Effect of dietary concentrate to forage ratio on growth performance, rumen fermentation and bacterial diversity of Tibetan sheep under barn feeding on Qinghai-Tibetan plateau

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This study aimed to research the effects of different dietary concentrate to forage (C:F) ratio on growth performance, rumen fermentation and bacteria diversity of barn feeding Tibetan sheep. The experiment contains fiver treatments (HS1, HS2 HS3, HS4 and HS5; n=8, respectively) based on dietary C: F ratios 0:100, 15:85, 30:70, 45:55, and 60:40, respectively. The ruminal bacterial community structure was investigated through high-throughput sequencing of 16S rRNA genes in V4 hypervariable region. The results showed that increasing dietary concentrate feed level from 0% to 60% exerted a positive effect on DMI, BW gain, gain rate and feed conversation ratio (F_{CR}) in Tibetan sheep. The increases dietary concentrate feed level elevatedNH_3-N, propionate and valerate concentrations, whereas, reduced molar ratio of acetate to propionate (A/P ratio) ($P<0.05$). For rumen bacterial diversity, increases in dietary concentrate content contributed to lower alpha diversity indexes including Shannon wiener, Chao1 and observed species, meanwhile, significantly increased the abundances of the phylum *Bacteroidetes* and the genus *Prevotella_1* ($P<0.05$). In conclusion, increases dietary concentrate content improved the growth performance and Tibetan sheep fed diets of 45% concentrate obtained a better performance; the inclusion of concentrate in feed changed rumen fermentation from acetate fermentation to propionate fermentation, and improved the energy utilization efficiency of Tibetan sheep; the increased in concentrate content significantly reduced rumen bacteria diversity and changed the abundance of some core bacteria.

1   **Effect of dietary concentrate to forage ratio on growth performance, rumen fermentation**

2   **and bacterial diversity of Tibetan sheep under barn feeding on Qinghai-Tibetan plateau**

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25 **Abstract:**

26   This study aimed to research the effects of different dietary concentrate to forage (C:F) ratio

27 on growth performance, rumen fermentation and bacteria diversity of barn feeding Tibetan

28 sheep. The experiment contains fiver treatments (HS1, HS2 HS3, HS4 and HS5; n=8,

29 respectively) based on dietary C: F ratios 0:100, 15:85, 30:70, 45:55, and 60:40, respectively.

30 The ruminal bacterial community structure was investigated through high-throughput sequencing

31 of 16S rRNA genes in V4 hypervariable region. The results showed that increasing dietary

32 concentrate feed level from 0% to 60% exerted a positive effect on DMI, BW gain, gain rate and

33 feed conversation ratio (F_{CR}) in Tibetan sheep. The increases dietary concentrate feed level

34 elevatedNH$_3$-N, propionate and valerate concentrations, whereas, reduced molar ratio of acetate

35 to propionate (A/P ratio) (P<0.05). For rumen bacterial diversity, increases in dietary concentrate

36 content contributed to lower alpha diversity indexes including Shannon wiener, Chao1 and

37 observed species, meanwhile, significantly increased the abundances of the phylum

38 *Bacteroidetes* and the genus *Prevotella_1* (P<0.05). In conclusion, increases dietary concentrate

39 content improved the growth performance and Tibetan sheep fed diets of 45% concentrate

40 obtained a better performance; the inclusion of concentrate in feed changed rumen fermentation

41 from acetate fermentation to propionate fermentation, and improved the energy utilization

42 efficiency of Tibetan sheep; the increased in concentrate content significantly reduced rumen

43 bacteria diversity and changed the abundance of some core bacteria.

44 **Keywords** Rumen bacterial diversity, Tibetan sheep, 16S rRNA, Concentrate-to-forage ratio,

45 Rumen fermentation parameter, Growth performance

46 **Abbreviation key**: C:F ratios= dietary concentrate to forage ratio, HS1= C:F ratio of 0:100,

47   HS2= C:F ratio of 15:85, HS3= C:F ratio of 30:70, HS4= C:F ratio of 45:55, HS5= C:F ratio of

48   60:40, DMI=dry matter intake, ADG=average daily BW gain, F_{CR} = feed conversation ratio.

49   **Introduction**

50   Tibetan sheep (*Ovis aries*) live exclusively on the Qinghai Tibetan Plateau (QTP) with a

51   altitude greater than 3,000 (An et al. 2005), they have adapted well to extremely harsh

52   environment and ingested grasses as their sole source of nutrition (Sasaki 1994; Wiener et al.

53   2003). Under traditional management, Tibetan sheep mainly grazed on natural pasture without

54   concentrate supplementation, and always suffered seasonal live-weight variations and viciously

55   cycled in "alive in summer, strong in autumn, thin in winter, tired in spring", due to seasonal

56   fluctuations in natural pasture supply and the contradiction between herbage supply and

57   livestock's requirement on the alpine pasture (Dong et al. 2006; Xue et al. 2005; Xu et al. 2017).

58   During growing season (June to Oct.), natural pasture can provide enough herbage for livestock,

59   the local people only drive livestock to alpine pasture for grazing. During non-growing season

60   (Nov to May) and vegetation green-up periods, grazing livestock shared low performance mainly

61   attributed to the decrement in herbage supply (both in quantity and quality). In addition,

62   irrational grazing during this period destroyed regular growth of alpine plants and function

63   recovery of alpine pasture. Previous studies have confirmed that spring short term rest-grazing is

64   an effective measure for the functional recovery of alpine meadows (Li et al. 2014; Li et al.2017;

65   Ma et al.2017), the local government encourages pastoralists to feed their livestock in warm shed

66   during spring grazing break. However, few studies concerning growth performance of barn

67   feeding Tibetan sheep during late non-growing and vegetation green-up periods were conducted,

68   but which was quiet important for providing pastoralists guidance in livestock breeding.

69   The rumen microorganisms play an important role in the digestion of proteins,

70   carbohydrates, starch, sugars and fats, which provides energy and proteins to the host by

71   producing volatile fatty acids and bacterial proteins through anaerobic fermentation (Ceceni et

72   al. 2015; Jiang et al. 2015) and finally affects ruminants performance. The rumen

73   microorganism can be affected by many factors, such as diet, hosts and geographic region

74   (Clark. 1975; Lee et al. 2012). The dietary nutrition level is a major factor affecting rumen

75   microbial diversity, healthy status and production capacity of ruminants (Clark. 1975). The

76   effect of diet on the structure of rumen microbial communities has been widely investigated in

77   Yak, Tan sheep, Holstein dairy cows, Mehshana buffalo and goats (Pitta et al. 2014; Jiao et al.

78   2015; Pitta et al. 2016; Wang et al. 2016; Xue et al. 2016). However, as the dominant ruminant

79   and living foundation for local herdsman, the studies of Tibetan sheep have only focused on

80   the growth performance, slaughter performance and economic benefit under different dietary

81   supplementation (Baruah et al. 2012; Chen et al. 2015c; Dodd et al. 2011; Feng et al. 2013;

82   Lee et al. 2012; Xu et al. 2017), but few comprehensive studies focused on the growth

83   performance, rumen fermentation and rumen microbial communities of Tibetan sheep.

84   Therefore, this study aimed to investigate the growth performance, rumen fermentation and

85   rumen bacterial diversity of Tibetan sheep under different dietary concentrate-to-forage ratios

86   during late non-growing and green-up periods. We hypothesized that different C: F ratios could

87   affect growth performance, rumen fermentation and rumen bacterial diversity in Tibetan sheep.

88   These results will be of great importance for providing guidance for local herdsmen in Tibetan

89   sheep breeding and for future research on rumen microbial metabolism in Tibetan sheep.

90   **Materials and Methods**

91   **Animals and Experimental Design**

92 This study was conducted at Haibei Demonstration Zone of Plateau Modern Ecological

93 Animal Husandry Scientific and Technology in Haibei Prefecture, Qinghai Province, China

94 (36°54' N, 100°56' E), from April 2016 to July 2016. All animal care procedures were consistent

95 with the guidelines from the Institution of Animal Care and the Ethics Committee of the

96 Northwest Institute of Plateau Biology, Chinese Academy of Sciences (NWIPB20160302). The

97 processing of the samples after collection was performed strictly in accordance with the

98 guidelines of NWIPB.

