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Effects of NDF Content on Protozoal Community and Grazing Rate in Rumen

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Abstract: The experiment was conducted to investigate the effects of NDF levers in diet on the community structure and the grazing rate of ruminal protozoa by Fluorescence-Labeled Bacteria (FLB) technique. Four Xuhuai goats with permanent cannulas were used in a 4×4 Latin squares and diets were divided into A (54.87%), B (44.23%), C (33.72%) and D (23.23%), on the basis of NDF content in diet. The research results showed that the NDF content in diet had a profound influence on the rumen fermentation and subsequently modified the structure and the grazing rate of protozoa. Percentages of *Entodiniinae* and *Isotrichidae* were much higher in group C and D, whose diets containing low NDF content, the reverse was true for *Diplodiniinae* or *Ophryoscolecinae*, their proportions were higher in group A and B, whose diets containing high NDF content while, *Entodiniinae* was the dominant population of protozoa in the rumen. Furthermore, significant differences were found in protozoa grazing rates between groups and the lowest rates (366.7 cells h^{-1}) falling in group B containing 44.23% NDF. And also, marked difference was found in the predation rates among genus and there were 361.9, 606.3 and 607.5 cells h^{-1}, respectively for *Entodiniinae*, *Diplodiniinae* and *Ophryoscolecinae* and the grazing rate of *Entodiniinae* were significantly lower than those of both *Diplodiniinae* and *Ophryoscolecinae*. Community structure and grazing rate of rumen protozoa were modified by dietary NDF content.

Key words: NDF content, rumen, protozoa, grazing rate, community

INTRODUCTION

Protozoa are the inherent community in ruminant rumen, their existence are very important for the stable inner environment and the cellulose digestibility in the rumen. It is however that as predators of bacteria in rumen, protozoa causes an insignificant recycling of bacterial protein and cycles significant amounts of organic nitrogen in rumen ecosystems and leading to a low utilization efficiency of nitrogen (Coleman, 1979; Jounay, 1996). Thus, it is a main channel for AA nutrition control and way for saving protein feedstuffs in ruminant, through regulating protozoa population and altering the bacterial recycling in the rumen (Santra *et al*., 2006). The level of forage inclusion in the ruminant diet might shift the rumen fermentation pattern and then manipulate the population and composition of rumen protozoa (Franzolin and Dehority, 1996; Brown *et al*., 2006). These changes of protozoa due to diet, on the one hand, will affect microbial protein production directly and on the other hand, will affect the microbial protein yield and the N utilization efficiency indirectly, through affecting the ruminal micro-recycling. Therefore, the determination of protozoan grazing rates on bacteria is necessary for estimating the microbial protein production and the efficiency of organic matter utilization. It is however that the ruminal micro-recycling of protozoa grazing on bacteria is far from clear. Thus in this study we present an essential technique of Fluorescence-Labeled Bacteria (FLB) to measure protozoa grazing on bacteria under different dietary NDF content conditions, the main objectives were to analyze the effects of dietary NDF content on protozoa population and grazing rate in goat's rumen and hoped to offer some references to researches on the technique of N nutrition regulating of ruminant and the way for saving protein feedstuffs.

MATERIALS AND METHODS

Animals and diets: This experiment was conducted at the experimental farm of Yangzhou University, Yangzhou, China and was approved by the Yangzhou University Animal Care Committee. Four Xuhuai goat wethers, 1 year old and weighing 27.8±2.5 kg BW, fitted with rumen cannulas were used in this experiment. The diet formulas used were showed in Table 1 and divided into A (54.87%),

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Table 1: Composition and nutrient levels of experimental diet

Ingredient	A	B	C	D
Corn (%)	0.97	17.39	38.65	60.87
Soybean meal (%)	8.70	11.59	9.66	6.76
Straw (%)	86.96	67.63	48.31	28.99
Urea (%)	0.97	0.97	0.97	0.97
Dicalcium phosphate (%)	1.06	1.06	1.06	1.06
Salt (%)	0.77	0.77	0.77	0.77
Premix[1] (%)	0.58	0.58	0.58	0.58
Total (%)	100.00	100.00	100.00	100.00
Nutrient level[2]				
Metabolism Energy (ME MJ kg^{-1})	5.78	7.17	8.62	10.14
Dry Matter (DM%)	91.84	91.26	90.63	89.99
Crude Protein (DM basis) (CP%)	9.20	10.91	11.03	10.82
Non-Structure Carbohydrate (NSC%)[3]	23.71	33.64	45.89	58.56
Structure Carbohydrate (SC%)[3]	54.87	44.23	33.72	23.23
NSC/SC	0.43	0.76	1.36	2.52

