Effects of Short Chain Fatty Acids (SCFAs) Modulation on Potentially Diarrhea Causing Pathogens in Yaks Through Metagenomics Sequencing

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Research

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Abstract

Yaks are of great importance on the plateau; however, an emerging endemic diarrheal disease during the last few years is posing a great threat to the health of these animals. Yaks have special gut microbiotal community and short-chain fatty acids (SCFAs) which are not only the principle nutrient substrates of intestinal epithelial cells but can also regulate the epithelial barrier. Until now, metagenomics sequencing has not been reported in diarrheal yaks. A scarce information is available regarding the levels of fecal SCFAs and diarrhea in yaks. The purpose of our study was to identify the potential pathogens that cause the emerging diarrhea and also to explore the potential relationship of short-chain fatty acids in this issue. We estimated diarrhea rate in yaks after collecting the equal number of fecal samples from affected animals. Metagenomics sequencing and quantitative analysis of SCFAs were performed which revealed 15-25% and 5-10% prevalence in diarrheal yak’s calves and adults yaks respectively. Significant difference was observed in GC contents (44.69%~46.08% vs 46.12%~46.38%) under two reference groups (p<0.05). Violin box plot also showed the higher degree of dispersion in gene abundance distribution of diarrhea yaks, while genes of normal yaks were relatively gathered. We found 366163 significant differential abundance genes in diarrheal yaks, with 141305 up-regulated and 224858 down-regulated genes as compared with normal yaks via DESeq analysis. Metagenomic binning analysis indicated the higher significant of bin 33 (Bacteroidales) (p<0.05) in diarrheal animals, while bin 10 (p<0.0001), bin 30 (Clostridiales) (p<0.05), bin 51 (Lactobacillales) (p<0.05), bin 8 (Lachnospiraceae) (p<0.05) and bin 47 (Bacteria) (p<0.05) were obviously higher in normal animals. At different levels, an obviously difference in Phylum, Class, Oder, Family, Genus and Species was noticed as 4, 8, 8, 16, 17 and 30 respectively. Compared with healthy yaks, Acetic acid (p<0.01), Propionic acid (p<0.01), Butyric acid (p<0.01), Isobutyric acid, Isovaleric acid (p<0.05) and Caproic acid (p<0.01) were all observed obviously at lower rate in diarrheal yaks. In conclusion, besides the increased pathogens level of Staphylococcus aureus, Babesia ovata, Anaplasma phagocytophilum, Bacteroides fluxus, viruses, Klebsiella pneumonia, and inflammation-related bacteria; the decreased of SCFAs caused by the imbalance of intestinal microbiota may potentially leads to emergence of diarrhea in yaks.

Introduction

The long-haired bovine specie i.e. yak is a well-known animal, which is an indispensable economic pillar on the Qing-Tibetan plateau [1]. There are approximately 15 million yaks in China accounting for over 90% of the world’s yak population [2, 3]. At the average of 3000~5000 m above the sea level, yak is depicted as a symbolic animal with the dependency of herdsmen’s lives [1]. Yaks serve as transportations, especially in the zigzag mountainy areas [4]. Meat, butter and milk from yaks are also considered as an essential food items for local Tibetans [3]. Its hide is used for making boots, rafts, aprons, leather bags and leather harness [2]. While the long hairs and dungs are commonly used for livelihood purpose [4].

Hongyuan is located in the eastern of Qinghai-Tibetan plateau and northwestern of Sichuan province, with the northern latitude of 31°51′-33°33′ and eastern longitude of 101°51′-103°22′. In this continental plateau the temperature is ranged from –22.8°C to 24.6°C with the average temperate of 2.9°C and 860.8 mm precipitation annually. In this region animal husbandry is the primary industry with 75383 yak’s population according to latest reports. About 7765 Kg of yak’s meat has also been produced annually accounting for 96.23%. However,
an emerging endemic diarrheal disease in yaks during the last few years (usually from May to August) not only has caused deaths to animals but also caused a huge constraint on the development of local economy.

Bovine diarrhea is a common disease throughout the farms with world-wide distribution. It has been causing a heavy economic loss in concern with fertility rate, milk production, and animals growth [5]. In yaks, diarrhea causing pathogens i.e. *Cryptosporidium parvum*, bovine viral diarrhea (BVD) virus, *Escherichia coli* have been reported continuously [6–8]. Although, many measures have employed to improve the hygiene and feeding management with the utilization of extensive drugs, but problem is still at peak [9].

The well-known complex intestinal tract is colonized by a large and diverse type of microbial microbiota [10]. This community produces extensive amount of metabolic products in intestine, which interact intimately with host cells to maintain physiological processes and functions i.e. nutrition absorption, host metabolism, and immunity [10–12]. Mainly, microbiota benefits the host through intestinal epithelium and by producing beneficial metabolites, that helps in food digestion and also against the pathogenic invasion. Gut microbiome has ability to convert fermentable dietary fibers into short-chain fatty acids (SCFAs) that provides additional energy to the host [13]. These SCFAs are organic carboxylic acids with less than 6 carbon atoms of acetate; among them propionate and butyrate are the most abundant protraction in intestine [14]. A previous study reported that diets containing alfalfa meal and commodity concentrated fiber could drop diarrhea rate via metabolic interactions between hindgut microbiota and SCFAs in piglets [15]. These SCFAs are also known for acting as ligands for G-protein coupled receptors by activating anti-inflammatory signaling [16]. Metagenomics sequencing is commonly utilized in microbial organisms, as it provides accurate classification of microbiota species and annotation to the bacteria at functional level rather than functional prediction [17–19].

