 |
| GH8           |                | 130.50   | 133.56   | 122.58   | 2.80| 0.270   |
| GH9           |                | 363.70   | 390.48   | 371.90   | 7.24| 0.320   |
| GH45          |                | 0.28b    | 0.45b    | 2.24a    | 0.33| 0.016   |
| Xylanases     |                |          |          |          |     |         |
| GH10          | GH10           | 481.40b  | 536.92a  | 505.02ab | 9.04| 0.030   |
| GH11          |                | 17.34b   | 27.13ab  | 35.54a   | 2.47| 0.003   |
| GH30          |                | 122.36   | 125.61   | 101.99   | 4.48| 0.055   |
| GH43          |                | 194.08   | 196.92   | 198.73   | 3.73| 0.889   |
| Mannase       |                |          |          |          |     |         |
| GH5           | GH5            | 111.47b  | 156.61b  | 212.09a  | 12.01| < 0.001 |
| GH26          |                | 268.66a  | 252.52a  | 217.97ab | 7.18| 0.005   |
| Xyloglucanases|                |          |          |          |     |         |
| GH5           | GH5            | 111.47b  | 156.61b  | 212.09a  | 12.01| < 0.001 |
| GH12          |                | 0.69     | 0.75     | 0.60     | 0.11| 0.879   |
| GH44          |                | 0.40c    | 1.30b    | 2.17a    | 0.20| < 0.001 |
| GH74          |                | 84.53    | 98.31    | 107.97   | 4.66| 0.115   |
| β-glucanases  |                |          |          |          |     |         |
| GH5           | GH5            | 111.47b  | 156.61b  | 212.09a  | 12.01| < 0.001 |
| GH16          |                | 311.07   | 347.48a  | 345.92a  | 5.37| 0.002   |
| GH17          |                | 0.29b    | 0.34b    | 0.90a    | 0.10| 0.011   |
| Acetyesterase |                |          |          |          |     |         |
| CE12          | CE12           | 305.64b  | 391.04a  | 416.44a  | 15.53| 0.003   |
| CE13          |                | 3.98a    | 1.16b    | 0.53b    | 0.45| < 0.001 |
| CE16          |                | 0.00     | 0.13     | 0.00     | 0.03| 0.160   |
| Amylase       |                |          |          |          |     |         |
| GH31          | GH31           | 655.08ab | 707.4a   | 643.81b  | 10.28| 0.015   |
| GH13          |                | 425.55   | 454.01   | 399.72   | 11.60| 0.163   |
| GH57          |                | 219.73a  | 185.77b  | 186.45b  | 4.86| 0.001   |
| GH77          |                | 270.10   | 270.45   | 263.52   | 3.46| 0.681   |
| GH15          |                | 3.20     | 3.33     | 1.89     | 0.33| 0.133   |
| GH19          |                | 4.55     | 1.87     | 4.07     | 0.59| 0.118   |

P-values were calculated using ANOVA (n = 6 per ruminant species).

Different superscripts in a row designate significant difference (P< 0.05).

We further compared the functional potentials of the rumen microbiota among the three ruminant species. Annotation of the metagenomic data using the KEGG database followed with LEfSe analysis showed that replication and repair and endocrine system were enriched in cattle (P< 0.05); dzo had more (P< 0.05) metabolic pathways of other amino acids; whereas metabolism of cofactors and vitamins was represented more abundantly (P< 0.05) in yak (Fig. 4a). At level 3 (Fig. 4b), cattle, yak, and dzo had 24, 18, and 5 metabolic pathways
enriched \((P < 0.05)\), respectively, compared with the other two ruminant species. The metabolism of pyrimidine, purine, starch and sucrose, cyanoamino acid, and aminoacyl-tRNA biosynthesis were the top five for cattle; amino acids biosynthesis, pantothenate and CoA biosynthesis, biofilm formation-\textit{Vibrio cholerae}, lipopolysaccharide biosynthesis, cysteine and methionine metabolism were the top five for yak, and carbon metabolism, butanoate metabolism, citrate cycle, sulfur relay system, and selenocompound metabolism were the top five for dzo. Differential modules based on LEfSe analysis were showed in Additional file 10: Figure S5.

