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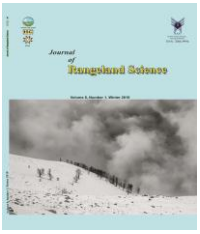

Research and Full Length Article:

Dynamic Changes of Main Rumen Microflora and Ruminal Fermentation in Sheep Supplemented with Molasses-Urea

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Abstract. The digestion and utilization of roughage by sheep depends on rumen digestion. The aim of this study was to evaluate the microbe quantity and fermentative efficiency of rumen supplement with molasses-urea. This experiment was conducted in 2014, 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 populations of majority bacteria were investigated by real-time PCR. The results showed that the populations 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 raised 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 was found 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 microbial crude protein (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 favorable effect on rumen fermentation by the microbes in ruminant.

Key words: Molasses-urea, Sheep, Rumen microflora, Rumen fermentation

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 *et al.* (2015) reported that the urea contented in molasses-urea could increase fodder intake by ruminants, so we can optimize the growth and nutrition metabolism of microbes in the rumen of ruminant animals 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), molasses-urea would promote maximum utilization ratio of roughage and reduce the cost of buying hay. Molasses-urea has 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 molasses-urea promotes rumen fermentation, through supplementation of molasses-urea in feedstuff. In this experiment, quantitative RT-PCR was used to detect the main representative bacteria we discovered, which includes *Ruminococcus albus* (*R. albus*), *Ruminococcus flavefaciens* (*R. flavefaciens*), *Fibrobacter succinogenes* (*F. succinogenes*), *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, NH3-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 molasses-urea. The results of this can provide a scientific basis for improving the utilization of low quality roughage and promoting molasses-urea product.

Materials and Methods

Animals and experimental 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 weight 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 molasses-urea added to it. The first 10 days served as the adaptation stage, of which the sheep adapted to the hay and the molasses-urea feed, then the test period began which lasted 5 days.

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 molasses-urea (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 molasses-urea was purchased from the Nippon Zenyaku Kogyo Co., Ltd in Japan.

Table 1. Composition and main nutrient indexes of sheep diets (DM basis)

Ingredients	Content (%DM)	Main nutrient levels	Content (%DM)
Corn	29	DM (%)	89.72
Soybean meal	11	ME(MJ/Kg)	8.721
Chinese wild rye	58	CP (%)	10.68
1% premix	0.8	Ca (%)	0.38
Limestone	0.4	P (%)	0.25
Salt	0.8		
Total	100		

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 HCl of 0.2 mol/L 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. succinogenes* 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.

Table 2. 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-CGAACGGAGATAATTTGAGTTTACTTAGG 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

Note F: Forward primer; R: Reverse primer

Measuring method of fermentation indices

The pH of the rumen liquids samples was measured using PHS-3B meter. NH_3-N was determined using the method by Wang *et al.* (2011); MCP was measured by referring the methods proposed by Cotta and Russell (1982) and Broderick and Craig (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 (Equation 1):

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

(Equation 1)

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.

Results

Influence of molasses-urea on the rumen bacteria of sheep

The rumen bacteria of sheep in the experimental group increased significantly ($P<0.5$) after supplying molasses-urea, 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 ($P<0.5$), other bacteria only showed a significant difference at 4-8 h.

Table 3. Effects of molasses-urea on rumen fermentation, protozoa and bacteria copies of sheep

Items	Control group	Test group
Ruminococcus albus	0.24±0.08	1.08±0.11**
Fibrobacter succinogenes	3.35±0.55	3.71±0.89**
Ruminococcus flavefaciens	2.26±0.35	3.23±0.82**
Anaerovibrio lipolytica	2.45±0.41	3.07±0.32**
Selenomonas ruminantium	2.68±0.44	3.08±0.49*
Protozoa($\times 10^4$)	19.06±2.27	22.64±1.86**
pH	6.47±0.22	6.09±0.29**
MCP (mg/100ml)	17.52±1.64	21.11±3.24*
NH_3-N(mg/100 ml)	11.87±1.30	18.55±2.54**

*, ** = the means of each rows has significant differences in ($P<0.05$) and ($P<0.01$), respectively

