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EFFECTS OF MANNAN OLIGOSACCHARIDES ON RUMEN FUNGAL FLORA OF DAIRY COWS

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ABSTRACT

To study the effects of mannan oligosaccharides (MOS) on rumen fungal flora of dairy cows, four lactating Chinese Holstein dairy cows were randomly divided into two groups in the two-stage 2 × 2 cross-over design. The experimental group (MT) was fed with a basal ration and MOS were perfused orally 60 g/(d head). The control group (CK) was fed with a basal ration. The rumen fluid was collected through the oral cavity of dairy cows using a negative pressure suction device. 50ml rumen fluid of every cow was collected each time respectively before feeding (0 h) and 2h, 4h and 6h after feeding in a day. The rumen fungi were tested by ITS sequencing technology. The results showed that four phyla were identified in CK group and five in MT group. The dominant phyla in both groups were *Ascomycota*, *Basidiomycota*, *Neocallimastigomycota,* whose abundances were more than 90% of the total rumen fungi. At the level of genus, 74 genera were identified in CK group, while 91 genera in MT group. Several cellulolytic fungi genera showed a decreasing trend or decreased significantly, such as *Neocallimastigaceae-NA*. The previous experiments that shows the addition of mannan oligosaccharides promoted the proliferation of rumen bacteria, while the growth of rumen fungi was inhibited in this result, which may be due to the antagonism between rumen bacteria and fungi. The growth of cellulose-degrading fungi was inhibited to some extend after the addition of MOS in the diet. *Candida* and *Zopfiella*, closely related to mycotic mastitis and immunosuppression respectively, had decreasing trends in rumen with supplemental MOS in the diets of the dairy cows, indicating that MOS might play a positive role in inhibiting harmful fungi. The experimental result shows that mannan oligosaccharides in the diet of dairy cows inhibited the growth of cellulolytic fungi and the proliferation of harmful fungi. In addition, the results showed that there might be the antagonistic effect between rumen fungi and bacteria potentially, which should be focused on for further study.

Keywords: Sequencing technology, fungi diversity, fiber degradation, antagonism, gastrointestinal health.

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INTRODUCTION

Mannan oligosaccharides (MOS) is an oligosaccharide composed of mannan or mannan with glucose through α-1,2, α-1,3, α-1,6 or β-1,3, β-1,4 glycosidic bonds, derived from outer layer of cell walls of S.cerevisiae. (Benites *et al*., 2008; Yan *et al*., 2008). MOS has been extensively researched in monogastric animals. According to the report of Chacher *et al*. (2017), MOS can improve cellular, humoral and skin immunity by activating macrophages in intestinal-related lymphoid tissue and also improve the growth speed and performance of broilers. From the slaughter experiments by Castillo *et al*. (2008), the MOS has beneficial effects on growth performance and nutrient digestibility of weaned piglets, and can also reduce diarrhea score of weaned piglets. MOS may protect intestine and improve immunity of animals. As a result, they have been widely used in research and production in numerous countries as a new feed additive. MOS also plays an active role in rumen fermentation, immunity and digestive function of ruminants. In recent years, researches on MOS in ruminants have been gradually carried out. Zheng *et al*. (2018) added 0%·kg^{-1}, 1.2%·kg^{-1}, 1.6%·kg^{-1} and 2.0%·kg^{-1} MOS in sheep diet respectively. It has been found that the addition of MOS can improve the antioxidant capacity of sheep, and also improve the fiber digestion of sheep. Franklin *et al*. (2005) supplied the MOS to the cows during the last 3 weeks of the dry period and then the cows were vaccinated against rotavirus at 4 weeks and 2 weeks before the expected parturition. The results have indicated that the addition of MOS to cows during the dry period can enhance their immune response to rotavirus.

The biggest difference between ruminants and monogastric animals is the rumen system, which contains ciliates, fungi and a large number of bacteria. Rumen fungi, which account for 5% to 20% of rumen microbial biomass, contain enzymes needed for digestion of plant feed, including cellulase, xylanase and other hydrolytic enzymes. Although rumen fungi do not account for much of the total rumen microorganisms, studies have showed their complexity and large amount of enzyme activities play a major role in digesting fiber (Akin and Borneman,

1990; Cammack *et al*., 2018; Rezaeian *et al*., 2004; Roger *et al*., 1992). As a result, it is of great significance to actual production to focus on the research on rumen fungus. Internal Transcribed Spacer (ITS) is a technique to obtain information on unknown fungi species by sequencing ITS DNA and then comparing the sequenced ITS sequences with known fungal ITS sequences. It is a method for analyzing the diversity of fungi (Vargas-Bello-Pérez *et al*., 2016). This study used ITS to investigate the effects of MOS on rumen microbial diversity in dairy cows focusing on the perspective of the diversity of rumen fungi.