99 A total of 40 female yearling Tibetan sheep with familiar body conditions (21.39±1.18 kg

100 BW, and not under current antibiotic treatment) were randomly divided into five treatments (8 in

101 each) under different C:F ratios (on dry matter basis):HS1(0:100), HS2 (15:85), HS3 (30:70),

102 HS4 (45:55) and HS5 (60:40), respectively. All sheep were allocated into 5 pens within a warm

103 shed. The concentrate and oat hay were manually mixed and fed (dry matter) based on 3.5% BW

104 of Tibetan sheep. Before study, all the animals were fed the experimental diet for a 15-day

105 adaptation phase. During formal 80 day experiment, the experimental animals were separately

106 fed twice dairy, at 8:00 am and 5:00 pm. The diets, salt brick and clean water were available

107 throughout the entire experiment. The ingredients and nutrient compositions of the animal diet

108 are shown in **Table 1 and Table S1**. The concentrate feed used in this study was produced by

109 Menyuan Yongxing Ecological Agricultural Development Co., Ltd., and oat hay are harvested,

110 bundled and stored in the year 2015.

111 **Measurement and sampling**

112 The diets and oats in each treatment were weighed and recorded daily to estimate individual

113 dry matter intake (DMI). Diet samples of approximately 500 g from each treatment were

114 collected, dried at 60 °C, ground through a 1-mm sieve and stored in a vacuum dryer for

115   nutritional analysis. To measure animal growth performance, Tibetan sheep were weighed before

116   feeding using an electronic balance at the beginning and end of formal experiment. We selected

117   four Tibetan sheep from each treatment group for rumen fluid sampling. Specifically, on 80 th

118   day, rumen content samples were obtained 3-4 h after the morning feeding using a stomach tube

119   attached to an electric pump, which has been confirmed to yield similar results for rumen

120   microorganisms and fermentation parameters as sampling using a rumen cannula (Ramos-

121   Morales et al. 2014). The rumen contents were filtered through four layers of sterilized gauze.

122   Approximately 67 mL of liquid was obtained from the rumen of each Tibetan sheep. The rumen

123   fluid was separated into three samples, one (approximately 2 mL) was transferred into sterilized

124   freezing tubes and stored in liquid nitrogen for DNA extraction; the second sample

125   (approximately 15 mL) was immediately used to measure the pH; and the third sample

126   (approximately 50 mL) was used for the assessment of rumen fermentation parameters, including

127   ammonia nitrogen (NH_3-N) and rumen volatile fatty acids (VFAs).

128   **Chemical analysis and calculation**

129   The feed samples were fried in an oven at 135 °C for 3 h to obtain the DM (AOAC, 1990;

130   Method No. 930.15). The total N was detected using Kjeldahl method; the crude protein content

131   was calculated as 6.25×N (Method No. 984.13); the ether extract (EE) was measured using the

132   Soxhlet system (Method No. 954.02); the acid detergent fiber (ADF) and neutral detergent fiber

133   (NDF) of diet were analysed using method described by Soest et al. (1991); and the starch was

134   measured according to PRC national standard (GB 5009.9-2016).

135   The body weight gain (BW gain), gain rate, average daily BW gain (ADG) and feed

136   conversion ratio (F_{CR}) were calculated according to the following equations:

137                              $$BW_{gain} \text{ (kg)} = BW_f - BW_i$$

138 $$\text{Gain rate (\%)} = 100 \times (BW_f - BW_i)/BW_i$$

139 $$\text{ADG (g/d)} = 1000 \times (BW_f - BW_i)/t$$

140 $$F_{CR} = DM_{consume}/(BW_f - BW_i)$$

141 where BW_f and BW_i are the final and initial body weights (kg), respectively, t is the

142 experimental time (d), and $DM_{consume}$ is the total feed consumed during the experiment (kg DM).

143 The ruminal fluid pH was measured using a portable pH meter (PHSJ-3F; Precision Instruments

144 Company, Shang Hai, China). For VFAs measurements, the rumen fluid was centrifuged at

145 12,000 g for 15 min, and the VFAs in the supernatant were analysed using a gas chromatograph

146 Agilent 7890A (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with a polar

147 capillary column (DB-WAX, 30 m×0.25 mm×0.25 μm) and a flame ionization detector (FID,

148 temperature set at 250°C). The temperature-programmed conditions were as follows: the

149 temperature was maintained at 60 °C for 2 minutes, increased to 180 °C at rate of 10 °C /min,

150 and increased to 250 °C at rate of 20 °C /min; the shunt ratio was 20:1;the flow rate was 1

151 mL/min; and the inlet temperature was 200 °C. The NH_3-N content in the supernatant was

152 quantified using a continuous flow analyser (SEAL Auto Analyser 3, Germany) described by

153 Rhine et al. (1998).

154 **DNA extraction and PCR amplification**

155   Genomic DNA was directly extracted from 0.2 g of each semisolid-state sample using cetyl

156 trimethylammonium bromide (CTAB) method (Porebski et al. 1997). The DNA quality was

157 assessed via 2% agarose gel elcectrophoresis, and metagenomic DNA concentratios were

158 determined with a NanoDrop 2000 (Thermo, Mass, USA). The DNA was then diluted to 1 ng/μL

159 using sterile water and stored at -4°C for PCR amplification.

160   For PCR amplification, the V4 hypervariable region of the bacterial 16S rRNA gene was

161 amplified using the universal primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R

162   (5'- GGACTACHVGGGTWTCTAAT-3') with unique barcodes. PCRs were performed in 25 μL

163   reactions consisting of 12.5 μL Phusion® High-Fidelity PCR Master Mix (New England

164   Biolabs), 1 μL of forward and reverse primers, 1 μL of template DNA and 9.5 μL of autoclaved

165   distilled water. The thermal cycling program consisted of initial denaturation at 94°C for 3 min,

166   30 cycles of denaturation at 94°C for 40 s, annealing at 56°C for 1 min, and elongation at 72°C

167   for 1 min and a final incubation step at 72°C for 10 min. For PCR product quantification and

168   qualification, we obtained mixtures of the same volumes of 1× loading buffer (containing SYBR

169   Green from Shanghai, SanoBio) and the PCR products were exmined ona 2% agarose gel

170   electrophoresis. Samples with a bright band between 400and 450 bp were mixed and purified

171   using a Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing libraries were then generated

172   using a TruSeq® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA) following the

173   manufacturer's recommendations, and the index codes were then added. The library quality was

174   assessed using a Qubit® 2.0 fluorometer (Thermo Scientific) and an Agilent Bio analyzer

175   system. The library was sequenced on an Illumina HiSeq PE250 platform (Novogene, Beijing,

176   China).

177   **Analysis of sequencing data**

178   The paired-end reads were assigned to samples based on their unique barcodes, and the

179   barcodes and primer sequences were then trimmed. The raw reads were filtered according to the

180   following rules: Removing reads containing more than 10% of unknown nucleotides (N);

181   removing reads containing less than 80% of bases with quality (Q-value)＞20. The FLASH

182   (version 1.2.7) was then used to merge paired-ends reads as raw tags with a minimum overlap of

183   10bp and mismatch error rates of 2% (Magoc & Salzberg 2011). The noisy sequences of raw

184   tags were filtered by QIIME (version 1.9.1) pipeline to obtain the high-quality clean tags

185   (Caporaso et al. 2010). The reads were then compared with a Gold database using the UCHIME

186   algorithm to detect and remove the chimaera sequences (Edgaret et al. 2011). All the sequences

187   were analysed using Uparse software (version 7.0.1001), and sequences with greater than or

188   equal 97% similarity were assigned to the same operational taxonomic unit (OTU). Taxonomic

189   information for each representative sequence was annotated using the Greengenes database

190   based on the RDP classifier algorithm (version 2.2) (Wang et al. 2007).

