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Analysis of the intestinal microbial community in healthy and diarrheal perinatal yaks by high-throughput sequencing

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ABSTRACT

Diarrhea, the most common disease of perinatal yaks greatly affects the growth of animals. Changes in the number and structure of intestinal flora can cause the disorder of the intestinal environment leading to diarrhea. A study was conducted to investigate the impact of diarrhea on the number and structure of intestinal flora in perinatal yaks. Fecal samples were collected from healthy and diarrhea-affected perinatal yaks; and changes in number and structure of intestinal flora were compared by 16S rDNA V3-V4 region high-throughput sequencing. A total of 272071 optimized sequences were acquired from bacteria, which were identified from 9 phyla, 13 classes, 17 orders, 36 families and 72 genera. The number of bacterial species among diarrheac intestinal flora was lower than the healthy group, with no significant difference between two groups ($P > 0.05$); however, significant differences were observed at phylum, class, order, family and genus level between two groups ($P < 0.05$). This study has provided for the first time an insight of the changes occurring in intestinal flora in perinatal yaks at high altitudes of the world.

1. Introduction

Diarrhea in cattle is a serious disorder affecting its fertility, milk production and weight gain resulting into economic losses [1,2]. Particularly, young calves especially perinatal ones are strongly affected by this condition leading to death due to malnutrition and dehydration [3]. To prevent the diarrhea, many measures are adopted to improve the hygiene and feeding management systems in the cattle industry; however, it still occurs world widely [5].

Diarrhea is generally accepted as a complicated disorder as many factors are attributed to both infectious and noninfectious factors causing this condition [3]. Approximately thousands of species from different genera of complicated microorganisms compose of the microbial community [8]. More than 15000 kinds of bacteria for a weight equal to 1 kg represents the first protection system of the gastrointestinal (GI) apparatus [9]. Intestinal bacteria serve the important role in the synthesis and metabolism of certain nutrients, hormones and vitamins, clearance of drugs and toxic metabolites, and defense against pathogens [9,10]. Previous researches have demonstrated that the gut microbial population interacts with the host immune system promoting the normal development and maturation of immune cells to produce cytokines thus influencing the host neurophysiology [11–13].

The yaks (*Bos grunniens*), a long-haired bovine specie is an inhabitant of cold environment and high plateau (about 3000 m above sea level) throughout the Himalayan region [4,14–16]. Approximately 90% of the world yak population is distributed in Qinghai, Tibet, and Sichuan provinces of China [15]. The yak rearing-farms are the important sources of milk, meat, hide and dung thus contributing to the socio-economic development of the local community and nomads [15,16]. So, any disease threat to the

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yaks in such areas may result into the significant economic losses also making it the animal welfare problem [14,16].

The intestinal microbial community information of animals and humans can be analyzed scientifically and annotated completely due to the rapid development of high-throughput sequencing and bioinformatics. To-date, scarce information is available in yaks at such high altitudes. So a study was designed to perform high-throughput sequencing analysis of the intestinal microbial in healthy and diarrheal affected-perinatal yaks in Hongyuan area of Sichuan province of China for the first time. The perspective results may lay a solid foundation for comparing the core bacterial strain between healthy and diarrheal yaks.

2. Materials and methods

2.1. Ethics statement

All procedures were performed under the instructions and approval of Laboratory Animals Research Centre of Hubei province in P. R. China and the Ethics Committee of Huazhong Agricultural University (Permit number: 4200695757).

2.2. Samples collection

Intestinal contents were collected from 4 healthy male perinatal yaks (N1-N4) and 3 diarrheal male perinatal yaks (D1-D3) from a livestock breeding station in Hongyuan, Sichuan of China. The selected yaks were of the same age (approximately 1 month) with similar characteristics. Before collection, the anal area of each perinatal yak was washed carefully with clean water and 70% alcohol solution. After collection, the intestinal contents were washed and centrifuged with phosphate buffer saline (PBS) and stored at −80 °C till further analysis.

2.3. DNA isolation

The gNDA of each sample was isolated by utilizing QIAam DNA Mini Kit (QIAGEN, Hilden, Germany) according to manufacturer's recommendations. Prior to the isolation protocol, each sample was preprocessed: the PBS-washed intestinal contents were centrifuged at 500 rpm for 4 min, the supernatant was separated and the sediments were rewashed twice. All of the collected supernatant was centrifuged at 13000 rpm for 5 min. Then the supernatant was discarded and the sediments were rewashed twice and re-suspended with 30 mL PBS. This procedure was repeated again and the final sediments were used for DNA isolation. The impurities which may hamper the PCR procedure were removed by utilizing AMPure. The integrities of gDNA were tested by agarose gel electrophoresis.

2.4. 16S rRNA gene amplification

The 16*S* rRNA gene with V3-V4 variable regions of PCR primers (F: CCTACGGGNGGCWGCA and R: GACTACHVGGGTATCTAATCC) with barcode on the forward primer were employed. The PCR mixture contained 33.8ul autoclaved distilled water, 5ul PCR Buffer (10×), 5ul dNTPs (2.5 mM), 1ul DNA, 0.2ul Taq E, 1ul of forward and reverse primer (working concentration: 10 m mol/L) in a 50ul reaction volume. Each of the 25 PCR cycles were set as: 94 °C for 20s, 55 °C for 30s, and 72 °C for 1 min after an initial hot start at 94 °C for 2 min and ending with 72 °C for 5 min. PCR products were analyzed on a 2% agarose gel stained with ethidium bromide.