MATERIALS AND METHODS

Experimental Animals and Feeding Management: Four lactating Chinese Holstein cows weighing about 550 kg in the similar parity were selected as experimental animals fed from August to October, 2018. The experimental animals were fed at a dairy farm in Yinchuan, Ningxia of China (106°27'E, 38°47'N). The daily milk production of the cows was about 30 kg. According to NRC (2001) dairy cattle feeding standard, the ratio of concentrate to roughage was 40:60 (DM basis, m:m). The composition and nutritional components of the diet were shown in Table 1. TMR feeding was used, and water was available *ad libitum.*

Table1: Dietary composition and nutritional composition of dairy cow rations [%].

Ingredient	Content	Dietary nutrition level	Content
Alfalfa hay	22.00	CP	17.35
Chinese wild rye	3.00	NDF	31.25
Corn silage	27.00	ADF	22.63
Corn	25.00	Ca	0.84
Soybean meal	14.50	P	0.40
Wheat bran	1.50	NE_L($MJ \cdot kg^{-1}$)	6.52
Whole cottonseed	4.50		
Mineral-vitamin premix	0.80		
Dicalcium phosphate	0.80		
Salt	0.70		
Magnesium oxide	0.20		

CP = crude protein; NDF= neutral detergent fiber; ADF= acid detergent fiber; NE_L = net energy for lactation;

Experimental Design*:* The cows were divided equally into control group (CK) and experimental group (MT). The cows in CK were fed with basal diet, while those in MT were fed with 60 g/head MOS in basal diet. MOS were perfused orally, twice in a day in the morning and evening before feeding. The experiment was designed as a 2 × 2 cross-over experiment. There were two stages in the whole experiment. Each stage lasted for 21d including 7 days of pre-feeding period and 14 days of the formal period. Finishing the first stage, the second stage of started after 14 days of recovery period. In the second stage, the treatments of previous control group and experimental group were exchanged to the other group.

Sample Collection and Processing: The rumen fluid was collected through the oral cavity of dairy cows using a negative pressure suction device. 50ml rumen fluid of every cow was collected each time respectively before feeding (0 h) and 2h, 4h and 6h after feeding in a day. In order to obtain representative samples, 5ml was taken out from every 4 samples of the same cows that were collected in one day and then mixed intensively. The samples were temporarily stored in freezing tube with liquid nitrogen and then transferred to the refrigerator at -80℃ in the laboratory for storage.

DNA Sample Extraction and Amplification

DNA Extraction: Rumen microbial DNA was extracted from 2 ml rumen fluid by kit method which were purchased from Nanjing Jiancheng Bioengineering Institute.

PCR Amplification: The purity and concentration of DNA samples were detected by agarose gel electrophoresis before amplification of PCR. After extracting genomic DNA from samples, ITS2 area of ITS rDNA was amplified with specific primers with barcode. The primer sequence was:

F : GCATCGATGAAGAACGCAGC;

R : ATATGTAGGATGAAGAACGYAGYRAA. (Ye *et al*., 2019)

Mixing and Purification of PCR Products: The PCR products were detected by electrophoresis with agarose gel with a gel concentration of 2%. The sample was mixed with equal concentration according to the concentration of PCR product. After mixing fully, the PCR product was detected by agarose gel electrophoresis with a gel concentration of 2%. Nanjing Jiancheng Bioengineering Institute provided the gel extraction kits to recover the products.

Hiseq Sequencing and Data: Samples were sent to Guangzhou GENE DENOVO Limited Company for Hiseq sequencing. Hiseq 2500 PE250 platform was used for the sequencing.

Statistical Software: The data were processed by Excel 2007, and the data were processed by ANOVA of two-stage cross design data in SAS 8.2. The data were showed by mean and standard error. $P < 0.05$ was regarded as the significant difference.

