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# Effects of Glycerol-Producing Yeast Supplementation on Production Performance, Biochemical Indexes, Subclinical Ketosis Incidence and Rumen Fermentation Performance of Dairy Cows

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**Abstract**

Glycerol could be used to alleviate negative energy balance in high-producing dairy cows as an important precursor participating of glucose synthesis. However, some industrial glycerol may do harm to cow health because of some mixed toxic chemicals. The purpose of this study was to investigate the effects of glycerol-producing yeast prepared in our laboratory on improving the production performance and reducing the subclinical ketosis incidence of transition dairy cows. The results showed that the concentrations of the β-Hydroxybutyric Acid (BHBA) and Non-Esterified Fatty Acid (NEFA) in the groups with glycerol-producing yeast were significantly decreased ($P<0.05$), while the concentrations of glucose, TP and the production of propionic acid were significantly increased ($P<0.05$) compared with the control groups on the 21st day. Moreover, the glycerol-producing yeast improved the milk quality by significantly increasing the rate of milk protein and milk fat ($P<0.05$). Rumen fermentation performance was improved by supplementation of glycerol-producing yeast with significant increase in propionic acid and Microbial Crude Protein (MCP) concentrations ($P<0.05$). Meanwhile, supplementation of glycerol-producing yeast reduced the incidence of subclinical ketosis by improving the blood glucose and NEFA concentration and decreasing the concentration of BHBA. In conclusion, glycerol-producing yeast supplementation benefits dairy cows on their production performance, some biochemical indexes, subclinical ketosis incidence and rumen fermentation performance. This study provided some supportive data for the application of glycerol-production yeast supplementation in dairy production.

**Keywords:** Glycerol-producing yeast; Dairy cows; Production performance; Biochemical indexes; Subclinical ketosis incidence

## Introduction

Dairy cows increase energy intake during lactation after delivery. However, Due to slow recovery of postpartum appetite, dry matter intake may be reduced [1]. Therefore, cows tend to have negative energy balance during perinatal period, where energy intake cannot meet energy demand. When cows are in negative energy balance, the body moves body fat to make up for the lack of energy [2]. Whereas excessive fat mobilization can lead to metabolic disorders in animals, leading to a range of energy metabolism disorders, especially clinical and subclinical ketosis. Compared with clinical ketones, subclinical ketones have no significant symptoms, but may reduce milk production, increase the incidence of other perinatal disease, causing serious economic losses [3-5].

Glycerol plays an important role in glucose metabolism and it is an important precursor to glucose synthesis, which can effectively alleviate the energy metabolism problem and prevent the occurrence of ketosis [6,7]. Adding glycerol to diet can improve the performance of dairy cows [8-10]. On the other hand, It is reported that glycerol can be absorbed by the gastrointestinal tract, into the glucose generation pathway to synthesize glucose, which better regulates the blood glucose and insulin levels of the ruminants [11-13]. In recent years, industrial glycerol has been widely used as feed additive [14]. However, industrial glycerol is mixed with a large number of harmful substances that can have a negative impact on animal health and food safety issuers. Fermented glycerol is produced by microorganisms made from sugar, starch, or agricultural by-products. Compared with industrial glycerol, fermented glycerol can reduce production cost and has a broad prospect in the breeding of ruminant animals [15].

In our laboratory, we used yeast to produce glycerol and obtained glycerol-producing yeast supplementation. In this study, we assessed the effects of glycerol-producing yeast on the production performance, blood biochemical indexes, incidence of subclinical ketosis and rumen fermentation performance of dairy Holstein cows.