191   **Statistical analysis**

192   Alpha and species diversity indexes, including the Shannon-Wiener indexe, Chao1 index,

193   Good's coverage and observed species were calculated by Qiime and graphed using Origin

194   (version 8.0). The correlation among growth performance, rumen fermentation parameters and

195   bacteria diversity were analyzed using SPSS (version 17.0), and heat map of correlations were

196   also prepared using Origin. The beta diversity based on weighted UniFrac distance, was

197   visualized through a principal coordinate analysis (PCoA). One-way ANOVA with Tuckey's test

198   was performed to compare the differences in relative abundances among different treatments,

199   and Duncan's multiple comparison test was used to determine the effects of dietary C: F ratios

200   on BW gain, gain rate, ADG, DMI, DM consume and F_{CR} using SPSS (version 17.0). Effects

201   were considered significant at P<0.05. The results are shown as the means± SEMs.

202   **Results**

203   **Growth performance of Tibetan sheep**

204   As shown in **Table 2,** treatments HS4 and HS5 significantly increased BW gain, gain rate

205   and ADG, whereas reduced F_{CR} as compared to treatments HS1 and HS2 (P<0.05). Tibetan

206   sheep fed diet in group HS1 showed the lowest growth performance. No significant difference

207   were detected in BW gain and F_{CR} between treatments HS4 and HS5, and the initial BW showed

208 no notably difference among the five groups (P=0.196).

209 Rumen fermentation parameters

210 As shown in **Table 3**, the NH_3-N, propionate and valerate concentrations in treatments HS4

211 and HS5 were significantly higher than that of other three groups (P<0.05). There was a

212 decreasing trend for total VFA, acetate and isovalerate concentrations, but no significant

213 differences were detected among the groups. The increases in dietary concentrate level

214 significantly decreased A:P ratio (P<0.05). The ruminal pH tended to decrease, but no significant

215 difference was detected (P>0.05), and the average pH value was approximately 6.61.

216 Sequencing and taxonomic composition of the rumen bacterial community

217 A total of 1,497,607 PE reads were obtained, and 1,461,673 clean reads from 20 different

218 samples were generated after quality control **(Table S2)**. All the sequences were aligned and

219 clustered into OTUs using a 97% sequence identity as a cut-off, which yielded a total of 49,216

220 OTUs **(Table S2)**. Rarefaction curves were generated with a 3% cut of for comparisons of

221 species and richness as shown in **Fig. 1.** All the curves of the observed species numbers tended

222 to saturate to a plateau at the minimum sequence number of 60,000 tags. In addition, the HS4

223 and HS5 groups tended to cluster together with a lower observed species number than the HS1,

224 HS2 and HS3 groups.

225 The taxonomic analysis detected a total of 41 phyla **(Table S3)**, and the most abundant

226 phyla were *Bacteroidetes* (52.18%), *Proteobacteria* (20.34%) and *Firmicutes* (14.34%). The top

227 ten phyla, which exhibited the highest relative abundances, were prevalent in all the samples,

228 accounting for nearly 98% of all sequences **(Fig. 2A)**. The moderate abundant phyla included

229 *Fibrobacteres* (1.25%), *Cyanobacteria (1.04%), Acidobacteria* (0.96%), *Tenericutes* (1.49%),

230   *Actinobacteria* (0.96%) and *Verrucomicrobia* (0.47%). The other known phyla accounted for

231   1.48%, whereas the remaining sequences that were unclassified accounted for 0.26%.

232   At the genus level, we detected 129 distinct genera across all the groups **(Table S4)**, and 10

233   genera whose relative abundances more than 1% were perceived as the most important bacteria

234   that affecting the rumen environment and digestive system **(Fig. 2B)**. The genus with relatively

235   high across all the samples were *Prevotella_1* (26.81%), *Succinivibrionaceae_UCG-002*

236   (7.11%), *Ruminobacter* (6.03%), *Rikenellaceae_RC9_gut_group* (4.73%), *Prevotellaceae_UCG-*

237   *003* (2.65%). Minor genera, such as *Prevotellaceae_UCG-001*, *Erysipelotrichaceae_UCG-004*,

238   *Fibrobacter*, *Christensenellaceae_R-7_group* and *Ruminococcaceae_NK4A214_group*

239   accounted for 1.23%, 1.09%, 1.01%, 1.22 and 1.09% of the sequences, respectively. The other

240   known genera accounted for 11.32% of the sequences, whereas sequences that were unclassified

241   accounted for 34.23% of the sequences.

242   **Effect of diets with different C:F ratios on the bacterial community**

243   To determine alpha diversity, we calculated Shannon-Wiener, Chao 1, observed species and

244   Good's coverage indexes, as shown in **Fig. 3**. The indexes of Shannon-Wiener, Chao 1 and

245   observed species in treatments HS1, HS2 and HS3 were significantly higher than treatments HS4

246   and HS5 **(Fig. 3A, B** and **C)**. The Good's coverage among five treatment were greater than 99%

247   (**Fig. 3D**). As shown in **Fig. 4**, the PCoA result showed that the rumen bacterial communities of

248   the five treatments were mainly classified into three clusters **(Fig. 4)**. The HS1 and HS2

249   treatments clustered very closely together, the HS4 and HS5, and the HS3 treatments formed two

250   clusters. In addition, HS3 treatment were closer to the cluster composed of the HS1 and HS2

251   treatments, which only represented 7.14% of the variability obtained with PC2.

252   The relative abundance of bacterial taxa was used to describe the impact of diets with

253   different C:F ratios on bacterial community. At the phylum level, top 8 phylum (relative

254   abundance >1%) were analyzed in **Table S5.** The ruminal compositions of the HS1 to HS3

255   treatments contained a significantly lower relative abundance of *Bacteroidetes*, whereas higher

256   relative abundances of *Proteobacteria*, *Acidobacteria*, and *Actinobacteria* than those of the

257   HS4 and HS5 treatments (P<0.05). No significant differences were found in the other phyla

258   (P>0.05). At the genus level, top 12 genera (relative abundance >1%) were analyzed in **Table**

259   **S6.** Treatments HS4 and HS5 shared a higher relative abundance of *Prevotella_1* than the other

260   three treatments (P<0.05). No significant differences were found in the rest of genera (p>0.05).

261   **Relationships between rumen bacterial diversity and host growth performance**

262   As shown in **Fig. 5**, a significant negative correlation was found between bacterial diversity

263   (including the Shannon-Wiener, Chao1, and observed species indexes) and growth performance

264   (including BW gain, ADG, and DMI) with increases in the C:F ratio (P<0.05). F_{CR} showed a

265   negative correlation with increases in the DMI, BW gain and ADG and a positive correlation

266   with the Shannon-Wiener, Chao1 and observed species indexes. In addition, growth performance

267   showed significant positive correlations with increases in the dietary C:F ratio (P<0.05).

268   **Relationships between rumen fermentation parameter and microorganisms**

269   A heat map of the correlations between the top 45 genera (relative abundance >0.1%) and

270   rumen fermentation was constructed (**Fig. 6**). A total of 360 correlation coefficients were

271   generated, and 33 of these coefficients (9.17% of the total correlation coefficients) showed

272   significant correlations (P<0.05). The fermentation parameters pH, acetate, isovalerate and A:P

273   were significantly positively correlated with most genera (e.g., *Victivalls*, *Thalassospira* and

274   *Sphaerochaeta*), whereas valerate and NH_3 showed a negative correlation with most genera (e.g.,

275   Victivalls, *Ruminococcaceae_UCG_02*, and *Thalassosphira*). *Prevotella_1*, which was the most

276   abundant genera, was significantly positively (P<0.05) correlated with NH_3, propionate and

277   valerate and negatively correlated with pH. No significant correlation were detected between

278   butyrate and the top 45 genera.

279   **Discussion**

280   **Growth performance of barn-fed Tibetan sheep**

281   Previous studies mainly aimed to ensure that Tibetan sheep live through grazing breaks (Ma

282   2008; Xie et al. 2014), and there is little knowledge on the BW gain and feed efficiency of

283   Tibetan sheep fed diets with different C:F ratios during late non-growing and vegetation green-

284   up periods. Our results revealed that increases in dietary concentrate feed levels within a certain

285   range (0% to 45%) exerted a positive effect on the feed intake, BW gain and F_{CR} of Tibetan

286   sheep during spring grazing breaks. This finding can be mainly attributed to the fact that high-

287   concentrate diet contains more digestive energy and nonstructural carbohydrates (Wang 2015),

288   which leads to increased nutrient intake, faster digestion through the digestive tract, and then

289   improved growth performance and higher feed efficiency (Haddad & Ata 2009; Haddad 2005).