It is generally accepted that microbiota composition and function contribute to the health status of the host [20]. Previously, dysfunctional gut microbiota was reported to be related with diseases like human inflammatory bowel disease, diabetes and cardiovascular disease [12, 21]. The imbalance of such intestinal microbiota may cause diarrhea due to the growing conditional pathogens, mucosal barrier damage, immunity dropping and intestinal permeability [20]. However, it is still unclear how the changed microbiota can cause the emerging diarrheal disease in yaks. Hence, this study was carried out to explore such potential pathogens and short chain fatty acid changes that cause emerging diarrheal disease in yaks.

**Results**
Table 1

Estimation of the prevalence of diarrhea in yaks in Hongyuan on the plateau.

| Farms | No. of yak calves (%) | No. of adult yaks (%) | Total No. of yak (%) |
|-------|-----------------------|-----------------------|----------------------|
| 1     | 20–25 (10–20%)        | 40–50 (2–8%)          | 60–75 (5–20%)        |
| 2     | 80–100 (25–30%)       | 220–250 (5–18%)       | 300–350 (10–20%)     |
| 3     | 30–40 (5–8%)          | 20–25 (0)             | 50–65 (2–10%)        |
| 4     | 25–30 (2–4%)          | 40–50 (0)             | 65–80 (0–4%)         |
| 5     | 150–200 (15–20%)      | 200–220 (5–10%)       | 350–420 (10–15%)     |
| 6     | 30–50 (10–15%)        | 30–40 (2–5%)          | 60–90 (5–10%)        |
| 7     | 20–30 (5–10%)         | 20–25 (2–4%)          | 40–55 (4–10%)        |
| 8     | 20–35 (10–15%)        | 30–35 (5–10%)         | 50–70 (8–12%)        |
| 9     | 60–70 (20–30%)        | 50–60 (5–10%)         | 110–130 (15–20%)     |
| 10    | 20–25 (10–15%)        | 30–40 (3–5%)          | 50–65 (8–10%)        |
| Total | 455–605 (15–25%)      | 680–795 (5–10%)       | 1135–1400 (10–15%)   |
Table 2
Parameters employed in GC-MS/MS.

| Procedure                  | Parameter                        |
|----------------------------|----------------------------------|
| Sample load                | 2 uL                             |
| Front Inlet Mode           | splitless                        |
| Carrier Gas                | Helium                           |
| Column                     | DB-FFAP (30 m x 0.25 mm x 0.25 µm) |
| Column Flow                | 1.2 min⁻¹                        |
| Oven Temperature Ramp      | 95 °C hold on 1 min, raised to 100 °C at a rate of 25 °C/min, raised to 130 °C at a rate of 17 °C/min, hold on 0.4 min, raised to 200 °C at a rate of 25 °C/min, hold on 0.5 min, after running for 3 min |
| Front Injection Temperature| 200 °C                           |
| Transfer Line Temperature  | 230 °C                           |
| Ion Source Temperature     | 230 °C                           |
| Quad Temperature           | 150 °C                           |

Table 3
Metagenomics binning analysis of yak intestines microbiota via Maxbin2 and Maxbat2

| Binner    | Bin   | Completeness (%) | Contamination (%) | GC   | lineage              | N50  | Size (bp)    |
|-----------|-------|------------------|-------------------|------|----------------------|------|--------------|
| Maxbat2   | bin.17| 99.31            | 1.284             | 0.348| Bacteria             | 54875| 2180769      |
|           | bin.34| 95.48            | 3.347             | 0.451| Selenomonadales      | 8117 | 1893381      |
|           | bin.64| 93.03            | 2.369             | 0.419| Clostridiales        | 11985| 1727329      |
|           | bin.2 | 95.04            | 1.436             | 0.384| Lactobacillus        | 18617| 1934486      |
|           | bin.41| 93.85            | 6.704             | 0.489| Bacteroidetes        | 11868| 1965257      |
|           | bin.58| 93.10            | 1.006             | 0.450| Clostridiales        | 21746| 1436082      |
| Maxbin2   | bin.44| 97.13            | 0.692             | 0.429| Clostridiales        | 18616| 1834347      |
|           | bin.38| 96.84            | 0.693             | 0.394| Selenomonadales      | 37174| 1818504      |
|           | bin.21| 96.81            | 2.259             | 0.318| Firmicutes           | 11181| 2115861      |
|           | bin.2 | 95.04            | 1.436             | 0.384| Lactobacillus        | 18617| 1934486      |
|           | bin.41| 93.85            | 6.704             | 0.489| Bacteroidetes        | 11868| 1965257      |
|           | bin.58| 93.10            | 1.006             | 0.450| Clostridiales        | 21746| 1436082      |
### Table 4
Equation of linear regressions detected by standard samples via GC-MS.

| SCFAs         | Equation                  | $R^2$  | Linearity range (mg/L) |
|---------------|---------------------------|--------|------------------------|
| Acetic acid   | $y = 10.001982 \cdot x - 3.631493$ | 0.998242 | 0.1–20                 |
| Propionic acid | $y = 4.991172 \cdot x - 1.707163$ | 0.995486 | 0.1–9.5               |
| Isobutyric acid | $y = 9.838497 \cdot x - 1.317041$ | 0.998669 | 0.1–8                  |
| Butyric acid  | $y = 69.122314 \cdot x - 21.545157$ | 0.997229 | 0.1–20                 |
| Isovaleric acid | $y = 72.284002 \cdot x - 14.156633$ | 0.998779 | 0.1–20                 |
| Valeric acid  | $y = 43.669973 \cdot x - 11.692827$ | 0.998961 | 0.1–20                 |
| Caproic acid  | $y = 34.683801 \cdot x - 7.396451$ | 0.994521 | 0.1–8                  |

#### Data deposition

The raw sequences data was deposited in BioSample database with the accession number: SAMN16091789-SAMN16091798.

