Wild environment and diet structure shape gut microbiome and functional composition in semi-feral Tibetan goats

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Abstract

Background : The gut microbiota composition is influenced by diet as well as the environment in both wild and domestic animals. Although the rumen microbiome in herbivorous ruminants has been studied, the gut metagenome and the underlying ecological mechanisms of different feeding systems in extreme environment have not been elucidated. Here, the influence of two feeding systems, grazing and drylot, on the gut microbiome composition of Tibetan goats was investigated. These goats are a semi-feral highland breed that lives at an altitude of ~4800 m. 16S rRNA gene sequencing and metagenomic analysis was conducted using the gastrointestinal tract lumen and mucosa (rumen, cecum, and colon) samples obtained from yearling animals.

Results: We observed distinct microbiome functions potential in the rumen and hindgut (cecum and colon). The peptidases, arginine and proline metabolism, oxidative phosphorylation, cysteine and methionine metabolism were highly enriched in the rumen microbiome. We demonstrated the proportion of *Methanobrevibacter* was significantly higher in the drylot group, thereby resulted in a higher abundance of enzymes involved in hydrogenotrophic methanogenesis. The core genera of *Clostridium*, *Prevotella* were observed in significantly different proportions between the two groups, these differences were reflective of the different nutrition metabolism between free-range and drylot animals. Although antimicrobial resistance in bacteria has been attributed to feeding conditions, the pasturing system did not affect the abundance of antibiotic resistance genes.

Conclusions: Together, these results highlight the importance of hindgut microbiota in the process of nutrient metabolism, and provide ecological insights into establishment of the GIT microbiome in ruminants under a unique environmental system.

Background

Ruminant livestock plays an important role in food security. Specifically, they convert low-nutrient lignocellulosic plant material into high-value animal proteins that include milk, meat, and fibers [1]. Regulation of the gastrointestinal tract (GIT) development of young ruminants through dietary structure will improve the GIT microbial digestive capacity. This in turn will improve the efficiency of nutrient absorption of GIT epithelium, as well as provide an alternative approach for the improvement of roughage utilization. One important factor in these parameters is the microbiota of the GIT, which is widely believed to be shaped by genetics, diet, geography [2], and social contact patterns [3]. For instance, dietary changes impact different bacterial species and functional properties in the fecal microbiome [4]. Social contact patterns are thought to shape the intestinal microbiota by affecting the horizontal transfer of the microbial [5].

It was determined that diet is a primary driving factor for changes in the gut microbial ecosystem in both wild animals [6–9] and domestic livestock [10, 11]. Compared to traditional nomadic pastoralism, modern livestock production sometimes relies on confined drylot feeding, in which animals are provided a protein

concentrate-based diet with a higher content of simple sugars. Investigating the effects of wild grazing and drylot strategies on the growth of the goats is of great importance not only to enhance our understanding of the role of GIT microbiota colonization and evolution, but also of the resulting changes in nutrient absorption and metabolism.

Previous studies have focused on the microbial diversity of the rumen in order to address global livestock challenges [12, 13]. However, the impact of ruminant hindgut microbiome should not be trivialized. Starting from the ileum, through the cecum, colon and rectum, these sites provide favorable conditions for fermentation, digestion and absorption, which allows for the microbial density and phylogenetic diversity to increase to levels comparable to that of the rumen [14, 15]. Mucosal-associated microbial communities are also important regulators of immune function and health [16].

The Tibetan Plateau, known as the roof of the world, is arguably one of the harshest environments on earth, boasting cold and hypoxic conditions, as well as low biomass. Humans that live on the Tibetan Plateau rely largely on animal husbandry as their main form of subsistence. Compared to other highland sites in the Qinghai-Tibetan Plateau (Fig. 1a), the study site used here, the Qiangtang National Natural Reserve, has an average altitude of 4,800 m and represents a largely intact ecosystem with little disturbance from human activities. In this study, the microbiome diversity across two feeding systems (free-ranging vs. drylot) in the nomadic areas of Tibetan Plateau were analyzed. Metagenomic and 16S rRNA data were analyzed to disentangle the relative contribution of the rumen and lower GIT microbiota, as well as the crosstalk with the host, and the impact of the environment on the microbiota. The microbiota diversity, composition, and potential functional characteristics were estimated using metagenomic data. The findings reported here provide novel ecological insights into the establishment of the GIT microbiome in animals under extreme environmental conditions.

