

Phylogenetic and functional adaptation of the gastrointestinal microbiome of goats kept at high altitude (4800 m) under intensive or extensive rearing conditions

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Abstract

Background: The gut microbiota composition is influenced by the diet as well as the environment in both wild and domestic animals. Although the rumen microbiome in herbivorous ruminants has been studied intensively, there is a lack of data regarding the simultaneous adaption of the rumen and hindgut metagenome as affected by different feeding systems in extreme environments. Therefore, we studied the effects of two feeding systems, grazing and drylot, on the rumen and hindgut microbiome composition of semi-feral Tibetan goats kept at high altitude (~4800 m). 16S rRNA gene sequencing and metagenomic analysis were conducted on DNA extracts from the contents and mucosal layer of different sections of the gastrointestinal tract (rumen, cecum, and colon).

Results: Intensive drylot feeding resulted in significantly higher zootechnical performance, narrower ruminal acetate: propionate ratios and a drop in the average rumen pH at slaughter to ~5.04. In response, the ruminal microbiome of drylot goats expressed a significantly lower diversity compared to the grazing animals. Otherwise, hindgut microbial adaption appeared to more diverse in the drylot group suggesting a higher influx of undegraded complex non-starch polysaccharides from the rumen. Despite their higher fiber levels in the diet, grazing goats exhibited lower counts of *Methanobrevibacter* and genes associated with the hydrogenotrophic methanogenesis pathway, presumably reflecting the scarce dietary conditions (low energy density) when rearing goats on pasture from extreme alpine environments. These conditions appeared to promote a relevant abundance of bacitracin genes, which potentially benefits the host's adaption to harsh environmental conditions. In parallel, we recognized a significant increase in the abundance of antibiotic resistance genes in the digestive tracts of drylot animals.

Conclusion: In summary, this study provides a deeper insight in the phylogenetic and functional adaption of the gastrointestinal microbiome of goats subject to intensive drylot and extensive pasture rearing conditions at high altitude.

Introduction

Ruminant livestock play an important role in food security. Specifically, they convert inedible lignocellulosic plant materials through ruminal microbial fermentation into high-value animal products including milk, meat, and fibers [1]. Therefore, the utilization rate of fiber-rich roughage within the ruminant's gastrointestinal tract (GIT) is an important measure of the nutrient conversion efficiency in ruminant production systems and a critical factor for the global food security in general [2]. The foundation of this trait is laid during the development of the young ruminant organism. In this context, the amount and composition of structural carbohydrates (fibers, non-starch polysaccharides) in the diet has a large impact on the development of the GIT, especially the rumen. An insufficient supply with these materials causes an impairment of ruminal development and, in consequence, a reduced (microbial) digestive capacity [3]. Another important factor is the GIT microbiome itself, which is believed to be shaped by animal genetics, diet, environmental (geographic) parameters [4], and social contact patterns [5, 6, 7].

The diet has been identified as a driving factor for changes in the gut microbial ecosystem in both wild [8, 9, 10, 11] and domestic animals [12, 13]. Compared to traditional nomadic pastoralism, modern high-performance livestock production very often relies on confined drylot feeding, in which ruminants are provided highly digestible diets with a much narrower ratio of simple sugars and proteins to non-starch polysaccharides compared to the diet of wildtype animals. This is supposed to induce dramatic differences in the functional and phylogenetic composition of the gastrointestinal microbiome. The majority of data in this regard reflect comparisons between cattle and sheep under drylot and grazing conditions, whereas reliable information on goats are scarce. However, extrapolating findings from bovines and ovines to goats is not easy because these species represent quite different types of ruminants and the latter have a more selective feeding behavior [14]. Given the importance of goat production systems on a global perspective [15], thorough investigation of the consequences of wild grazing versus drylot feeding strategies 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.

Another important question refers to the alterations in gut microbial composition in comparison between captive and free-ranging animals. It has been shown in wild animals from different species that captivity induces dramatic changes in the metagenome [16, 17, 18]. Furthermore, presenting woodrats collected from wild habitats with diets very close to their natural feeding habits showed significant mitigation of microbial alterations with a 90% retention of native microbial communities across the experiment [19]. These results again confirmed the diet to be the major driver of gut microbial composition. Otherwise, it also suggested a significant proportion of changes (~ 10%) were due to the captive environment itself. Such data is yet not available for livestock. In addition, ruminants have a more complex digestive system compared with monogastric animals and it is still unclear how environmental interaction with dietary factors affects the gut microbiota as well as its interaction with the host. Additionally, the increasingly widespread presence of antibiotic resistance has made it imperative to consider diverse environments as sources of emerging resistance [20, 21]. Resistance was identified as being present in microbial communities before the widespread clinical and agricultural use of antibiotics [22]. Whether grazing ruminants compared to such drylot conditions express differences in the composition and abundance of antibiotic resistance genes (ARGs) remains unclear. Furthermore, this issue was never investigated in an animal cohort under extreme environmental conditions.

This study investigated the phylogenetic and functional composition of the rumen and hindgut microbiome of goats kept under free-range and drylot conditions, respectively, in an extreme environment. Therefore, we selected the Tibetan plateau (Qiangtang National Natural Reserve), also referred to the “roof of the world”, as testing location. This is unarguably one of the harshest environments on earth due to the high altitude above sea level, boasting cold and hypoxic conditions, as well as low available plant biomass [23]. 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 Tibetan plateau (Fig. 1 a), 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. We`ve analyzed the microbial diversity across two feeding

systems (free-ranging vs. drylot) in the nomadic areas of the Tibetan plateau. Metagenomic and 16S rRNA data were collected 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 findings reported here provide novel ecological insights into the establishment of the GIT microbiome in animals under extreme environmental conditions.

