Effects of dietary NFC/NDF on rumen microbiomes of Karakul sheep based on Three Generations of Full-length Amplifiers sequencing

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The research was supported by the National Natural Science Foundation Project (31760680) and Xinjiang Production and Construction Group with the young and middle-aged innovation talents fund (No. 2016BC001).

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Abstract

An study was was conducted to investigate the effects of dietary(non fibrous carbohydrate) NFC/(neutral detergent fiber)NDF on ruminal bacteria in Karakul sheep. Twelve Karakul sheep were assigned randomly to four dietary treatments of NFC/NDF (0.78, 1.23, 1.61 and 2.00 respectively) as group 1, 2, 3 to 4. The experiment lasted for four periods, period I (1~18 d), II (19~36 d), III (37~54 d) and IV (55~72 d). Ruminal digesta were collected consecutively for three days to measure pH and bacteria per period. The results indicated that the average ruminal pH and amounts of OTUs were decreased with the increase of dietary NFC/NDF for four periods. At phylum level, Bacteroidetes and Firmicutes were the predominant bacteria of four periods, Bacteroidetes were decreased, while the relative abundance of Firmicutes was increased with dietary NFC/NDF for four periods, but the difference wasn't significant (P>0.05). At genus level, the most relative abundance genus was unidentified-Lachnospiraceae which reached the highest in group 3 for four periods, but the difference wasn't significant (P>0.05). Conclusion: ruminal pH and bacteria were decreased with the increase of dietary NFC/NDF and the most dominant bacteria were not change with dietary NFC/NDF and periods in Karakul sheep.

Introduction

Rumen plays an important role in the growth and production of ruminants and it contains a large number of rumen microorganisms (*Bacteria*, *Protozoa*, *Eukarya* and *Archae*a[1-3]). Rumen microorganisms breakdown food to provide volatile fatty acids, bacterial protein, and so on to the host animal[4]. Yang et al.[5] confirmed that 80% of the starch, 50% of the fibre and 60% of the organic matter in the diet were fermented in the rumen to provide energy for the host. Meanwhile, after a long-term selection and evolution, rumen microbiomes and the host have formed a symbiotic relationship to maintain the host's health[6]. Dietary regulation has an important effect on rumen fermentation[7-9] and microbiome[10-11]. Wei et al.[12] showed that with the increase of dietary NFC/NDF, ruminal pH decreased significantly, furthermore the composition of rumen microbial flora also changed, and the total number of rumen bacteria decreased in goats. However, there are scarce studies on rumen microbiome structure changes with dietary NFC/NDF in Karakul sheep and the rumen is very complex in which microbiome may change again with prolong of periods. NDF plays an important role in dry matter intake (DMI) and feed digestibility[13-14], and NFC in diets is another factor that affect DMI, Hall et al. reported that NFC would be degraded rapidly in the rumen[14]. The development of technology makes it more accurate to study on rumen microbiomes, Three Generation of Full-length Amplifiers sequencing is one of them, which can improve the resolution of species identification, and improve the accuracy of microbial species composition identification in the samples[15-16]. In this experiment, ruminal pH and microbiome were measured for four periods to investigate effects of dietary NFC/NDF on ruminal microbiome in Karakul sheep.

Materials and Methods

63 # Animals and Dietary composition

64 All experimental procedures were approved by Tarim University Animal Care and Use
65 Committee, and humane animal care were followed throughout the experiment. Twelve Karakul
66 sheep with similar age and weight (35.3 ± 3.3 kg) were fitted with permanent fistula and were
67 randomly assigned into four dietary treatments of NFC/NDF (0.78, 1.23, 1.61, 2.00 respectively)
68 as group 1, 2, 3 and group 4, each group with three replicates. They all received vaccines for
69 parasites before the adaption period, and were fed meeting the standards for raising meat and
70 sheep in the People's Republic of China[17]. All sheep were housed individually in metabolic
71 cages (1.2 m × 1.5 m) and fed the experimental diet individually twice a day at 9:00 a.m. and 8:00
72 p.m with free access to water. The ingredients and nutrient level of the diet were shown in S1
73 Table.

74 # The experimental design and sample collection

75 The experiment lasted for 72 d including four periods, including period I (1~18 d), II (19~36 d),
76 III (37~54 d) and IV (55~72 d). Each period lasted for 18 d. The first 15 d for adaption and 3 d for
77 samples, feed intake and defecation rule were studied in the adaption period. The ruminal digesta
78 were sampled consecutively before morning feeding for three days, the sheep of one group were
79 collected together for 50 mL, and then, were stored in -80 ℃ to investigate on rumen
80 microorganisms. Meanwhile, the ruminal pH was measured after feeding of 0, 1, 3, 6 and 9 h
81 using pH meter (FE22).

82 # DNA Extraction, PCR and Pacio sequencing

The total genetic DNA was extracted using QIAamp Fast DNA Stool Mini Kit (QIAGEN, Shanghai) according to the illustration and the extracted DNA was detected by 1% agarose gel. The V1-V9 regions of 16S rDNA were amplified by PCR from the extracted DNA using the universal primers: F, 5'-AGAGTTTGATCCTGGCTCAG-3'; R, 5'-GNTACCTTGTTACGACTT-3' (synthesized by Biological engineering co., Ltd). PCR was carried out in triplicate 50-μL reactions which containing 2 μL Primer Mix (1uM), 5 ng gDNA, 1 μL Trans Fastpfu, 10 μL 5× Buffer, 5 μL 5× StimuLate, 5 μL dNTPs (2.5mM each), 27 μL NFW. Thermocycling parameters were as follows: 2 min predenaturation at 95 ℃; 35 cycles of denaturation at 95 ℃ for 30 s, Annealing at 60 ℃ for 40 s, extension at 72 ℃ for 90 s; and a Final extension at 72 ℃ for 10 min. The production was detected by 2% agarose gel. PCR products was purified with Gel Extraction Kit (QIAGEN, Shanghai), and the productions were sequenced on PacBio platform.

The sequence analysis

The 16S rDNA reads were firstly processed to get clean reads by discarding the reads that are shorter than 1340 bp, longer than 1640 bp, and not matching the expected barcodes. Using Uparse software [18] to cluster all Clean Reads of all samples. Operational taxonomic units (OUTs) were formed at the similarity of 97%[19]. The OUTs were annotated by the Mothur and SILVA （http://www.arb-silva.de/） [20] according to the reference taxonomy provided by SSUrRNA detabase[21]. The OTUs were analyzed by Qiime pipeline (Version 1.9.1) to calculate the richness and diversity indices i e. observed OTUs, Chao1, Shannon, Simpson, ACE.