290   Under traditional grazing, Tibetan sheep exhibit low growth performance (ADG＝36~55

291   g/d) during spring, and more seriously, unsustainable grazing during this period decreases the

292   plant species richness and biomass in alpine pasture (Ma 2008). In contrast, shortened rest-

293   grazing increases the standing herbage biomass by 77%~189% (Li et al. 2017; Ma et al. 2017).

294   Herein, we confirmed that a diet with diet with a 45% concentration supplementation

295   significantly improves the performance of Tibetan sheep. Therefore, the use of spring rest-

296   grazing combined with barn feeding could increase livestock performance and promote the

297   functional recovery of alpine pasture.

Effect of different C: F ratios on rumen fermentation parameters

The pH, NH$_3$-N concentration and VFA molar ratio are the main internal environmental indicators of rumen fermentation. If the rumen pH was in the range of 6.2 to 7.0, the ecological environment of rumen microorganisms could be relatively stable, which could ensure normal rumen fermentation (Weng et al. 2013; Zhang et al. 2013). In our study, the ruminal pH ranged from 6.42 to 6.86, and this finding indicated that the increased proportion of concentrate did not induce acidosis, which is usually defined as a decrease in the rumen pH to less than the threshold value of 6.0 (Nocek 1997). The ruminant pH values obtained in the present study are inconsistent with previous studies (Cerrillo et al. 1999; Chen et al. 2015a). The pH value can be affected by many factors, such as higher doses of flour or starches (Lettat et al. 2010; Minuti et al. 2014), the time from concentrate feed supplementation to ruminal liquid collection (Lettat et al. 2010), and the insertion depth of the rumen catheter (Li et al. 2009). In our study, increases in the dietary concentrate level tended to decrease the ruminal pH, but no significant difference was detected (P>0.05), which could be due to the starch content. The HS1 and HS2 groups had a lower starch content (25.58 and 26.95 mg/g, respectively), which led to a higher pH value, whereas the HS3, HS4 and HS5 groups had a higher starch content (32.55, 33.72 and 39.37mg/g, respectively), which resulted in a relatively lower pH value. The dietary C:F ratios did not significantly affect the total volatile fatty acids, which is in accordance with previous research (Chen et al. 2015a). This result might be due to the ability of the rumen system that can adapt to appropriate dietary C:F ratios through the self-adjustment of rumen microorganisms. Ammonium nitrogen which is the final product through the decomposition of protein and nonprotein in feed, and it is also the main nitrogen source for the synthesis of bacterial protein by rumen microorganisms. In our study, high concentrate feed level elevated NH3-N concentration, which is consistent with the

321   former study (Reddy & Reddy. 1985; Yang et al. 2001). The high dietary concentrate increased

322   nitrogen content in the rumen, thereby increasing ruminal ammonia nitrogen (Moorby et al.

323   2006). As for individual VFA concentrations in current study, diets with high-concentrate levels

324   slightly reduced acetate concentration, whereas significantly increased propionate proportion,

325   thereby resulting in a significant reduction of A: P ratio. (Andrade & Schmidely 2006; Polyorach

326   et al. 2014). From the point of view of energy utilization, a decrease in the A:P ratio reflects an

327   improvement in the feed energy utilization efficiency, which also explains the improved growth

328   performance of Tibetan sheep in the high-concentrate groups (HS4 and HS5).

329   **Core bacterial communities in the rumen**

330   Although rumen microbial community composition varies with diet and host, a 'core

331   microbiome' is found across a wide geographical range (Herdson et al. 2015). The dominance of

332   *Bacteroidetes* or *Proteobacteria* could be attributable to variations in diet, environment, hosts

333   and farming practices over a wide geographical range (Amato et al. 2013; Henderson et al.

334   2013). In our study, different dietary concentrate level induced *Bacteroidetes* (55.02%) to

335   become the most abundant phyla, followed by *Proteobacteria* (22.10%), and this microbial

336   distribution of major phyla was similar to that obtained in previous studies on Yaks (Chen et al.

337   2015b). These results might be related to the functions of rumen bacteria. Members of the

338   phylum *Bacteroidetes* have a greater ability to degrade protein and carbohydrates than species

339   belonging to *Proteobacteria* (Huo et al. 2014; Pitta et al. 2014). Our study also found that the

340   relative abundance of *Firmicutes* was the third largest phylum and was lower than that of

341   *Proteobacteria*. Similar results were observed in bovines and cattle during the transition from

342   forage to concentrate (Jami et al. 2013). However, Xue et al. (2016) observed that the most

343   abundant phyla in the rumen of natural pasture-grazing Tibetan sheep are *Bacteroidetes*,

344   followed by *Firmicutes*. One possible reason for this difference is that the hosts investigated by

345   Xue et al. (2016) were grazed on natural pasture and that the forage nutrition and types differed

346   from those in our study, and these differences might have resulted in a higher abundance of

347   *Firmicutes*.Additionally, the phylum *Fibrobacteres*, with an abundance less than 1.5%,

348   comprised only a small fraction of the community composition compared with those of other

349   phyla, and these results agree with previous studies (Petri et al. 2013; Wang et al. 2016).

350   In our study, although different C:F ratio did not change the core structure of the rumen

351   microbiome, the relative abundances of *Bacteroidetes* and *Proteobacteria* showed noticeable

352   shifts at phylum level. Tibetan sheep fed high-concentrate diets significantly increased the

353   relative abundance of *Bcateroidetes*, whereas, reduced the relative abundance of *Proteobacteria*.

354   Pervious research revealed that *Bacteroidetes* were the major rumen microorganisms in

355   degrading non-fibrous carbohydrates and contained genes related to the degradation of non-

356   fibrous polysaccharides (Russell & Diezgonzalez. 1998). In our study, high concentrate feed

357   contained more non-fibrous carbohydrates and polysaccharides, thus, increased the abundance of

358   *Bacteroidetes*. At the genus level, *Prevotella_1* was the predominant genus, and the relative

359   abundances of this genus significantly increased with increases in dietary C:F ratio. This finding

360   was consistent with that previously obtained by Stevenson & Weimer et al. (2007). The high

361   abundance of this genus can perhaps be explained from two points of view. Firstly, it if possible

362   that this bacterial genus has a wide metabolic niche due to genetic relatedness or to the high

363   genetic variability that enable this genus to occupy different ecological niches within the rumen.

364   Secondly, *Prevotella_1* strains play an important role in the degradation and utilization of plant

365   noncellulosic polysaccharides, protein, starch and xylans. The increase in the abundance of this

366   genus could be attributed to dietary nutrition changes, such as increased dietary protein and

367   starch. In addition, *Prevotella_1* is also considered to be associated with propionic acid

368   production (Strobel 1996), which might also explain the increasing proportion of propionate

369   obtained with increase in the C:F ratio.

370   **Relationship between bacterial diversity and growth performance of Tibetan sheep**

371   In the past 50 years, humans have drastically altered the diet that ruminants consume. The

372   use of grain feed increases the productivity of animals and the economic benefit of animal

373   husbandry. In our study, the diversity and richness was significantly lower in high dietary C:F

374   ratios treatments than low dietary C: F ratios (HS1, HS2 and HS3). Lin et al. (2015) found that

375   the Shannon diversity index of bacteria were higher in low concentrate diet group than in high

376   concentrate diet group concentrate diet group (Lin et al., 2015). Liu et al. (2015) discovered the

377   diversity of ruminal epithelial bacterial community from goats fed the hay diet were significantly

378   higher than those fed the high-grain diet (Liu et al., 2015). Diet composition may effect on the

379   diversity index of microorganisms.The increase in microbial diversity could be because low

380   dietary concentrate diets provide a greater range of carbohydrate substrates (e.g., cellulose and

381   many heteropolysaccharides) than high dietary concentrate treatments and/ or because

382   microorganisms grow faster in high pH conditions (Hobson & Stewart. 2012).The growth

383   performance results indicated that increases in the concentrate feed level from 0 to 45%, exerted