Materials And Methods

Study sites, participating animals, and sample collection

A total of 50 half-sibling female 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, the drylot group was allowed to be with the dams until weaning (Fig. 1a, Table S1). The grazing group was allowed to free-range, following their dams without any artificial feeding (Nima, Tibetan, China, altitude > 4,800 m; Fig. 1a). Weaning age was the same between both systems at 90 days postpartum. The goats in spring mainly consumed various perennial grasses such as *Stipa purpurea*, *Kobresia tibetica Maxim*, *Leontopodium pusillum* and *Stipa purpurea Griseb*. The herbage intake and forage digestibility of the half-sibling Tibetan goats in spring were determined by alkane technology in the same place before [24]. The dry matter, crude protein, calcium, and phosphorus intakes of the half-sibling Tibetan goats were 450, 32, 5.23, 0.46 g per day, respectively, and the average dry matter digestibility was $45.62 \pm 1.65\%$. The NDF and ADF content (DM basis) of diet for grazing goats was $55.98 \pm 0.10\%$ and $34.53 \pm 0.10\%$, respectively [24]. From each group, 5 goats were randomly selected and slaughtered on day 365 postpartum. Some rumen bacteria that are essential for mature rumen function showed stability as early as 1 year after birth of ruminant animals [25], this is the main reason why goats are 1 year old as the end point of this experiment. 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 phosphate-buffered saline (PBS, pH 7.0) to remove the digesta. The 15 ml of ruminal, caecal, and colon contents were strained through cheesecloth and immediately subjected to pH measurement. Subsequently, 5% HgCl₂ were added to the samples and stored into liquid nitrogen for the determination of short chain fatty acids (SCFA) concentrations. The ruminal, caecal, and colon contents were collected and stored into liquid nitrogen for the extraction of microbial DNA.

Analysis of short chain fatty acids

Concentrations of SCFA were measured in content samples from the rumen, cecum, and colon using an Agilent 7820A gas chromatograph (Agilent Technologies, Santa Clara, USA), See the supplementary materials (Supplementary materials-MATERIALS AND METHODS) for detailed measurement procedures.

DNA extraction, PCR amplification, and 16S rRNA sequencing

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 the grazing and drylot group, respectively. Negative control (DNA-free water and buffer; n = 3) was used for DNA extraction and sequencing after using “decontam” [26] to remove the negative control contamination. Total DNA was extracted from the tissues and lumen using the E.Z.N.A.® stool DNA Kit (Omega Bio-tek, Norcross, GA, USA) according to the 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 supplementary materials (Supplementary materials-MATERIALS AND METHODS) for detailed measurement procedures.

Metagenomic analyses, assembly, and construction of the gene catalog

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. 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 as well as their mated reads were removed. Metagenomics data were assembled using MEGAHIT [27]. 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. Open reading frames (ORFs) from each assembled contig were predicted using Metagene [28]. The predicted ORFs with lengths of at least 100 bp were retrieved and translated into amino acid sequences using the National Center for Biotechnology Information (NCBI) translation table. See the supplementary materials for detailed procedures.

Quantitative PCR (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) [29]. See the supplementary materials 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 zootechnical data were

analyzed by one-way ANOVA with a Tukey's test using SPSS 21.0. β diversity indices (Bray-Curtis) were calculated in QIIME [30] 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).

Results

Zootechnical performance and parameters of microbial fermentation

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, and pH measurements, as well as SCFA in GIT, 16S rRNA gene sequencing, and metagenome sequencing are summarized in 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), increased by 48.5%. 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).

Microbiota composition and function feature change in foregut and hindgut

We obtained a total number of 2,098,000 clean reads from metagenomic sequencing data. 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 included *Unclassified_O_Bacteroidales*, *Norank_C_Cyanobacteria*, *Lachnoclostridium_1*, *Ruminiclostridium*, *Lactobacillus* and *Staphylococcus* (Table S3). Additionally, metagenomic 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 ORF length of 478 bp.

Based on the read abundances at the level of phylum, eggNOG orthologous groups (OG) and gene levels (Fig. 2a) were investigated for microbial diversity (Shannon index) in different compartments. For phyla and cluster of OG level, it was observed that the luminal microbial diversity of hindgut was lower than that observed in rumen (Fig. 2a). However, at the gene level, the hindgut diversity was higher compared

with the rumen (Fig. 2a). 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 (PCoA) of operational taxonomic units (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 functional potentials for nutrient metabolism due to community structure differences. Next, PCoA was conducted on the lumen and mucosa samples separately (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).

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 S4), whereas *Spirochaetae* was prevalent and highly specific for the cecum mucosa (average abundance 26.98% and 27.46%; Fig. 2d and Table S4). At the genus level, the predominant members in the hindgut were *Treponema_2*, *Ruminococcaceae_UCG-005*, *Ruminococcaceae_UCG-010*, *Alistipes*, *Bacteroides*, *Prevotellaceae_UCG-004*, and *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 S5). To determine relationships between the differential abundances of the gut bacteria with pH and SCFA, a correlation analysis was conducted (Fig. S3). *Clostridium*, *Alistipes*, and *Ruminiclostridium* were positively correlated with pH and acetic acid production, whereas *Methanobrevibacter* and *Barnesiella* were positively correlated with propionic acid production, as well as *Prevotella* and *Butyrivibrio* have positively correlated with butyric acid production (Fig. S3).

Furthermore, we determined that rumen and hindgut have distinct functional 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 (Fig. 2e). In contrast to the rumen, 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).

