Exploring Yak (*Bos grunniens*) Rumen Bacterial and Fungal Communities from 5 Days after Birth to Adulthood

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Abstract

The gut microbial community of mammals, especially ruminants, plays an important role at different ages of the host. Ruminants have a unique compartment in their digestive tract; the rumen, comprising microorganisms that can effectively decompose plant fibers for the host to transform into milk and for growth and development, which is important for meat production. Colonization of rumen microorganisms is closely related to host developmental stage and affects host performance production. There is little information regarding initial colonization and subsequent changes of the rumen microbial population in wild grazing animals, from birth to adulthood. This study investigated the rumen bacterial and fungal populations of grazing yaks in five experimental groups, ranging from a few days after birth to adulthood using amplicon sequencing. Results indicated that rumen microbial communities of these yaks undergo a gradual change from 5 to 180 days after birth, with the bacterial and fungal diversity stabilizing at the age of 2 years. Additionally, *Ruminococcus* was detected in 5-day-old yak rumens, with a high percentage of *Penicillium* and other microbial species are important for normal rumen function detected in the adult rumen. The changes to the yak rumen microbial community after birth were reflected in the increased anaerobic fiber degradation group, and decreased aerobic and facultative anaerobic bacteria. Microbial diversity and abundance in the yak rumen increased with age. Rumen microbial composition of 6-month and 2-year-old yaks had obvious homogeneity. There were some differences in dominant rumen microorganisms among the different age groups. Further studies are required to confirm the functions of these differential and dominant microorganisms in each age group.

Introduction

In recent years, studies have found there is an inseparable relationship between intestinal microbial community and the animal host (Guo et al. 2019; Quinn et al. 2020), which affects the host's well-being and proper functioning. The rumen of the ruminant digestive tract fully demonstrates these relationships. Rumen microbes degrade complex plant polysaccharides into compounds that can be absorbed and digested by ruminants (McGovern et al. 2018). This critical process is important in human development as it converts plant fiber in forage grass into milk and meat for human consumption. Through the process of rumination, the contact time between plant forage and rumen microorganisms can be prolonged, providing favorable conditions for full plant fiber degradation, and promoting microorganism reproduction and growth. Rumen bacteria and fungi are important for the development of a strong immune system in yaks, playing important roles in immune metabolism [7]. The rumen ecosystem changes from the time the calf is born, mainly so that aerobic and facultative anaerobic microorganisms decreased and the number of anaerobic microorganisms is increased (Jami et al. 2013). The study showed that the microbial community found in goat rumens from one week after birth to adulthood varied significantly with age and diet (Wang et al. 2016). Rumen development is not only affected by age but also dietary and rumen microbial diversity (Beharka et al. 1998). Proper physical stimulation, including better forage, can promote calm rumen growth and development (Mirzaei et al. 2015). Co-occurrence analysis of rumen microbial taxa revealed a syntrophic interaction within and between

microbial domains with changes in the diet and age of calves (Kumar et al. 2015). The number of facultative anaerobes was high in calves 1 d after birth gradually decreasing to a stable level within 6–8 weeks (Minato et al. 1992). In the establishment and colonization of rumen microbial communities in young ruminants; groups of archaea, bacteria, and fungi exist in 1-week-old animals (Li et al. 2012; Dias J et al. 2017). Calves host similar microbial families and genera, and the microbial diversity gradually changes with forage intake after weaning (Dill-McFarland et al. 2017). Bacterial and fungal changes in calve rumens were also affected by the natural grazing patterns of cows. Until presently, researchers have explored changes in newborn animals to maturity in cows and goats raised in captivity, with changes and mechanisms of intestinal flora composition being a subject of interest in recent years. However, an understanding of microbial colonization in wild grazing yak calves developmental stages and microbial changes at mature stages remains elusive. By understanding the overall rumen bacterial and fungal communities of 5-day-old yaks and other age groups under grazing conditions, changes in these communities could be traced from the 5th day after birth and their remnants during subsequent growth stages. Hence, with increased grazing time, rumen microorganism composition is gradually affected by the environment.