Results And Discussion

Animal measurements

To determine the relationship between the GIT microbiota and feeding condition of animal growth performance, phenotypic data for animals from the two feeding groups were collected. An overview of the analyses of birth weight, yearling weight, pH and short chain fatty acid (SCFA) in GI, 16S rRNA gene sequencing and metagenome sequencing are summarized in additional files (Table S2). No differences in birth weights of kids were observed between the grazing and drylot groups (Fig. 1b). However, the average yearling weight (at day 365) of the drylot group was significantly higher than that of the grazing group ($p = 0.007$, Fig. 1c), indicating that the nutritionally optimized feed resulted in significant better animal growth. To further investigate the effects of the environment on rumen, cecum, and colon fermentation patterns, the pH and SCFA levels were determined in the rumen, cecum, and colon fluids. It was observed that the pH in the drylot group was significantly decreased in the rumen ($p = 0.001$, Fig. 1d), and significantly increased in colons ($p = 0.03$, Fig. 1d). However, the pH in cecum was not altered ($p > 0.05$, Fig. 1d). Additionally, it was observed that drylot feeding significantly decreased acetic acid levels ($p <$

0.001, Fig. 1e), and significantly increased the concentration of propionic acid ($p < 0.001$, Fig. 1e). The concentrations of butyric acid in the rumen, cecum, and colon were not affected by the animal's diet ($p > 0.05$, Fig. 1g). The main metabolites of GIT microbiota, SCFAs can affect GIT mucosal immune responses. They can bind Toll-like receptors, activate G-protein coupled receptors, and inhibit histone deacetylase activity by affecting the function of different immune cells in the walls of the GIT [17]. Gluconeogenesis in the GIT has been demonstrated to mediate beneficial metabolic effects through the intermediary's butyrate and propionate. Propionate has been described as an efficient hepatic gluconeogenic substrate, it also serves as a gluconeogenic substrate in intestines before reaching the liver [17, 18]. These results indicate that the diet composition is likely to significantly alter the growth performance by influencing bacterial metabolites within the GIT. Since significant differences in SCFAs were observed between the two feeding treatments, it was assumed that the composition and function potential of the rumen and intestinal microbes were affected by different diets.

Microbiota composition and function of grazing and drylot goats

To determine how the feeding conditions altered the global microbiota structures of different GIT compartments, 60 luminal and mucosal samples from 3 compartments (rumen, cecum, and colon) were collected from grazing and drylot raised groups, as well as 5 soil samples from where the goats resided, negative control (DNA-free water and buffer; $n = 3$) were used for DNA extraction and sequencing, after using "decontam" [19] to remove the negative control contamination, 2,098,000 clean reads were obtained. These sequences included an average of 32,276 reads per sample. Further analysis revealed that removal of the contaminating bacteria had a large effect on the samples with low microbial abundance. The contaminating bacteria including *Unclassified_O_Bacteroidales*, *Norank_C_Cyanobacteria*, *Lachnoclostridium_1*, *Ruminiclostridium*, *Lactobacillus* and *Staphylococcus* (Table 1). Additionally, shotgun sequencing of 30 luminal samples generated a total of 289.7 Gb of Illumina HiSeq clean metagenomic data after removing low-quality reads and host contaminants, with an average of 9.65 Gb per sample. Based on the assembled contigs with an N50 contig length of 790.93 bp, a total of 3.78 million non-redundant genes were identified, with an average open reading frame (ORF) length of 478 bp.

Based on the read abundances at the level of phylum, eggNOG orthologous groups (OGs), and gene levels (Fig. 2a), were investigated for microbial diversity (Shannon index) in different compartments. For phyla and COG level, it was observed that the microbial diversity of hindgut was lower than was observed in rumen (Fig. 2a). However, at the gene level, the hindgut diversity was higher compared with the rumen (Fig. 2a). These results are in agreement with a previous study reporting that the rumen bacterial community had greater diversity compared to the hindgut in ruminants [20]. In addition, we observed that drylot feeding improved hindgut microbial diversity and reducing rumen microbial diversity (Fig. 2a). The total number of bacteria in the lumen was significantly higher than that of the mucosa (Fig. 2b, $P < 0.05$), indicating that drylot feeding significantly increased bacterial numbers in the hindgut (Fig. 2b, $P < 0.05$). The principal coordinates analysis of OTUs indicated that the microbiota is significantly different between the rumen and hindgut (ANOSIM, Bray-Curtis metric: $R^2 = 0.64$, $p = 0.001$; Fig. 2c). Interestingly,

the mucosa and lumen microbiota of the hindgut formed 2 distinct clusters (ANOSIM, Bray-Curtis metric: $R^2 = 0.43$, $p = 0.001$; Fig. 2c). These results suggested that the hindgut lumen and mucosa microbiota may have different functions potential for nutrient metabolism due to community structure differences. Next, PCoA was conducted on the lumen and mucosa samples separately. These data suggested that the feeding condition significantly altered the community structure of the rumen lumen (ANOSIM, Bray-Curtis metric: $R^2 = 0.67$, $p = 0.001$; Fig. S1a). Interestingly, we observed that the feeding system had little influence on the mucosa microbial structure in the same compartment (ANOSIM, Bray-Curtis metric: $R^2 = 0.57$, $p = 0.001$, Fig. S1b). Previous study found that grain-rich diets altered the colonic fermentation and mucosa-associated bacterial communities and induced mucosal injuries in goats [21], compared with the present study, no high proportion of concentrate was added to the diet of the drylot group, which resulted in less difference in the composition of mucosal microbiota. In addition, a previous study compared the gut microbial communities of wild and captive black rhinos, and found that there was no significant difference in alpha diversity levels between wild and captive black rhinos, but significant differences in beta diversity, this study also found that bacterial groups traditionally associated with the ruminant gut of domestic animals have a higher relative abundance in captive rhinos. Functional profiling results showed greater abundance of glycolysis and amino acid synthesis pathways in captive rhino microbiomes, representing an animal receiving sub-optimal nutrition with a readily available source of glucose but possibly an imbalance of necessary macro and micronutrients [22].