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

The PCoA revealed significant differences in the microbiota between the lumen and mucosa in rumen of the two feeding conditions in the OTU level (ANOSIM, Bray-Curtis metric: $R^2 = 0.46$, $p = 0.001$. Figure 3a). 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, Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis discriminated the ruminal lumen metagenomes (Fig. S5) 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 were differentially enriched, including *Methanobrevibacter* and *Selenomonas* (Fig. 3d). *Methanobrevibacter* is mainly involved in hydrogenotrophic methane production pathway [31]. The 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).

In grazing goats, *Ruminococcus* was determined to be a core genus that positively facilitated two different clusters in the rumen. On the other hand, *Treponema* 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 simpler in the drylot group and was not as complicated as was observed in the rumen of grazing goats (Fig. 3f).

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 gene families are involved in plant cell wall degradation. In addition, the families of GH77, GH23, GH13, G32, 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). These gene families promote the transformation of starch and glycogen into dextrin that uses EC3.2.1.10 to further break the molecule down to transform into D-Glucose. Additionally, EC 3.2.1.20 promotes 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.

Analyses of metagenomic sequencing data of hindgut microbiota between grazing and drylot goats

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. S5a). Specifically, *Spirochaetes* and *Fibrobacteres* were significantly higher ($p = 0.03$. Fig. S5b), and *Firmicutes* were significantly lower in cecum lumen of drylot goats ($p = 0.03$. Fig. S5b). 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. S5b). The core genera of *Clostridium*, *Prevotella*, and *Treponema* were observed in significantly different proportions between the two groups (Fig. 4a). 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, as well as 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).

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. S6). However, in the drylot group, 30 genera were complexly 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 S6).

Similar change of core genera patterns was 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. S7). As a result, alanine, aspartate and glutamate metabolism, as well as glyoxylate and dicarboxylate metabolism were highly enriched in the colon of grazing goats (Fig. S8a). 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. S8b). 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. S8b).

The difference of foregut and hindgut ARGs between grazing and drylot goats

Of particular interest is the difference of rumen, cecum and, colon antibiotic type between the free-range grazing and drylot goats. The PCoA revealed a list of significantly expressed antibiotic type in each of the two feeding systems (Additional files 2: Fig. S9a). The grazing goats harbored lower abundances of ARGs. These results were confirmed by the ARGs levels, in which bacitracin were significantly higher in the grazing group (average abundance 97.84%, $p = 0.01$, Fig. 4e, Fig. S9), whereas the resistance genes of lincosamide, tetracycline, macrolide, cephalosporin, and streptomycin were significantly enriched in the drylot group (Fig. 4e, Fig. S9).

Discussion

In the present study, the difference in the daily weight gain and gut fermentation patterns reflected the narrower energy: fiber ratio in the drylot feed in comparison to the free-ranging animals that were mainly fed with fiber-rich grass. This also resulted in increased concentrations in propionic and butyric acid and lower acetic acid levels, which is in line with the current literature [32]. In fact, propionyl-CoA transferase, lactoyl-CoA dehydratase, and acryloyl-CoA reductase have been shown to be the key enzymes that mediate the ruminal lactate metabolism pathway [33]. Under conditions of a high-concentrate diet, lactic acid mainly passes through the acrylic acid pathway. D-lactic acid is first converted into L-lactic acid under the action of lactate racemase (LR) and then further metabolized. L-lactic acid is transformed into lactoyl-CoA by propionyl-CoA transferase and subsequent dehydration produces acryloyl-CoA and finally acryloyl-CoA, which is then hydrogenated to propionate [34]. The changed SCFA pattern promoted a drop in the ruminal pH of drylot animals down to an average of 5.04. According to the current literature, this may indicate rumen acidosis, which is a common pathology in high performance ruminant production systems where fermentable fiber sources are partially replaced by simple carbohydrate substrates (starch, monosaccharides). It is accompanied with systemic inflammatory processes [35] and has been associated with a drop in fibrolytic bacteria and an increase in gram-negative bacteria [36]. These reports are in line with our data on the functional annotation of the metagenome, which pointed to increased counts of genes associated with starch breakdown in drylot goats and, in parallel, a decrease in genes involved in the digestion of non-starch polysaccharides. It is intriguing that neither the feed intake behavior nor the growth performance or visual appearance and behavior of the drylot goats indicated any pathological problems. This again highlights that pathologies and especially such of a subacute nature are not necessarily reflected by the performance level of animals, hence, such parameters are not suitable to serve as biomarkers of an animal's wellbeing. Future studies should regularly screen the circulation of inflammatory markers in the blood throughout such studies to identify the onset of systemic inflammation. Most importantly, the current standard drylot feeding regime for Tibetan goats must be improved to avoid impairments of animal wellbeing in the future.