### Prevalence of diarrhea in yaks

Diarrhea in yaks was found in all farms (10/10) especially in calves farm (100%), while 80% (8/10) of farms reported diarrhea in adult yaks. The overall prevalence of diarrhea was ranged from 0–4% to 15–20%. While in calves and adults prevalence was ranged 15–25% and 5–10% respectively (Table 1). Significant difference was observed in both upper limit prevalence ($p < 0.001$) and lower limit prevalence ($p < 0.01$) (Fig. 2).

### Sequencing data of yak microbiota samples and Gene abundance distribution

Overall, 445199120 total reads and 445089080 clean reads were obtained from diarrheal yaks; while 285976660 and 285951940 total and clean reads were obtained respectively in normal yaks. Moreover, 66295299314 and 4267183625 clean bases were found in diarrheal and normal yak groups respectively. The Q20 and Q30 in both groups was more than 97% and 92%, which confirmed the reliable and accurate base recognitions [22]. Though no significant difference ($p > 0.05$) was observed in total reads, clean reads, Q20, and Q30. However, obvious difference was found in GC content between diarrheal (44.69%–46.08%) and normal yaks (46.12%–46.38%) ($p < 0.05$) (Fig. 3). According to violin box plot, the degree of dispersion in gene abundance distribution was higher in diarrheal than normal yaks (Fig. 4).

### Species composition and abundance analysis

Annotated analysis was preformed via MetaPhlAn2 ([http://huttenhower.sph.harvard.edu/metaphlan2](http://huttenhower.sph.harvard.edu/metaphlan2), Version 2.0) by comparing with database. Results were showed through Krona [23] which revealed the abundance of Firmicutes and Proteobacteria in normal yaks. In comparison with normal, yaks with diarrhea were observed with a significant drop of Firmicutes ($p < 0.05$) (Fig. 5).
At Phylum level, Firmicutes and Bacteroidales were found primarily in both groups (Fig. 6a). Principal component analysis (PCA) found the left side location of D1, D2, D3, D4, D5, and D6 groups. While NA, NB, NC, and ND groups were found to be located on the right side in two-dimensional graphic representation. Samples in normal yaks were concentrically distributed as compared to the diarrheal yaks (Fig. 6b). Compared with normal animals, Bacteroidetes ($p < 0.01$) and Apicomplexa ($p < 0.05$) were significantly higher in diarrheal yaks, while Firmicutes ($p < 0.05$) and Euryarchaeota ($p < 0.001$) were obviously at lower levels (Fig. 6c).

At class level, Clostridia and Bacteroidia were observed mainly in normal yaks, while Bacteroidia, Clostridia and Bacilli were dominated in diarrheal animals (Fig. 7a). PCA showed that samples in diarrheal and normal yaks were sporadic and adjacent respectively (Fig. 7b). Compared with normal animals, Bacteroidetes ($P < 0.01$) and Aconoidasida ($p < 0.05$) were obviously higher in diarrheal yaks, while Clostridia ($p < 0.05$), Methanobacteria ($p < 0.001$), Flavobacteriia ($p < 0.001$), Deltaproteobacteria ($p < 0.001$), Alphaprotebacteria ($p < 0.01$), and Cytophagia ($p < 0.001$) were clearly lower in number (Fig. 7c).

At Order level, Clostridiales was higher in normal yaks. While Bacteroidales was higher in diarrheal animals (Fig. 8a). PCA showed that the samples in diarrheal animals were far from each other than normal animals (Fig. 8b). Compared with normal animals, Bacteroidales ($p < 0.01$) and Piroplasmida ($p < 0.05$) were significantly higher, while Clostridiales ($p < 0.05$), Methanobacteria ($p < 0.001$), Flavobacteriales ($p < 0.001$), Cytophagales ($p < 0.001$), Spirochaetales ($p < 0.001$), and Marinilabiliales ($p < 0.001$) were obviously lower in diarrheal yaks (Fig. 8c).

At Family level, Ruminococcaceae and Lachnospiraceae were found mainly in normal yaks, while Bacteroidaceae was found in diarrheal yaks with high abundance (Fig. 9a). PCA showed that samples in diarrheal animals were located far from each other than normal animals (Fig. 9b). Compared with normal animals, Bacteroidaceae ($p < 0.001$), Staphylococcaceae ($P < 0.05$) and Babesiidae ($p < 0.05$) were higher in diarrheal yaks, while Ruminococcaceae ($p < 0.001$), Eubacteriaceae ($p < 0.001$), Methanobacteriales ($p < 0.001$), Flavobacteriaceae ($p < 0.001$), Clostridiales Family XIII. Incertae Sedis ($p < 0.001$), Erysipelotrichaceae ($p < 0.05$) and Eggerthellaceae ($p < 0.001$) were remarkably lower (Fig. 9c).