\textbf{Rumen metabolite profiles are different among the three ruminant species.}

We also used metabolomics to comparatively examine the metabolite profiles of the rumen microbiome among the three ruminant species. A total of 185 individual metabolites were identified. Principal component analysis (PCA) analysis of all the samples and three quality control (QC) samples showed that the analysis system was stable (Additional file 11: Figure S6a). As shown by orthogonal partial least squares discriminate analysis (OPLS-DA), cattle, dzo, and yak had distinct rumen metabolite profiles (Additional file 11: Figure S6b-d). After filtering using the student's t-test \((P < 0.05)\) and variable importance in projection \((VIP > 1)\), 27 metabolites were found to differ between two ruminant species (Table 4). Notably, 16 metabolites differed \((P < 0.05)\) between cattle and dzo: Caproic acid, benzenepropanoic acid, 2-deoxyerythritol, lithocholic acid, dehydroascorbic acid, 4-(dimethylamino) azobenzene, phenylacetamide, uridine 5’-monophosphate, and z-phthalate were higher \((P < 0.05)\) in cattle than dzo, and the most upregulated metabolites in cattle were lithocholic acid \((FC = 25.65)\) and phenylacetamide \((FC = 22.74)\). On the other hand, ethylene glycol, glycine, aminomalonate, 3-hydroxyphenylacetic acid, chenodeoxycholic acid, 2-picolinic acid, 3,6-anhydro-D-glucose were higher \((P < 0.05)\) in dzo than cattle, with glycine \((FC = 17.86)\), chenodeoxycholic acid \((FC = 12.82)\), 2-picolinic acid \((FC = 22.22)\), 3,6-anhydro-D-glucose \((FC = 15.39)\) being the most upregulated in dzo. Compared to the rumen of cattle, the rumen of yak had a higher \((P < 0.05)\) ethylene glycol, 2-hydroxyypyrazinyl-2-propenoic acid, sebacic acid, 2-picolinic acid, 1,3-dihydroxypyridine, chenodeoxycholic acid, and 3,6-anhydro-D-glucose, but a lower \((P < 0.05)\) serine, z-phthalate, 2-deoxyerythritol, undecanedioic acid, lithocholic acid, dehydroascorbic acid, glucose-6-phosphate, uridine 5’-monophosphate, and isochlorogenic acid. When compared to the rumen of dzo, the yak rumen had higher valeric acid, boric acid, 1,3-dihydroxypyridine, benzenepropanoic acid, and sebacic acid, but a lower 5'-deoxy-5'-methylthioadenosine, glycine, aminomalonate, pimelic acid, 3-hydroxyphenylacetic acid, and isochlorogenic acid.
Table 4
Rumen fluid metabolites that differed between two of the three ruminant species (n = 6 per species)

| Metabolite                        | Cattle vs Dzo | Cattle vs Yak | Dzo vs Yak |
|-----------------------------------|---------------|---------------|------------|
| | RT (min) | VIP | P value | FC | VIP | P value | FC | VIP | P value | FC | |
| Ethylene glycol                   | 9.45          | 2.549 | 0.002 | 0.353 | 2.193 | 0.014 | 0.502 | 1.435 | 0.110 | 1.422 |
| Phenylacetamide                   | 9.47          | 2.229 | 0.025 | 22.741 | 1.678 | 0.186 | 2.422 | 1.148 | 0.095 | 0.106 |
| Valeric acid                      | 9.57          | 1.795 | 0.051 | 1.522 | 0.113 | 0.551 | 0.882 | 2.589 | 0.014 | 0.579 |
| Boric acid                        | 9.71          | 0.740 | 0.116 | 2.196 | 0.214 | 0.897 | 0.961 | 1.015 | 0.034 | 0.438 |
| 5'-Deoxy-5'-Methylthioadenosine   | 9.99          | 1.935 | 0.100 | 0.278 | 1.461 | 0.258 | 15.17 | 2.829 | 0.015 | 54.566 |
| 2-Picolinic Acid                  | 10.5          | 1.736 | 0.001 | 0.045 | 2.415 | < 0.001 | 0.041 | 0.287 | 0.602 | 0.900 |
| Caproic acid                      | 11.16         | 1.662 | 0.018 | 2.426 | 1.094 | 0.649 | 1.168 | 1.451 | 0.097 | 0.481 |
| Dehydroascorbic Acid              | 11.59         | 1.096 | < 0.001 | 3.332 | 1.572 | < 0.001 | 4.656 | 0.389 | < 0.001 | 1.397 |
| 2-Deoxyerythritol                 | 12.32         | 1.843 | < 0.001 | 7.787 | 2.457 | < 0.001 | 9.590 | 0.238 | 0.055 | 1.231 |
| Glucose-6-Phosphate               | 15.19         | 0.941 | 0.001 | 2.083 | 1.351 | < 0.001 | 2.175 | 0.068 | 0.619 | 1.044 |
| Glycine                           | 15.5          | 4.329 | 0.001 | 0.056 | 1.584 | 0.071 | 0.351 | 4.127 | 0.002 | 6.274 |
| Serine                            | 16.32         | 0.733 | < 0.001 | 3.044 | 1.035 | < 0.001 | 4.202 | 0.236 | 0.050 | 1.381 |
| 1,3-Dihydroxyphryidine            | 16.71         | 0.343 | 0.180 | 2.424 | 1.080 | 0.035 | 0.275 | 1.149 | 0.010 | 0.113 |
| Benzenepropanoic acid             | 17.34         | 6.440 | 0.030 | 1.666 | 3.032 | 0.739 | 1.065 | 7.269 | 0.003 | 0.639 |
| Aminomalonate                     | 18.17         | 1.386 | 0.026 | 0.716 | 0.413 | 0.816 | 1.031 | 1.461 | 0.030 | 1.441 |
| 3,6-Anhydro-D-Glucose             | 18.42         | 1.527 | < 0.001 | 0.065 | 1.345 | 0.002 | 0.094 | 0.649 | 0.107 | 1.459 |
| Pimelic Acid                      | 19.07         | 0.721 | 0.181 | 0.041 | 0.116 | 0.683 | 1.064 | 1.048 | 0.022 | 1.266 |
| 2-Hydroxypyrazinyl-2-Propanoic Acid | 19.07       | 1.236 | 0.665 | 0.823 | 6.336 | 0.048 | 0.505 | 3.694 | 0.168 | 0.613 |
| 3-Hydroxyphenylacetic acid        | 20.25         | 1.452 | 0.004 | 0.515 | 0.132 | 0.799 | 0.948 | 1.396 | 0.007 | 1.839 |
| 4-[(Dimethylamino)azobenzene      | 21.56         | 1.149 | 0.002 | 17.479 | 0.689 | 0.249 | 1.752 | 0.713 | 0.090 | 0.100 |
| Isochlorogenic Acid               | 21.97         | 0.506 | 0.124 | 1.396 | 1.881 | < 0.001 | 16.819 | 1.158 | 0.001 | 12.044 |
| Sebacic acid                      | 23.99         | 0.214 | 0.005 | 1.547 | 1.292 | 0.021 | 0.106 | 1.054 | 0.017 | 0.068 |
| Undecanedioic acid                | 25.96         | 0.414 | 0.530 | 1.056 | 1.016 | 0.004 | 1.396 | 0.746 | < 0.001 | 1.322 |
| Lithocholic Acid                  | 25.60         | 1.376 | < 0.001 | 25.649 | 1.228 | 0.021 | 3.142 | 0.420 | 0.268 | 0.123 |
| Chenodeoxycholic Acid             | 25.96         | 1.434 | 0.019 | 0.078 | 1.747 | 0.023 | 0.099 | 0.397 | 0.629 | 1.268 |