Certain ruminal microbes (so called methanogens) use different substrates (such as hydrogen, formate, methyl compounds, and acetic acid) to produce methane [37]. Since acetate from cellulose breakdown is readily absorbed through the rumen wall, hydrogenotrophic methanogenesis based on hydrogen and carbon dioxide as substrates appear to be the most important methanogenic pathway within animal digestive tracts [38, 39]. In this context, *Methanobrevibacter* is the most important archaeobacteria of the hydrogenotrophic methanogenesis pathway [40]. The ruminal methane production is affected by various factors (such as digestive tract pH, feed composition, feeding level, digestive tract microbial composition, etc.), however, it mainly depends on the rate and pathway of hydrogen production and hydrogen discharge in the digestive tract [41]. During the traditional feeding process of sheep and goats, the methanogens and cellulolytic bacteria in the rumen establish very early [42]. Furthermore, methanogens can affect the number of hydrogen-producing bacteria and protozoan communities [43]. The number of methanogens themselves is affected by soluble dietary carbohydrates (starch, sugar). In the present study, genes associated with the methanogenesis pathway were significantly enriched in the rumen of

drylot goats relative to the grazing group, which was in accordance with higher counts of *Methanobrevibacter*. This was an interesting observation since their diet contained less fiber and more starch and sugar, which was also reflected by their ruminal fermentation characteristics. In earlier studies, increasing concentrate ratios in complete feed have been associated with curvilinear decrease in methane emission in cattle [44] due to the increasing ruminal propionate proportion and associated drop in pH [45]. In contrast, higher cell wall fibers in the diet are promoting methane emissions by increasing the acetate proportion in the rumen [46, 47]. The question remains, why the group with the wider starch: fiber ratio in the diet exhibited relatively higher counts of methanogenesis-associated genes. Ruminal methanogenesis is an energy-dependent process [48]. It has been already pointed out that hydrogenotrophic methanogenesis is the most important methanogenic pathway within animal digestive tracts [38, 39]. The ruminal availability of the necessary hydrogen and carbon dioxide as well as the proliferation of methanogens very much depends on the availability of soluble energy substrates like starch and sugar [48, 49]. The availability of soluble energy substrates was obviously much higher in the drylot diet compared to that of grazing animals. In fact, our grazing goats just received the natural pasture of the Tibetan Plateau, which appeared to be very scarce in terms of energy and nutrient density. This seemed to not only result in significantly lower zootechnical performance but, most interestingly, also in an energy shortage for microbial methanogenesis. Literature reports on higher methanogenesis from cattle fed diets with a wider fiber: concentrate ratio compared to those with high dietary concentrate ratios [44] are not directly referring to our situation since diets from high-performing cattle are usually balanced in their starch and sugar contents to provide enough energy to the microbes for optimal feed breakdown. Under such conditions, the amount of fiber and associated breakdown into acetic acid indeed makes a difference in terms of methanogenesis.

A previous study subcategorized gut commensal bacteria into four populations: luminal commensal bacteria, mucus-resident bacteria, epithelium resident bacteria, and lymphoid tissue-resident commensal bacteria [50]. Mucosal microorganisms have been shown to modulate animal immune function [51]. Meanwhile, the luminal microbes facilitate most of the fermentation of substrates passing alongside the gastrointestinal tract [52]. The present study suggests that the mucosal community is shaped by the host rather than the available substrates [53]. In this study, the mucosal community of cecum did not differ between grazing and drylot animals. It has been proposed earlier that the ruminal mucosal microbiota may represent some sort of “nursery” for the luminal microbial community [54]. It appears quite logical that these microbiota with constant physical contact to the host cells are also in close interaction with these. Presumably, they are more dependent on the availability of substrates at the mucosal interface and this spectrum is mainly shaped by absorptive and excretory activity of host cells. Furthermore, the composition cell surface structures (glycocalyx) to which these bacteria attach is also shaped by host genetics [55]. Overall, the ruminal mucosa-associated microbes may explain a large proportion of the effects the host genetics are expressing on the metagenome [55]. In strong contrast to the caecum mucosal community, our results indicate that drylot feeding resulted in more diverse and complex luminal microbial community in the caecum, but more independent and simple rumen luminal microbial communities. Our results indicate occurrence of rumen acidosis in the drylot goats. The classic view of

rumen acidosis is that the rapid fermentation of feed produces more lactic acid, propionate, and butyrate [56] and the rumen pH drops to a certain level. This results in an inhibition of the cellulose bacteria and an increase in acid-tolerant bacteria [57]. The overall consequence is a decrease in rumen microbial community richness and diversity, which renders the interaction network between bacteria to become more independent and simpler. We therefore postulate that an insufficient fiber degradation in the rumen of drylot goats increased the influx of non-starch polysaccharides into the lower intestinal segments, thereby resulting in an increase of the cecum and colon microbial abundance and diversity. These hypotheses need to be proven in future experiments.

The grazing goats from the present study showed an increased abundance of the bacitracin gene within their ruminal, caecal and colon contents, simultaneously, a lower abundance (close to the lowest level of detection) of other types of bacterial antibiotic genes were detected. Bacitracin is a mixture of high molecular weight polypeptides of microbial origin (*Bacillus sp.*) that possess antimicrobial activity against gram-positive microorganisms by interfering with bacterial cell wall formation and peptidoglycan synthesis [58]. A previous study has reported that bacitracin in addition to its antibacterial activity, neutralizes a variety of pathologically relevant bacterial (protein) toxins and protects cultured host cells from intoxication [59]. Furthermore, bacitracin prevents the pH-mediated transport of the enzyme subunits of these toxins across endosomal membranes into the cytosol of target cells, most likely by inhibiting the essential membrane transport function of their binding/transport subunits [59]. In addition, a dietary supplementation of bacitracin increased the amount of indole-3-acetic acid, 3-indoxyl sulfate, and 5-hydroxyindoleacetate as well as decreased indole-3-carboxylic acid within the ceca of turkeys [60]. These metabolites have been shown to exert significant positive effects on the nutrient utilization and immunological status and health of the host [61]. In light of these reports, we conclude that the upregulation of bacitracin in the microbiome of grazing goats from the present study represents a measure to adapt to harsh environmental conditions. In contrast to grazing, drylot goats exhibited a significantly higher abundance of genes which establish resistance against specific types of antibiotics, including tetracycline, macrolide, cephalosporin, and streptomycin. Since all animals were treated equally except for the basal diet, environment and housing, we suspect that either dietary or environmental factors from the stable were promoting the establishment of a wide array of antibiotic resistance genes. Horizontal gene transfer is an important way of antibiotic resistance gene transmission and is one of the reasons for the increasingly serious environmental pollution by antibiotic resistance genes [62]. Potentially, horizontal gene flow also contributed to the establishment of antibiotic resistance in our drylot animals. Future research should be focused on the dissection of the various dietary and environmental factors within the drylot systems that may promote antibiotic resistance. For example, environmental factors such as light, temperature, and oxygen have been shown affect the spread of resistance genes in the environment and should therefore be considered in respective follow-up studies [63, 64].