2.5. Library preparation and sequencing

Sequencing libraries were generated using NEB Next Ultra DNA Library prep Kit for Illumina (NEB, USA) following the manufacturer's instructions, and index codes were added. The library quality was assessed on the Qubit 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. Then each of the libraries was performed with high-throughput sequencing on an Illumina MiSeq platform; and the paired-end reads were generated. The barcodes and primers were trimmed from the sequences and the short sequences <200bp were removed from the raw data. The sequences containing 6bp and bigger homopolymer regions and ambiguous base calls were removed. Sequences were then denoised and chimeras were removed. Operational taxonomic units (OTUs) were identified after removal of sequences clustering at 3% divergence (97% similarity). OTUs were then taxonomically grouped and classified using BLASTn tool against a curated GreenGenes database [17] and compiled into each taxonomic level into both “counts” and “percentage” files. Counts files contain the actual number of sequences while the percent files contain the relative (proportion) percentage of sequences within each sample that map the designated taxonomic classification. In order to compute alpha diversity, the OTU table was verified and calculated the three metrics: Chao1 estimated the species abundance; Observed Species estimated the amount of unique OTUs found in each sample and Shannon index. Rarefaction curves were generated based on these three metrics.

2.6. Bioinformatics and statistical data analyses

Relative abundance of identified organisms was normalized prior to analysis based on maximum read counts per sample. The organisms with low relative frequencies (<0.1%) were filtered. The remaining 100 different types (at the genus level) were used for group comparison analysis. All analyses used the RStudio-software package (v.0.98.1091). Significance of differences in the relative abundances between experiment and control groups were assessed by ANOVA (analysis of variance) method. Group based comparisons between the experiment and control microbial groups with a confidence interval 95% were carried out using Tukey's Test using the “TukeyHSD” function of the R package “stats”. Hierarchical clustering based on the R package “cluster” was generated using average linkage. Heatmaps used the heatmap.2 function based on R package “gplots” (see Fig. 1).

3. Results

3.1. The diversity of the intestinal microbial community between healthy and diarrheal perinatal yaks

In current study, the gDNA was purified and the fragments of 16S rDNA V3-V4 were amplified successfully (Figs. 2 and 3). Illumina MiSeq sequencing platform has been adopted to sequence the V3-V4 hypervariable region of 16S rRNA for the microbial community in samples. The sequencing data was filtered to get the valid data of 272, 071 tags; the mean length were approximately 449.2 bp tags (Table 1; Fig. 4). The effective tags of all samples were clustered and those sequences with over 97% similarity are considered as one OTU. On average, over 2000 OTUs have been detected from every sample (Table 2). The VENN of samples OUT distribution showed that there were 176 and 281 bacterial species were found in diarrheal and healthy groups respectively; while a total 136 bacterial species were observed in both groups (Fig. 5). Chao1 index was 281 in diarrheal group and 870 in healthy ones (Table 3). The intestinal microbial community diversity of the

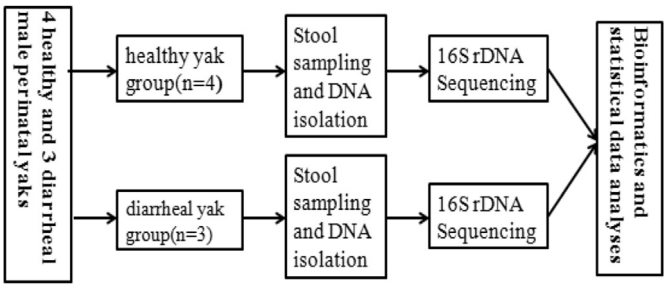

Fig. 1. Flow diagram of the study design.

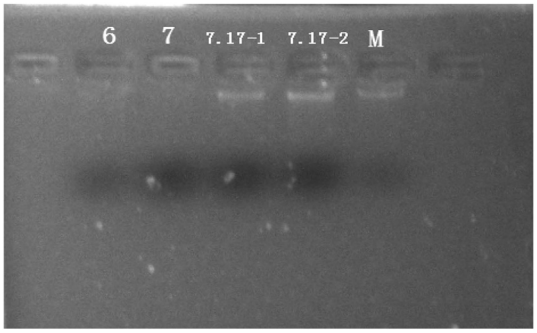

Fig. 2. gDNA purification on 2 agarose gel.

diarrheal and healthy groups was evaluated with the Shannon index: 2.8933 and 3.9475 respectively and Simpson index: 0.1152 and 0.0525 respectively (Table 3). No significant differences of the Shannon and Simpson indices were found between the two groups by Mann-Whitney *U* test ($P > 0.0$). Moreover, Shannon index got saturated but the rarefaction curve of every sample could not enter the plateau phase (Fig. 6). From the rarefaction curve at 97% similarity level of index of different samples (Fig. 7) and the rank-abundance curve of different samples (Fig. 8), it was observed that the sample numbers, abundance and evenness of the intestinal microbial species had met the sequencing and analysis requirements.

3.2. Composition of the intestinal microbial community of the healthy and diarrheal perinatal yaks

At the phylum level, 9 of the bacterial phyla were identified in both groups (Fig. 9). The main bacterial phyla in diarrheal group were Bacteroidetes, Firmicutes, Fusobacteria and Proteobacteria

Table 1
The statistics of optimized sequence after removed of barcodes, primers, short sequences <200bp, bigger homopolymer regions and ambiguous base calls.