## Materials and Methods

### Animals and management

All animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the U.K. Animals (Scientific




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Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments. Twenty female Holstein cows were fed in double row with tail tether and free access to water. TMR basic diet (Table 1) was used in the experiment, which was fed three times a day and fixed column and fixed position. Cows averaged calves 2.1 ± 0.13, body condition score 3.27 ± 0.63, total amount of lactation in the last cycle (305 days) 9961 ± 103.12 kg at the beginning of the study. In order to evaluate the overall effect of glycerol producing yeast preparation, twenty cows were randomly divided into two groups. The experimental period was 21 days. The control group was fed with basic diet, and each cow in the test group was fed with 1 L of glycerol-producing yeast supplementation (containing 108.2 g/L of glycerol, and $2.8\times10^9$ CFU/mL of viable yeast) every day.

### Sample collection, Milk yield detection and Milk composition detection

Milk samples were taken on the 0th, 7th, 14th and 21st day after delivery (30 mL of mixed milk was collected at the ratio of 4:3:3 for three times of each cow), then put into a centrifuge tube containing preservative (potassium dichromate), and stored at 4°C for inspection. The milk composition was determined by a multi-functional dairy composition rapid analyzer (FOSS, Shanghai, China), and the number of somatic cells was determined by SCC analyzer (LACTOSCAN, Nanjing, Jiangsu, China).

### Calculation of Body Condition Score (BCS) and Dry Matter Intake (DMI)

Each cow's physical condition was assessed on the first and twenty-first days after delivery on a 5-point scale (1-5 points), with a grade difference of 0.25. The target score of the perinatal cow is 3 to 4 points. If the score is lower than 3 points, it means that the energy supply of the cow is insufficient. If the score is higher than 4 points, it means that the energy intake of the cow is excessive [16]. The total amount and residue of TMR feed per cow was measured and recorded daily, and the dry matter intake per cow was calculated during experiment.

### Serum biochemical indexes assay

Blood was collected from the tail vein using a disposable blood collector on the 0th, 7th, 14th and 21st day after delivery. The samples were placed in a centrifuge tube filled with heparin sodium. Then they were centrifuged at 3500 rpm for 10 minutes by low speed centrifuge (David science and Education Instrument, Hangzhou, China). The upper plasma was transferred to a 2 mL centrifuge tube with a pipette and stored at -20°C. The indexes of plasma determination included BHBA, NEFA, glucose, Total Protein (TP), Triglyceride (TG), Creatinine (CREA), Alkaline Phosphatase (ALP), Aspartate Transaminase (AST), Phosphorus (P) and Calcium (CA).

Blood glucose, TP, TG, Creatinine, ALP, AST, Ca and P in cow plasma were measured by automatic biochemical analyzer bs-300 (Mindray biomedical, Shenzhen, China).

### NEFA, BHBA concentrations assay

BHBA and NEFA in plasma were determined by the kit according to the manufacturer's instructions (Jiancheng, Nanjing, Jiangsu, China). The diagnosis of cow ketosis is based on the concentration of BHBA in the plasma. It will be determined as clinical ketosis when the concentration of BHBA in the plasma is higher than 2.6 mmol/L, and subclinical ketosis when the concentration of BHBA is between 1.0 to 2.6 mmol/L.

### Rumen fluid components assay

Four dairy cows with fistula were randomly divided into a control group and experimental group. This experiment included two periods. Each period was 15 days, including 12 days of pre-feeding period and 3 days of sampling period. The control group was fed with basic diet, and each cow in the test group was fed with 1 L of glycerol-producing yeast supplementation (containing 108.2 g/L of glycerol, and $2.8\times10^9$ CFU/mL of viable yeast) every day.

The rumen fluid was collected on the 13th, 14th and 15th day after noon feeding. About 150 mL of rumen fluid was collected each time, then filtered with three layers of gauze. The filtrate was put into a 10 mL sterile centrifuge tube and stored at - 20°C. Then concentrations of volatile fatty acids (acetic acid, propionic acid, butyric acid), total volatile fatty acids, bacterial protein and ammonia nitrogen in rumen fluid were detected.

### NH3-H concentration assay

The NH3-H concentration was determined by phenol hypochlorite calorimetry [7]. Absorbance was read by a spectrophotometer at 630nm (Bio-Rad, Hercules, CA, United States), and ammonia nitrogen concentration in the sample was calculated according to the standard curve.