[1]The composition of premix: $FeSO_4 \cdot 7H_2O$ 170 g kg^{-1}, $CuSO_4 \cdot 5H_2O$ 70 g kg^{-1}, $MnSO_4 \cdot 5H_2O$ 290 g kg^{-1}, $ZnSO_4 \cdot 7H_2O$ 240 g kg^{-1}, $CoCl_2 \cdot 6H_2O$ 510 mg kg^{-1}, KI 220 mg kg^{-1}, Na_2SeO_3 130 mg kg^{-1}, VA1: 620,000 IU kg^{-1}, VD3: 324,000 IU kg^{-1}, VE: 540 IU kg^{-1}, VK3: 150 mg kg^{-1}, VB1: 60 mg kg^{-1}, VB2: 450 mg kg^{-1}, VB12: 0.9 mg kg^{-1}, VB5: 1,050 mg kg^{-1}; Pantothenic acid calcium: 750 mg kg^{-1}; Folacin 15 mg kg^{-1}; [2]Analyzed value, [3]SC = NDF - NDFN, NSC = 100 (NDF + CP + EE + ASH - NDFN)

B (44.23%), C (33.72%) and D (23.23%), according to NDF ratio in diet. Amounts of energy offered were calculated to meet energy requirements recommended by NRC (1981). The goats were fed experimental diet at 1.3 times their Metabolizable Energy (ME) requirement for maintenance (1.3 M). The animals were given in equal meals day^{-1} at 8:00 and 20:00; they also had free access to clean drinking water.

Experimental design and sample manipulating: The animals were randomly assigned to 4 dietary treatments in a 4×4 Latin square design. Each experimental period consisted of a 14 days preliminary period and then followed by 7 days for rumen fluid sampling. Approximately, 20 mL digests mixture was taken from rumen at an interval of 2 h on the 1st day (8:00, 10:00, 12:00, 14:00, 16:00, 18:00 and 20:00, respectively) by using stomach tube and then the rumen liquor collection was squeezed through two layer of gauze. Immediately after collection, pH value, ammonia-N concentration was measured using 10 mL sample; the remaining 10 mL of mixture was also immediately fixed and stained with an equal volume of MFS solution (containing 8 g L^{-1} NaCl, 0.6 g L^{-1} methyl green and 100 mL L^{-1} formaldehyde) at room temperature for 30 min and after fully mixing all the samples of different sampling point during the whole day, the well-mixed liquid effluent was ready for protozoal enumeration test. Mixture obtained from rumen on the second, 3rd day, was used for making the bacteria-free rumen fluid and the Fluorescence-Labeled Bacteria (FLB), respectively and rumen fluid obtained on the following sampling day was for grazing rate testing. The sampling time of the consecutive 6 days (2nd-7th) was all set for 11:00 am.

Rumen fermentation parameters and protozoal enumeration: Ruminal pH value in rumen was tested immediately using a standardized laboratory pH meter (pHS-3C, Huanyu Comp. Jingtan, China) at each sampling time point. Ammonia -N concentration was determined by the colorimetric spectrophotometric method (Chaney and Marbach, 1962). The Dacron bag technique described by Ørskov and Hovell (1977) as a method for measuring NDF digestibility.

The staining samples were observed under microscope (400×) to count protozoa cells and then calculated the protozoa number using the following equation.

$$N \times D \times 4 \times 10^4 = \frac{\text{Protozoa number}}{\text{mL}}$$

where:

N = Total number of protozoa in 4 middle panes
D = Dilution times

Grazing experiment: Grazing experiment was carried out following Wang *et al.* (2008).

Bacteria-free rumen fluid: Rumen liquor were collected from each goat during each experimental period and centrifuged at 29000× g for 20 min and then the bacteria free rumen fluid was numbered in serial number and stored at 4°C within 1 week before use.