RESULTS AND DISCUSSION

Sample Sequencing Depth Analysis: The Shannon curve of the rumen fluid samples in this experiment was shown in Figure 1. As shown in the figure, when the depth of sequencing exceeded 5000 reads, the curve tended to be flat and the samples reached a saturation state, indicating that the depth of sequencing in this experiment was sufficient to cover most of the microorganisms in each sample.

OTUs Comparison: After Illumina Hiseq sequencing, the low-quality sequences were removed. The effective sequences were spliced and clustered into operational taxonomic units (OTUs) by Mothur according to 97% similarity. According to the results of OTU clustering analysis, the clustering information of different samples was analyzed, and then Venn graph was drawn based on the common and unique OTUs information. Figure 2 showed that the OTUs were 245 in MT group and 291 in CK group. There were 97 same OTUs shared in the both groups.

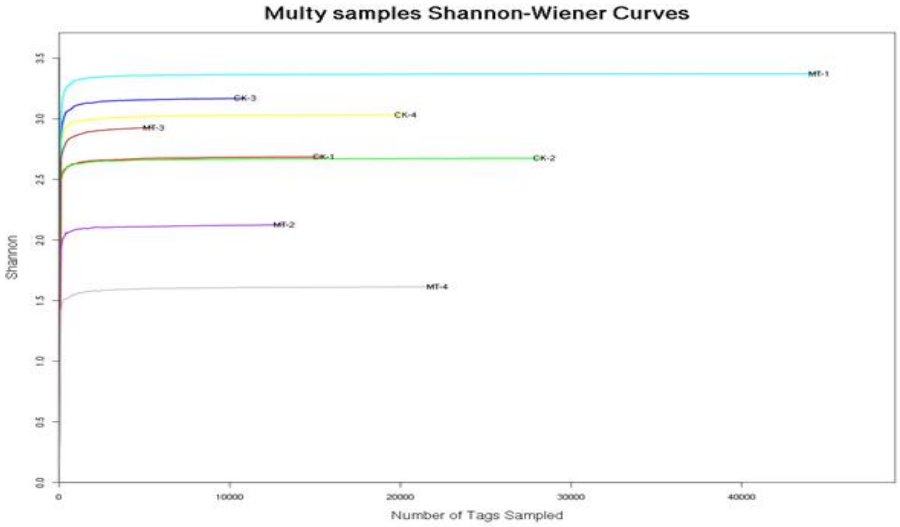

Figure 1. Shannon curve to evaluate the fungus species diversity in samples of the dairy cows. The higher the value is, the higher the species diversity is. When the curve tends to flatten or reaches the plateau stage, it can be considered that the increase of sequencing depth has no effect on species diversity and the amount of sequencing tends to be saturated. CK = the control group; MT = the experimental group.

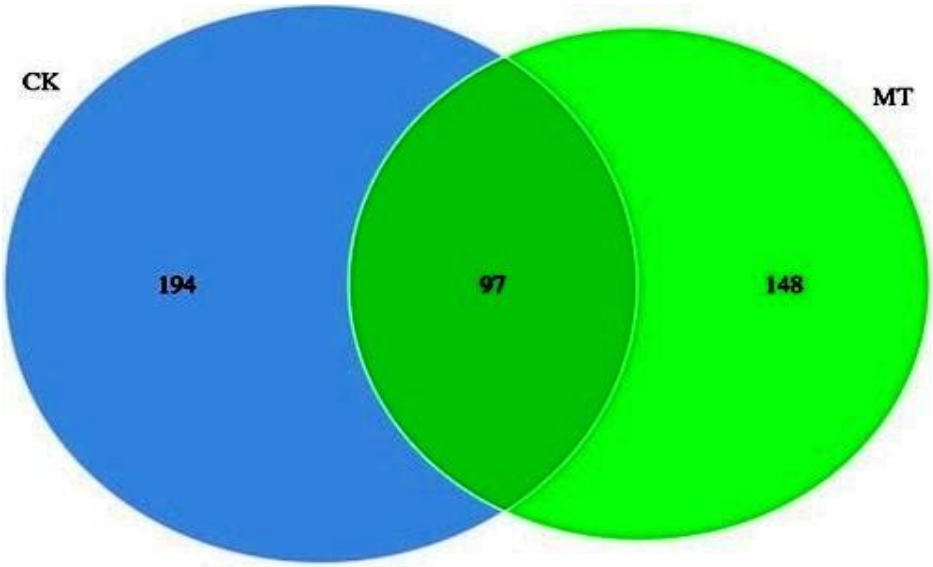

Figure 2. Venn graph to describe operational taxonomic units of rumen fungi of the dairy cows between CK and MT groups. CK = the control group; MT = the experimental group.