The phenotypic differences between the feeding systems examined here were primarily affected by the GIT lumen microbial structure. The relative abundances of phyla and genera showed distinct microbial structures between the lumen and mucosa in both the rumen and hindgut (Fig. 2d, Fig. S2). In addition, *Bacteroidetes* and *Firmicutes* were the advantage phyla. In the rumen mucosa, *Proteobacteria* was in high abundance for both the grazing and drylot environments (average abundance 6.62% and 8.44%; Fig. 2d and Table S3), whereas *Spirochaetae* was prevalent and highly specific for the cecum mucosa (average abundance 26.98% and 27.46%; Fig. 2d and Table S3). These results are consistent with a previous study showing *Bacteroidetes* was the second most prevalent phyla in the colonic mucosa, whereas *Proteobacteria* was the second most prevalent in the ruminal mucosa. *Firmicutes* and *Spirochaetae* were the second most dominant phyla in mucosal samples of the cecum [23].

At the genus level, the predominant members in the hindgut were *Treponema_2*, *Ruminococcaceae_UCG-005*, *Ruminococcaceae_UCG-010*, *Alistipes*, *Bacteroides*, *Prevotellaceae_UCG-004*, *Ruminococcaceae_UCG-013*. However, the predominant members of the rumen were *Prevotella_1*, *Bacteroidales_BS11*, *Butyrivibrio_2*, and *Prevotellaceae_UCG-001* (Fig. S2 and Table S4). These bacteria are crucial for the degradation and metabolism of plant structural carbohydrates, especially *Prevotella*, *Bacteroidales*, *Ruminococcaceae*, and *Butyrivibrio* [24]. To determine relationships between the differential abundances of the gut bacteria with pH and SCFA, a correlation analysis was conducted (Fig. S3). *Clostridium*, *Alistipes*, *Ruminiclostridium* were positively correlated with pH and acetic acid production, whereas *Methanobrevibacter* and *Barnesiella* were positively correlated with propionic acid production, and *Prevotella* and *Butyrivibrio* have positively correlated with butyric acid production (Fig.

S3). These findings provided new insights into the relationship between SCFAs, intestinal microbiota, and intestinal mucosal immune-related diseases [17]. Linear discriminant analysis effect size (LEfSe) was used to determine the top genus-level biomarkers distinguishing lumen and mucosa of different compartments from the two feeding groups (Fig. S4). We found *Bacteroidetes*, *Prevotellaceae* and *Prevotella_1* were biomarker in the rumen lumen of goats in the grazing group, *Lachnospiraceae*, *Butyrivibrio_2* were biomarker in the rumen mucosa of goats in the grazing group, *Ruminococcaceae_UCG_005*, *Rikenellaceae* were biomarker in the colon lumen of goats in the grazing group, *Ruminococcaceae_UCG_013*, *Verrucomicrobia* and *Akkermansia* were biomarker in the colon mucosa of goats in the grazing group (Fig. S4).

In addition, the effects of pasture soil microbes on the GIT microbial structure of grazing goats were investigated. A total of 20 soil samples were randomly collected from grazing areas. Every 4 samples were pooled for sequencing. Ternary Plot analysis indicated that the soil microbiota had no influence on mature GIT microbiota for grazing goats (Fig. S5).

Furthermore, we determined that rumen and hindgut have distinct function potential. Specifically, those involving peptidases, arginine and proline metabolism, oxidative phosphorylation, cysteine and methionine metabolism, energy metabolism and other ion-coupled transporters were highly enriched in the rumen microbiome relative to that of the hindgut. Interestingly, these pathways were enriched at extremely low levels in the hindgut (Fig. 2e), studies of these pathways related to rumen physiology need further validation. Pathways involved in chloroalkane and chloroalkene degradation, peroxisome, lysosome, ethylbenzene degradation, pertussis, neurotrophin signaling, TGF-beta signaling, focal adhesion, vascular smooth muscle contraction, clavulanic acid biosynthesis, and leukocyte transendothelial migration were highly enriched in the hindgut microbiome (Fig. 2e). These results are indicative of the specialized roles of the rumen and hindgut microbiomes play in metabolism and immunity potential.

