scientific reports

Check for updates

OPEN

Traditional Chinese herbal formulas modulate inflammatory mediators, antioxidant enzyme levels, and ruminal microbiota composition in postpartum female Yaks

Yanbin Zhu[1], Jia Wang[2], Xiaojuan Zhang[3], Yangji Cidan[1], Hongzhuang Wang[1], Panduo Pubu[3], Munwar Ali[2], Kun Li[2]✉ & Wangdui Basang[1]✉

Traditional Chinese Medicine (TCM) is an emerging area due to increased antimicrobial resistance (AMR). The objective of this research was to explore the antioxidant, and anti-inflammatory potential of three traditional Chinese herbal formulas (TCHFs), along with variations in rumen bacteria. In this study, forty postpartum (80 ± 15) female yaks after the calves had weaned (PWFs) were divided into three experimental groups, which were offered basal feed with 5% (95% basal diet) TCHF1 (DE group), 5% TCHF2 (DF group) and 5% TCHF3 (DG group), and fourth, control group (DH group), fed only a basal diet for 30 days. Following blood and rumen fluid samples on the 15th and 30th day, ELISA testing was performed to check antioxidant enzyme levels and inflammatory mediators. The results indicated that TCHF2 significantly upregulated the interleukin-10 (IL-10) ($p < 0.05$). Additionally, 16 S rRNA sequencing results showed that TCHF2 significantly enhanced Firmicutes to Bacteroidetes ratio (F/B) at the phylum level. On day 15th, phylum Actinobacteria, SR1, Cyanobacteria, and Armatimonadetes were found to be significantly ($p < 0.05$) different, while, at the genus level, *Butyrivibrio*, *CF231*, *YRC22*, *Moraxella*, *Clostridium*, etc. were significantly different ($p < 0.05$). On day 30, phylum SR1, Armatimonadetes, Chlorobi, and genus *Coprococcus*, *Oscillospira*, *Selenomonas*, *L7A_E11*, *Clostridium*, etc. were found to be significantly different ($p < 0.05$). This study concluded that TCHF2 is the most effective one among all.

Keywords Antioxidant, Rumen, 16S rRNA sequencing, Microbiome, *Bos grunniens*, Herbal

Yak (*Bos grunniens)* in the hostile ecological environment of the Qinghai-Xizang Plateau with low temperature, high altitude (average altitude over 4000 m), low oxygen level, and strong ultraviolet radiations, is comparatively well adapted[1]. Yaks play a significant role in the livelihoods of local herders, meeting about 50% of meat, and 90% of local milk demand, also a source of dairy products, fiber, leather, and dung (fuel) and act as a means of transportation[2–4]. China houses approximately 16 million yaks, representing over 90% of the global yaks[5]. So, their reproductive management and health are of great importance.

Literature has demonstrated the beneficial effect of TCM on antioxidant enzyme levels. For instance, the formula consisting of *Codonopsis pilosula*, *Ophiopogon japonicus*, and *Schisandra chinensis* has shown effectiveness against damage in pheochromocytoma-12 cells by enhancing the activity of superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px)[6]. Similarly, Qing Huo Yi Hao (QHYH) has been shown its scavenging mechanism free radical effectively in high glucose-treated endothelial cells[7]. Also, herbs such as *Coptis chinensis* and *Scutellaria baicalensis* have been noted for their ability to enhance the body's antioxidant defenses by modulating the activities of antioxidant enzymes[8]. Researches indicated that Sini Tang (SNT), a TCM formula has demonstrated its ability to reduce the levels of pro-inflammatory cytokines like tumor necrosis factor-alpha (TNF-α), IL-6, and IL-1β in rats[9]. Another study focused on the Kouyanqing Granule

[1]Institute of Animal Husbandry and Veterinary Medicine, Tibet Academy of Agriculture and Animal Husbandry Sciences, Lhasa 850009, China. [2]College of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095, China. [3]Agriculture and Animal husbandry science and technology service station in Seni district, Naqu 852000, China. ✉email: bw0891@163.com; lk3005@njau.edu.cn

(KYQG) formula which significantly reduced the pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α, and effectively modulated inflammatory response[10]. Healthy gastrointestinal (GI) flora enhance immunity by influencing cytokines production, priming innate immune response, and ultimately activating adaptive immune response (over 80% of immune cells are located in the gut)[11,12].

To adapt to the harsh plateau environment, the rumen microbiome of yaks has also co-evolved with the host[13]. Various factors including age, sex, disease, pregnancy, milk feeding, water intake, pre- and probiotics usage, environmental stress, genetic modification, and different feeding practices influence the biodiversity of gut microbiota in yaks[14]. Pieces of literature indicated that fermented traditional Chinese medicine (TCM) as a feed additive, increased the abundance of *Butyrivibrio*, which plays a role in fiber digestion and butyrate production[8]. Similarly, TCM formula containing *Atractylodes rhizome* and *Amur cork-tree* increased Firmicutes and decreased Bacteroidetes at phylum level with a notable increase in genera like *Ruminococcus* and *fibrobacter* which play an important role in cellulose digestion[7]. Additionally, TCM formulas containing Ginseng and Licorice were noted to improve overall microbial composition by increasing genera like *Prevotella* and *Lactobacillus*[15]. Diverse, and metabolically vibrant rumen microorganisms, ferment plant proteins and polysaccharides into short-chain fatty acids (SCFAs), which provide the primary carbon and energy for ruminants[16,17].

Traditional medicine based on Chinese herbal remedies has proved a sustainable and effective alternative to conventional antibiotics[18,19]. These natural therapeutics possess a variety of diverse bioactive compounds including antimicrobial, anti-inflammatory, and immunomodulatory effects, with the potential to mitigate infections without contributing to AMR[20–22], providing a cost-effective antibiotic alternative such as TCHFs. According to the best of our knowledge, this is the first study demonstrating the effectiveness of three different TCHFs with specific compositions in addressing postpartum heat challenges in PWFs, employing 16 S rRNA sequencing technology, correlating inflammatory markers, antioxidant enzymes, and microbiota variations, providing the foundation for future research regarding the effectiveness of TCHFs and their potential in the livestock industry.

Results

Effects of TCHF on inflammatory mediators and antioxidant enzyme level in PWFs

On the 15th day of the experiment, no significant differences in the antioxidant enzyme levels were detected in all four groups (Fig. 1A-D). However, on day 30, compared to the DH group (control group), the total antioxidant capacity (T-AOC) in the TCHF1 group increased significantly in serum ($p < 0.001$). Furthermore, TCHF2 feeding significantly increased ($p < 0.01$ or $p < 0.0001$) levels of GSH-Px and decreased levels of malondialdehyde (MDA). Meanwhile, MDA activity in the treated group was significantly decreased ($p < 0.05$). No significant alterations were detected regarding IL-6, TNF-α, IL-1β, and IL-10 on either the 15th or 30th day (Fig. 1E-F). However, on the 30th day, the levels of IL-1β in the TCHF2 group were significantly up-regulated ($p < 0.05$).

Analysis of rumen bacterial diversity in PWFs

48 rumen fluid samples were subjected to amplicon sequencing. In total 4,481,091 raw sequences (DE: 1103181, DF: 1136858, DG: 1136823, and DH: 1104229) and 4,204,635 filtered sequences (DE: 1034452, DF: 1066468, DG: 1068629 and DH: 1035086) were obtained from the V3/V4 regions of given samples (3). The effective square quantity regarding bacteria was 93.81%, (Table 1).

Alpha diversity

On day 15 Chao1 showed that the highest variation was found in group DFZ1 followed by DGZ1, DHZ1, and DEZ ($p = 0.15$), however, on day 15 DHZ2 and DFZ2 ($p = 0.38$) showed the highest variations in rumen microbiota. Regarding the Simpson index at day 15, DGZ1 showed the highest variations ($p = 0.029$), however

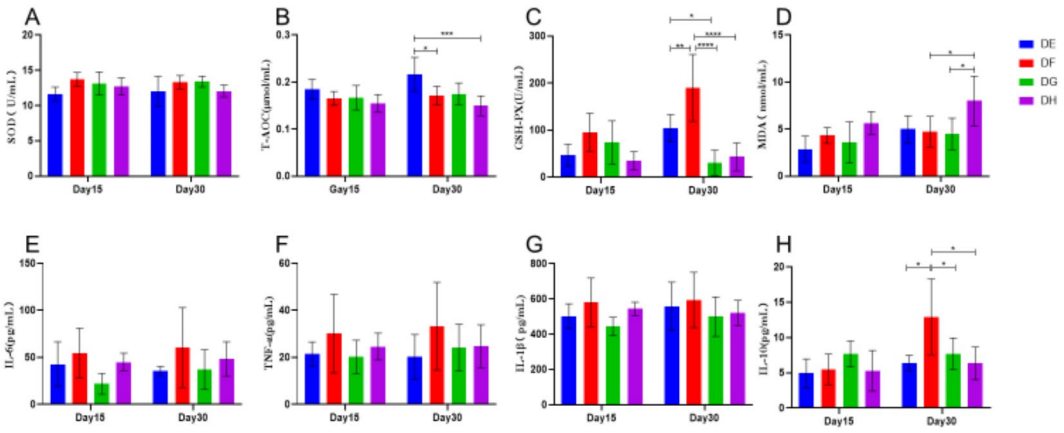

Fig. 1. Effects of TCHFs on oxidative stress and inflammation in PWFs. (**A**–**D**) indicate the serum antioxidant-related indicators, including SOD, T-AOC, GSH-Px, and MDA. (**E**–**H**) represent the serum inflammatory mediators including IL-6, TNF-α, IL-1β, and IL-10. Data are represented as means ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Sample group	Input	Filtered	Denoised	Merged	Non-chimeric	Non-singleton
DEZ1	551,162	516,958	487,533	316,609	255,963	251,967
DEZ2	552,019	517,494	487,958	323,980	254,770	250,131
DFZ1	553,465	520,195	492,876	336,104	282,068	278,953
DFZ2	583,393	546,273	519,162	362,895	295,472	291,586
DGZ1	552,829	519,560	492,964	337,749	274,330	271,456
DGZ2	583,994	549,069	524,149	377,704	286,450	281,850
DHZ1	556,710	521,965	494,673	342,538	285,612	281,982
DHZ2	547,519	513,121	487,554	344,343	275,476	271,271

Table 1. The bacterial sequencing analysis statistics of Yaks in different groups.

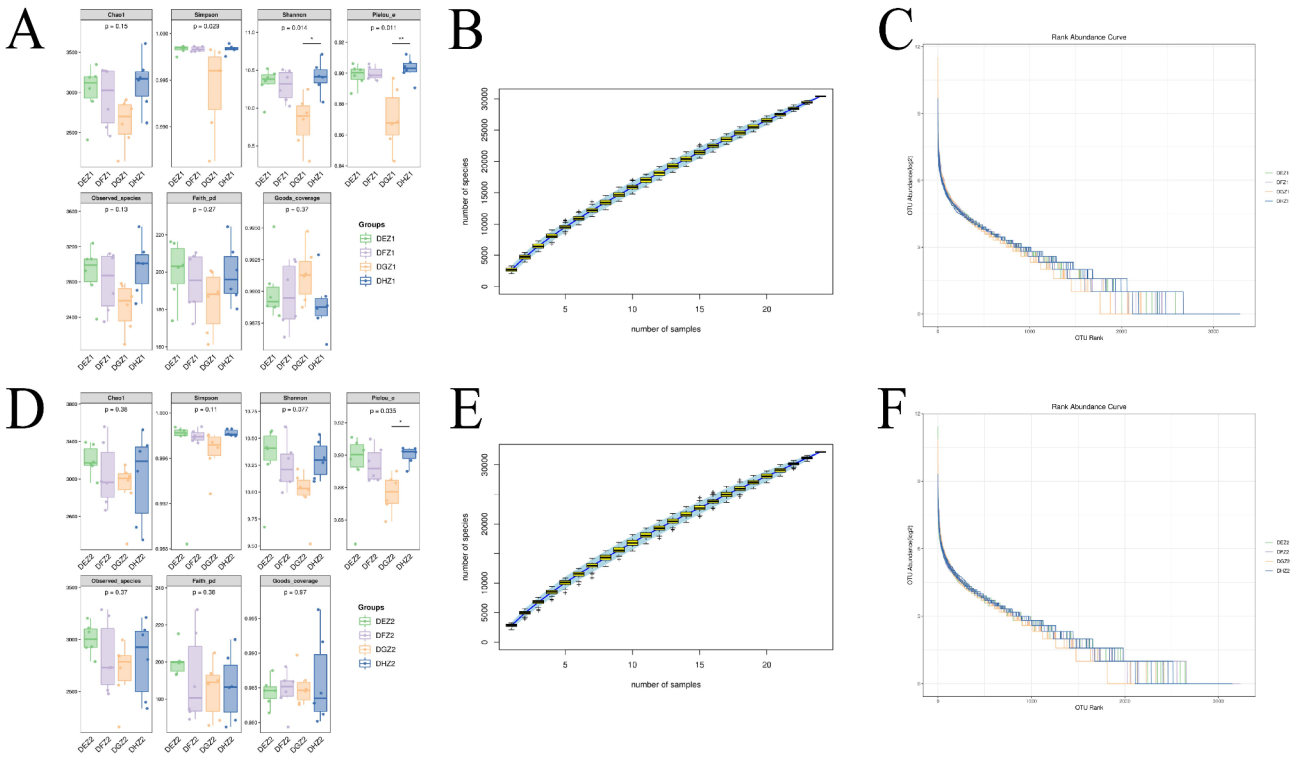

Fig. 2. Alpha diversity analysis of rumen bacteria after provision with TCHFs in different groups. (**A**,**D**) alpha-diversity index of rumen bacteria from the group provided with TCHFs and sampled on days 15 and 30 of the study respectively.)**B**,**E**) Species accumulation curves of all samples collected on the days 15 and 30 of the study respectively. (**C**,**F**) Rank abundance curve of different samples collected on the days 15 and 30 of the study, respectively.

variability decreased until day 30. Overall, significant variations ($p < 0.05$) were observed between samples on days 15 and 30 regarding Pielou-e, Observed species, Faith-pd, and Goods-coverage.