Fluorescence-Labeled Bacteria (FLB): Rumen fluid were collected from each goat during each experimental period and went through cellulose filter (pore size, 2 μm), then centrifuged at 22000× g for 15 min. Sediment was collected and washed 2 times using saline solution and 2 mL DTAF (5-((4,6-dichlorotriazin-2-yl)amino) fluorescein hydrochloride, Sigma) dyeing solution was added, the sample was incubated in a water bath kept at 60°C for 2 h,

followed by centrifugation to throw away the DTAF dyeing solution, the sediment was washed 2 times and resuspended in half volume of respective bacteria free rumen fluid with the same number and the FLB solution was stored at -20°C within 1 week before use.

Ingestion experiment: Rumen fluid were collected from each goat during each experimental period and went through cellulose filter (pore size, 80 μm), then centrifuged at 150× g for 15 min. Sediment was washed 2 times and resuspended in half volume of respective bacteria-free rumen fluid with the same number and subsequently, the samples were incubated in similar condition as rumen inner environment (O_2-free, shaking and 39°C water bath) for 30 min to reactivate protozoa and then added equal volume of respective FLB solution with the same number to begin the ingestion experiment, 1 mL subsamples were taken at 5 min intervals for 40 min after incubation, the time series samples were inspected by epifluorescence microscopy, 3 repeats were set for each sampling-time point.

Covert coefficient and data statistics: Number of FLB were converted to biomass N using an average bacterial biovolume of 0.1 μm^3 and a nitrogen conversion factor of 0.054 pg N of μm^3 (Wang *et al*., 2008). The data of the experiment was subjected to Analysis of Variance (ANOVA) (SPSS, ver. 11.5) and the means were compared for significance by Least Significant Difference test (LSD) at the $p<0.05$ and regression analysis was subjected to the curve estimation procedure.

RESULTS

Ruminal fermentation: The pH-value range throughout the sampling period was between 6.17 and 7.16. The lowest mean pH was found in D, while the highest data was found in A ($p = 0.021$) as shown in Table 2 and there were no significant differences between B-D. The daily variation patterns of pH value were clearly illustrated by Fig. 1. It is worth mentioning that slight fluctuations of pH occurred in group A and fluctuated at the high levels with comparisons to those exhibited by the groups B-D, which fluctuation at the low levels, especially D.

As Fig. 2 shows the continuous recording of NH_3 concentration changed from 3.63-28.35 mg/100 mL Significant differences were observed in the average NH_3 concentration between groups ($p = 0.000$) and group A, interpreted the lowest average NH_3-N concentration (6.09 mg/100 mL), while group B, demonstrated the highest peak (17.99 mg/100 mL). As shown in Fig. 2 the daily variation patterns of NH_3 concentration were quite different from each other. For example, compared to group B-D, the NH_3 concentration of group A fluctuated smoothly while, NH_3 concentration of B rose rapidly after feeding and reached the highest peak at 2 h postprandial and then declined sharply in the post-feeding period form 2-8 h and finally, changed at a low and stable level, with the comparison of C and D, till the second meal.

The results of the NDF degradability were also shown in Table 2 and revealed there were remarkable differences between treatments ($p = 0.000$). The NDF degradability of A was extremely low and significantly lower than that of D, B and C while, no significant difference was detected in NDF degradability between B and C.

Protozoal population: The results of protozoal cell densities were shown in Table 2. It was clearly that the highest protozoal density was observed in group C while, the opposite was found in group A, interpreting the lowest protozoal density. Further classification of protozoa (Table 2 and Fig. 3) revealed that the percentages of *Entodiniinae* and *Isotrichidae* increased, while percentages of *Diplodiniinae* and

Table 2: Effects of dietary NDF content on protozoa population and grazing rate on bacteria