Unique composition and functions of the rumen microbiota of grazing and drylot goats

To investigate the effect of different feeding systems on the rumen microbial communities and their functions potential, the PCoA of the OTU level revealed significant differences in the microbiota between the lumen and mucosa in rumen of the two feeding conditions (ANOSIM, Bray-Curtis metric: $R^2 = 0.46$, $p = 0.001$. Fig. 3a). The total number of rumen bacteria was significantly lower in drylot goats (Fig. 3). *Firmicutes*, *Bacteroidetes*, *Spirochaetes*, and *Proteobacteria* were observed to be the dominant phyla in the ruminal lumens from both groups (Table S5). In the ruminal mucosa, *Proteobacteria* was more abundant than *Spirochaetes* (Fig. 2d). These results corroborate previous reports as these being the predominant phyla in the rumen [25, 26]. At the genus level, for the relative abundances of the core genera, *Methanobrevibacter* was significantly higher in the drylot group ($p = 0.01$, Fig. 3b), while *Alistipes* was significantly lower ($p = 0.01$, Fig. 3b). Furthermore, KEGG pathway analysis discriminated the ruminal lumen metagenomes (Fig. S6), and it was observed that methane metabolism was significantly enriched in the drylot group ($p = 0.03$, Fig. 3c). Furthermore, the core genera that are significant contributors to the

methane pathway and were differentially enriched included *Methanobrevibacter* and *Selenomonas* (Fig. 3d). *Methanobrevibacter* is a hydrogenotrophic methanogen [27], and the hydrogenotrophic methane production pathway for enzymes are involved in formate dehydrogenase (EC 1.2.1.2), formylmethanofuran dehydrogenase (EC 1.2.99.5), formylmethanofuran-tetrahydromethanopterin N-formyltransferase (EC 2.3.1.101), methenyltetrahydromethanopterin cyclohydrolase (EC 3.5.4.27), methylenetetrahydromethanopterin dehydrogenase (EC 1.5.98.1), 5,10-methylenetetrahydromethanopterin reductase (EC 1.5.98.2), tetrahydromethanopterin S-methyltransferase (EC 2.1.1.86) and coenzyme-B sulfoethylthiotransferase (EC 2.8.4.1) [28]. It was also observed that an increased number of *Methanobrevibacter* genes in drylot goats prompted an examination of the enzyme abundance for each of the enzymes involved in hydrogenotrophic methanogenesis (Fig. 3e). We determined that the enzymes involved in the hydrogenotrophic methane production pathway were significantly enriched in the drylot group (Fig. 3e). Unfortunately, methane emissions were unable to be measured in this study. Previous study comparisons of gene and transcript abundance for enzymes involved in methanogenesis between high and low CH₄ yield sheep, found that similar abundance of methanogens and methanogenesis pathway genes in high and low methane emitters. However, transcription of methanogenesis pathway genes was substantially increased in sheep with high methane yields [29]. These genes are consistent with our result.

In grazing goats, *Ruminococcus* was determined to be a core genus that positively facilitated two different clusters in the rumen. *Treponema*, on the other hand, was a competitively inhibited cluster of bacteria, with negative correlations calculated for these genera (Fig. 3f). In contrast to the grazing group, a co-occurrence network was found to be more independent and simple in the drylot group, and was not as complicated as was observed in the rumen of grazing goats [30]. *Ruminiclostridium* and *Clostridium* were core genera, actively promoting interactions between different clusters (Fig. 3f). Interestingly, *Alistipes* appeared to actively restrain the relative abundance of *Methanobrevibacter*, which may be responsible for the observed differences in the methane pathway between the grazing and drylot groups.

Since ruminants require a method to efficiently digest lignocellulose in order to satisfy their energy requirements, the CAZyme profiles of different degradation efficiencies were examined in the context of varied feeding systems. The family of GH3, GH2, GH78, and GH9 were significantly higher in grazing goats (Fig. 3g). These families include endoglucanase (EC 3.2.1.4), beta-glucosidase (EC 3.2.1.21), xylan 1,4-beta-xylosidase (EC 3.2.1.37), beta-glucosylceramidase (EC 3.2.1.45), and exo-beta-glycosaminidase (EC 3.2.1.165) [31]. Of these enzymes involved in plant cell wall degradation, EC 3.2.1.4 has been demonstrated to promote cellulose degradation to cellodextrin, and EC 3.2.1.21 promotes the degradation on cellodextrin to cellobiose and D-Glucose. In addition, the families of GH77, GH23, GH13, GH32 and GH25 were significantly higher in drylot raised goats (Fig. 3g). Furthermore, the family consisted of alpha-amylase (EC 3.2.1.1), oligo-alpha-glucosidase (EC 3.2.1.10), and alpha-glucosidase (EC 3.2.1.20). It has been demonstrated that EC 3.2.1.1 promotes starch and glycogen transformed to dextrin, and dextrin uses EC 3.2.1.10 to further break the molecule down to transformed into D-Glucose. In addition, EC 3.2.1.20 promoted the conversion of maltose to D-Glucose (Fig. 3g). As a result of the high-grain diets optimized to maximize growth rates and feed efficiency in the drylot, digestible carbohydrate

supplementation of the diet promotes changes in the ruminal microbiome, ultimately reducing the diversity of the microbial communities. There is a clear pattern of gene abundance reflecting microbial crosstalk with the host, highlighting the different nutrition metabolism in grazing and drylot goats.