Species accumulation curves, which are used to measure and predict the increase of species richness as the sample size increases, showed that the curves tended to be flattened, and Good's coverage index for each group was close to 100%, indicating sufficient sequencing depth to cover all species (Fig. 2A, B, D, E) (Table 2). The Venn diagram based on the OTUs, can be used to understand the composition of microorganisms in the sample. The results showed that the DEZ1 and DEZ2 group (TCHF1 sample collected on the 15th and 30th day) had the highest number of rumen bacterial OTUs in PWFs, accounting for 37.24% and 35.96% respectively. In contrast, the DGZ1 and DGZ2 groups (TCHF3 samples collected on the 15th and 30th day) had the lowest number of rumen bacterial OTUs, accounting for 33.67% and 34.38% respectively. Additionally, the OTUs sharing number of rumen bacteria collected from yaks treated with different TCHFs was lower, indicating significant differences in the composition of rumen bacteria due to provision with different TCHFs (Fig. 3A, B).

The flatness of the rank abundance curve can reflect the evenness of community composition. The results showed that the fold lines in the DGZ1 and DGZ2 groups were steeper, and Pielou's Evenness indexes were significantly lower ($p < 0.05$, $p < 0.01$), indicating lower uniformity of community composition. In contrast, the

Group	Chao1	Simpson	Shannon	Pielou_e	Observed_species	Faith_pd	Goods_coverage
DEZ1	3014.35 ± 333.22	0.998 ± 0.0004	10.33 ± 0.2	0.9 ± 0.01	2904.1 ± 301.95	200.5 ± 16	0.99 ± 0.003
DFZ1	2936.95 ± 378.39	0.998 ± 0.0002	10.3 ± 0.2	0.9 ± 0	2798.45 ± 342.55	194.4 ± 16.2	0.99 ± 0.003
DGZ1	2626.63 ± 293.49	0.994 ± 0.0048	9.83 ± 0.34*	0.87 ± 0.02**	2517.05 ± 272.08	184.29 ± 16.47	0.99 ± 0.002
DHZ1	3122.86 ± 338.05	0.998 ± 0.0004	10.41 ± 0.21	0.9 ± 0.01	2974.35 ± 310.42	199.21 ± 16.24	0.99 ± 0.002
DEZ2	3200.68 ± 160.54	0.997 ± 0.0041	10.31 ± 0.33	0.89 ± 0.02	3005.53 ± 150.99	200.32 ± 8.01	0.98 ± 0.002
DFZ2	3050.14 ± 352.25	0.998 ± 0.0005	10.25 ± 0.23	0.89 ± 0.01	2828.33 ± 349.77	191.25 ± 24.84	0.98 ± 0.003
DGZ2	2897.84 ± 306.13	0.997 ± 0.002	9.98 ± 0.24	0.88 ± 0.01*	2689.6 ± 296.22	185.17 ± 15.19	0.99 ± 0.003
DHZ2	3016.18 ± 486.48	0.998 ± 0.0003	10.3 ± 0.17	0.9 ± 0.01	2815.52 ± 370.92	186.79 ± 18.38	0.99 ± 0.006

Table 2. Alpha diversity indices representing variations between and within different groups.

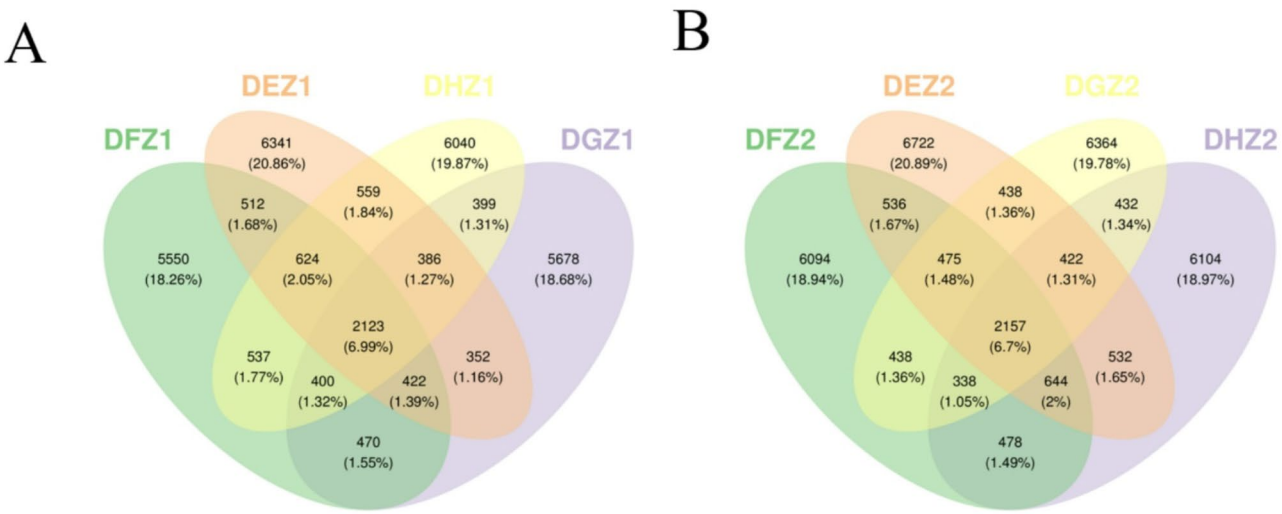

Fig. 3. Venn diagram of bacterial OTUs distribution of rumen fluid, collected from different groups fed with TCHFs on the 15th (**A**) and 30th day (**B**) respectively. Each ellipse stands for a sample group (DEZ1, DFZ1, DGZ1 and DHZ1 sampled on the 15th day; DEZ2, DFZ2, DGZ2, and DHZ2 sampled on the 30th day). Numbers indicate the count and percentage of unique and shared elements within each dataset.

abundance difference between OTUs in the DHZ1 and DHZ2 groups was smaller, showing higher evenness (Fig. 2A, C, D, F).

Beta diversity

Beta diversity indices; Principal Coordinates Analysis (PCOA) and Non-Metric Multi-Dimensional Scaling (NMDS), were used to assess differences in gut microbial community structure among samples. The distribution of samples in the continuous sorting axis showed significant changes in the bacterial communities over time in each group after the provision of three TCHFs (Fig. 4A, C). The stress values of NMDS analysis (0.162 for the 15th day and 0.0959 for the 30th day) indicated reliable results for further research and analysis (Fig. 4B, D).

Analysis of similarities (ANOSIM) was used with NMDS to check whether differences between groups were significantly greater than differences within groups. All p-values were between − 1 and 1 after pair comparison, and all except for the DE vs. DF group were more than 0, indicating that the difference between groups was greater than the difference within groups, except for the comparison of TCHF1 and TCHF2 treated groups on the 15th and 30th days (Table 3).

Rumen bacterial composition analysis in PWFs.

The relative abundances of dominant bacterial taxa at different levels were determined through the classification of microbial taxa. The taxonomic composition of different groups showed significant variations. The top three rumen bacteria in terms of relative abundance at the class, order, and family level for each group sampled on the 15th and 30th days were not significantly different (Fig. 5B, C, D, G, H, I). However, there were significant differences ($p < 0.05$) in the relative abundances of rumen bacteria at the phylum and genus levels.

A total of 29 phyla and 323 genera were recognized from 24 rumen fluid samples collected on the 15th day of the experiment. Thirty phyla and 336 genera were recognized from 24 samples collected on the 30th day of the experiment. The bacterial composition of rumen fluid sampled on days 15 and 30 was compared to analyze whether different TCHFs significantly affected the ruminal bacterial composition of PWFs.

At day 15, at the phylum level, Firmicutes (DFZ1 = 46.92%, DGZ1 = 44.22%) were the most dominant bacteria in DFZ1 and DGZ1 groups, followed by Bacteroidetes (DFZ1 = 41.4%, DGZ1 = 43.87%) and Verrucomicrobiota

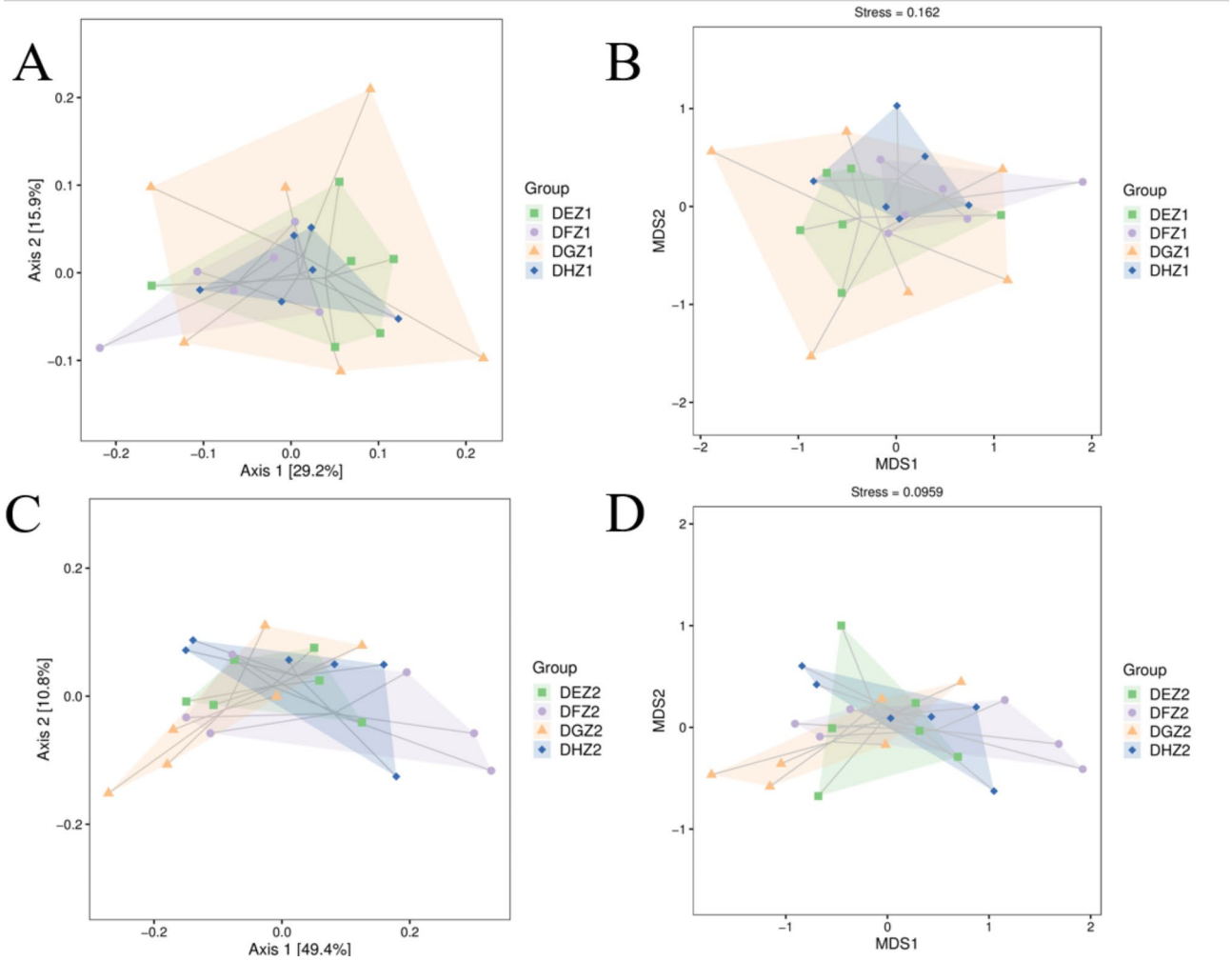

Fig. 4. Beta diversity analysis of rumen bacteria from different groups treated with TCHFs. (**A**, **C**) PCoA analysis of bacterial OTU distribution of rumen fluid collected from different TCHFs fed groups on the 15th and 30th days respectively. (**B**, **D**) NMDS analysis of bacterial OTU distribution of rumen fluid, collected from different groups fed with TCHFs on the 15th and 30th day respectively.