An understanding of the dynamic change in microflora from 5-day-old to adult yaks can aid in the establishment of relationships between microbial interactions in rumen development, thus an understanding of rumen function in providing certain microbe pivotal for early intestinal flora changes that later influence host health and specificity in microbial configuration. Therefore, this study attempted to describe rumen bacterial and fungi compositions and the changes occurring with age (newborn calves to adult yaks) using Amplicon sequencing.

Material And Methods

All animals studied in this experiment were approved by the animal protection and utilization committee of Yunnan Agricultural University, China (contract 2007-0081), and there was compliance with the guidelines of the Laboratory Animal Ethics Committee in experimental animal sources and sample collection.

The test site was located in the natural pasture of Tiancheng Lun Zhu Agricultural Products Development Co., Ltd. in the north of Shangri-La County, with an average altitude of 3600 m, and a temperate monsoon climate. Maximum average daily temperature was 13 °C, and minimum was 1 °C, annual precipitation was 600 mm, and relative humidity was 65%. Grass vegetation types were mainly Compositae, Gramineae, Sedge family, Buttercup family, and Polygonaceae. Plant types were *Blysmus sinocompressus*, *Anemone rupestris*, *Kobresia setchwanensis*, *Kobresia pygmaea*, and *Penetilla saundersiana*, with a forage yield of 182 g/m². Breast milk components were as follows: milk fat 6.14% ± 0.19%, milk protein 4.38% ± 0.06%, lactose 5.07% ± 0.05%, total milk solids 17.83% ± 0.15%, non-fat 10.92% ± 0.10%, urea nitrogen 14.24% ± 0.67%, and somatic cells 39.63% ± 2.68% (10³).

Experimental animals were provided by the Tiancheng Lunzhu Company, Shangri-La City, China. Three Zhongdian yaks (*Bos grunniens*) that had been following their mothers to graze naturally in the wild from birth to 6 months of age were selected, and six 2-year-old free-grazing Zhongdian yaks were selected to collect samples at 45, 90, and 180 days after birth. Two hours after morning grazing, a catheter was inserted into the rumen, and a vacuum sampler was used to collect rumen fluid samples. For each animal, 30 mL of rumen fluid was collected, divided into three parts, placed in 10 mL polypropylene tubes, rapidly stored in liquid nitrogen, and brought back to the laboratory for storage in a refrigerator at -80 °C.

DNA extraction and sequencing

Microbial community DNA was extracted using the EZNA Stool DNA Kit (Omega Bio-Tek, Norcross, GA, USA) following the manufacturer's instructions. DNA was quantified with a Qubit Fluorometer using the Qubit dsDNA BR Assay kit (Invitrogen, USA), and the quality was checked by running an aliquot on 1% agarose gel. Variable regions V1–V9 of the bacterial 16S rRNA gene were amplified with degenerate PCR primers, 27F (5'-AGRGGTYGATYMTGGCTCAG-3') and 1492R (5'-RGYTACCTTGTTACGACTT-3')(Takahashi et al. 2014). The ITS2 of the internal transcribed spacer (ITS) region was amplified using degenerate PCR primers, ITS3 (5'-GCATCGATGAAGAACGCAGC-3') and its4 (5'-TCCTCCGCTTATTGATATGC-3')(Toju et al. 2012). Both forward and reverse primers were tagged with Illumina adapter, pad, and linker sequences. PCR enrichment was performed in a 50 µL reaction containing 30 ng template, fusion PCR primer, and PCR master mix. PCR cycling conditions were as follows: 94 °C for 3 min; 30 cycles of 94 °C for 30 s, 56 °C for 45 s, 72 °C for 45 s, and a final extension for 10 min at 72 °C for 10 min. PCR products were purified using AmpureXP beads and eluted in elution buffer. Libraries were qualified using the Agilent 2100 Bioanalyzer (Agilent, USA). Validated libraries were used for sequencing on the Illumina MiSeq platform (BGI, Shenzhen, China) following standard pipelines of Illumina, and generated 2 × 300 bp paired-end reads. Sample Numbers and links in the NCBI database:SRP260807 :PRJNA630991