The importance of hindgut microbiota for growth between grazing and drylot goats

Although the microbial composition in cecum has been thoroughly reported on [25, 32, 33], the function of this microbiota remains poorly understood. The PCoA of the OTU suggested significant differences between the microbiota of the cecum lumen and mucosa in the two feeding systems examined here (ANOSIM, Bray-Curtis metric: $R^2 = 0.45$, $p = 0.001$. Fig. S7a). Specifically, *Spirochaetes* and *Fibrobacteres* were significantly higher ($p = 0.03$. Fig. S7b), and *Firmicutes* were significantly lower in cecum lumen of drylot goats ($p = 0.03$. Fig. S7b). In the cecum mucosa, the proportion of *Spirochaetae* accounts more than 27% of the total microbial population, but accounts for only about 1.5% in the lumen (Fig. S7b). The core genera of *Clostridium*, *Prevotella* and *Treponema* were observed in significantly different proportions between the two groups (Fig. 4a). This finding is in agreement with a previous study in which *Prevotella*, *Bacteroides*, *Ruminococcus*, and *Clostridium* were consistently identified in hindgut samples, and were therefore considered to be part of the core microbiota [34, 35]. Consistently, significant differences in the top proportions of functional levels are due to difference in the abundances of the core genera (Fig. 4c). The grazing goats were enriched for several microbial pathways, including quorum sensing, aminoacyl-tRNA biosynthesis, peptidoglycan biosynthesis, carbon metabolism, pentose phosphate pathway and propanoate metabolism. In general, these pathways are involved in translation, replication and repair, and cellular processes (Fig. 4d). In comparison, the drylot group was significantly enriched for pathways related to amino acid metabolism (e.g. alanine, aspartate and glutamate metabolism, biosynthesis of amino acids, arginine biosynthesis, glyoxylate and dicarboxylate metabolism, fatty acid biosynthesis, lysine biosynthesis and fatty acid metabolism) (Fig. 4c). *Prevotella* has more diverse functional isomers than *Clostridium* in genes involved in specific metabolic processes [12]. Furthermore, *Prevotella* possess a greater diversity of functional isoforms than *Clostridium* for peptide digestion, which may be related to the essential production of the SCFAs propionate and butyrate used as nutrients by the host [12]. *Clostridium* has significantly higher functional diversity than *Prevotella*, involving a range of metabolic processes including cysteine biosynthesis and formaldehyde assimilation/serine pathways [12]. In general, the differences in bacterial metabolites are directly related to the differences in abundance of the core genera of the hindgut, which also leads to differences in pathways associated with nutrition metabolism.

Of particular interest is the difference of intestinal antibiotic resistance genes (ARGs) between the free-range grazing and drylot goats. The PCoA revealed a list of significantly expressed ARGs in each of the two feeding systems (Additional files 2: Fig. S8). The grazing goats harbored lower abundances of ARGs. It is possible, even likely that the administration of antibiotics in the feed is associated with a significant increase in microbiota richness in the drylot goats. These results were confirmed by the ARGs levels, in which bacitracin resistance genes was significantly higher (average abundance 97.84%, $p = 0.01$, Fig. 4e) in the grazing group, whereas the resistance genes of tetracycline, macrolide cephalosporin and

streptomycin were significantly enriched in the drylot group (Fig. 4e). Bacitracin is a mixture of high molecular weight polypeptides that possess antimicrobial activity against gram-positive microorganisms by interfering with bacterial cell wall formation and peptidoglycan synthesis. Bacitracin may also interfere with additional cellular processes [36, 37]. Previous studies demonstrated that bacitracin-treated chickens had significant changes in their cecum microbiota. Of particular interest was the significant increase in abundance of *Clostridium* [38, 39]. In order to improve immune function and adapt to harsh environments, grazing goats produce high levels bacitracin (by *Bacillus sp*), thereby promoting the healthy growth of the body and achieving the goal of adapting to the environment. In addition, antimicrobial resistance in bacteria was significantly correlated with feeding conditions. Importantly, pasturing did not lead to an increase in the abundance of tetracycline, macrolide cephalosporin and streptomycin antibiotic resistance genes.

Subsequently, it was observed that the co-occurrence network was more independent of grazing group in cecum. *Ruminococcus* and *Paenibacillus* showed positive correlations with one another, and demonstrated a relatively independent and stable cluster (Fig. S9). However, in the drylot group, 30 genera are complex correlated with each other, and formed a large co-occurrence network in the cecum. *Eubacterium* and *Butyrivibrio* are important nodes, suggesting that they competitively inhibit colonization by *Phascolarctobacterium* and *Blautia* (Fig S9). Interestingly, *Eubacterium* and *Butyrivibrio* have the ability to ferment SCFA in the animal gut [40]. These results suggest that the diet provided in the drylot feeding strategy results in more diverse and complex cecum microbial communities, but more independent and simple rumen microbial communities.

