Effects of Molasses and Urea Supplement in Winter Season for Protecting Grassland and the Effective Use of the Resources of Grasses on the Growth Performance and Ruminal Microbial Flora in Ruminants in Inner Mongolia

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Abstract

The malnutrition of sheep is often observed in northern area of China, because the animals are generally raised under the condition of the natural grassland throughout a year. In order to improve the performance of sheep production in this region, it might be essential to keep a constant nutritional level in sheep throughout a year. The present experiment, therefore, was conducted to clarify the seasonal(the spring, summer and late winter)changes of the nutrition of grass and sheep rumen fermentation in natural grassland of this region, then the follow experiment improved the nutrients digestibility via promoted growth of microorganism utilizing non-protein nitrogen and sugar of molasses-urea supplementation. The following four experiments were carried out:

The first experiment revealed the seasonal dynamics of herbage intake, diet composition and digestibility and clarified the relationship of those with herbage nutrient and botanical composition of grazing sheep in grassland of Inner Mongolia, n-alkanes technique was used to test in sheep that herded in June, August and December respectively. The results showed that the sheep mainly intaked *Artemisia frigid*, *Stipa krylovii* and *Carex sp.* with the proportions of 33.5%, 17.9% and 21.2% respectively in spring. In summer, the sheep main intaked *Cleistogenes*, *potentilla tanacetifolia*, *Thymus*, the intake of *Artemisia frigid*, *carex sp.* and *stipa krylovii* reduced. In winter, *Artemisia frigid* accounted for 50.1% of herbage intake, and the intakes of *Cleistogenes sp.* and *Stipa krylovii* increased to 15.3% and 18.4% respectively. Herbage intake by the sheep in spring was 1.8 kg DM d^{-1}, and digestibility was 71.4%. Herbage intake and digestibility decreased slightly to 1.7 kg DM d^{-1} and 68.4% during the summer, respectively and decreased significantly to 1.2 kg DM d^{-1} and 36.4% in winter. There were significant of correlation on diet composition with CP in winter and botanical composition in summer. And the high positive correlation between herbage intake and digestibility was observed in grazing sheep.

In the second experiment, based on high throughput sequencing, 70898 reads of valid base sequence were obtained. Among those, there were 2613 OUTs types, 32 dominant types were got eventually after the data processing. In different seasons, it was different in the ratio of OUTs types from various types of grazing sheep. *Firmicutes* and *Bacteroidetes* can be identified as dominant bacteria within the rumen of grazing sheep, with a percentage of 90% in all bacteria. Meanwhile, there were small amount of *Candidatus saccharibacteia*, *Tenericutes*, and *Proteobacteria* in

certain types. The rumen bacterial diversity in grazing sheep decreases gradually from the herbage growth period in August to the withering period in January of the next year, and gradually increasing from April.

The third experiment was conducted to determine the effects of molasses-urea supplementation (MUS) on weight gain, ruminal fermentation and microbial populations of sheep grazing in a winter. Forty sheep, allowed free consumption of MUS after grazing, served as a treatment group, while 30 sheep, fed only by pasture, served as a control group. Ruminal fermentation were measured, which consisted of pH, bacterial crude protein (BCP) and ammonia nitrogen (NH_3-N). In addition, populations of five major bacteria were investigated. The results showed that, the average daily weight gain, concentration of NH_3-N and population of protozoa was significantly higher ($P < 0.05$) in the treatment group than those in the control group. Contrastingly, no significant difference was found in BCP concentration and pH value between the two groups. At the end of the experiment, the populations of the *Selenomonas ruminantium*, *Anaerovibrio lipolytica*, *Fibrobacter succinogenes*, *Ruminococcus flaveciens* and *Ruminococcus albus* in the treatment group were significantly higher than those of the control group ($P < 0.05$). These results demonstrate that greater weight gain can be induced during winter in Inner Mongolia by improved nutritional status through promotion of microbial populations using urea and sugar.

The aim of the fourth experiment was to evaluate the microbe quantity and fermentative efficiency of rumen supplement with molasses-urea. Eight sheep were selected and divided into two groups (a control group and a treatment group), and only the treatment group animals were supplied with molasses-urea for ad libitum consumption. Rumen fluid was collected every 2 h and rumen fermentation parameters were measured. The population of majority bacteria was investigated by real-time PCR. The results showed that the population of majority bacteria increased in the rumens of treatment group animals ($P<0.05$). Each bacterium quantity decreased gradually after feeding, and reached the lowest level 2 h after intake. It then slowly increased and reached the highest level at 8 h after intake. Finally, each bacterium quantity returned to the same level as before intake. In contrast, the protozoa number rose to the highest at 4 h after intake and declined gradually. The concentration of protozoa in the treatment group sheep was significantly higher than that of control group ($P<0.05$). The pH of rumen liquids found was in a normal range and was not different between both groups. However, the pH decreased from the highest level before feeding to the lowest level within 4 h, and it increased after

intake for 8 h. The concentration of NH_3-N and MCP synthesis, in the rumen liquids, were both significantly higher than that of control group($P<0.05$), the highest concentration of NH_3-N and MCP was reached after feeding by 2 h and 4 h, respectively. Molasses-urea has a positive effect on the rumen, due to their useful effect on rumen fermentation by the microbes in ruminant.

Based on above study, the molasses-urea can maintain the pH of rumen in sheep and improve the concentration of ammonia nitrogen and MCP yields. However beyond this, it is also able to increase the quantity of microbes in rumen, therefore promote a productive and conducive environment inside the rumen. Consequently, it improves the utilization of low quality roughage and the nutrition intake of sheep.

Key words：grazing sheep; rumen; molasses-urea supplementation; herbage intake; Inner Mongolia

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List of Abbreviations

￥	China Yuan(CNY)
ADF	Acid Detergent Fiber
Ash	Crude Ash
CF	Crude Fiber
CP	Crude Protein
ddNTP	Fluorescent Dideoxynucleotide Triphosphate
DGGE	Denatured gradient gel electrophoresis
DM	Dry Matter
DMI	Dry Matter Intake
dNTP	Deoxynucleoside Triphosphates
EE	Ether Extract
IVDMD	Dry Matter Digestibility
MCP	Microbial Protein
MUS	Molasses-urea supplementation
NDF	Neutral Detergent Fiber
NFC	Non-fiber Carbohydrate
NH_3-N	Ammonia Nitrogen
OM	Organic Matter
OTUs	Operational Taxonomic Units
PCoA	Principal Coordinate Analysis
PCR	Polymerase Chain Reaction
RDP	Ribosomal Database Project
VFA	Volatile Fatty Acid

CHAPTER Ⅰ Introduction

1.2 Background of the study

The sheep farming is a major sector in China. According to China Statistical Yearbook (2016), Total livestock in year-end has reached 303 million, and the number of slaughtered sheep has reached 287million in 2015. The number of sheep has been steadily rising since 2011(Table 1-1). In 2014, mutton production reached 4280 thousand tons, comprising 5% of total meat production. Mutton output value was ¥238 billion, comprising 8% of the total output value of animal husbandry, and this amount increased year over year(Table 1-2). Inner Mongolia, which is located north of China (Fig.1-1), has a wide grassland area of 78.8 million hectares, constituting 22% of overall grassland area in China. It has a total annual biomass reserve of 68 billion kg produced by the grassland, among which, the edible hay has a total reserve of about 40.9 billion kg. Thus, Inner Mongolia is regarded as an important livestock husbandry base and a livestock production area in China. Livestock husbandry in grassland is the predominant and basic industry in Inner Mongolia, particularly owing to the rapid development of sheep husbandry. Inner Mongolia has the largest sheep production area. Statistical data (Inner Mongolia Statistical Yearbook 2016) reveal that 53.2 million sheep were reared in Inner Mongolia, comprising 14.5% of the overall sheep kept in China in 2014. It also ranked first in mutton output for three consecutive years, with a total mutton output of 603.6 thousand tons, comprising 15.1% of overall domestic mutton output (4.0 million tons) and 29.9% of the total meat output of Inner Mongolia (2.0 million tons).

Along with mutton consumption, the price of mutton markedly increased year by year because of improvement in living standards and the dietary adjustment of Chinese residents. The price of mutton significantly increased from ¥46.23/kg in 2012 to more than ¥50/kg; almost up to ¥53/kg in 2013.Such a large increase drew attention from the government. As of 2015, although the price of mutton slightly decreased, it remained relatively high. This increase in the price of mutton may be mainly attributed to the increment in both the cost of feed and household consumption, mostly consisting of feed cost. Therefore, both the increase in sheep husbandry efficiency and reduction in sheep feed cost can effectively alleviate the cost of mutton and further meet the mutton consumption demand of Chinese

residents.

Primary grazing of sheep on the vast prairie in Inner Mongolia year-round may help reduce costs in sheep feed. However, a dry period lasted for 7 to 8 months in winter and spring in northern China. Fan (2008) demonstrated that a large increase in crude protein (CP), marked a decrease in neutral detergent fiber (NDF), 50% decrease in *in vitro* dry matter digestibility (IVDMD), and decrease in pasture intake were in winter and spring, compared with summer. Meanwhile, the nutritional deficiency caused by the large herbage intake with increased fiber content and reduced nourishment would lead to "fat in summer, strong in autumn, thin in winter, dead in spring" of livestock.

Therefore, to increase the economic benefits of grazing sheep in northern grassland area, nutritional balance has to be achieved throughout the year. The best method to attain this objective is supplementary feeding to livestock during seasons with nourishment shortage; this technique allows alteration in the traditional feeding model. Consequently, studies on livestock husbandry in Inner Mongolia grassland have mainly concentrated on the nutritional intake, nutritional requirement, supplementary feeding cost, and efficiency of grazing sheep.

1.2 History of the studies

Research progress of the herbage intake and diet composition of grazing sheep by using n-alkanes

The herbage intake of grazing livestock is difficult to determine. The n-alkanes method, a simple and accurate technique for measuring the herbage intake of grazing animals was developed. N-alkane is not only an organic hydrocarbon compound; it is also a component of epidermal wax on the surfaces of plant leaves and stems. Mayes (1986) indicated that as a homolog series, saturated n-alkanes are generally easy to analyze, always consisting of 21–37 carbon atoms and widely distributed on the herbage surface.

In the study by Grace (1986), increased n-alkane content with an even number of carbon atoms in herbage was observed. In addition, n-alkanes with an odd number of carbon atoms could hardly be absorbed in the gastrointestinal tract of livestock, which led to a relatively high recovery rate of n-alkanes in the feces of livestock. Thus, n-alkanes with an odd number of carbon atoms can be treated as an indicator for different plants.

Dove (1992) and Mayes (1995) demonstrated that the longer the carbon chain of n-alkanes, the higher the recovery rate, further revealing the marked variation in the gradient mode of saturated n-alkane content among different herbage species (n-alkane content mode varied with the epidermis of species). The n-alkane so-called "fingerprint for plant," which denotes a unique n-alkane content mode for different plants, provides a solid material basis for the evaluation of feeding habits and forage intake of livestock.

In their experiments, herbage intake was evaluated by feeding the livestock with known concentrations of saturated n-alkane capsule with an even number of carbon atoms because the recovery rate of synthetic alkane (with an even number of carbon atoms) in the livestock excreta was extremely similar to that of the natural alkane (with an odd number of carbon atoms). Therefore, the herbage intake and diet composition of the livestock could be accurately estimated. Dove (1990) demonstrated that forage intake by herbivores could be more accurately estimated using the saturated n-alkane method than applying either the Cr_2O_3 marker method or the *in vitro* digestion method. For the saturated alkane method, variation in the digestibility of herbage intake among individual livestock could be approximated; in addition, the Cr_2O_3 content in livestock feces sharply fluctuated. In the experiments conducted by Piasentier (1995), the herbage intake of grazing ewe was determined by simultaneously performing two methods: the saturated n-alkane method and the method in which the defecation quantity is determined using the Cr_2O_3 marker. Meanwhile, herbage digestibility was evaluated by *in vitro* digestion. The experimental results showed a significant difference between the two methods.

In the study by Liu (2001), n-alkanes with C31 and C35 were selected as endogenous tracers to determine dry matter digestibility; no significant difference was found between the experimental results and those obtained by full excrement analysis. The average amount (80.5%) obtained by the n-alkanes method was exactly the same as that obtained by the total collection method. The n-alkane method more accurately estimated digestibility, providing a more reliable means of determining digestibility to determine livestock intake (e.g., free grazing livestock), which was difficult to determine by the total collection method. Moreover, Dove (1995) used the least squares method to estimate plant composition in mixed herbage. All above mentioned experimental results demonstrated that the saturated n-alkane method exhibited broad potential application in estimating herbage intake, diet composition, and digestibility of livestock.

Research progress on rumen microbial polymorphism in grazing sheep

Studies associated with seasonal variation in ruminal microflora have rarely been conducted. Orpin (1985) evaluated the effect of seasonal variation on the quantity of rumen methanogen and forage utilization in the high-arctic Svalbard reindeer. The results indicated that the methanogen quantity varied significantly with the seasons. The quantity of cultivable microorganisms in summer was $2.9 \pm 1.26 \times 10^{10}$/mL, whereas that in winter was only 0.36×10^{10}/mL or 17% of the quantity in summer. *Butyrivibrio fibrisolvens* comprised 22% of the microorganisms in summer and 30% in winter. *Streptococcus bovis* comprised 17% of the microorganisms in summer and 4% in winter. Hodgson (1982) determined that the gross quantity of rumen microbes and protozoa varied with the seasons. Fermentation activity was the weakest in winter because of the least quantity of rumen microbes in winter; however, the microbe composition varied slightly with the seasons. Seasonal variation in the fermentation activity of the sika deer rumen was demonstrated in the study by Asano (2007). The results indicated the following: the digestibility of organic matter and fiber from pasture was lower in summer and autumn than in the other seasons; the pH of rumen was lowest in autumn; and the rumen NH_3-N content increased in winter and spring but decreased in summer.

The change in the physical status of livestock, which was thought to have been brought about by seasonal variation on rumen microbes, was actually caused by forage because forage composition varied with the seasons. Numerous studies on the effect of different forage and quality of forage on rumen fermentation and microbe polymorphism have been conducted. The findings indicated that for grazing ruminants, degradability of carbohydrate was the predominant factor affecting the quantity of ruminal microbe and microflora; as for the fermentation of diet with high-fiber content, both the ratio of acetic acid to propionic acid and methane loss increased (Beever *et al*. 1989).

Tajima (2001) used the 16S rDNA clone method to examine the variation in the rumen bacterial community structure of cow, with the forage changing from roughage to a high-concentrate diet. In the experiment, the microbe changed in both cases, indicating that forage significantly affected the microbial flora. Real-time PCR was employed by Tajima (2000) to determine the variation in quantity in *Pratt Coli*, *Bacteroides succinogenes*, *Ruminococcus flavefaciens* with forage. The results suggested that the variation in quantity between two kinds of major cellulose decomposition microbes, *F.succinogenes and R.Flavefaciens*, was identical to the

previous results obtained using the culture method.

Most studies on the rumen microbe polymorphism of ruminants, such as cattle, sheep, and yaks have been conducted in China. Traditional microbe separation and molecular biotechnology were employed. Variation in microbial population in goat rumen and pig manure was investigated by Yao (2004) and Zhu (2003).

Few studies concerning Mongolian sheep have been conducted; in particular, the seasonal variation in the rumen microbes of grazing Mongolian sheep has not been systematically examined. Studies on grazing sheep are currently focused on the diet composition and herbage intake (Wang 2000); no studies related to rumen microbes have been conducted. Research concerning the seasonal variation in sheep rumen microbes has solely focused on Tibetan sheep. Dan (2009) evaluated the seasonal variation in sheep rumen microbes and the herbage nutrient of Tibetan sheep, in addition to the seasonal dynamic factor analysis of the rumen microbial flora of Tibetan sheep. Moreover, correlations in the population dynamics of ciliates in different seasons were determined in the study by Yao (2002). Unfortunately, no in-depth study on rumen microbes has been conducted. Seasonal variation in digestibility and intake of forage was evaluated in the study by Liu (2001), which provided a basis for the rational utilization of pasture and feeding resources.

Studies related to the regulation of rumen function mostly focused on the effect exerted on the rumen function by the nutrition levels in diet and the concentrate-to-roughage ratio. Li (2008) indicated that with a dietary concentrate level of 0.60 kg/d, the degradation efficiency of cornstalk in sheep rumen was significantly affected; thus, the recommended dietary concentrate level should not exceed 0.50 kg/d. However, the research conducted by Li (2008) demonstrated that the NH_3-N level and gas yield produced by *in vitro* fermentation of cashmere increased with high-calorie, high-protein diet feeding. Similar results were obtained for the digestion of dry matter and organic matter. The effect of various nitrogen sources on the internal environment and microbe community of sheep rumen was evaluated by Wang (2007) by using 3 kinds of dietary nitrogen sources: soybean meal, fish meal and sunflower meal. The results demonstrated that the cellulose-decomposing microorganism community in sheep rumen varied with the nitrogen source. Similar results were obtained for the quantity and species of the ciliate, among which are most rumen cellulose- decomposing microorganisms. The highest activity of the corresponding cellulose enzyme in the rumen of the livestock was obtained for the soybean meal group; similar outcomes were obtained for the overall utilization and digestion efficiency of nitrogen.

Research Progress on Optimized feeding of grazing sheep

The feeding methods of grazing sheep in natural grassland have been adopted for mutton sheep feeding in China, which significantly influenced the development of sheep industry and augmented the income of farmers and herdsmen. As for the fundamentals in mutton sheep breeding, a number of misconceptions and undetermined areas related to mutton sheep breeding have yet to be corrected. Sheep breeding in pastoral areas have not been taken advantage of scientifically and reasonably; thus, the production performance of grazing sheep has not been optimized. Determining the factors affecting mutton sheep production involves complexity; regardless, nutrition is identified as the main factor.

The nutritional requirements for confined and semi-confined breeding of mutton sheep have been widely investigated; however, the nutritional requirements of breeding mutton sheep in free grazing have not been researched, particularly, studies have not been conducted on the livestock–forage combination on the basis of the current grassland resource. In recent years, with a dramatic increase in supplementary feeding cost caused by an increase in the price of fodder, gaining maximal economic benefit with minimal expense became a serious problem confronting us. Supplementary feeding is necessary to improve the efficiency of sheep grazing; the forage quantity, digestibility and nutritional intake of grazing sheep intaked from grassland should first be determined.

Most animal experiments demonstrated that the production performance of sheep grazing could be significantly modified by supplementary feeding. Meanwhile, several points were unknown, such as how each nutrient worked, which kind of nutrient played the major role, and how the nutrients interacted with one another. These factors make further fine supplementary feeding-including the feeding formula, quantity, and intervals of periodic supplementary feeding-unavailable.

The most predominant characteristic of ruminant digestion was the catabolism effect of rumen microbes, owing to a wide variety of rumen microbes for sheep. The decomposition of forage fiber by microbes played a significant role in fodder utilization and nutrient absorption. For the livestock, reasonable and efficient rumen microbial population structure and species composition were finally formed via long periods of natural selection, and by their synergic cooperation, fodder digestion and utilization were achieved. Thus, a supplementary feeding scheme solely based on the type of nutritional intake could not be generalized. Most studies indicated that under

different breeding conditions, significant modification of internal rumen environment and reduction of methane emission could be achieved by increasing the amount of rumen microbes and modifying the microbial population structure, resulting in daily gain for the sheep.

Therefore, the internal environment and the microbial population of rumen significantly influenced ruminant livestock nutrition. In particular, for the dry period in winter, the forage generally had high fiber content. The utilization efficiency of limited forage could be effectively measured using the synergic effect of rumen microbes by which the increase in supplementary feeding efficiency and decrease in breeding cost of grazing livestock could be obtained.

1.3 Objective of the study

Year-round primary grazing of sheep on the vast prairie in Inner Mongolia may help reduce the cost of sheep breeding. However, a dry period lasts for 7–8 months in winter and spring in the northern prairie. Fan (2008) showed that compared with the green period, the dry period in winter showed a marked increase in the CP content in hay, a marked decrease in the NDF content in hay, a 50% decrease in IVDMD, and a decrease in forage intake. Meanwhile, the nutritional deficiency in the livestock, caused by the large intake of herbage with higher fiber content and reduced nutrients, led to “fat in summer, strong in autumn, thin in winter, dead in spring” of livestock.

Thus, the economic benefits of grazing sheep in the northern grassland area have to be increased, and nutritional balance throughout the year has to be achieved. The best method to attain this aim is the supplementary feeding of livestock during seasons of nourishment shortage. This method alters traditional breeding. Therefore, the ultimate objective of livestock husbandry in Inner Mongolia grassland is focused on the nutritional intake, nutritional requirement, improvement of forage digestibility, reduction in supplementary feeding cost, and improvement in the supplementary feeding efficiency of sheep grazing.

It was well know that the rumen is an important digestive organ of ruminant, it is important to the digestibility of forage, so we can provide necessary nutrition for ruminal bacteria, improve the digestibility of forage by controlling ruminal fermentation, then the nutrition of grazing sheep have been improved, thereby reduce supplemental feed, and reduce overgrazing on grassland.

So this study aims to (i) determine the feed intake and forage digestibility of

grazing sheep in different seasons,(ii)to analyze natural pasture of grassland influence on rumen microbes,(iii)to supplement feeding for necessary nutrition through the control and use of rumen microbes,(iv)to enhance the digestibility of low-quality forage in order to improve the energy utilization ratio of ruminants and thus improve animal production performance, and(v)to reduce the cost of supplementary feeding and improve production efficiency.