(<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA630991>)

Sequence analyses

Raw data was filtered to eliminate adapter pollution and low-quality readings to obtain clean reads, with paired end reads with overlaps merged into tags. Subsequently, tags were clustered into operational taxonomic units (OTUs) at a 97% sequence similarity. Taxonomic ranks were assigned to OTU representative sequences using Ribosomal Database Project (RDP) Naive Bayesian Classifier v.2.2. Alpha diversity, beta diversity, and screening of different species were then analyzed based on OTU and taxonomic ranks.

Tags were clustered into OTUs using USEARCH (v7.0.1090) software. OTU representative sequences were taxonomically classified using Ribosomal Database Project (RDP) Classifier v.2.2, trained on the Greengene_2013_5_99 database, using 0.5% confidence values as the cutoff. Filtered tags were clustered

into operational taxonomic units (OTUs) at 97% similarity, and the OTU number per sample primarily represented sample diversity degree. Based on OTU abundance, the OTU of each group was listed, Venn diagrams were drawn using Venn diagram software R (v3.1.1), and common and specific OTU IDs were summarized. Based on the OTU abundance information, relative abundance of each OTU in each sample was calculated, and principal component analysis (PCA) of OTUs was performed with the relative abundance value. The software used in this step was package ade4 software R (v3.1.1). Good's coverage, Alpha diversities including Inverse Simpson and Shannon index, richness (observed number of OTUs) and evenness (Shannon evenness) were calculated using Mothur V.1.31.2. Beta diversity analysis was performed using QIIME (v1.80) software. There were differences in sequencing depth in different samples; thus, normalization was introduced, with sequences extracted randomly according to the minimum sequence number for all samples. Extracted sequences formed a new 'OTU table biom' file, and the beta diversity distance was calculated based on the 'OTU table biom' file. Kyoto Encyclopedia of Genes and Genomes (KEGG); Version: 2018.01.

Results

Rumen bacterial and fungal composition across different age groups

Sample bacteria 16S rRNA and fungal ITS amplicon were sequenced. Bacterial species were generated with 150240 mass readings, with an average of $12,520 \pm 5,108$ per sample, and fungal species with 702,674 mass readings, with an average of $58,556 \pm 4,650$ per sample. After tags were optimized, the minimum tag number all samples was selected and clustered into OTUs for species classification at 97% similarity. The abundance of each sample in each OTU was counted. OTUs abundance preliminarily indicated sample species richness. Bacteria in 18 samples produced a total of 1872 OTUs. Fungi from 18 samples produced a total of 2599 OTUs. To assess whether the sampling effort provided sufficient OTU coverage to accurately describe the bacterial and fungal composition of each group, sample- and individual-based rarefaction curves were generated for each group. From 5 days to 2 years old, with the increased age, bacteria abundance and evenness in the rumen increased significantly, reaching a significant level of difference. This was also apparent with the Shannon and Simpson diversity indices, which were significantly different between the groups ($P < 0.05$, Kruskal–Wallis test). Species richness of rumen fungi was different in different age groups, however the difference was not significant. There was no significant difference between the Shannon and Simpson diversity indices ($P > 0.05$). Overall, 19 phyla were detected in rumen bacterial samples. Among them, *Firmicutes* and *Bacteroidetes* were detected as dominant phyla regardless of age group (Figure 1(a)), but their ratio and composition among the groups varied considerably. *Proteobacteria* was found in samples taken from the 5-day-old group compared to other groups. *Fusobacteria* were found in calf groups but were more prominent in newborn calves. *Tenericutes* were more abundant in older animals. *Elusimicrobia* abundance increased with age. Four phyla were detected in rumen fungal samples. *Ascomycota* and *Basidiomycota* were the most abundant phyla of rumen fungi present in all age groups. *Zygomycota* appeared in the 5-day-old group, and almost

did not appear in the 45-day, 90-day and 180-day-old groups until it was rediscovered at 2 years of age. *Chytridiomycota* was found in all age groups, especially in the 2-year-old group. Unclassified phylum in fungus accounted for 23.9% of the 45-day-old group. *Ascomycota* proportion in the calf stage increased until after 2 years of age.