Effective sequence	Samples number	Total sequences	Total bases	Mean length (bp)
16S	7	272071	122223291	449.2

accounting for 99.21% of the microbial population (Fig. 9). Among those, Firmicutes and Bacteroidetes were the main phyla in healthy group accounting for 94.22% (Fig. 9). Through comparison of diarrheal and healthy groups, Archaea and Candidatus Sacchari bacteria were found of significant differences, respectively ($p < 0.05$), while Bacteroidetes and Firmicutes were found at extremely significant differences level, respectively ($p < 0.01$). At class level, a total 13 classes were found in two groups with 11 and 10 classes in diarrheal and healthy group, respectively (Fig. 10). The intestinal microbial community of diarrheal group was mainly composed of Bacteroidia; Clostridia; Fusobacteriia, accounting for 88.73%, while Clostridia and Bacilli had made up of the main intestinal microbial community of health group, accounting for 74.34% (Fig. 10). Differentiation analysis of diarrheal and healthy groups have revealed that Gammaproteobacteria and Verrucomicrobiae were at significant differences in both groups respectively ($p < 0.05$); Bacteroidia and Clostridia were also highly significant ($p < 0.01$) (Fig. 10). At order level, a total 17 orders were found in both groups; and 13 orders in both diarrheal and healthy groups (Fig. 11) with Bacteroidales (40.62%), Clostridiales (30.76%), Fusobacteriales (17.25%) and Enterobacteriales (4.63%) in diarrheal group accounting for 93.26%, while the health group was mainly consisted of Clostridiales (69.03%), Bacteroidales (17.29%) and Lactobacillales (5.20%) accounting for 91.52% (Fig. 11). Enterobacteriales; Bacillales and Verrucomicrobiales were found of significant differences in both groups ($p < 0.05$) with Bacteroidales and Clostridiales at higher significantly different levels ($p < 0.01$). At family level, a total 36 families were found in both groups, and there were 24 and 31 families in the diarrheal and healthy groups (Fig. 12). The intestinal microbial community of diarrheal group was mainly composed of Bacteroidaceae (27.38%), Fusobacteriaceae (17.25%), Ruminococcaceae (14.97%), Lachnospiraceae (12.68%) and Prevotellaceae (10.75%) accounting for 83.03%. While Ruminococcaceae (45.83%), Lachnospiraceae (7.34%), unclassified "Bacteroidales" (7.79%) and Peptostreptococcaceae (6.14%) were consisted of the intestinal microbial community of healthy group accounting for 67.1%. Among those, Bacteroidales_incertae_sedis; Bacteroidaceae; Enterobacteriaceae; Ruminococcaceae and Verrucomicrobiaceae were found at significantly different level between both groups ($p < 0.05$). At genera levels, a total 72 genera were found in two groups; 53 in diarrheal group and 59 in the healthy one (Fig. 13). The main genera

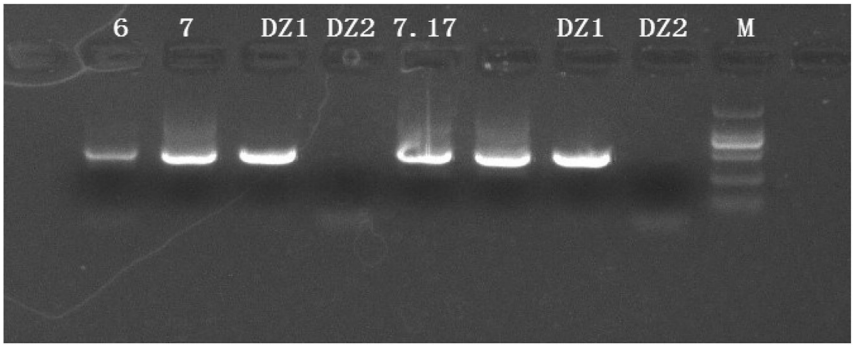

Fig. 3. PCR amplification in 16SrDNA V3-V4 variable region on 2 agarose gel. (positive control: DZ1; negative control: DZ2) Marker: 2000 1000 750 500 250 100 bp DNA ladder.

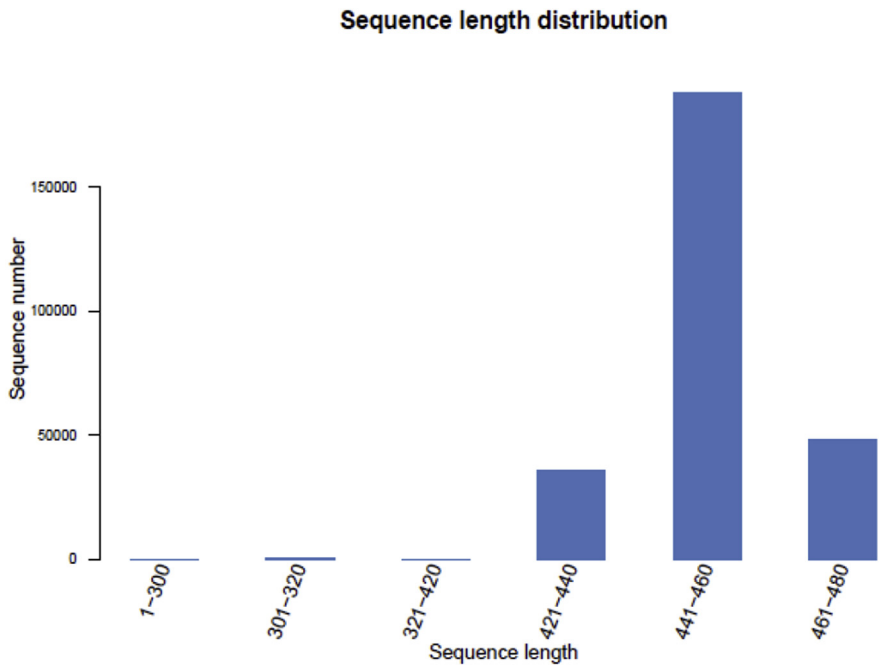

Fig. 4. The distribution length of optimized sequence.