At Genus level, Bacteroides and Clostridium were found higher in normal yaks, while Bacteroides was the main genus in diarrheal yaks (Fig. 10a). PCA indicated that samples in diarrheal animals located far than normal animals (Fig. 10b). Compared with normal animals, Bacteroides ($p < 0.001$), Staphylococcus ($p < 0.05$), Blautia ($p < 0.05$), Babesia ($p < 0.05$) and Butyricoccus ($p < 0.05$) were outstandingly higher in diarrheal yaks, while Clostridium ($p < 0.01$), Alistipes ($p < 0.001$), Ruminococcus ($p < 0.001$), Eubacterium ($p < 0.001$), Methanobrevibacter ($p < 0.001$), Oscillibacter ($p < 0.001$), Butyrivibrio ($p < 0.001$), Bacillus ($p < 0.05$), Paenibacillus ($p < 0.001$), Anaerotruncu ($p < 0.001$), Roseburia ($p < 0.05$), Treponema ($p < 0.01$) and Lachnoclostridium ($p < 0.05$) were lower (Fig. 10c).

At Species level, Firmicutes bacterium; CAG:110 and Clostridiales were found most abundantly in normal yaks, while Staphylococcus aureus was the main species in diarrheal yaks (Fig. 11a). PCA indicated that samples in diarrheal animals located separately as compared to normal animals (Fig. 11b). Compared with normal animals, Staphylococcus aureus ($p < 0.05$), Bacteroides coprophilus ($p < 0.01$), Bacteroides plebeius ($p < 0.01$),
Butyricicoccus pullicaecorum ($p < 0.01$), Babesia ovata ($p < 0.05$), Fusobacterium mortiferum ($p < 0.05$), [Ruminococcus] gnavus ($p < 0.01$), Anaplasma phagocytophilum ($p < 0.05$), Bacteroides fluxus ($p < 0.05$), Firmicutes bacterium CAG:424 ($p < 0.05$), Viruses ($p < 0.05$), Fournierella massiliensis ($p < 0.05$), Bacteroides vulgatus ($p < 0.05$) and Klebsiella pneumoniae ($p < 0.05$) were higher in diarrheal yaks, while Firmicutes bacterium CAG:110 ($p < 0.01$), Clostridiales bacterium ($p < 0.001$), Ruminococcaceae bacterium ($p < 0.001$), Clostridium sp. CAG:413 ($p < 0.001$), Clostridia bacterium ($p < 0.001$), Firmicutes bacterium CAG:137 ($p < 0.001$), Methanobrevibacter olleyae ($p < 0.001$), Bacteroidales bacterium WCE2008 ($p < 0.001$), Ruminococcus avefaciens ($p < 0.001$), Methanobrevibacter ruminantium ($p < 0.001$), Anaerotruncus sp. Cag:390 ($p < 0.001$), Clostridium sp. CAG:448 ($p < 0.001$), Firmicutes bacterium ($p < 0.01$), Methanobrevibacter olleyae ($p < 0.001$), Bacteroidetes bacterium ($p < 0.001$), Anaerotruncus sp. Cag:390 ($p < 0.001$), Clostridium sp. CAG:448 ($p < 0.001$), Firmicutes bacterium CAG:124 ($p < 0.001$) and Firmicutes bacterium CAG:170 ($p < 0.001$) were significantly lower in number (Fig. 11c). The statistics of compositional significant species in comparison with normal animals is shown in Fig. 12. Circus map indicated that the Phylum level of two groups is mainly consisted of Firmicutes, while obvious difference was found in case of Fusobacteria, Bacteroidetes and Proteobacteria in both groups. While difference was found in Bacteroidia, Bacilli and Gammaproteo at the species level (Fig. 13).

**Functional analysis of yak intestine microbiota**

Functional profile analysis was performed via annotating against the GO, KEGG, EggNOG and CAZy databases [24–27]. Annotated scores of one having HSP > 60 bits were selected for analyzing relative abundance at different functional levels [28–31]. In total, 354 990, 486 219, 778 943, 867 820, 366 984 and 188 719 non-redundant genes were found in GO, eggnog, KEGG, NR, swissport and CAZy, respectively. In KEGG, non-redundant genes were related to cellular community, energy metabolism and 40 more metabolic pathways in all yaks. Slightly lower of nervous system, development, and nucleotide metabolism was found in diarrheal yaks (Fig. 14a). In eggnOG, about 24 cell metabolic pathways i.e. wall biogenesis, chromatin structure and dynamics were reported in all animals. In secondary metabolites biosynthesis, signal transduction mechanisms were a bit higher in diarrhea animals, while translation, ribosomal structure and biogenesis were slightly higher in healthy yaks (Fig. 14b). In CAZy, carbohydrate-binding modules, glycosyl transferases, glycoside hydrolases, polysaccharide lyases, auxiliary activities and carbohydrate esterase were seen in both of the two groups. Moreover, glycoside hydrolases was found higher in normal yaks, while glycosyl transferases was higher in diarrhea yaks (Fig. 14c).

DESeq analysis was employed to uncover significant differential abundance genes between the two yak groups at Fold Change ≥ 2 and $p$-value < 0.01 [32]. Compared with normal yaks, there were 366163 obviously significant differential abundance genes in diarrheal yaks, with 141305 up-regulated and 224858 down-regulated (Fig. 15a). Differential abundant genes were compared against cluster of orthologous proteins database. Most of the genes were found related with amino acid metabolism, replication, recombination, cell wall biogenesis, carbohydrate transportation & metabolism, translation, ribosomal structure and biogenesis (Fig. 15b). Annotation of differential abundant genes in KEGG path showed the genes relationship with metabolism (Fig. 15c). Enrichment analysis of differential abundant genes in KEGG pathway revealed the top 24 obvious over-presentation genes. Enrichment Factor in the X-axis represented the significant enrichment level of differentially expressed abundant genes. Lipopolysaccharide biosynthesis gen was at the highest level of differentially expressed abundant genes (Fig. 15d). Most significantly different genes were related to ribosome ($p < 0.001$), peptidoglycan biosynthesis ($p < 0.001$), homologous recombination ($p < 0.001$).
**Binning analysis of the metagenome of intestinal microbiota in yaks**