Group1	Group2	Sample size	Permutations	*R*	*p*-value
DEZ1	DFZ1	12	999	−0.014814815	0.587
	DGZ1	12	999	0.134259259	0.049*
	DHZ1	12	999	0.172222222	0.028*
DFZ1	DGZ1	12	999	0.092592593	0.121
	DHZ1	12	999	0.17962963	0.011*
DGZ1	DHZ1	12	999	0.222222222	0.006**
DEZ2	DFZ2	12	999	−0.044444444	0.582
	DGZ2	12	999	0.221296296	0.042*
	DHZ2	12	999	0.081481481	0.164
DFZ2	DGZ2	12	999	0.040740741	0.265
	DHZ2	12	999	0.008333333	0.367
DGZ2	DHZ2	12	999	0.240740741	0.013*

Table 3. ANOSIM of the rumen bacteria collected from different TCHF supplement groups on the 15th and 30th days through pairwise comparison.

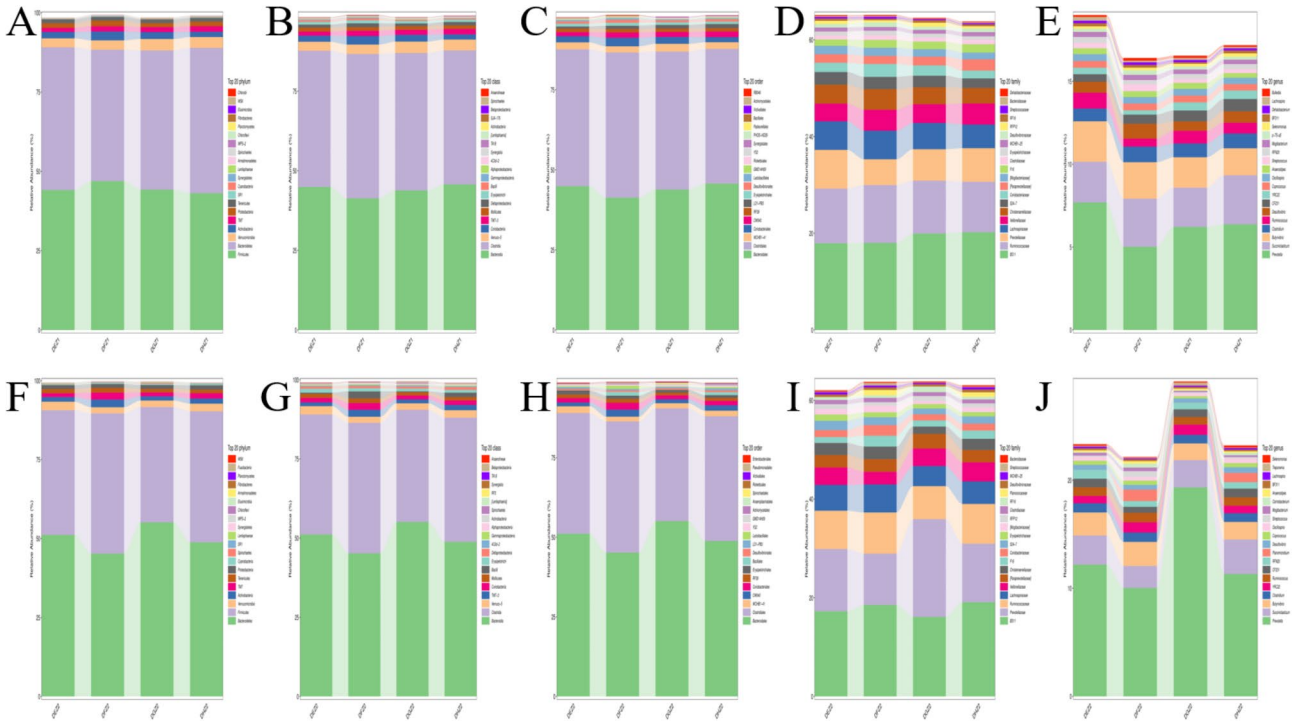

Fig. 5. The relative abundances and distribution of significant rumen bacteria in different TCHFs treated groups, collected on the 15th (**A**–**E**) and 30th days (**F**–**J**) respectively. Rumen bacterial composition at the phylum (**A**, **F**), class (**B**, **G**), order (**C**, **H**), family (**D**, **I**), and genus (**E**, **J**) levels.

(DFZ1 = 2.95%, DGZ1 = 3.6%). However, the phyla Bacteroidetes (DEZ1 = 44.93%, DHZ1 = 45.79%), Firmicutes (DEZ1 = 44.14%, DHZ1 = 43.13%) and Verrucomicrobiota (DEZ1 = 2.85%, DHZ1 = 3.4%) were the three most dominant phyla in the DEZ1 and DHZ1 groups, accounting for a total of 91.92% and 92.31% of the bacterial composition, respectively (Fig. 5A).

The relative abundance of rumen fluid bacteria sampled on day 30th showed significant changes at the phylum level. The phyla Bacteroidetes (DEZ2 = 51.15%, DFZ2 = 45.23%, DGZ2 = 55.13%, DHZ2 = 48.85%), Firmicutes (DEZ2 = 39.5%, DFZ2 = 44.43%, DGZ2 = 36.53%, DHZ2 = 41.46%) and Verrucomicrobiota (DEZ2 = 2.69%, DFZ2 = 1.93%, DGZ2 = 2.05%, DHZ2 = 2.34%) were the three most dominant phyla among the four groups sampled on the day 30, accounting for approximately 93.34%, 91.59%, 93.71% and 92.7% of the total taxonomic groups identified, respectively (Fig. 5F).

At the known genus level, *Prevotella* (DEZ1 = 7.69%, DFZ1 = 5.02%, DGZ1 = 6.22%, DHZ1 = 6.37%), *Succiniclasticum* (DEZ1 = 2.46%, DFZ1 = 2.9%, DGZ1 = 2.37%, DHZ1 = 2.96%) and *Butyrivibrio* (DEZ1 = 2.44%, DFZ1 = 2.2%, DGZ1 = 1.83%, DHZ1 = 1.63%) were the top three dominant genera among the four groups collected on the 15th day (Fig. 5E). On the 30th day, *Prevotella* accounted for 12.19%, 10.03%, 19.33%, and 11.33%; *Succiniclasticum* accounted for 2.69%, 2.04%, 2.53% and 3.19%; *Butyrivibrio* accounted for 2.14%, 2.22%, 1.54% and 1.62% in DEZ2, DFZ2, DGZ2 and DHZ2 group, respectively (Fig. 5J), consistent with the samples collected on the 15th day.

Metastatic analysis was performed to compare rumen bacteria collected on the 15th and 30th days in the different treatments- (DE, DF, and DG) and control group (DH) at the phylum and genus levels. This was done to analyze the effects of TCHFs on the classification of rumen flora of yaks. Compared with the DHZ1 group at the phylum level, the relative abundances of phylum SR1 and WPS-2 in the DEZ1 group were significantly lower. Actinobacteria had a higher proportion in the DFZ1 group, while Cyanobacteria was significantly reduced in the DFZ1 group. Armatimonadetes and SR1 in the DGZ1 group were significantly lower (Fig. 6). Meanwhile, compared with the DHZ2 group, the phyla SR1 and Chlorobi in the DEZ2 and DGZ2 group occupied a smaller percentage. Additionally, the relative abundance of Armatimonadetes in the DGZ2 group was noticeably lower (Fig. 7).

Compared to the control group (DHZ1, DHZ2), the TCHFs treated groups showed significant differences in the relative abundances of many bacteria at the genus level. Among the groups sampled on day 15, the relative abundance of genera *unidentified_[Mogibacteriaceae]*, *Veillonella*, and *Candidatus Arthromitus* in the DEZ1 group was significantly higher than the DHZ1 group. Besides, *CF231*, *unidentified_SR1*, *unidentified_YS2*, *unidentified_WPS-2*, *Clostridium*, *unclassified_Erysipelotrichaceae* and *unclassified_[Paraprevotellaceae]* occupied a lower percentage in the DEZ1 group. Meanwhile, in the DFZ1 group, the richness of *unidentified_Ruminococcaceae*, *Butyrivibrio*, *unidentified_Coriobacteriaceae*, *unclassified_Coriobacteriaceae*, *p-75-a5*, *Veillonella*, *unclassified_Erysipelotrichaceae*, *Thiobacillus* and *unidentified_Mollicutes* was obviously higher than DHZ1 group, but the richness of *YRC22*, *unidentified_Prevotellaceae*, *unidentified_YS2*, *[Prevotella]*, *unclassified_[Paraprevotellaceae]*

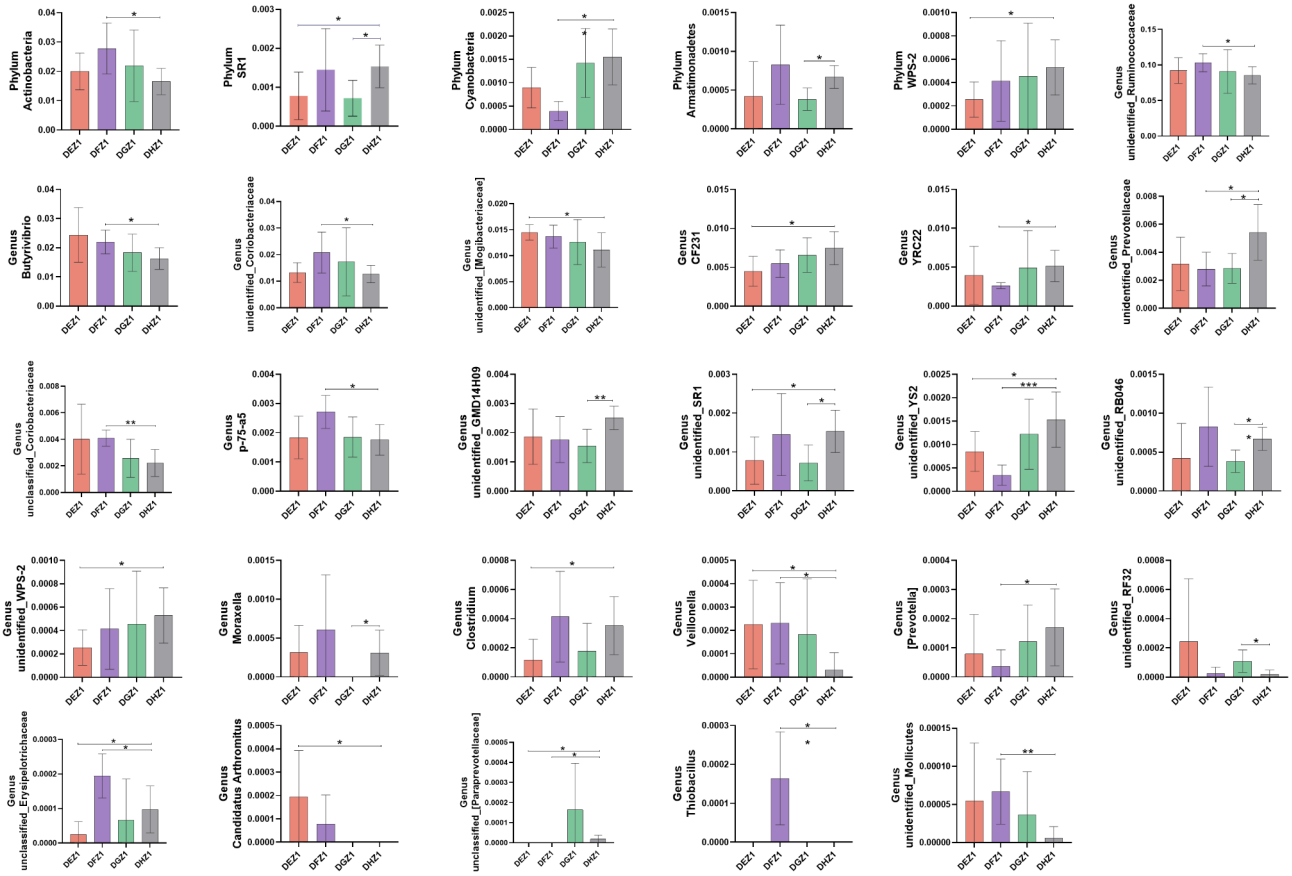

Fig. 6. Metastatic analysis of the rumen bacteria collected on the 15th day in the different groups treated with TCHFs (DEZ1, DFZ1, and DGZ1) compared with the control group (DHZ1) at the phylum and genus levels, showing the significantly different phylum and genera among four groups at day 15. Data are represented as means ± SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

were significantly lower. Besides, *unidentified_RF32* in the DGZ1 group was more dominant than in the DHZ1 group; by contrast, the genera *unidentified_Prevotellaceae*, *unidentified_GMD14H09*, *unidentified_SR1*, *unidentified_RB046*, and *Moraxella* accounted for a smaller proportion (Fig. 5).