Table 2
The quantity of OTU in different parts of samples.

OTU name	D1	D2	D3	…	OTUsize
OTU1	2313	3	0	…	2462
OTU2	2382	0	1157	…	3558
OTU3	2205	133	225	…	2589
OTU4	1792	8	20	…	2123

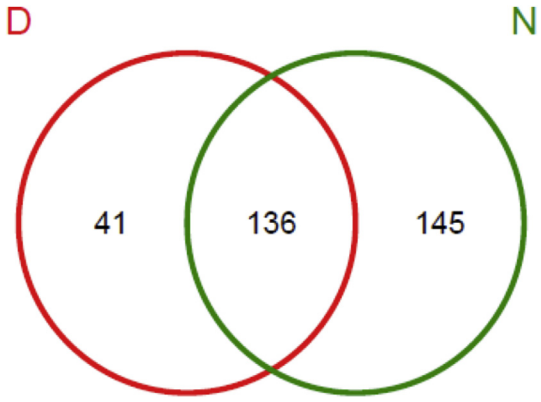

Fig. 5. VENN of the comparison of samples OUT distribution. (D: diarrheal group; N: healthy group).

in diarrheal group were Bacteroides (27.37%), unclassified "Fusobacteriaceae" (17.25%), Blautia (7.65%), unclassified "Prevotellaceae" (6.62%), Faecalibacterium (6.40%) and Escherichia/Shigella (4.63%) accounting for 69.92%. In healthy group, the unclassified Ruminococcaceae (29.00%), Clostridium XI (11.08%) and Acetivibrio (6.14%) were observed accounting for 46.22% (Fig. 13). Bacteroides, Clostridium IV, Escherichia/Shigella, Faecalibacterium, Flavonifractor, Mogibacterium, Olsenella, Phocaeicola, Pseudoflavonifractor, Saccharibacteria genera incertae sedis, Sporobacter; Turicibacter; unclassified Rikenellaceae, unclassified Coriobacteriaceae and unclassified Ruminococcaceae were found at significantly different levels in both groups ($p < 0.05$).

4. Discussion

Previously, many studies have been conducted to investigate the intestinal bacterial community in a wide range of vertebrates (humans, zebrafish, and rainbow trout) and invertebrates (drosophila, butterfly, black tiger shrimp and white shrimp) [10,18–24]. However, till now little is known about this community in yaks, an economically important bovine species of the high altitude and remote plateau. In this study, we documented the changes in the intestinal bacterial community of healthy and diarrheal perinatal yaks through high-throughput sequencing.

The richness and diversity of intestinal bacterial species play important roles in maintaining the intestinal ecological balance [8]. In present study, the samples OTU distribution showed 176 bacterial species in diarrheal group and 281 in healthy group; however, a total 136 bacterial species were found in both of the two groups by VENN (Fig. 5) demonstrating the bacterial species of diarrheal yaks

Table 3
The 97% similarity level of diversity index of the two groups sample sequences.

Group	Reads	OTUs	Coverage	ACE	Chao1	Shannon	Simpson
diarrheal group	55318	257	0.998498	283	281	2.8933	0.1152
healthy group	100331	838	0.999414	877	870	3.9475	0.0525

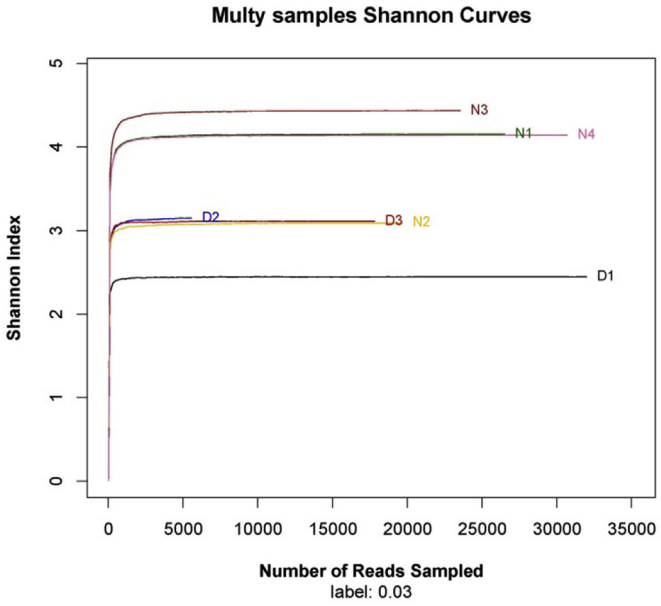

Fig. 6. The diversity index curve of Shannon-Wiener of different samples (D: diarrheal group; N: healthy group).