Metagenomics binning analysis revealed 9 bins with genomic integrity > 50% and contamination rate < 10% in yaks (Table 1). The genomic integrity was 93.10–99.31% containing Bacteria, Selenomonadales, Clostridiales, Firmicutes, Lactobacillus and Bacteroidetes. Through heat map of 76 bins, bin 33 ($p < 0.05$) was significantly higher in diarrheal group, while bin 10 ($p < 0.0001$), bin 30 ($p < 0.05$), bin 51 ($p < 0.05$), bin 8 ($p < 0.05$) and bin 47 ($p < 0.05$) was obviously higher in normal group (Fig. 16a & b). The genomic of those bins were Bacteroidales (bin 33), Clostridiales (bin 30), Lactobacillales (bin 51), Lachnospiraceae (bin 8) and Bacteria (bin 47).

**Quantitative analysis of SCFAs in yaks**

Sample quality control analysis showed that the TIC from different samples nearly overlapped completely, which indicated that the current data were repeatable and reliable (Fig. 17). The TIC from yak mixture samples showed several single waves without overlapping that reveals valid results of SCFAs (Fig. 18). In present study, all the correlation coefficient ($R^2$) of each equation of linear regressions were over 0.994 $\approx$ 1.000, which ensured the accuracy of SCFAs values (Table 4).

A significant difference of the seven SCFAs except Valeric acid was found between normal and diarrheal yaks. Acetic acid, Propionic acid, Butyric acid, Isobutyric acid and Caproic acid were found in normal yaks which were obviously higher than diarrheal yaks ($p$-value < 0.01). It was also admirable that Isovaleric acid in normal yaks was also a slightly higher than diarrhea yaks ($p$-value < 0.05) (Fig. 19).

**Discussion**

As a commonly reported disease, cattle diarrhea causes considerable economic losses for cattle producers world-wide [33]. In Norway, about 10 million US dollars loss was noted due to calves death effected by diarrhea in 2006 [34]. As an agricultural country, the development of animal husbandry is important especially in Hongyuan (China) like plateau areas. In our study, the prevalence of diarrhea in yaks was estimated about 15–25% and 5–10% in yak calves and adults yaks respectively (Table 1). Diarrhea in yaks was significantly higher in yak calves (Fig. 2), which was in line with the widely accepted knowledge that morbidity and mortality of diarrhea in calves is more serious [35]. Therefore, discovering the potential causes of this emerging diarrhea is urgent and meaningful, especially on the remote plateau.

The intestinal microbiota is also considered as an additional organ, which comprises of billions of microorganisms. Intestinal microbiota is not only important in the synthesis and metabolism of nutrients, hormones, vitamins, but also plays role in drugs utilization, pathogen's fortification, and immune system maturation [13, 36, 37]. Therefore, the imbalance of intestinal microbiota may lead to serious disease. Previously, we performed high-throughput sequencing of intestinal microflora from diarrheal yaks, our study found 41 genera of bacteria in perinatal healthy yaks, while 145 genera of bacteria were only tested in healthy perinatal yaks [5]. Moreover, 212 genera of fungus were found in healthy yaks, 373 and 208 genera of fungus were found in calves and diarrheal yaks respectively [38]. However, 16 s RNA sequencing was limited to genus level. In the current study, metagenomics sequencing was employed to explore the potential pathogens of diarrhea in yaks. Obvious difference was found in GC content (44.69%–46.08% vs 46.12%–46.38%) of two yak
groups ($p < 0.05$) (Fig. 3). Violin box plot also showed the higher gene abundance in diarrhea yaks in concern with the degree of dispersion than normal yaks (Fig. 4). Such results may predict the different composition of microbiota in diarrhea and normal animals. In different levels, we found more significant lower species composition in diarrhea yaks (Fig. 12). Overall, 366163 obvious significant differential abundance genes with 141305 up-regulated and 224858 down-regulated genes was found in diarrheal yaks as compared with normal yaks via DESeq analysis (Fig. 15a). Metagenomics binning analysis with bin 33 (Bacteroidales) ($p < 0.05$) was significantly higher in diarrheal animals, while bin 10 ($p < 0.0001$), bin 30 (Clostridiales) ($p < 0.05$), bin 51 (Lactobacillales) ($p < 0.05$), bin 8 (Lachnospiraceae) ($p < 0.05$) and bin 47 (Bacteria) ($p < 0.05$) was obviously higher in normal animals (Fig. 16a & b).

*Staphylococcus aureus* is commonly known bacterium related to human and animal foodborne diseases [39]. This pathogen also causes orthopedic implant-associated infection, especially methicillin-resistant bacteria [40]. As infected animals are commonly treated with antimicrobial agents, thus the serious antimicrobial resistance is becoming a public concern world-widely [39]. Diseases such as gastroenteritis, nausea, vomiting, abdominal cramps and etc. are usually seen in infected individuals [41]. The increasing of *Staphylococcus aureus* in diarrheal yaks may indicate a potential threaten for local herdsmen. *Bacteroides coprophilus* was previously reported as pro-inflammatory in ankylosing spondylitis [42], which may infer with inflammatory status of diarrhea yaks. *Bacteroides plebeius* was previously found significantly higher in type 2 diabetes mellitus patients [43], which was also regarded as a biomarker of this disease. Thus the increase of *Bacteroides plebeius* in diarrhea animals means the abnormal glucose metabolism in yaks.