In addition, compared to the control group (DHZ2), the difference in the relative bacterial abundance in rumen fluid sampled on day 30 indicated that genera *unclassified_Enterobacteriaceae*, *Pseudomonas*, *Serratia*, and *Sphaerochaeta* in the DEZ2 group; *L7A_E11*, *Pseudomonas*, *Atopobium*, and *unidentified_Spirobacillales* in the DFZ2 group; *unidentified_RF16*, *Pseudomonas*, *Haemophilus* and *Paludibacter* in the DGZ2 group accounted for more scale than the DHZ2 group. Furthermore, the genera *unidentified_SR1*, *Desulfobulbus*, and *unidentified_OPB56* in the DEZ1 group; *unidentified_GMD14H09*, *Selenomonas*, *unidentified_ML615J-28*, *[Prevotella]*, *unidentified_Synergistaceae*, *Staphylococcus* in the DFZ2 group; and *unclassified_Ruminococcaceae*, *Coprococcus*, *Oscillospira*,*unidentified_SR1*, *Moryella*, *unidentified_RB046*, *unidentified_Synergistaceae*, *Staphylococcus* and *unidentified_OPB56* in the DGZ2 group were significantly less than in the DHZ2 group (Fig. 7). Given that this discriminant analysis may not detect the whole taxa, Linear discriminant analysis Effect Size (LefSe) analysis (LDA score > 2) known as biomarkers was able to detect microorganisms with higher abundance in each group relative to the other groups. The results showed that the biomarker of DEZ1 group was *g_ Haemophilus*, the biomarker of DFZ1 group was o_Pseudomonadales, the biomarkers of DGZ1 group were f_Erysipelotrichaceae, o_Erysipelotrichales, c_Erysipelotrichi, and *g_Clostridium* was detected in the DHZ1 group (Fig. 8A, C). Meanwhile, p_Firmicutes was observed in DFZ2 group, *g_Clostridium*, f_Lachnospiraceae, o_Clostridiales, c_Clostridia served as biomarkers in the DHZ2 group (Fig. 8B, D).

Discussion

Yak are seasonal breeders, with the estrus period concentrated in summer and fall and the calving peak period is from March to May. However, postpartum female yak often faces negative energy balance, and oxidative and inflammatory stress, interfering with the secretion of reproductive hormones[23]. As a result, only 3.03% of postpartum yaks show natural estrus during the breeding season[24,25]. Therefore, successful induction of estrus in yaks is crucial for better reproductive performance. In this context, serum antioxidant enzyme levels and inflammatory mediators reflect the body's health state[26,27].

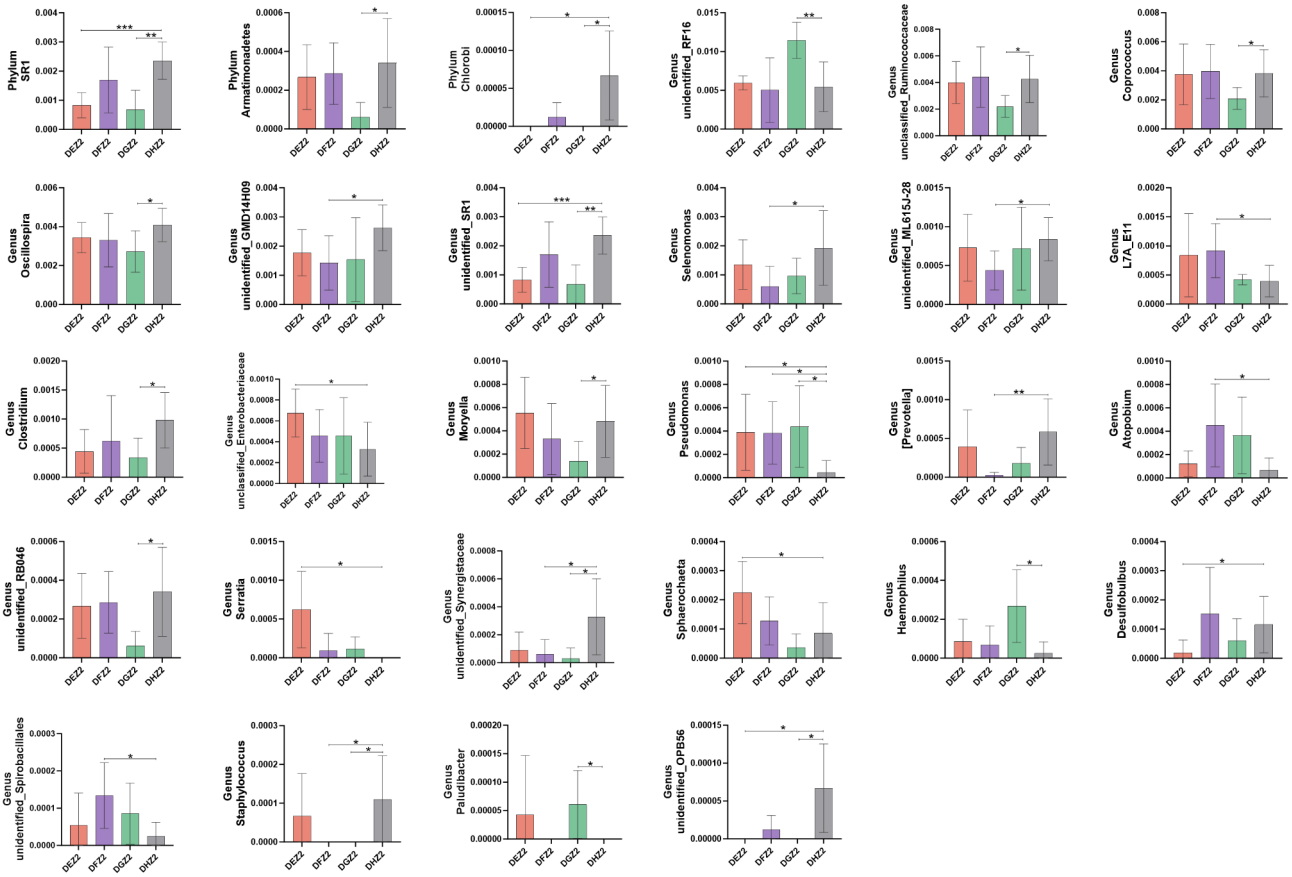

Fig. 7. Metastatic analysis of the rumen bacteria collected on the 30th day in the different groups (DEZ2, DFZ2, and DGZ2) compared with the control group (DHZ2) at the phylum and genus levels, showing the significantly different phylum and genera among four groups at day 30. Data are represented as means ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

In the current study, antioxidant enzymes increased in the groups provided with TCHF1 and TCHF2, while significantly decreased in groups provided with TCHF3. Additionally, IL-10 in PWFs provided with TCHF2 significantly increased, indicating that TCHF2 benefits both by reducing oxidative stress and inflammation[28–30]. Jiang et al. (2024) found that the contents of T-AOC and GSH-Px in the serum of layers, fed with compound-fermented Chinese medicine significantly increased, while the level of MDA was significantly decreased[31]. In this study, results indicated that intake of TCHF1 resulted in a substantial increase in the T-AOC compared to the control group. These findings are consistent with previous studies where herbal formulations enhanced antioxidant defense associated with various diseases[32]. This increase in T-AOC may be the compensatory mechanism to effectively counteract oxidative damage[33,34].

While TCHF3 intake resulted in a significant decrease in the MDA level, it did not show a corresponding increase in GSH-Px or T-AOC level. This raises the question about the specific mechanism through which TCHF3 exerted its effects. However, this observation was supported by an investigation that different antioxidant compounds can exert varied effects on the oxidative stress pathways[35,36]. In TCHF2 there was a marked increase in the GSH-Px activity and a marked decrease in MDA level, indicating a decrease in the oxidative damage and is consistent with previous findings where antioxidants were effective against lipid peroxidation[37]. The increase in GSH-Px is important as it neutralizes the toxic effects of H_2O_2, and protects cells from oxidative damage[38].

Interestingly, inflammatory markers such as IL-6, TNF-α, IL-1β, and IL-10 did not show significant alterations on either day 15 or day 30, except for an upregulation of IL-1β in the TCHF2 group on day 30. This finding is in contrast to existing literature, suggesting that increased oxidative stress often correlates with elevated inflammatory markers[39,40]. This lack of significant changes raises important questions about the interrelations between oxidative stress and inflammation[41]. It leads to a reassert gap and needs further clarity in this regard.

The rumen bacteria are integral to maintain homeostasis, immune system performance, digestion, absorption, and metabolic balance[42]. In the given study, β-diversity results indicated that the group provided with TCHF3 exhibited greater intra-group differences. It can be speculated that the absorption of TCHF3 in PWFs varied significantly, which was not conducive to recovery and estrus. Studies have shown that natural remedies like *Lycium barbarum* polysaccharides (LBP) derived from the fruit of the *Lycium barbarum* plant and *Scutellaria baicalensis* can upregulate beneficial gut bacteria[43–46].

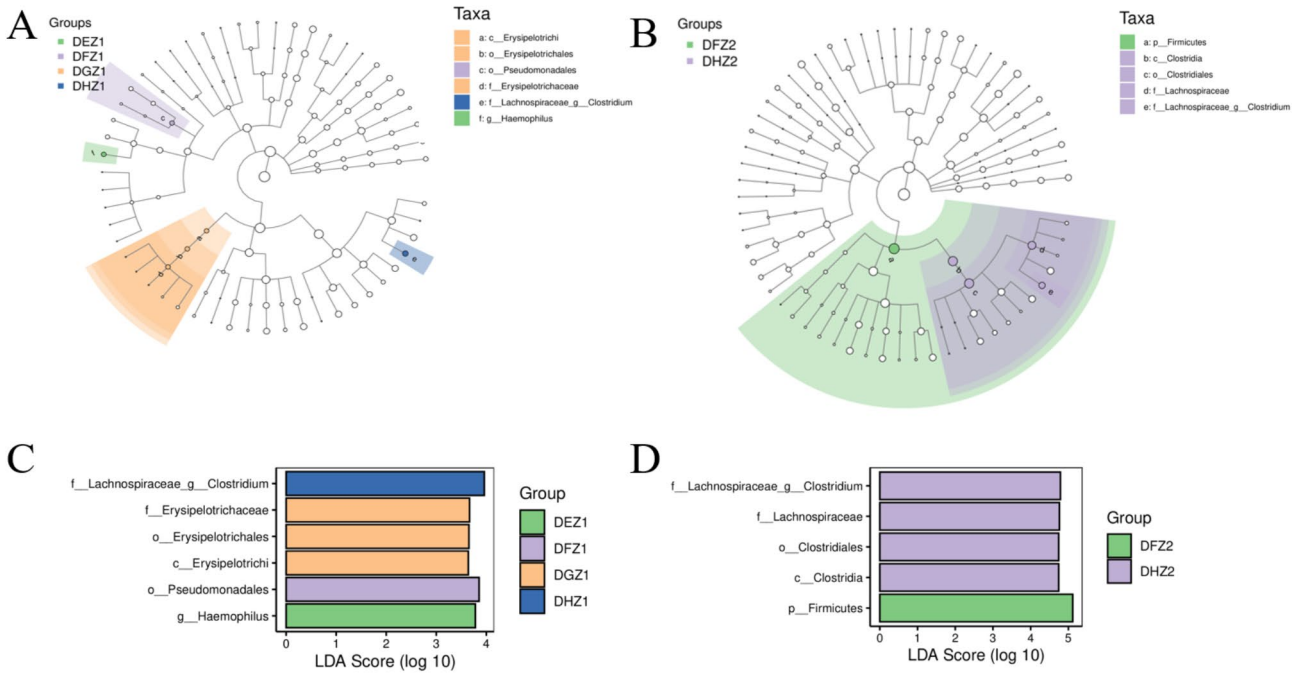

Fig. 8. LefSe analysis of rumen fluid bacteria collected from different groups provided with TCHFs on the 15th (**A**, **C**) and 30th (**B**, **D**) days respectively. (**A**, **B**) Cladograms showing taxa distribution across groups. (**C**, **D**) LDA scores highlight differentially abundant taxa within each group. LDA scores > 2 were considered significantly different.