Analysis of Alpha Diversity of Rumen Fungi

Table2: Indexes of Alpha diversity of rumen fungi of the dairy cows between CK and MT groups.

Item	chao	Shannon	Simpson	coverage
CK	330.964 9	2.891 4	0.092 1	0.995 1
MT	321.641 3	2.509 7	0.208 1	0.993 5
P-value	0.261 3	0.605 9	0.617 8	0.495 8

CK=control group; MT=experimental group.

Alpha diversity is the analysis of species diversity in a single sample, including chao1, shannon and Simpson (Kemp *et al*., 2004). The chao1 predicts the species of microorganisms in the sample (the number of OTUs) based on the measured number of tags and OTUs and their relative proportions. It is relative values based on the known results. Shannon is a diversity index that reflects both OTU abundance and OTU uniformity. The larger the Shannon is and the closer the Simpson is to 0, the richer the species in the sample are.

Table 2 showed that, the Chao1 value and the Shannon value were both lower in MT group (321.64 and 2.51, respectively) than CK group (330.96 and 2.89, respectively), but there were no significant group differences (P = 0.26 and 0.61, respectively). The Simpson was higher in MT group (0.21) than CK group (0.09), but not significantly (P = 0.62).

Changes in the Structure of Rumen fungi

Analysis at the Level of Phylum

Table 3: The structure of ruminal fungi at phylum level of the dairy cows between CK and MT groups.

	CK (%)		MT (%)			
Phylum	Mean	Std.err	Mean	Std.err	*P*-value	log2 (Mean_MT/Mean_CK)
Ascomycota	72.34	4.44	83.24	4.56	0.091	0.20
Zygomycota	0.00	0.00	5.71	3.40	0.149	10.39
Neocallimastigomycota	15.18	5.28	5.72	3.70	0.211	-1.41
Basidiomycota	12.36	5.11	5.15	1.52	0.247	-1.27
Fungi-NA	0.12	0.024	0.18	0.056	0.343	0.64

CK=control group; MT=experimental group; NA = no annotation.

Using ITS rDNA sequencing technology, four phyla of fungi in the rumen fluid were identified in CK group and five phyla in MT group. According to Table 3, the preponderant phyla in both groups of rumen fungi were *Ascomycota, Basidiomycota* and *Neocallimastigomycota* . The abundance of these three phyla exceeded 90% of the total fungi in the rumen.The dominant rumen fungus *Ascomycota* showed an increasing trend in MT group (M = 83.24 ± 4.56%) than CK group (M = 72.34 ± 4.44%), but the difference was not significant (P = 0.091). The abundances of dominant fungi *Basidiomycota* and *Neocallimastigomycota* in MT group (M = 5.15 ± 1.52% and 5.72 ±3.70%, respectively) were 1.41 and 1.27 times lower respectively than those in CK group (M = 12.36 ± 5.11% and 15.18 ± 5.28%, respectively), but the differences were not significant (P = 0.247 and P = 0.211, respectively). The mean of *Zygomycota* abundance was 5.71% in MT group while there were little *Zygomycota* identified in CK group, but the group difference was not significant (P = 0.149).