The butyrate-producing bacteria *Butyricicoccus pullicaecorum* is commonly linked with inflammatory conditions of intestinal ecosystem [44], which may cause inferred inflammatory response during diarrhea in yaks. Though *Babesia ovata* is a low pathogenic species, but its infection may lead to severe damages in cattle when co-infected with *Theileria orientalis* [45]. Previous study reported that the prevalence of *T. orientalis* in yaks was 9.7% on the plateau [46]. The infection of *T. orientalis* may be the main reason for bloody diarrhea in yaks (Fig. 1). *Fusobacterium mortiferum* was usually isolated from Crohn's and Behcet's disease patients [47], also *Ruminococcus gnavus* is a Crohn's disease-associated pathobiont [48], which was in line with the diarrhea symptom in yaks. *Anaplasma phagocytophilum* is a commonly reported emerging tick-borne zoonotic pathogen causing anaplasmosis [49]. This bacterium primarily infects host neutrophils, which break the first-line immune defensive barrier in mammals [50]. The infected animals show typically anemia [51], which reveal that *A. phagocytophilum* may contribute to diarrhea in yaks. *Bacteroides fluxus* is a pathogenic species of *Bacteroides* that displays numerous and high rate of antibiotic resistance. Higher abundance of *Bacteroides fluxus* means, this bacterium acting as potential role in diarrhea. *Firmicutes bacterium* was reported to be associated with lipogenesis metabolism in animals with nonalcoholic fatty liver disease [52]. The increased *Firmicutes bacterium* (CAG:424) in diarrhea yaks may cause dyslipidemia. Bovine viral diarrhea and Rotavirus were also reported in yaks [53, 54], which could be inferred that the increased abundance of these virus may cause diarrhea in yaks.

*Fournierella massiliensis* is described as a new human-associated member of the family Ruminococcaceae [55], which may have little relationship with diarrhea. *Bacteroides vulgatus* was found a main cause of polycystic ovary syndrome through disrupted ovarian functions and aggravated insulin resistance [56]. This means increment of *Bacteroides vulgatus* in yaks may cause diarrhea via affect glycol metabolism. *Klebsiella*
pneumonia causes many infections i.e. pneumonia, urinary tract infection, meningitis and bacteremia [57], which indicates the infection status of diarrheal yaks. While Firmicutes bacterium CAG:110 (p < 0.01), Clostridiales bacterium (p < 0.001), Ruminococcaceae bacterium (p < 0.001), Clostridium sp. CAG:413 (p < 0.001), Clostridia bacterium (p < 0.001), Firmicutes bacterium CAG:137 (p < 0.001), Methanobrevibacter olleyae (p < 0.001), Bacteroidales bacterium WCE2008 (p < 0.001), Ruminococcus flavefaciens (p < 0.001), Methanobrevibacter ruminantium (p < 0.001), Bacteroidete bacterium (p < 0.001), Anaerotruncus sp. Cag:390 (p < 0.001), Clostridium sp. CAG:448 (p < 0.001), Firmicutes bacterium (p < 0.01), Firmicutes bacterium CAG:124 (p < 0.001) and Firmicutes bacterium CAG:170 (p < 0.001) were significantly lower (Fig. 11c). Firmicutes bacterium CAG:110 was found to be potentially associated with swine feed efficiency variation in cecum microbiota via the utilization of dietary polysaccharides and dietary protein [58]. Its mean the dropped abundance of Firmicutes bacterium CAG:110 decrease the efficiency of feeds. Firmicutes bacterium CAG:137, Firmicutes bacterium (p < 0.01), Firmicutes bacterium CAG:124 (p < 0.001) and Firmicutes bacterium CAG:170 (p < 0.001) belongs to host energy uptake or storage limiting related Firmicutes, which are one of the most abundant bacteria in animals and human beings [59]. The lower of Firmicutes bacteria may linked to glycol metabolism, which may further cause diarrhea.

Butyrate producing Clostridiales bacterium is associated in protection of host from colorectal cancer, immune, and metabolic disorders [60]. It means dropped Clostridiales bacterium in yaks made contribute to diarrhea. Ruminococcus flavefaciens works with noncellulolytic Treponema or Butyrivibrio species that can accelerates the digestion of cellulose [61]. The lower Ruminococcus flavefaciens in diarrheal yaks may decrease the cellulose efficiency. Previously lower Ruminococcaceae bacterium was found in hospitalized patients, Cirrhosis [52], and diarrhea foals [62]. The deceased of this bacterium may insight that Ruminococcaceae bacterium has relationship with diarrhea in yaks. CAG:413, CAG:448 and Clostridia bacterium are belongs to Clostridium genus, which were recognized as beneficial bacteria to the host [63]. The lower of these three Clostridium spp. may promote diarrhea in yaks. Methanobrevibacter olleyae and Methanobrevibacter ruminantium composed the M. ruminantium clade, which belongs to ruminant Methanobrevibacter genus [64]. These two bacteria with other Methanobrevibacter spp. compose the rumen methanogenic community [65], which indicates that diarrheal yaks also have decreased production of methane. Bacteroidales bacterium WCE2008 is a Bacteroidales specie, which was accepted as “beneficial” microbes [66]. Previously dropped abundance of Bacteroidales was found in pediatric patients with CD [66], which may infer that the imbalance of this bacterium is related to diarrhea in yaks. Higher abundance of Bacteroidete bacterium is related to healthy lean of host, as it can generate three main SCFAs, butyrate, acetate and propionate [67]. The decreased Bacteroidete bacterium in animals contribute to diarrhea. Anaerotruncus can utilize cheese whey to produce acetic and butyric acids [68]. The decreased Anaerotruncus sp. Cag:390 in diarrhea may effect fatty acid metabolism in ruminants.