In the current study, Bacteroidetes and Firmicutes were found to be predominant phyla in the rumen in all groups, which is consistent with the previous studies[47–49]. Firmicutes degrade fiber and convert cellulose into VFAs, while Bacteroidetes facilitate protein and carbohydrate digestion and absorption and strengthen the enteric immune system[50,51]. SCFAs (propionate, butyrate, acetate) are the major byproduct of gut bacteria, break carbohydrates that are indigestible and are recognized as vital energy materials, have anti-cancer and anti-inflammatory characteristics and can lower cholesterol and fat storage, regulate the pH of the intestine, and also avoid predatory harmful germs from entering and sticking[52,53]. F/B is a critical indicator for assessing interrelations between gut microbes and host energy metabolism; a higher F/B ratio is positively correlated with nutrient absorption and fat deposition[50,54,55]. In the current study, at day 15, no significant effect on the F/B ratio, but after 30 days, the F/B value of PWFs fed TCHF2 significantly increased, while the F/B value in PWFs fed TCHF1 and TCHF3 decreased significantly. This indicates the significant impact of prolonged TCHFs on yaks' rumen microbial composition.

Firmicutes as a biomarker in group DFZ2 suggested that an increased relative abundance of Firmicutes in the GI tract was found to be positively correlated with energy metabolism[4], indicating that TCHF2 positively influenced feed digestion and ultimately growth in PWFs[56,57]. Following the previous results, the current study showed that compared to the control group, the relative abundance of Actinobacteria was significantly higher, and Cyanobacteria was significantly lower in the rumen fluid of PWFs fed with TCHF2 for 15 days. Actinobacteria's secondary metabolites, primarily antimicrobial peptides, have a strong probiotic effect, improving immunity[58–60]. Also, a negative relationship was detected between an improved immune response and an abundance of Cyanobacteria[61].

The reduction of Cyanobacteria in the DFZ1 group pointed out the selective pressure exerted by TCHFs that could affect the overall metabolic output of the rumen microbiome[62,63]. Additionally, the existence of some lesser recognized phyla like Chlorobi and WPS-2, distinct from mammalian fecal/rumen samples or insect samples[64], and their specific functions need further exploration. In this study metastatic analysis and LefSe analysis, showed that provision of TCHF2 for 15 days significantly enhanced the relative abundance of cellulolytic bacteria such as *Butyrivibrio*, *unidentified_Ruminococcaceae*, and *p-75-a5*, indicating TCHF2 could promote fiber decomposition and maintain GI flora homeostasis in PWFs[64–66].

Beneficial genera like *unclassified_Ruminococcaceae*, *Coprococcus*, *Oscillospira*, and *Moryella* which produce butyrate, decreased significantly after 30 days of feeding with TCHF3, indicating that TCHF3 may suppress microbial homeostasis and energy absorption[56,67,68]. Moreover, the proportion of *Proteobacteria* increased significantly after feeding TCHF1 for 30 days. The relative abundance of *Proteobacteria* serves as a key indicator of gut microbial homeostasis[69,70], indicating that TCHF1 may have disrupted the rumen microbes. However, a more detailed study is needed in this regard.

Genera such as *Veillonella*, *Candidatus*, and *Arthromitus* were significantly increased in the DEZ1 group compared to the DHZ1 group, suggesting enhanced fermentation capabilities and possible improved energy availability for the host. The presence of *unidentified_[Mogibacteriaceae]* showed a shift towards more specialized

microbial communities that may lead to increased nutrient absorption and metabolism[58]. Contrary to this, several genera such as *Clostridium*, showed decreased relative abundance, which may reflect a competitive exclusion effect and change in the substrate availability after TCHF supplementation[51,58].

On day 30 of treatment further differences emerged; genera like *Pseudomonas*, *Serratia*, and *Sphaerochaeta* showed higher abundance in groups DEZ2, DFZ2, and DGZ2 compared to the DHZ2 group. This proved that the TCHFs not only influence immediate microbial response but also promote long-term shifts that could alter the rumen functions over time. The decrease in the relative abundance of certain genera like *Desulfobulbus* and some others in the treated group indicates a potential stabilization of beneficial microbial populations at the expense of less advantageous ones[58,63,71]. These findings are in line with other studies that indicate that dietary intervention could alter the rumen microbial populations and their functioning[51,72]. Literature indicated that *Scutellaria baicalensis* (in TCHF1) could help modulate the immune response in the gut, potentially promoting the beneficial bacteria while inhibiting the pathogenic ones[73], *Radix Astragali* is associated with the growth of gut-friendly bacteria[74]. In TCHF2 *Lycii Fructus* has antioxidant properties, and can protect against oxidative stress in the gut, promoting a healthier environment for microbial growth[45]. Similarly, *Angelicae Sinensis*, or Dangui, could enhance nutrient absorption and positively influence microbial diversity[74]. *Chuanxiong Rhizoma* present in TCHF3 has been shown to promote circulation, and hence increase the nutrient availability to microbes[74]. The differences in herbal composition in different formulas affect the bioavailability of nutrients and phytochemicals due to better substrate[73].

This study provides insights into the use of TCHFs with basal feed to improve the health and reproductive performance of PWFs by enhancing antioxidant capacity, immunomodulation, and beneficially altering rumen microbes. This research is significant with a perspective to enhanced AMR and can lead to better antibiotic alternatives in the form of herbal products. Limitations of the study include small sample size, short intervention period, lack of detailed mechanisms to understand individual herbs' contribution, lack of inclusion of comprehensive inflammatory markers pathways like NF-κB, toll-like receptor signaling pathway, ambiguity in effects of TCHF3, and the impact of environmental and genetic factors not well justified here. Future research can focus on these parameters to explore the host-microbiome interactions more quantitatively.

Conclusion

In conclusion, serum antioxidant enzyme levels increased after providing TCHFs in a time-dependent manner. TCHF2 increased antioxidative potential and proved as anti-inflammatory, and enhanced immunity in PWFs. Prolonged intake of TCHF2 significantly increased the F/B ratio and the relative abundance of other gut-friendly bacteria. However, adding TCHF1 or TCHF3 to the diet tended to promote the propagation of harmful bacteria and disrupted the rumen microbiome. Further research is needed to know the quantified effect of each ingredient included in these herbal formulas and also regarding the more detailed modes of action of individual components of these three TCHFs.

Methods

Formulation of TCHFs

According to the nutritional requirements of PWFs and the medicinal properties of Chinese herbs, we formulated three traditional Chinese herbal formulas (TCHF1, TCHF, TCHF3). Following good agricultural practices (GAP) standards, all herbs were purchased from Bozhou Herbal Medicine Market. After drying and crushing each herb, they were mixed in the appropriate proportions to create the three TCHFs (Table 4) and then stored in dark dry conditions at a temperature of 20–25 °C, with a relative humidity of 50–60%, in a sealed (air-tight) container to avoid external exposure and resultant degradation.

Experimental design

The experiment was conducted from July 2023 to September 2023 in Linzhou Jingmu Agricultural and Animal Husbandry Development Company (29°58'52"N, 91°16'47"E,) in Lhundup, Lhasa City, Tibet Autonomous Region, with having average altitude of 4200 m (Fig. 9). Forty PWFs (80 ± 15 days postpartum, Fig. 10) were equally divided into a control group and three test groups randomly. Each PWF was fed 5 kg on dry matter basis composed of alfalfa hay 30.0%, oat hay 20.0%, corn 30.5%, wheat bran 12.0%, soybean meal 1.5%, cottonseed meal 1.0%, rapeseed oil 1% and 4% premix with DM 88.03%, ME 9.85%, CP 11.67%, NDF 31.83%, ADF 18.50%, Ca 0.88% and P 0.60% per day in the control group (DH group), and each PWF in three experimental group DE, DF and DG, was fed 5 kg basal diet with 5% TCHF1, TCHF2 and TCHF3, respectively. Environmental conditions such as enclosure, lighting, and ventilation were kept consistent across all groups (Table 5).

Animals were allowed to 7-day adaptation period followed by a 7-day pre-test period. During the pre-test period 1/4th of the test dose of TCHFs was administered to three treatment groups from day 1 to 4 and 1/2 of the test dose was administered from day 5 to 7, to get an idea about the safety of TCHFs by observing adverse reactions. After the TCHFs were safe, a test dose (5% TCHFs) was administered for 30 days. After fine mixing of TCHFs in basal feed, the feed was offered twice daily: 9:30 am and 3:00 pm daily, with ad libitum water.

Ethics approval statement

All procedures performed in this research were approved by the Laboratory Animal Welfare and Ethics Committee of the Institute of Animal Husbandry and Veterinary Medicine, Tibet Academy of Agriculture and Animal Husbandry Science, and the ethics committee of Nanjing Agricultural University (NJAU.No20220305025). All methods were, carried out by relevant guidelines and regulations. This study followed ARRIVE guidelines. All relevant experimental details, including study design, sample size determination, randomization, and blinding, have been reported per the ARRIVE checklist.

Formula Code	Common Name	Herbal Ingredient	Latin Name (with Authority and Family)	Amount Used	Probable Composition (Key Constituents)	Role in Formula	Growing Conditions and Source Details
TCHF1	Huang Qin	Baical Skullcap Root	*Scutellaria baicalensis* Georgi [Lamiaceae]	15 g	Baicalin, Baicalein, Wogonin, Flavonoids	Anti-inflammatory, antioxidant, antibacterial, Regulating the Qi and Blood	Grown in Anhui Province, following GAP standards; purchased from Bozhou Herbal Medicine Market
	Zang Dang Shen	Hairy Codonopsis Herb	*Codonopsis mollis* Nannf. [Campanulaceae]	15 g	Polysaccharides, Saponins, Flavonoids, Alkaloids	Immunomodulatory, tonifying Qi and spleen enhancing vital energy	Grown in Tibet Province, following GAP standards; purchased from Bozhou Herbal Medicine Market
	Huang Qi	Astragalus Root	*Astragalus membranaceus* (Fisch.) Bunge [Fabaceae]	15 g	Astragaloside IV, Flavonoids, Polysaccharides	Qi tonifier, immunomodulatory, anti-inflammatory	Grown in Shanxi Province, following GAP standards; purchased from Bozhou Herbal Medicine Market
	Bai Shao	White Peony Root	*Paeonia lactiflora* Pall. [Ranunculaceae]	15 g	Paeoniflorin, Albiflorin, Monoterpenes, Phenolic compounds	Anti-spasmodic, nourishing blood, anti-inflammatory	Grown in Anhui Province, following GAP standards; purchased from Bozhou Herbal Medicine Market
	Gan Cao	Licorice Root	*Glycyrrhiza uralensis* Fisch. ex DC. [Fabaceae]	10 g	Glycyrrhizic acid, Liquiritin, Flavonoids, Isoflavonoids	Harmonizer/enhancer, tonic for Qi and spleen	Grown in Gansu Province, following GAP standards; purchased from Bozhou Herbal Medicine Market
TCHF2	Gou Qi Zi	Wolfberry Fruit	*Lycium barbarum* L. [Solanaceae]	15 g	Polysaccharides, Betaine, Zeaxanthin	Antioxidant/anti-aging	Grown in Ningxia Province, following GAP standards; purchased from Bozhou Herbal Medicine Market
	Dang Gui	Chinese Angelica Root	*Angelica sinensis* (Oliv.) Diels [Apiaceae]	15 g	Polysaccharides, Flavonoids, Zeaxanthin, Carotenoids	Blood circulation enhancer, immunomodulatory, boosting Qi, tonifying blood	Grown in Gansu Province, following GAP standards; purchased from Bozhou Herbal Medicine Market
	Zang Dang Shen	Hairy Codonopsis Herb	*Codonopsis mollis* Nannf. [Campanulaceae]	15 g	Polysaccharides, Saponins, Flavonoids, Akaloids	Immunomodulatory, tonifying Qi and spleen, enhancing Vital Energy	Grown in Tibet Province, following GAP standards; purchased from Bozhou Herbal Medicine Market
	Bai Zhu	Largehead Atractylodes Rhizome	*Atractylodes macrocephala* Koidz. [Asteraceae]	15 g	Atractylenolides, Polysaccharides, Sterols, Essential Oils	Digestive aid, Qi tonifier, immune enhancement	Grown in Anhui Province, following GAP standards; purchased from Bozhou Herbal Medicine Market
	Huang Qin	Baical Skullcap Root	*Scutellaria baicalensis* Georgi [Lamiaceae]	15 g	Baicalin, Baicalein, Wogonin, Flavonoids	Anti-inflammatory, antioxidant, antibacterial, regulating the Qi and blood	Grown in Anhui Province, following GAP standards; purchased from Bozhou Herbal Medicine Market
	Gan Cao	Licorice Root	*Glycyrrhiza uralensis* Fisch. ex DC. [Fabaceae]	10 g	Glycyrrhizic acid, Liquiritin, Flavonoids, Isoflavonoids	Harmonizer/enhancer, tonic for Qi and spleen	Grown in Gansu Province, following GAP standards; purchased from Bozhou Herbal Medicine Market