384   a positive effect on the feed intake, BW gain, gain rate and F_{CR} in Tibetan sheep. Therefore,

385   based on our findings, we can conclude that improved growth performance is not linked to a

386   higher diversity of rumen microorganisms. It is well known that a decrease in the bacterial

387   diversity can induce the evolution of a certain bacterial group and thereby its dominance in the

388   community. Thus, in our study, a decrease in bacteria diversity is associated with a higher

389   dominance index (phyla *Bacteroidetes* and genera *Prevotella_1*), which ensured that the host

390 could receive more nutrition and ultimately exhibits an improved growth performance. In

391 addition, in our study, increases in the dietary C:F ratio from 0:100 to 30:70 were associated with

392 increases in both the rumen bacterial diversity and the host growth performance, whereas, dietary

393 C:F ratios of 45:55 and 60:40 could only promote growth performance of Tibetan sheep.

394 Briesacher et al. (1992) observed that the digestive of the rumen is influenced by the number of

395 bacterial species and the total abundance of bacteria. Wanapat et al. (2003) investigated the

396 microorganisms in cattle and swamp buffaloes and observed that swamp buffaloes exhibit a

397 stronger ability to digest cellulose than cattle due to their greater abundance of bacteria

398 $(1.6 \times 10^9/\text{mL})$ compared with that of cattle $(1.36 \times 10^9/\text{mL})$. In our study, the HS3 group (C: F

399 ratio 30:70) exhibited a richer microbial community and an increased in microbial

400 diversity.Therefore, the inclusion of 30% concentrate in feed might be a good choice of diet that

401 can be provided to Tibetan sheep. However, we did not perform digestibility experiments with

402 all the treatments, which is a limitation of this study. Therefore, whether a rumen environment

403 with a high bacterial richness and a high bacterial diversity is associated with a higher digestive

404 capacity of Tibetan sheep remains to be investigated.

405 **Conclusion**

406 The different dietary C: F ratios affected the growth performance, rumen fermentation and rumen

407 bacterial diversity of Tibetan sheep. Increasing the dietary concentrate feed level from 0% to

408 60% exerted a positive effect on the DMI, BW gain, gain rate and F_{CR} of Tibetan sheep during

409 late non-growing and green-up period, and Tibetan sheep fed with 45% concentrate level for

410 barn feeding can result in a better improvement in animal performance. Moreover, high C:F

411 ratios significantly increased ammonium nitrogen , reduced the A:P molar ratio and changed the

412 composition of the bacterial community.

Acknowledgement

413

We would like to thank Yinfa Ji for his help in sampling. We also thank Hanzhong Ji,

414

senior livestock engineer from Haibei Prefecture, for providing the experiment site.

415

Competing Interests

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The authors declare no competing interests.

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Funding

418

This work was supported by National Key R&D Plan (2016YFC0501905,

419

2016YFC0501805), the "Strategic Priority Research Program" of CAS, "Key Technology

420

Support Program" of Qinghai province (2018-S-2, 2015-SF-A4-2, 2016-NK-148, 2017-SF-A6,

421

2017-NK-153), the Qinghai innovation platform construction project (2017-ZJ-Y20) and NSFC

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31402120.

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Data Availability

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The sequencing data for the 16S rRNA genes are publicly available in the NCBI

425

(https://www.ncbi.nlm.nih.gov/) Short Read Archive under accession. No. PRJNA477411.

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Figure 1

Figure 1 The rarefaction analysis anomg the 20 different samples.

The group HS1 samples included sheep HS1.1, HS1.2, HS1.3 and HS1.4. The treatment HS2 samples included sheep HS2.1, HS 2.2, HS2.3 and HS4. The treatment HS3 samples included sheep HS3.1, HS3.2 HS3.3 and HS3.4. The treatment HS4 samples included HS4.1, HS4.2, HS4.3 and H4.4. The treatment HS5 samples included HS5.1, HS5.2, HS5.3 and H5.4. The treatment HS1= concentrate to forage ratio 0:100, HS2= concentrate to forage ratio 15:85, HS3= concentrate to forage ratio 30:70, HS4= concentrate to forage ratio 45:55, HS5=concentrate to forage ratio 60:40.

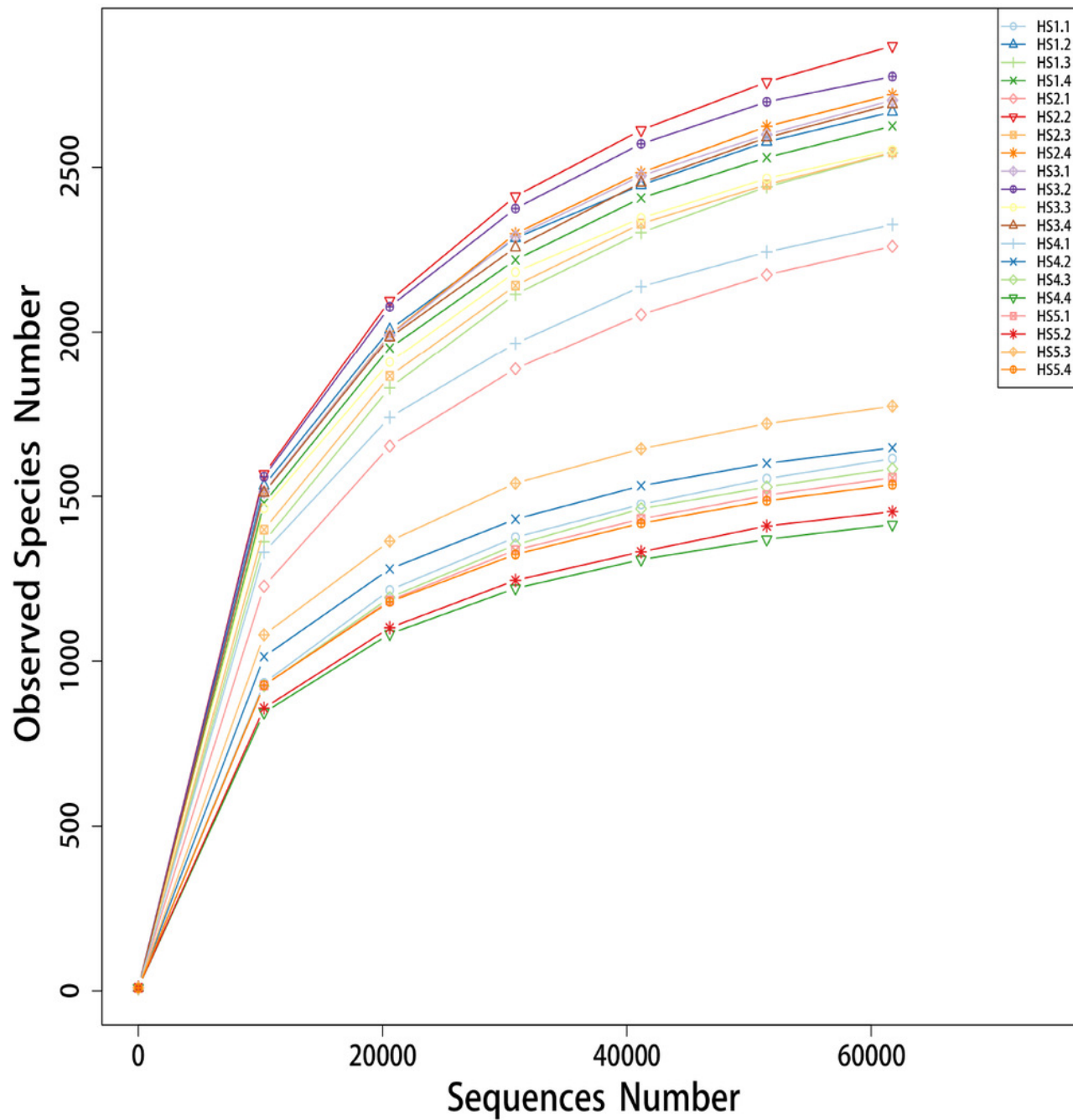



Figure 2

Dominant bacterial phylum in individual samples and the shared genera across the ruminal samples.