Conclusions

In summary, the current study presents the establishment of GIT microbiome in semi-feral goats under challenging environmental and dietary conditions. Strong correlations were observed between feeding conditions and the abundance of genes related to microbial methanogenesis in goats. Drylot goats exhibited enhanced expression levels of genes of the hydrogenotrophic methanogenesis pathway as well as ruminal counts of *Methanobrevibacter* compared to free-ranging grazing animals. This presumably reflected the scarce dietary conditions in the grazing group that resulted in a significantly reduced (soluble) energy intake and associated lower zootechnical performance. Furthermore, drylot feeding resulted in more diverse and complex hindgut microbial communities but more independent and simple rumen microbial communities. High-abundances of bacitracin genes were observed in grazing compared to drylot goats, which are believed to improve the host's adaption to harsh environmental conditions. At the same time, the digestive systems of drylot animals harbored more genes that are supposed to promote resistance to tetracycline, macrolide, cephalosporin and streptomycin (Fig. 5). Future studies should be designed to identify how the natural defense mechanisms for goats can be stimulated by a more beneficial dietary design. Taken together, this study provides new insights into the colonization pattern of microbes in two feeding systems under cold and hypoxic conditions.

Declarations

Ethics 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 2017XZ0513007.

Consent for publication

Not applicable.

Availability of data and material

The samples of 16S rRNA gene sequencing and shotgun metagenomic data are available from the NCBI under accession No. SRP188060.

Competing interests

The authors declare that they have no competing interests.

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fundere had any role in the design and conduct of the study; collection, management, analysis, and interpretation of the data, as well as preparation, revision, or approval of the manuscript.

Authors' contributions

Yulin Chen: Conceptualization, Funding acquisition, Methodology, Writing - review & editing. **Ke Zhang:** Conceptualization, Methodology, Investigation, Data Curation, Writing - review & editing. **Chong He:** Data Curation. **Yangbin Xu:** Investigation. **Chenguang Zhang:** Investigation. **Chao Li:** Visualization. **Xu Jing:** Visualization, Software. **Meili Wang:** Visualization, Software. **Yuxin Yang:** Conceptualization, Methodology. **Langda Suo:** Conceptualization, Investigation. **Peter Kalds:** Writing - review & editing. **Jiuzhou Song:** Writing - review & editing. **Xiaolong Wang:** Conceptualization, Funding acquisition, Writing - review & editing. **Daniel Brugger:** Writing - review & editing. **Yujiang Wu:** Conceptualization, Investigation, Funding acquisition.

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Abbreviations

GIT: Gastrointestinal tract; ARGs: Antibiotic resistance genes; NDF: Neutral detergent fiber; ADF: Acid detergent fiber; SCFA: Short chain fatty acids; ORFs: Open reading frames; OG: eggNOG orthologous groups; PCoA: Principal coordinates analysis; OTUs: Operational taxonomic units