Similar core genera patterns were observed in colons as were found in the cecum (Fig. 4b). For example, the proportions of *Intestinimonas*, *Paenibacillus*, *unclassified_o_Clostridiales*, *unclassified_f_Ruminococcaceae*, *Ruminiclostridium*, and *Roseburia* were significantly higher in grazing goats (Fig. S10). As a result, alanine, aspartate and glutamate metabolism and glyoxylate and dicarboxylate metabolism were highly enriched in the cecum of grazing goats (Fig. S11a). Furthermore, when focusing on the differences of the CAZy family in colon, it was observed that GT2, GT4, CE1, GH10, AA6, GH9, and GH16 were significantly enriched in the drylot group (Fig. S11b). These genes encode for enzymes involved in plant cell wall degradation, such as endo-1,4-beta-xylanase (EC 3.2.1.8), endoglucanase (EC 3.2.1.4) and sucrose synthase (EC 2.4.1.13). In addition, the genes GH109, GH78, CE3, GH29, GH28, GH127 and CE9 were significantly enriched in the grazing group (Fig. S11b). Interestingly, GH109 and GH 29 were reported to be involved in mucin synthesis [41]. Among the CAZy genes, beta-L-arabinofuranosidase (EC 3.2.1.185); 3-C-carboxy-5-deoxy-L-xylose hydrolase polygalacturonase (EC 3.2.1.15) and alpha-L-rhamnosidase (EC 3.2.1.40) have been implicated as those contributing to the metabolism of different carbohydrate substrates [24]. These results suggest that the cecum microbes had a completely different metabolic pattern. For example, the cecum microbes in drylot goats were mainly involved in plant cell wall degradation, whereas in the grazing goats, the cecum microbes primarily contributed to the immune functions of the host. The pasture conditions significantly enhanced the metabolic interaction of the rumen strains, weakened the microbial metabolism interactions of the hindgut. The opposite was true under the drylot feeding conditions. Compared with

single stomach animals (e.g. humans, chicken, and pigs), hindgut microbial fermentation was positively correlated with the growth rate of individuals.

Conclusions

The current study presents the establishment of GIT microbiome in semi-feral goats under differing environmental conditions. Strong correlations were observed between feeding condition and CH₄ yields in goats. Drylot feeding conditions enhanced the expression levels of the hydrogenotrophic methanogenesis pathways in ruminal methanogens (Fig. 5). The metagenomic data indicated that the hindgut lumen and mucosa microbial communities serve completely different functions potential for nutrient metabolism, such as arginine and proline metabolism, which were observed to be performed entirely in the rumen. In addition, our analysis also unveiled the underlying functions of the hindgut microbiota in grazing and drylot goats (Fig. 5). Furthermore, high-abundance bacitracin resistance gene was enriched in grazing goats, which are believed to improve host immunity, and to better adapt the animals to the extreme environment in which they live. Also, evidence of drylot feeding aggravation of the expression of tetracycline, macrolide cephalosporin and streptomycin antibiotic resistance genes was presented. Taken together, we provide new insights into the colonization pattern of microbes in two feeding systems under cold and hypoxic conditions.

Methods

Ethics statement

This study was conducted at the experimental facilities of the Animal Husbandry and Veterinary Institute of Tibet Autonomous Region. The experiment was approved by the Institutional Animal Care and Use Committee of the Northwest A&F University under permit number 2016ZX08008002.

Study sites, participating animals, and sample collection

A total of 50 half-sibling Tibetan goats were selected for the comparison of different feeding systems (grazing vs. drylot feeding, n=25 each) after birth. Animals in the drylot group were housed in feedlots and provided feed from concentrates (Fig 1a, Table S1). The grazing group was allowed to free-range, following their dam without any artificial feeding (Nima, Tibetan, China, altitude > 4,800 m; Fig. 1a). The grassland mainly includes various perennial grasses such as *Stipa purpurea*, *Kobresia tibetica Maxim*, *Leontopodium pusillum*, *Carex moorcroftii Falc.Ex Boott*, *Saussurea tibetica C. Winkl* and *Artemisia wellbyi Hemsl. et Pears. ex Deasy*. The herbage intake and forage digestibility of the half-sibling Tibetan goats in spring were determined by alkane technology [42]. The dry matter intake of the half-sibling Tibetan goats was 450g per day, and the dry matter digestibility was 45.85%. From each group, 5 goats were randomly selected and slaughtered on day 365. Samples of mucosal and luminal tissues were collected from the rumen, cecum, and colon, Mucosal tissue is gently scraped with a sterile glass slide, and rinsed 3 times with sterile PBS (pH 7.0) to remove the digesta, and were then immediately frozen in

liquid nitrogen. Colon and cecum tissues were either snap-frozen or immersed in 4% paraformaldehyde for determination of brush-border enzyme activities and histology, respectively.

VFA Analysis

Concentrations of volatile fatty acids (VFAs) were measured in the rumen, cecum, and colon lumen samples using an Agilent 7820A gas chromatograph (Agilent Technologies, Santa Clara, USA), See the supplemental material for detailed measurement procedures.

DNA extraction, PCR amplification, and 16S rRNA sequencing

Total DNA was extracted from the tissues and lumen using the E.Z.N.A.[®] soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) according to manufacturer's protocol. The DNA concentration and purity were determined using the Nanodrop 2000 UV-VI spectrophotometer (Thermo Scientific, Wilmington, USA). The quality of the extracted DNA was assessed using 1% agarose gel electrophoresis. The V3-V4 region of the DNA was then amplified using the primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') on a thermocycler PCR system (Gene Amp 9700, ABI, USA). Purified amplicons were pooled in equimolar ratios, and subjected to paired-end sequencing (2 × 300) on an Illumina MiSeq platform (Illumina, San Diego, USA) according to the standard protocols by Major BioBio-Pharm Technology Co. Ltd. (Shanghai, China). See the supplemental material for detailed measurement procedures.