1RT = retention time (min);
2VIP, variable importance in projection;
3FC, fold change. Take cattle vs dzo for example, if a FC value is less than 1, it means that there is less metabolite in cattle than in dzo.
Metabolite pathway enrichment analysis based on the significantly different rumen metabolites revealed the pathways that potentially contributed to the observed difference in rumen metabolite profiles (Fig. 5). The pathways for styrene degradation, primary bile acid biosynthesis, glyoxylate and dicarboxylate metabolism, chloroalkane and chloroalkene degradation, and thiamine metabolism were different \((P < 0.05)\) between cattle and dzo (Fig. 5a). Between cattle and yak (Fig. 5b), the pathways involved in streptomycin biosynthesis, chloroalkane and chloroalkene degradation, starch and sucrose metabolism, inositol phosphate metabolism, and primary bile acid biosynthesis differed \((P < 0.05)\). The pathways for biotin metabolism, styrene degradation, thiamine metabolism, glutathione metabolism, and phosphonate and phosphinate metabolism differed \((P < 0.05)\) between dzo and yak (Fig. 5c).

### Different rumen microbiota may lead to different metabolites.

We assessed the relationship between the relative abundance of bacterial species (shown in Fig. 2) and rumen metabolites (shown in Table 4) using Pearson correlation analysis. Lithocholic acid and glucose-6-phosphate were positively correlated with \textit{Bacteroides} sp. CAG.1060, \textit{Bacteroides} sp. CAG.545, \textit{Bacteroides} sp. CAG.709, \textit{Bacteroides} sp. CAG.770, \textit{Alistipes} sp. CAG.435, \textit{Alistipes} sp. CAG.514, and \textit{Lachnospiraceae bacterium} AC2028. Chenoexoxycholic acid, 3,6-Anhydro-D-glucose were positively correlated with eight \textit{Prevotella} species (\textit{P. oralis}, \textit{Prevotella} sp. CAG.487, \textit{P. buccae}, \textit{P. oryzae}, \textit{P. albensis}, \textit{P. brevis}, \textit{Prevotella} sp. CAG.732, and \textit{Prevotella} sp. CAG.1092) and two \textit{Ruminococcus} species (\textit{R. flavefaciens} and \textit{R. albus}).