Continued

Formula Code	Common Name	Herbal Ingredient	Latin Name (with Authority and Family)	Amount Used	Probable Composition (Key Constituents)	Role in Formula	Growing Conditions and Source Details
TCHF3	Zhu Ma Gen	Ramie Root	*Boehmeria nivea* (L.) Gaudich. [Urticaceae]	50 g	Flavonoids, Phenolic acids, Tannins	Hemostatic, strengthening the tendons and bones, promoting circulation	Grown in Hubei Province, following GAP standards; purchased from Bozhou Herbal Medicine Market
	Chuan Xiong	Szechuan Lovage Rhizome	*Ligusticum chuanxiong* Hort. [Apiaceae]	20 g	Ligustilide, Ferulic acid, Essential oils, Polysaccharides	Blood circulation enhancer, regulating Qi, promoting menstrual flow	Grown in Sichuan Province, following GAP standards; purchased from Bozhou Herbal Medicine Market
	Shu Di Huang	Prepared Rehmannia Root	*Rehmannia glutinosa* (Gaertn.) DC. [Orobanchaceae]	50 g	Catalpol, Acteoside, Rehmanniosides, Stachyose	Nourishing yin, tonifying blood, antioxidant	Grown in Henan Province, following GAP standards; purchased from Bozhou Herbal Medicine Market
	Bai Shao	White Peony Root	*Paeonia lactiflora* Pall. [Ranunculaceae]	30 g	Paeoniflorin, Albiflorin, Monoterpenes, Phenolic compounds	Anti-spasmodic, nourishing blood, anti-inflammatory	Grown in Anhui Province, following GAP standards; purchased from Bozhou Herbal Medicine Market
	Huang Qi	Milkvetch Root	*Astragalus membranaceus* (Fisch.) Bunge [Fabaceae]	30 g	Astragaloside IV, Flavonoids, Polysaccharides	Qi tonifier, immunomodulatory, anti-inflammatory	Grown in Shanxi Province, following GAP standards; purchased from Bozhou Herbal Medicine Market
	Zang Dang Shen	Hairy Codonopsis Herb	*Codonopsis mollis* Nannf. [Campanulaceae]	40 g	Polysaccharides, Saponins, Flavonoids, Akaloids	Immunomodulatory, tonifying Qi and spleen, enhancing vital energy	Grown in Tibet Province, following GAP standards; purchased from Bozhou Herbal Medicine Market
	Bai Zhu	Largehead Atractylodes Rhizome	*Atractylodes macrocephala* Koidz. [Asteraceae]	60 g	Atractylenolides, Polysaccharides, Sterols, Essential Oils	Digestive aid, Qi tonifier, immune enhancement	Grown in Anhui Province, following GAP standards; purchased from Bozhou Herbal Medicine Market
	Zhi Shi	Bitter Orange Fruit	*Citrus aurantium* L. [Rutaceae]	30 g	Flavonoids, Synephrine, Alkaloids, Volatile Oils,	Digestive aid, promoting Qi flow, cardiovascular support	Grown in Hunan Province, following GAP standards; purchased from Bozhou Herbal Medicine Market
	Chen Pi	Dried Tangerine Peel	*Citrus reticulata* Blanco [Rutaceae]	30 g	Hesperidin, Polymethoxyflavones, Naringin	Digestive aid, Qi regulator, antioxidant	Grown in Guangdong Province, following GAP standards; purchased from Bozhou Herbal Medicine Market
	Huang Qin	Baical Skullcap Root	*Scutellaria baicalensis* Georgi [Lamiaceae]	30 g	Baicalin, Baicalein, Wogonin, Flavonoids	Anti-inflammatory, antioxidant, antibacterial, regulating the Qi and blood	Grown in Anhui Province, following GAP standards; purchased from Bozhou Herbal Medicine Market
	Zi Su Ye	Perilla Leaf	*Perilla frutescens* (L.) Britton [Lamiaceae]	30 g	Perillaldehyde, Rosmarinic acid, Limonene	Anti-inflammatory, dispersing cold and relieving the exterior, regulating Qi	Grown in Anhui Province, following GAP standards; purchased from Bozhou Herbal Medicine Market
	Ai Ye	Mugwort Leaf	*Artemisia princeps* Pamp. [Asteraceae]	20 g	Essential oils, Flavonoids, Sesquiterpenes, Coumarins	Dispersing cold, warming the uterus, anti-inflammatory	Grown in Anhui Province, following GAP standards; purchased from Bozhou Herbal Medicine Market
	Gan Cao	Licorice Root	*Glycyrrhiza uralensis* Fisch. ex DC. [Fabaceae]	20 g	Glycyrrhizic acid, Liquiritin, Flavonoids, Isoflavonoids	Harmonizer/enhancer, tonic for Qi and spleen	Grown in Gansu Province, following GAP standards; purchased from Bozhou Herbal Medicine Market

Table 4. Composition of three TCHFs, detailed ingredients's description, their functions and growing conditions.

Sample collection

Samples were obtained from the group DE, DF, DG, and DH (control) on the 15th and 30th days of the experiment considering each animal in the given group (classified as DEZ1 and DEZ2, DFZ1 and DFZ2, DGZ1 and DGZ2, DHZ1 and DHZ2, for group DE, DF, DG and DH, respectively). Blood samples from the jugular vein were collected from all experimental yaks, subsequently, centrifuged at 1378 xg for 15 min to separate the serum from cellular components of blood, and then serum was stored at -20℃. Also, in the morning before feeding, 50mL of rumen fluid was collected from each yak's rumen using an oral stomach tube (OST) on days 15th and 30th. The rumen fluid was filtered through sterilized 4-layer gauze, aliquoted, and stored at -80℃ for further analysis.

Estimation of inflammatory mediators and antioxidant enzyme levels in serum

Using the serum samples in ELISA kits, levels of inflammatory mediators including IL-6 (Bovine-IL-6, ml064296-2, Shanghai Enzyme-linked Biotechnology Co., Ltd., China, Shanghai), IL-10 (Bovine-IL-10, ml002476-2, Shanghai Enzyme-linked Biotechnology Co., Ltd., China, Shanghai), IL-1β (Bovine- IL-1β, ml064295-2, Shanghai Enzyme-linked Biotechnology Co., Ltd., China, Shanghai) and TNF-α (Bovine-TNFα, ml077389-2, Shanghai Enzyme-linked Biotechnology Co., Ltd., China, Shanghai). Using specific assay kits the activities of SOD (A001-3-2, Nanjing Jiancheng Bioengineering Institute, China, Nanjing), MDA (A003-1-2, Nanjing Jiancheng Bioengineering Institute, China, Nanjing), GSH-Px (A005-1-2, Nanjing Jiancheng Bioengineering Institute, China, Nanjing) and T-AOC (A015-1-2, Nanjing Jiancheng Bioengineering Institute, China, Nanjing) were estimated by using assay kits.

DNA extraction and 16 S rRNA sequencing analysis

Twelve rumen fluid samples (6 collected on the 15th and 6 collected on the 30th day; 6 samples collected from each DEZ1, DEZ2, DFZ1, DFZ2, DGZ1, DGZ2, DHZ1, and DHZ2 group, respectively) were randomly

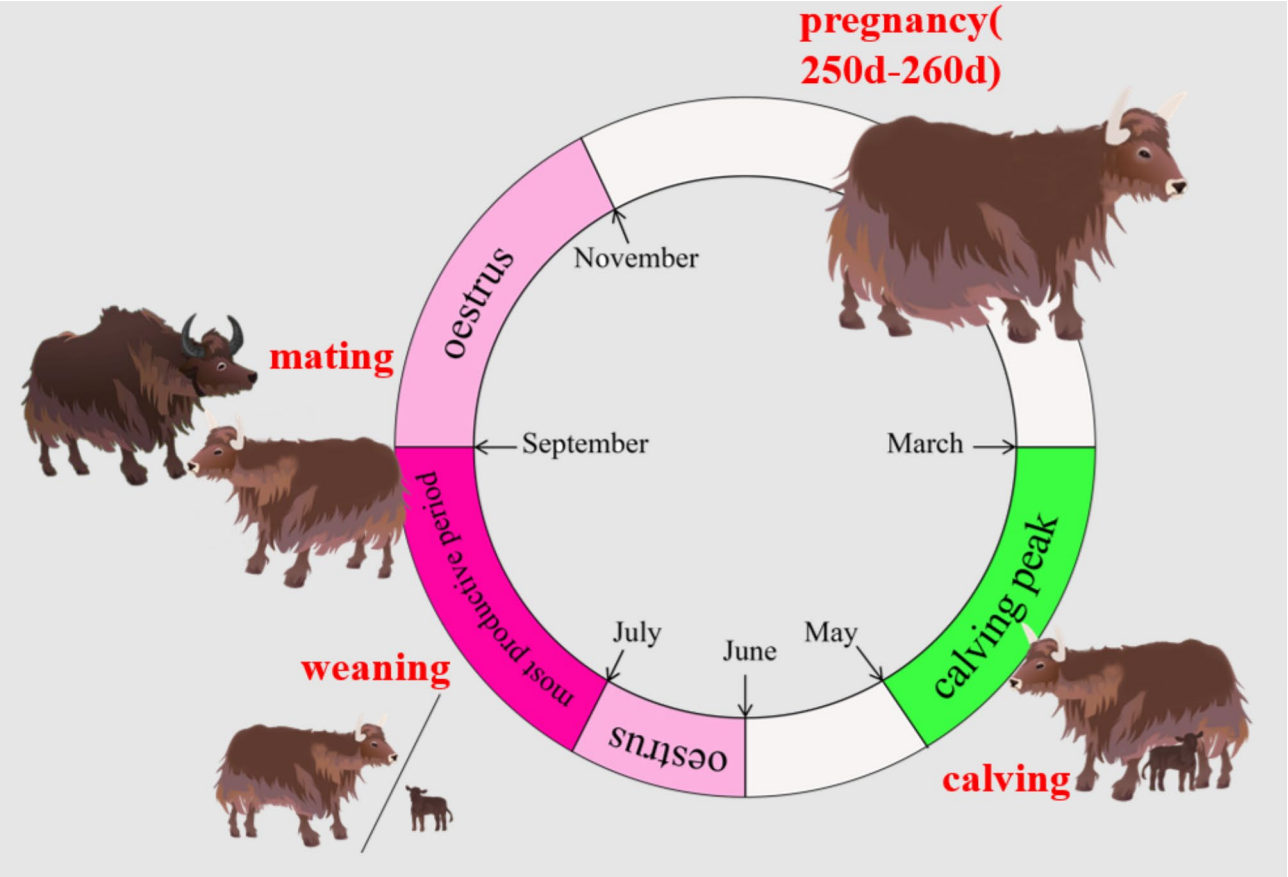

Fig. 9. This diagram illustrates the key stages in the reproductive cycle of yaks over a year, offering a comprehensive overview of yak breeding and management. The cycle includes periods of estrus (heat), mating, pregnancy, calving, and weaning, along with the peak calving period and most productive time of the year. Mating occurs following the estrus period in late autumn. Pregnancy in yaks lasts approximately 250–260 days. The calving peak occurs in spring, followed by the weaning of calves.The period between summer and early autumn represents the most productive time for yaks.

selected from each group (DE, DF, DG, and DH) for 16 S rRNA sequencing analysis. Total DNA was extracted from 48 frozen rumen fluid samples DNA Kit (Solarbio Science & Technology Co., Ltd.) and tested the purity and DNA was quantified using Nanodrop. Hypervariable regions (V3/V4) were amplified by specific bacterial 16 S rRNA gene primers; the forward primer (5'-ACTCCTACGGGAGGCAGCA-3') and the reverse primer (5'-GGACTACHVGGGTWTCTAAT-3'), with high-fidelity DNA polymerase[75,76] provided by Beijing Quanshijin Biotechnology Co., Ltd.