(A) The top 10 shared bacterial taxonomic composition across ruminal samples at the phylum level. (B) The top 10 shared bacterial toxonomic composition across ruminal samples. Percentage is shown on the X-axis. The box represent the interquartile range between the first and the third quartiles, and the symbol"——" represent the max value, "×" represent the variation range from 1% to 99% and "□"represent the mean value. The treatment HS1= concentrate to forage ratio 0:100, HS2= concentrate to forage ratio 15:85, HS3= concentrate to forage ratio 30:70, HS4= concentrate to forage ratio 45:55, HS5=concentrate to forage ratio 60:40.

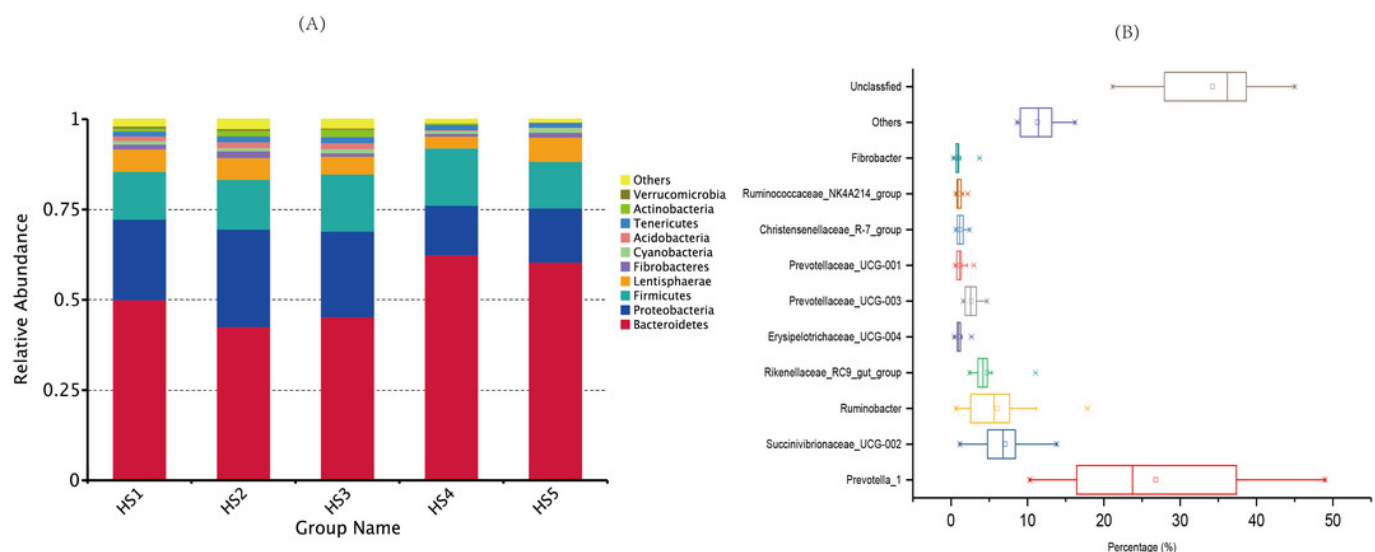



Figure 3

The differences in bacteria community diversity and richness indexes among different feeding treatment.

(A) The Shannon-Wiener diversity among each treatment. (B)The Chao1 richness estimator among each treatment. (C) The observed species in each treatment. (D) The good coverage in samples among each treatment. The treatment HS1= concentrate to forage ratio 0:100, HS2= concentrate to forage ratio 15:85, HS3= concentrate to forage ratio 30:70, HS4= concentrate to forage ratio 45:55, HS5=concentrate to forage ratio 60:40.

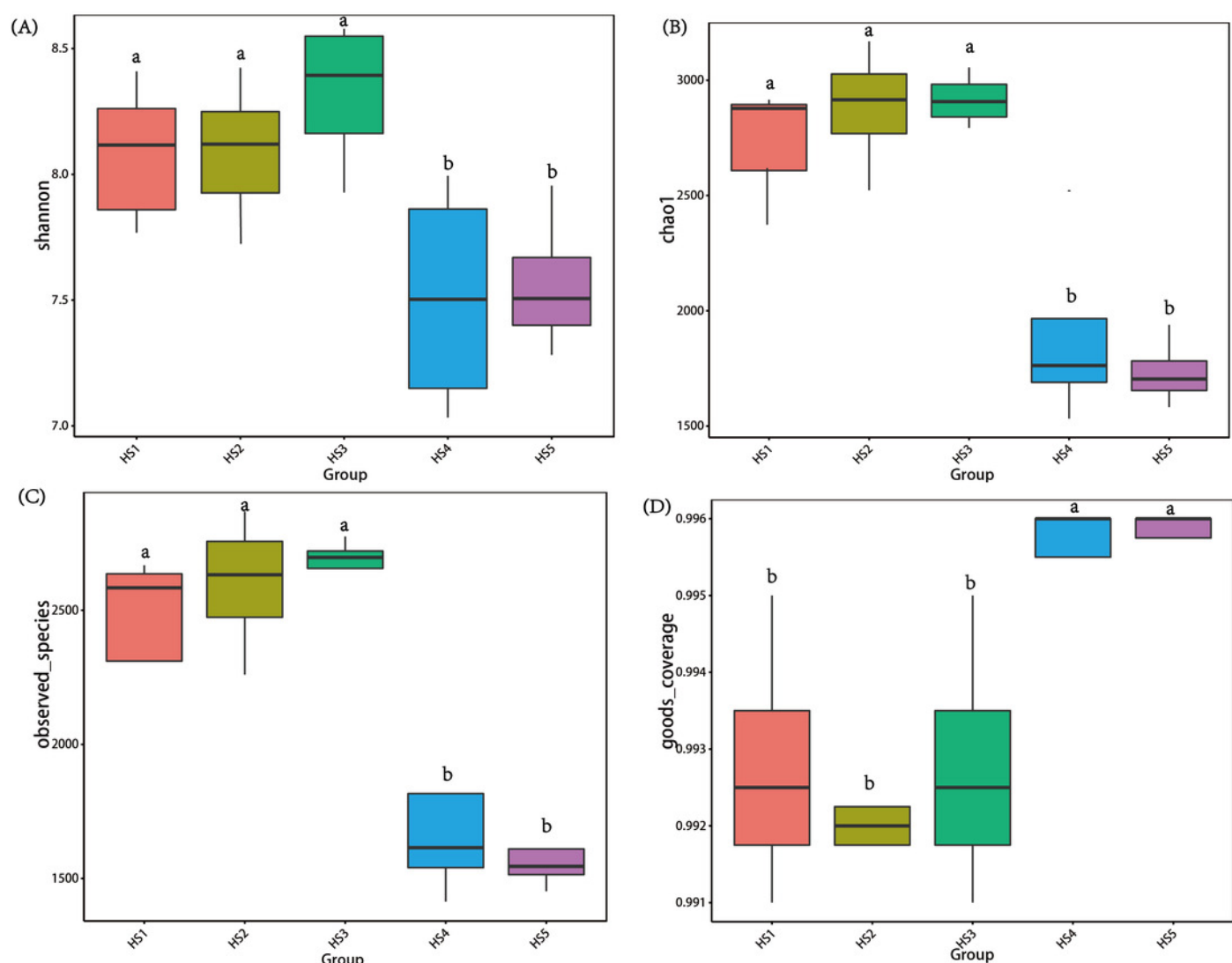



Figure 4

The principal coordinate analysis (PCoA) using weighted UniFrac metrics.

The treatment HS1= concentrate to forage ratio 0:100, HS2= concentrate to forage ratio 15:85, HS3= concentrate to forage ratio 30:70, HS4= concentrate to forage ratio 45:55, HS5=concentrate to forage ratio 60:40.