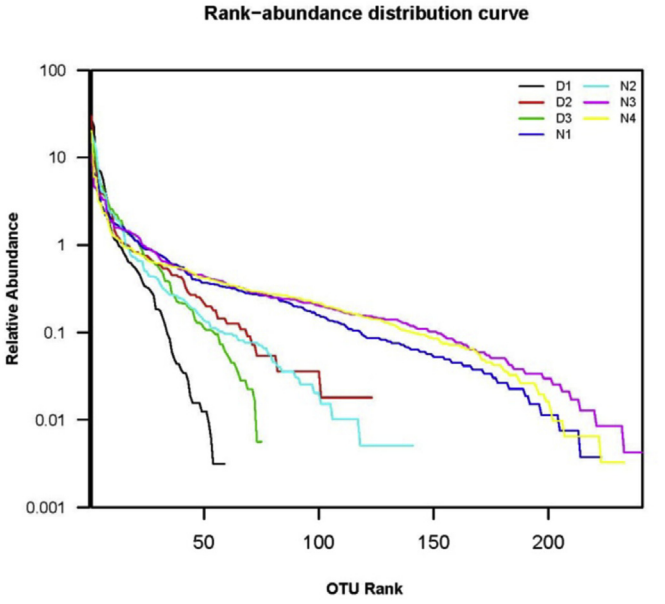

Fig. 8. The Rank-abundance curve of different samples (D: diarrheal group; N: healthy group).

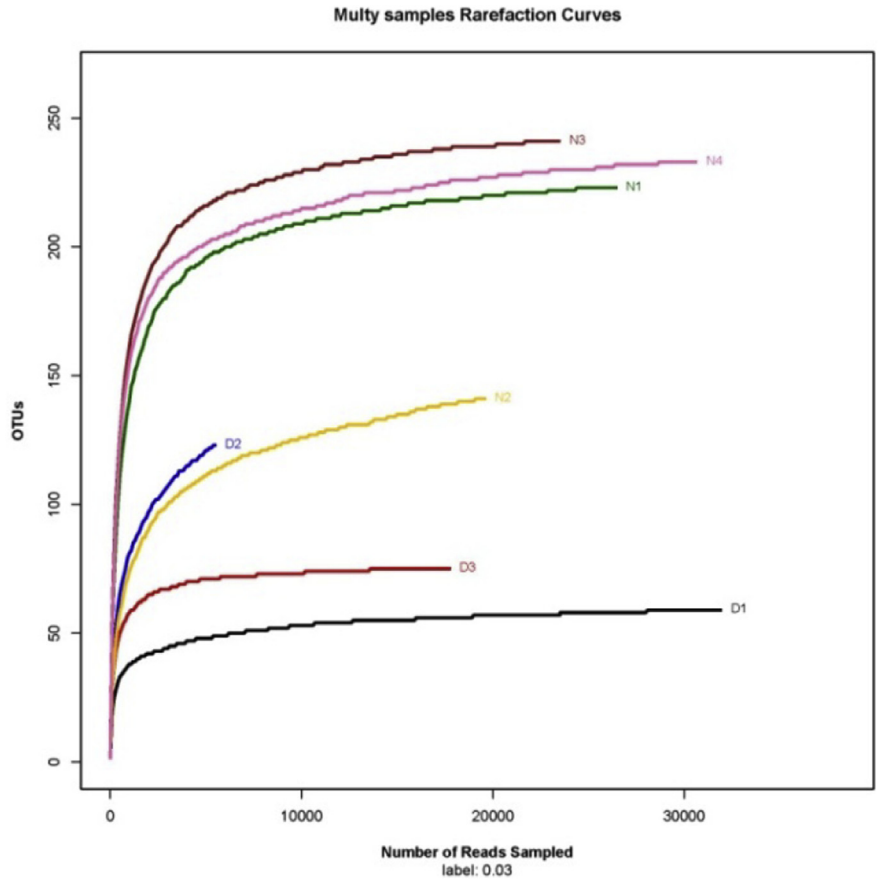

Fig. 7. The Rarefaction curve at 97% similarity level of index of different samples (D: diarrheal group; N: healthy group).