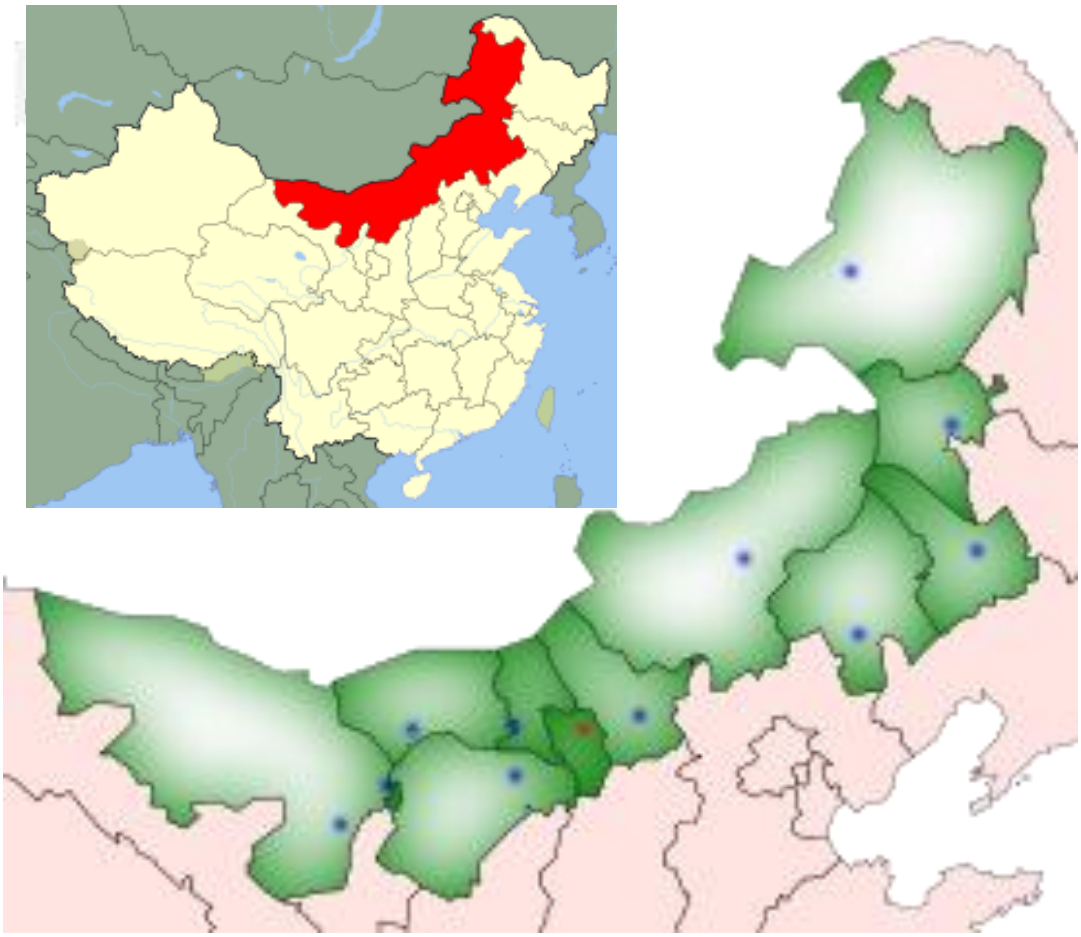
The accurate prediction of nutritional intake and utilization through different periods were achieved by determining the quantity of herbage intake, intake plant composition, and herbage digestibility of mutton sheep in different seasons. On the basis of the effects of nutrition on rumen microbes, supplementary feeding of necessary nutrition was provided by adjusting and using rumen microbes. Both low-quality forage digestibility and the energy utilization efficiency of ruminants were improved. These changes enhanced the livestock production performance, reducing the supplementary feeding cost and enhancing the production efficiency. Thus, the following will be presented in the thesis.

First, supplementary feeding is necessary for the improvement of grazing sheep. The quantity of forage intake, digestion rate, and nutritional intake of grazing sheep has to be determined first. The seasonal variations in the intake of grazing sheep, chemical components, and digestibility of plants in the typical steppe and desert steppe (which is the main steppe type in Xilingol) have to be evaluated. This study provides information on the quantity of nutrients needed in grazing, the type of nutrients to be supplied, and the time to provide the supply.

Second, most studies indicate that rumen microbes significantly influence ruminantal digestibility and nutrient absorption. Thus, before the feeding supply is provided, we should consider the rumen microbe function. The needs of rumen microbes have to be satisfied, and they have to play their role. Thus, the experiment is conducted to analyze rumen microflora and rumen fermentation in different seasons. This study provides basic data for the regulation of rumen bacteria.

Third, understanding the nutritional intake and seasonal variation in the conditions of grazing sheep, particularly in winter, and in accordance with the nutritional requirement of sheep, the molasses urea that supply nutrients for rumen microbes are developed to increase the quantity of microbes and modify the rumen microflora. By so doing, the utilization efficiency of forage is enhanced, further resulting in daily livestock gain. In addition, supplementary efficiency is enhanced and the cost of grazing sheep is reduced. It also reduces the pressure of grazing in natural steppe, thus protecting the ecological environment.

Fourth, this study showed that the molasses urea supplement could obviously improve the production performance of grazing sheep. The molasses urea improves the production performance of grazing sheep by increasing the number of rumen microbes, thus improving the digestibility of forage or increasing bacterial protein production, or both. This study helps determine the forage digestion rate, rumen microorganisms, rumen fermentation parameters, and so on after supplying molasses urea. From this information, the mechanism of improving the production performance of grazing sheep is revealed.



(Reference on http://www.luventicus.org/ditu/zhongguo/neimenggu/baotou.html)

Fig.1- 1 The site of Inner Mongolia in China

Table1-1 Total livestock in year-end and slaughter of sheep in China

Year	2011	2012	2013	2014	2015
Total livestock in year-end（million sheep）	281	282	285	290	303
Slaughter（million sheep）	272	267	271	276	287

(China statistical yearbook, 2016)

Table1-2 The status of sheep production in animal husbandry in China

Year	2010	2011	2012	2013	2014
Mutton production （thousand ton）	3990	3930	4010	4080	4280
The proportion of mutton production in the total meat production（%）	5	5	5	5	5
Output value of sheep （billion ¥）	140	171	201	229	238
The proportion of output value of sheep in the total animal husbandry（%）	7	7	7	8	8

(China statistical yearbook, 2016)

CHAPTER Ⅱ The Seasonal Changes of Chemical Component and Digestibility of Plants in the Grassland of Inner Mongolia

2.1 Introduction

The malnutrition of sheep during autumn to winter season is often observed in the northern area of China because the animals are generally raised under the condition of the natural grassland throughout the year. In order to improve the performance of sheep production in this region, it is essential to know the nutritional level in sheep throughout a year. Nutrient intake depends on the plant species, herbage intake and digestibility of grazing sheep. However, it is very hard to estimate the herbage intake, diet composition and digestibility of livestock in grazing condition. Although many scientists performed plenty of research on many proposed estimating methods, most methods have shown numerous limitations, such as low accuracy, complicated procedure, tedious and expensive (Wang 1995). The n-alkane technique, on the other hand, has proven to be effective and accurate for estimating herbage intake, diet composition and digestibility of grazing sheep (Mayes *et al*. 1986, Dove 1992, Mayes and Dove 2000). In previous experiments, estimates of herbage intake using the radio C33:C32 were the most accurate among the other combination of C31:C32 (Zhang 2002). So in the present study, the alkane pair C32:C33 with C33 as internal marker was used to estimate herbage intake, diet composition and digestibility in grazing sheep.

It was well documented herbage intake is influenced by many factors, including the species of grass, location, growth stage and region, environmental condition, and analytical method (Laredo *et al*. 1991, Olivan and Osoro 1999, Kelman *et al*. 2003, Dove *et al*. 1996). Considering the alkane recovery rate in the feces, the sampling for multiple plants and the growing period of herbage (Liu *et al*. 2006), only 8 species of herbage were chosen in this study. Herbage intake, diet composition and digestibility of sheep grazing on the arid grassland were determined in a recent study in Siziwang Banner, in western Inner Mongolia (Hu *et al*. 2014). The results of n-alkane patterns were consistent with those reported by other researchers (Wang 2000), proving the feasibility of detecting diet composition of grazing sheep using the n-alkane technique in Inner Mongolia. The results of Hu (2014) showed that daily dry matter intake and DM digestibility of sheep decreased significantly from summer to winter, and a diet composition analysis indicated that *Artemisia frigida* was the most dominant diet component in the arid steppe of Siziwang Banner. However, information on seasonal

changes of herbage intake and digestibility in sheep is not available in Xilingol League and Bayannaoer which were the main sheep raising area in Inner Mongolia. Traditionally, Zhenglan Banner, located in the grassland of Xilingol; Wulatezhong Baner, located in the grassland of Bayannaoer, were considered to be the typical steppe and desert steppe, respectively (Hu *et al*. 1995). However, the herbage species are complex. There is no study that has estimated the intake and digestibility of herbage in sheep grazed in above two sites.

So the aim of this study was to verify the seasonal changes of herbage intake, diet composition and digestibility of grazing sheep, and clarify the relationship of those with herbage nutrient and botanical composition in the grassland of Xilingol League and Bayannaoer.

2.2 Materials and methods

Experimental site

The experiment was carried out in two locations, which are located in Inner Mongolia's central and Western.

The first experimental site, Zhenglan Banner (E 116.02°, N 42.25°), is located in the central of Inner Mongolia, China, is a typical steppe. The dominant species there include *Stipa krylovii*, *Artemisia frigid*, *Leymus chinensis*, *Cleistogenes sp.*, and *Carex sp.*; the major accompanying species are *Thymus sp.*, *Potential bifurca*, *Potentilla tanacetifolia*, *Agropyron cristatum*, etc. The plants are generally 6 to 25 cm in height with average vegetation coverage of 25 to 35%.

The second experimental site, Wulatezhong Banner(E108 °51 ', N41 °30 '), locates in the western of Inner Mongolia, with the altitude of 1300 m, annual average temperature 4.2 ℃, annual average rainfall of 200 mm except for July and August, the evaporation in which was 10 times of the precipitation, and the typical plant in the meadow was *Stipa breviflora* and *Cleistogenes songorica*, the type of which was desert steppe.

Treatment of n-alkane methods

Six grazing Mongolian female sheep (one year old with an average body weight of 45.0±1.0 kg) were on pasture from June to early December on the experimental site. The sheep were free to roam all day long from summer to winter in three experimental

periods: spring (from middle May to early June), summer (August) and winter (December). No supplementary feed was supplied throughout the experiment periods. In each experimental period, all sheep were given one n-alkane capsule (97% purity, Acros Organics, NJ, USA; including 60 mg C32) every 15 d from the start of experiment to the end of experiment. Digestibility and diet composition in the grazing sheep were determined using the n-alkane technique as described by Hu (2014).

Collection of herbage and fecal samples

Herbage samples were collected manually. The species, positions and heights of the herbage that the sheep ate were observed for 5 min every 2 h in grazing for 5 d. Meanwhile, the herbage samples mimicking what the sheep were grazing on were collected, weighed, mixed and reserved at -20℃. Fecal samples were taken from the rectums of the sheep. Fecal sample (10 g) of each tested sheep was collected 3 times a day for 5 d in 12 time points, with an interval of 2 h. The samples were reserved in sealed bags at -20℃.

The herbage and fecal samples were analyzed for crude protein (CP), dry matter (DM), Ca and P according to Association of Official Analytical Chemists (1990). Neutral detergent fiber (NDF), acid detergent fiber (ADF) and ether extract (EE) following Van (1991). ME concentration in the diet was calculated from apparent DM digestibility estimated using C33 alkane.

N-alkane determination method

The samples were pretreated using the method proposed by Dove and Mayes (2005) and assayed using a Shimadzu GC-9A gas chromatograph.

The herbage composition was calculated using the suggested program (Eat What) proposed by Dove and Moore (1995).The dry matter intake was calculated from the pair of alkane C_{33} (naturally present in the herbage) and C_{32} alkane (dosed):
$DMI=D_{32}*F_{33}/ (F_{32}*H_{33}-F_{33}*H_{32})$.

Where, DMI is daily dry matter intake (kg/d), D_{32} is amount of C_{32} alkane dosed daily (mg/d). F_{33} and F_{32} are fecal concentrations of C_{33} and C_{32} alkanes (mg/kg DM), H_{33} and H_{32} are the herbage concentrations of C_{33} and C_{32} alkanes (mg/kg DM), respectively.

DM digestibility (DMD) was calculated using C_{33} as internal marker using the following formula: $DMD=1-(I_{33}*FR_{33})/F_{33}$.

Where, FR_{33} represents the faecal recovery of C_{33} alkane, and I_{33} is the dietary C_{33} concentration.

The recovery rates, as provided by Nigel (2000) for C_{31}, C_{32}, C_{33}, and C_{35} were 0.76, 0.87, 0.85 and 0.81, respectively.

Statistical analysis

Statistical analyses were performed using SPSS19 (SPSS Inc., Ireland). Effect of season on DM intake, dry matter digestibility and diet composition was analyzed by means of one-way analysis of variance (ANOVA). Significance levels were taken at $P<0.05$ to $P< 0.01$. Multiple comparisons between means were made using the Duncan's multiple range tests.

2.3 Results

Alkane patterns of herbage and feces

The average n-alkane content of the herbage consumed by the sheep is shown in Table2- 1 and Table2- 2. As expected, the contents of C_{29}, C_{31} and C_{33} were the high in all herbage species. The content of C_{32} alkane in the most species was very low. It showed that each herbage species had a specific alkane patterns. *Stipa krylovii*, *Artemisia frigida* and *Artemisia annua* had the highest concentration of C_{31} in all seasons. *Potentilla tanacetifolia* and *Erodium sp.* had a higher concentration of C_{33} than those of other species. The concentration of alkanes differed in different seasons. The concentration of C_{31} in *Stipa krylov*ii and *Artemisia frigida* declined from spring to winter, whereas the opposite was true from spring to summer. But the concentration of C_{31} in *Artemisia annua* increased from spring to summer, and declined from summer to winter. The fecal n-alkane concentrations in different seasons are presented in Table 2-3.

Diet composition in the grazing sheep

The diet composition in the grazing sheep was changed dramatically in different seasons for two experiments (Table2-4 and Table2-5).

In the first experimental site, Zhenglan Banner, *Artemisia frigida* was the most important dietary component for sheep in three seasons. *Artemisia frigida* constituted 33.5, 28.7 and 50.1% of the sheep's diet in summer, autumn and winter, respectively.

Carex sp. was a minor component of sheep diet in three seasons. In summer, the sheep showed major preference for *Artemisia frigida* which together with *Carex sp.*, *Stipa krylovii* and *Leymus chinensis* comprised more than 90% of the diet. In summer and winter, the sheep consumed mostly *Artemisia frigida* (28.7 and 50.1%), *Carex sp*. (18.4 and 19.2%) and *Stipa krylovii* (14.8 and 15.4%). The *Leymus chinensis* and *Potentilla tanacetifolia* were consumed only in spring, whereas *Potential acaulis* and *Thymus* were consumed only in summer. In spring and summer, the botanical composition of experiment site was dominated by *Artemisia frigid*, *Carex sp.*, *Stipa krylovii*, rare and inedible herbage species. The rare and inedible herbage species accounted for 42 and 41% in spring and summer, when the diversity of herbage species is higher. In winter, only *Artemisia frigida* (36%), *Cleistogenes sp.* (15%), *Carex sp.* (10%*)* and *Stipa krylovii* (25%) were detected in the botanical composition. The botanical composition of the experiment site was scarce where the proportion of rare and inedible herbage species was only 14% of the pasture.

The second experimental site, Wulatezhong Banner belongs to desert and semi-desert steppe. Same with the Zhenglan Banner, the diet composition of grazing sheep significantly varied in different season. During the spring, the predominant component of sheep diet were *Stipa breviflora* and *Carex duriuscula*, and the intake percentage of which were 71.8%, 13.4%, respectively. In the summer, the predominant component of sheep diet were *Peganum harmala*, *Poa annua* and *Allium mongolicum*, the intake percentage were 42.3%, 20.4%,12.1%, respectively. In the winter, the pasture turned yellow, the predominant component of sheep diet were *Artemisia scoparia*, *Carex duriuscula* and *Artemisia annua*, the intake percentage were 68.9%,17.8%,13.3% respectively.

Herbage intake and digestibility of the grazing sheep in Zhenglan Banner

It was observed that herbage intake reached 1.8 kg/d in spring, followed by 1.7 kg/d in summer and 1.2 kg/d in winter in Zhenglan Banner (Table2-6). Herbage intake did not differ in spring and summer ($P>0.05$) but it was significantly different in winter ($P<0.05$) (Table2-6). As pasture grows, its digestibility deteriorates. The digestibility of herbage species in spring, summer and winter were 71.4, 68.4 and 36.4%, respectively. The dry weight/fresh weight of grass increased from spring to winter, and it differed significantly between summer and winter. As expected, grass production was significantly low in winter ($P<0.05$). Herbage digestibility in spring and summer did not show any difference, but it differed significantly in winter ($P<0.05$). Herbage intake

correlated significantly with digestibility, but not with dry weight/fresh weight or with grass production.

Herbage intake and digestibility of the grazing sheep in Wulatezhong Banner

It was observed that herbage intake reached 0.8 kg/d in spring, followed by 0.9 kg/d in summer and 1.6 kg/d in winter in Wulatezhong Banner. Herbage intake did not differ in spring and summer (P>0.05) but it was significantly different in winter (P<0.05) (Table2-7). It is same with Zhenglan Banner. The digestibility of herbage was also same as Zhenglan Banner with the changes of season. The digestibility of herbage in spring, summer and winter were 61.4, 57.1 and 54.3%, respectively. The body weight of grazing sheep increased from spring to winter, and it differed significantly among seasons. As first experiment, plant production was significantly low in winter (P<0.05). Herbage digestibility in spring and summer did not show any difference, but it differed significantly in winter (P<0.05). Herbage intake correlated significantly with grass production and body weight, but not with DM digestibility. This result was diferent with it in Zhenglan Banner.

Dynamic variation of the nutritional components of the pastures in different seasons

The nutritional components of the pastures were presented in Table2-8 and Table2-9. In the present experiment, as the season changed from summer to autumn and then to winter, crude protein (CP) and ME values decreased in all herbage species, while the neutral detergent fiber (NDF) and acid detergent fiber (ADF) levels increased. The experimental period had a significant effect on the CP, NDF, and ADF of pastures. The present research showed a significant correlation between diet composition and CP content of pasture during winter (Table2-10). There was higher correlation between diet composition and proportion of grass in all season, but it was only significant in summer. There was no s correlation between diet composition and NDF.

Nutrient intake of the grazing sheep in different seasons

As shown in Table2-11, digestibility of DM, CP and OM in herbage decreased as the change of seasons. Meanwhile, digestibility of CP and OM in winter was significantly lower than that in spring and summer (P <0.05), and it was no significant

difference observed was between spring and summer with CP and OM digestibility ($P>0.05$). But the digestibility of NDF and ADF in herbage in spring and summer was significantly lower than that in winter ($P<0.05$). Similarly, no significant difference between spring and summer was observed about the digestibility of NDF and ADF ($P>0.05$).

Nutrient intake of the grazing sheep in different seasons was been calculated according to the herbage intake and nutritional components of the pastures (Table2-12). The results show that the nutrient intake of CP by grazing sheep in spring and summer meet the demand with feeding standard of meat-producing sheep. But there is a gap between nutrient intake and nutrient requirement, especially the gap in CP is larger in winter. The nutrient intake of calcium and phosphorus in the spring meet the demand of nutrient requirement, but it was lack in summer and winter. The nutrient intake of ME by grazing sheep was insufficient in all three seasons.

2.4 Discussion

The lack of knowledge of diet composition and nutritional status of sheep in different seasons has restricted the development of sustainable grazing systems in China. In northern China, sheep suffer from exposure to extend periods of nutrient deficiencies, in some cases, up to 7 months of the year. Therefore, it is important that grazing systems are designed to make the best use of the nutrients supplied by the native pasture during the short period of abundance during the spring and summer. The feed intake of grazing sheep is difficult to estimate. However, the discovery that different herbage species contain unique fingerprints of alkanes has revolutionized the ability for scientists to determine the intake of different pasture species by grazing animals (Malossini 1990, Dove and Mayes 1991, Hameleers and Mayes 1998). Since the original work quoted above, the alkane technique has been widely used to assess herbage intake, DM digestibility and diet composition in grazing animals (Kelman *et al*. 2003, Newman *et al*. 1995, Zhang 2002). The alkane recovery rates tend to increase with carbon-chain length (Sun *et al*. 2008). A similar trend was observed in a recent study in Inner Mongolia (Hu *et al*. 2014) and this study.

To our knowledge, there are no available data on the botanical composition of the diet selected by sheep grazing on the Inner Mongolia typical steppe. The results clearly demonstrated that diet components selected by sheep differed between seasons. *Artemisia frigid, Carex sp.* and *Stipa krylovii* were the main herbage species for all seasons, presenting 90% of the diet during the spring. *Leymus chinensis* and *Potentilla*

were selected by sheep only in the spring. *Thymus sp.*, *Potential acaulis* and *Cleistogenes sp.* became the main proportion of the diet in the summer. These seasonal changes might be modulated by plant palatability, particularly plant proportion and the radio of dry weight/fresh weights. Dziba (2003) and Rosa (2002) thought it might be modulated by plant height and aromatic compounds present in it. However, in this study, the effect of height and flavors on diet selection was not investigated. Nevertheless, there was a significant correlation between botanical composition and CP content. The characterization of diet components of sheep grazed on an Inner Mongolian steppe was the key aim of this study. The experiment site chosen for this study would have been classified as a typical steppe. But in the present study, *Artemisia frigida* was the most dominant diet component, a species normally dominant in desert steppes (Hu *et al*. 2014). Of course, this may reflect the fact that over the past few decades, the Inner Mongolian grasslands have been undergoing drastic changes with desertification becoming widespread even in areas like Xilingol. There are numerous reasons for it. Firstly, it was due to over grazing; and secondly, the general deterioration of the land has arisen because of population increase and mining activities.