Statistical Analyses

104 The results of pH were expressed as means using SPSS 17.0. Comparisons between groups were
105 performed with ANOVA followed by Duncan test. The difference of ruminal bacteria was
106 measured and expressed using SPSS 17.0. As well, and the differences were considered to be
107 significant at $P<0.05$.

108 Results

109 pH

110 The average pH of 0, 1, 3, 6, 9 h after feeding for four periods were shown in Table 1, the pH was
111 that: group 1> group 2> group 3> group 4 for four periods. There was no significant difference
112 between four groups in period I ($P>0.05$), while there was significant difference between four
113 groups in period II, III and IV ($P<0.05$), which showed that the ruminal pH decreased
114 significantly as the dietary NFC/NDF increased.

115 **Table 1. Effects of dietary NFC/NDF on ruminal pH**

116 Extraction DNA of rumen bacteria

117 The DNA extraction results were shown in Fig. 1. The main band was clear and there was no
118 concentrated band below 500 bp, which indicated that the purity of DNA was well and it could
119 meet the requirement of sequencing.

120 **Fig. 1. Extraction DNA of rumen bacteria**

121 The analysis of basic sequencing data

122 The OTU number of each group in one period was shown in Venn graph as Fig. 2. The result

123 showed that the OTUs was that: group 1> group 2 > group 3> group 4 for four periods, which
124 showed that the diversity of rumen microbiome decreased with the increase of NFC/NDF, and the
125 number of each group became more stable with prolong of periods

126 **Fig. 2. Vene graph of OTUs in Karakur sheep**

127 The analysis of OTU Alpha diversity

128 The Alpha diversity is a kind of analysis in the diversity of microbiome, which involves the
129 abundance index of Chao1[22] and ACE[23] and the diversity index of Shannon and Simpson[24].
130 Before diversity analysis, the dilution curve was drawn by R software (Version 2.15.3) to detect
131 whether the obtained data could fully reflect the distribution of rumen fluid flora in Karakul sheep.

132 OTU dilution curve

133 As showed in Fig. 3, the dilution curve of each group keep increasing with the increase of the
134 depth of sequencing, which is indicated that new bacteria had been found. The results showed that
135 the sequencing quantity of each sample could be used to analyze the diversity of flora.

136 **Fig. 3. OTU dilution curve of bacteria in the rumen of Karakul sheep**

137 Sample diversity index

138 The results of Alpha diversity were shown in Table 2. The results showed that there was some
139 difference in the number, richness and diversity of rumen bacterial species in different dietary of
140 NFC/NDF.

141 **Table 2. Analysis of Alpha diversity of rumen liquid samples at 0.03 distance**

Structural analysis of rumen microbiome

At phyla level, seventeen different phyla were detected. The S2 table and Fig. 4 showed that the main phylum microbiome didn't change with the dietary NFC/NDF and periods, and Bacteroidetes (52%~72%) and Firmicutes (19%~46%) was the main microbiome for four periods. The relative abundance of Tenericutes was 3% to 8% and the others was less than 1%. Fig. 5 was composed by four main dominant phylum to investigate effects of NFC/NDF on their relative abundance, the abundance of Bacteroidets and Proteobacteria were that: group 1> group 2> group 3> group 4 for four periods, but the difference wasn't significant(P>0.05). While the abundance of Firmicutes was that : group 4> group 3> group 2> group 1, the difference wasn't significant(P>0.05) as well. The abundance of Tenericutes reached the highest in group 4 for four periods.

Fig. 4. The column chart of the main dominant phylum in Karakul sheep

Fig. 5(a~d) . Effects of dietary NFC/NDF on relative abundance (% reads) of rumen phylum in Karakul Sheep

At the genus level, a total of 77 genera were obtained from sequence alignment. It can be seen from S3 Table and Fig. 6. The main genus microbiome didn't change with the dietary NFC/NDF and periods. The highest relative abundance of genus was a kind of semi-cellulose degrading bacteria, *unidentified-Lachnospiraceae* (1.86%~16.68%). The following relative abundance of genus was *Succiniclasticum* (0.12%~17.03%). Fig. 7 was composed by four main dominant genus to investigate effects of dietary NFC/NDF on their relative abundance, the abundance of *Succiniclasticum* was that: group 2> group 1> group 3> group 4, and the difference wasn't

significant (P>0.05). The relative abundance of *unidentified-Lachnospiraceae*, *Anaeroplasma* and *unidentified-Bacteroidales* reached the highest in group 3 for four periods, and the difference wasn't significant (P>0.05) as well.

Fig. 6. The column chart of the main dominant genus in Karakul sheep

Fig. 7(a~d) . Effects of dietary NFC/NDF on relative abundance (% reads) of rumen genus in

Discussion

Effects of dietary NFC/NDF on ruminal pH

pH is the directest index affecting rumen fermentation[25] and diets are key factors affecting pH. The results showed that the ruminal average pH decreased with the increase of NFC/NDF for four periods, which was approved with T.Ma et al.[26]. Agle et al. [27] and Pina et al. [28] also reported that with adding of concentration, ruminal pH decreased. This is mainly due to that the increase content of NFC resulted in the increase of VFA, while the low content of NDF resulted in the decreased rumination in sheep and decreased saliva to the rumen. Thus, the average pH of group IV was significantly lower than the other groups. The results were consistent with other studies[29-30]. Yang et al [31] pointed out that when pH was lower than 6.0 for a long time, the sheep would be in a long-term pathological state. The results showed that the average pH in group IV was near to 6.0 , it may have negative effects on Karakul sheep, and needs to be further verified.

Effects of dietary NFC/NDF on rumen bacteria in Karakul

183 **sheep**

184 Diets have crucial effects on rumen microorganisms, Jin et al. [32] showed that the number and
185 diversity of rumen bacteria in goats feed under high grain (71.5%) diet were lower than those with
186 high forage diet (0% grain). In this study, the results showed that the rumen bacteria diversity in
187 Karakul sheep decreased with the increase of dietary NFC/NDF, which was consistent with the
188 results of Liu [33], However Yong et al. [34] showed that there was no significant difference in
189 the number of rumen bacteria when the sheep were fed with different ratio of forge to
190 concentration diets, which might be caused by the difference of diets and species.