### Bacterial protein concentration and volatile fatty acids assay

Bacterial protein concentration was detected according to the instruction of matched test kit, according to the manufacturer's instructions (Jiancheng, Nanjing, Jiangsu, China). Volatile fatty acids were detected by Nanjing Jiancheng Bioengineering Institute.

### Statistical analysis

SPSS 21.0 software was used for statistics, and two-way ANOVA was used for the significance of the differences. All results were expressed as means ± SEM. On the 21st day, in glycerol-producing yeast treated group, $^*P<0.05$, indicating a significance compared with the control group.

## Results and Discussion

### Effects of glycerol-producing yeast on the production performance of dairy cows

In order to determine the effect of glycerol-producing yeast on production performance of dairy cows, milk composition, milk yield and the dry matter intake were detected on the 0th, 7th, 14th and 21st day.

As shown in Figure 1, body condition score of the treated group slightly increased (P=0.08) compared with the control group. The intake of dry matter increased from the first week to the third week in both groups, but there was no significant difference between the two groups. Colostrum contains high protein (especially whey protein and casein) and a large number of immune factors. Therefore, the study did not calculate the milk composition on the first day after delivery. As shown, the milk protein rate and fat rate of each group showed a downward trend. On the 21st day, the milk protein rate and fat rate of the treated group were significantly higher ($P<0.05$)