### Discussion

The yak is indigenous livestock on the Qinghai Tibetan Plateau, and it is raised at altitudes between 3,000 and 5,000 m [29]. The high altitude and the environmental conditions are not suitable for domestic cattle. The cattle used in this experiment are Qaidam yellow cattle, which is well adapted to an altitude of 2,800 meters, but its pulmonary artery pressure is still significantly higher than that of indigenous yak and Tibetan sheep [29]. Currently, little is known whether the rumen microbiome is co-evolved tighter with ruminants living in high-altitude environments. In this study, we integrated rumen fermentation profiles, metataxonomics, metagenomics, and metabolomics of the rumen microbiome comparatively to explore how the rumen microbiome might help yak to adapt to its living conditions, especially its poor dietary conditions unique to the Qinghai-Tibet high plateau. Compared to the rumen microbiome of cattle, the yak rumen microbiome enriched cellulase and hemicellulase, and PL families, which can help improve fiber degradation. On the other hand, yak had decreased amylase-containing GH families and CBM families, which might slow down starch degradation in the rumen and allow more starch to reach the small intestine. Additionally, the increased succinate-producing and utilizing bacterial species might promote propionate production. All the above are consistent with the ability of yak to live on the poor diet available on the high plateau.

Rumen microbiota is primarily responsible for the energy acquisition of ruminants, and many studies showed that differences in rumen microbiota might result in different energy efficiency [6, 30]. We first compared the rumen microbiota among the three ruminant species. Of the 21 phyla identified by metagenomics in this study, \textit{Bacteroidetes} and \textit{Firmicutes}, which are considered the important microbes in providing the energy required by ruminant animals [31], were the most predominant. Studies have shown that with the increase in dietary energy level and concentrate proportion, the relative abundance of \textit{Firmicutes} could increase [32, 33]. Similarly, the gut microbiota of obese people and mice caecum contained more \textit{Firmicutes} and less \textit{Bacteroidetes} [34, 35], which agrees with that the relative abundance of \textit{Firmicutes} can be positively correlated with dietary energy concentration. Compared with the study of Ahmad et al., [33] the relative abundance of \textit{Bacteroidetes} in the three ruminant species was higher, but that of \textit{Firmicute} was lower. The discrepancy might be partially attributable to the concentrate proportions (30%, day matter) in the diet of the cited study. We also detected many phyla, such as \textit{Proteobacteria} and \textit{Verrucomicrobia}, whose relative abundance was significantly higher in yak than in the other two ruminant species. Members of the phylum \textit{Proteobacteria} are metabolically flexible modifying their gene repertoire in response to changes in available substrate and energy sources [36, 37], and their relative abundance was significantly higher in the rumen of cows with high milk yield and

|                | Cattle vs Dzo | Cattle vs Yak | Dzo vs Yak |
|----------------|--------------|--------------|------------|
| Uridine 5'-Monophosphate | 26.75 1.746 0.017 2.455 | 2.833 < 0.001 5.819 | 0.693 0.338 2.370 |
| Z Phthalate     | 31.29 2.933 < 0.001 6.861 | 4.010 < 0.001 8.566 | 0.396 0.110 1.248 |

\(^1\)RT = retention time (min);

\(^2\)VIP, variable importance in projection;

\(^3\)FC, fold change. Take cattle vs dzo for example, if a FC value is less than 1, it means that there is less metabolite in cattle than in dzo.
milk protein content, suggesting that *Proteobacteria* is positively correlated with feed efficiency [6]. *Verrucomicrobia* contains species that are highly specialized degraders of fucoidans and other complex polysaccharides [38], and they may play an important role in polysaccharide degradation. In a previous transcriptomic study, *Ruminococcus* and *Fibrobacter* were shown to express most of the cellulase transcripts, they, together with *Prevotella*, probably expressed most of the hemicellulose transcripts in the rumen [28]. The high abundance of *Ruminococcus*, *Fibrobacter*, and *Prevotella* and the activities of carboxymethyl cellulase and avicelase in the rumen of yak corroborate its greater ability to digest crude fiber. Xue et al. [6] compared the microbial composition between cows with high and low milk yield and milk protein content, and they found that the rumen species enriched in the high-yielding cows were mainly members of *Prevotella*, and *P. oralis*, *P. buccae*, *P. albensis*, and *R. flavefaciens* were significantly higher in the high-yielding cows. We conclude that the above four bacterial species are positively correlated with feed efficiency. Interestingly, they were also significantly more predominant in the rumen of yak than the other two ruminant species. These species, probably among others, may help enable yak to adapt to its poor diet.