The intake and digestibility of herbage changed depending on seasons, especially in the winter, when herbage intake decreased greatly. The results are consistent with those previously reported by Hu (2014) for the Inner Mongolian desert steppe. But in the current research, herbage digestibility was generally higher than that for a typical desert steppe pastures in the spring and autumn, lower in the winter. This was probably due to the fact that sheep grazed on desert steppe pastures are adapted to dry grass in terms of their rumen functions. There was a strong correlation between herbage intake and digestibility. This was not really surprising because the crude fiber level in forage is higher in the winter and the protein and ME values are lower, grass is not easy digestion and leave rumen (Li *et al*. 2015), so a large number of fiber stayed in the rumen, the sheep feel satiety, and didn't intake more feed. Herbage intake of grazing sheep have certain relationship with grass production (Liu *et al*. 2013), but in this study, the grass production is lower and the dry weight/fresh weight of grass also is lower, there are bad palatability, which lead it difficulty to intake more herbage.

Herbage species with a higher nutritional value appear to affect sheep's diet selection. With the decrease of herbage availability and nutritive value across the grazing season (spring to winter), the feed intake of sheep decreased. In addition, digestibility remained similar across different seasons. In the present study, diet composition significantly correlated with the CP content in the winter, and correlate with the proportion of grass in summer; this indicates that sheep have the ability to

select grass. Furthermore, the diet composition of grazing sheep was not been affected by the NDF content of herbage, probably due to the heavy diet selection by the sheep that maintained the rumen environment within a certain physiological and microbiological range (Morand-Fehr 2005).

In brief, during the spring all the herbage species were lush, with a lot of twigs and tender leaves, making them highly nutritious. All these contributed to a intake and digestibility of herbage by sheep, which did not exhibit herbage selection. In the summer, as precipitation increased, herbage growth also accelerated, leading to with increased biomass and fiber level. The palatability of the pastures decreased somewhat, resulting in a slight reduction of herbage intake and digestibility. With the onset of winter, there was hardly any plant growth, ending up with coarse stems and stubbles that were rich in fibre and poor in nutrients. Under these circumstances, sheep attained a minimum of nutrition during the winter (Li and Wang 1998). Our findings call for ways to manipulate pasture management and to promote sheep performance.

2.5 Conclusion

The results from the present study showed that the characteristics of alkanes in herbage are species specific within each season. This research revealed that *Artemisia frigida* was the most dominant diet component, with different proportions in different seasons. A positive correlation between diet component and botanical composition was observed only in summer. There was a significant correlation between diet component and CP in winter. Thus, it is clear that herbage intake of sheep depends on the botanical origin and nutrient content of pasture. The limited nutrient factors of grazing sheep in spring and winter were energy and soluble nitrogen. The nutrient intake of CP and ME by grazing sheep reduced, it can't meet the demand of its normal growth. At the same time, the intake of calcium and phosphorus in the summer and winter was also insufficient, so the limited nutrient factors of grazing sheep in spring and winter were energy and soluble nitrogen.

Table2-1 N-alkanes concentration of different herbages species of Zhenglan Banner in different seasons (mg/kg DM)

Season	Herbages species	C25	C27	C29	C31	C32	C33	C35
Spring	*Carex sp.*	14.5	23.2	87.9	169.8	1.6	154.7	23.7
	Cleistogenes sp.	25.5	76.3	421.4	554.8	0.4	258.0	9.3
	Artemisia frigida	31.3	34.3	149.5	993.9	0.4	292.7	5.4
	Leymus chinensis	37.4	94.6	165.5	232.3	0.8	116.9	23.7
	Potential bifurca	5.1	58.9	75.4	172.8	9.0	305.1	55.7
	Potentilla tanacetifolia	19.5	53.2	233.5	590.3	12.8	390.5	8.0
	Stipa krylovii	17.4	41.9	306.1	1021.7	0.9	208.6	19.2
	Thymus sp.	53.4	88.7	302.8	286.7	13.6	363.3	31.7
Summer	*Carex sp.*	6.4	15.4	224.7	412.3	0.6	496.1	9.6
	Cleistogenes sp.	83.3	151.0	183.8	352.9	2.6	413.3	14.9
	Artemisia frigida	24.7	53.5	165.8	357.2	1.8	141.3	9.0
	Leymus chinensis	26.2	66.9	136.7	203.6	2.5	232.3	14.9
	Potentilla bifurca	16.8	49.5	129.3	116.7	2.2	282.3	8.5
	Potentilla tanacetifolia	25.7	49.3	176.8	246.1	1.6	367.6	8.7
	Stipa krylovii	24.8	76.6	172.8	553.0	1.4	201.1	4.9
	Thymus sp.	36.2	66.4	321.0	459.5	9.2	471.4	23.8
Winter	*Carex sp.*	14.5	33.9	99.6	213.2	0.6	127.0	15.7
	Cleistogenes sp.	10.8	33.5	86.4	345.1	1.9	76.7	10.0
	Artemisia frigida	19.0	31.1	97.4	234.2	0.8	20.8	7.5
	Leymus chinensis	7.3	22.7	165.4	254.6	0.4	113.6	9.8
	Stipa krylovii	15.4	49.2	253.2	264.5	0.7	564.3	31.3

Table2-2 N-alkanes concentration of different herbages species of Wulatezhong Banner in different seasons (mg/kg DM)

Season	Herbages species	C25	C27	C29	C31	C32	C33	C35
Spring	*Allium mongolicum*	83.4	140.5	179.4	1000.2	63.3	78.9	63.1
	Artemisia annua	47.0	76.6	101.6	85.8	50.0	34.0	1.8
	Artemisia frigida	18.0	13.0	32.3	66.0	8.1	27.1	7.8
	Carex duriuscula	12.6	17.3	98.8	150.4	6.8	19.7	1.5
	Erodium sp.	29.2	62.4	201.8	305.1	30.4	148.6	33.5
	Iris bungei	61.3	118.1	125.1	67.5	69.2	35.7	0.2
	Peganum harmala	67.1	89.4	124.2	72.2	48.0	10.1	7.4
	Stipa breviflora	105.8	117.0	165.6	216.0	36.0	21.2	5.8
Summer	*Allium mongolicum*	101.6	190.1	241.7	843.2	135.2	74.0	23.7
	Artemisia annua	101.0	190.3	357.3	219.8	26.5	15.4	1.0
	Artemisia scoparia	6.6	16.5	47.2	42.9	10.6	17.6	13.5
	Carex duriuscula	45.0	81.6	129.8	147.4	52.5	43.7	12.4
	Erodium sp.	31.5	100.7	582.7	1183.1	58.0	596.8	21.4
	Peganum harmala	125.5	147.9	319.4	175.5	8.0	64.0	32.3
	Poa annua	76.4	78.9	98.8	178.2	47.7	50.2	20.0
	Setaira viridis	71.2	163.2	400.4	216.5	76.9	55.7	11.8
	Tribulus terrestris	76.4	78.9	98.8	178.2	47.7	50.2	20.1
Winter	*Artemisia annua Linn*	55.2	66.1	145.6	133.4	24.7	29.2	5.3
	Artemisia scoparia	6.6	7.7	76.4	0.4	59.6	20.0	1.1
	Carex duriuscula	238.0	310.2	767.9	389.2	208.6	39.9	9.1
	Peganum harmala	7.3	10.7	22.9	13.2	3.7	2.3	1.4
	Setaira viridis	318.8	331.9	480.4	352.2	228.6	172.6	5.6

Table2-3 N-alkanes concentrations of grazing sheep fecal in different seasons (mg/kg DM)

Site	Season	C25	C27	C29	C31	C32	C33	C35
Zhenglan Banner	Spring	20.9±7.3^{b}	51.7±23.0^{b}	372.1±50.3^{a}	480.0±190.9^{a}	195.9±46.6	359.4±148.6^{a}	8.0±3.1
	Summer	26.1±8.0^{b}	70.8±14.9^{a}	31.7±2.5^{b}	318.0±42.0^{b}	186.7±23.4	242.4±80.4^{b}	10.9±2.7
	Winter	15.5±8.1^{a}	40.1±9.4^{b}	41.2±7.0^{b}	465.1±94.0^{a}	115.8±10.7	119.6±26.8^{c}	9.9±2.6
Wulatezhong Banner	Spring	23.6±1.5	45.3±3.2	84.8±3.3	182.3±10.5	29.2±1.7	82.8±3.4	15.1±0.8
	Summer	127.6±22.9	151.8±16.3	460.3±50.9	553.8±64.9	49.6±5.1	168.6±26.7	35.0±6.5
	Winter	53.9±9.6	146.4±29.2	675.4±112.6	524.6±61.2	68.9±8.4	77.9±3.7	7.2±2.0

The different letters (a,b,c) indicate significant differences in different seasons at $P < 0.05$.

Table2-4 The diet composition of grazing sheep and botanical composition in different seasons in Zhenglan Banner (%)

Name	Item	*Artemisia frigida*	*Cleistogenes sp.*	*Potentilla acaulis*	*Carex sp.*	*Stipa krylovii*	*Leymus chinensis*	*Potentilla tanacetifolia*	*Thymus sp.*	Rare and inedible herbage
Spring	Diet composition	33.5±1.3[b]	—	—	21.2±1.1	17.9±1.0[a]	17.6±1.0	9.8±0.5	—	
	Botanical composition	13			12	14	9	10		42
Summer	Diet composition	28.7±2.1[b]	10.8±0.9[b]	8.1±1.0	18.4±0.9	14.8±1.2[b]	—	—	19.2±0.8	
	Botanical composition	15	8	7	13	10			6	41
Winter	Diet composition	50.1±1.4[a]	15.3±0.9[a]	—	19.2±1.2	15.4±1.1[a]	—	—	—	
	Botanical composition	36	15		10	25				14

The different letters (a, b) indicate significant differences in different seasons at $P < 0.05$.Bobannical composition: the proportion of each grass in the steppe. Diet composition: the proportion of each grass in the diet. The diet composition was calculated using the suggested program (Eat What) proposed by Dove and Moore (1995).

Table2-5 The diet composition of grazing sheep and botanical composition in different seasons in Wulatezhong Banner (%)

Item	*Carex duriuscula*	*Artemisia frigida*	*Stipa breviflora*	*Iris bungei*	*Peganum harmala*	*Tribulus terrestri*	*Erodium sp.*	*Allium mongolicum*	*Setaira viridis*	*Poa annua*	*Artemisia soparia*	*Artemisia annua*
Spring	13.4	6.4	71.8	3.9	4.4	—	—	—	—	—	—	—
Summer	—	—	—	—	42.3	7.90	8.9	12.1	8.6	20.4	—	—
Winter	17.8	—	—	—	—	—	—	—	—	—	68.9	13.3

Table2-6 The herbage intake and digestibility in grazing sheep，dry fresh ratio and grass production of natural pasture in different season

Season	DM intake,kg DM/d	DM digestibility, %	Dry weight/fresh weight, %	Grass production, g/m^2
Spring	1.8 ± 0.1^a	71.4 ± 1.6^a	47.5 ± 5.8^a	93.74 ± 5.4^a
Summer	1.7 ± 0.1^a	68.4 ± 1.3^a	59.3 ± 4.2^b	111.39 ± 6.0^a
Winter	1.2 ± 0.1^b	36.4 ± 1.2^b	63.3 ± 3.3^b	58.67 ± 4.0^b
Correlation with DM intake, kg DM/d		0.997*	-0.798	0.882

The different letters (a, b) indicate significant differences in different seasons at $P < 0.05$.
DM= Dry matter.The star letters (*) indicate significant differences of correlation in different seasons at $P < 0.05$.

Table2-7 The herbage intake and digestibility in grazing sheep，dry fresh ratio and grass production of natural pasture in different seasons

Season	DM intake,kg DM/d	DM digestibility, %	Body weight, %	Grass production, g/m^2
Spring	0.8 ± 0.2^{a}	61.4 ± 2.1^{a}	25.7 ± 1.3^{a}	41.1 ± 26.4
Summer	0.9 ± 0.3^{a}	57.1 ± 2.1^{a}	34.0 ± 2.5^{b}	322.7 ± 131.7
Winter	1.6 ± 0.2^{c}	54.3 ± 2.0^{b}	41.7 ± 3.8^{c}	115.6 ± 44.4
Correlation with DM intake, kg DM/d		-0.84	0.89*	0.92*

The different letters (a, b) indicate significant differences in different seasons at $P < 0.05$. The star letters (*) indicate significant differences of correlation in different seasons at $P < 0.05$.

Table2-8 The nutritional components of the pastures in Zhenglan Banner in different seasons

Season	Specie	DM, %	CP, %	EE, %	NDF, %	ADF, %	Ca, %	P, %	ME, KJ/kg
Spring	*Carex sp.*	85.1±0.1	11.1±1.3	6.8±2.5	41.1±5.3	24.9±1.5	1.0±0.3	0.5±0.1	6.7±0.3
	Artemisia frigida	86.7±0.0	12.1±0.8	7.5±4.6	46.1±3.6	28.8±0.9	1.3±0.2	0.5±0.1	7.0±0.2
	Leymus chinensis	87.8±0.9	11.2±0.5	5.1±0.5	47.1±0.5	26.2±1.9	1.0±0.1	0.5±0.0	7.1±0.4
	Potentilla tanacetifolia	85.0±0.3	11.2±1.5	5.9±2.8	42.6±0.4	24.6±0.6	0.8±0.1	0.5±0.1	6.8±0.0
	Stipa krylovii sp.	84.3±3.0	14.0±4.1	5.5±2.4	54.8±6.5	27.9±3.7	0.9±0.0	0.5±0.1	6.6±0.3
Summer	*Carex sp.*	85.2±1.0	9.4±0.5	4.6±0.1	47.2±0.0	26.1±1.9	1.4±0.0	0.5±0.0	7.1±0.0
	Cleistogenes sp.	88.7±1.4	12.2±0.9	2.9±0.1	42.09±2.8	31.2±1.3	1.0±0.2	0.5±0.0	7.1±0.1
	Artemisia frigid	86.8±2.2	11.5±0.9	2.8±0.2	55.1±6.3	24.7±5.1	1.5±0.3	0.6±0.0	7.1±0.2
	Potential bifurca	81.2±0.2	11.6±0.3	3.4±0.1	41.4±2.2	22.0±3.6	0.8±0.0	0.9±0.0	8.3±0.6
	Stipa krylovii	87.5±1.0	10.0±0.4	3.5±0.5	62.6±0.5	32.5±0.1	0.9±0.0	0.4±0.0	6.8±0.1
	Thymus sp.	86.5±0.3	8.8±0.1	5.1±0.1	40.6±0.3	27.9±0.2	0.8±0.0	0.4±0.0	7.3±0.1
Winter	*Carex sp.*	85.3±34.8	6.9±3.5	3.4±1.5	52.3±21.7	33.1±14.3	1.2±0.5	0.7±0.3	4.7±1.9
	Cleistogenes sp.	86.4±4.2	8.4±2.5	1.9±0.5	61.4±11.7	40.4±6.5	1.0±0.2	0.7±0.1	3.9±1.1
	Artemisia frigid	88.3±1.5	11.7±0.3	2.6±0.3	52.9±3.3	32.1±3.3	1.1±0.0	0.7±0.1	5.2±0.2
	Stipa krylovii	91.8±4.6	7.8±1.4	2.6±0.6	66.5±5.2	34.7±3.1	0.9±0.0	0.5±0.1	5.2±0.6

The different letters (a, b) indicate significant differences in different seasons at $P < 0.05$. DM=Dry matter; CP=Crude protein; NDF=Neutral detergent fiber; EE=Ether extract; ADF=Acid detergent fiber; ME=Metabolizable energy

Table2-9 The nutritional components of the pastures in Wulatezhong Banner in different seasons

Season	Specie	DM/%	CP/%	NDF/%	ADF/%	ADL/%	Ash/%	OM/%
Spring	*Allium mongolicum*	90.9	22.6	20.5	15.9	2.2	17.7	73.2
	Artemisia annua	93.4	18.4	24.2	18.3	2.1	23.1	70.3
	Artemisia frigida	92.9	12.6	36.7	27.2	2.7	19.2	73.7
	Carex duriuscula	92.9	16.0	49.0	21.5	1.8	8.8	84.2
	Erodium sp.	92.7	13.9	22.6	17.3	2.8	15.8	76.9
	Iris bungei	92.1	10.0	48.1	37.9	4.6	10.3	81.8
	Peganum harmala	91.4	20.4	23.2	17.3	3.9	12.9	78.6
	Stipa breviflora	93.0	11.5	66.3	32.5	3.8	5.0	88.0
Summer	*Allium mongolicum*	94.5	18.8	32.9	25.7	5.6	7.9	86.6
	Artemisia annua	92.0	25.3	34.3	25.1	9.6	10.0	82.1
	Artemisia scoparia	95.7	16.6	36.6	24.7	6.7	14.2	81.5
	Carex duriuscula	92.8	15.6	57.4	23.0	1.2	8.6	84.2
	Erodium sp.	93.5	15.5	25.2	18.8	4.2	10.7	82.9
	Peganum harmala	93.6	16.1	31.7	22.4	4.1	18.8	74.8
	Poa annua	94.1	10.7	46.5	23.0	2.5	18.3	75.7
	Setaira viridis	93.4	11.2	53.8	28.3	2.1	13.2	80.2
	Tribulus terrestris	91.9	19.9	23.3	16.5	2.7	16.8	75.1
Winter	*Artemisia annua*	91.5	14.8	42.5	30.9	8.2	7.7	83.8
	Artemisia scoparia	91.9	9.2	54.5	41.0	13.4	7.1	84.8
	Carex duriuscula	91.3	11.9	61.1	33.2	12.9	8.7	82.6
	Peganum harmala	92.2	7.4	36.4	27.0	8.9	23.3	68.9
	Setaira viridis	92.6	8.4	56.0	31.5	10.7	15.4	77.2

Table2-10 The correlation between diet composition and CP, NDF or botanical composition in grassland of Zhenglan Banner in different seasons

Season	CP	NDF	Botanical composition
Spring	0.17	0.05	0.52
Summer	-0.26	0.53	0.97*
Winter	0.92*	-0.55	0.85

The star letters (*) indicate significant differences of Correlation in different season at $P < 0.05$. CP=Crude protein; NDF=Neutral detergent fiber.

Table2-11 Nutrient digestibility of pasture groups in different seasons

Item	Spring	Summer	Winter
DM，%	61.4±2.1[a]	57.1±2.1[a]	54.3±0.0[b]
CP，%	69.6±1.9[a]	68.2±2.0[a]	52.9±1.2[b]
NDF，%	49.9±2.2[a]	45.1±2.2[a]	56.4±2.5[b]
ADF，%	47.8±2.3[a]	42.4±2.3[a]	58.2±2.1[b]
OM，%	66.5±2.4[a]	58.6±2.2[a]	53.5±2.1[b]

Note：In the same column, same small letter superscripts mean no difference($P > 0.05$), adjacent letter superscripts mean significant difference($P < 0.05$), secluded superscripts letter mean significant difference ($P < 0.01$).

Table2-12 The diet nutritional components of the pastures of grazing sheep in different seasons

Nutrition		DM（Kg/g）	CP(g)	EE(g)	NDF(g)	ADF(g)	Ca(g)	P(g)	ME(MJ/Kg)
Nutrient Requirement		1.6	206.5				5.95	4.7	22.7
Spring	Nutrition Intake	1.8 ± 0.1^{a}	259.0 ± 5.3^{a}	16.9 ± 3.1^{a}	738.9 ± 42.5^{a}	470.8 ± 53.2^{a}	6.8 ± 0.5^{a}	4.9 ± 0.6^{a}	12.1 ± 0.5^{a}
	Gap	0.2	52.5				0.8	0.2	-10.6
Summer	Nutrition Intake	1.7 ± 0.1^{a}	246.1 ± 4.3^{a}	6.2 ± 0.4^{b}	851.2 ± 50.0^{a}	440.9 ± 68.4^{b}	4.8 ± 0.1^{b}	4.1 ± 1.0^{a}	16.4 ± 2.1^{a}
	Gap	0.1	39.6				-1.2	-0.6	-6.3
Winter	Nutrition Intake	1.2 ± 0.1^{b}	88.3 ± 3.0^{b}	2.5 ± 0.1^{b}	971.9 ± 109.9^{b}	470.7 ± 95.4^{a}	3.4 ± 0.5^{b}	3.5 ± 1.7^{b}	5.6 ± 1.8^{b}
	Gap	-0.4	-118.2				-2.6	-1.2	-17.1

Note：In the same column, same small letter superscripts mean no difference（$P > 0.05$）,adjacent letter superscripts mean significant difference（$P < 0.05$）,secluded superscripts letter mean significant difference （$P < 0.01$）.

Gap = nutrient intake –nutrient requirement.