191 Roughage is the main feed source for ruminants, and rumen bacteria play a crucial role in the
192 utilization of roughage. Research showed that diets with easily fermentable carbohydrates would
193 decrease fiber degradation[35], resulting the imbalance of cellulolytic bacterial species.
194 Bacteroidetes play an important role in the degradation of non-fiber substances and Firmicutes
195 mainly degrade fiber substances. A large number of studies have shown that Bacteroidetes and
196 Firmicutes are the most dominant flora in the gastrointestinal tract of mammals[36-39]. Li et al.
197 [40] showed that when the calves were fed with two kinds of NFC/NDF diets, Bacteroidetes and
198 Firmicutes were still the main dominant flora. In this study, the results showed that the relative
199 abundance of Bacteroidetes and Firmicutes in different dietary NFC/NDF were still the main
200 dominant phylum. Some results showed that the abundance of bacteria in the same sample would
201 be different if the gene region was sequenced different[41], In our experiment, the region of
202 V1-V9 was sequenced and the results showed that the relative abundance of rumen bacteroidetes
203 decreased with the increase of dietary NFD/NDF in Karakul sheep, which was consistent with
204 Ellison[42]. However, when Kim et al. [43] researched on the content of Bacteroidetes in beef

cattle by sequencing V1-V3 region, the content of Bacteroidetes in high forage group was significantly lower than that of high proportion cereal group, which may be due to the difference of species differences and the sequence regions measured. In addition, the degradation rate of dry matter and organic matter was higher in group 3, 4 than which in group 1, 2 (the results were found previously by our team) so other bacteria except Bacteroidetes in Karakul sheep might have digested non-fiber substances and needs to be further studied.

Improving the fiber degradation rate is very important for ruminants. Bacteria and fungi play a crucial role in the decomposition and utilization of cellulose. In this study, the relative abundance of Firmicutes reached the highest when the dietary of NFC/NDF were 1.61 and 2.00, which was consistent with the results that had done before (The result was that: the NDF degradation rate in dietary NFC/NDF of 1.61 was the highest).U*nidentified-Lachnospiraceae* was the most dominant genus and its relative abundance reached the highest in group 3 for four periods, which further identified the results that: NDF degradation rate in dietary NFC/NDF of 1.61 was the highest.

In addition, there were many unidentified bacteria in the rumen of Karakul sheep, which might mean that there were some new species in Karakul sheep and needs to be further studied.

Conclusions

The ruminal pH and total diversity of rumen bacteria decreased with the increase of dietary NFC/NDF. The most dominant phylum, genus and species didn't change with dietary NFC/NDF and the ruminal bacteria became more stable with prolong of periods in Karakul sheep.

Acknowledgments

We are grateful for other tutors and classmates in our department that helped in the experiment.

Author contributions

Contributed reagents/materials/analysis/ tools: X.G.; Performed the experiments: X.P; Analyzed the data: X.P; Writing- original draft: X.P.; Writing–review editing: X.P., X.G., C.J., J.L., X.Z., S.Z., C.L., and A.S..

Supporting information

S1 Table. The ingredients and nutrient composition of the diet (% of DM). ①The premix provided the following per kg of diets: VA 1800 IU, VD3 600 IU, VE 30 mg, Fe 65 mg, Se 0.15 mg, I 0.6 mg, Cu 10 mg, Mn 28 mg, Zn 45 mg, Cu 12 mg. ②Nutrition level was a calculated value. ③NFC=（1NDFCPFatAsh）× 100%。

S2 Table Effects of dietary NFC/NDF on relative abundance of phylum in Karakul sheep

Period I (1~18 d), II (19~36 d), III (37~54 d) and IV (55~72 d) and Group 1, 2, 3, 4 means four groups of sheep treated with four dietary levels of NFC/NDF (0.78, 1.23, 1.61, 2.00 respectively). The same as below.

S3 Table Effects of dietary NFC/NDF on relative abundance of genus in Karakul sheep

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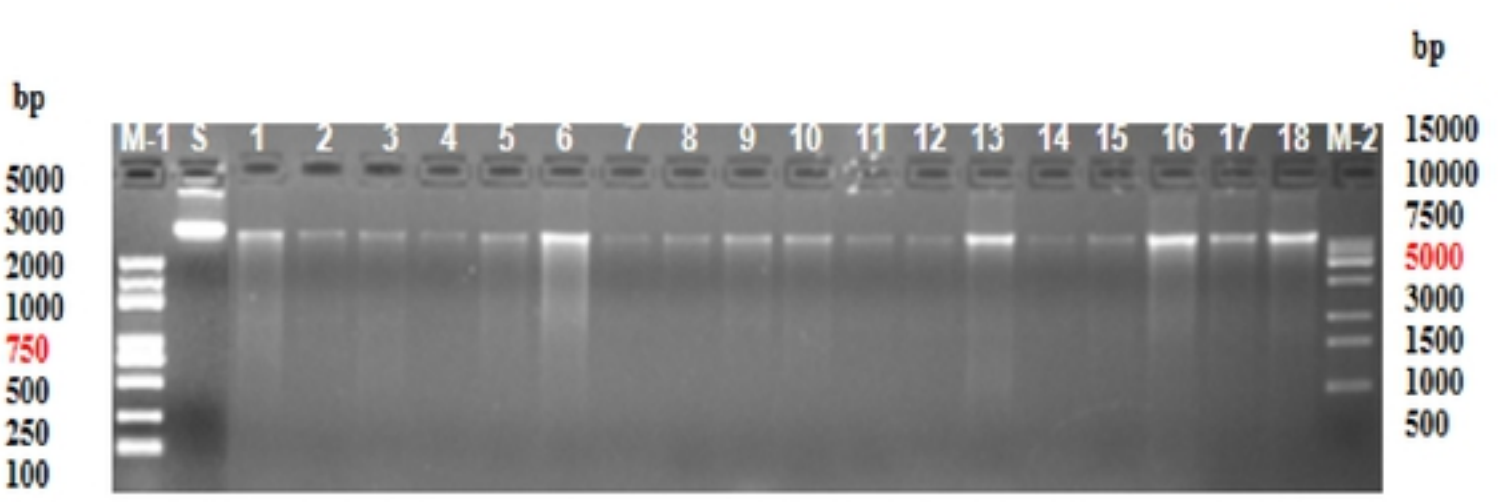
1 **Table 1. Effects of different NFC/NDF on rumen fluid pH**

Items	pH				SEM	*P*-value
	1	2	3	4		
Period I	6.36	6.32	6.26	6.05	0.07	0.507
Period II	6.42a	6.28ab	6.03bc	6.01c	0.06	0.015
Period III	6.68a	6.48ab	6.34bc	6.19c	0.06	0.012
Period IV	6.52a	6.27ab	6.16b	6.05b	0.06	0.016

2 Note: In the same row, values with no letter or the same letter superscripts mean no significant
3 difference ($P>0.05$), while with different small letter superscripts mean significant difference (P
4 <0.05). Period I (1~18 d), II (19~36 d), III (37~54 d) and IV (55~72 d) and Group 1, 2, 3, 4
5 means four groups of sheep treated with four dietary levels of NFC/NDF (0.78, 1.23, 1.61, 2.00
6 respectively). The same as Table 3.