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Figures

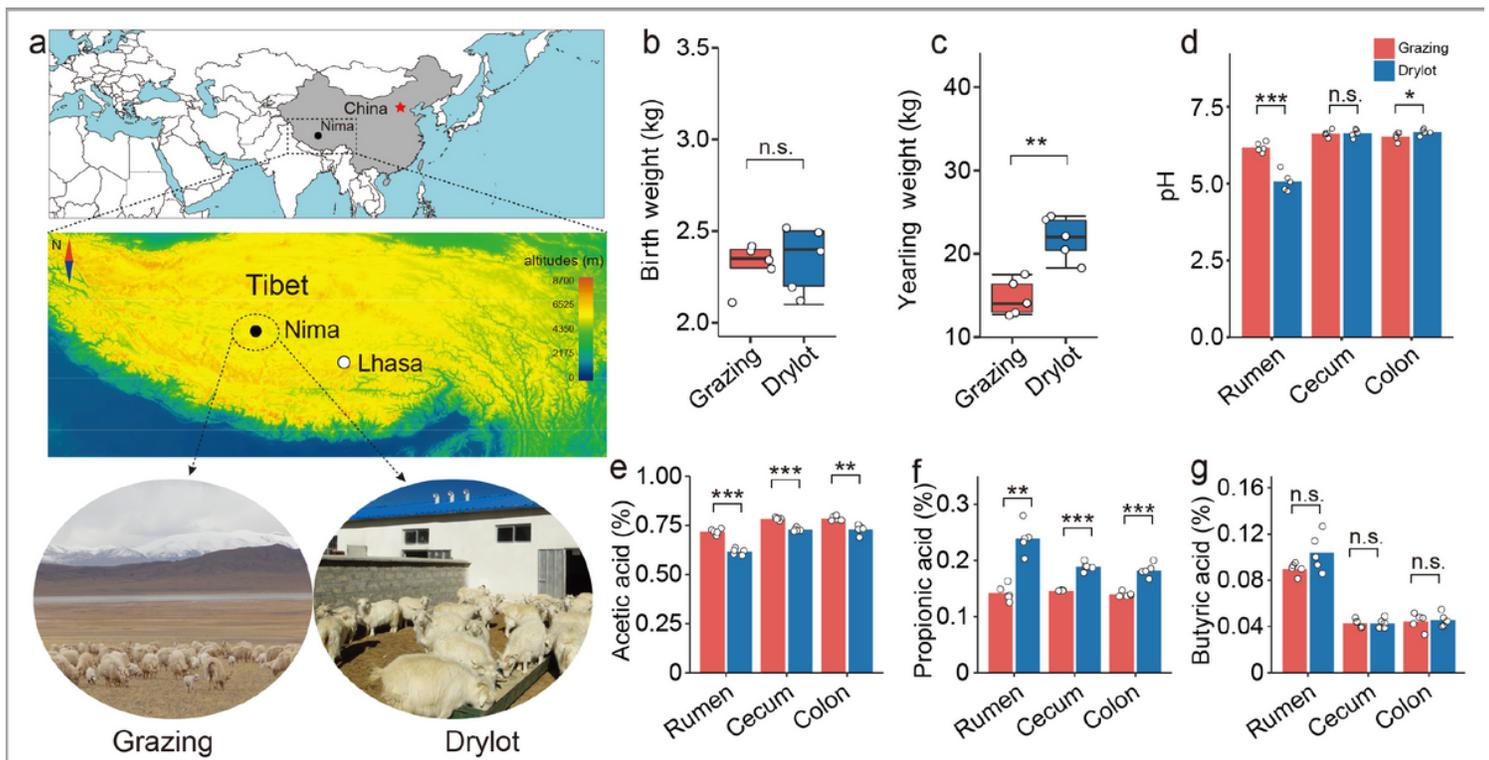


Figure 1

Geographical locations of the study sites and body measurements of Tibet goats. (a) Geographic locations of the 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.

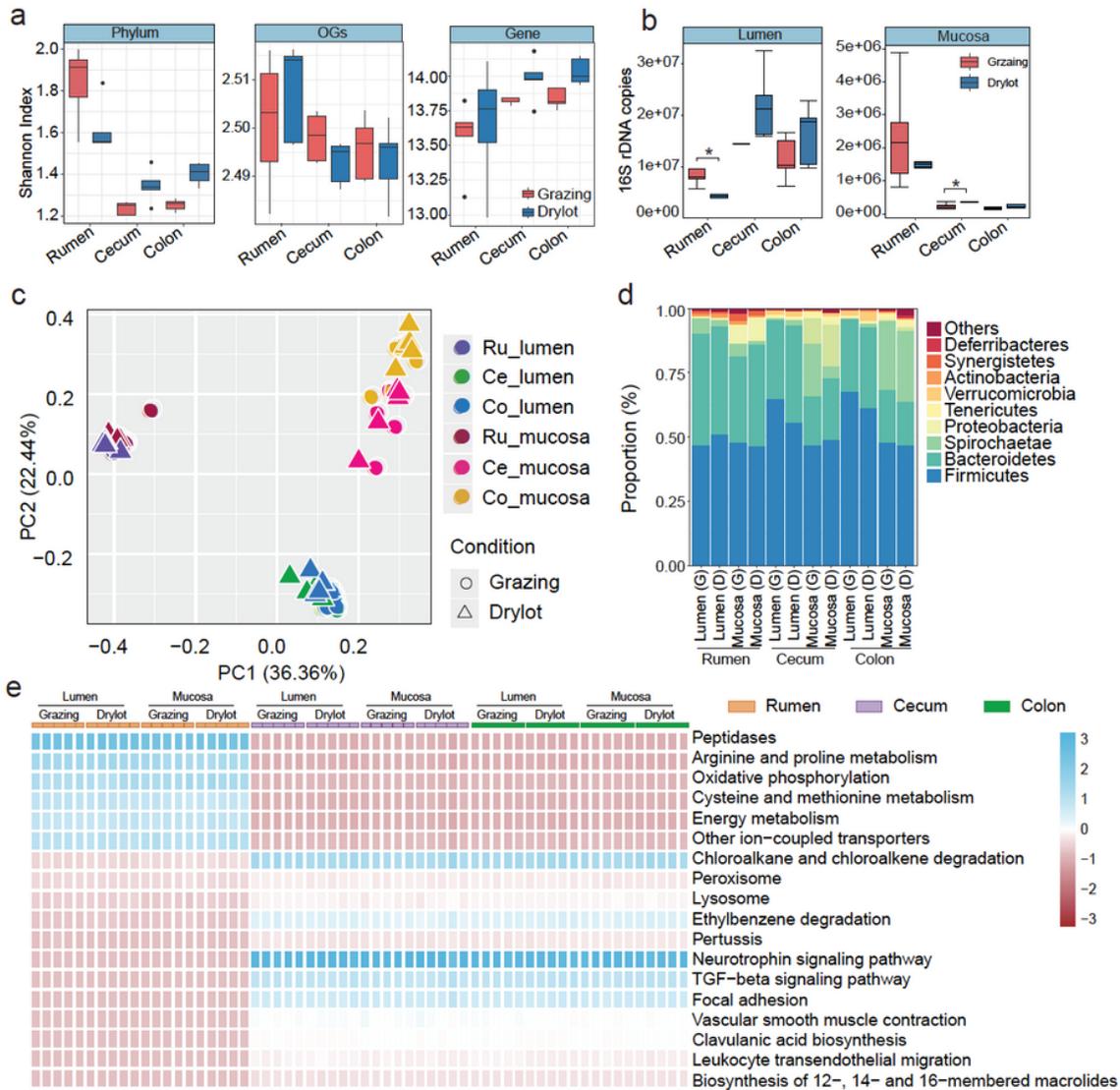


Figure 2

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 \log_{10}).