CHAPTERⅢ The Seasonal Changes of Types and Numbers of Ruminal Bacteria and Fermentation Pattern in Livestock Grazing on the Grassland of Inner Mongolia

3.1 Introduction

Ruminants often obtain their food from herbages, agricultural products, and byproducts in China. Various microorganisms inhabiting the rumen are well-coordinate to influence roughage use and fiber digestion. Particularly in northern China, ruminants are pastured and fed in natural grassland all year round. The quantities, types, and nutrients of herbages in natural grasslands vary each season. Meanwhile, microbes in the rumen exert important effects. Molecular biological techniques based on the 16S rDNA have greatly improved the development of the micro-ecological research. Specifically, high-throughput sequencing, also known as next-generation sequencing, can effectively analyze complex microbial communities without culture, which is widely used in studies on microflora in animal digestive tracts. In northern prairie regions with harsh environment, sheep rely on natural herbages for their main feed. The digestive effects of large numbers of microorganisms in the rumen provide protection for their survival. The nutritional contents of herbages through different seasons influence the types and quantities of microorganisms in the rumen, thus influencing the digestion and absorption of herbages by grazing sheep. However, studies on seasonal changes in the rumen microflora of grazing sheep are still rarely reported. Thus, with high-throughput sequencing, this study investigated the changes in rumen bacterial communities in grazing sheep to provide a scientific basis for nutritional regulation for grazing sheep in northern grasslands. These changes are attributed to nutritional changes in herbages through different seasons.

3.2 Materials and methods

Experimental animals and management

The experimental animals consisted of 6 Mongolian sheep of the same species, weight, and age, and of good physical condition. The sheep were grazed in natural grassland all year round in Zhenlan Banner, Xilinguole. The experimental daily diet was sourced from natural pasture; the experimental sheep were not fed other types of hay.

Collection of samples and treatment

In the middle of January, April, June, August, and October during the experimental period, rumen fluid (50 mL) was collected from the sheep via the

mouth, using a stomach tube before morning, filtered by 4 layers of gauze and preserved at -80 ℃. The purpose was to extract DNA to determine the microbial flora of the rumen.

DNA Extraction

DNA from different samples was extracted using the MicroElute Genomic DNA Kit (D3096-01; Omega, Guangzhou, China) according to the manufacturer's instructions. Sample blanks consisting of unused swabs were processed through DNA extraction for verification of no DNA. The total DNA of swabs was eluted in 20 μl of elution buffer (Omega D3096) and stored at -80 ℃ until measurement in the PCR (Lianchuan Biotech, Hangzhou, China).

PCR Amplification and 16S rDNA Sequencing

Using the total DNA from the samples as a template, the primer (319F 5'-ACTCCTACGGGAGGCAGCAG-3'; 806R 5'-GGACTACHVGGGTWTCTAA T--3'), we amplified the V3-V4 regions of the bacterial 16S rDNA. All reactions were carried out in 25μl (total volume) of mixtures containing approximately 25 ng of genomic DNA extract, 12.5μl of PCR premix, 2.5μl of each primer, and PCR-grade water to adjust the volume. PCR reactions were performed in a Master cycler gradient thermocycler (Eppendorf, Hamburg, Germany) under the following conditions:initial denaturation at 98 ℃ for 30 seconds; 35 cycles of denaturation at 98 ℃ for 10 seconds，annealing at 54 ℃/52 ℃ for 30 seconds, and extension at 72 ℃ for 45 seconds;final extension at 72 ℃ for 10 minutes. The PCR products were confirmed with 2% agarose gel electrophoresis. Throughout the DNA extraction process, ultrapure water, instead of a sample solution, was used to exclude the possibility of false-positive PCR results as negative controls.

The PCR products were normalized by AxyPrep ™ Mag PCR Normalizer (Axygen Biosciences, Union City, CA，USA), which allowed for the skipping of the quantification step regardless of the PCR volume submitted for sequencing. The amplicon pools were prepared for sequencing with AMPure XT beads (Beckman Coulter Genomics, Danvers, MA, USA), and the size and quantity of the amplicon library were assessed on the LabChip GX (Perkin Elmer, Waltham, MA, USA) and with the Library Quantification Kit for Illumina (Kapa Biosciences, Woburn, MA, USA), respectively. PhiX Control library (Illumina) was combined with the amplicon library (expected at 30%) and sequenced on 300PE MiSeq runs using the standard Illumina sequencing primers.

Data Analyses

The sequence reads were pre-processed with removal of primer sequence. The high-quality 300PEs was assembled using FLASH, without preliminary quality

trimming, as the reads were expected to overlap by approximately 90 bp. The assembled sequence reads processing was performed using QHME (version 1.6.0), the criteria for QHME quality trimming were as follows: 1) truncation of sequence before three consecutive low-quality bases and re-evaluation for length; 2) no ambiguous base calls; and 3) minimum sequence length of 150 bp after trimming. Between 5% and 10% of the reads were filtered out when these quality criteria were applied. Then the filtered sequences were clustered by using the CD-hit-est based clustering method. Software PyNAST (http:/qiime.org/pynast/) was used to analyze and calculate the numbers of sequence and operational taxonomic units (OTUs) for each sample by comparing the representative sequence in the Greengene core set database. Then the species abundance and distribution were analyzed before cluster analysis. Next, equences were grouped into various OTUs using Felsentein-corrected similarity matrices, and those within an operational taxonomic unit shared at least 97% similarity. The Ribosomal Database Project (RDP) classifier was used to classify the 16S rDNA into distinct taxonomic categories by aligning sequences to a curated database of taxonomically annotated sequences. All 16S rDNA sequences were mapped to the RDP database using BLASTN in order to achieve taxonomic assignments. Sequences greater than 97% identity were used to associate a group of OTUs to specific species, while those with less than 97% identity were considered novel reads. The microbial diversity in individual conjunctival samples was estimated using rarefaction analysis, Shannon diversity index, and Simpson diversity index.

Statistics and analysis

All data were subjected to single factor experiment design and one-way ANOVA using the GLM program of the SPSS19 software, and multiple comparisons were conducted using the Duncan's method. $P < 0.05$ was considered statistically significant.

3.3 Results

DNA isolated from the sample

Samples for monitoring DNA contamination did not produce 16S rDNA gene amplicons for sequencing analysis (Fig.3-1), indicating the sampling process was controlled effectively. The PCR technology was used to produce objective DNA for monitoring DNA quality (Fig.3-2).

Rumen microbial diversity in different months

A rarefaction analysis, Shannon, Simpson, and Chao richness analysis were performed to assess the number and the diversity of microbial communities

(Fig.3-3, Table3-2). In this study, 70898 reads were obtained, which were distributed in 2613 OTUs (Operational Taxonomic Units). The rarefaction curve illustrates that the number of reads reached the platform period after 20000 (Fig.3-3), and the number of experiments met the experimental objective. The results indicated that the number of rumen microbial OTUs varied over the months, increasing in January, reaching its highest in August, and then decreasing after October. No significant difference in the number of OTUs was observed among the months of January, October, June, and August; however, a significant difference between the OTUs in January and October and those in June and August ($p<0.05$). Rumen bacterial diversity in grazing sheep gradually increased from January to August and then decreased starting October. By contrast, the Simpson's index declined from January and rose in October (Table 3-2). The results indicated that more types of rumen microbes in sheep were found in spring and summer. However, the number of bacterial species varied; some bacteria were more dominant than others.

Analysis of bacterial diversity at the phylum level

By analysis of bacterial diversity at the phylum level, *Firmicutes* and *Bacteroidetes* were identified as the dominant bacteria in the rumen of grazing sheep, comprising 56.9% and 21.5% of the total bacteria, respectively. These bacteria significantly influenced rumen digestion. The number of *Firmicutes* began to rise in January, but no significant difference was found among the months; the number reached its highest in August, and this number was significantly higher than that in June. The number began to decline significantly in October. *Bacteroidetes* exhibited a change in trend similar to that shown by *Firmicutes* but reached their highest number in June; this number was significantly larger than those in other months. The number of *Bacteroidetes* in other months was not significant (Fig.3-4, Table3-3).

Analysis of bacterial diversity at the family level

By analysis of bacterial diversity at the family level, *Ruminococcaceae*, *Lachnospiraceae*, *Christensenellaceae*, *Prevotellaceae*, and *Rikenellaceae* were identified as the dominant bacteria in the rumen of grazing sheep, comprising 19.1%, 17.0%, 12.1%, 8.5%, and 8.3% of the bacteria, respectively; these microorganisms play an important role in rumen digestion. All bacterial counts increased from January, reaching their highest in June or August. No significant difference in the bacterial count of *Christensenellaceae* was observed among the months; by contrast, significant differences were indicated between the bacterial counts of other families in June or August and those in other months (Table3-4).

Phylogenetic tree analysis of rumen bacteria

Analysis of the phylogenetic tree indicated that the bacteria were clustered together in June and August. Except for that in the individual sheep, bacteria clustering were more dispersed in other months (Fig3-4, Fig3-5). Bacterial count was higher in June and August than in other months; however, more dominant species were also present in those months. The number of bacteria decreased in other months; however, the number of dominant families also decreased, the number of bacteria in other families increased, and overall diversity increased.

3.4 Discussion

In northern prairie regions with harsh environment, sheep mainly feed on natural herbages, and the digestive effects of a large numbers of microorganisms in the rumen provide protection for their survival. The nutritional contents of the herbages in different seasons can influence the types and quantities of microorganisms in the rumen, influencing the digestion and absorption of herbages by the grazing sheep. The diversity analysis of rumen bacteria in grazing sheep in different months indicates that the rumen bacterial diversity of grazing sheep decreased gradually from the herbage growth period in August to the withering period in January of the following year, and gradually increased from April of the following year. Dan (2009) also found that the number of rumen bacteria was significantly higher in summer than in the other 3 seasons; the results were higher in autumn and winter than in spring, which is consistent with the results of the current study.

We analyzed the OTUs of bacterial species more than once. *Firmicutes* and *Bacteroidetes* were identified as the main bacteria at the phylum level; *Ruminococcaceae*, *Lachnospiraceae*, *Christensenellaceae*, *Prevotellaceae*, and *Rikenellaceae* were the main bacteria at the family level in the rumen of grazing sheep. In general, the composition of bacterial flora in the present study was similar to that of other ruminants, showing typical bacterial flora characteristics for fiber digestion (Singh 2012, Lee 2012, Kim 2014). Thus, only few bacterial floras affect digestion in the rumen. In general, all bacterial counts were reduced from January to August and then increased from October at the phylum level. *Firmicutes* and *Proteobacteria* were identified as the dominant bacteria in August, whereas *Bacteroidetes* were the dominant bacteria in June. *Cyanobacteria* were the dominant bacteria in August and October, whereas *Spirochaeta* were the dominant ones in April, June, and August. Similar rules were set at the family level. These results showed that different bacteria significantly influenced ruminal digestion in different seasons.

The study also demonstrated that most samples were aggregated into June and August by seasons, except for a few sheep; bacteria clustering in other months were more dispersed. The number and species of bacteria were more similar in

June and August than in other months; however, more dominant species were also identified in those months.

Analysis of the phylogenetic tree showed similar results. The bacteria in June and August were clustered together; the predominant bacteria had more OTUs in other months. Except for those in the individual month, the bacteria in June and August were more widely dispersed in other months. The number of bacteria decreased in other months; however, the number of dominant families decreased, other bacteria family increased, and overall diversity increased.

Nitrogen and carbon sources necessary for the growth of rumen bacteria mainly come from the feeds. Under traditional grazing conditions, the dietary crude protein at 12%-13% can meet the needs for maximum microbial growth. With the changes in months and seasons, the leaf dry matter content in northern grasslands also increases as the herbages change from green to yellow, crude protein content decreasing and fibers increasing with the growth process. These findings suggest that the protein content exhibits a single-peak change with fluctuations during seasonal changes, whereas the crude fiber content exhibits the opposite trend. In the current study, the rumen microbes of grazing sheep exhibited the highest diversity in August, which is consistent with the results obtained by Dan (2009). *Firmicutes* were the dominant type in each season. Several studies indicate that the rumen microbes in ovines mainly consisted of *Firmicutes* and *Bacteroidetes*; this result slightly varies from the results of the present study. In this study, *Bacteroidetes* only constituted 18% of the total bacteria and were not the main bacteria in the rumen, which could be attributed to feeding conditions. Grazing sheep consume feeds provided by grasslands year-round, mostly consisting of fiber-rich herbages; therefore, the bacteria were mainly *Firmicutes* for fibrinolysis. However, ovines in drylot feeding also consume a large amount of starch-rich concentrates, hence the dominance of *Bacteroidetes* with starch and soluble carbohydrates as substrates.

3.5 Conclusion

The rumen bacterial diversity in grazing sheep decreases gradually from the herbage growth period in August to the withering period in January of the following year, and gradually increasing from April of the following year. Various types of rumen bacteria exist, most of which tend to be categorized; meanwhile, some specific dominant bacterial communities are distinctive and different in each season. The bacterial communities in months other than August exhibit considerably closely spaced, indicating that dominated by *Firmicutes*, the rumen microbes of grazing sheep exhibit the highest diversity in August.

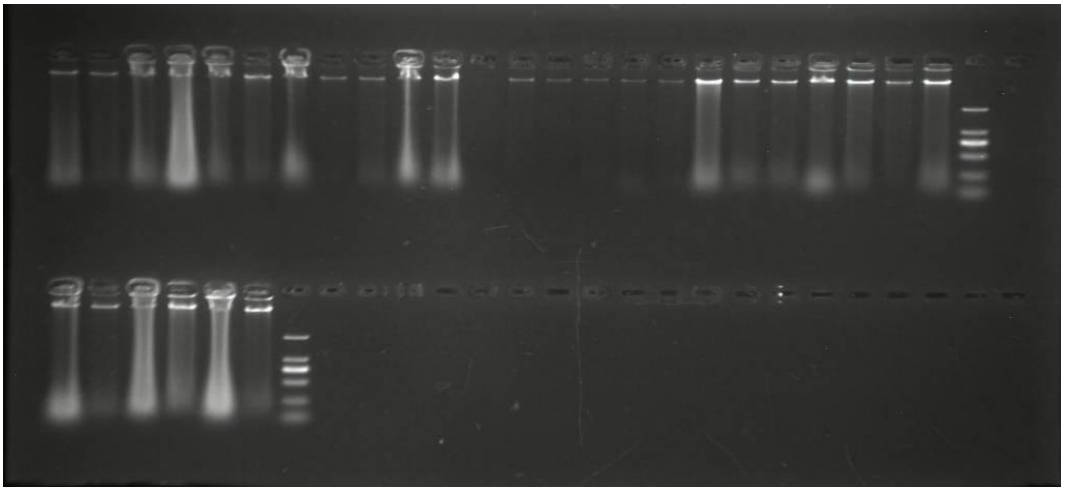


Fig.3- 1 A fraction of screenshot of the running gel of genomic DNA extraction.

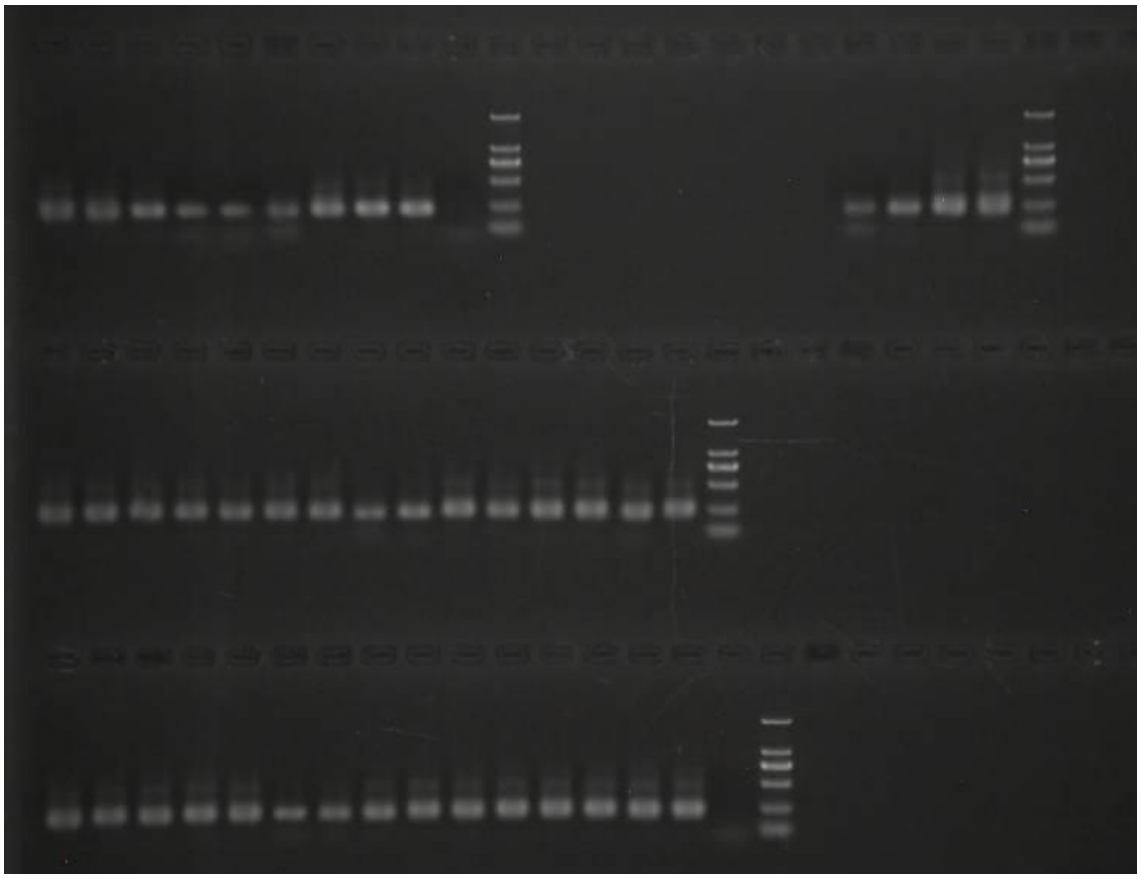


Fig.3- 2 A fraction of screenshot of the running gel of objective DNA PCR from grazing sheep

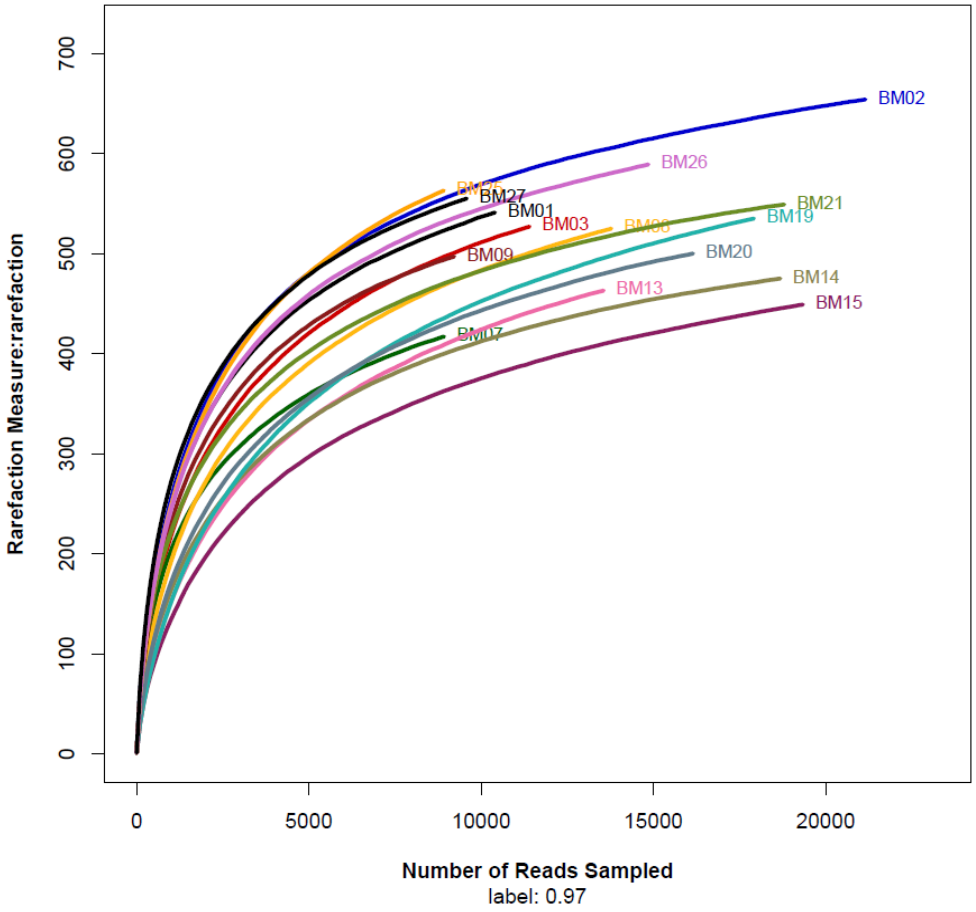


Fig.3- 3 The rarefaction curve of rumen microbe from grazing sheep in different months