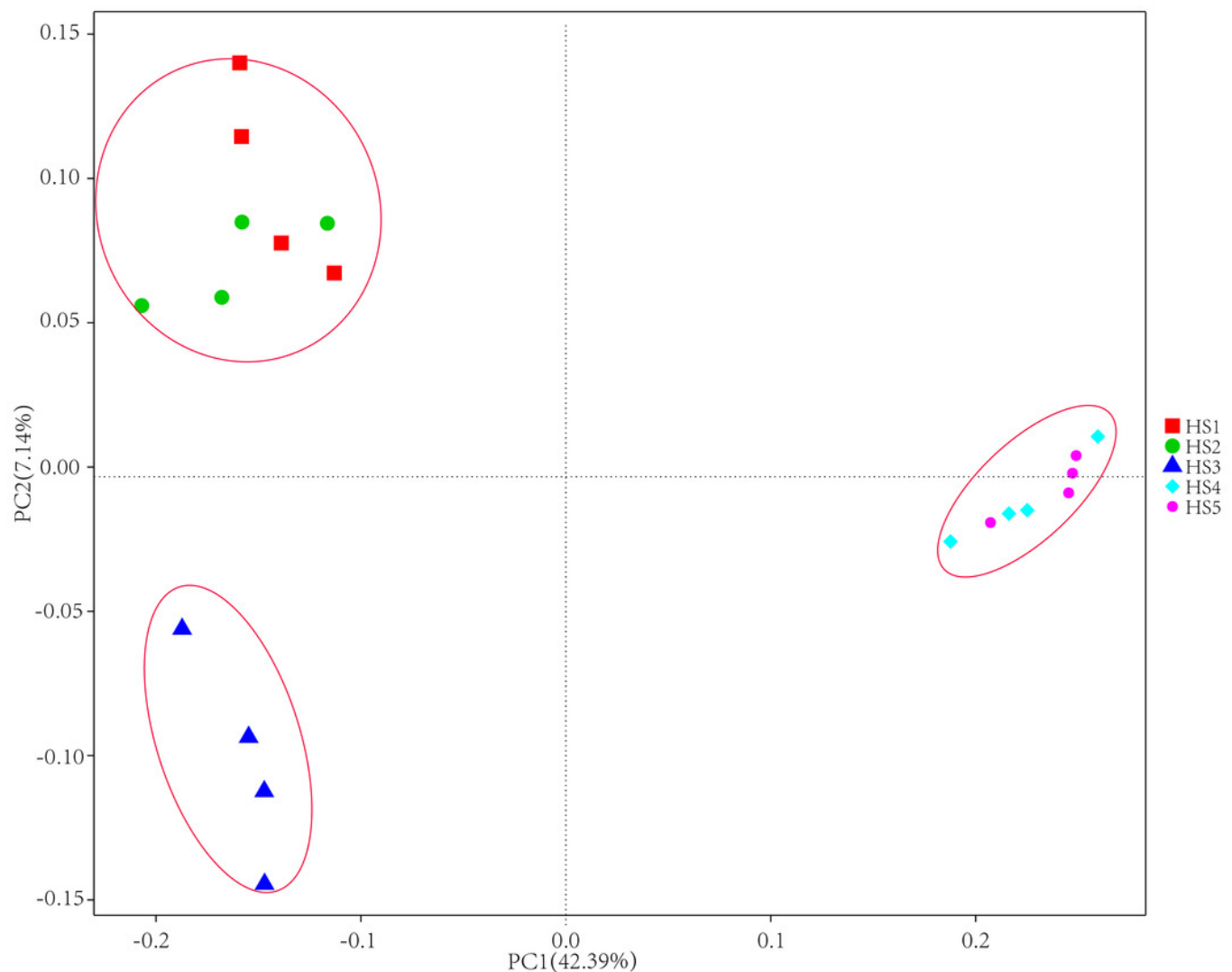



Figure 5

The heat map of the correlations between bacterial diversity and growth performance of Tibetan sheep.

DMI=dry matter intake; ADG=average daily body weight gain; FCR=feed conversion ratio; BW again= body weight gain. The treatment HS1= concentrate to forage ratio 0:100, HS2= concentrate to forage ratio 15:85, HS3= concentrate to forage ratio 30:70, HS4= concentrate to forage ratio 45:55, HS5=concentrate to forage ratio 60:40. * significant at P<0.05.

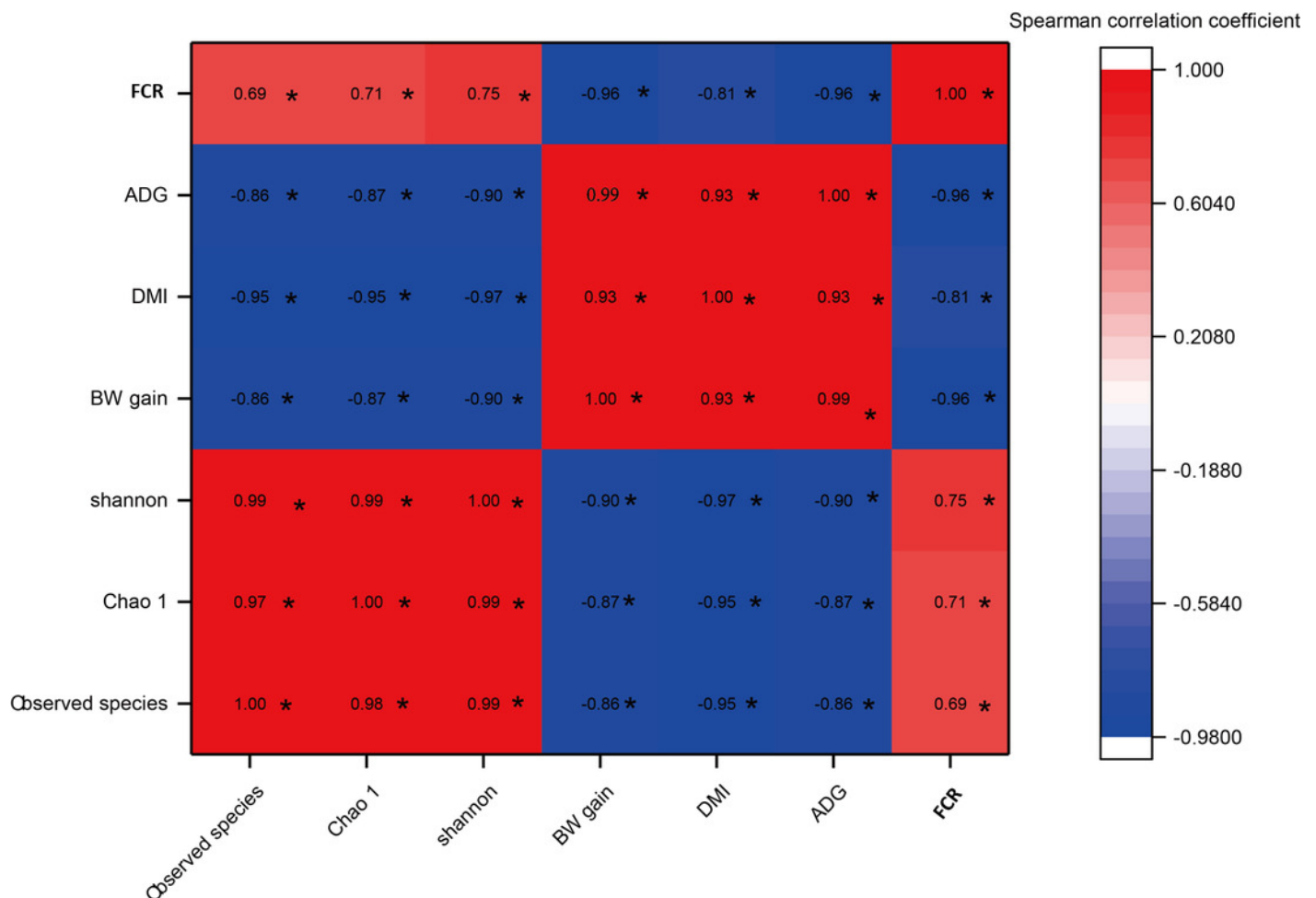
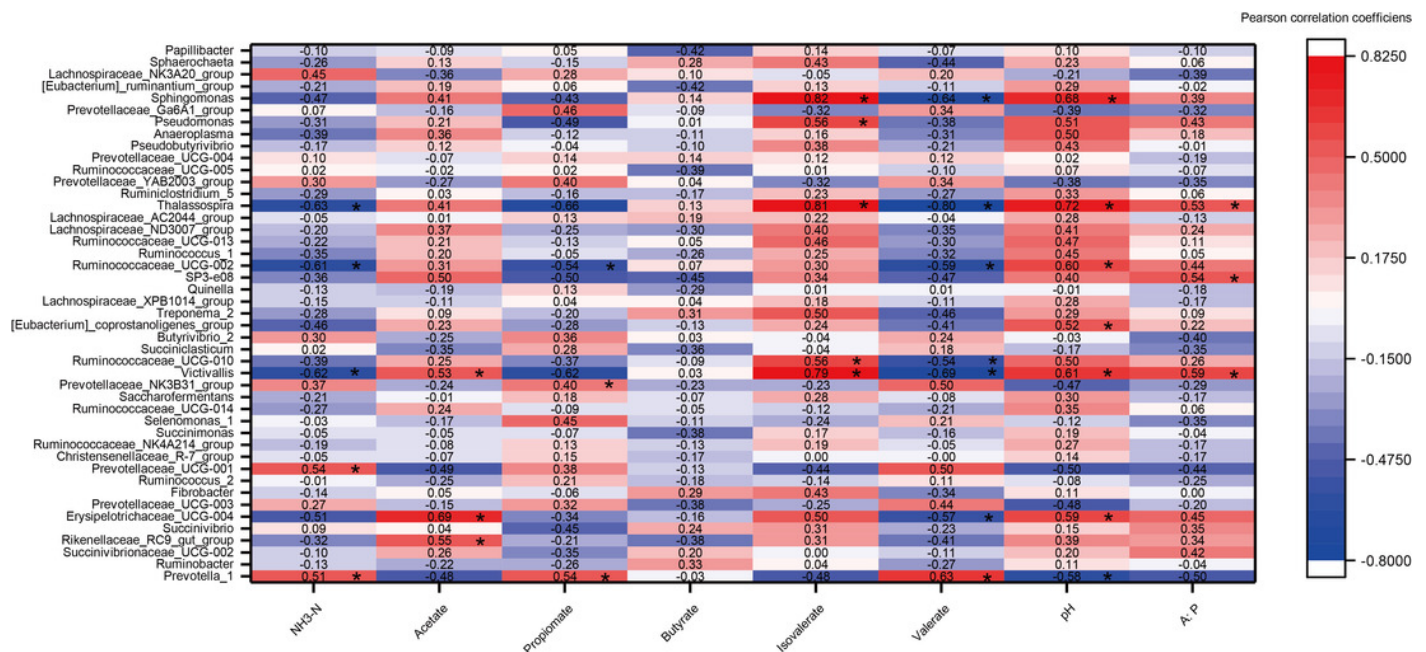