In the present study, yak was found to possess more hemicellulases (e.g., GH11, GH5, GH44, GH16, GH17) and rhamnogalacturonan endolyase (e.g., PL11 and PL4-4) than cattle. Many CBMs associated with galactose binding (CBM61, CBM62, CBM51) and L-rhamnose binding (CBM67) were also enriched in yak. These results indicate that yak probably has a stronger activity in removing galactose from the main chain of hemicellulose. Rapid hemicellulose digestion can facilitate cellulose digestion in forage because the removal of hemicellulose can expose cellulose for microbial attachment and digestion. Although GH13, which contains the well-known amylases, did not differ among the three ruminant species, its subfamilies GH13-8 (starch branching enzyme) and GH13-12 and GH13-14 (pullulanase) and their associated CBM (CBM48) [39] were more abundant in cattle (Additional file 12: Table S6). Another starch binding CBM, CBM41 [40, 41], was also enriched in cattle. The yak rumen microbiome probably has increased its ability to degrade forage not only by enhancing cellulose degradation (as evidenced by enriched GHS involved in cellulose hydrolysis) but also by enhancing hemicellulose and pectin degradation. On the other hand, the rumen microbiome of cattle has more abundance of GHS and CBM involved in starch digestion and binding, respectively. Even though both the yak and cattle used in the present study were fed the same diet, the rumen microbiome of yak probably has evolved to be more adapted to the forage that yak consumes, whereas the rumen microbiome of cattle, which are domesticated and has been fed diets containing starch, likely has evolved to better utilize starch. Yak produces milk of desirable characteristics (e.g., high milk fat, milk protein, milk total solids), but its milk yield is very low (about 1 kg per day) [42, 43]. The lack of sufficient dietary energy is one limiting factor. It will be interesting to test if the rumen microbiome of yak can evolve and gradually adapt to starch-rich diets over long-term adaptation.

It is well-known that forage typically increases acetate concentration in the rumen than a diet containing starch. In the present study, acetate molar proportion reached 75.1–77.9%, significantly higher than that reported in other studies [44–46], and the acetate: propionate ratio was also much higher than that reported by others [32, 33]. Both were likely attributed to the forage-only diet used in the present study. One previous study showed that rumen fermentation to propionate could increase energy efficiency [2]. Indeed, glucose fermentation to acetate, propionate, and butyrate can provide 62%, 109%, and 78% of the energy stored in glucose, respectively [47]. The gain of energy in propionate fermentation is because it incorporates hydrogen. This in turn reduces the amount of energy wasted via methane production from hydrogen. Two fermentation pathways are mainly responsible for the propionate production in the rumen: the acrylate pathway and succinic acid pathway [25], with the former using lactate as the input substrate and acryloyl-CoA as an intermediate, while the latter using oxaloacetate as the starting substrate and succinate as an intermediate. In this study, the increased relative abundance of succinate-producing and utilizing bacterial species seen in yak might be attributable to the higher propionate proportion detected in that species. On the other hand, because lactate-producing and utilizing bacterial genera did not differ in relative abundance among the three ruminant species, the acrylate pathway of propionate production might be similar across the three ruminant species. Isobutyrate, isovalerate, and 2-methyl-butanate are considered essential growth factors for most fiber-degrading microorganisms [48–50]. The increased isobutyrate and isovalerate concentration (data was not shown) noted in the yak might facilitate the growth of some fiber-degrading bacteria, which corroborates the increased relative abundance of the three well-known cellulosolytic bacteria: *R. flavefaciens*, *R. albus*, and *F. succinogenes*, in the yak. Branched-chain volatile fatty acids are used in synthesizing branched-chain amino acids [48]. The higher branched-chain volatile fatty acids concentration in the yak rumen may increase valine, leucine, and isoleucine biosynthesis. It should be noted that although all the three ruminant species had acetate as the major VFA, yak had the highest molar proportion of propionate but the lowest molar proportion of acetate. Thus, we speculated that in the absence of high-quality forage, the yak rumen microbiome is adapted to change the rumen fermentation and enhance energy harvest.

Our metabolomic results showed that cattle enriched styrene degradation and streptomycin biosynthesis, while yak and dzo enriched primary bile acid biosynthesis. Bile acids are important components of the bile, and they play an important role in fat metabolism, mainly in the intestinal and liver circulatory systems where they play a protective role through recirculation [51]. The presence of primary and secondary bile acids in the rumen suggests that the rumen microbiome may play an important role in the conversion of primary to secondary bile acids in ruminants [52]. Further studies are needed to explore the mechanisms by which bile acids circulate in ruminants.
Uridine 5'-monophosphate consists of phosphoric acid, ribose, and uracil, the latter of which is a nucleobase for RNA biosynthesis. The enriched pyrimidine metabolism in the cattle rumen might explain the increased uridine 5'-monophosphate therein. The enhanced nucleotide metabolism pathway in the cattle rumen microbiome may provide more substrates for replication and repair, translation, and transcription. The upregulated starch and sucrose metabolism in the cattle rumen might be attributable to the higher concentration of glucose-6-phosphate therein. Taken together, metabolite alterations were consistent with the metagenome results, and they jointly corroborate the adaption of yak to the high plateau environments.