OTU diversity and similarity of rumen bacteria and fungi

PCA was used to construct 2-D graphs to summarize factors mainly responsible for the difference; similarity was high where two samples were closely located. Based on the OTU abundance information, relative abundance of each OTU in each sample was calculated, and the PCA of OTUs was carried out with relative abundance value. Community OTU comparisons were carried out using non-metric multi-dimensional scaling (NMDS; OTU X 97% identity, species level similarity) of each group using the Bray Curtis similarity metric, which revealed that samples clustered together according to their particular age group, suggesting that each group hosts ITs with distinct bacterial and fungal communities (Figure 2). Bacteria composition in 5-day-old group was significantly different, and bacteria composition in other age groups was highly similar (Figure 2 (a)). Fungal composition in the 45-day-old and 2-year-old group was significantly different, and other age groups were highly similar (Figure 2 (b)). Venn diagrams visually displayed the common and unique OTUs number in multiple samples/groups. Core microbiomes of different environments could be obtained if combined with OTUs representing species. Observed differences were between the 5-day-old group and other age groups and their low similarity. Additionally, only a few genera were shared between the bacterial community during primary stages of colonization and the community found in mature animals (Figure 3 (a)). In each age group, unique bacterial species number first decreased and then increased with age (Figure 3 (a)). Exclusive fungi number in the 5-day and 180-day-old groups was significantly lower than that in other age groups (Figure 3 (b)). The five age groups used in this study showed several unique OTUs (Figure 3 (b)). Fungal OTUs numbers varied among the five age groups and did not change with age.

Alpha-Diversity Measures

Alpha diversity was applied to analyze the complexity of species diversity for a sample through several indices, including observed species, Chao1, Ace, Shannon, and Simpson. Complexity of the sample is proportional to the first four values, whereas a negative correlation is indicated by the Simpson value. Rumen bacteria were more diverse and had greater evenness in the 2-year-old group than in ruminal bacteria in the 5-day, 45-day, 90-day, and 180-day-old groups, as indicated by Chao1, ACE, Shannon, and Simpson indices ($P < 0.05$) (Figure 4 (a)). Rumen bacterial richness and diversity of adult yaks in natural grazing conditions were higher than those in calf stages. There were significant differences in rumen fungi richness among different age groups, but no significant difference in diversity as indicated by Chao1, ACE ($P < 0.05$), and Shannon and Simpson indices ($P > 0.05$) (Figure 4 (b)). Yak rumen fungi abundance was affected by age. Additionally, pronounced differences in bacterial and fungal

composition among individual animals in a given age group under the same grazing conditions were evident. A large variation in the population of selected microbial communities among individual adult yaks was also documented.

Genus associated with age

In the case of bacteria, the five groups shared 23 genera that were present in all animals, but their levels were radically different between the 5-day-old and older groups (Figure 5 (a)). There are three unique genera in the 5-day-old group: *Bacteroides*, *Peptostreptococcus*, and *Veillonella*. We examined genera such as *Streptococcus*, *Prevotella*, *Selenomonas*, and *Fibrobacter*, which were substantially represented in one of the age groups but were at potentially low levels in all animals from the other groups; in the case of fungi, the five groups shared 32 genera that were present in all animals, but their levels were radically different between the 5-day-old and 2-year-old groups (Figure 5 (b)). We examined the genera exhibiting more changes between the 5-day-old and 2-year-old groups, considering only those that were present in all samples. We examined genera such as *Acrostalagmus*, *Cryptococcus*, *Mucor*, *Penicillium*, *Plenodomus*, and *Thelebolus*, which were substantially represented in one of the age groups but were at potentially low or undetectable levels in all animals from the other groups. The composition of both aerobic and anaerobic combinations was found to vary significantly from calf to adult.