Items	A	B	C	D	Note (%)	SEM	p-value
pH-value	6.95^{A}	6.57^{B}	6.51^{B}	6.38^{B}	-	0.05700	0.021
NH_3-N concentration (mg/100 mL)	6.09^{A}	17.99^{B}	16.07^{B}	15.90^{B}	-	1.18500	0.000
NDF degradability (%)	33.59^{A}	43.12^{C}	45.09^{C}	36.82^{B}	-	1.29510	0.000
Protozoa density $\times 10^5$(cells mL^{-1})	6.13^{A}	19.41^{B}	24.07^{C}	21.13^{BC}	-	1.69625	0.000
Profiles of protozoa (%)	**A-D increase**						
Entodiniinae	71.98^{A}	73.36^{A}	78.35^{B}	77.48^{B}	7.64	0.89829	0.000
Diplodiniinae	10.20^{A}	10.19^{A}	7.81^{B}	7.08^{B}	-30.59	0.48794	0.000
Ophryoscolecidae	4.10^{A}	3.51^{A}	2.50^{B}	2.42^{B}	-40.98	0.30636	0.000
Isotrichidae	5.10^{A}	5.40^{A}	6.27^{B}	7.22^{C}	29.36	0.38940	0.001
	91.39	92.45	94.93	94.20	-	-	-
Grazing rate	**Mean**						
Grazing rate (cells h^{-1})	429.500^{D}	366.70^{A}	389.500^{B}	402.200^{C}	-	2.7345	0.000
N recycling rates (pg $cell^{-1}$ h)	2.319	1.98	2.103	2.172	-	-	-
Entodiniinae	378.600^{C}	345.30^{A}	358.400^{B}	365.400^{B}	361.90	5.2389	0.000
Diplodiniinae	627.000^{C}	586.20^{A}	603.900^{B}	608.100^{B}	606.30**	6.1677	0.000
Ophryoscolecidae	629.300^{C}	588.50^{A}	603.200^{B}	609.200^{B}	607.50**	6.5761	0.000

A-D were diets containing 54.87, 44.23, 33.72 and 23.23% NDF, respectively; $^{A-D}$The means were not significantly different ($p>0.05$); neighbor letters indicated that the means were significantly different ($p<0.05$); parted letters indicated that the means were extremely different ($p<0.01$)

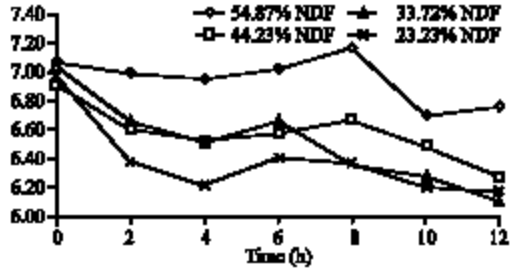

Fig. 1: The dynamics of pH change

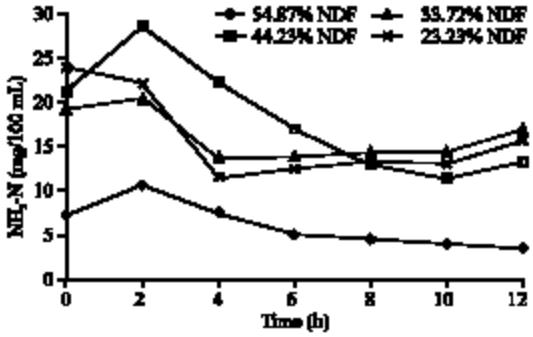

Fig. 2: The dynamics of NH_3-N concentration change

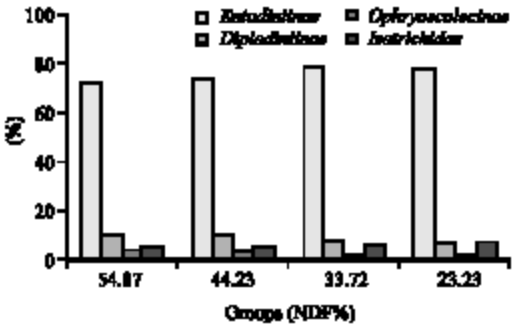

Fig. 3: Effects of diets on protozoal community structure

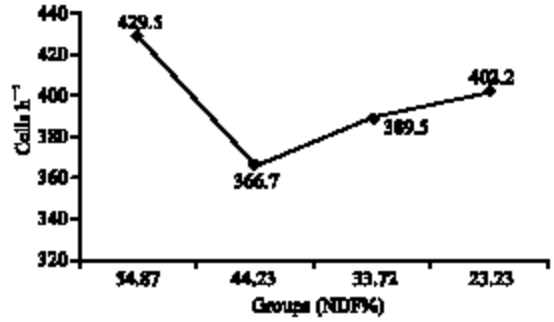

Fig. 4: Effects of diets on protozoal grazing rates

Ophryoscolecidae decreased with the decrease of NDF level. In addition, the size of *Entodiniinae* population was much bigger than that of the other 3 genuses.