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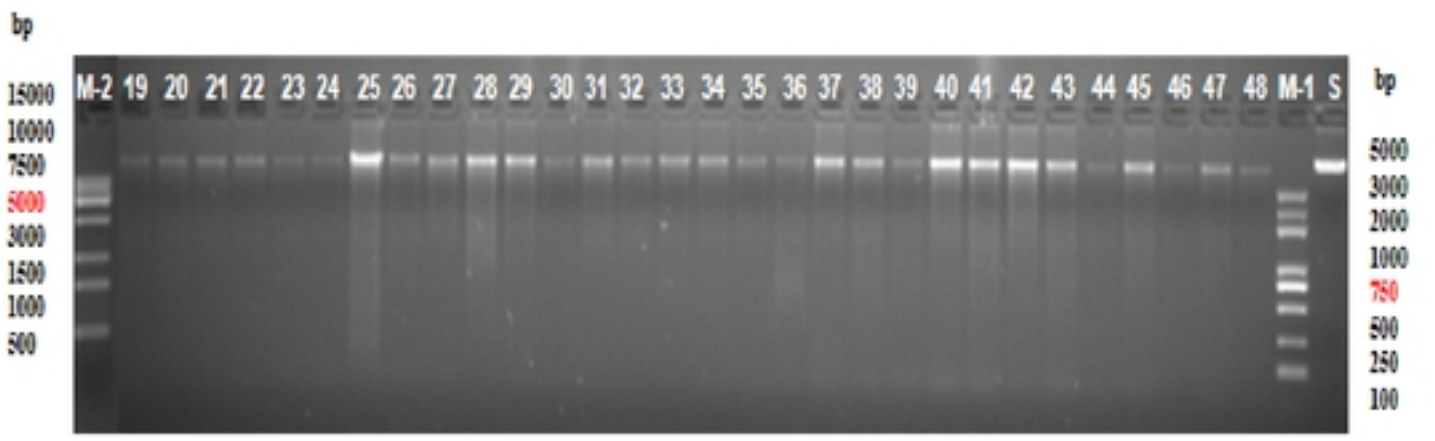
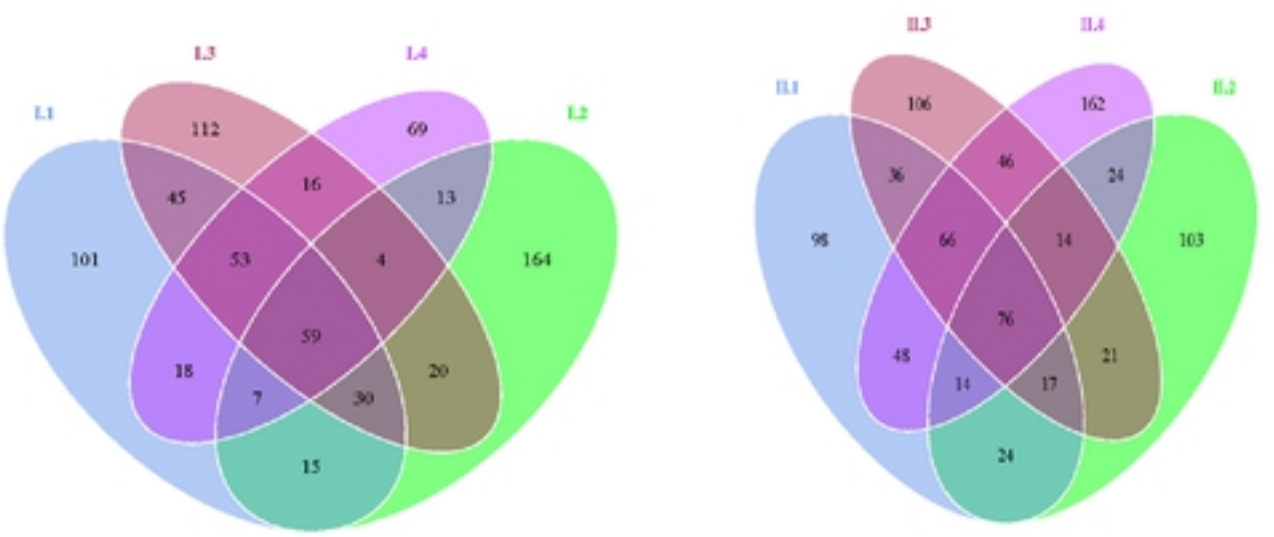


Fig. 1. Extraction of rumen bacterial DNA. The DNA of forty-eight samples from four periods samples were extracted, the order of 1~3, 4~6, 7~9 and 10~12 means samples in group 1, 2, 3 and group 4 of period I respectively, each group with three replicates. The following order are as period I.