Figure 6

The heat map of the correlations between bacterial diversity and growth performance of Tibetan sheep.

DMI=dry matter intake; ADG=average daily body weight gain; FCR=feed conversion ratio; BW again= body weight gain. The treatment HS1= concentrate to forage ratio 0:100, HS2= concentrate to forage ratio 15:85, HS3= concentrate to forage ratio 30:70, HS4= concentrate to forage ratio 45:55, HS5=concentrate to forage ratio 60:40. * Significant at P<0.05.

The nutritional composition of the whole diets among different treatments (% DM basis).

[a] The tratments HS1, HS2, HS3, HS4 and HS5 refer to the C:F ratios of 0:100, 15:85, 30:70, 45:55 and 60:40, respectively. [b] DM= dry matter; CP= crude protein; EE= ether extract; ADF= acid detergent fibre; NDF= neutral detergent fibre. [c] Manufactured by Menyuan Yongxing Ecological Agricultural Development Co., Ltd. Contained corn (48%), wheat (30%), soybean meal (7%), colza cake (6%)cottonseed meal (5%), , salt (1%), pre-mix (1%),CaHPO4 (1%) and $CaCO_3$ (1%).

Items	Groups [a]				
	HS1	HS2	HS3	HS4	HS5
Ingredient (%)					
Concentrate feeds [c]	0	15	30	45	60
Oats hay	100	85	70	55	40
Nutrient content					
DM [b]	93.42	92.70	91.82	91.23	90.35
CP	6.31	7.69	9.37	10.38	11.94
Starch (mg/g)	25.58	26.95	32.55	33.72	39.37
EE	2.13	2.21	2.36	2.37	2.49
ADF	34.1	30.68	27.33	23.79	19.64
NDF	57.64	52.04	44.85	39.05	33.12
Calcium	0.35	0.40	0.48	0.55	0.61
Magnesium	0.24	0.27	0.32	0.35	0.42
Phosphorus	0.22	0.27	0.33	0.38	0.45

The effects of different dietary C: F ratios on the growth performance of Tibetan sheep.

[a] The treatments HS1, HS2, HS3, HS4 and HS5 refer to concentrate-to-forage ratios of 0:100,15:85, 30:70, 45:55 and 60:40. DMI=dry matter intake; DM consume= DMI×t, t is experimental time(d), BW=body weight; ADG= average daily BW gain . FCR= feed conversion ratio. Values in the same row with different superscripts are significantly different (P<0.05).

Items	Groups [a]					p-value
	HS1	HS2	HS3	HS4	HS5	
Initial BW (kg)	21.31±0.92	22.13±3.06	21.25±1.69	19.88±1.1.81	22.19±2.34	0.196
Final BW(kg)	23.81±1.25[d]	26.44±3.07[cd]	28.38±2.23[bc]	29.75±2.62[b]	32.94±3.49[a]	<0.01
BW gain (kg)	2.50±0.76[d]	4.31±1.92[c]	7.13±1.71[b]	9.88±1.43[a]	10.75±1.79[a]	<0.01
Gain rate (%)	11.73±3.50[d]	19.93±6.54[c]	33.78±8.63[b]	49.98±7.13a	48.59±7.56[a]	<0.01
ADG (g/d)	31.25±9.45[d]	53.91±12.91[c]	89.06±21.33[b]	123.44±17.91[a]	134.38±22.41[a]	<0.01
DMI (g/d)	670.30±45.26[c]	719.16±120.09[bc]	741.21±71.22[abc]	807.68±32.29[ab]	851.93±98.86[a]	<0.01
DM consume (kg)	53.62±3.62[c]	57.53±8.98[bc]	59.30±5.70[bc]	64.61±4.98[ab]	68.15±7.91[a]	<0.01
FCR	23.86±9.75[a]	13.94±3.38[b]	8.92±3.06[c]	6.67±1.15[c]	6.44±1.00[c]	<0.01

The effects of different dietary C: F ratios on the rumen fermentation parameters of Tibetan sheep.

[a] The Treatments HS1, HS2, HS3, HS4 and HS5 refer to concentrate to forage ratios of 0:100, 15:85, 30:70, 45:55 and 60:40, respectively. [b] Values in the same row with different superscripts are significantly different ($P<0.05$).

Parameters	Groups [a]					P [b]
	HS1	HS2	HS3	HS4	HS5	
Ammonia nitrogen (mg/L)	46.20±12.37[c]	46.20±12.37[c]	64.00±3.88[b]	67.10±8.21[b]	107.06±15.07[a]	<0.05
Acetate (%)	77.82[a]	71.70[b]	64.10[b]	60.14±8.55[b]	64.55±2.15[b]	0.32
Propionate (%)	12.60±0.71[b]	16.02±0.18[b]	22.79±1.13[a]	26.81±3.60[a]	29.38±0.95[a]	<0.05
Butyrate (%)	8.25±0.38	10.66±1.73	11.30±0.40	11.51±2.20	12.02±0.40	0.23
Isovalerate (%)	0.79±0.10[a]	0.92±0.07[a]	0.83±0.02[a]	0.58±0.03[b]	0.72±0.09[b]	0.23
Valerate (%)	0.52±0.02[b]	0.70±0.04[b]	0.98±0.03[b]	1.11±0.04[a]	1.50±0.12[a]	<0.05
Total volatile fatty acids (mmol/L)	74.94±10.76	66.12±2.95	65.21±1.00	67.44±1.56	61.24±7.67	0.11
Acetate to propionate ratio (A:P)	6.38±1.55[a]	4.47±0.16[a]	2.85±0.24[b]	2.24±0.39[b]	2.19±0.69[b]	<0.05
pH	6.86±0.01	6.83±0.03	6.83±0.04	6.63±0.06	6.42±0.10	0.41