Microflora is a key regulator of digestion, extraction, synthesis, and absorption of many nutrients and metabolites i.e. bile acids, lipids, amino acids, vitamins, and short-chain fatty acids (SCFAs) [37]. SCFAs are not only principle nutrient substrates of intestinal epithelial cells, but also can regulate the epithelial barrier [69]. Previously, concentrations of SCFAs was related with diarrhea-predominant irritable bowel syndrome patients [69]. SCFAs could mitigate adenine-induced chronic kidney disease [70]. Preoperative fecal levels of SCFAs had an important impact on the occurrence of postoperative infectious complications in patients with esophageal cancer [71]. SCFAs in fecal samples was commonly used as an approximation of gut levels, which can infer the
relationship between intestinal SCFAs production and fecal levels [72]. In the current study, 6 out of 7 SCFAs were uncovered significantly lower in diarrhea yaks from 100 mixed fecal samples by employing GC-MS/MS (p < 0.05) (Fig. 5). It reveals that the imbalance of gut microbiota dropped the levels of SCFAs in diarrhea due to the extensive immunological and regulatory functions of SCFAs in the host-microbe interactions [73], activating anti-inflammatory signaling via acting as ligands of G-protein coupled receptors e.g. GPR109A, GPR41, and GPR43 [16]. The current results are in line with diarrhea-dominant IBS with lower levels of SCFAs [74]. Among the common SCFAs, acetate (C2), propionate (C3) and butyrate (C4) are the most in number, produced by anaerobic fermentation of dietary fibers in intestine [16]. Those three SCFAs are accounted for 90% of SCFAs produced by gut microbiota, which depicts the beneficial effects on intestinal epithelial cells and immune cells in the intestinal mucosa [75, 76].

Acetate was reported to mediate joint inflammation in a murine gout model via inflammasome assembly and IL-1β [77]. Propionic Acid was found to be increased in gut-associated Treg cells (relates to systemic immune reaction and disease amelioration) [78]. Butyrate is not only a primary energy source for colonocytes, but also can maintain intestinal homeostasis through anti-inflammatory actions via inhibiting nuclear factor kappa β, and histone deacetylation by promoting epithelial barrier function [16, 79]. Previously, lower abundance of butyrate-producing bacteria and fecal butyrate were found in stroke patients as higher risk factors [80].

Bacteroidetes from Fimcutes mainly produce acetate and propionate, while Butyrate is mainly produced by phylum Fimcutes i.e. Faecalibacterium prausnitzii, Clostridium leptum, Eubacterium rectale and Roseburia spp. [81]. In a previous study, Fimcutes phylum was found clearly lower in diarrheal yaks (p < 0.05) [5]. Also genus of Clostridium_IV (p < 0.01) and Clostridium_XI (p < 0.05) were found obviously lower in diarrheal yaks except Clostridium_XVIII (p < 0.01). Genera of Bacteroides (p < 0.05) and Faecalibacterium (p < 0.05) were found significantly higher in diarrhea yaks, while no significant difference was found in genera of Eubacterium, Eubacterium and Roseburia [5]. However, among all those genera, Clostridium_IV and Clostridium_XI were the dominant [5], which may uncover that the decreasing of clostridium may cause the drop of SCFAs (C2-C4). In a previous study, Isobutyric acid, Isovaleric acid and Caproic acid were found significantly lower in diarrheal animals (p < 0.05), which was in line with patients suffering from cirrhosis and neuromyelitis optica spectrum disorders [82, 83]. As SCFAs plays a critical role in mucosal integrity and immune response [84]. So, the dropping of SCFAs (C4-C6) may mean the damage of mucosal and inflammation response. In a sentence we can say, SCFAs generation bacteria of Anaerotruncus sp. Cag:390, Clostridiales bacterium and Butyricicoccus pullicaecorum are lower in number in diarrheal yaks. Although Fusobacterium mortiferum producing butyric and acetic acids increase obviously [85]. But, it could not affect the dropping trend of SCFAs in diarrheal animals. Statistical analysis of SCFAs; relevant to dominant KEGG signal pathways related to SCFA Acetic acid (53.85%) (Fig. 19); which was the primary level of acetate (50–70%) in the intestine [86].

In conclusion, we estimated the prevalence of emerging diarrhea disease in yak calves (15–25%) and adults (5–10%). Besides the high prevalence of Staphylococcus aureus, Babesia ovata, Anaplasma phagocytophilum, Bacteroides fluxus, viruses, Klebsiella pneumonia, and inflammation-related bacteria, the decreased of SCFAs may potentially lead to emerging diarrhea in yaks. Our results will make insights to the prevention and treatment of emerging diarrhea disease in yaks on the cold plateau.

Materials And Methods
Ethics statement

Fecal samples were collected under the permission of the relevant institutions. All procedures were performed under the instructions and approval of Laboratory Animals Research Centre of Hubei province and Sichuan province, and also under the ethics committee of Huazhong Agricultural University in P. R. China.

Sample collection

We visited 10 family yak farms with diarrhea out-break during June and July, 2019 (Table 1) in Hongyuan, Sichuan, China. The prevalence of diarrhea at farms was estimated by consulting animal owners as these bovines were free-ranged having grasslands without concentrated feed on the plateau. A total of 120 fresh fecal samples were collected from diarrheal (n = 60) and healthy (n = 60) yak calves. All the fecal samples were frozen immediately in liquid nitrogen and then transported to the laboratory of Huazhong Agricultural University. Samples were kept at -80 °C for further appraisement.