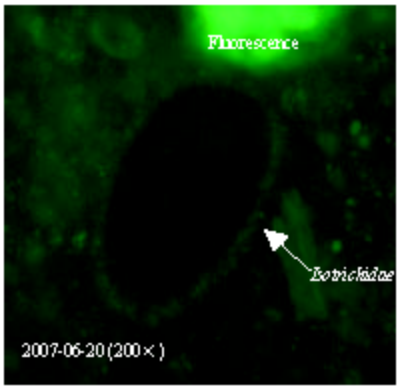

Fig. 5: The fluorescent photo of *Isotrichida*

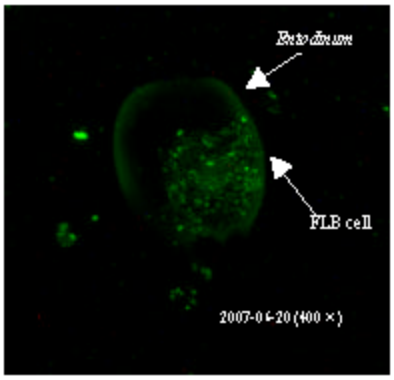

Fig. 6: The fluorescent photo of *Entodinum* with FLB cell, FLB was rumen bacteria stained with DTAF (5-((4,6-dichlorotriazin-2-yl)amino) fluorescein hydrochloride)

Protozoal grazing rate: Results presented in Table 2, Fig. 4 revealed that grazing rates varied according to diet ($p = 0.0000$) and the highest peak was observed in group A; while, lowest value was recorded by group B. Except group A, grazing rates had a tendency of increase with dietary NDF ratio decreasing. Significant differences were found in grazing rate across protozoal genuses and the grazing rates were respectively, 361.9, 606.3 and 607.5 cells h^{-1} for *Entodiniinae*, *Diplodiniinae* and *Ophryoscolecinae*. Furthermore, the grazing rate of *Entodiniinae* was significantly lower than those of *Diplodiniinae* and *Ophryoscolecinae*. It was however, that as Fig. 5 showed that no FLB cell was observed within *Isotrichida* body and failed to measure its grazing rate, compared to the other genus showed in Fig. 6 in this experiment.

DISCUSSION

Rumen fermentation: NDF content is one of factors affecting pH value in the rumen and a certain NDF level is

necessary to avoid a fast decline in pH and to ensure the microbial fermentation. Meanwhile, the changes of pH have a profound effect on the activity of rumen microbe, especially cellulose catabolic bacteria and protozoa, due to acid intolerance. It is well recognized that rumen microbial organisms only thrive within a narrow pH value range suitable for cellulose degradation. So, it is very important that pH value is within the suitable range for microbe fermentation, if pH value is out of the range of 5.5-7.5, then cellulose degradation will be severely affected. Calsamiglia *et al.* (2002) reported low pH would decrease the degradability of cellulose and feed protein. Although, the pH values in present experiments were all within the desired range for microbe's growth, they were also characterized with relatively great fluctuation ranged between 6.17 and 7.16 and had a tendency of decrease with the decrease of NDF level, this then even resulted in the decrease of NDF degradability, which conforming to the previous report.

Ammonia is a colligation indicator of the degradation of nitrogen source and the ammonia usage by rumen microbes. As a nitrogen source of the main microbes in rumen, such as cellulose-catabolic bacteria, the low NH_3-N concentration could inhibit the microbial activity and decrease the cellulose degradability. In current study, the NH_3-N concentration of B was highest and upgraded rapidly after feeding. The probable reason might be ascribed to the high microbial activity in this group, this then promoted the cellulose degradability and the result of high cellulose degradability in this group concurred with the high microbial activity. Additionally, protozoa could degrade protein into NH_3, but seldom utilized NH_3 to synthesize their body protein, thus the existence of protozoa might lead to a high NH_3-N concentration, while regulating protozoa could decrease the NH_3-N concentration. And this in turn might decrease the cellulose degradability (Jounay, 1996). This agreed with the results of this study, which showed that group A had the lowest protozoal density, the lowest NH_3-N concentration and the lowest NDF degradability among groups.