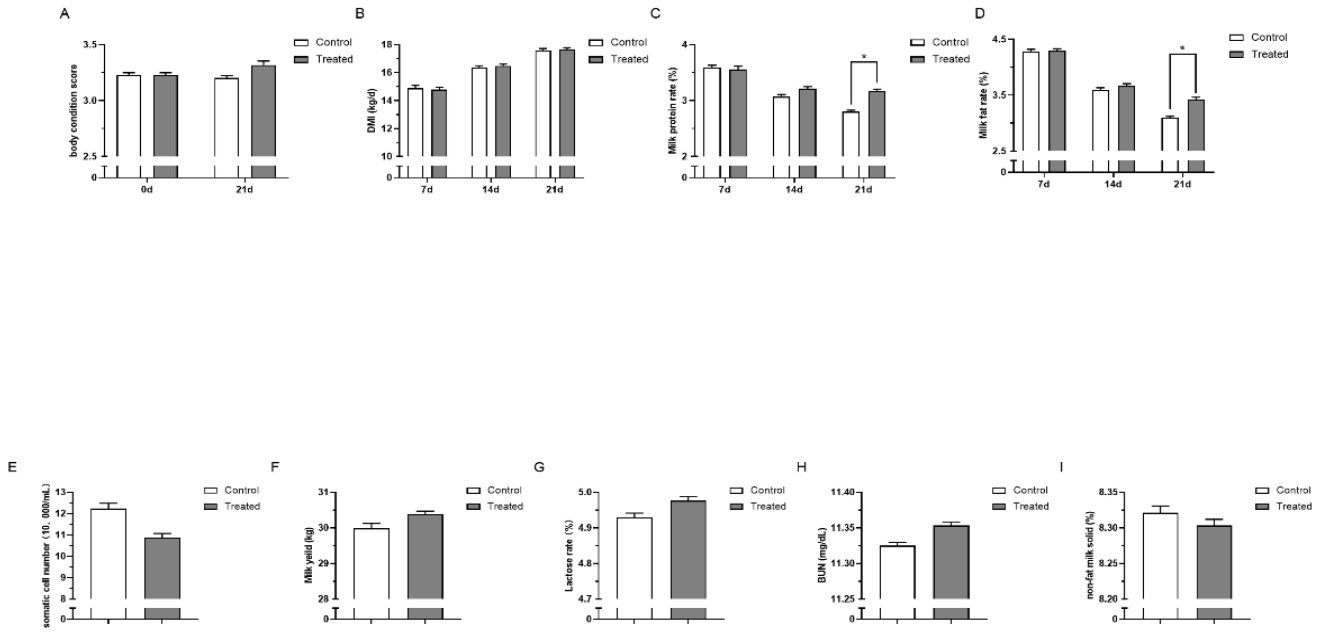


**Figure 1: Effects of glycerol-producing yeast on the production performance of dairy cows.** The control group was fed with a basal diet daily, while 1 L of glycerol-producing yeast was added in the treatment group. Body condition score (A) was calculated on the 0th and 21st day and dry matter intake (B) was calculated on the 7th, 14th, 21st day. Milk samples were collected on the 7th, 14th and 21st days after delivery. The milk protein rate (C) and the milk fat rate (D) were detected. On the 21st day, somatic cell number (E) slightly decreased (P=0.08) compared with the control group. There showed no significance compared with the control group in milk yield (F), lactose rate (G), BUN (H) and non-solid lipid's concentration (I) in treated group. All results were expressed as means ± SEM. in glycerol-producing yeast treated group, *P<0.05, indicating a significance.

than those of the control group. Meanwhile, as shown in Figure 1, on the 21st day, the number of somatic cells slightly decreased (P=0.08) compared with the control group. There was no significance in milk yield, the concentration of lactose, BUN and non-solid lipid in the treated group.

## Effects of glycerol-producing yeast on biochemical indexes of dairy cows

As shown in Figure 2, the concentration of NEFA in plasma increased on the 7th and 14th day after delivery and then decreased on the 21st day in each group. However, the concentration of NEFA in the treated group was significantly lower ($P<0.05$) compared with the control group. Moreover, the concentration of glucose in blood plasma decreased on 7th and 14th day after delivery in each group, then they turned to an increase and the concentration of glucose in the treated group was significantly higher ($P<0.05$) compared with the control group on the 21st day. Furthermore, the total protein concentration in the plasma of each group of dairy cows has been on the rise, it significantly increased ($P<0.05$) on the 21st day compared with the control group. As shown, there was no significant different in the concentrations of AST, ALP, Ca, P, Creatinine and TG in the glycerol-producing yeast treated group.

## Adding glycerol-producing yeast reduce the incidence of subclinical ketosis of dairy cows

As shown in Figure 3, the concentration of BHBA in the blood plasma increased on the 7th and 14th day after delivery of each group, then they turned to a downtrend. Moreover, the concentration of BHBA in the treated group was significantly lower ($P<0.05$) compared with the control group on the 21st day.

**Table 1:** Ingredient and nutrient composition of the diet.

| Ingredient | %of DM | Nutrient composition | |
|---|---|---|---|
| Newly produced feed | 39.1 | $NE_L$ (Mcal/kg) | 1.73 |
| Supplement feed | 10.3 | CP (%of DM) | 17.2 |
| Pressed corn | 4.2 | NFC (%of DM) | 35.3 |
| Cottonseed wool | 7 | NDF (%of DM) | 33.1 |
| Beet meal | 5 | ADF (%of DM) | 20.31 |
| Alfalfa hay | 15.4 | Ca (%of DM) | 0.98 |
| Oats hay | 5 | P (%of DM) | 0.45 |
| Corn silage | 14 | Ash (%of DM) | 8.6 |

**Table 2:** Effect of adding glycerol-producing yeast on the incidence of ketosis in perinatal dairy cows.

| n=10 | | | | |
|---|---|---|---|---|
| Group | Control (cases/n) | | Treated (cases/n) | |
| Type | subclinical ketosis | clinical ketosis | subclinical ketosis | clinical ketosis |
| Before | 0% (0/10) | 0% (0/10) | 0% (0/10) | 0% (0/10) |
| After | 30% (3/10) | 10% (1/10) | 10% (1/10) | 0% (0/10) |

As shown in Table 2, there are cases of subclinical ketosis in both groups. The incidence of subclinical ketosis was 30% in the control group and 10% in the treated group, while the incidence of clinical ketosis was 10% in the control group and 0% in the treated group. The incidence of subclinical ketosis in the treated group was significantly lower compared with the control group.