DNA extraction and mixing of samples

The genomic DNA from fecal samples were extracted by QIAamp Fast DNA Stool Mini Kit (QIAGEN) in accordance with the manufacturer's instructions. Genomic DNA samples were stored at -20 °C prior to further assessment. The quantity and quality of extractions were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA,USA), agarose gel electrophoresis, and Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen). Each 10 samples were then mixed and gained 6 diarrheal group samples (D1, D2, D3, D4, D5, D6) and 4 normal group samples (NA, NB, NC, ND).

Library construction and sequencing

Metagenome shotgun sequencing libraries (400 bp) were constructed by using Illumina TruSeq Nano DNA LT Library Preparation Kit. Each library was sequenced by employing Illumina HiSeq X-ten platform (Illumina, USA) with PE150 strategy (Shanghai, China).

Sequence Analysis

Further analysis to achieve quality-filtered reads, the sequencing adapters were removed from raw sequencing reads by using Cutadapt (v1.2.1) [87]. Low quality reads were trimmed by performing a sliding-window algorithm. Reads were aligned to the host genome via BWA (http://bio-bwa.sourceforge.net/) to remove host gene contamination [88]. Then quality-filtered reads were de novo assembled to construct the metagenome for each mixed sample by IDBA-UD (Iterative De Bruijn graph Assembler for sequencing data with highly Uneven Depth) [89]. All coding regions (CDS) of metagenomic scaffolds longer than 300 bp were predicted by MetaGeneMark (http://exon.gatech.edu/GeneMark/metagenome) [90]. CDS sequences of current samples were clustered by CD-HIT at 90% protein sequence identity, to obtain non-redundant gene catalog [91]. Gene abundance in each sample was estimated (http://soap.genomics.org.cn/) on the base of aligned reads number. The lowest common ancestor taxonomy of the non-redundant genes was obtained by aligning them against the NCBI-NT database by BLASTN (e value < 0.001). Similarly, the functional profiles of the non-redundant genes were obtained by annotated against the GO, KEGG, EggNOG and CAZy databases by utilizing DIAMOND (Buchfink) alignment algorithm [92].
Comparing of the difference of intestine microbiota between normal and diarrheal yaks

Based on the taxonomic and functional profiles of non-redundant genes, LEfSe (Linear discriminant analysis effect size) was performed to detect differentially abundant taxa and functions across the groups by using default parameters [93]. Beta diversity analysis was performed to investigate the compositional and functional variation of microbial communities across diarrheal and healthy yak samples through Bray-Curtis distance metrics [94]. Visualization was done via principal coordinate analysis (PCoA), nonmetric multidimensional scaling (NMDS) and unweighted pair-group method with arithmetic means (UPGMA) hierarchical clustering [95]. Differential gene (Up-regulated gene and Down-regulated gene) abundance analysis was preformed via DESeq at Fold Change ≥ 2 and p-value < 0.01 [28]. Metagenomics binning analysis was carried out by using Maxbin2 and Maxbat2 [96] at > 50% genomic integrity and < 10% contamination rate.

Extraction of fatty acids from fecal samples

Firstly 20 mg fecal from each sample was taken out to mix with 1 mL Phosphoric acid (0.5% v/v) in a sterile 2 mL EP tube, and then mixed up thoroughly via vortex and ultra-sonication for 10 and 5 minutes respectively. Secondly, 0.1 mL sample was taken out and then putted into a sterile 1.5 mL EP tube with the edition of 0.5 mL MTBE (CAS NO. 1634-04-4). Final product was mixed up thoroughly via vortex and ultra-sonication for 3 and 5 minutes respectively. Thirdly, at 12000 rpm sample was centrifuged at 4°C for 10 min. After taking out 0.2 mL from supernatant. Mixed 10 extracted samples from the same group and vortex for 1 minute. At the end, 0.2 mL mixture sample was taken out into sample detection vial for further analysis through GC-MS/MS (Agilent).

Qualitative and quantitative analysis of SCFAs

Total ions current (TIC) and standard quality of all mixture samples were detected through GC-MS/MS (Agilent) by using the procedures and parameters showed in Table 2. Sample's quality control analysis was carried out to ensure the validity of the method. Standard of quality samples was checked thrice in concern with instrument stability. This standard quality sample was tested in every ten samples to monitor the repeatability of the analysis process. Qualitative and quantitative analyses of SCFAs were performed by Agilent MassHunter. Standard curves for all SCFAs were generated by detecting standard quality control samples which were Caproic acid (CAS No. 64-19-7), Isovaleric acid (CAS No. 79-09-4), Valeric acid (CAS No. 79-31-2), Butyric acid (CAS No. 107-92-6), Propionic acid (CAS No. 503-74-2), Acetic acid (CAS No. 109-52-4), Isobutyric acid (CAS No. 142-62-1), 2-methylpentanoic acid (CAS No. 97-61-0), MTBE (CAS No. 1634-04-4), and Phosphoric acid (CAS No. 7664-38-2).

Statistical Analysis

The prevalence of diarrhea at different farms was analyzed through IBM SPSS Statistics (SPSS 22.0) by using chi-square (results were followed as upper and lower limits prevalence). Quantitative analyses of SCFAs were expressed as means ± standard deviation (SD). While the difference of SCFAs among the groups were analyzed via Wilcoxon test and Fold changes through piloting SPSS (IBM, 22.0). Significance level was kept as p < 0.05. T-test was also performed to compare intestinal microbiota difference by using IBM SPSS Statistics.

Declarations
Conflict of interest

The authors state that there are no competing interests.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

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