Analysis at the Level of Genus: Table 4 showed the structure of rumen fungi at the level of genus. 74 genera of fungi in rumen fluid were identified in CK group, while 91 genera of fungi in MT group. Because there were plenty of genera of fungi identified, Table 4 only listed the dominant fungi (the abundance was more than 5%), the subdominant fungi (the abundance was 0.5% - 5%) and the fungi which had significant group differences. According to Table 4, five dominant genera of rumen fungi were identified in CK group, which were *NA, Nectriaceae_NA, Piromyces, Saccharomyces* and *Aspergillus*, and 9 subdominant genera, which were *Candida, Pyronemataceae-NA, Neocallimastigaceae-NA, Mycosphaerella, Cladosporium, Zopfiella, Neocallimastix* and *Robillarda*. Meanwhile, four dominant genera were identified in MT group, which were *NA, Nectriaceae_NA, Aspergillus* and *Mortierella*, and 10 subdominant genera, which were *Piromyces, Saccharomyces, Neocallimastigaceae-NA, Mycosphaerella, Cladosporium, Neocallimastix, Sarocladium, Mortierella, Chaetomidium* and *Phaeosphaeriaceae-NA*. In addition, *Sarocladium* increased in MT group (M = 1.01 ± 0.57%) compared to CK group (M = 0.02 ± 0.01%), a significant group difference (P < 0.05). The abundance of *Mortierella* was higher in MT group (M = 5.71 ± 3.40) than CK group (M = 0.00), but the group difference was not significant (P = 0.59). *Neocallimastigaceae-NA* (0.69 ± 0.31 vs 3.96 ± 1.33%), *Phialosimplex* (0.00 vs 0.02 ± 0.01%) and *Chaetomiaceae-NA* (0.00 vs 0.01 ± 0.00%) decreased in MT group compared to CK group, significant group differences (P < 0.05). The abundances of Candida (0.36 ± 0.33 vs 4.31 ± 2.41%), *Zopfiella* (0.06 ± 0.06 vs 3.27 ±

2.06), *Neocallimastix* (0.53 ± 0.47 vs 3.04 ± 1.05%) and *Cryptococcus* (0.00 vs 0.12 ± 0.08%) trended to be lower in MT group than CK group (P = 0.068, 0.086, 0.077 and 0.069, respectively). Besides, *Pyronemataceae-NA, Robillarda* and *Geastrum* appeared in CK group alone, while *Mortierella, Chaetomidium* and *Phaeosphaeriaceae-NA* appeared in MT group alone, but there were no significant group differences.

Table 4: The structure of rumen fungi at genus level of the dairy cows between CK and MT groups.

Genus	CK (%)		MT (%)			
	Mean	Std.err	Mean	Std.err	*P*-value	og2 (Mean_MT/Mean_CK)
NA	23.83	8.47	13.85	1.95	0.173	-0.78
Nectriaceae_NA	7.18	5.25	12.28	11.91	0.876	0.77
Piromyces	6.37	2.56	2.56	2.08	0.172	-1.31
Saccharomyces	5.92	1.24	4.72	2.26	0.845	-0.33
Aspergillus	5.75	2.15	19.77	15.32	0.712	1.78
Candida	4.31	2.41	0.36	0.33	0.068	-3.58
Pyronemataceae-NA	4.15	4.14	0.00	0.00	0.285	-28.63
Neocallimastigaceae-NA	3.96[a]	1.33	0.69[b]	0.31	0.010	-2.53
Mycosphaerella	3.88	3.84	3.44	3.01	0.974	-0.18
Cladosporium	3.55	1.94	1.28	1.19	0.299	-1.48
Zopfiella	3.27	2.06	0.06	0.06	0.086	-5.82
Neocallimastix	3.04	1.50	0.53	0.47	0.077	-2.51
Robillarda	1.85	1.85	0.00	0.00	0.291	-27.46
Geastrum	0.59	0.59	0.00	0.00	0.280	-25.82
Cryptococcus	0.12	0.08	0.00	0.00	0.069	-23.55
Sarocladium	0.02[a]	0.01	1.01[b]	0.57	0.037	5.60
Phialosimplex	0.02[a]	0.01	0.00[b]	0.00	0.047	-20.71
Chaetomiaceae-NA	0.01[a]	0.00	0.00[b]	0.00	0.042	-19.54
Mortierella	0.00	0.00	5.71	3.40	0.059	10.39
Chaetomidium	0.00	0.00	1.22	1.20	0.224	26.86
Phaeosphaeriaceae-NA	0.00	0.00	1.77	1.76	0.268	27.40

CK = the control group; MT = the experimental group, NA = no annotation.
[a,b] Values within a row with different superscripts differ significantly at P<0.05.