The regulation mechanism of glycerol-producing yeast on the ruminal fermentation performance was revealed by investigating

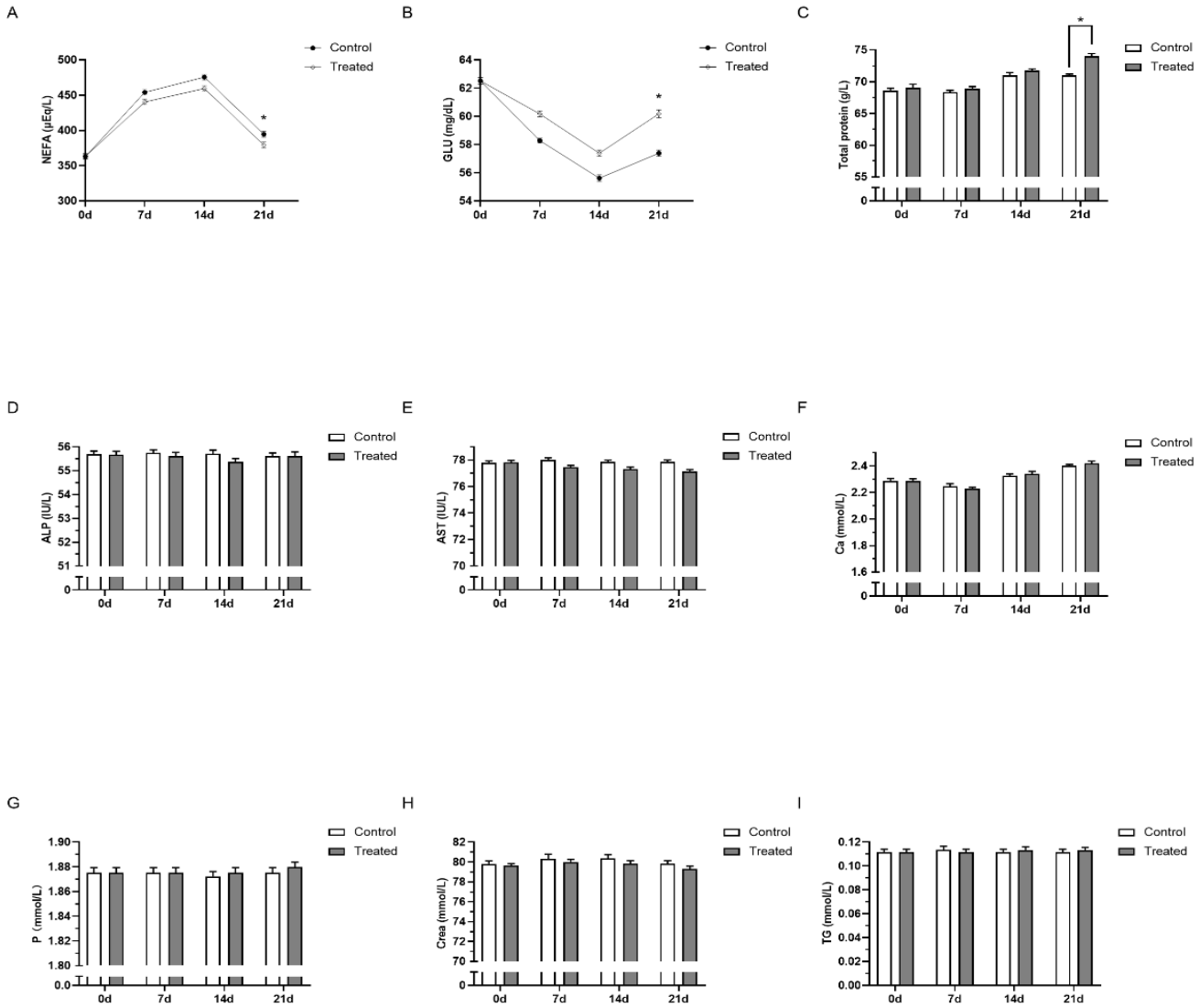


**Figure 2: Changes of the concentrations of biochemical indexes of dairy cows.** Blood samples were collected from the tail vein before the noon feeding on the 0th, 7th, 14th and 21st day. Then the concentrations of NEFA (A), Glucose (B), TP (C), ALP (D), AST (E), Ca (F), P (G), Creatinine (H) and TG (I) were determined. All results were expressed as means ± SEM, in glycerol-producing Yeast treated group, *P<0.05, indicating a significance.

the change of ruminal fermentation parameters. As shown in Figure 4, the addition of glycerol-producing yeast in the diet significantly increased ($P$<0.05) the concentration of propionic acid in the rumen of dairy cows and significantly reduced ($P$<0.05) the ratio of ethylene to propylene compared with the control group, while there was no significant effect on the proportion of acetic acid and butyric acid in the total volatile fatty acids. Compared with the control group, the concentration of total volatile fatty acids in rumen fluid of the treated group has an increasing trend, but there was no significant difference (P=0.06). As shown in Figure 4, compared with the control group, there was no significant effect on the concentration of ammonia nitrogen in rumen fluid. The addition of glycerol-producing yeast in the diet significantly increased the concentration of bacterial protein in rumen fluid of dairy cows ($P$<0.05).

## Discussion

Ketosis mainly affects the milk yield and composition of dairy cows, causing serious economic losses. It has been reported that the decrease in the peak period of lactation is the main reason for the decline in milk production in dairy cows after having ketosis [4]. First, we measured the indicators related to the production performance of the perinatal cows. BCS and DMI in the treated group was found to be slightly higher than