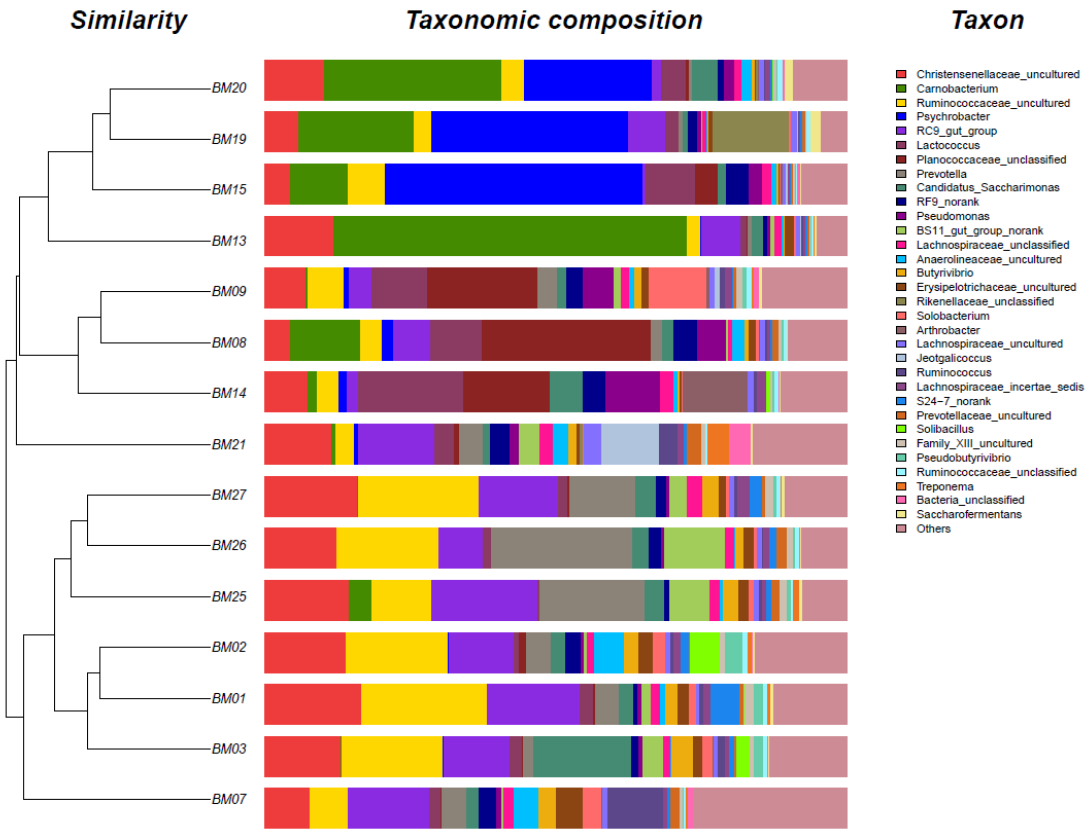


Fig.3- 4 Phylogenetic tree analysis of rumen bacteria

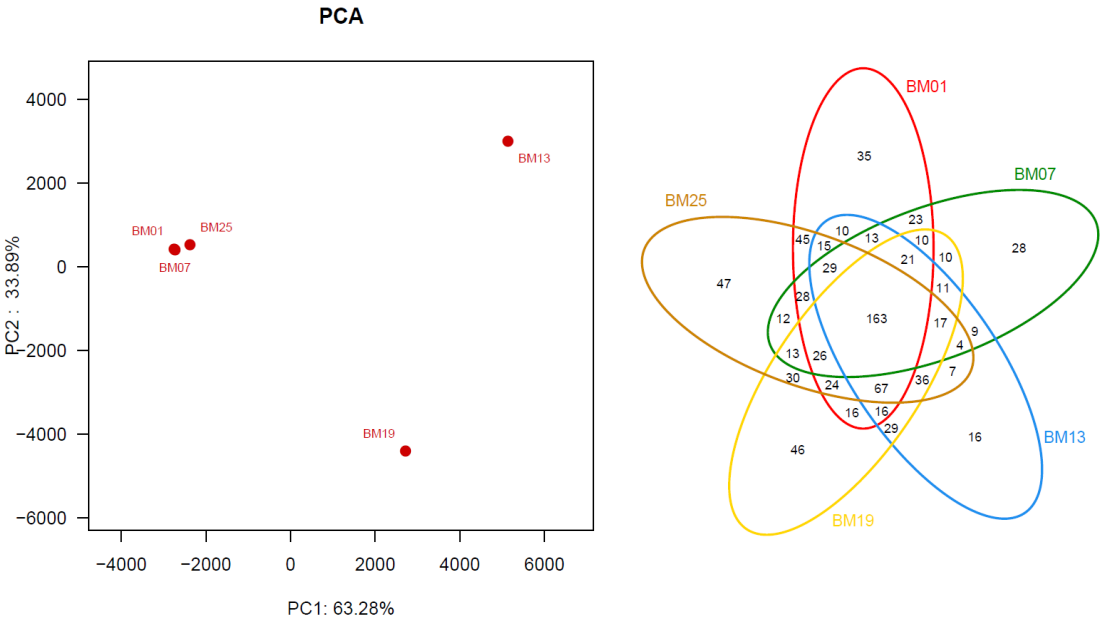


Fig.3- 5 Principal component analysis and venn cluster analysis

Table3-1 Grouping table of grazing sheep in different months

Sheep number	Sample number	Sampling date
1	BM01	August
2	BM02	
3	BM03	
4	BM04	
5	BM05	
6	BM06	
1	BM07	October
2	BM08	
3	BM09	
4	BM10	
5	BM11	
6	BM12	
1	BM13	Janua ry
2	BM14	
3	BM15	
4	BM16	
5	BM17	
6	BM18	
1	BM19	April
2	BM20	
3	BM21	
4	BM22	
5	BM23	
6	BM24	
1	BM25	June
2	BM26	
3	BM27	
4	BM28	
5	BM29	
6	BM30	

Note: we collect 6 sample every month from grazing sheep.

Table3-2 Rumen microbial diversity index of grazing sheep in different months

Months	January	April	June	August	October
Reads	17193±1828	17622±778	11118±1878	14322±3429	10641±1571
OTU	462.3±7.5^{a}	528.0±14.6^{ab}	569.0±10.3^{b}	574.0±40.2^{b}	479.7±32.4^{a}
Ace	546.0±12.8^{a}	617.0±15.6^{ab}	655.0±11.0^{b}	658.0±34.5^{b}	567.3±36.7^{a}
Chao	559. 0±9.3^{a}	639.7±26.6^{ab}	664.0±5.5^{b}	676.0±38.5^{b}	562.0±37.3^{a}
Shannon	3.1±0.4^{a}	3.7±0.5^{ab}	4.9±0.1^{c}	4.8±0.1^{c}	4.3±0.3^{bc}
Simpson	0.22±0.09^{a}	0.11±0.04^{ab}	0.02±0.00^{b}	0.02±0.00^{b}	0.06±0.03^{b}
Coverage	0.99±0.00	0.99±0.00	0.99±0.00	0.99±0.00	0.99±0.00

The different letters (a, b) indicate significant differences in different months at $P < 0.05$.

Note: OTUs, operational taxonomic units. Ace, Chao, Shannon and Simpson all were diversity index, the higher the value, the higher the diversity.

Table3-3 Analysis of bacterial diversity at Phylum level

Bacterial taxonomy	January	April	June	August	October
Spirochaetae	1.3±0.9^{b}	7.0±2.1^{a}	8.3±1.8^{a}	9.3±2.4^{a}	4.7±0.9^{ab}
Lentisphaerae	4.3±0.9	11.0±4.0	9.7±1.7	6.0±1.7	6.3±1.2
Cyanobacteria	9.7±2.6^{ab}	5.3±1.2^{b}	6.0±1.5^{b}	12.0±0.6^{a}	14.7±1.7^{a}
Candidate division TM7	13.3±1.2	13.7±0.7	12.0±1.0	14.7±0.3	12.7±1.3
Bacteria norank	16.3±2.9	16.7±3.0	15.3±0.9	23.3±2.9	21.0±1.5
Proteobacteria	26.0±2.5^{b}	25.3±0.9^{b}	19.0±2.0^{c}	33.7±2.6^{a}	26.3±0.3^{b}
Tenericutes	27.7±2.6	32.3±2.6	29.3±2.0	26.7±3.8	28.7±1.7
Bacteroidetes	84.0±13.0^{a}	115.3±17.2^{a}	152.0±0.6^{b}	109.0±6.0^{a}	95.3±7.4^{a}
Firmicutes	270.7±6.2^{ab}	289.0^{ab}	309.7±5.5^{ab}	329.0±22.1^{a}	260.3±22.8^{b}

The different letters (a, b, c) indicate significant differences in different months at $P < 0.05$.

Table3-4 Analysis of bacterial diversity at Familly level

Bacterial taxonomy	January	April	June	August	October
Defluviitaleaceae	5.0±0.6	6.0±1.2	6.0±0.6	6.0±1.2	4.3±0.9
Streptococcaceae	6.0±1.0^{ab}	5.7±0.3^{ab}	4.3±0.3^{b}	6.7±0.3^{a}	5.3±0.7^{ab}
Spirochaetaceae	1.3±0.9^{b}	7.0±2.1^{a}	8.3±1.8^{a}	9.3±2.4^{a}	4.7±0.9^{ab}
Moraxellaceae	5.7±0.3^{b}	6.3±0.3^{b}	6.0±0.6^{b}	7.7±0.3^{a}	5.7±0.3^{b}
Gastranaerophilales norank	7.7±2.6^{b}	4.7±0.8^{b}	5.0±1.5^{b}	10.0±0.6^{ab}	13.0±1.5^{a}
S24-7	6.0±1.2^{b}	6.7±0.3^{b}	12.3±1.5^{a}	13.7±0.3^{a}	8.0±1.5^{b}
BS11 gut group	7.7±0.3^{a}	10.7±2.6^{a}	18.3±0.3^{b}	9.3±0.9^{a}	7.3±0.3^{a}
Unknown Family	12.7±0.9	13.3±0.3	12.0±1.0	13.7±0.3	12.3±1.2
Coriobacteriaceae	12.7±2.7^{b}	14.3±2.6^{ab}	14.0±1.0^{ab}	20.7±2.4^{a}	18.3±1.2^{ab}
Family XIII	19.7±0.3	23.3±1.2	24.0±0.6	24.7±2.9	18.3±2.6
Erysipelotrichaceae	21.7±3.5^{b}	20.7±3.4^{b}	31.0±1.5^{a}	32.0±3.1^{a}	22.0±1.5^{b}
RF9 norank	26.7±3.0	30.7±2.3	27.7±1.8	25.7±4.4	26.3±1.5
Rikenellaceae	30.0±8.0^{a}	42.3±4.2^{ab}	45.7±1.8^{b}	37.7±1.2^{ab}	28.7±4.4^{a}
Prevotellaceae	22.0±7.1^{a}	36.0±7.5^{a}	61.0±2.0^{b}	37.0±6.6^{a}	36.0±2.1^{a}
Christensenellaceae	55.0±1.2	55.3±2.3	56.3±3.2	56.0±2.9	44.0±6.5
Lachnospiraceae	68.7±1.5^{b}	72.0±2.5^{b}	84.7±1.9^{a}	86.3±4.8^{a}	64.7±1.9^{b}
Ruminococcaceae	71.3±1.9^{b}	86.7±7.4^{ab}	86.0±6.0^{ab}	94.3±5.8^{a}	83.3±8.2^{ab}

The different letters (a, b, c) indicate significant differences in different months at $P < 0.05$.

CHAPTERⅣ The Effects of Molasses and Urea Supplement on the Weight Gain and Rumen Indexes in Sheep Grazing Winter Pasture of Inner Mongolia

4.1 Introduction

Sheep traditionally graze for six to seven months a year, from spring to winter in northern China. Crude protein (CP) content in grass decreases dramatically and digestibility of grass decreases, although crude fiber (CF) content increases significantly during this period (Li *et al*. 2015). Thus, the nutrition of grasses in winter is characterized by the year's lowest CP with high CF, low soluble nutrients and an imbalance of energy/nitrogen (Johnson *et al*. 2001). Previous study indicates that the sheep, especially for pregnant, cannot obtain sufficient energy from winter pasture only to meet their requirements for reducing weight loss and increasing lamb birth weight (Stateler *et al*. 1995), which is likely to become more serious as the general deterioration trend of grasslands increases. Moreover, it was demonstrated that development of a winter supplement for grazing sheep is key to achieving good performance for livestock farmers in northern China. However, feed shortage in China was serious, and the price of concentrates was high, thus the input of the herdsman has increased and the benefit has reduced. On the other hand, large amounts of molasses are produced as a by-product of sugar production in China, while its supply is expected to substantially increase in the near future, due to the expansion of the existing factories and a number of new plants currently under construction and in the planning phase.

Roughage, including pasture and crop residues, which forms the basis of livestock diet, is usually rather low in nutrient content. So supplementation is needed for grazing sheep, particularly it is important to supply with fermentable energy and protein sources in winter. Molasses-urea supplementation (MUS) has been used as a livestock feed supplement in comparable environments in a number of countries with reports of encouraging results (Plaizier *et al*. 1999, Martin *et al*, 1981, Seyoum and Fekede 2006). A positive response to dietary MUS can therefore be expected in productive and reproductive performance of livestock, as well as to the benefit-cost ratio for local and crossbred dairy cows in different dairy production systems. Nevertheless, data are lacking for the effects of MUS under grazing conditions in China, particularly in regard to Mongolian sheep. Therefore, the objective of this study is to evaluate the effect of MUS on the performance of grazing in natural grassland systems. The studying targets involved in weight gain, ruminal fermentation and ruminal microbial populations in grazing sheep in the Inner Mongolian winter.

4.2 Materials and methods

Experimental animals

Seventy male Inner Mongolian fine wool sheep (1 year old, 27.3±0.5 kg) of similar body weight were selected as experimental animals.

Experimental Design

The experimental site, Zhenglan Banner (E 116.02°, N 42.25°), is located in the south of Inner Mongolia, China, and is a typical steppe. Seventy sheep were selected as experimental animals and were random divided into two groups; 40 served as a treatment group and 30 served as a control group. The two study groups were mixed during pasture time (08:00 to 17:00) throughout the experimental period from 17th December to 24th February, free drinking before grazing and after grazing. All the animals of treatment group were fed MUS block after grazing and free consumption, and the control group were feed according to the traditional mode in the pastoral areas of China, won't feed any other feeding. The experiment included 2 periods; the first 10 days was the preliminary period for acclimation, the next 60 days was experimental period. The MUS blocks(weight 2 kg, 14×14×10 cm) used for this study were provided by Tianjin Zenoaq Animal Health Company (Tianjin, China), the composition of MUS blocks see the table4-1.

Sample collection and processing

At the beginning and end of the experiment, body weight for each experimental animal was measured before grazing. Six sheep from each group were random selected, from whom 50 ml of rumen fluid was collected orally in the morning during the last three days of the experimental period, immediately measured the pH using PHS-3C,then added 5 drops of concentrated sulfuric acid and stored at -80 ℃ in a refrigerator. The rumen fluid of each sheep for three consecutive days were been mixed, and weighed 15ml, 4000rmp 20 min. The mixture was centrifuged at 16 000 g for 20 min at 4 ℃ to separation of supernatant and the precipitation, bacterial crude protein (BCP) and ammonia nitrogen (NH_3-N), were measured using colorimetric and Kjeldahl determination methods (Cotta and Russell 1982, Broderick and Craig 1989). In addition, the populations of *Selenomonas ruminantium*, *Anaerovibrio lipolytica*, *Fibrobacter succinogenes*, *Ruminococcus flaveciens*, *Ruminococcus albus* were investigated using real-time PCR (Koike and Kobayshi 2001, Tajima *et al* 2001). Real-time PCR was performed using a standard SYBR PCR protocol on a Roch 480 System. The 10 μL of PCR mixture included 5.0 μL of SYBR premix EX Taq (Takara), 1.0 μL of DNA template, 0.5 μL of forward amplification primer(10 μmol/μL), 0.5 μL of reverse amplification primer (10 μmol/μL) and 3 μL of autoclaved distilled water. The reaction mixtures were incubated at 95℃ for 5 min, followed by 40 amplification cycles of 95℃for 30 s and 60℃for 30 s. The threshold cycle (CT) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold.

Protozoa counts

The number of protozoa was determined by the blood corpuscle count plate method (Wang *et al.* 2009).

Statistics

Statistical analyses were performed using SPSS19 (SPSS Inc., Ireland). Effects of control and treatment groups were analyzed by means of one-way analysis of variance (ANOVA). Significance levels ranged from 0.05 to 0.01.

4.3 Results

Weight gain

The average daily consumption of MUS was 43.2 g per sheep per day in the treatment group. The results showed that average daily gain in the treatment group (64.8 g) was significantly higher ($P < 0.05$) than that (30.9 g) in the control group (Table4-2).

Rumen microbial changes

At the end point of the experiment, data for populations of *S. ruminantium*, *A. lipolytica*, *F. succinogenes*, *R. flaveciens* and *R. albus* were collected, which are presented in Table4-3. The populations of major symbiotic microbes in the treatment group were significantly higher than those in the control group ($P < 0.05$). The populations of *S. ruminantium*, *R. albus* and *A. lipolytica* respectively increased up to 132.0%, 113.0% and 92.4%, while the populations of *F. succinogenes* and *R. flaveciens* respectively increased up to 45.7% and 36.7%.

Rumen fermentation, protozoa changes

The results showed that rumen fermentation and protozoa populations are different between the treatment and control groups (Table4-4). Fine-wool sheep rumen pH value was between 6.0-7.0, and no significant difference in pH value was found between the two groups. Ammonia nitrogen concentration in the treatment group (18.2 ± 0.4 mg/100 mL) was significantly higher ($P < 0.05$) than that in the control group (16.2 ± 0.5 mg/100 mL). Bacterial crude protein concentration in the treatment group was lower than that in the control group, but without significant difference ($P > 0.05$). Protozoa numbers (given as figures $\times 10^4$) in the treatment group (35.9 ± 3.4) was significantly higher ($P < 0.05$) than that in

the control group (26.3 ± 4.3).

4.4 Discussion

Comprehensive analysis, conducted during the grass withering period, indicates that grazing sheep supplied with MUS significantly improved daily gain, which results are consistent with other researcher's findings (Wan *et al*. 2009). Improved results are due to the soluble sugar, nitrogen and minerals for microbial rumen that MUS blocks provide, all of which change the rumen environment. By improving the density of rumen bacteria and protozoa, especially *R. flavefaciens* and *R. albus* (Kostenbauder *et al*. 2007, Rumsey *et al*. 1971), ruminant digestion of CF and bacterial protein synthesis improve and provide more nutrients for the body.

First, pH level is an important indicator of rumen fermentative status, so it is important to maintain the pH within a normal range to ensure normal fermentation in the rumen. Chase (1988), Piwonka and Firkins (1996) reported that molasses may increase the digestibility of dry matter by reducing rumen pH and increasing the hydrolysis of cellulose in circumstances that do not improve feed intake. In this study, pH decline after supplement, but it is not significant between two groups; the value is still in the normal level of 6-7. It indicated that MUS supply easily fermented molasses for the major function bacteria in the rumen, which more suitable for the growth of rumen microorganisms, the major function bacteria proliferated and digested a large amount of crude fiber, produced large amounts of organic acid, and then pH slightly drops.

Wang (2009) reported that ewe rumen NH_3-N concentration averages only 4.2-7.7 mg/100 mL in the winter and spring. In our test sheep rumen, NH_3-N concentration of 18.2 mg/100 mL was measured after supplementing with MUS, which lies within the concentration range of 15- 25 mg/l00 mL required for rumen microbial fermentation. Therefore, MUS is improve rumen environment, and beneficial to rumen microorganisms and bacterial protein synthesis. Soder(2010)also found that MUS contains significant amounts of urea and molasses provides energy for animals, remains rumen stable and avoids urea toxicity; thus, the MUS is beneficial to livestock. In addition, the urea contented in MUS can increase fodder intake by ruminants (Emmanuel *et al*. 2016), which is an important cause that MUS can improve the ruminant production performance.

The *F. succinogenes* numbers in grazing sheep rumen are similar with that of feedlot sheep; though *R. flavefaciens* and *R. albus* numbers were lower (Liu *et al*. 2008, Li *et al*. 2011). Confirming that MUS increases rumen microbial count, the numbers of *R. flavefaciens* and *R. albus* increase after supplying MUS, which increases bacterial population, and may improve fiber digestion via increased NH_3-N in the rumen. As a result, the measured higher daily gain may be achieved in the treatment group through higher DM digestibility. Similar results are often found with other energy-dense supplements (Leupp *et al*. 2005).

Under MUS, fine-wool sheep rumen bacterial protein content did not increase, this results opposite with the increase of major bacterial populations. This result was strange. But the number of rumen protozoa significantly increased, possibly because protozoa predated heavily on other rumen microorganisms, resulting no significant net change in bacterial protein content (Newbold and Hillman 1990). In further, we need to consider the total bacteria number and microorganism crude protein (MCP) for a further study on this subject.

4.5 Conclusion

After supplying MUS blocks to sheep, an obvious weight gain trend was found, which was due to provision of fermentable carbohydrates and non-protein nitrogen for bacteria, maintained stable pH of rumen, and enriched ammonia. Conditions produced by MUS were beneficial for major rumen microbial growth and reproduction, promote the increase of major rumen microbes, improve rumen environment, enhance rumen digestion, and improve feed digestibility, thereby enhancing the production ruminants on poor-quality feed.

Table4-1 The composition of MUS blocks (each 1 kg)

Component	Molasses	Urea	$Fe_2(SO_4)_3$	$CoSO_4$	$CuSO_4$	$ZnSO_4$	$MnSO_4$	$Ca(IO_3)_2$	corncob powder
Content (g)	500	100	1.7	6.1×10^{-3}	0.8	4.5	2.26	19.4×10^{-3}	Up to 1000

Table4-2 Weight gain of grazing sheep supplied with molasses-urea supplementation (MUS) in winter in north China

Item	Control group (mean ± SE)	Treatment group (mean ± SE)
Number	30	40
Days	60	60
Daily consumption，g	0	43.2
Initial weight, kg	27.3 ± 0.5	27.4 ± 0.7
Final weight, kg	29.2 ± 0.6[b]	31.4 ± 0.8[a]
Weight gain, kg	1.9 ± 0.5[b]	3.9 ± 0.4[a]
Average daily gain, g	30.9 ± 8.9[b]	64.8 ± 5.2[a]

[a,b] Means in a row with different superscripts significantly differ (P< 0.05).

Note:Control group: the grazing without supplied MUS; Treatment group: supply with MUS block after the grazing.