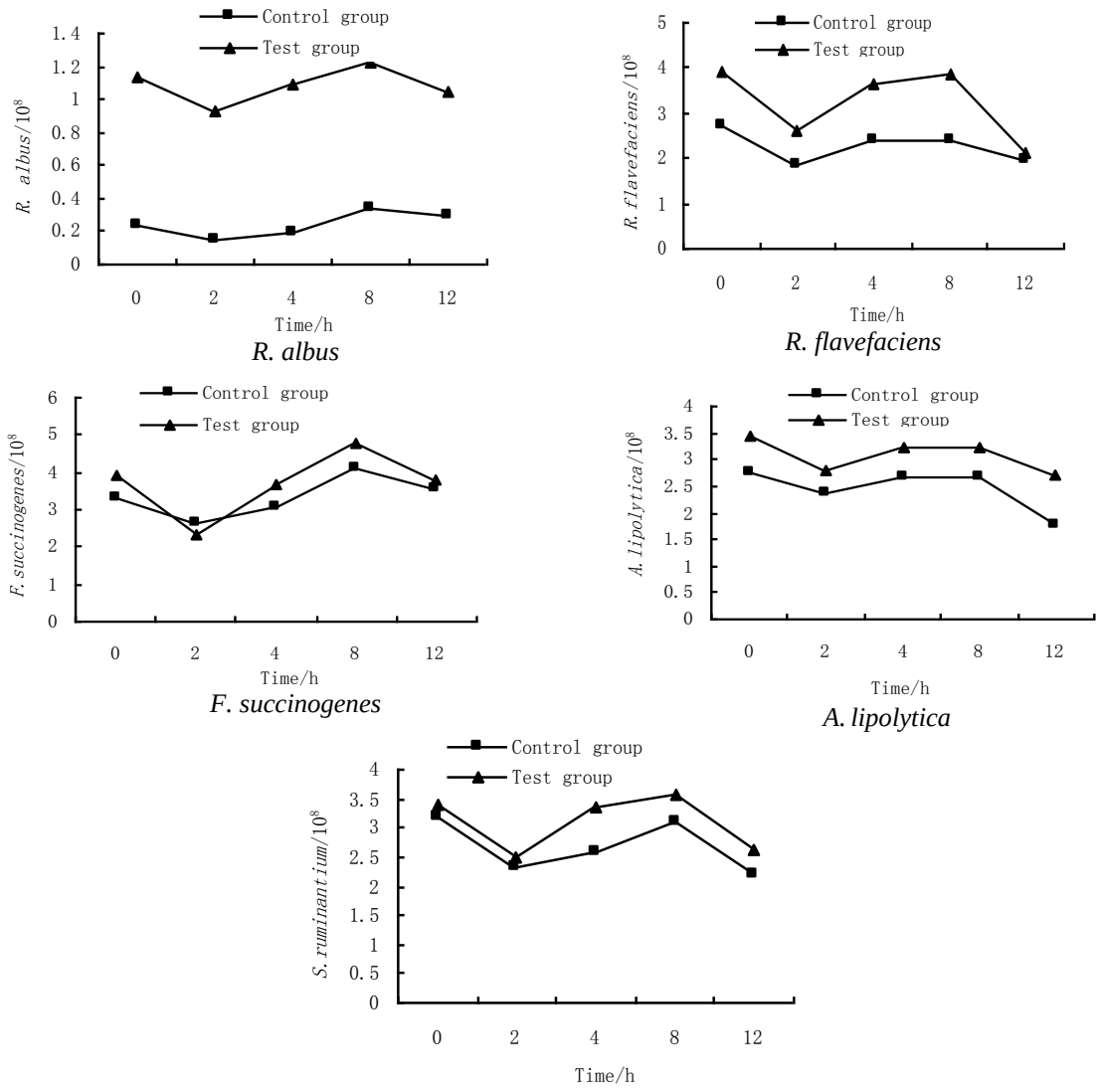

Fig. 1. Effects of molasses-urea on rumen bacteria copies of sheep

Effects of the molasses-urea on the rumen Protozoa of sheep

The protozoa quantity in the sheep rose gradually after each intake of the molasses-urea, 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 molasses-urea 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 molasses-urea on NH3-N in rumen of sheep

Through the intake of the molasses-urea, 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 molasses-urea 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 molasses-urea (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).