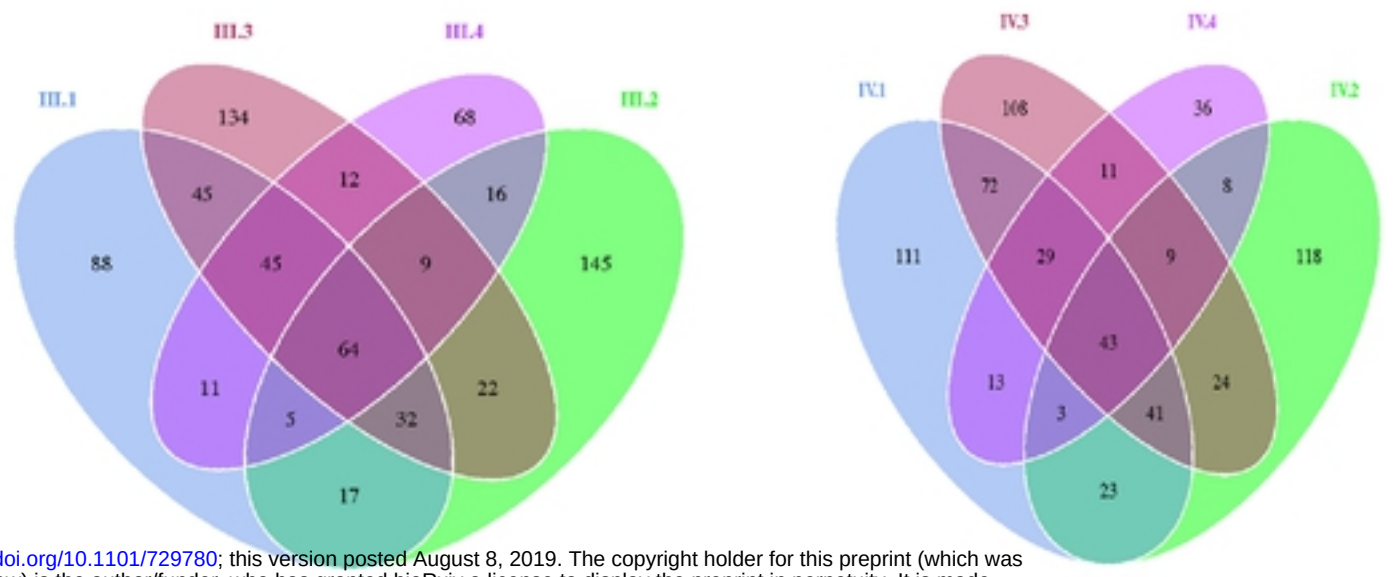
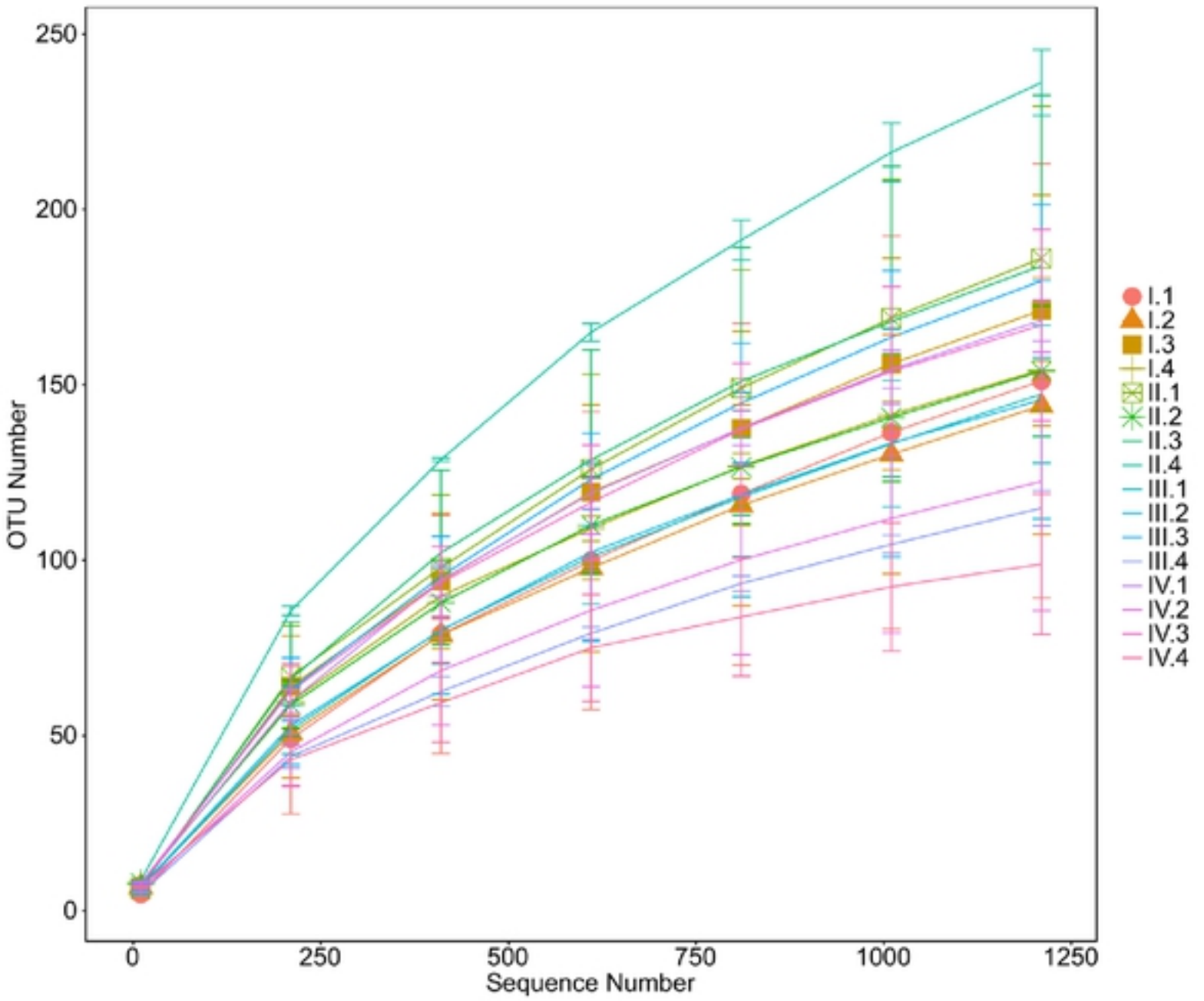


Fig. 2. Vene graph of microflora in rumen fluid of Karakul sheep. The amounts of OTUs in each group were shown and four groups of one period were formed in one Vene graph.



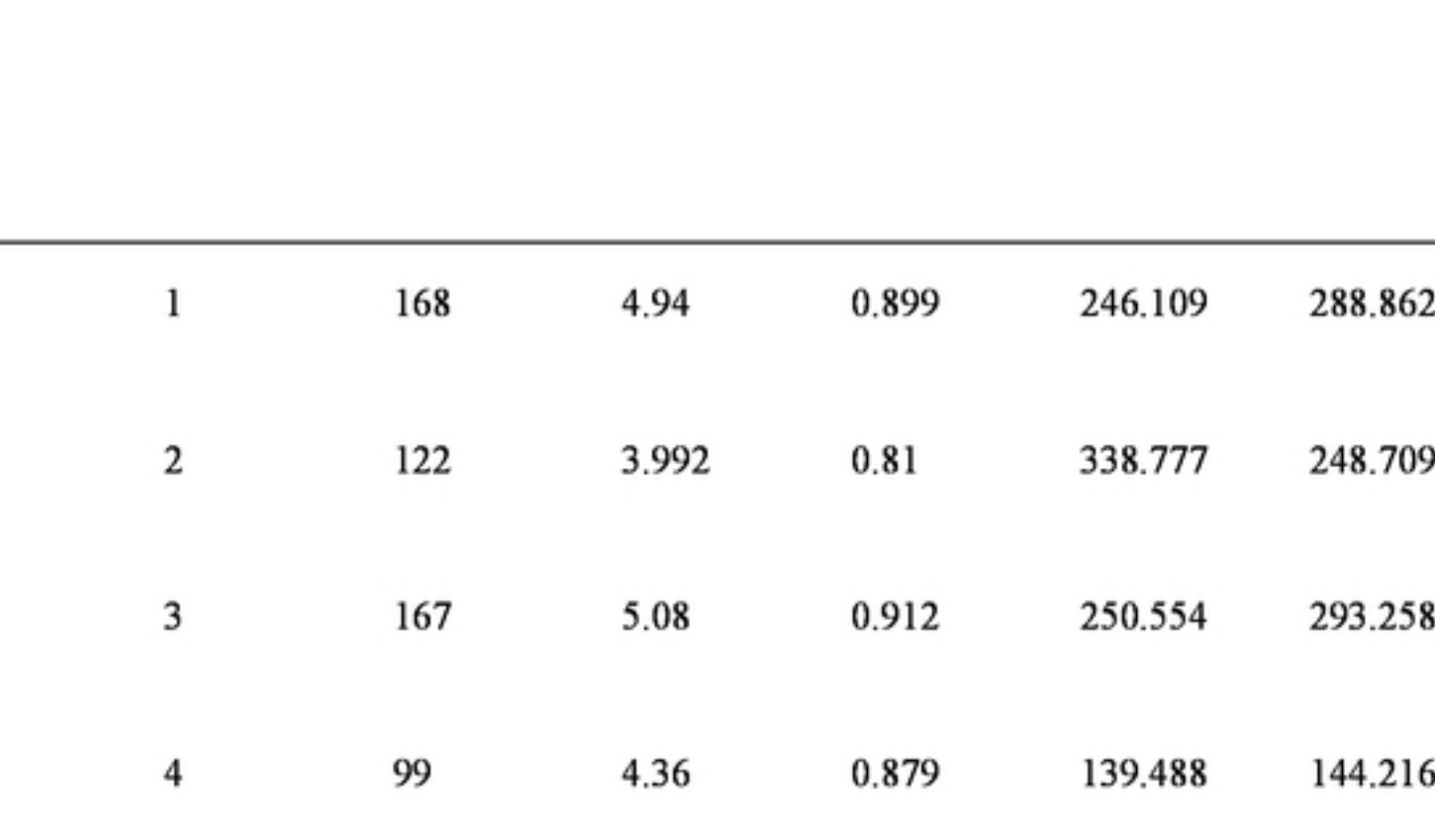
19 **Fig. 3. OTU dilution curve of bacteria in the rumen of Karakul sheep.** Rarefaction curves of