Annotation of Gene Function

KEGG Orthology was used to study the function of rumen bacterial genes in yaks of different ages. A total of 150,240 genes were enriched into 31 KEGG pathways (KEGG Orthology Level 2). The top 10 KEGG pathways represented by these genes are related to molecular metabolism—Carbohydrate metabolism—Metabolism of cofactors and vitamins—Amino acid metabolism—Metabolism of terpenoids and polyketides—Metabolism of other amino acids—Replication and repair—Energy metabolism—Glycan biosynthesis and metabolism—Lipid metabolism—Translation—The proportion of genes in each pathway did not differ significantly among different age groups (Figure 6).

Discussion

Early intestinal flora colonization can be revealed by amplicon sequencing of microorganisms in different age groups of yaks. In this analysis, age, environment, and lactation factors contributed to persistent differences in community composition, suggesting the important role of preferential succession in shaping intestinal communities. The objective of this study was to evaluate the composition of ruminal bacteria and fungi in newborn yaks and to explore how the composition changes during normal development.

There were more than hundred shared species between the bacterial and fungal communities during the primary stages of colonization and the communities found in mature yaks (Figure 3). This could be

because in the first days of life, the rumen is active and is involved in the digestion of plant material. In the early stage, the intestinal flora of calves showed a changing pattern where the number of microorganisms with oxygen tolerance and flagella decreased gradually, while the number of microorganisms with slow growth and spore formation increased gradually. The diversity index and OTU number of bacterial communities in the samples of each age group increased with age, and the within-group similarity of bacterial communities also increased with age. The horizontal composition of the rumen fungal phylum in calves was the same as that in adult yaks, but the proportion was significantly different. This indicates that the rumen environment of grazing adult yaks, although more functional, is also a closed and independent ecosystem with more specific and more homogeneous bacterial and fungal communities, compared to primary communities with more heterogeneity among the younger age groups. The gradual increase in bacterial diversity in Holstein cattle and goats from birth to adulthood is associated with a gradual change in community diversity (Jami et al. 2013; Wang et al. 2016). Interestingly, similar studies have been reported in recent years on the gut microbes of human infants. It has been found that the intestinal flora of infants starts from the early colonizers who are variable and good at rapid proliferation, but the functional traits gradually converge and stabilize in the first year of life; the gut microbiota adapts to the anoxic environment in the intestine and spreads among individuals through spores, while the taxonomic composition of the flora continues to change (Guittar et al. 2019).

The dominant phyla of bacteria found in each age group were *Bacteroidetes* and *Firmicutes*, and the dominant phylum of fungi was *Ascomycota*. These phyla varied greatly in number and the number of genera that compose them. Compared with yaks in other age groups, *Proteobacteria* is more prominent in 5-day-old calves. *Chytridiomycota* is less abundant in the calf stage, mainly composed of *Piromyces*, but more prominent in adult yaks. In cows and goats, the gastric juices of these mature ruminants are dominated by *Bacteroides* or *Firmicutes* (Cunha et al. 2011; Jami and Mizrahi 2012). *Proteobacteria* accounted for 28.7% of the bacterial abundance in the 5-day-old group, which decreased to 2.6% after 2 years of age. In a previous study, *Proteobacteria* accounted for 70% of 2-day-old Holstein calves and declined with age (Rey et al. 2013). *Fusobacterium nucleatum* has been extensively researched in recent years and can promote the development of colorectal cancer, but at the same time, it is a symbiotic and opportunistic pathogen (Brennan and Garrett 2019). *Fusobacteria*, which account for 7.2% of bacterial abundance in the calf stage, were not found in the mature yak group. Yak rumen fungi have undergone a long period of natural selection and efficiently degrade forage grass to provide energy and nutrition for yaks. The composition of yak rumen fungi in adult yaks has a unique advantage because of the ratio of the fungi present and the ability to efficiently degrade lignocellulose. Our results show that fungal taxa commonly found in the rumen of the 2-year-old group were already established in the rumens of calves at 5 days, regardless of diet composition. With the change in age, the composition ratio of *Ascomycota*, *Basidiomycota*, and *Chytridiomycota* in the fungal phyla of grazing yaks changed gradually. *Ascomycota*, *Basidiomycota*, and *Chytridiomycota* changed from 51.9%, 40.1%, 5.1% to 48.7%, and 12.7% to 34.3%, respectively. The change in the ratio is beneficial for the degradation and utilization of plant fibers. Rumen anaerobic fungi are affected by the diet and age (Kumar et al. 2015). A recent study