As demonstrated in the literature [23, 24, 53], many factors, including feed, age, and management, can affect the gut microbiome composition and its functions in animals. Because yak is not domesticated, and the yak animals used in the present study were not confined, we could not weigh each of the study animals for the exact bodyweight or track the specific feed each animal consumed. Differences in body weight and feed consumption might be confounding factors that could have affected the results. Apparently, the yak rumen microbiome features, as determined by metataxonomics, enzymatic assays, metagenomics, and metabolomics, can help the host to adapt to its harsh environments, especially the poor diet available to it, but it remains to be determined how the body of yak affects its rumen microbiome. Future studies are required to explore the adaption relationship between high-altitude animals and the symbiotic microbiome. Such information will be useful to understand the adaptability of high-altitude animals to various environments and their domestication.

**Conclusion**

Our study revealed a clear difference in the composition, functions, and metabolome of the rumen microbiome among yak, dzo, and domesticated cattle. Although the relative abundance of the genes that code for amylases and their associated CBM families were lower in yak than in cattle, the yak rumen microbiome increased the abundance of cellulolytic bacteria and the genes encoding cellulase and hemicellulase, making yak better adapted to digest its roughage-based diets. Besides, the yak rumen microbiome had more succinate-producing and -utilizing bacterial species, supporting more pyruvate fermentation to propionate. These findings can help better understand how the yak rumen microbiome aids in yak's adaption to its poor diet in high plateau environments, and provide a foundation for further studies on microbial roles in the physiology and health of agricultural animals.

**Methods**

**Experiment design and sample collection**

Ten Qaidam yellow cattle, dzo, and yak each with similar body weight (about 200 kg) and age (5-6 years old) were used in this study. Because of the difficulty to weigh each of the grazing animals, one herdsman estimated the bodyweight of each animal visually. All the study animals were raised in the Qinghai-Tibet plateau and had never received any supplementary feed. In summer, they were grazing in the mountain grassland, while they were grazing at the foot of the mountain in winter, where *Kobresia myosuroides* and *Phragmites communis* were the predominant pasture species. The sampling site was located in the central part of Qinghai Province, in the eastern part of the Qaidam Basin (36° 49' -37° 20' N, 98° 87' -99° 27' E). The annual average temperature in this area is 3.5°C, and the average altitude is 4,000 meters. Rumen uid samples were collected from each animal using an oral stomach tube and a pump, both of which were thoroughly cleaned using fresh warm water between sample collections. The first 10-15 ml of sample from each animal was discarded to avoid contamination from saliva. Approximately 200 mL ruminal uid sample was collected from each animal, and half of it was immediately frozen in liquid nitrogen and then stored at -80°C until analysis. The rest of each ruminal sample was filtered through four layers of cheesecloth, and the filtrate was used for pH measurement and then preserved at -20°C for VFA analysis and fibrolytic enzyme activity assay.

**Analysis of VFA and plant cell-degrading enzyme activity**

The rumen fluid samples were thawed at 4°C, and the solid particles and protein were removed according to the procedures of Li et al.[54]. The VFA concentrations were analyzed using gas chromatography (Agilent Technologies 7820A GC system, Santa Clara, CA) equipped with a 30 m × 0.25 mm × 0.33 μm fused silica column (AE-FFAP, Atech Technologies Co. Ltd., Shanghai, China).

One aliquot of each thawed rumen fluid sample was centrifuged at 1,000× g for 10 min at 4°C. The supernatant was immediately collected and analyzed for the activity of carboxymethyl cellulase (CMCase), avicelase, xylanase, and acetyl esterase using carboxymethyl cellulose, avicel, birchwood xylan, and p-nitrophenyl acetate, respectively, as the substrates. The enzyme assay reaction was incubated at 39°C and pH 7.0 for 30 min except for the xylanase assay (for 15 min). The amounts of released reducing sugars were quantified using the dinitrosalicylic acid colorimetry method [55], and the production of p-nitrophenol was determined at 415 nm [56, 57]. One unit of enzyme
activity was defined as the amount of enzyme releasing 1 µmol of reducing sugar (e.g., xylose or glucose equivalent) or p-nitrophenol per min per ml.

**Metataxonomic analysis of rumen prokaryotes and fungi**

Microbial DNA was extracted from each rumen sample using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.) according to the manufacturer's protocols. The final DNA concentration and purity were determined using a NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA), followed by visual quality checking using agarose gel (1%) electrophoresis. Individual amplicon libraries were prepared for prokaryotes using PCR amplification of the V3-V4 hypervariable regions of the 16S rRNA gene with primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGG TWTCTAACT-3'). The ITS1 region of the fungal rRNA operon was amplified with primers 1737F (5'-GGAAG TAAAA GTCGT AACAA GG-3') and 2043R (5'-GCTGC GTTCT TCATC GATGC-3') to prepare individual amplicon libraries for fungi. The PCR products were gel purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using QuantiFluor™-ST (Promega, USA) according to the manufacturer's protocol. Purified amplicons were pooled at equimolar ratio and paired-end sequenced (2 x300 bp) on an Illumina MiSeq platform (Illumina, San Diego, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The raw reads have been deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: PRJNA744001, PRJNA744022).