Metagenomic analyses of rumen, cecum, and colon luminal samples

The paired-end library was constructed using TruSeq[™] DNA Sample Prep Kit (Illumina, San Diego, CA, USA). Adapters containing the full complement of sequencing primer hybridization sites were ligated to the blunt-end of fragments. Paired-end sequencing was performed using the Illumina HiSeq 4000 platform.

Metagenome assembly and construction of the gene catalog

Adapter sequences were removed from the 3' and 5' ends of the paired end Illumina reads using SeqPrep. Low-quality reads (length < 50 bp, quality values < 20, or containing N bases) were removed using Sickle. Reads were aligned to the goat reference genome (ID 10731) by BWA, and any hit associated with the reads and their mated reads were removed. Metagenomics data were assembled using MEGAHIT [43]. Contigs with a length of over 300 bp were selected as the final assembling result. The contigs were then used for further gene prediction and annotation.

Gene prediction, taxonomy, and functional annotation

Open reading frames (ORFs) from each assembled contig were predicted using Metagene [44]. The predicted ORFs with lengths of at least 100 bp were retrieved and translated into amino acid sequences using the NCBI translation table. See the supplemental material for detailed procedures.

qPCR analysis

The qPCR reactions were performed using the primers F: CCTACGGGAGGCAGCAG and R: ATTACCGCGGCTGCTGG on a Bio-Rad CFX Manager Real-Time PCR System (Bio-Rad, Hercules, CA, USA) [45]. See the supplemental material for detailed measurement procedures.

Statistical Analyses

16S rRNA gene sequencing and metagenomics statistics data are presented as box-and-whiskers plots based on two-tailed *p*-values derived from a Wilcoxon rank-sum test. Statistics of animal measurements were analyzed by one-way ANOVA with a Tukey's test using *SPSS* 21.0. β diversity indices (Bray-Curtis) were calculated in QIIME [46], and Bray-Curtis distance was calculated using the VEGAN package. For taxonomic data, FDR correction of the *p* values was conducted in R environment (www.r-project.org).

Availability of data and materials

The samples 16S rRNA gene and shotgun metagenomic data are available from the National Center for Biotechnology Information (NCBI) under accession No. SRP188060.

Declarations

Ethical Approval and Consent to participate

This study was conducted at the experimental facilities of the Animal Husbandry and Veterinary Institute of Tibet Autonomous Region. The experiment was approved by the Institutional Animal Care and Use Committee of the Northwest A&F University under permit number 2016ZX08008002.

Consent for publication

Not applicable.

Availability of supporting data

The samples 16S rRNA gene and shotgun metagenomic data are available from the National Center for Biotechnology Information (NCBI) under accession No. SRP188060.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

K.Z., X.W., Y.Y., Y.C., and Y.W designed the research; K.Z., Y.X., C.Z., G.B., J.D., D.C., L.Z. and L.S. performed the research; C.H., K.Z, X.J., P.K., and C.L. analyzed the data; K.Z., W.X., and J.S wrote the paper; and all authors reviewed the manuscript before submission.

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Abbreviations

GIT, gastrointestinal tract; SCFA, short-chain fatty acid; ORF, open reading frame; KEGG, Kyoto Encyclopedia of Genes and Genomes; OTU, operational taxonomic unit; PCoA, principal coordinate analysis; PICRUSt, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; LEfSe, Linear discriminant analysis effect size; ARGs, antibiotic resistance genes; COG, Clusters of orthologous groups.

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Tables

Genus name	Before remove-Mean (%)	Before remove-Sd (%)	After remove-Mean (%)	After remove-Sd (%)	P	FDR
<i>Unclassified_o_Bacteroidales</i>	0.178	0.2097	0.1232	0.2021	0.049	0.942
<i>Norank_c_Cyanobacteria</i>	0.1249	0.2894	0.133	0.3344	0.043	0.936
<i>Lachnospirillum_1</i>	0.0561	0.1681	0.0011	0.003	0.001	0.331
<i>Ruminoclostridium</i>	0.0414	0.051	0.0257	0.0512	0.002	0.373
<i>Lactobacillus</i>	0.0068	0.0118	0.0013	0.0046	0	0.001
<i>Staphylococcus</i>	0.0057	0.0138	0.0036	0.0103	0.044	0.936
<i>Ornithinibacter</i>	0.0053	0.0247	0	0	0.047	0.936
<i>Gillisia</i>	0.0025	0.0132	0	0	0.026	0.936
<i>Pseudomonas</i>	0.0021	0.0079	0	0	0.002	0.373
<i>Ruminococcaceae_UCG-012</i>	0.0018	0.0093	0	0	0.047	0.936
<i>Exiguobacterium</i>	0.0012	0.0085	0	0	0.047	0.936
<i>Inquilinus</i>	0.0011	0.0061	0	0	0.047	0.936
<i>Norank_f_env.OPS_17</i>	0.0011	0.0047	0	0	0.047	0.936
<i>Norank_o_Rhizobiales</i>	0.001	0.0047	0	0	0.026	0.936
<i>Pseudoxanthomonas</i>	0.0009	0.0046	0	0	0.047	0.936

Table 1 Environmental contaminants were identified and removed by “decontam”.