20 OTUs clustered at 97% sequence identity across different samples.

21 **Table 2. Analysis of Alpha diversity of rumen liquid samples at 0.03 distance**

Items		observed_species	Shannon	Simpson	Chao1	ACE
I	1	151	3.488	0.631	295.35	309.08
	2	144	4.447	0.853	259.414	271.994
	3	171	4.594	0.799	332.167	340.381
	4	154	4.946	0.897	232.5	252.125
II	1	186	4.536	0.787	321.413	357.069
	2	154	5.088	0.927	246.385	274.676
	3	184	4.865	0.838	315.519	348.306
	4	236	6.127	0.961	399.36	417.33
III	1	147	4.465	0.863	243.969	295.035
	2	146	4.545	0.874	233.092	263.23
	3	180	5.024	0.906	275.943	318.957
	4	115	3.786	0.798	198.268	208.16

	1	168	4.94	0.899	246.109	288.862
IV	2	122	3.992	0.81	338.777	248.709
	3	167	5.08	0.912	250.554	293.258
	4	99	4.36	0.879	139.488	144.216



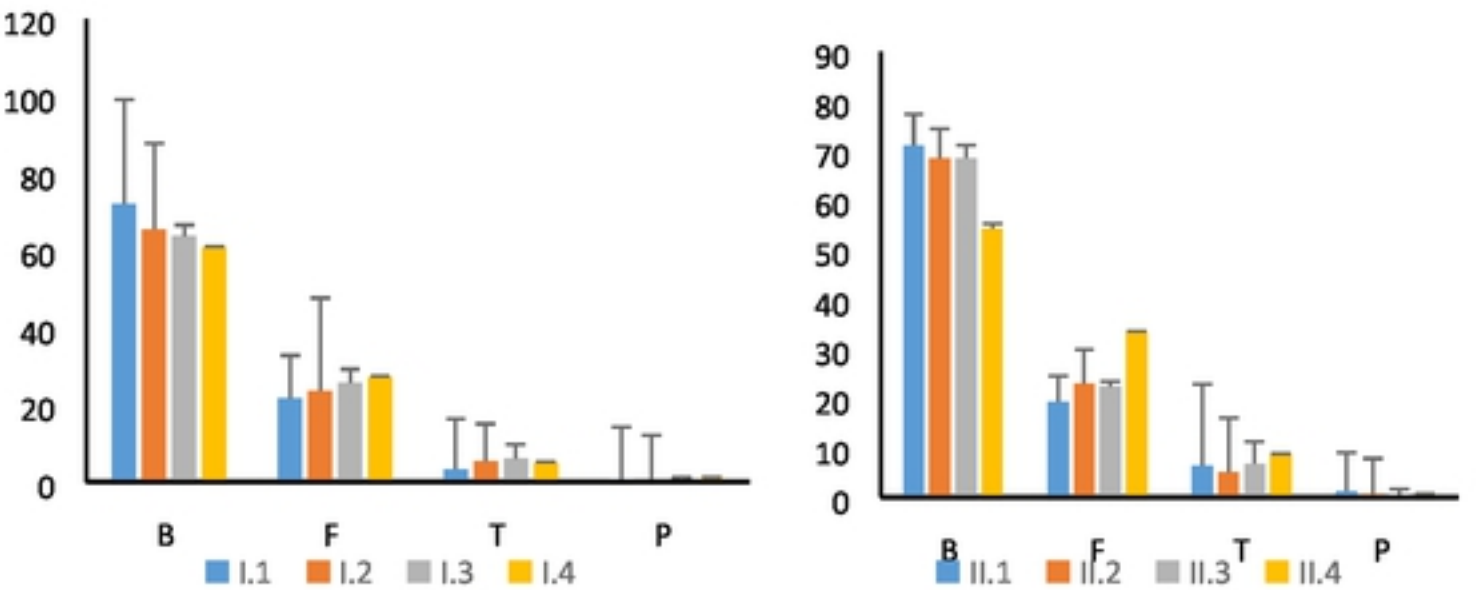
22

23 **Fig. 4. The column chart of the main dominant phylum in Karakul sheep fed with different**

24 **NFC/NDF diets.** A color-coded bar plot showing the average bacterial phylum distribution across

25 the different age groups that were sampled.