Table4-3 Effects of molasses-urea supplementation (MUS) on rumen bacteria copies ($\times 10^8$) of sheep in winter in north China

Item	Control group (mean ± SE)	Treatment group (mean ± SE)
Selenomonas ruminantium	2.1 ± 0.8^b	4.9 ± 0.1^a
Anaerovibrio lipolytica	2.4 ± 0.0^b	4.6 ± 2.7^a
Fibrobacter succinogenes	2.3 ± 0.7^b	4.0 ± 1.3^a
Ruminococcus flavefaciens	3.8 ± 0.6^b	5.6 ± 2.3^a
Ruminococcus albus	0.5 ± 0.1^b	1.0 ± 0.2^a

[a,b] Means in a row with different superscripts significantly differ ($P < 0.05$).

Note:Control group: the grazing without supplied MUS; Treatment group: supply with MUS block after the grazing.

Table4-4 Effects of molasses-urea supplementation (MUS) on rumen fermentation, protozoa after sheep grazing in winter in north China

Item	Control group (mean ± SE)	Treatment group (mean ± SE)
Protozoa ($\times 10^4$)	26.3 ± 4.3^b	35.9 ± 3.4^a
pH	6.8 ± 0.1	6.9 ± 0.2
BCP, mg/100 mL	16.1 ± 1.5	16.0 ± 1.0
NH_3-N, mg/100 mL	16.2 ± 0.3^b	18.2 ± 0.4^a

BCP = bacterial crude protein; NH_3-N= ammonia nitrogen.

^{a,b} Means in a row with different superscripts significantly differ ($P < 0.05$).

Note: Control group: the grazing without supplied MUS; Treatment group: supply with MUS block after the grazing.

CHAPTER Ⅴ The Mechanism of Molasses and Urea Supplement Can Improve the Production Performance of Grazing Sheep on Winter Grassland of Inner Mongolia

5.1 The effects of molasses and urea supplement on the digestibility and rumen microflora in sheep grazing hay

5.1.1 Introduction

Sheep is traditionally grazed for 6–7 months from spring to winter in north China. Plant was evaluated to have the lowest nutritional value in winter, with high crude fiber (CF), low soluble nutrients, and unbalanced energy/nitrogen (Johnson *et al*. 2001). Crude protein (CP) content in grass decreased markedly, digestibility of grass decreased by 50% in winter. However, CF content increased significantly during this period (Fan and Jin 2009). Sheep were found unable to rely solely on pasture for sufficient energy to meet the requirements for good performance in winter (Moore and Kunkle 1995). The development of a supplement for grazing sheep in winter could be a key to achieving good performance among livestock farmers in north China. Thus, the present study was conducted to determine the effect of MUS on the weight gain, ruminal fermentation, and microbial populations of grazing sheep in winter in Inner Mongolia.

5.1.2 Materials and methods

Experimental animals and management

The experimental animals consisted of 8 Mongolian sheep of the same species, weight, and age and good physical condition. Equipped with permanent rumen fistula, the sheep were randomly divided into 2 groups, with 4 sheep in each group. The control group was fed hay, whereas the experimental group was fed hay with molasses–urea. The experimental sheep were caged separately, with regular access to feeding and drinking ad libitum daily. The experimental daily diet (0.8 kg in total) was supplied in the morning and evening. In addition, the experimental sheep were fed with 60 g of molasses–urea particles daily. The pre-feeding period was 15 d, whereas the normal feeding period was 7 d.

Collection of samples and treatment

During the experimental period, fecal samples at 20% each time were regularly collected for 7 consecutive days. All samples that were collected over 7 d were mixed; 500 g of the samples was heated at -20 ℃ until use. Rumen fluid (50 mL) was collected *via* the mouth of the sheep, using the stomach tube before morning feeding before and on the last day of the experimental period. The fluid was filtered using 4 layers of gauze and then preserved at -80 ℃ for the purpose of

extracting DNA to determine the microbial flora.

Analysis of microbial diversity in rumen

DNA extraction, PCR amplification, 16S rDNA sequencing, and statistical analyses were conducted as described in chapter 3.2, the information of primers used to microbe composition sequencing analysis showed in Table5-1-1.

5.1.3 Results

Effect of MUS on the digestibility of hay

The digestibility of DM with MUS was significantly increased by 7% ($P<0.01$) (Table5-1-2). In addition, the digestibility of CP was significantly increased ($P<0.05$) by 3.45% on average, whereas the difference in digestibility of EE was not extremely significant ($P>0.05$). The digestibility of NDF in the experimental group was 3.2% higher than that in the control group, whereas the digestibility of ADF in the experimental group was 0.14% higher than that in the control group. A significant difference was indicated in the digestibility of NDF between the experimental group and the control group ($P<0.05$), whereas no significant difference in ADF was determined ($P> 0.05$).

Diversity analysis

After quality control of high-throughput sequencing data, the sequence number of bacteria, number of OTU, and species diversity index were determined, as shown in Table5-1-3. After sequencing of bacteria in the rumen of sheep, the following were found: sequence number, 33,747; average number of OTUs, 4085; and coverage rate of sequencing, above 0.88. The average Shannon bacterial diversity index was 7.6, and the average Chao diversity index was 2344; significant differences in bacterial indexes were found among the groups ($P>0.05$).

Effect of molasses–urea on bacterial flora

The effects of MUS on the composition of bacteria in the rumen at the phylum level were presented in Fig.5-1-1. A total of 22 phyla were found in sheep rumen bacteria, mainly consisting of bacteria in the *Bacteroidetes* and *Firmicutes* families, comprising 90.7% of all bacteria. The effects of MUS on the composition of bacteria in the rumen at the family level are shown in Figure5-1- 2. A total of 123 families, mainly consisting of unclassified *Bacteroidetes*, were identified as main bacterial species, comprising 31% of all bacteria; this number was followed by unclassified *Armatimonadetes* and *Prevotellaceae*, comprising 5.1% and 3.7% of all bacteria, respectively.

Table5-1-4 showed that the proportion of *Firmicutes* in the rumen supplemented with MUS significantly decreased ($P<0.05$) at the phylum level. The proportions of *Proteobacteria* were significantly increased, whereas the proportions of other bacteria that comprised 1% of all bacteria were not affected ($P>0.05$). At the family level, the proportion of *Bacteroidales* in the rumen significantly increased after supplying with MUS ($P<0.05$), whereas the proportions of *Porphyromonadaceae* and *Prevotellaceae* were decreased, and the proportions of other bacteria comprising over 1% of all bacteria were not affected ($P>0.05$).

5.1.4 Discussion

With the development of the feed industry and the aquaculture industry, demand for various types of feed resources has increased. The lack of feeds and the serious shortage of protein feed resources seriously influence the development of animal husbandry. These inadequacies prompt the use of a large number of non-protein nitrogen feed products in ruminants to save protein feed resources, reduce feed costs, and enhance the productivity of animals. MUS can increase the digestibility of OM, DM, CP, and CF among animals, owing to its comprehensive nutrition, palatability, low cost, convenience, long-term storage, and absence of deliquescence. These properties significantly improve the conversion rate of feeds and exert beneficial effects to replenish the lack of nutrients or imbalance in nutritional intake in ruminants, such as sheep, dairy goats, and deer.

The digestibility of DM was higher in the experimental group supplement withn MUS, which was similar to the experimental results by Adamu in 1988. Adamu added different levels of urea to the daily feeds, and the results indicated that with an increase in urea, the digestibility of DM in the whole digestive tract also improved. Wu (2005) also reported that the digestibility of DM was significantly higher in rumen after sheep supplementation with MUS than in the control group. The digestibility of CP supplemented with MUS was significantly increased by 3.2% on average. The experimental studies by Milton (1995) and Zinn (2003) demonstrated that the digestibility of CP in rumen was increased when urea added to the daily diets was increased. In 1997, the study by Milton also showed that compared with the basal diet, the diet supplemented with urea of up to 0.5% linearly increased the digestibility of CP in the rumen as the amount of urea was increased. The study by Milton (1997) indicated that compared with the basal diet, when urea comprised 0.5% of DM in the diet, the true digestibility of CP in the rumen increased with increasing urea. The reason for such a phenomenon was that the addition of urea provided nitrogen sources for microorganisms, thus increasing the microbial proteins in the rumen.

MUS can significantly increase the digestibility of fibers. The experiment by Wan (2009) showed that supplement with 100 g MUS, the digestibility of DM was

increased from 42.7% to 44.2%. Ayasuriya (1983) and Abidin (1987) separately reported that straws treated with urea could significantly increase the digestibility of fibers. The results of this experiment were consistent with the above mentioned findings. Urea produces ammonia after decomposition by urease in rumen, which promotes the growth and reproduction of microorganisms (including cellulolytic bacteria) in the rumen, improves the ecological environment of the rumen, and effectively softens hay and straw feeds. These processes increase the intake of fiber feeds, enhancing their digestibility and improving the intake of nutrition. William (1988) sprayed straws with molasses–urea, which significantly increased the digestibility of OM, cellulose, hemicellulose, and nitrogen. However, straws treated using urea exhibited higher *in vivo* digestibility than *in vitro* digestibility. This difference could be attributed to the effective use of ammonia by the microorganisms in the rumen.

Effect of MUS on bacterial composition in sheep rumen

Bacteria comprise about 50% to 80% of the biomass in the rumen. Studies have shown the abundant presence of bacterial populations in the rumen of ruminants. The microbial floras were similar to those in the digestive tract of other ruminants, with *Firmicutes* and *Bacteroides* being the main two categories, comprising about 90% of the microorganisms. Other categories comprised small proportions, including *Candidatus Saccharibacteria*, *Proteobacteria*, *Tenericutes*, and *Verrucomicrobia* (Kittelmann 2013). High-throughput sequencing was employed in the current exreriment, and the results showed the presence of many bacterial species in the sheep rumen, and 123 families of bacteria were found. Shannon's diversity index at the species level was 7.61 on average, and the MUS significantly affected the bacterial diversity in the rumen. The main bacteria in sheep rumen were *Bacteroides* and *Firmicutes* at the phylum level, *Bacteroidates*, unclassified *Armatimonadetes* and *Prevotellaceae* at the family level. The results of this study were similar to those of other studies on the bacterial floras in sheep rumen. On the whole, the composition of bacterial floras was similar to that in other ruminants; it also manifested the characteristics of bacterial floras dominated by typical fiber digestion (Singh 2012). Existing studies have shown that the addition of exogenous molasses–urea could further promote microbial growth and protein synthesis in the rumen (Ottou 1996). Meanwhile, this study also indicated that molasses–urea could increase the digestibility of DM, CP and fibers in sheep. Currently, few studies have been conducted on the impact of molasses–urea on bacterial composition in the rumen. In the current study, the MUS reduced the proportion of Gram-positive bacteria in *Firmicutes* in sheep rumen and increased the proportion of *Proteobacteria*, and the proportion of bacteria was significantly changed in *Bacteroidales* and unclassified *Clostridiales* closely associated with fiber digestion in the rumen. MUS clearly affected the composition of bacterial floras in the rumen to a certain extent. Therefore, the association of increased

digestibility of hay nutrients in this study is associated with changes in bacterial floras in the rumen is worthy of further studies.

5.1.5 Conclusion

The digestibility of DM, NDF, and ADF was significantly increased in sheep supplement with MUS, indicating that MUS helps sheep improve the digestibility of CF. The ruminal microbial flora results suggest that the main bacteria in the sheep rumen at the phylum level were *Bacteroides* and *Firmicutes*, whereas those at the family were *Bacteroidales*, *Ruminococcaceae*, and unclassified *Clostridiales*. Sumarry, the composition of bacterial floras in the sheep rumen was similar to that in other ruminants, such as cows, and manifested the characteristics of bacterial flora dominated by typical fiber digestion. MUS reduced the proportion of *Firmicutes* in the rumen and increased the proportion of *Bacteroidales*. These results could lead to a change in rumen microflora and improve the function of rumen digestion.

Taxonomy	Total count	Total %	BC1 %	BC2 %	BC3 %	BT1 %	BT2 %	BT3 %	BT4 %	FC1 %	FC2 %	FT1 %	FT2 %	FT3 %
Archaea;p__Euryarchaeota	49	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Bacteria;p__Actinobacteria	2462	0.6%	0.3%	0.9%	0.2%	0.6%	0.7%	0.6%	0.7%	0.3%	0.6%	1.4%	0.5%	0.4%
Bacteria;p__Armatimonadetes	8	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Bacteria;p__Bacteroidetes	167383	41.3%	53.9%	25.3%	48.0%	39.9%	52.3%	43.4%	40.5%	27.7%	37.7%	30.6%	48.8%	45.1%
Bacteria;p__Candidatus Saccharibacteria	7524	1.9%	0.9%	1.6%	1.1%	1.5%	1.3%	2.9%	2.9%	1.8%	1.9%	2.1%	0.6%	3.3%
Bacteria;p__Chlorobi	28	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Bacteria;p__Chloroflexi	1811	0.4%	0.3%	0.4%	0.1%	1.2%	0.4%	1.3%	0.4%	0.4%	0.2%	0.3%	0.2%	0.1%
Bacteria;p__Cyanobacteria	3200	0.8%	1.5%	1.2%	0.3%	2.5%	0.8%	1.1%	1.6%	0.1%	0.1%	0.1%	0.0%	0.1%
Bacteria;p__Cyanobacteria/Chloroplast	641	0.2%	0.1%	0.2%	0.0%	0.0%	0.0%	0.1%	0.1%	0.2%	0.2%	0.4%	0.3%	0.4%
Bacteria;p__Elusimicrobia	341	0.1%	0.0%	0.0%	0.0%	0.1%	0.1%	0.1%	0.3%	0.0%	0.1%	0.0%	0.1%	0.0%
Bacteria;p__Fibrobacteres	49	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Bacteria;p__Firmicutes	200171	49.4%	37.9%	61.3%	45.8%	44.9%	38.9%	41.8%	45.2%	66.2%	55.4%	63.7%	47.3%	48.0%
Bacteria;p__LD1	29	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Bacteria;p__Lentisphaerae	2190	0.5%	1.1%	1.8%	0.4%	0.9%	0.7%	0.8%	0.7%	0.1%	0.1%	0.1%	0.1%	0.0%
Bacteria;p__Planctomycetes	227	0.1%	0.0%	0.1%	0.0%	0.1%	0.0%	0.2%	0.0%	0.1%	0.0%	0.0%	0.1%	0.0%
Bacteria;p__Proteobacteria	5725	1.4%	1.7%	1.6%	0.4%	2.9%	1.4%	2.5%	3.4%	0.2%	1.7%	0.3%	0.6%	0.2%
Bacteria;p__Spirochaetes	2061	0.5%	0.3%	0.3%	0.9%	0.5%	0.8%	0.9%	0.8%	0.1%	0.5%	0.2%	0.3%	0.5%
Bacteria;p__Synergistetes	873	0.2%	0.1%	0.2%	0.0%	1.0%	0.3%	0.4%	0.2%	0.0%	0.0%	0.0%	0.1%	0.1%
Bacteria;p__Tenericutes	6149	1.5%	0.8%	3.0%	2.1%	1.0%	1.2%	2.1%	1.2%	2.8%	1.3%	0.8%	0.8%	1.5%
Bacteria;p__Verrucomicrobia	3886	1.0%	0.8%	2.1%	0.6%	2.8%	0.9%	1.6%	1.9%	0.1%	0.1%	0.1%	0.2%	0.2%
Bacteria;p__WPS-2	5	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Bacteria;p__unclassified	150	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.2%	0.1%	0.0%	0.0%	0.0%	0.0%	0.0%

Fig.5-1- 1 Effects of MUS on ruminal bacteria composition at phyla level of grazing sheep

Taxonomy	Total		BC1	BC2	BC3	BT1	BT2	BT3	BT4	FC1	FC2	FT1	FT2	FT3
	count	%	%	%	%	%	%	%	%	%	%	%	%	%
_Archaea;p__Euryarchaeota;c__Methanobacteria;o__Methanobacteriales;f__Methanobacteriaceae	49	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
_Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Actinomycetaceae	6	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
_Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Brevibacteriaceae	5	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
_Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Corynebacteriaceae	300	0.1%	0.0%	0.1%	0.0%	0.0%	0.1%	0.1%	0.1%	0.0%	0.3%	0.0%	0.1%	0.0%
_Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Dietziaceae	27	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
_Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Microbacteriaceae	27	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
_Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Micrococcaceae	42	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
_Bacteria;p__Actinobacteria;c__Actinobacteria;o__Bifidobacteriales;f__Bifidobacteriaceae	283	0.1%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.6%	0.1%	0.0%	
_Bacteria;p__Actinobacteria;c__Actinobacteria;o__Coriobacteriales;f__Coriobacteriaceae	1754	0.4%	0.3%	0.8%	0.1%	0.5%	0.6%	0.5%	0.5%	0.3%	0.2%	0.8%	0.3%	0.4%
_Bacteria;p__Actinobacteria;c__Actinobacteria;o__unclassified;f__unclassified	12	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
_Bacteria;p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae	6	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
_Bacteria;p__Armatimonadetes;c__SJA-176;o__RB046;f__unclassified	8	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
_Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__BS11	20580	5.1%	0.4%	0.9%	3.2%	0.3%	7.4%	17.2%	7.5%	0.5%	2.8%	4.7%	4.5%	10.1%
_Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae	99	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.1%	0.1%	0.0%
_Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__ML635J-40	22	0.0%	0.0%	0.0%	0.0%	0.1%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
_Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Paraprevotellaceae	757	0.2%	0.0%	0.1%	0.2%	0.2%	0.5%	0.3%	0.3%	0.1%	0.0%	0.1%	0.1%	0.1%
_Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae	4975	1.2%	0.2%	0.4%	2.2%	0.4%	0.6%	0.3%	0.6%	2.0%	1.5%	1.5%	3.2%	2.0%
_Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	14926	3.7%	0.7%	0.5%	8.3%	2.3%	4.3%	1.6%	1.5%	1.1%	2.7%	7.0%	8.8%	3.9%
_Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__RF16	129	0.0%	0.0%	0.0%	0.0%	0.1%	0.0%	0.0%	0.1%	0.0%	0.0%	0.0%	0.0%	0.0%
_Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__S24-7	253	0.1%	0.0%	0.0%	0.5%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.1%	0.0%	0.0%
_Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__SB-1	39	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
_Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__p-2534-18B5	5	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
_Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__unclassified	125451	31.0%	52.5%	23.3%	33.4%	36.5%	39.4%	24.0%	30.4%	23.9%	30.5%	17.0%	32.0%	28.9%
_Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae	87	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.2%	0.0%	0.0%	0.0%	0.0%
_Bacteria;p__Bacteroidetes;c__Sphingobacteriia;o__Sphingobacteriales;f__Sphingobacteriaceae	28	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
_Bacteria;p__Bacteroidetes;c__Sphingobacteriia;o__Sphingobacteriales;f__unclassified	7	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
_Bacteria;p__Bacteroidetes;c__unclassified;o__unclassified;f__unclassified	25	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
_Bacteria;p__Candidatus Saccharibacteria;c__Saccharibacteria_genera_incertae_sedis;o__Saccharibacteria_genera_incertae_sedis;f__Saccharibacteria_genera_incertae_sedis	7524	1.9%	0.9%	1.6%	1.1%	1.5%	1.3%	2.9%	2.9%	1.8%	1.9%	2.1%	0.6%	3.3%
_Bacteria;p__Chlorobi;c__OPB56;o__unclassified;f__unclassified	28	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
_Bacteria;p__Chloroflexi;c__Anaerolineae;o__Anaerolineales;f__Anaerolinaceae	1014	0.3%	0.2%	0.2%	0.0%	0.7%	0.2%	0.9%	0.3%	0.3%	0.1%	0.0%	0.0%	0.0%
_Bacteria;p__Chloroflexi;c__Anaerolineae;o__Anaerolineales;f__Anaerolineaceae	797	0.2%	0.1%	0.2%	0.1%	0.5%	0.2%	0.4%	0.1%	0.1%	0.0%	0.3%	0.2%	0.1%
_Bacteria;p__Cyanobacteria;c__4C0d-2;o__YS2;f__unclassified	3200	0.8%	1.5%	1.2%	0.3%	2.5%	0.8%	1.1%	1.6%	0.1%	0.1%	0.1%	0.0%	0.1%
_Bacteria;p__Cyanobacteria/Chloroplast;c__Chloroplast;o__Chloroplast;f__Chloroplast	641	0.2%	0.1%	0.2%	0.0%	0.0%	0.0%	0.1%	0.1%	0.2%	0.2%	0.4%	0.3%	0.4%
_Bacteria;p__Elusimicrobia;c__Elusimicrobia;o__Elusimicrobiales;f__Elusimicrobiaceae	199	0.0%	0.0%	0.0%	0.0%	0.0%	0.1%	0.1%	0.2%	0.0%	0.0%	0.0%	0.0%	0.0%
_Bacteria;p__Elusimicrobia;c__Endomicrobia;o__Candidatus Endomicrobium;f__Candidatus Endomicrobium	142	0.0%	0.0%	0.0%	0.0%	0.1%	0.0%	0.0%	0.0%	0.0%	0.1%	0.0%	0.1%	0.0%
_Bacteria;p__Fibrobacteres;c__Fibrobacteria;o__Fibrobacterales;f__Fibrobacteraceae	49	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
_Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae 1	1579	0.4%	0.3%	1.2%	0.1%	0.3%	0.2%	0.4%	0.4%	0.5%	0.3%	0.2%	0.3%	0.5%
_Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae 2	18	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
_Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Paenibacillaceae 1	214	0.1%	0.1%	0.2%	0.0%	0.1%	0.0%	0.0%	0.0%	0.1%	0.0%	0.0%	0.0%	0.1%
_Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Planococcaceae	82	0.0%	0.0%	0.0%	0.0%	0.2%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
_Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Staphylococcaceae	23	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
_Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Enterococcaceae	142	0.0%	0.0%	0.1%	0.0%	0.0%	0.0%	0.0%	0.0%	0.1%	0.1%	0.0%	0.0%	0.0%
_Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae	15	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
_Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae	56	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
_Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Christensenellaceae	10018	2.5%	4.4%	5.7%	3.0%	1.6%	2.0%	2.9%	1.9%	1.3%	2.0%	3.5%	0.8%	1.1%

Fig.5-1- 2 Effects of MUS on ruminal bacteria composition at family level of grazing sheep

Table5-1-1 Information of primers used to microbe composition sequencing analysis

Prime	Name	Sequence
Forward Primer	319F	5'-ACTCCTACGGGAGGCAGCAG-3'
Reverse Primer	806R	5'-GGACTACHVGGGTWTCTAAT-3'

Table5-1-2 Nutrient digestibility of sheep

Group	DM（%）	CP（%）	EE（%）	NDF（%）	ADF（%）	ADL（%）
Treatment group	49.0±7.4**	46.2±12.8*	72.4±5.4	53.3±4.2*	44.0±4.8*	16.8±5.8
Control group	41.0±6.7	42.8±10.0	70.7±5.4	46.8±1.3	38.9±9.7	16.8±5.40

The star letters (*) indicate significant differences of correlation in a culumn at $P < 0.05$, the doble star letters (**) indicate significant differences of correlation in a culumn at $P < 0.01$.