The raw sequence reads were demultiplexed, quality-filtered using Trimmomatic, and then merged using FLASH. Operational taxonomic units (OTUs) were clustered (de novo) with a 97% similarity cutoff using UPARSE (version 7.1 http://drive5.com/uparse/) and chimeric sequences were identified and removed using UCHIME. The representative 16S rRNA gene sequences of the OTUs were taxonomically classified using the RDP Classifier algorithm (http://rdp.cme.msu.edu/) against the Silva 128 database at a confidence threshold of 70%. The ITS sequences were taxonomically assigned using the UNITE7.0 database (https://unite.ut.ee/).

**Metagenomic sequencing and analysis**

Each of the microbial DNA extracts was fragmented to an average size of about 300 bp using Covaris M220 (Gene Company Limited, China). Individual sequencing libraries were prepared using the TruSeqTM DNA Sample Prep Kit (Illumina, San Diego, CA, USA). Paired-end sequencing was performed on Illumina NovaSeq (Illumina Inc., San Diego, CA, USA) at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) using the NovaSeq Reagent Kit according to the manufacturer's instructions (www.illumina.com). The raw reads have been deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: PRJNA744415).

Adapter sequences were trimmed off from the paired-end reads using SeqPrep (https://github.com/jstjohn/SeqPrep). Low-quality reads (length < 50 bp, a quality value < 20, or having any Ns) were removed using Sickle (https://github.com/najoshi/sickle). Host DNA was identified and removed after comparing all the reads with the genomes of cattle (https://www.ncbi.nlm.nih.gov/genome/?term=cattle) and yak (https://www.ncbi.nlm.nih.gov/genome/?term=yak) using BWA (http://bio-bwa.sourceforge.net). The cleaned metagenomic sequence reads were assembled using MEGAHIT [58] (https://github.com/voutcn/megahit). Contigs with a length of ≥ 300 bp were used for further analysis.

ORFs from each contig were predicted using MetaGene [59] (http://metagene.cb.k.u-tokyo.ac.jp/). All ORFs sharing ≥ 95 % sequence identity over ≥ 90% of their length were clustered using CD-HIT [60] (http://www.bioinformatics.org/cd-hit/), and the longest sequence from each cluster was selected as its representative sequence to construct a non-redundant gene catalog. The quality-filtered sequence reads were mapped to the representative sequences with 95% identity using SOAPAligner [61] (http://soap.genomics.org.cn/), and gene abundance in each sample was calculated as transcripts per million (TPM).

Representative sequences of the non-redundant gene catalog were compared to the NCBI NR database using BLASTP (Version 2.2.28+, http://blast.ncbi.nlm.nih.gov/Blast.cgi) [62] for taxonomic assignment, while KEGG annotation was conducted also using BLASTP (Version 2.2.28+) against the KEGG database [63] (http://www.genome.jp/kegg/). Carbohydrate-active enzyme annotation was predicted using hmmscan (http://hmmer.janelia.org/search/hmmscan) against the CAZy database Version 5.0 (http://www.cazy.org/). The minimum e-value cutoff for all the annotations was set at 1e-5.

**Metabolomic analysis of rumen fluid**

One hundred µL of each rumen fluid sample was subjected to metabolite extraction using 500 µL methanol: water (4:1, v/v) solution containing 2% L-2 chlorophenylalanine (as internal standard). Then the mixtures were vortexed for 10 s and centrifugation at 12,000 rcf at 4°C for 20 min. The supernatant was carefully transferred to a glass-derived bottle and vacuum-dried. After 80 µL methoxy amine hydrochloride (15 mg/mL in pyridine) was added, the samples were vortex-mixed for 2 min and incubated at 37°C for 90 min to carry out
oximation reaction. Eighty µL of trifluoroacetamide reagent containing 1% trimethylchlorosilane and 20 µL n-hexane were added to each sample, and then all samples were vortex-mixed for 2 min and incubated at 70°C for 60 min. The samples were cooled to room temperature and analyzed using gas chromatography and mass spectrometry (GC-MS).

The rumen metabolome was analyzed using gas chromatography (Agilent 7890A, Agilent Technologies, Inc., Santa Clara, CA, USA) coupled to an Agilent 5975C mass selective detector (Agilent, USA) with an inert electron impact ionization (EI) source and ionization voltage at 70 eV. Briefly, metabolites were separated with an HP-5MS capillary column (30 m × 0.25 mm × 0.25 um) using helium (99.999% purity) as the carrier gas at a constant flow rate (1 mL/min). The GC column temperature was programmed to hold at 60°C for 0.5 min, rise to 310°C at a rate of 8°C/min, and then hold at 310°C for 6 min. A QC sample was prepared by pulling an equal volume of each sample, and the QC sample and rumen fluid samples were analyzed in the same manner. To assess the repeatability of the analysis, the QC sample was injected once every 10 rumen fluid samples. The GC-MS data were processed using the MassHunter workstation Quantitative Analysis package (version v10.0.707.0) to extract raw peaks, filter and calibrate data baselines, align peaks, deconvolute, identify peaks, and integrate peak areas. The resulting matrixes detected in at least 80% of the samples were retained. After filtering, the missing values of the raw data were filled up by half of the compound minimum. The peak area was normalized in the data analysis. The internal standard was used for data quality control (reproducibility), and the metabolic features whose relative standard deviation (RSD) exceeded that of the QC by >30% were discarded. Mass spectra of these metabolic features were identified using the Fiehn database (https://fiehnlab.ucdavis.edu/projects/fiehnlib).