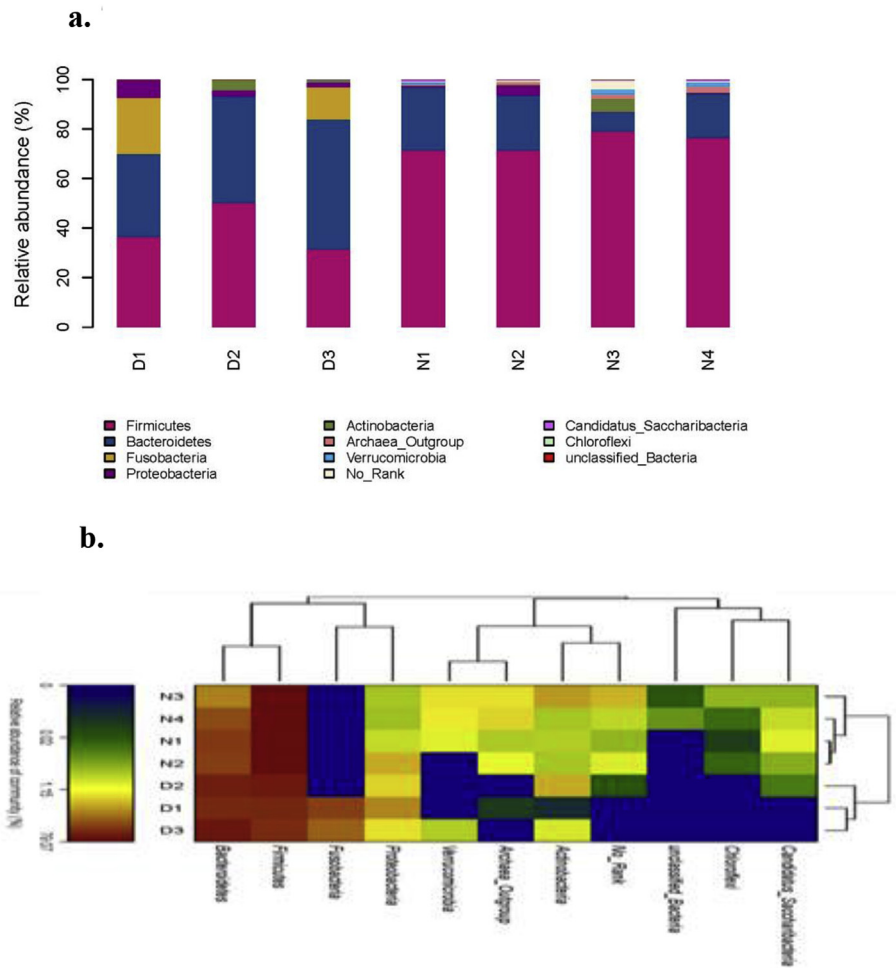

Fig. 9. (a) The community structure map of samples at the Phylum level in two groups (D: diarrheal group; N: healthy group). (b) The community structure heatmap of samples at the Phylum level in two groups (D: diarrheal group; N: healthy group).

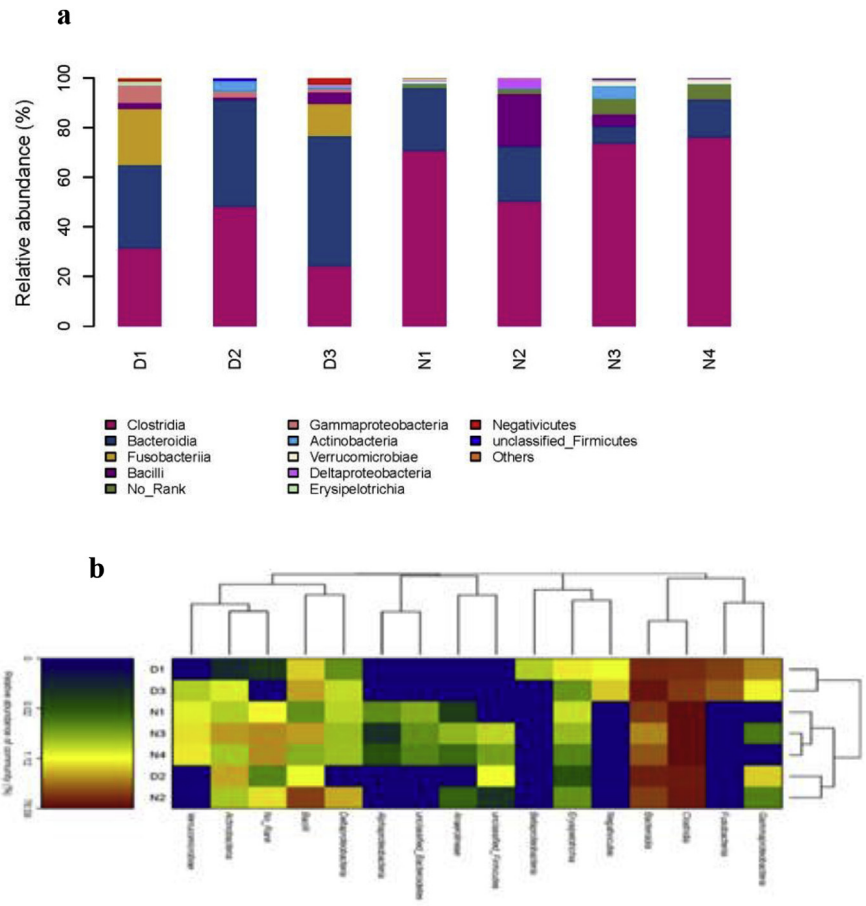

Fig. 10. (a) The community structure map of samples at the Class level in two groups (D: diarrheal group; N: healthy group). (b) The community structure heatmap of samples at the Class level in two groups (D: diarrheal group; N: healthy group).