Table5-1-3 Quality control of high-flux sequencing and diversity indexes of bacteria

Items	Test group	Control group
Observed species	1972*	1631
Shannon	8.0*	7.5
Simpson	0.97	0.97
Chao1	2614*	2138

The star letters (*) indicate significant differences of correlation in a row at $P < 0.05$.

Table5-1-4 Effects of molasses urea on ruminal bacteria composition (the percentage of bacteria in total bacteria＞1%) at phyla and family levels of sheep

Taxonomy levels	Microbes	Control%	Test%
Phyla	*Bacteroidetes*	42.4	44.0
	Candidatus saccharibacteria	1.20	2.2
	Firmicutes	48.3	42.7*
	Proteobacteria	1.23	2.6 *
	Tenericutes	2.0	1.4
	Verrucomicrobia	1.2	1.8
Family	*Bacteroidales; BS11*	1.5	8.1 *
	Porphyromonadaceae	0.9	0.5*
	Prevotellaceae	3.2	2.4*
	Bacteroidales; unclassified	36.4	32.6
	Saccharibacteria_genera_incertae_sedis	1.2	2.2
	Christensenellaceae	4.4	2.1
	Lachnospiraceae	8.6	6.9
	Ruminococcaceae	17.4	18.3
	Clostridiales;unclassified	13.3	12.4

The star letters (*) indicate significant differences of correlation in a row at $P < 0.05$.

5.2 The dynamic changes of main rumen microbs and ruminal fermentation in sheep supplemented with molasses-urea

5.2.1 Introduction

Sheep digest and utilize nutrients through the effects of microbes in their rumen. The reproduction and growth rate of microbes are mainly determined by the available nutrient and energy levels in the rumen. Therefore, the rumen's environment directly affects the microbial crude protein (MCP) synthesis (Deng *et al.* 2008). Sucrose and monosaccharides in molasses are the most easily absorbed by animals. Their active ingredients are able to improve the productive performance, reproductively and immunity of sheep (Meng *et al.* 1995). Adding urea into feedstuff is considered an effective way of supplementing proteins in the diets of ruminants (Wan *et al.* 2009). Emmanuel (2015) reported that the urea contented in MUS supplement (MUS) could increase fodder intake by ruminants, so we can optimize the growth and nutrition metabolism of microbes in the rumen of ruminant animal's supplements with molasses and urea. The nutritive value and yield of pasture is low in winter and spring of the China's natural grassland (Li *et al.* 2015), MUS would promote maximum utilization ratio of roughage and reduce the cost of buying hay. MUS have the effect of improving rumen fermentation, which in turn improves productive performance and has significant economic effects (Zhang *et al.* 2013; Wang *et al.* 2010; Kostenbauder *et al.* 2007; Zhang 1998). However, it needs to be further study on how MUS promotes rumen fermentation, through supplementation of MUS in feedstuff. In this experiment, quantitative RT-PCR was used to detect the main representative bacteria we discovered, which includes *Rumiaococcus albus* (*R.albus*), *Rumiaococcus flavefaciens* (*R.flavefaciens*), *Fibtobacter succinogene* (*F.succinogene*), *Anaerovibrio lipolytica* (*A.lipolytica*) and *Selenomonas ruminantium* (*S. ruminantium*) in the rumen of sheep. Meanwhile, the rumen fermentation of sheep was studied by determining the protozoa papulation, and pH, NH_3-N, MCP values. The purpose of this was to research the dynamic changes in the main rumen microflora and ruminal fermentation in sheep that had diets supplemented with MUS. The results of this study can provide a scientific basis for improving the utilization of low quality roughage and promoting MUS product.

5.2.2 Materials and methods

Experimental animals and design

Eight sheep were used in this experiment and each came from a first filial generation between Ujumqin and Dorper sheep (called Ujumqin×Dorper F1)

in October 2014. The weights of the chosen sheep were 40±1kg. They were equipped with permanent rumen fistula and divided in two groups randomly with 4 sheep in each group. The control group was fed hay, while the experimental group was fed hay with MUS added to it. The first 10 days served as the adaptation stage, of which the sheep adapted to the hay and the MUS feed, then the test period began which lasted 5 days.

Management for experimental sheep

The experimental sheep were fed in an isolated house. Each sheep was fed 1.2 kg of hay for one time per day at 9 am. The MUS (50% molasses and 10% urea) were added to the experimental group's feed, and consisted of 60g per day. The sheep could drink water freely during this period. The feedstuff was composed of concentrates and hay with a ratio of 30:70. This was compressed into particles with its concrete compositions and nutrients displayed in Table 1. The MUS was purchased from the Nippon Zenyaku Kogyo Co., Ltd in Japan.

Collection and processing of the rumen liquid sample

After a 10 days adaptation period, the rumen liquids were collected, before feeding, through the rumen fistula at 9 am, and then rumen liquids were collected every 2 h and 4 h in succession, respectively (that is 0, 2, 4, 6, 8, 12 h after feeding). The pH levels of rumen liquids collected were measured immediately. Some of the rumen liquid was filtered using four layers of gauze. Then, the centrifugation was conducted at 4000 r/min for 15 min to remove the protozoa and large particle of feed. The supernatants of 0.5 mL were put into the sampling tubes with added 0.2 mol/L of HCl, respectively, and then shook uniformly to prepare for the measurement of NH_3-N. The rest of the supernatants were immediately stored in a refrigerator at -20°C to detect the MCP. The others rumen liquids were stored at -80°C to measure the protozoa and the bacteria contents in the rumens.

The measurement of rumen bacteria

The TIANamp Stool DNA Kit, developed by TIANGEN biotech Beijing Co., Ltd., was used to extract the total DNA in the rumen liquids. Afterwards, the DNA product purification Kit, by TIANGEN, was applied to purify the DNA we extracted. Primer design and experimental methods of *R. albus*, *R. flavefaciens* and *F. succinogene* were designed based on the method by Koike and Kobayshi (2001); while *A. lipolytica* and *S. ruminantium* were designed based on the method proposed by Tajima *et al.* (2001). The primer was synthesized by Takara biotechnology (Dalian) Co., Ltd. and the primer sequences are shown in Table 2.

Measuring method of fermentation indexes

The pH of the rumen liquids samples was measured using PHS-3B meter. NH_3-N was determined using the method by Wang (2011); MCP was measured by referring the methods proposed by Cotta (1982) and Broderick (1989); and a differential centrifugation method was used to isolate MCP.

Protozoa quantity

To detect the quantities of protozoa in the rumen liquids, 10 ml samples were required. They were dyed using MFS solutions, including NaCl of 8 g, Methyl green of 0.6 g and Formalin of 100 ml from volume of 1000 ml, respectively. A hemocytometer was used to count the protozoa quantity, specifically by using a microscope (Olympus CKX41, 100×). The calculating formula is:

$$\text{Protozoa quantity/ml} = N/4 \times D \times 16 \times 10 \times 1000 = N \times D \times 4 \times 10^4$$

Where:
N is the total protozoa quantity;
D is the dilution multiple.

Statistical analysis

Statistical analyses were performed using SPSS19 (SPSS Inc., Ireland). Effects of control and treatment groups were tested by means of one-way analysis of variance (ANOVA). Significance levels ranged from 0.05 to 0.01.

5.2.3 Results and analysis

Influence of MUS on the rumen bacteria of sheep

The rumen bacteria of sheep in the experimental group increased significantly ($P<0.5$) after supplying MUS, as shown in Table 3. This indicates that the dynamic change law of bacteria quantity was consistent in all the sheep, as shown in Fig.1. The quantity of bacterium each group reduced gradually after feeding, reached the minimum quantity 2h after intake. Then it rose step by step and achieved maximum quantity at 8h after intake, and it returned to the normal level that was detected before intake.

The *R. albus* and *A. lipolytica* in the experimental group, at each time period, were remarkably higher than that detected in the control group ($P<0.05$), other bacteria only showed a significant difference at 4-8 h.

Effects of the MUS on the rumen protozoa of sheep

The protozoa quantity in the sheep rose gradually after each intake of the MUS, rose to maximum levels after 4 h, and then decreased with insignificant change (Fig. 2). These results show the average quantity of protozoa in the experimental group is 22.6×10^4/ml, which was 18.8% higher than that of control group ($P<0.05$), as shown in Table 3.

The effects of the MUS on pH in rumen

Result showed that the pH levels of rumen liquid for the sheep in experimental group were in the range of 5.86-6.83. Although pH levels in experimental group were significantly lower than that of control group ($P<0.05$), they remained in the normal physiological range. As shown in Fig 2, the pH level was recorded at its highest level before intake and then was seen to decrease gradually after intake for 2~4. It then reached its lowest levels at 4 h before rising again at 8 h. Consequently, these results show the pH levels returned it to the level before intake.

Influence of the MUS on NH_3-N in rumen of sheep

Through the intake of the MUS, the change trends of the NH_3-N concentration for the rumens were the same in both groups. After intake since 0h, NH_3-N begun to increase, and was at its maximum levels for 2h. It then decreased gradually until reaching the level before intake, as shown in Fig 2. The mean NH_3-N value in the experimental group was higher than that of control group, showing a remarkable difference ($P<0.01$) (Table 3).

Effects of the MUS on MCP in rumen of sheep

The MCP concentration in the experimental group was greatly higher ($P<0.05$) than that of the control group after intake of the MUS (Table 3). MCP concentration decreased gradually after intake, and was at the minimum level at post-prandial 2 h; the MCP in the experimental group increased rapidly and reached the maximum amount at 4h. It then decreased gradually, whilst the increase speed in the control group was much slower, reaching the peak level 8 h after intake. Consequently, MCP both in the control group and experimental group restored to the level before intake (Fig. 2).

5.2.4 Discussion

This research investigated the influence of the MUS on the microbe quantities in the rumen of sheep. The microbes in the rumen of ruminants are characterized by a variety of complex population and structure. They have great effects on the

organisms' health and forage digestion. However, due to the harsh conditions found *in vitro* culture, they are difficult to be quantified. With rapid development of quantitative RT-PCR technology, 15 kinds of bacteria have been detected (Tajima *et al.* 2001; Li *et al.* 2008; Li *et al.* 2011). Quantitative RT-PCR technology is able to present the varying trend of bacteria quantity, found in rumen, with the change in the amount of ration provided. It is more visual, rapid and accurate than previously employed, traditional counting methods. As the representative bacteria in rumen microbes, *R. albus, R.flavefaciens, F.succinogenes, A.lipolytica* and *S.ruminantium* reflect the health conditions of rumen and their capability for digesting nutrients. So, in this research, they were analyzed using quantitative RT-PCR technology.

Bacteria quantity of the rumen, in the ruminant specimens, showed a dynamic change law to some extent. Within 1-2h of feeding, the gastric contents of the rumen increased, and the concentration of bacteria was diluted. However, alongside this dilution, the bacteria quantity presented a decreasing trend. With the digestion of forage, mass reproduction of the microbes in the rumen was found. Concentrations of bacteria reached their maximum levels within 4-8h. At 8h, the nutrients were depleted, the reproduction speed of microbes deceased, and then the quantity reduced due to gastric emptying effects. These results showed the same change trend as that of adding soybean oil and linseed oil to beef, previously executed by Yang (2007). According to the research by Leedle (1982), when the sheep were fed a diet that had high forage content and a diet with high concentrate successively, the quantity of each bacterium in the rumen was lowest after feeding for 2 h and 4 h. After this it began to rise stably again. However, supplied with MUS, the quantity of microbes increased rapidly while, decreasing slowly. This suggested that MUS could facilitate the reproduction of rumen microbes, and maintain a high level of microbe quantity in the rumen. Dehority (1989) reported that cellulolytic bacterium took up a great proportion in rumen bacteria of sheep. This was conducive for digestion of low quality forage.

It is generally known that feeds with high intake fiber are favorable for maintaining a high pH in rumen (Wang and Zhang 2011). In this experiment, the MUS were a high-energy feed. It contained large amounts of carbohydrates, which provides sufficient energy for synthesizing MCP by rumen microbes. So, the MUS led to rapid fermentation which leads to a decrease in pH. However, this decrease in pH levels remained within a normal range. This phenomenon suggests that the rumen pH value remained at a low level; it is conducive to the normal function of rumen microbial reproduction and various enzymes activity. Therefore, the function of rumen fermentation was enhanced, and the digestive ability of poor forage was increased.

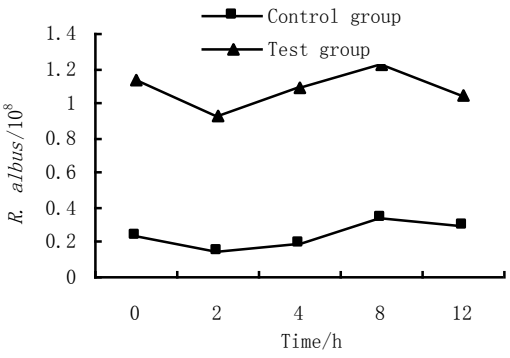
NH_3-N level is influenced by factors such as nitrogen intake, degradation extent of feed protein, synthetic speed of rumen microbes and the transportation and energy level of endogenous nitrogenous substances (Wang and Zhang 2011). In this experiment, the NH_3-N concentration was measured, and it reached peak levels at 2h after intake for the sheep in experimental group. After this, it decreased slowly. The

results showed the same varying trend with that of feeding beef only with molasses, as seen in previous research by Greenwood (2000). The MCP of the rumen, for the experimental sheep, increased fast after intake for 2 h, and reached the maximum after intake for 4h. However, in the control group, the MCP rose slowly after intake for 2h, but did not research maximum until feeding for 8 h, after which it reduced very quickly. These results showed that the MUS provided microbes with sufficient ammonia nitrogen and other nutrition, and promoted the microbial activity and synthesis of MCP.

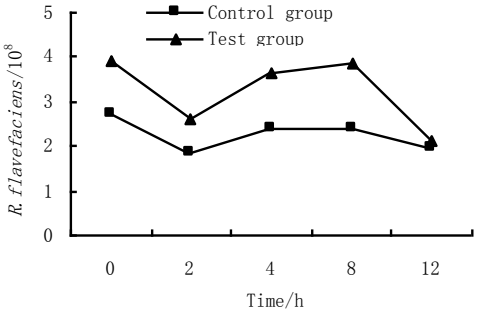
Supplying with the MUS, the protozoa quantity of the rumen increased significantly, while its concentration showed little change with the intake time. This phenomenon indicated that protozoa are sensitive to nutrients. After intake, the protozoa quantity increased rapidly without being influenced by rumen dilution. This was one of the reasons that led to an overall decrease of bacteria in the rumens of the sheep specimens, because of the phagocytosis of bacteria. But the dynamic change of protozoa was stable after intake, this indicates that protozoa quantity would not increase and further reduce the phagocytosis of bacteria. This means that the bacteria quantity in rumen could be increase and the quantity of cellulolytic bacterium increased accordingly. This is significantly important and helpful for improving the rumen fermentation and degradation of cellulosic feeds in rumen.

5.2.5 Conclusion

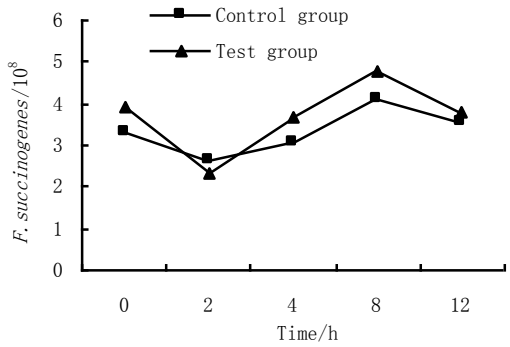
Based on consistent diet and management conditions, the MUS can maintain the pH of rumen in sheep and improve the concentration of ammonia nitrogen and MCP yields. However, beyond this, it is also able to increase the quantity of microbes in rumen, therefore promote a productive and conducive environment inside the rumen. Consequently, it improves the utilization of low quality roughage and the nutrition intake of sheep.



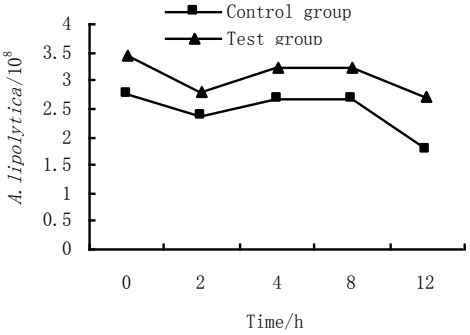
R. albus



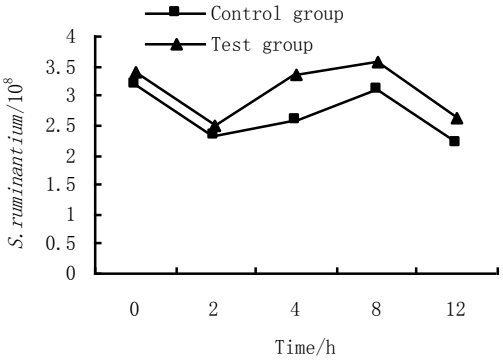
R.flavefaciens



F.succinogenes



A.lipolytica



S.ruminantium

Fig.5-2- 1 Effects of MUS on rumen bacteria copies of sheep

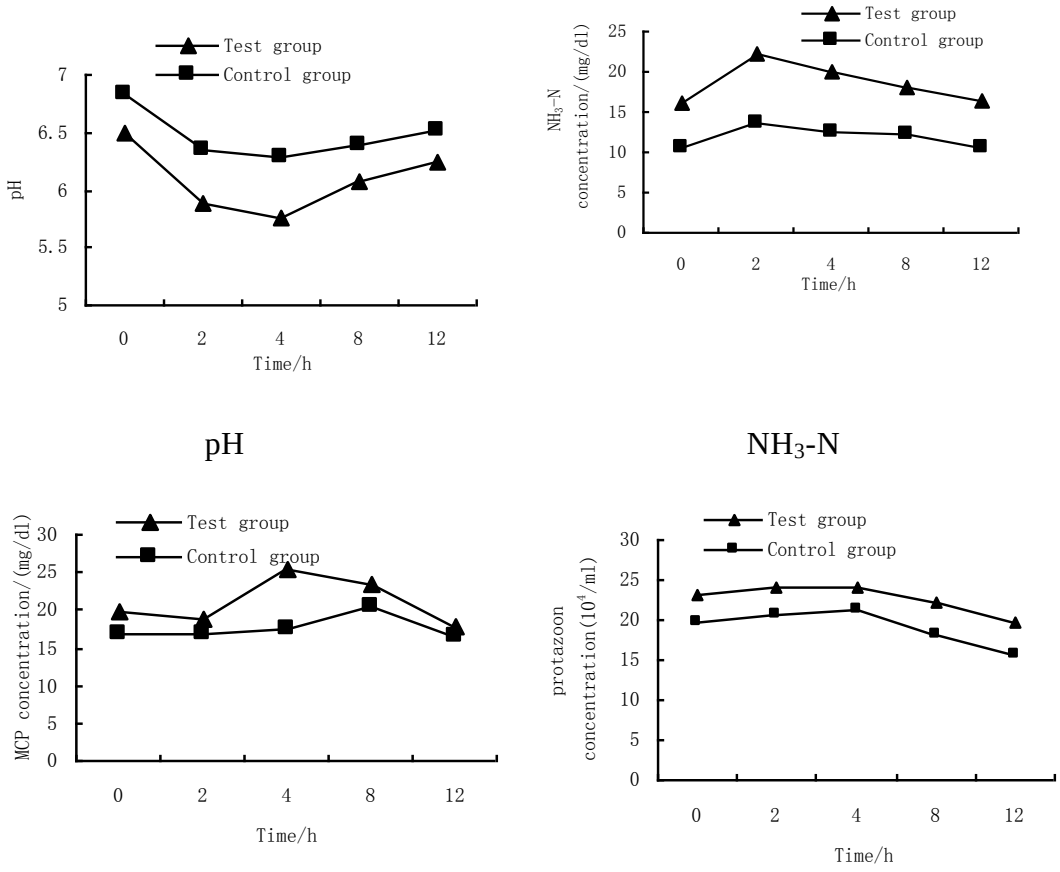


Fig.5-2- 2 Effects of MUS on ruminal fermentation of sheep

Table5-2-1 Composition and main nutrient indexes of sheep diets (DM basis)

Ingredients	Content (%DM)	Main nutrient levels	Content (%DM)
Corn	29	DM (%)	89.7
Soybean meal	11	ME(MJ/Kg)	8.7
Chinese wildrye	58	CP (%)	10.7
1% premix	0.8	Ca (%)	0.4
Limestone	0.4	P (%)	0.3
Salt	0.8		
Total	100		

Table5-2-2 The sequences of primers of *R. albus, F.succinogenes, R.flavefaciens , A.lipolytica and S.ruminantium*

Target strains	Sequences of primers and probes(5'-3')	Annealing Tm/℃	Product size/bp
R. albus	F- CCCTAAAAGCAGTCTTAGTTCG R -CCTCCTTGCGGTTAGAACA	60℃	176
R.flavefaciens	F-CGAACGGAGATAATTTGAGTTTACTTAG G R- CGGTCTCTGTATGTTATGAGGTATTACC	60℃	132
F.succinogenes	F- GTTCGGAATTACTGGGCGTAAA R- CGCCTGCCCTGAACTATC	60℃	121
A.lipolytica	F-TGGGTGTTAGAAATGGATTC R- CTCTCCTGCACTCAAGAATT	57℃	597
S.ruminantium	F- TGCTAA TACCGAATGTTG R-TCCTGCACAAGAAAGA	53℃	513

注：F:forward primer;R:reverse primer

Table5-2-3 Effects of MUS on rumen fermentation, protozoa and bacteria copies of sheep

Items	Control group	Test group
R. albus	0.2±0.1	1.1±0.1**
F.succinogenes	3.4±0.6	3.7±0.9**
R.flavefaciens	2.3±0.4	3.2±0.8**
A.lipolytica	2.5±0.4	3.1±0.3**
S.ruminantium	2.7±0.4	3.1±0.5*
pH	6.5±0.2	6.1±0.3**
BCP（mg/100ml）	17.5±1.6	21.1±3.3*
NH_3-N(mg/100 ml)	11.9±1.3	18.6±2.5**
Protozoa($\times10^4$)	19.1±2.3	22.6±1.9**

Values with asterisk superscripts in the same row means significant difference($P<0.05$), while a double asterisk superscripts means extremely significant difference($P<0.01$).