Note: $P < 0.05$ by Wilcoxon rank-sum test, FDR of Corrected P value.

Figures

Figure 1

Geographical locations of study sites and body measurements of Tibet goats. (a) Geographic locations of Tibet goats study sites. (b) Comparison of birth weights of grazing and drylot goats. (c) Comparison of yearling weights (D365) of grazing and drylot goats. Changes in GIT lumen of pH (d), acetic acid (e), propionic acid (f), and butyric acid (g). SCFAs were measured using gas chromatography. n.s. $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ by one-way ANOVA with Tukey's test for intra- and intergroup comparisons. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

Figure 2

Comparison of the GIT microbiome gene catalogues, composition, and functions in grazing and drylot goats. (a) Alpha diversity at the phylum, OGs and NR gene counts of the metagenomic analyses. (b) Bacterial 16S rDNA copies in the lumen or mucosa of grazing and drylot raised goats. * p < 0.05. (c) PCoA of the lumen and mucosa community composition of grazing and drylot goats using unweighted UniFrac of 16S rRNA sequences. (d) The relative abundances of the most dominant phyla (%). The color-coded bar plot shows the average abundances of GIT lumen and mucosa of grazing (G) and drylot (D) goats. (e) Rumen and hindgut show different functional roles of the gut microbiome (abundance shown as log10).

Figure 3

Differences in the ruminal microbiota and metabolism pathways of grazing and drylot goats. (a) PCoA of rumen luminal and mucosal community composition of grazing and drylot goats using unweighted UniFrac of 16S rRNA sequences. (b) Differences in abundance of genera *Methanobrevibacter* and *Alistipes*. * $p < 0.05$. (c) Differences in expressed pathways of Methane metabolism in grazing and drylot groups. * $p < 0.05$. (d) The relative contributions of dominant bacterial genera for methane metabolism

(%). (e) Diagram of the hydrogenotrophic methane production pathway illustrates the enzymes involved in each biochemical reaction between grazing and drylot goats. * $p < 0.05$. (f) Goats rumen microbial co-occurrence network analysis based on core genera. Only the top 30 genera are presented. Spearman's rank correlation coefficient > 0.50 ; p -value < 0.05 . Different colors represent different phyla in the rumen. The size of nodes is proportional to the relative abundance of the genera, the solid line indicates a positive correlation between species, the dotted line indicates a negative correlation between species. The thickness of the line indicates the magnitude of the correlation coefficient value. (g) The genetic differences of the GH family involved in completely different cellulose degradation pathways. Red represents the rumen-derived cellulose degradation pathway in grazing goats, and green represents drylot goats. * $p < 0.05$ by Wilcoxon rank-sum test.

Figure 4

The composition of the hindgut microbiota, functional distributions and ARGs expressed of grazing and drylot goats. The abundances of core genera in (a) cecum and (b) colon of grazing and drylot raised goats. * $p < 0.05$ by Wilcoxon rank-sum test. (c) Shotgun metagenomic sequencing reveals differences in functional microbial pathways of cecum. * $p < 0.05$ by Wilcoxon rank-sum test. (d) The log-transformed LDA scores and cladogram illustrate significant functions in cecum of grazing and drylot goats. The LDA score obtained by LDA analysis (linear regression analysis). The larger the LDA score, the greater the effect of the functional abundance on the observed functional differences. (e) Relative abundances of ARGs found in each group of goats (%). * $p < 0.05$, ** $p < 0.01$ by Wilcoxon rank-sum test.

Figure 5

The putative mechanism differences in the rumen and hindgut microbiota under varied feeding conditions. (Left) Grazing significantly increased the abundance of *Methanobrevibacter* in the rumen, resulting in enhanced hydrogenotrophic methane production pathway, grazing significantly increased the acetic acid synthesis, and the proportion of Bacitracin resistance gene significantly increased under grazing conditions, and related to the body's immunity. (Right) Drylot feeding significantly increased the propionic acid synthesis, enhanced the abundance of resistance genes such as Tetracycline, Macrolide, Cephalosporin, and increased the abundance of *Rike_RC_9*, *Prevote_UCG_003* and *Prevotella*. Grazing significantly enhanced the synthesis of acetic acid in the hindgut, enhanced the abundance of *Clostridium* and *Rumi_UCG_005*, and enhanced the proportion of the resistance gene Bacitracin, which enhanced the pathway of Carbon metabolism and peptidoglycan synthesis, and significantly improved the cellulose degradation pathway. The red arrow indicates a significantly enhanced in the grazing group; the green arrow indicates a significantly enhanced in the drylot group.

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