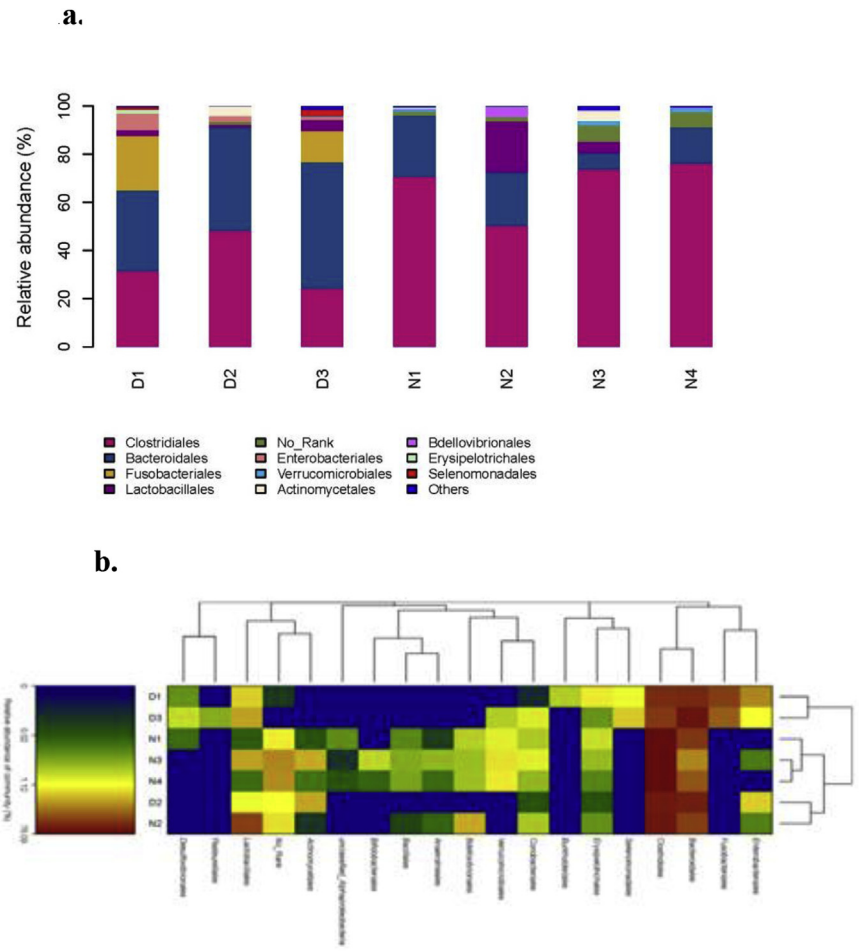

Fig. 11. (a) The community structure map of samples at the Order level in two groups (D: diarrheal group; N: healthy group). (b) The community structure heatmap of samples at the Order level in two groups (D: diarrheal group; N: healthy group).

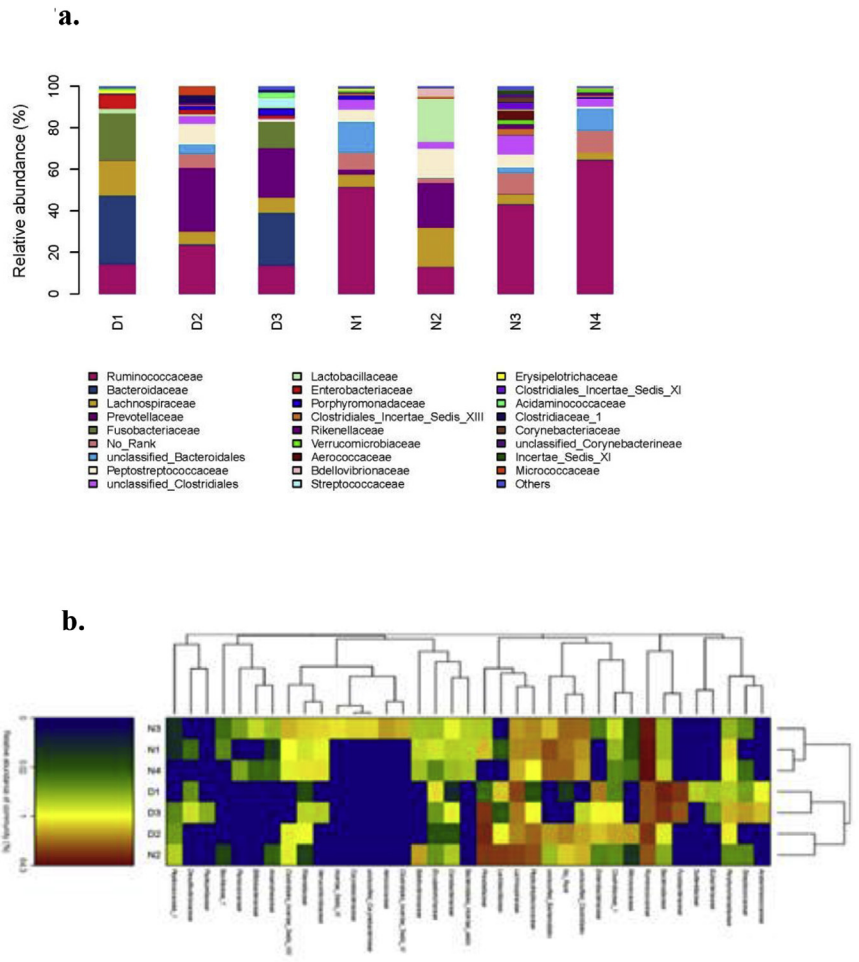

Fig. 12. (a) The community structure map of samples at the Family level in two groups (D: diarrheal group; N: healthy group). (b) The community structure heatmap of samples at the Family level in two groups (D: diarrheal group; N: healthy group).

more obviously less than that of healthy yaks. Chao1 index was 281 in diarrheal group and 870 healthy group, respectively (Table 3). The intestinal microbial community diversity of the two groups was evaluated with the Shannon index (2.8933 and 3.9475) and Simpson index (0.1152 and 0.0525) (Table 3); no significant difference of both indices were found between two groups by Mann-Whitney *U* test ($P > 0.0$) which concluded that the species richness and diversity of diarrheal yaks and healthy yaks were nearly the same. These results are in accordance with the study between juvenile idiopathic arthritis patients and control children [15]; however, not in line with the previously reported studies describing the diversity in IBD patients' intestinal microorganism which was much lower than that of healthy people [25]. The species richness and diversity of long-living elderly people are usually higher than that of healthy people with the average age of 50 [8].