showed the presence of a fungal community in the first meconium of a newborn, which becomes more complex with gestational age. These findings suggest that colonization by intestinal fungi may occur before birth (Willis et al. 2019). This study suggested that gut microbes were colonized before the fetus was born, and their formation was related to the microbial composition of the mother. After the calf is born, the fungal abundance in the gastrointestinal tract is greatly affected by breast milk and the environment. The diversity and richness of fungi found in breast milk are highly similar and are affected by geographical location, delivery mode, and interaction with bacteria (Boix-Amorós et al. 2019).

The diversity of yak species in each group changed significantly with age. The OTU-level richness indices ACE and Chao1, as well as measures of evenness, such as the Shannon and Simpson indices, differed between the calf stage and the 2-year-old group. Ruminal microbiota at the calf stage became less diverse and more uneven as a result, whereas the ruminal microbiota had a greater richness and evenness in the 2-year-old group. With the change in diet, rumen microbial richness and diversity of calves changed to a mature ruminant state (Meale et al. 2016). The animals in this study were grazed in the wild for a long time without artificial feeding. Newborn calves began to eat the plant fiber slowly at an early stage to promote the development of the rumen and affect microbial colonization at the early stage of rumen development.

The dominant genus of rumen bacteria found in all age groups was *Prevotella*, which varies in number and composition. Animals of all age groups were born in the wild, and newborn calves were in a grazing state. Calves were fed with plant fiber after 2 days of birth, so the proportion of *Prevotella* in each group was relatively close. In a study of intestinal microbes in children, the genus *Platella* dominated the *Bacteroidetes* in African children, accounting for 53% of the total intestinal bacteria. In European children, the genus *Platella* does not exist, whereas the genus *Bacteroidetes* is dominant. The differences in diet contributed to the result: the African diet consisted mainly of plant fiber, while the European diet was high in animal protein, sugar, starch, and fat, but low in fiber (Carlotta et al. 2010). Both bacteria and fungi differ in composition in all age groups and have unclassified genera to varying degrees. There was a significant increase in the number of important anaerobes in rumen samples collected from calves that were considered permanent species of the mature rumen microbial community. *Prevotella* is considered a major component of the genetic and metabolic diversity of rumen microbes (Purushe et al. 2010). *Ruminococcus* is higher in calves than in the 2-year-old group, and there are two main cellulose decomposition bacteria of this genus in the adult rumen: *Ruminococcus flavefaciens* and *R. Albus* (Flint and Bayer 2008). *Ruminococcus flavefaciens* was detected in all age groups. This is indirect evidence that 5-day-old calves feed on plant fibers. *Penicillium* increased 26-fold in 2-year yaks compared to 5-day yaks. *Penicillium* isolated from the gastric juice of cow rumen has a high capacity for cellulose degradation (Andriani et al. 2013).

Our findings led us to conclude that these yaks underwent a gradual process of change from 5 to 180 days after birth and that the bacterial and fungal community diversity in the rumen stabilized at 2 years of age. In addition, *Ruminococcus* was detected in the rumen of 5-day-old yaks, and a high percentage of *Penicillium* and other microbial species important for normal rumen function were detected in the adult

rumen, which increased as the yak matures. Future research will entail the use of trait-based method to explore the succession mechanism of the rumen microbial community and its influence on host immunity, to aid in better understanding the functional roles of the microbial community.