Effects of Mannan Oligosaccharides on Rumen Fiber Degrading Bacteria: Some rumen anaerobic fungi can secrete cellulase with high activity and degrade cellulose, hemicellulose and even lignin in many plant cells walls (Wei *et al*., 2016; Wood *et al*., 1986). *Sarocladium* can secrete cello-oligosaccharide oxidase (Lee *et al*., 2006). *Neocallimastigaceae-NA* and *Neocallimastix* belong to Neocallimastigaceae family, which plays a major role in the degradation of fibers in the rumen (Boots *et al*., 2013; Zhou *et al*., 2010). In this study, *Neocallimastigaceae-NA* (phylum *Neocallimastigomycota*), the dominant cellulolytic fungi in CK group, decreased significantly in MT group. In addition, Neocallimastix (phylum *Neocallimastigomycota*) showed a decreasing trend in MT group. Although *Sarocladium* (phylum *Ascomycota*) was higher significantly in MT group than CK group, its content was in a lower level than those decreasing in MT group. It was indicated that adding MOS to diets mostly inhibited the growth of cellulose-degrading fungi in general.

Researchers suggest that MOS significantly improve the DM, ADF and NDF degradation rates of corn silage, peanut vine, chinensis and alfalfa in rumen(Lin *et al*,2014). While the supplementation of MOS in the diet inhibited the growth of rumen fiber degrading fungi in this experiment. The results of our previous experiments show that adding mannan oligosaccharides to the diets of dairy cows obviously effects the amount of cellulose-degrading or hemicellulose-degrading bacterial flora. Among them, the relative abundance of hemicellulose-related species such as *Anaerobiospirillum, Ruminococcus, Pseudobutyrivibrio and Lachnospira* increase significantly or tend to increase with the same level supplementation of mannan oligosaccharides (Guo *et al*. 2018). The different results between rumen bacteria and fungi might be due to their antagonism. Bernalier *et al*. (1993) find that rumen coccus has antagonistic effect on cellulase activity of *Neocallimastix*. Dehority and Tirabasso (2000) have found that rumen bacteria can significantly inhibit the growth of rumen fungi by in vitro culture. The study of removing fungi from the goat rumen by Mao *et al*. (2002) has shown that the presence of anaerobic fungi in goat rumen can affect the protein concentration of rumen microbial, which suggests that

there can be a strong antagonism between anaerobic fungi and other microorganisms in the rumen. In this experiment, the results of indexes of Chao1, Shannon and Simpson were consistent between the groups. Combined alpha diversity analysis and OTUs comparison, the results showed that adding MOS in diet trended to reduce the rumen fungi flora abundance of dairy cows to a certain extent, but there was no significant group difference. Further researches are needed.

Effects of Mannan Oligosaccharides on Gastrointestinal Health of Dairy Cows: MOS can promote the proliferation of beneficial bacteria, inhibit the reproduction of intestinal pathogens, reduce the incidence of gastrointestinal diseases, and enhance animal immunity (Bagheri *et al*., 2009; Uyeno *et al*., 2015; Westland *et al*., 2017). Studies have shown that the addition of MOS can improve the IgA of calves (Heinrichs *et al*., 2013). IgA, as the antibody secreted by the gut mucosa, plays an important role in gut barrier function (Planer *et al*., 2016). The results of this study showed that the abundance of *Zopfiella* had a decreasing trend with the addition of MOS in the diets. It has been found that *Zopfiella* has immunosuppressive characteristics (Fujimoto *et al*., 2004). In addition, *Candida* species are the most common microorganisms which can be isolated from infected mammary glands among the mycotic mastitis agents (Şeker and Özenç, 2011). In this experiment, *Candida* in rumen had a decreasing trend after the supplemental mannan oligosaccharides in the diets of the experimental cows. Furthermore, it has been reported that *Mortierella* can accumulate detectable amounts of arachidonic acid, one of the essential fatty acids, which is necessary nutrients for animals (Shinmen *et al*., 1989). It was shown in this study that he abundance of *Mortierella* in rumen had an increasing trend after the supplemental MOS in the diets of the experimental cows. This research indicated that MOS might play a positive role in inhibiting harmful fungi.

Conclusion: The study used ITS rDNA sequencing technology to analyze the changes of rumen fungal flora in dairy cows fed with or without mannan oligosaccharides. The results showed that mannan oligosaccharides in the diet of dairy cows inhibited the growth of cellulolytic fungi and the proliferation of harmful fungi. In addition, the results showed that there might be the antagonistic effect between rumen fungi and bacteria potentially, which should be focused on for further study.

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