that of the control group. As expected, the addition of glycerol-producing yeast increased the milk protein and fat rate, and showed significant differences on the 21st day ($P$<0.05). The number of somatic cells in the milk of the treated group decreased, but there was no significant difference. Our results were consistent with the previous reports [6,17,18]. The improvement of energy balance in dairy cows may be due to the glycerol, as a glycogen precursor, participates in glucose synthesis to reduce the body's fat breakdown [12,19]. Glycerol can also improve the concentration of insulin in the blood of dairy cows. Insulin and essential amino acids are activators of mTORC1 mechanism targets in mammary epithelial cells. The increase of insulin concentration can promote the activation

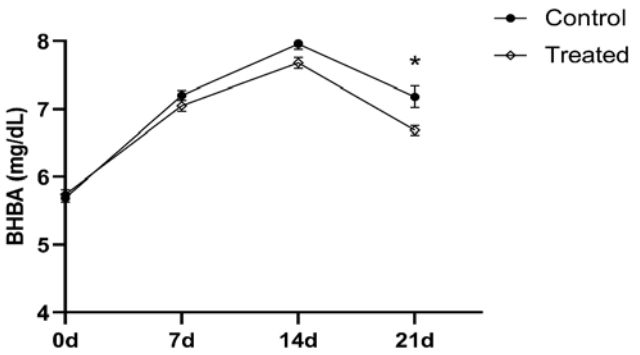


**Figure 3:** Changes of the concentration of BHBA in blood of dairy cows. Blood samples were collected from the tail vein before the noon feeding on the 0th, 7th, 14th and 21st day. Then the concentrations of BHBA were determined. All results were expressed as means ± SEM, in glycerol-producing Yeast treated group, *P<0.05, indicating a significance.

of mTORC1 mechanism, stimulate mammary gland to synthesize protein, and then increase the content of milk protein [8]. The increase of somatic cells in milk indicates the occurrence of breast infections and then reduces the production performance [20,21]. The decrease of the number of somatic cells and increase of the milk protein and fat rate in our experiment indicated that the production performance was improved. The addition of glycerol to the diet of dairy cows has been found no increase in milk production of dairy cows, which was consistent with the results of this experiment [18]. The reason may be that the trial period was relatively short and no subsequent change in milk production was detected. It may also be that the cows were in the perinatal period and the slow recovery of appetite.