The metabolomic data were analyzed using PCA, and OPLS-DA was used to determine the global metabolic differences among the three ruminant species. Statistical significance among species was declared at a VIP value of >1 and a P-value of <0.05. P-values were estimated using paired Student’s t-test for single-dimensional statistical analysis. A total of 27 differential metabolites among two of the three ruminant species were mapped into their biochemical pathways through metabolic enrichment and pathway analysis based on search against the KEGG database (http://www.genome.jp/kegg/) [63]. These metabolites were classified according to the pathways into which they were mapped or the functions that they could perform. Differential metabolites were cross-listed with the pathways in the KEGG database, and the top differential pathways were identified [64]. The relationship between different species and metabolites was visualized as a heat map using the “pheatmap” package in R (www.r-project.org).

**Declarations**

**Ethics approval**

Animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee (protocol number: NWAFAC1008) of the College of Animal Science and Technology of the Northwest A&F University (Yangling, Shaanxi, China).

**Consent for publication**

No applicable.

**Availability of data and material**

All sequencing data are available from the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under accession number PRJNA744001, PRJNA744022 and PRJNA744415.

**Competing interests**

The authors declare that they have no conflict of interests.

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**Authors’ contributions**

JY, ZY, XC and YC designed the study. CZ, YQ and ZC collected rumen samples. FZ, YL and LW analyzed the rumen samples for VFA. CZ, JW and DW performed the enzyme assays for rumen fibrolytic enzyme activities. LW did the DNA extraction, CZ, SK, YC and WS performed
the bioinformatics and statistical analysis. LW, YC, AK, XW and JZ performed rumen metabolomic analysis. CZ and ZY wrote the manuscript, and all other authors revised the manuscript. All authors read and approved the final manuscript.

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Figures
Figure 1

Plots of principal coordinates analysis (PCoA) comparing the overall rumen microbiota among the three ruminant species. A (bacteria) and B (fungi) were based on Bray-Curtis dissimilarity determined by metataxonomics (n=9 per species), and C was based on Bray-Curtis dissimilarity determined by metagenomics (n=6 per species). The ellipses represent 95% of the samples belonging to each group. Statistical significance of difference was tested using ANOSIM with 999 permutations.
Microbial species (identified in the rumen metagenome) significantly differed in relative abundance among the three ruminant species. The difference was tested using the Kruskal-Wallis H test, with post hoc test done using the Tukey-Kramer test (n=6 per species). Only the microbial species each with a relative abundance > 0.1% in all samples were shown. *P < 0.05, ** P < 0.01.
Figure 3

Identified bacterial species likely involved in succinate production (A) and succinate utilization (B). The difference in relative abundance among the three ruminant species was tested using the Kruskal-Wallis H test, with post hoc test done using the Tukey-Kramer test (n=6 per species). Abbreviations: P, Prevotella; R, Ruminococcus; F, Fibrobacter; Sucm, Succinimonas; Sucv, Succinivibrio; Rumb, Ruminobacter; M, Mitsuokella; Po, Porphyromonas; A, Actinobacillus. * P < 0.05, ** P < 0.01.
Figure 4

Differential KEGG pathways at level 2 (A), level 3 (B) among the three ruminant species as determined using LEfSe. Only the pathways that differed significantly (P < 0.05) between two of the three ruminant species with an LDA >2 are shown.
Figure 5

Metabolic pathways significantly differing in rumen metabolites between cattle and dzo (A), between cattle and yak (B), and between dzo and yak (C). The X-axis and Y-axis represent the pathway impact and pathway enrichment, respectively. The darker the color indicated the smaller the P-value from enrichment analysis, and the larger abscissa indicates greater impact from the pathway topology analysis, respectively (n = 6 per species).
Figure 6

Pearson correlations between significantly different rumen microbial species and microbial metabolites. The color and the color intensity of the squares correspond to the direction and strength of the correlation based on the scale to the right.
Figure 7

A schematic depicting the microbial processes of carbohydrate utilization and VFA formation in yak vs cattle. Red upward arrows (↑) indicate increase in yak compared with cattle, while blue downward arrows (↓) indicate decrease in yak.

Supplementary Files

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