Conclusion

The rumen microbial diversity of yaks at 5 days of age was relatively low, but increased significantly with increased age, more especially the unclassified microbial community. Various factors contributed to growth period of calves compared to adult yolks, resulting in relative microflora instability. Although yaks in the 5-day-old and 180-day-old groups were grazed under similar conditions, significant differences were observed in their microbiota, indicative that rumen microbiota underwent developmental changes at 180 days of age, independent of the feeding method. There were core microorganism species and specific microorganisms in the calf rumen at each age stage, however there were significant changes in rumen microorganism abundance. Changes in diet and age-driven effects synergistically influenced temporal and spatial changes of rumen bacteria and fungi in grazing yaks. Early ingestion to roughage was beneficial in the establishment of fiber-degrading bacteria in calves.

Declarations

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

WDW, MHM, YSL made substantial contributions to the conception or design of the experiments. WDW, ZGR, DMY, SLY Performed the experiments. WDW, HSC analyzed the data. WDW, YSL wrote the paper. All authors read and approved the final manuscript and ensure that issues relating to the accuracy or completeness of any part of the work are properly investigated and resolved.

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Figures

Figure 1

Overall, 19 phyla were detected in rumen bacterial samples. Among them, *Firmicutes* and *Bacteroidetes* were detected as dominant phyla regardless of age group (Figure 1(a))

Figure 2

Bacteria composition in 5-day-old group was significantly different, and bacteria composition in other age groups was highly similar (Figure 2 (a)). Fungal composition in the 45-day-old and 2-year-old group was significantly different, and other age groups were highly similar (Figure 2 (b)).

Figure 3

only a few genera were shared between the bacterial community during primary stages of colonization and the community found in mature animals (Figure 3 (a)). In each age group, unique bacterial species number first decreased and then increased with age (Figure 3 (a)). Exclusive fungi number in the 5-day and 180-day-old groups was significantly lower than that in other age groups (Figure 3 (b)). The five age groups used in this study showed several unique OTUs (Figure 3 (b)). Fungal OTUs numbers varied among the five age groups and did not change with age.

Figure 4

Alpha diversity was applied to analyze the complexity of species diversity for a sample through several indices, including observed species, Chao1, Ace, Shannon, and Simpson. Complexity of the sample is proportional to the first four values, whereas a negative correlation is indicated by the Simpson value. Rumen bacteria were more diverse and had greater evenness in the 2-year-old group than in ruminal bacteria in the 5-day, 45-day, 90-day, and 180-day-old groups, as indicated by Chao1, ACE, Shannon, and Simpson indices ($P < 0.05$) (Figure 4 (a)). Rumen bacterial richness and diversity of adult yaks in natural grazing conditions were higher than those in calf stages. There were significant differences in rumen fungi richness among different age groups, but no significant difference in diversity as indicated by Chao1, ACE ($P < 0.05$), and Shannon and Simpson indices ($P > 0.05$) (Figure 4 (b)).

Figure 5

In the case of bacteria, the five groups shared 23 genera that were present in all animals, but their levels were radically different between the 5-day-old and older groups (Figure 5 (a)). There are three unique genera in the 5-day-old group: *Bacteroides*, *Peptostreptococcus*, and *Veillonella*. We examined genera such as *Streptococcus*, *Prevotella*, *Selenomonas*, and *Fibrobacter*, which were substantially represented in one of the age groups but were at potentially low levels in all animals from the other groups; in the case of fungi, the five groups shared 32 genera that were present in all animals, but their levels were radically different between the 5-day-old and 2-year-old groups (Figure 5 (b)).

Figure 6

KEGG Orthology was used to study the function of rumen bacterial genes in yaks of different ages. A total of 150,240 genes were enriched into 31 KEGG pathways (KEGG Orthology Level 2). The top 10 KEGG pathways represented by these genes are related to molecular metabolism—Carbohydrate metabolism—Metabolism of cofactors and vitamins—Amino acid metabolism—Metabolism of terpenoids and polyketides—Metabolism of other amino acids—Replication and repair—Energy metabolism—Glycan

biosynthesis and metabolism—Lipid metabolism—Translation—The proportion of genes in each pathway did not differ significantly among different age groups(Figure 6).