Protozoal community structure: The favorite substrates of rumen protozoa are starch and soluble polysaccharides. Therefore, the increase of proportion of dietary concentrate in some degree could lead to an increase in protozoa density. However, too much concentrate in diet will reduce rumen pH, consequently, result in protozoa population shrinking, or disappear finally in the rumen (Grubb and Dehority, 1975). Similarly, current research showed that protozoa population varied with dietary NDF contents. Group A recorded the lowest protozoal density and group C interpreted the highest density of protozoa. It was however that there was a decline in group D, when the concentrate level sustained increase, which agreed with the pervious study. *Entodinium* and *Isotricha* sp. was the dominant flora of microbial diversity for a high-grain diet with dissoluble carbohydrate, whereas, *Diplodinium* sp. and *Ophryoscolecinae* seemed to be the dominant protozoa for a high-forage diet with a high proportion of fibre (Han *et al.*, 2002; Wang *et al.*, 2007). Currently, the structure of protozoa community also changed with diets, the proportions of *Entodiniinae* and Isotrichida increased by 7.64 and 29.36%, respectively while, proportions of *Diplodiniinae* and *Ophryoscolecinae* decreased by 30.59 and 40.98%, respectively when, NDF content in diets decreased from 54.87-23.23%. These results indicated that protozoa community structure subjected to dietary NDF contents and concurred with the previous research described above. These results also proved that it was feasible to control rumen fermentation through protozoa regulation. In addition, Although, there were significantly different in the proportion of *Entodiniinae* between groups in this research, while the percents of *Entodiniinae* were all above 71.98% (the average value was 75.30%), much higher than those of the other 3 groups and was 8.54, 24.04 and 12.55 times as much as *Diplodiniinae*, *Ophryoscolecinae* and Isotrichida, respectively, which indicated that the *Entodiniinae* was the dominant group in rumen protozoa and conforming to the reports by Dehority and Odenyo (2003) and Dobicki *et al.* (2006).

Grazing rate: Previous researches showed that bacteria recycling rates ranged between 0.3 and 2.7% without protozoa while, the range of bacteria recycling rates were between 2.4 and 3.7% with protozoa (Zhu, 2004) these revealed that the existence of protozoa would cause the insignificant recycling of bacteria, subsequently, led to a decrease of contribution of microbial protein to duodenum and improved the maintenance requirement of protein of host animal consequently (Wallace and McPherson, 1987; Ivan *et al.*, 2000; Koenig *et al.*, 2000). Therefore, it was a main channel for AA nutrition control in ruminant, through regulating protozoa population to regulate the microbial protein yield reaching duodenum (Leng, 1982; Santra *et al.*, 2006). And for ruminant AA control, it is necessary to conduct further experiments to study the recycling rule of rumen bacteria by protozoa predation.

Many factors would disturb the protozoan grazing rate, including the ruminant diet, physicalchemical property of growth environments (pH, Eh, NH_3 and NH^{4+}, etc.), protozoa itself (genus, growth period, life propagation cycle, cell density, etc.), food density and so

on Hong *et al.* (2001), Lebaron *et al.* (2001), Lang *et al.* (2003) and Xu *et al.* (2004). It was well documented that the activity, population sizes and composition of rumen protozoa were manipulated by diets (Hristov *et al.*, 2004; Nhan *et al.*, 2007) and this then altered the protozoal grazing rate. As far as dietary NDF content was concerned, the higher the NDF content were, the lower the activity of protozoa might be and then leading to a lower grazing rate finally. Currently, the grazing rates of all groups falling within the range of 10^2-10^4 cells h^{-1} wasgenerally recognized as the range of grazing rates of rumen protozoa. But the recorded grazing rates still varied widely across diets and tended increased with decreasing NDF content, agreeing with the previous study.

The one probable reason was that the diet might influence the rumen inner environment and inhibit protozoal activity and then affected the grazing rate of protozoa. Another possible reason was estimated as follow: different protozoa genus would be selected by different diets and different genus had different grazing rate (Hong *et al.*, 2001). These then might lead to various grazing rates depending on diets. The above estimation was supported by the results of current study, which showed that significant differences existed in grazing rate between genuses. Anyway, the rumen micro ecosystem is complex and the factors that affect the protozoa grazing rate are not very clearly, so these results or estimations should be verified by further experiments.

CONCLUSION

It could be concluded that the NDF content in diet had an important influence on the rumen fermentation and subsequently modified the structure and the grazing rate of protozoa. The percentages of *Entodiniinae* and *Isotrichidae* increased, while percentages of *Diplodiniinae* and *Ophryoscolecinae* decreased with the decrease of NDF content. And *Entodiniinae* was the dominant population in protozoa. In addition, significant differences were found in grazing rate between NDF contents, or across protozoal genuses. And the lowest grazing rate was observed in B containing 44.23% NDF while, grazing rate of *Entodiniinae* was much lower than those of *Diplodiniinae* and *Ophryoscolecinae*.

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