The PCR reaction mixture for each amplification reaction consisted of 12.5μL PCR Mix, 1.0μL DNA, 1.0μL of each forward and reverse primers, 9.5μL dd.H2O, with a total reaction volume of 25μL. PCR amplification was consisting a total of 35 PCR cycles, with each cycle having an initial hot start pre-denaturation temperature of 95℃ (5 m), then 95℃(15 s); the primer annealing temperature Tm was 50℃ (15s); the elongation at 72℃ (45s) followed by extension at 72℃ (10 m) and finally stored at 4℃. After performing PCR, the resultant amplified PCR products were assessed using 2% agarose gel electrophoresis. PCR products then, were purified and recovered with magnetic beads (Vazyme VAHTSTM DNA Clean Beads).

Referring to the preliminary quantitation of the electrophoresis, then detected and quantified PCR amplification recovered products (PCR amplified products were quantified with the Quant-iT PicoGreen dsDNA Assay Kit and the Microplate reader (BioTek, FLx800) were mixed in corresponding ratios following the sequencing requirements of each sample. The library was constructed via the Illumina Novaseq 6000 sequencing platform; TruSeq Nano DNA LT Library Prep Kit from Illumina (Illumina Software; Version1.3)[77], was examined and then sequenced after it passed the quality assessment (the library was quality-checked on the Agilent Bioanalyzer, using the Agilent High Sensitivity DNA Kit). The library was quantified on the Promega QuantiFluor fluorescence quantification system with the Quant-iTPicoGreen dsDNA Assay Kit. Pre-processed paired-end reads were performed with a MiSeq sequencer using the corresponding reagent (reagent is MiSeq Reagent Kit V3 (600 cycles)) to detect and remove the adapter.

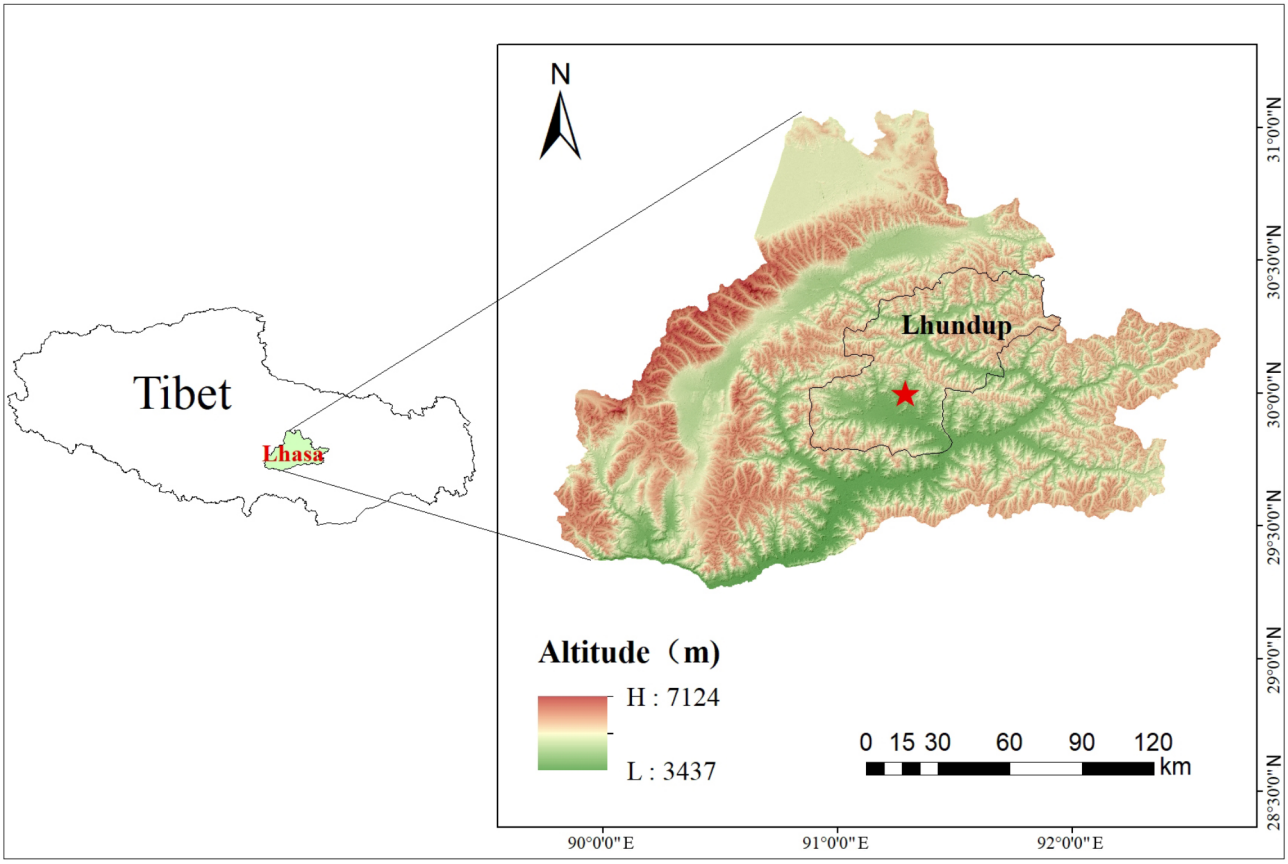

Fig. 10. The inset map demonstrates the location of Lhasa within Tibet, underscoring the regional context. The main panel presents a detailed topographic map of Lhundup County, delineated by a black border, highlighting the mountainous terrain and geographical positioning of the study area.

Parameter		Groups			
		DE(TCHF1)	DF(TCHF2)	DG(TCHF3)	DH (control)
Feed	Basic diet(per/kg) (alfalfa hay 30.0%, oat hay 20.0%, corn 30.5%, wheat bran 12.0%, soybean meal 1.5%, cottonseed meal 1.0%, rapeseed oil 1% and 4% premix with DM 88.03%, ME 9.85%, CP 11.67%, NDF31.83%, ADF 18.50%, Ca 0.88% and P 0.60%)	4.75	4.75	4.75	5
	TCHF additive (kg/per) (TCHF1/TCHF2/TCHF3)	0.25 (TCHF1)	0.25(TCHF2)	0.25(TCHF3)	/
	Feed intake total (kg/per)	5	5	5	5
	Water	sufficient	sufficient	sufficient	sufficient
Environmental conditions	Farming pattern (Intensive/Pastoral)	Intensive	Intensive	Intensive	Intensive
	Temperature (℃)	7–21	7–21	7–21	7–21
	Humidity (%)	65–73	65–73	65–73	65–73
	Light/Dark cycle (hours)	13:11	13:11	13:11	13:11

Table 5. Basal feed composition and environmental conditions for all experimental Yaks.

Bioinformatics and functional analysis

After high-throughput screening, the original sequencing information was in FASTQ form. The paired-end reads were then preprocessed with cutadapt software to check and remove the adapter. After paired-end reads filtered low-quality sequences, denoised, and merged after trimming, and Divisive Amplicon Denoising Algorithm 2 (DADA2; Version: 1.26.0) was used to detect and cut off chimera reads. Due to the short reading length of MiSeq sequencing and to ensure the quality of sequencing, the optimal sequencing length of the target fragment was 200–450 bp. Finally, the software generated the representative readings and ASV abundance graphs.

After choosing exemplary sequences for each ASV using the Quantitative Insights Into Microbial Ecology (QIIME 2 Version: 2024.10) software package, all representative sequences were annotated and blasted against Silva database Version 138, utilizing classify-sklearn (Version: 1.6.1) with the standard parameters. According

to taxonomic data, a statistical evaluation of microbial structure was carried out[78]. Statistical Analysis of Metagenomic Profiles (STAMP; Version: 2.1.3) and LefSe (Version: 1.0) differential analysis were employed to assess the abundance of bacterial and fungal species. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2; Version: 2.6.) was used to predict the metabolomics based on the marker gene (16 S/ITS) sequence[79]. The ANOSIM and Wilcoxon tests were employed to determine variations among treatments.

Alpha diversity (α-diversity), including species accumulation box charts, rank abundance curves, and a range of statistical analysis metrics were used to evaluate differences in microbial species' richness and diversity among samples. PCoA and NMDS served as typical statistical methods regarding beta diversity (β-diversity) to comparatively analyze the microbial community composition among different samples.

Statistical analysis

Using GraphPad Prism (Version: 8.0) and SPSS (Version: 26.0), the experimental data were analyzed via Student's t-test, chi-square test, ANOVA, Kruskal-Wallis test, and Dunn's test, with P-value < 0.05 was considered to be statistically significant.

Data availability

The datasets generated and/or analyzed during the current study are available in the NCBI Sequence Read Archive (SRA) database under accession number: PRJNA1139658, (https://submit.ncbi.nlm.nih.gov/subs/sra/SUB14621292/overview)

Received: 17 December 2024; Accepted: 19 February 2025
Published online: 28 May 2025

References

1. Wen, Y. et al. Intestinal microbial diversity of free-range and captive Yak in Qinghai Province. *Microorganisms* **10**, 754 (2022).
2. Wiener, G., JianLin, H. & Ruijun, L. The Yak second edition. *Food Agric. Organ. United Nations Reg. Off Asia Pac. Bangkok Thail.* (2003).
3. Pang, K. et al. Ruminal microbiota and muscle metabolome characteristics of Tibetan plateau Yaks fed different dietary protein levels. *Front. Microbiol.* **15**, 1275865 (2024).
4. Wang, W. et al. Linkages between rumen microbiome, host, and environment in Yaks, and their implications for Understanding animal production and management. *Front. Microbiol.* **15**, (2024).
5. Wang, H. et al. A global analysis of CNVs in diverse Yak populations using whole-genome resequencing. *BMC Genom.* **20**, 61 (2019).
6. Yang, X. et al. The role of traditional Chinese medicine in the regulation of oxidative stress in treating coronary heart disease. *Oxid. Med. Cell. Longev.* 3231424 (2019). (2019).
7. Xu, Q., Zhang, B., Li, X. & Gao, X. Traditional Chinese medicine formula Qing Huo Yi Hao as superoxide anion scavenger in high glucose-treated endothelial cells. *Acta Pharmacol. Sin.* **33**, 496–502 (2012).
8. Prieto, J. M. & Schinella, G. R. Anti-inflammatory and antioxidant Chinese herbal medicines: links between traditional characters and the skin lipoperoxidation Western model. *Antioxidants* **11**, 611 (2022).
9. Liu, J. et al. Anti-Inflammatory Effects of the Chinese Herbal Formula Sini Tang in Myocardial Infarction Rats. *Evidence-Based Complement. Altern. Med.* 309378 (2014). (2014).
10. Liu, H. et al. Discovery of anti-inflammatory ingredients in Chinese herbal formula Kouyanqing granule based on relevance analysis between chemical characters and biological effects. *Sci. Rep.* **5**, 18080 (2015).
11. Sittipo, P., Lobionda, S., Lee, Y. K. & Maynard, C. L. Intestinal microbiota and the immune system in metabolic diseases. *J. Microbiol.* **56**, 154–162 (2018).
12. Negi, S., Das, D. K., Pahari, S., Nadeem, S. & Agrewala, J. N. Potential role of gut microbiota in induction and regulation of innate immune memory. *Front. Immunol.* **10**, 2441 (2019).
13. Liu, Z., Li, J. & Niu, K. Biodiversity in mosaic communities: soil microbial diversity associates with plant functional groups relating to soil available phosphorus in Tibetan alpine meadow. *Eur. J. Soil. Biol.* **116**, 103479 (2023).
14. Arshad, M. A. et al. Gut Microbiome colonization and development in neonatal ruminants: strategies, prospects, and opportunities. *Anim. Nutr.* **7**, 883–895 (2021).
15. Liao, H., Banbury, L. K. & Leach, D. N. Antioxidant activity of 45 Chinese herbs and the relationship with their TCM characteristics. *Evidence-based Complement. Altern. Med.* **5**, 429–434 (2008).
16. Beecher, M. et al. Gastrointestinal tract size, total-tract digestibility, and rumen microflora in different dairy cow genotypes. *J. Dairy. Sci.* **97**, 3906–3917 (2014).
17. Liu, C. et al. Dynamic alterations in Yak rumen bacteria community and metabolome characteristics in response to feed type. *Front. Microbiol.* **10**, 1116 (2019).
18. Salam, M. A. et al. Antimicrobial resistance: a growing serious threat for global public health. in *Healthcare* 11, 1946. (MDPI, 2023).
19. Organization, W. H. WHO bacterial priority pathogens list, 2024. *World Heal. Organ.* (2024).
20. Venditto, V. J. et al. Immunomodulatory effects of Azithromycin revisited: potential applications to COVID-19. *Front. Immunol.* **12**, 574425 (2021).
21. Cristofori, F. et al. Anti-inflammatory and Immunomodulatory effects of probiotics in gut inflammation: a door to the body. *Front. Immunol.* **12**, 578386 (2021).
22. Velho, T. R. et al. Immunomodulatory effects and protection in sepsis by the antibiotic Moxifloxacin. *Antibiotics* **13**, 742 (2024).
23. Zi, X. D. Reproduction in female Yaks (*Bos grunniens*) and opportunities for improvement. *Theriogenology* **59**, 1303–1312 (2003).
24. Arthington, J. D. & Minton, J. E. The effect of early calf weaning on feed intake, growth, and postpartum interval in thin, brahman-crossbred primiparous cows. *Prof. Anim. Sci.* **20**, 34–38 (2004).
25. Liu, P. et al. Postpartum oestrous cycling resumption of Yak cows following different calf weaning strategies under range conditions. *Anim. Sci. J.* **89**, 1492–1503 (2018).
26. Arulselvan, P. et al. Role of antioxidants and natural products in inflammation. *Oxid. Med. Cell. Longev.* (2016). (2016).
27. Li, L. et al. Multi-omics analysis and the remedial effects of Swertiamarin on hepatic injuries caused by CCl4. *Ecotoxicol. Environ. Saf.* **282**, 116734 (2024).
28. Lee, S. E. & Park, Y. S. The emerging roles of antioxidant enzymes by dietary phytochemicals in vascular diseases. *Life* **11**, 199 (2021).