CHAPTER Ⅵ The Comprehensive Analysis of MUS on the Rumen Function in Sheep Grazing on the Winter Grassland of Inner Mongolia

6.1 General discussion

6.1.1 Determination of herbage intake and digestibility of grazing sheep using n-alkanes

The determination of herbage intake by grazing livestock has always been an important field of research. For grazing livestock, it was difficult to identify herbage intake, and it was even more difficult to determine herbage intake quantity according to the plant type or the proportion of different herbages. Although the nutritional value of each type of herbage was clear, the herbage intake of each type of herbage and types of ingestion herbage still remain unclear; thus, the correct evaluation of both the nutrient demand and nutrition balance of grazing livestock could not be performed. Therefore, the precise determination of herbage intake and the evaluation of diet composition of grazing livestock are of the utmost significance for the nutrition research of grazing livestock.

Most researchers have already dedicated large resources to methods of determination of herbage intake by grazing livestock, and certain achievements has been obtained; however, several shortages could be found in such methods, e.g., too much subjectivity, recovery problems, and difficulties in the determination of herbage intake by individual livestock. The ideal means to solve these issues was to introduce the n-alkanes method, in which, the alkanes that are contained in the epidermal wax on the surface of plants, was selected as an endogenous indicator. Addition of alkanes with an even number of carbon atoms was selected as exogenous indicator and thus, the herbage intake could be determined based on the proximity of recovery. However, the method here was widely used throughout the world. Most experiments conducted by Mayes and Dove proved that the C_{33} contained in plants exhibited a very similar recovery rate with artificially synthesized C_{32}. Consequently, the accurate determination of herbage intake by sheep, goats, and cows was achieved, and C_{32} alkane capsules and based on mathematical method, was employed for the determination of herbage intake and diet composition. The results complied with most experimental results obtained by previous researchers. Moreover, the scientific nature and reliability of herbage intake and intake plant composition were once again confirmed in the present study. Wider development prospect of such a method will be obtained with further development of the research.

6.1.2 Importance of the rumen microbe for grazing sheep

The rumen microflora is a complex microbial community, which sojourned in the rumen of ruminants. The rumen provides sustained and stable environmental conditions for the survival and reproduction of microbes, and these microbes provide the ruminants with beneficial nutrients *via* necessary biochemical reactions. The digestion and utilization of various nutrients by ruminants are influenced by rumen microbial species and composing proportions. Rumen microflora plays an important role in the nutritional metabolism and especially in the use of roughage by ruminants. Furthermore, it is of great significance for the maintenance of normal nutrition and physiological functions of ruminants. The rumen microbial population mainly consists of bacteria, fungi, protozoa, archaebacteria, and a small amount of accompanying bacteria. A complex and perfect ecological system was formed among rumen microbial populations, ruminants, and various microbes, which ensured the coordination and stability of microorganisms with the host, in addition to the coordination and stability among various microbes.

Among various rumen microbes, rumen bacteria exhibit maximal specificity, accounting for 50%-80% of the overall rumen biomass, and the bacterial content was up to 10^{11} in 1 ml of rumen liquid. According to their function, rumen bacteria can be divided into starch degradation bacteria, hemicellulose degradation bacteria, cellulose degrading bacteria, and fat degradation bacteria. These microbes had functions that the organs of host cannot provide, such as the degradation of indigestible nutrients in the rumen, such as pectin, cellulose, and hemicellulose. In addition, most of the rumen bacteria are obligatory anaerobic bacteria, and the enzyme activity involved in lignin and cellulose decomposition contained in herbage requires such a type of bacteria. Among all bacteria, *Amber fibrobacters*, *Ruminococcus flavefaciens*, and *Ruminococcus albus* were the main decomposition bacteria of cellulose and hemicellulose. It was interesting that such a type of bacteria only accounted for less than 4% of the overall rumen bacteria.

Beyond cellulose decomposing bacteria, the rumen also contains bacteria for the degradation of proteins, fat, and starch, among which, the function of bacteria protease was similar to that of trypsase. By its reaction, the dietary protein could be degraded into metabolites including amino acids, ammonia, and peptide; complex cell walls and starch were degraded by primary fermentation bacteria, and secondary fermentation bacteria could then utilize the products of primary fermentation bacteria. Entering the rumen, with the deaminase generated by protozoa and bacteria, the herbage is then degraded to produce NH_3, which can be used by bacteria for the synthesis of protein, while NH_3 cannot be used by protozoa for the synthesis of amino acids. Thus as for protozoa, protein only sourced from the intake of protein contained in bacteria or herbage.

Another important role of rumen microbes is the synthesis of microbial protein (MCP) with ammonia, peptides, and amino acids using herbage degradation as nitrogen source, with organic matter degradation products of VFA and ATP as carbon skeleton and energy source, respectively. Rumen microbial protein was the main

source of absorbable protein for ruminant intestines, occupying 40%-60% of the total protein required by livestock (Welch 1993), which played an important role in the protein nutrition system for ruminants.

At present, diet formulation for ruminant is mainly based on an animal's demand for energy and protein. An increase of concentrate proportion was mostly introduced by culturists to improve the output performances of sheep, which in turn caused an increment of breeding cost. Most research results revealed that, it was more reasonable to improve the quantity of the rumen microbial synthesis than to solely increase the concentrate proportion. Since microbial protein is a high quality animal protein, the amino acid composition was more beneficial for the absorption by the host. Furthermore, the final microbial fermentation product can provide a large quantity of energy for the demand of the host.

6.1.3 Effects of different seasonal diets on rumen microflora

The number and species of rumen microorganisms are closely related to their own condition and diet structure. Studies have reported that the number of cellulose-degrading bacteria and starch-degrading bacteria greatly affect the dietary herbage-to-concentrate ratio. The number and types of cellulose-degrading bacteria increased in a high roughage diet; the number and types of starch-degrading bacteria increased when animals receive a high concentrate diet, and total rumen microorganisms are also a significantly increased (Coe 1999, Goad 1998). However, few reports exist about the effects of grazing on rumen microflora in ruminants during different seasons. In this study, the results were similar to those of different roughage diets.

Tajima (2001) and Fernando (2010) pointed out that when a high roughage diet shifts into a high concentrate diet, the number of *Amyloidoma gastric bacillus*, *Lactobacillus acidophilus*, *Selenomonas ruminantium*, and *Megasphaera elsdenii* increased in the rumen of bovis; however, the number of *Rumincoccus flavefaciens*, *Filamentous succinate*, *Bacillus*, and some other cellulose-degrading bacteria decreased. Weimer (1990) and Tajima (2001) found that the numbers of cellulolytic bacteria increased when the cellulose content increased in rumen, while low cellulose content and high starch content in the diet decreased the number of cellulose-degrading bacteria.

The current experiment first studied the effects of natural pasture in different seasons on rumen microflora of grazing Mongolia sheep. The results showed that the content of *Bacteroidetes* and *Firmicutes* in the Mongolia sheep rumen were maximal in different seasons, verifying that *Bacteroidetes* and *Firmicutes* were advantageous microflora in the ruminant rumen (Ley *et al*. 2008, Qin *et al*. 2010, Singh *et al*. 2012, Oliveira *et al*. 2013). However, there is a small difference: The level of *Firmicutes* was much higher than that of *Bacteroidetes* grazing in the grasslands of Mongolia sheep; however, *Firmicutes* and *Bacteroidetes* in the rumen are similar in the

Mongolia sheep that are only feeding on hay. With the grassland turning from withered to green, the intake of nutrients changes, herbage intake increases, digestibility increases, *Ruminococcaceae, Lachnospiraceae,* and *Prevotellaceae* also increase. With the grassland turning from green to withered, the *Gastranaerophilales* in the rumen increased. However, the *Christensenellaceae* did not show significant differences during different seasons, which may be due to *Christensenellaceae* being the main bacteria in the rumen, and intake nutritional changes do not cause them to change.

NH_3-N is a degradation product of feed protein and non-protein nitrogen in the rumen nitrogen metabolism, and the appropriate NH_3-N concentration is the primary condition to ensure normal growth of microorganisms. The protein of herbage was rich in summer, and the protein intake of grazing sheep was high, while fermentable nitrogenous substances in green pasture increased, which can increase the NH_3-N concentration of the rumen. During winter, the green feeding declines, soluble nitrogen in dietary decreases significantly and consequently, the NH_3-N concentration in rumen decreased significantly. At the same time, NH_3-N is the raw material for growth and reproduction of rumen microbes as well as for the synthesis of BCP. With the decrease of NH_3-N concentration, total bacterial content in the rumen decreased and the concentration of BCP decreased. This is the reason that concentrations of NH_3-N and BCP in rumen in the summer are significantly higher than in winter in this study ($P < 0.05$).

6.1.4 Study on nutrition limiting factors

As mentioned above, seasonal changes of the grassland pasture and rumen microbial populations caused an intake of nutrition of grazing sheep to lessen during winter. However, it is a key period of pregnancy, lambing, and lactation for Mongolian sheep in winter and spring in Inner Mongolia. As a result, it is particularly important for sheep to know the accurate amount of supplementary feeding and nutritional restrictive factors during this period. The grey relational analysis method was employed by Liu (2001) for the study of 18 types of limiting factors that are associated with seasonal variation of herbage nutrients in the Alpine meadow and the dynamic relationship between digestion&metabolism and production property of grazing sheep. The analysis results revealed that digestion&metabolism of the herbage was the key factor affecting herbage intake of grazing sheep, which is identical with the results of our study. Atmospheric temperature and protein content of herbage were the main factors, affecting the daily gain of sheep. A similar conclusion was obtained by Wang (2011) in a study of the nutrient limiting factor, which affected grazing sheep production with grey relational analysis method. It was reported by Wang (2001) that the shortage of protein, fermentation capability, P, Zn, and Se were the main nutrient limiting factors for grazing Tan sheep in Ningxia during spring and winter. Wang (2011) considered that the shortage of energy and

protein, low quality of hay, cold stress response, and the interaction among the factors above during spring and winter were the main nutrient limiting factors for grazing sheep in north China. The determination of the overall nutritional status of grazing sheep *via* the nutrient detection method and the combination of livestock and herbage was conducted in the present study. Lower protein and herbage intake in addition to poor digestion&metabolism capability were the main nutrient limiting factors for grazing sheep during spring and winter. All experimental results proved that the nutrient limiting factors for grazing sheep was miscellaneous, and that a single nutrient limiting factor could be rarely observed in practice. In general, the study of nutrient limiting factors for grazing livestock is extremely complicated and satisfactory results can only be obtained *via* systematic study, which is conducted by comprehensive consideration of all aspects of factors, without leaning on or ignoring of certain factors. Therefore, integrating the above research results and the experimental results of this study, energy and soluble nitrogen were the main nutrition limiting factors, and the necessary trace elements should also be considered.

6.1.5 Change of rumen microflora supplement with molasses-urea

According to the above results, the limited nutrient factors of grazing sheep during spring and winter were energy and soluble nitrogen. Due to the cold climate of the grassland of northern China, the intake of nutrients decreased, resulting in a change of rumen microbial population, a decrease of their number, and a lower digestibility of herbage. Several studies showed that *Bacteroidetes* in a grain fed control group were significantly higher than those in treatment group that were fed with herbage (Ellison 2014, Shanks 2011, Kim 2014, Petri 2013). This study shows that supplementation with MUS led to significantly increase of *Bacteroidetes*, indicating MUS and grain feed to have the same effect in rumen. Petri (2013) shows that *Prevotella* in the grain group was significantly higher than in the hay group on beef cattle bacterial community structure and Kim (2014) also found similar results in the study on cattle ruminal bacterial diversity. However, this study shows that *Prevotella* change is not obvious supplemented with MUS, which is not consistent with the above results and may be a small effect of MUS on *Prevotella*.

Results of Tajima (2001) showed that *Ruminococcus flavefaciens* decreased by approximately 10% after feeding turned from hay to grain. Pitta (2014) reported that when the cellulose content increased in the diet, *Fibrobacter*, *Ruminococcus* also increased; *Fibrobacter* and *Ruminococcus* are higher in rumen of Indian buffalo fed with hay. Petri (2013) showed that *Fibrobacter succinogenes* in the hay-fed group of cattle rumen was significantly higher than that of the grain-fed group, *Ruminococcus spp*. has a decreasing trend with the increase of concentrate; however, the difference did not significantly. Similarly, this study revealed that supplementation with MUS, did not lead to a significant decrease of *Ruminococcus* (cellulolytic bacteria) and this result differs when animals are fed with grain. Under the condition of high

concentrate, rumen microbial ferment, and large production of amounts of VFA, the pH fell sharply, which could be the cause of rumen fiber-degradation bacteria reduction. However, the MUS provide necessary nutrition supplies for microbial, playing the function of the rumen microbial digestion, and microbial quantity does not reduce, thus enhancing the usage rate of roughage and bacteria protein synthesis.

6.2 Scientific optimization for supplementary feeding of grazing sheep

The purpose of supplementary feeding in grazing sheep is to improve the production level *via* an increase of nutrient input. However, the final purpose of supplementary feeding cannot be achieved without the consideration of individual consumption and nutrient utilization rate of sheep. Therefore, modern breeding theory is based on the combination of supplementary feeding with nutrition regulation, by aiming at the main nutrient limiting factors of grazing, with overall nutrient regulation measures to improve the herbage utilization rate as well as the overall efficiency of current supplementary feeding. Hence, according to systematic nutrition regulation theory, an optimization of breeding design was proposed by Lu (2014) in which, based on certain production targets and nutrition regulation purposes, results relied on local available resources and various influencing factors to optimize and design a optimum breeding mode. Based on this mode, the design of diet could be optimized and finally, based on the satisfaction of the nutrient demand of livestock, an economic and qualified diet with balanced nutrients, safe breeding, and special regulation function was provided. The present experiment was the actual practice of this theory, by aiming at herbage intake and diet composition of winter grazing sheep. Based on the analysis, the results obtained were that the main nutrient limiting factors were protein and energy for grazing sheep in the grassland of Inner Mongolia. This enabled a breakup of the traditional diet formula preparation, which was only based on the nutrient demand of livestock provided by standard breeding, and hence a supplementary feeding plan based on the actual nutrient demand of livestock was proposed. Furthermore, the scientific nature and feasibility of "optimization of breeding design" was once again proved by the experimental results. According to the current status of sheep production, herbage, and herbage in grassland, main nutrition regulation measures are introduced below:

A. Supplementary feeding with fermentable nitrogen sources; the typical fermentable nitrogen source was urea.

B. Increase of fermentation energy, provided fermentation energy by supplementary feeding of non-structural carbohydrates.

6.3 Sustainable development of Grassland animal husbandry

Currently, the Chinese grassland livestock husbandry encounters unprecedented challenges and opportunities: How to explore solutions for the dilemma of grassland

degradation requires the grasping of the direction and mode of the development of grassland livestock husbandry with simultaneous consideration of environment protection, and the improvement of life level of herdsman. Here, the promotion of economic and social development of grassland was a crucial topic associated with economy, living, and food security in grassland areas. Chinese grassland is not only the most important green ecological barrier, but also the basic means of production for the development of livestock husbandry, thus enriching herdsmen, and exhibiting important strategic significance for the development of economy and ecological society.

Due to constant population growth and constant development of market demand, nomadic economy system of self-reclusive, self-sustaining, self-regulation, inefficient, and extensive in the grassland have transformed to an open ecological economic system. Along with the dramatic growth of livestock quantity (e.g., the livestock amount increased from 1.1 million in the early 1950s to 16 million in 2000 in the Xilingol grassland) and immigration of agricultural population, large areas of grassland were left uncultivated after reclamation. The problem of grassland degradation emerged as a result of decrease of grassland area. According to statistics, the degraded grassland area of Inner Mongolia reached up to 1.67 square kilometers annually, and the annual loss of ecosystem services value was estimated to be 3.2 billion RMB. In the past 30 years, the loss of ecosystem services value was 1.42 times that of the livestock husbandry output of Inner Mongolia for 50 years (Zhang 2016).

Solely decreasing livestock quantity in breeding will lead to a loss of herdsmen income, with a contradiction between economy and ecological development. Thus, how to maintain the benign circulation of grassland ecology system became an important investigation direction for most researchers. From current study we know, the intake nutrient of grazing sheep didn’t meet the damand of nutrient requirement in spring and winter, and the nutrient limit factor was soluble energy and nitrogen. the traditional feed supplement, corn is more expensive, many farmers are not willing to feed it for sheep, they prolong the grazing time; result in grassland overgrazing and grassland degradation. While the MUS are rich in nitrogen and energy, is relatively cheaper. Provision necessary nutrients for bacteria, it was beneficial for major rumen microbial growth, maintaining stable fermentation, and improves the digestibility of forage and enrich the yields of MCP. So the weight gain of grazing sheep increase, sheep shorten the grazing time in grassland, grassland have been effectively protected.

CHAPTER Ⅶ General conclusion

7.1 The innovation of this study

Dynamic changes of the flora of rumen microbes were systematically studied for the first time in Inner Mongolia grassland during different season. We analyzed the effect of herbage nutrients to rumen predominant bacteria during different seasons, and provide theoretical support to grazing sheep rumen regulation.

The mechanism to improve the performance of grazing sheep supplemented with molasses-urea was conducted for the first time from the perspective of rumen microbes. Several experiments were conducted, including daily change of rumen microbes, herbage digestibility, and changes of rumen microflora supplements with molasses-urea. The results indicated that molasses-urea reduced the proportion of bacteria in the *Firmicutes* in the rumen and increased the proportion of bacteria in the *Bacteroidales*, thus increasing the nutrition of sheep and consequently improving its daily weight.

From the molecular level, we provide another feasibility for supplementary feeding of grazing sheep during winter and spring in northern china, that provides nutrition for rumen microorganisms, thus increasing the number of rumen microbes, changing the microflora, and improving inferior herbage digestibility, while increasing the output of BCP, improving ruminant production performance, and ultimately reducing the cost of supplementary feeding.

7.2 General conclusions of the thesis

The type and proportion of the intake herbage by Inner Mongolia sheep differed with seasons. There was a significant correlation between diet component and CP during winter. Therefore, following the changes of season, the nutrition and digestibility of grassland in northern China gradually decreased from late spring to winter. Thus, it became clear that the CP and ME of pasture were seriously scarce and inefficient during winter and spring, which were major limiting nutritional factors in grazing sheep.

Firmicutes and *Bacteroidetes* can be identified as dominant bacteria within the rumen of grazing sheep and different herbage nutrition of grazing sheep intake led to changes in the rumen microflora. At Phylum level, *Firmicutes* and *Bacteroidetes* were dominant bacteria. The number of *Firmicutes* began to rise in January and reached the highest value in August, and began to significantly decline in October; *Bacteroidetes* was similar to that of *Firmicutes*. At Family level, *Ruminococcaceae*, *Lachnospiraceae*, *Christensenellaceae*, *Prevotellaceae*, and *Rikenellaceae* were dominant bacteria, the number of bacteria all increased from January, reaching the highest level during June or August.

From these results, fermentable carbohydrates and non-protein nitrogen were insufficient in grazing sheep. After supplying fermentable carbohydrates and non-protein nitrogen to sheep in the form of MUS, an obvious weight gaining trend was observed, which was due to provision necessary nutrients for bacteria, maintaining stable pH of rumen, and enriching ammonia. These conditions were beneficial for major rumen microbial growth and reproduction, promoting the increase of *bacteroidales* and unclassified *clostridiales*, and reducing the proportion of *Firmicutes* in the rumen. This may result in the change of rumen microflora, improve the function of rumen digestion, and improve the rumen environment, thus enhancing the digestibility of ruminants on poor-quality feed. However, beyond this, the non-protein nitrogen in MUS provides ammonia nitrogen for ruminal bacteria and bacteria rapidly reproduce and increase the quantity of microbes in rumen, providing CP for grazing sheep, then improving the production performance of sheep. MUS were cheaper in China; therefore, the MUS were revealed to be the optimum supplemental feed formula during winter and spring. It can lead to great efforts of spreading the sales of MUS in the grassland area of north China.

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