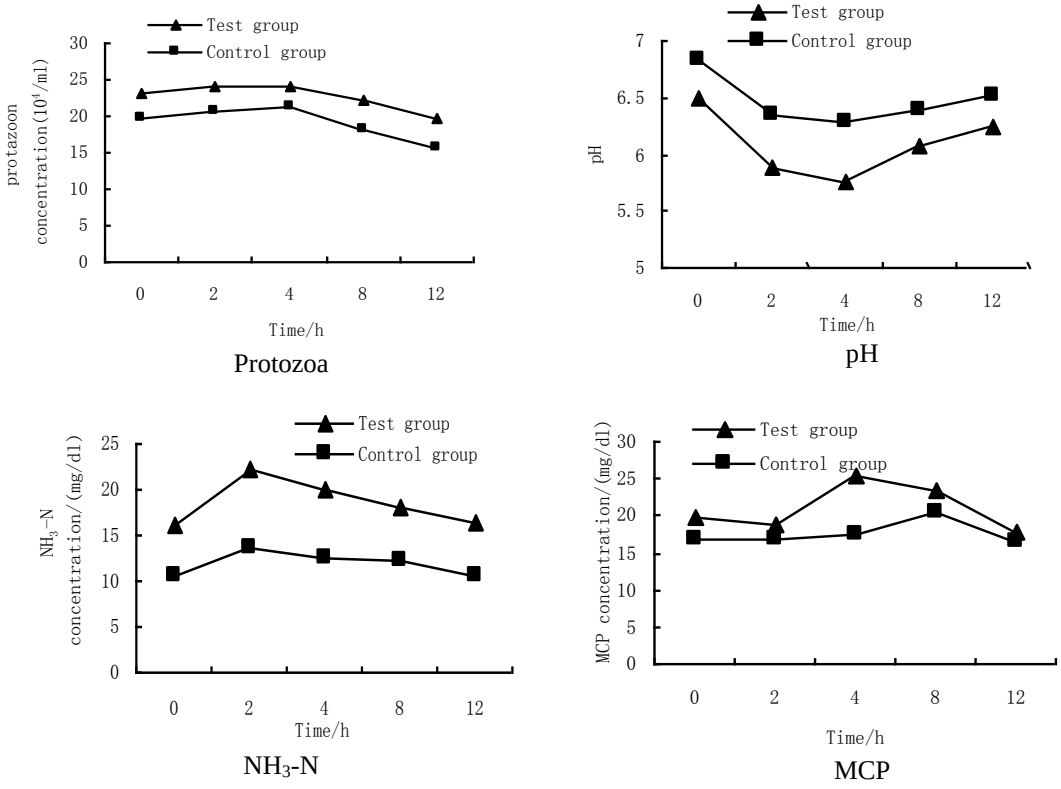

Fig. 2. Effects of molasses-urea on Protozoa, pH, MCP and NH_3-N of rumen in sheep

Discussion

This research investigated the influence of the molasses-urea 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 *et al*. (2007). According to the research by Leedle *et al.* (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 molasses-urea, the quantity of microbes increased rapidly while, decreasing slowly. This suggested that molasses-urea could facilitate the reproduction of rumen microbes, and maintain a high level of microbe quantity in the rumen. Dehority *et al.* (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 molasses-urea was a high-energy feed. It contained large amounts of carbohydrates, which provides sufficient energy for synthesizing MCP by rumen microbes. So, the molasses-urea 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 *et al.* (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 molasses-urea provided microbes with sufficient ammonia nitrogen and other nutrition, and promoted the microbial activity and synthesis of MCP.

Supplying with the molasses-urea, 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.

Conclusion

Based on consistent diet and management conditions, 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.

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تغییرات فعال فلور میکروبی شکمبه اصلی و تخمیر شکمبه در مکمل‌های غذایی گوسفند حاوی ملاس اوره

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چکیده. تعلیف علوفه توسط گوسفندان و هضم آن گوسفند بستگی به هضم در شکمبه دارد. هدف از این مطالعه ارزیابی مقدار تخمیر میکروبی شکمبه، مکمل غذایی حاوی ملاس اوره بود. این آزمایش در سال ۱۳۹۳ انجام شد، هشت گوسفند انتخاب شدند و به دو گروه (گروه شاهد و گروه آزمایش) تقسیم شدند و فقط حیوانات گروه مورد آزمایش به صورت آزادانه ملاس اوره مصرف کردند. مایع شکمبه در هر ۲ ساعت جمع آوری شد و پارامترها و تخمیر شکمبه اندازه‌گیری شد. بیشتر جمعیت باکتری توسط روش زمان واقعی PCR مورد بررسی قرار گرفت. نتایج نشان داد که به طور معنی‌داری اکثریت جمعیت باکتری در شکمبه حیوانات گروه آزمایش است ($P<0.05$). همچنین نتایج نشان داد که مقدار باکتری پس از تغذیه به تدریج کاهش یافته و به پایین‌ترین سطح در ۲ ساعت پس از مصرف رسید. سپس به آرامی افزایش یافته و به بالاترین سطح در ۸ ساعت پس از مصرف رسیده است. در نهایت، مقدار باکتری به همان سطح قبل از مصرف باز می‌گردد. در مقابل، تعداد تک یاخته‌ها به بالاترین مقدار در ۴ ساعت پس از مصرف رسیده و به تدریج کاهش یافته است. غلظت تک یاخته در گوسفند گروه آزمایش به طور قابل توجهی بالاتر از گروه شاهد بود ($P<0.05$). اسیدیته مایعات شکمبه در یک محدوده نرمال بود و بین دو گروه متفاوت بود. با این حال، میزان اسیدیته از بالاترین سطح قبل از تغذیه به پایین‌ترین سطح در ۴ ساعت پس از مصرف کاهش یافت، و سپس ۸ ساعت پس از مصرف زیاد شد. غلظت NH3-N و پروتئین خام میکروبی، در مایعات شکمبه، به طور قابل توجهی بالاتر از گروه شاهد بود($P<0.05$) ، بالاترین غلظت از NH3-N و MCP پس از تغذیه به ترتیب بعد از ۲ ساعت و ۴ ساعت بود. نتیجه‌گیری می‌شود که ملاس اوره با توجه به اثر مطلوبی که بر روی تخمیر در شکمبه توسط میکروبها در نشخوارکنندگان دارد اثر مثبتی بر روی هضم در شکمبه دارد.

کلمات کلیدی: ملاس اوره، گوسفند، فلور میکروبی شکمبه، تخمیر شکمبه