Next, we measured the concentrations of partial serum biochemical indexes of dairy cows. In this study, the average concentrations of blood glucose in both groups were in the normal range (31-77 mg/dL), but that in the treated group was significantly higher than the control group ($P$<0.05), and the concentration of NEFA was significantly lower ($P$<0.05), which was consistent with the previous study [22]. Moreover, the concentration of BHBA in the plasma was significantly reduced ($P$<0.05) and the incidence of subclinical ketosis and clinical ketosis was lower in the treated group than that in the control group. BHBA is the main component of plasma ketone body. Under normal circumstances, the ketones body produced by the liver and the ketones used in peripheral tissues are dynamically balance. However, the utilization of ketone body by peripheral tissue is limited. When the body is short of sugar, it breaks down fat to make up for energy. However, the metabolite NEFA produced by excessive fat decomposition can produce a large amount of ketones after β oxidation. If the production of ketones exceeds the limit of liver decomposition of the ketone body, the ketone body level will rise, leading to subclinical ketosis [23]. BHBA concentration has become an important indicator for determining clinical ketosis or subclinical ketosis in dairy cows. It has been reported that adding glycerol to the diet of dairy cows can reduce the concentrations of NEFA, BHBA and urone [24-27], which was consistent with our test results. The results showed that adding glycerol producing yeast to the diet could slow down the decrease of blood glucose within two weeks after delivery. The body didn't need to decompose fat then produce NEFA and BHBA, so that alleviated the negative balance of energy, and reduced the incidence of subclinical ketosis.

Glycerol is a fast-metabolic carbohydrate that also involves in the synthesis of phospholipid and triglyceride. Adding glycerol to the diet increases the accumulation of lipid in the liver. If lipids are produced