26 a b



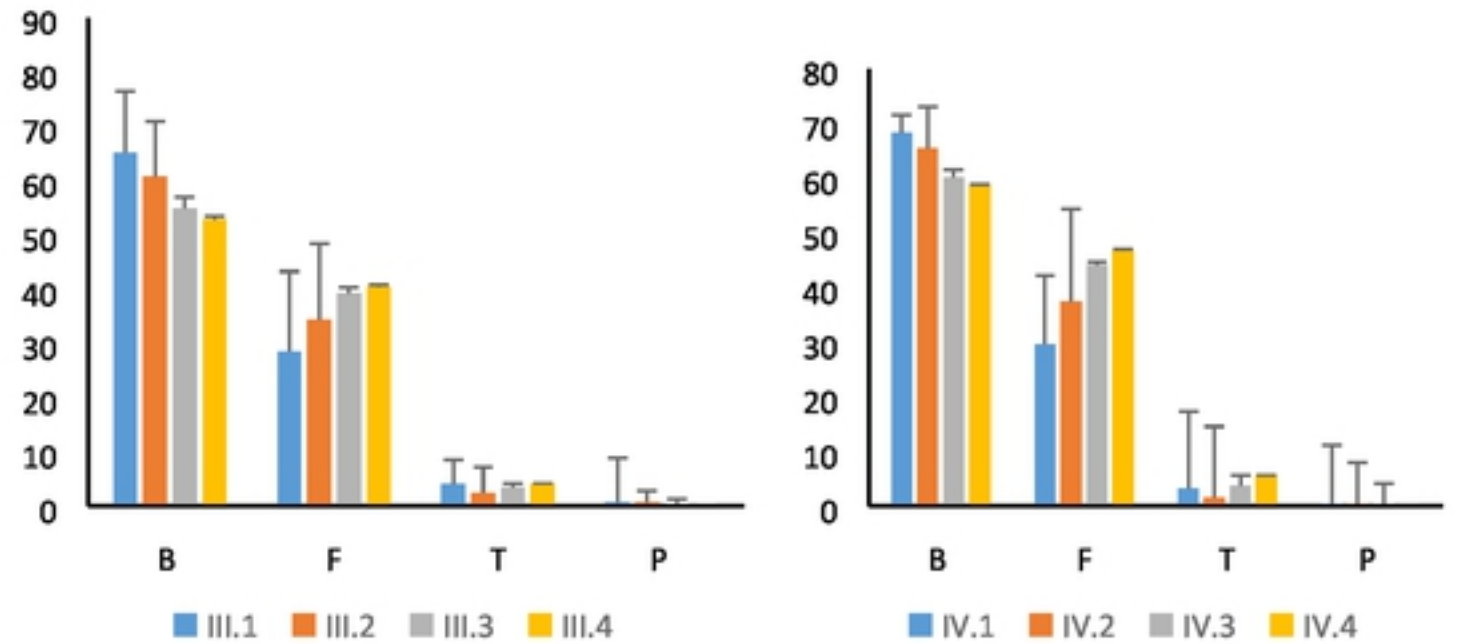
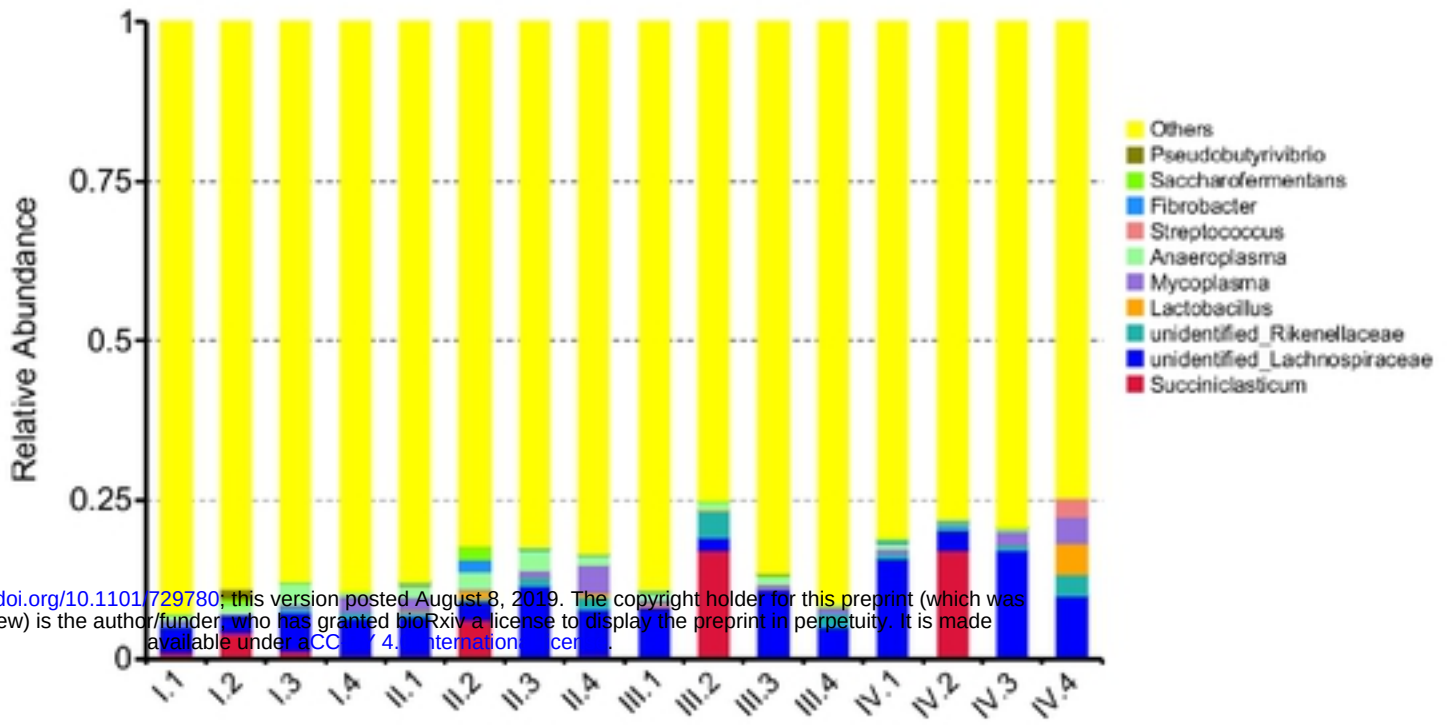


Fig. 5(a~d) . Effects of different NFC/NDF diets on relative abundance (% reads) of rumen phylum in Karakul Sheep

Note: B means Bacteroidets, F means Firmicutes, T means Tenericutes, P means Proteobacteria; a, b, c, d represents the experiment period of I, II, III, IV respectively.