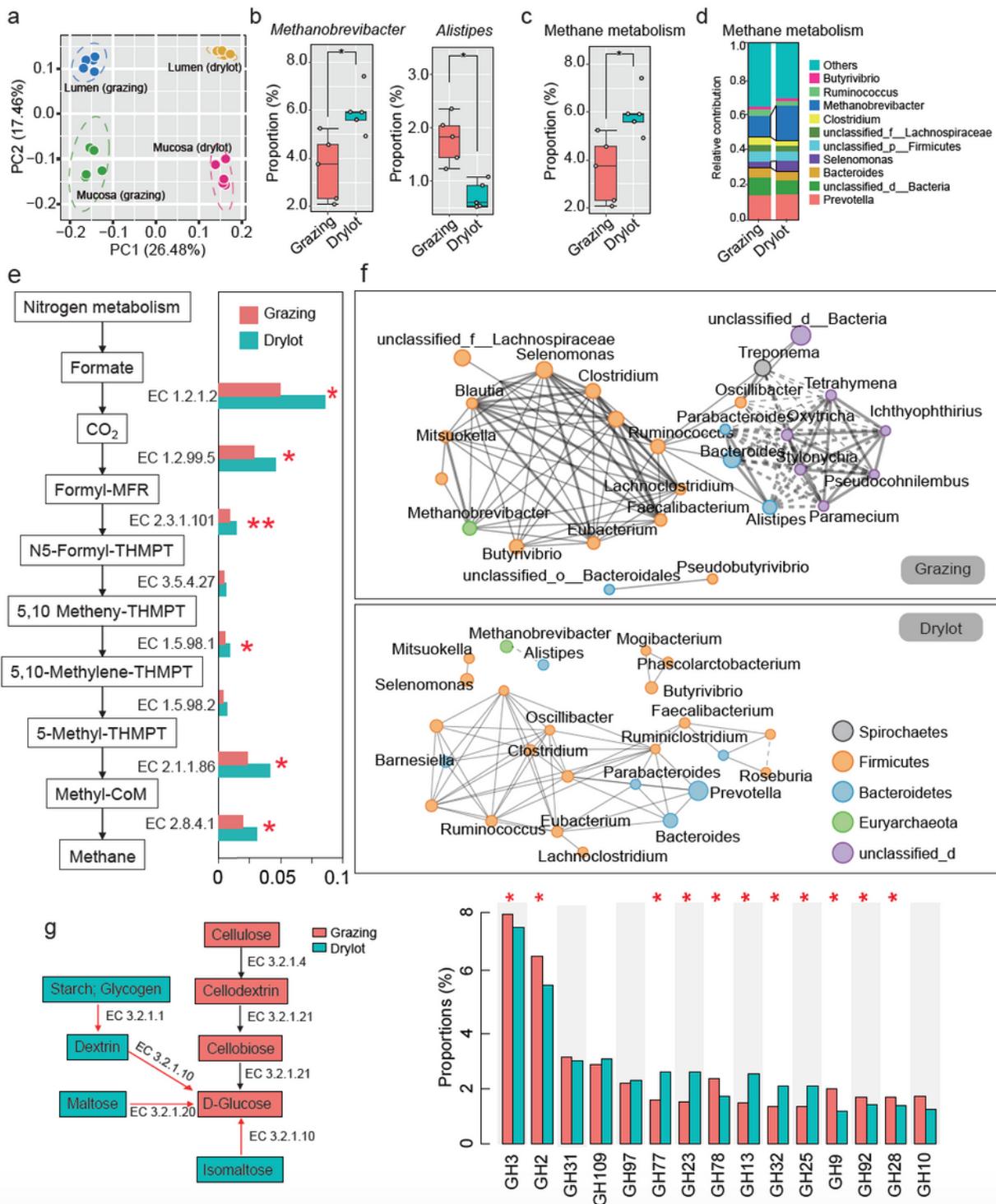


Figure 3

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.