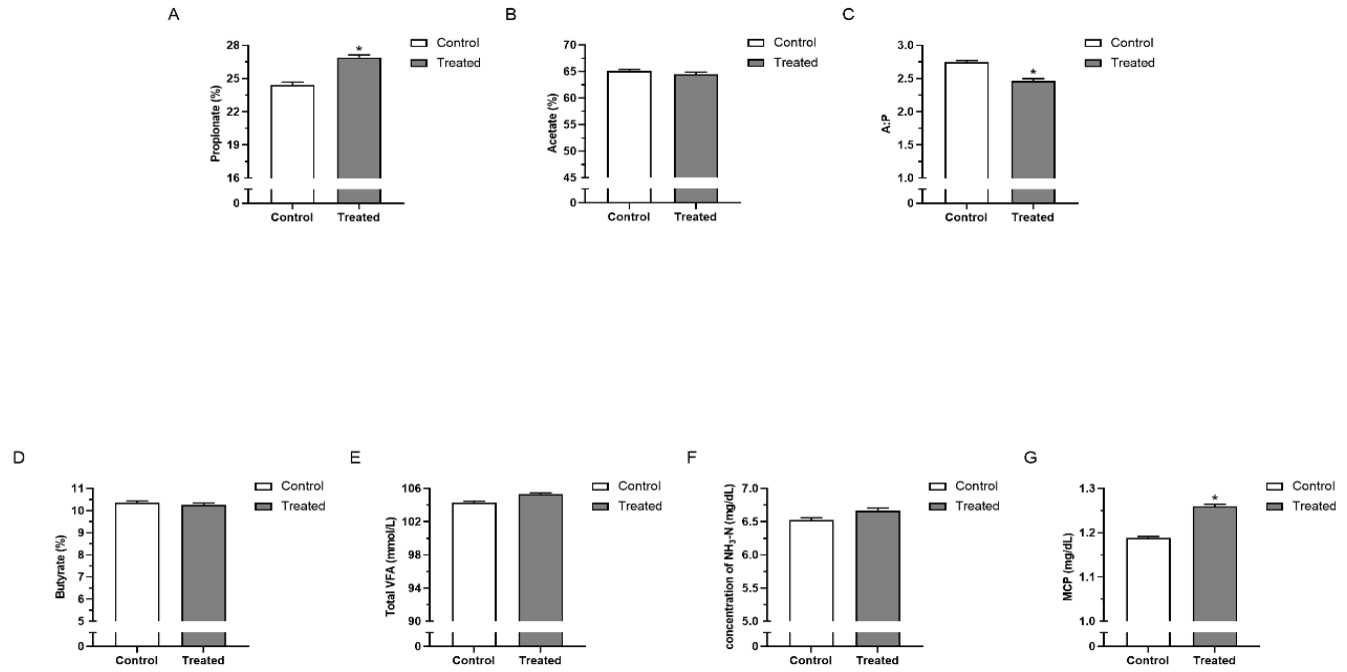


**Figure 4:** Effects of glycerol-producing yeast on ruminal fermentation performance of dairy cows. On the 21st day, the molar percentage of propionic acid (A), acetate (B) and butyrate (D) in TVFA and concentrations of total VFA (E), NH3-N (F), MCP (G) were measured. The ratio of acetic acid to propionic acid (C) was calculated. All the results were expressed as means ± SEM, in glycerol-producing yeast treated group, *P<0.05, indicating a significance.

more than the liver's ability to metabolize, it can lead to lipid accumulation and production of fatty liver [28]. The results showed that there was no significant change in triglyceride concentration compared with the control group. In addition, there was no significant effect on the concentration of ALP, AST and creatinine in the plasma of dairy cows, indicating that the amount of glycerol added to this experiment did not affect the normal function of the liver, and did not increase liver lipid metabolism. The adding of glycerol-producing yeast increased the concentration of TP in the plasma, which may be due to the increased of feed digestibility in rumen and the promotion of bacterial protein synthesis.

Therefore, we tested the rumen fermentation performance related indicators. The volatile fatty acids in rumen fluid of dairy cows are mainly fermented by rumen microorganisms. The yield and proportion of volatile fatty acids in ruminants are important indicators to determine the nutritional value of the diet and the energy conversion rate of ruminants. The results showed that the proportion of propionic acid in the total volatile fatty acids in rumen fluid was significantly increased ($P<0.05$), which was consistent with the previous report [29-31]. The demand of propionic acid for glucose synthesis is reduced due to the absorption of glycerol by the rumen gastric wall into the gluconeogenesis pathway, which makes more propionic acid used for the synthesis of odd chain fatty acids in breast [32]. The increase of propionic acid ratio in rumen fluid of the treated group may be due to the fermentation of 30-69 % glycerol in rumen [12]. The reason for the decrease of the ratio of ethylene to propylene may be that the concentration of propionic acid was increased and the concentration of acetic acid was decreased after glycerol enters the rumen. Bacterial protein concentration is an ideal index to measure rumen microbial activity and protein synthesis efficiency. The concentration of available ammonia nitrogen in rumen can reflect the balance between protein degradation and protein synthesis. Adding glycerol to the diet of dairy cows had no significant effect on the concentration of bacterial protein in rumen fluid, which indicated that glycerol would not have adverse effects on the synthesis of bacterial protein in rumen and the supply of nitrogen source in rumen [33]. In this study, adding glycerol-producing yeast supplementation to diet increased the concentration of ammonia nitrogen in rumen fluid and the synthesis of bacterial protein. It has been reported that live yeast can promote growth of microorganisms and reduce the loss of nitrogen sources in condition where enough soluble nitrogen and carbohydrates are available [34].

## Conclusion

In summary, glycerol-producing yeast could alleviate the negative energy balance of the perinatal cows by increasing the concentrations of blood glucose and propionic acid. Meanwhile, it could also improve production performance and milk quality. Our study may provide supportive data for the application of glycerol-production yeast supplementation in dairy production.

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