29. Zhou, Z. J. *The Effectiveness of Acupuncture and Herbal Medicine Combined Treatment for Female Infertility With Polycystic Ovary Syndrome*. (A Narrative Review, 2022).
30. Miao, K., Liu, W., Xu, J., Qian, Z. & Zhang, Q. Harnessing the power of traditional Chinese medicine monomers and compound prescriptions to boost cancer immunotherapy. *Front. Immunol.* **14**, (2023).
31. Jiang, M. H. et al. Effects of enzymolysis and fermentation of Chinese herbal medicines on serum component, egg production, and hormone receptor expression in laying hens. *Anim. Biosci.* **37**, 95–104 (2024).
32. Balahoroğlu, R., Dülger, H., Özbek, H., Bayram, İ. & Şekeroğlu, M. R. Protective effects of antioxidants on the experimental liver and kidney toxicity in mice. *Electron. J. Gen. Med.* **5**, 157–164 (2008).
33. Du, N. et al. Combination of ginsenoside Rg1 and Astragaloside IV reduces oxidative stress and inhibits TGF-β1/Smads signaling cascade on renal fibrosis in rats with diabetic nephropathy. *Drug Des. Devel Ther.* **12**, 3517–3524 (2018).
34. Cordiano, R. et al. Malondialdehyde as a potential oxidative stress marker for allergy-oriented diseases: an update. *Molecules* **28**, 5979 (2023).
35. Halliwell, B. & Gutteridge, J. M. C. *Free Radicals in Biology and Medicine*. https://doi.org/10.1093/acprof:oso/9780198717478.001.0001 (Oxford University Press, 2015).
36. Anqi et al. Protective effect of lentinan against LPS-induced injury in mice via influencing antioxidant enzyme activity, inflammatory pathways and gut microbiota. *Pakistan Vet. J. J.* **44**, 647–665 (2024).
37. Diepenhorst, N. A. et al. High throughput, quantitative analysis of human osteoclast differentiation and activity. *Anal. Biochem.* **519**, 51–56 (2017).
38. Huang, Z. et al. Application of natural antioxidants from traditional Chinese medicine in the treatment of spinal cord injury. *Front. Pharmacol.* **13**, (2022).
39. Biswas, S. K. Does the interdependence between oxidative stress and inflammation explain the antioxidant paradox? *Oxid. Med. Cell. Longev.* (2016). (2016).
40. Gambini, J. & Stromsnes, K. Oxidative stress and inflammation: from mechanisms to therapeutic approaches. *Biomedicines* **10**, 753 (2022).
41. González-Carnicero, Z. et al. Regulation by Nrf2 of IL-1β-induced inflammatory and oxidative response in VSMC and its relationship with TLR4. *Front. Pharmacol.* **14**, (2023).
42. Pan, C. et al. Dietary supplementation with *Bupleuri Radix* reduces oxidative stress occurring during growth by regulating rumen microbes and metabolites. *Animals* **14**, 927 (2024).
43. Wang, Y. et al. Effects of *Lycium Barbarum* polysaccharides on immunity and the gut microbiota in cyclophosphamide-induced immunosuppressed mice. *Front. Microbiol.* **12**, (2021).
44. Liu, Y. et al. Rumen Microbiome and metabolome of high and low residual feed intake Angus heifers. *Front. Vet. Sci.* **9**, (2022).
45. Neelam, K., Dey, S., Sim, R. & Lee, J. Au Eong, K.-G. Fructus lycii: A natural dietary supplement for amelioration of retinal diseases. *Nutrients* **13**, 246 (2021).
46. Zhang, S. et al. Baicalin promotes appetite by regulating gut Microbiome and immunity? *J. Funct. Foods.* **105**, 105557 (2023).
47. Pinnell, L. J. et al. Bacteroidetes and Firmicutes drive differing microbial diversity and community composition among micro-environments in the bovine rumen. *Front. Vet. Sci.* **9**, (2022).
48. Gharechahi, J. et al. Lignocellulose degradation by rumen bacterial communities: new insights from metagenome analyses. *Environ. Res.* **229**, 115925 (2023).
49. Zhang, R., Ye, H., Liu, J. & Mao, S. High-grain diets altered rumen fermentation and epithelial bacterial community and resulted in rumen epithelial injuries of goats. *Appl. Microbiol. Biotechnol.* **101**, 6981–6992 (2017).
50. Brulc, J. M. et al. Gene-centric metagenomics of the fiber-adherent bovine rumen microbiome reveals forage specific glycoside hydrolases. *Proc. Natl. Acad. Sci.* 106, 1948–1953 (2009).
51. Omondi, V. O. et al. Multi-omics analyses reveal rumen microbes and secondary metabolites that are unique to livestock species. *mSystems* 9, (2024).
52. Lee, J. et al. Gut microbiota–derived short-chain fatty acids promote poststroke recovery in aged mice. *Circ. Res.* **127**, 453–465 (2020).
53. Moniri, N. H. & Farah, Q. Short-chain free-fatty acid G protein-coupled receptors in colon cancer. *Biochem. Pharmacol.* **186**, 114483 (2021).
54. Liu, L. et al. Research progress on the regulation of production traits by Gastrointestinal microbiota in dairy cows. *Front. Vet. Sci.* **10** (2023).
55. Spence, C., Wells, W. G. & Smith, C. J. Characterization of the primary starch utilization Operon in the obligate anaerobe *Bacteroides fragilis*: regulation by carbon source and oxygen. *J. Bacteriol.* **188**, 4663–4672 (2006).
56. Den Besten, G. et al. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *J. Lipid Res.* **54**, 2325–2340 (2013).
57. Liu, W. et al. Comparison of gut microbiota of Yaks from different geographical regions. *Front. Microbiol.* **12** (2021).
58. Guo, T. et al. Rumen bacteria abundance and fermentation profile during subacute ruminal acidosis and its modulation by *Aspergillus oryzae* culture in RUSITEC system. *Fermentation* **8**, 329 (2022).
59. Derrien, M., Belzer, C. & de Vos, W. M. *Akkermansia muciniphila* and its role in regulating host functions. *Microb. Pathog.* **106**, 171–181 (2017).
60. Qi, Y. et al. High-throughput sequencing provides insights into oral microbiota dysbiosis in association with inflammatory bowel disease. *Genomics* **113**, 664–676 (2021).
61. Zhao, L. et al. Effects of nitrate addition on rumen fermentation, bacterial biodiversity and abundance. *Asian-Australasian J. Anim. Sci.* **28**, 1433–1441 (2015).
62. Wu, R. et al. Research progress in isolation and identification of rumen probiotics. *Front. Cell. Infect. Microbiol.* **14** (2024).
63. Guo, J. et al. Characterization of the rumen microbiota and volatile fatty acid profiles of weaned goat kids under shrub-grassland grazing and indoor feeding. *Animals* **10**, 176 (2020).
64. Camanocha, A. & Dewhirst, F. E. Host-associated bacterial taxa from Chlorobi, Chloroflexi, GN02, synergistetes, SR1, TM7, and WPS-2 phyla/candidate divisions. *J. Oral Microbiol.* **6**, 25468 (2014).
65. Guo, C. et al. Effects of different forage types on rumen fermentation, microflora, and production performance in peak-lactation dairy cows. *Fermentation* **8**, 507 (2022).
66. Gagen, E. J., Padmanabha, J., Denman, S. E. & McSweeney, C. S. Hydrogenotrophic culture enrichment reveals rumen Lachnospiraceae and Ruminococcaceae acetogens and hydrogen-responsive Bacteroidetes from pasture-fed cattle. *FEMS Microbiol. Lett.* **362**, fnv104 (2015).
67. Tian, H. et al. Depletion of butyrate-producing microbes of the Firmicutes predicts nonresponse to FMT therapy in patients with recurrent *Clostridium difficile* infection. *Gut Microbes* **15**, (2023).
68. Soliman, M. M., Ahmed, M. M., Salah-eldin, A. & Abdel-Aal, A. A.-A. Butyrate regulates leptin expression through different signaling pathways in adipocytes. *J. Vet. Sci.* **12**, 319 (2011).
69. Amato, K. R. et al. The role of gut microbes in satisfying the nutritional demands of adult and juvenile wild, black howler monkeys (< scp > *A Louatta pigra*). *Am. J. Phys. Anthropol.* **155**, 652–664 (2014).
70. Scarpellini, E. et al. The human gut microbiota and Virome: potential therapeutic implications. *Dig. Liver Dis.* **47**, 1007–1012 (2015).

71. Huuki, H. et al. Long-term effects of early-life rumen microbiota modulation on dairy cow production performance and methane emissions. *Front. Microbiol.* **13**, (2022).
72. Stevenson, D. M. & Weimer, P. J. Dominance of *Prevotella* and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quantification real-time PCR. *Appl. Microbiol. Biotechnol.* **75**, 165–174 (2007).
73. Cui, Y. et al. Chinese herbal formula (CHF03) attenuates non-alcoholic fatty liver disease (NAFLD) through inhibiting lipogenesis and anti-oxidation mechanisms. *Front. Pharmacol.* **10**, (2019).
74. Wu, Y. C. & Hsieh, C. L. Pharmacological effects of *Radix Angelica sinensis* (Danggui) on cerebral infarction. *Chin. Med.* **6**, 32 (2011).
75. Patel, J. B. 16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory. *Mol. Diagnosis* **6**, 313–321 (2001).
76. Geng, J. et al. 16S rRNA gene sequencing reveals specific gut microbes common to medicinal insects. *Front. Microbiol.* **13**, (2022).
77. Lundberg, D. S., Yourstone, S., Mieczkowski, P., Jones, C. D. & Dangl, J. L. Practical innovations for high-throughput amplicon sequencing. *Nat. Methods* **10**, 999–1002 (2013).
78. Lozupone, C. A., Hamady, M., Kelley, S. T. & Knight, R. Quantitative and qualitative B diversity measures lead to different insights into factors that structure microbial communities. *Appl. Environ. Microbiol.* **73**, 1576–1585 (2007).
79. Douglas, G. M. et al. PICRUSt2 for prediction of metagenome functions. *Nat. Biotechnol.* **38**, 685–688 (2020).

Acknowledgements

The work was supported by the Nagqu city major science and technology project (NQKJ-2023-10), the Department of Science and Technology of the Tibet Autonomous Region (XZ202101 ZD0002N), the breeding and efficient propagation of yaks in Gesangtang in Linzhou county (QYXTZX-LS2020-01), and the Ministry of Agriculture and Rural Affairs of China (CARS-37).

Author contributions

Author contributions statementY.B.Z., K.L. M.A. and W.D.B.S.- Conceptualization and Methodology. Y.B.Z., J.W., X.J.Z., Y.J.C.D., H.Z.W., M.A., and P.D.P.B- Methodology, Formal Analysis, Software. Y.B.Z., J.W., M.A., and KL- Data curation, Writing–original draft, Writing–review and editing, Validation. K.L. and W.D.B.S.- Writing–original draft, Supervision, Project administration, Funding acquisition, Resources, Validation. W.D.B.S-Visualization and Supervision. All authors reviewed and approved the final manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Correspondence and requests for materials should be addressed to K.L. or W.B.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.