An ecosystem with rich species can maintain the system stability and balance due to its stronger anti-disturbing ability against external environment [26]. In our study, the results indicating the rich diversity of the intestinal flora of both groups might not be one reasonable explanation for diarrhea; since the composition of microorganisms in two groups were found apparently different. As mentioned above, the significant differences ($p < 0.05$) at all levels; from phyla to species, demonstrate the composition of bacterial percentage to be the main factor causing the diarrhea in yaks.

Bacteroides, as a dominant bacterial genus in the intestinal microbial community of diarrheal yaks can absorb nutrition, produce short-chain fatty acid [27] restore & promote the maturation of epithelial cells and are related to the metabolism of fat. Faecalibacterium can fight against inflammation [28], generate butyrate acid, and protect the intestinal mucosa [29]. In our study, it can be hypothesized that the yaks were under infection with inflammation mainly due to these two genera which are negatively correlated and in accordance with previous studies of IBS and IBD [25,28]. As *Escherichia coli* and *Salmonella sp*. are widely recognized as the infectious pathogens of diarrhea [6,7]; so *E. coli* has been observed as high as 4.63% in the diarrheal yaks.

The phylum Fusobacteria is commonly present in the human and animal gastrointestinal tract and is usually associated with the mucous membrane. The rectal mucosa of patients with colon carcinoma is also enriched with the bacteria [30,31]. In current study, the Fusobacteria was found in 17.25% of cases as in the study of Kostic et al. (2012) [32].

The Prevotellaceae bacteria are frequently found in subgingival plaque from patients of periodontal diseases and acute necrotizing ulcerative gingivitis. The association of periodontitis with systemic diseases; cardiovascular diseases and preterm birth has also been reported [33]. However, Prevotellaceae were found in 6.62% cases in the diarrheal yaks deducing that this genus had acted as a pathogen of negatively correlated in diarrheal yaks.

Ruminococcus can generate short-chain fatty acid which can enhance the protective functions of intestinal barrier and reduce the colonization of opportunistic pathogens in the intestines

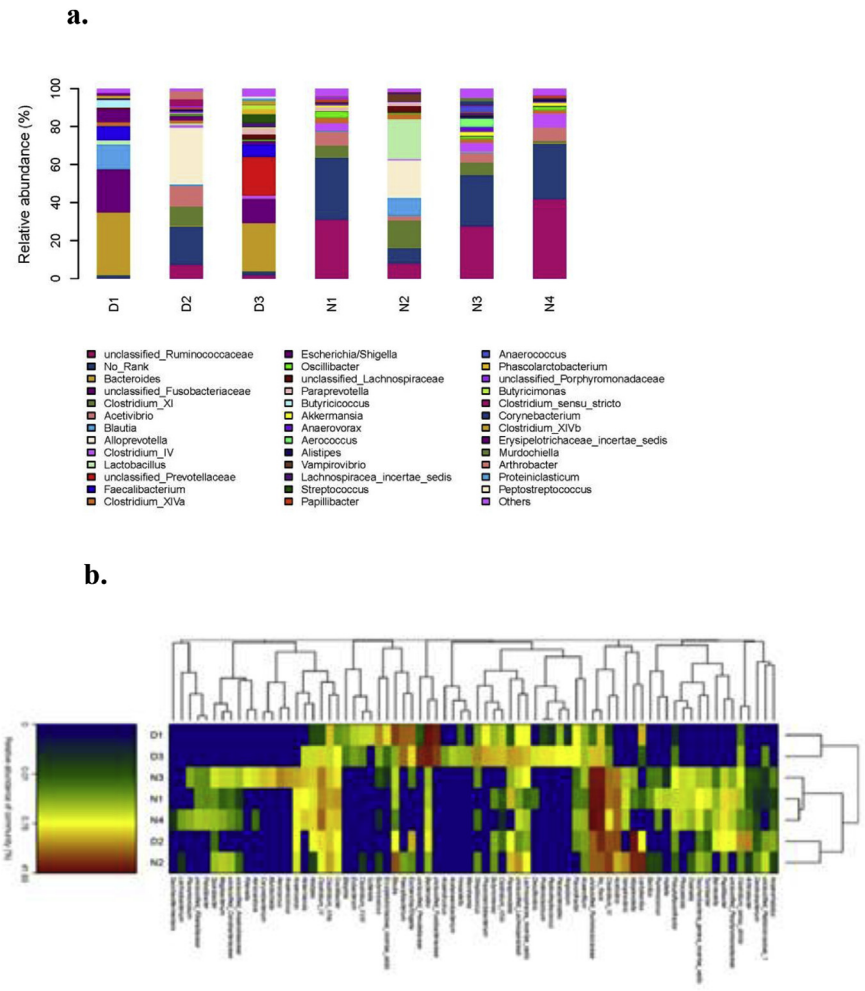

Fig. 13. (a) The community structure map of samples at the Genus level in two groups (D: diarrheal group; N: healthy group). (b) The community structure heatmap of samples at the Genus level in two groups (D: diarrheal group; N: healthy group).

[34,35]. The healthy yaks were abundant of unclassified-Ruminococcaceae (29.0%); however, it was seldom found in diarrheal yaks demonstrating its severe damage during diarrhea. Such type of situation also occurs in case of Clostridium and Acetivibrio species (11.08% & 6.14% respectively in healthy yaks); however, not found in diarrheac cases exhibiting its severe harm in intestinal tract.

In conclusion, this study has showed for the first time the changing pattern of different bacterial species in intestinal flora of healthy and diarrheal perinatal yaks which may lay a solid foundation for the pathogenic bacteria in this condition.

Conflict of interest

The authors declare that they have no competing interests.

Acknowledgements

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