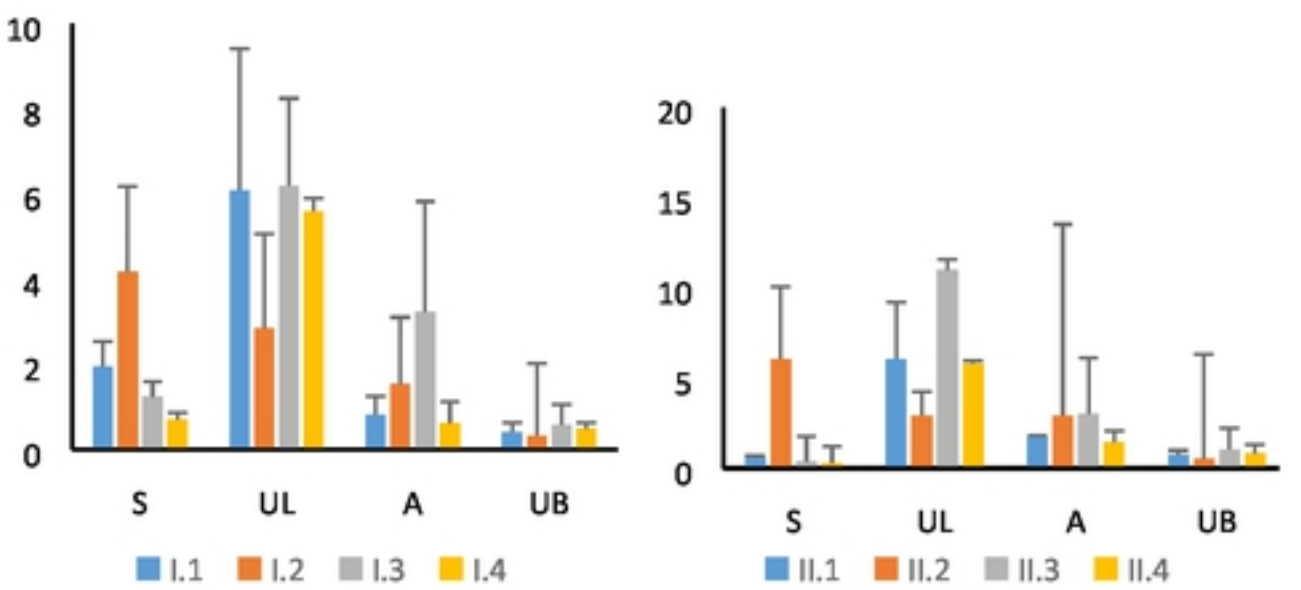
35

36 **Fig. 6. The column chart of the main dominant genus in Karakul sheep fed with different**

37 **NFC/NDF diets.** A color-coded bar plot showing the average bacterial genera distribution across

38 the different age groups that were sampled

39 a b



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41 c d

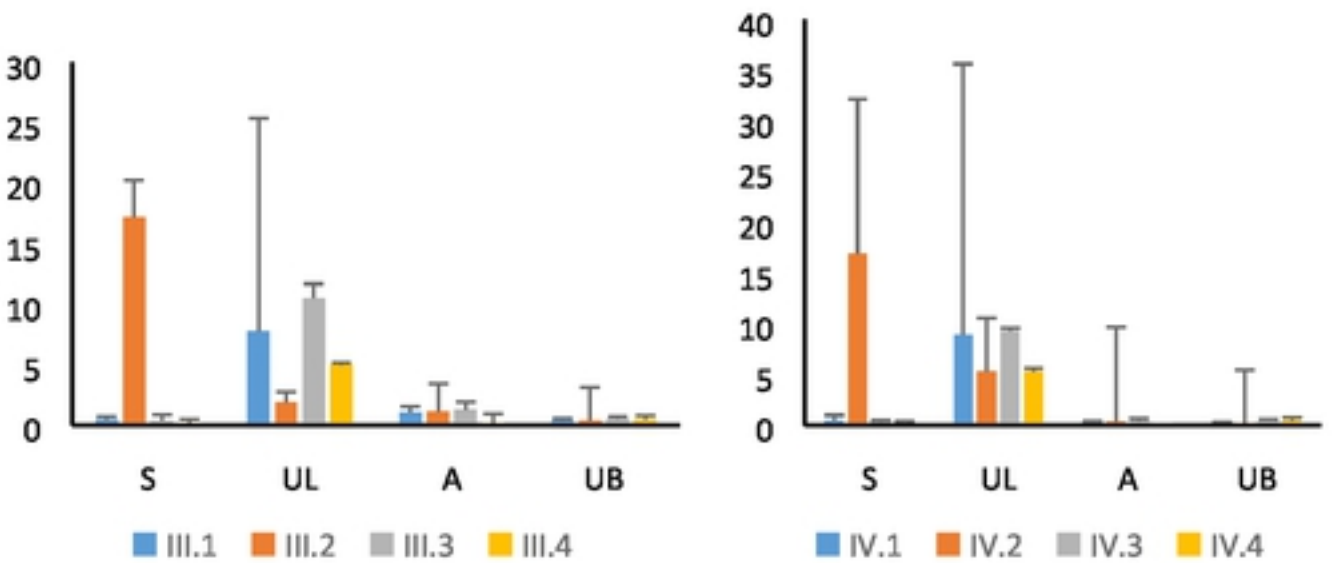

Fig. 7 (a~d) : Effects of different NFC/NDF diets on relative abundance (% reads) of rumen genus in Karakul Sheep. Note: S means *Succiniclasticum*, UL means *unidentified-Lachnospiraceae*, A means *Anaeroplasma*, and UB means *unidentified Bacteroidales*, a, b, c, d represents the experiment period of I, II, III, IV respectively.

Relative abundance of community (%)

Fig. 8. Heat mop of the rumen bacteria composition at species level. The heat map indicates the relative percentage of each species for the different dietary NFC/NDF group sampled.

Table 3. Effects of different NFC/NDF on the relative abundance (%) of cellulose-degrading

53 **bacteria**

	Period I					
Species	1	2	3	4	SEM	*P*-value
Butyrivibrio-fibrisolvens	2.664a	0.466b	2.990a	2.123a	0.371	0.035
Fibrobacter-sp-UWCM	-	0.027	-	-	0.006	0.441
Ruminococcus-flavefaciens	0.027	0.054	0.051	-	0.038	0.216
Ruminococcus-albus	-	0.055	-	-	0.009	0.052
	Period II					
Species	1	2	3	4	SEM	*P*-value
Butyrivibrio-fibrisolvens	2.689a	0.548b	3.123a	2.406a	1.132	0.027
Fibrobacter-sp-UWCM	0.082	0.411	0.027	-	0.350	0.294
Ruminococcus-flavefaciens	-	0.055	0.050	0.027	0.036	0.290
Ruminococcus-albus	0.027	0.055	-	-	0.01	0.561
	Period III					
Species	1	2	3	4	SEM	*P*-value
Butyrivibrio-fibrisolvens	6.785a	0.657c	7.687a	3.292b	1.143	<0.01

Fibrobacter-sp-UWCM	0.025	0.027	-	-	0.009	0.596
Ruminococcus-flavefaciens	0.027	0.082	0.055	0.027	0.021	0.627
Ruminococcus-albus	-	-	-	-	-	-

Period IV

Species	1	2	3	4	SEM	*P*-value
Butyrivibrio-fibrisolvens	7.234a	2.301c	8.694a	5.975b	1.581	<0.01
Fibrobacter-sp-UWCM	-	0.082	-	-	0.07	0.441
Ruminococcus-flavefaciens	-	0.082	0.079	-	0.016	0.052
Ruminococcus-albus	-	0.085	-	-	0.013	0.063