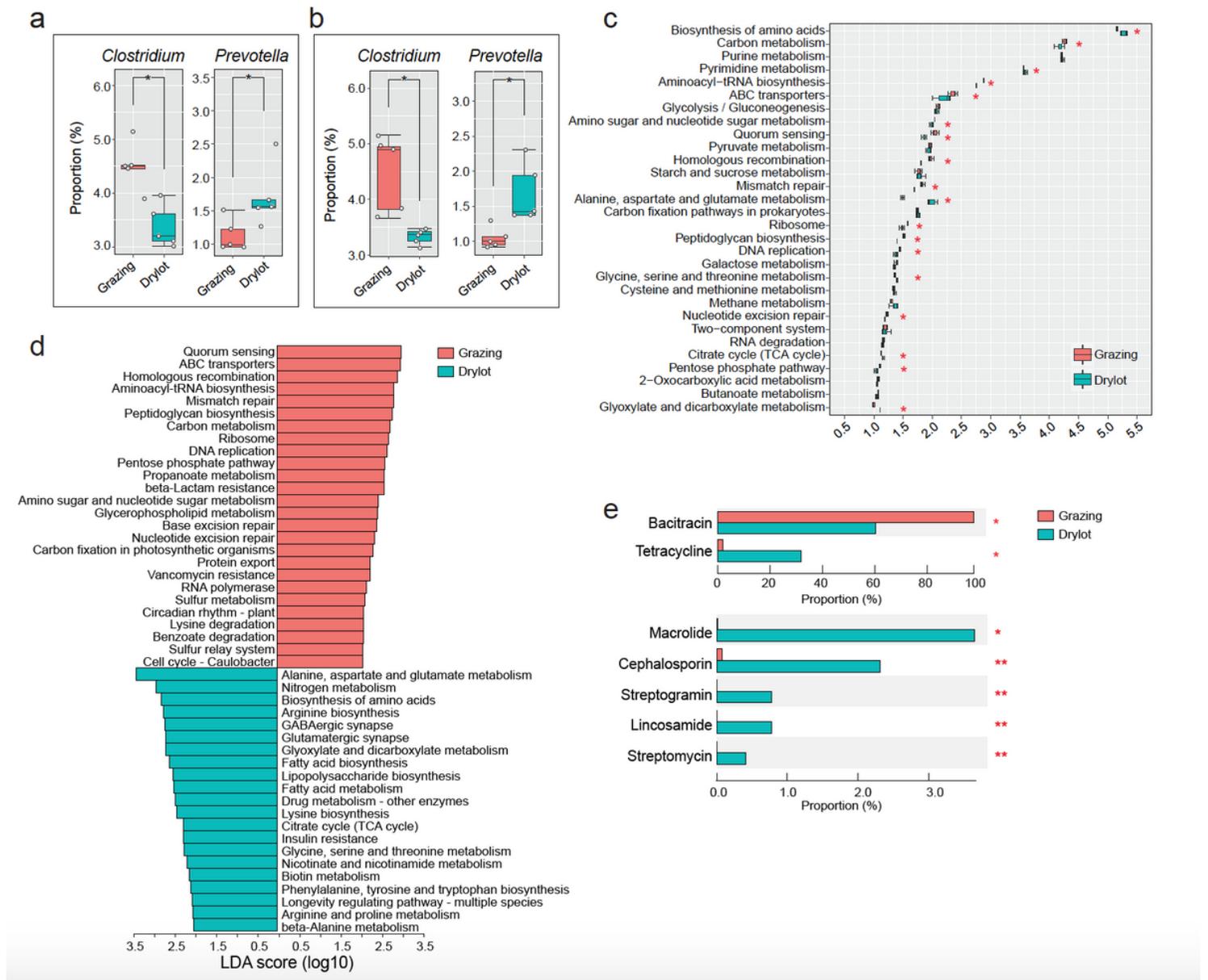


Figure 4

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.

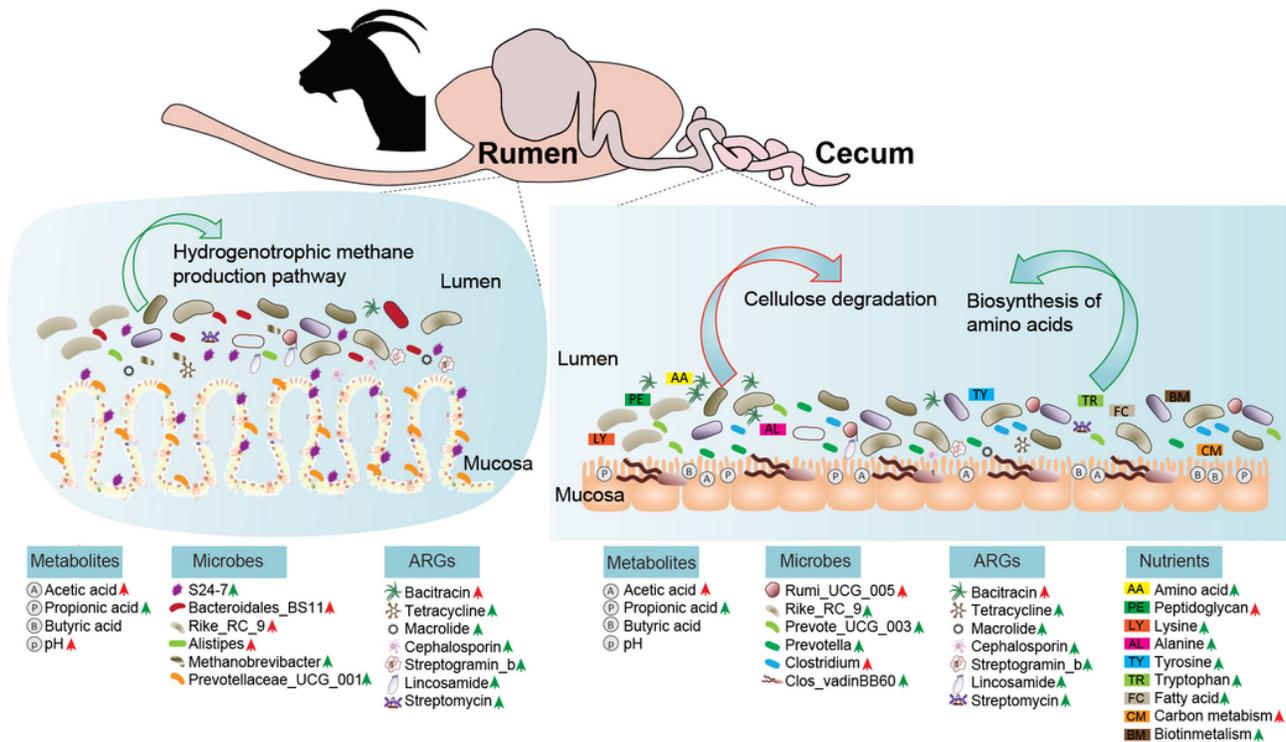


Figure 5

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. (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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