Effects of Candida tropicalis and Mulberry Leaf Flavonoids on Nutrient Metabolism and Rumen Fermentation in Calves (Postprint)

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Abstract

This study was conducted to investigate the effects of dietary supplementation with Candida tropicalis (CT) and mulberry leaf flavonoids (MLF) on energy and nitrogen metabolism, rumen microbial protein synthesis, and rumen fermentation in pre- and post-weaning calves. Forty-eight Chinese Holstein bull calves aged (20$±$2) days were randomly allocated to 4 groups: the control group (CON) was fed a basal diet consisting of milk replacer and starter feed before weaning and starter feed after weaning; the CT group received CT supplementation in the basal diet; the MLF group received MLF supplementation; and the CM group received both CT and MLF supplementation. Calves were weaned from milk replacer at 56 days of age, and the experimental period lasted 60 days. Rumen fluid was collected at 28, 42, 56, and 80 days of age, and digestion and metabolism trials were conducted at 35 and 63 days of age. The results showed that compared with the CT group, the metabolizable energy of calves in the CM group was significantly increased before weaning ($P<0.05$). Compared with the CON group, the nitrogen utilization rate of the CM group was significantly increased after weaning ($P<0.05$). Before weaning, the biological value of nitrogen in the MLF group was significantly higher than that in the CON and CT groups ($P<0.05$). Compared with the CON group, the CT group significantly increased rumen fluid pH from 28 to 80 days of age and rumen microbial protein content at 56 and 80 days of age ($P<0.05$), while rumen fluid ammonia nitrogen concentration showed no significant changes in the CT, MLF, and CM groups ($P>0.05$). Compared with the CON group, total volatile fatty acid concentration and butyric acid content in the MLF group at 56 days of age were significantly increased ($P<0.05$). At 42 days of age, the acetate/propionate ratio in the CT and MLF groups showed an increasing trend compared with the CON group ($P=0.090$), and acetic acid and valeric acid contents showed no significant differences among groups ($P>0.05$). These

results suggest that dietary CT supplementation helps improve rumen fluid pH and promotes rumen microbial protein synthesis in pre-weaning calves. CT and MLF supplementation helps increase dietary metabolizable energy and nitrogen biological value in pre-weaning calves, improve gross energy metabolic rate and nitrogen utilization rate in post-weaning calves, and reduce fecal energy and total nitrogen excretion, while also improving rumen fermentation. Furthermore, the combined use of CT and MLF is superior to single supplementation.

Full Text

Effects of Candida tropicalis and Mulberry Leaf Flavonoids on Nutrient Metabolism and Rumen Fermentation of Calves

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Abstract: This study investigated the effects of dietary supplementation with *Candida tropicalis* (CT) and mulberry leaf flavonoids (MLF) on energy and nitrogen metabolism, rumen microbial protein synthesis, and rumen fermentation in pre- and post-weaning calves. Forty-eight Chinese Holstein bull calves aged (20$±$2) days were randomly assigned to four groups (12 calves per group). The control group (CON) received a basal diet consisting of milk replacer and starter before weaning and starter only after weaning. The CT, MLF, and CM groups received the basal diet supplemented with CT, MLF, and the combination of CT and MLF, respectively. Calves were weaned from milk replacer at 56 days of age, and the experimental period lasted 60 days. Rumen fluid was collected at 28, 42, 56, and 80 days of age, and digestion-metabolism trials were conducted at 35 and 63 days of age. The results showed that compared with the CT group, the metabolizable energy of pre-weaning calves in the CM group was significantly higher ($P<0.05$). Compared with the CON group, the nitrogen utilization of post-weaning calves in the CM group was significantly improved ($P<0.05$), while the nitrogen biological value of pre-weaning calves in the MLF group was significantly higher than that in the CON and CT groups ($P<0.05$). Compared with the CON group, the CT group significantly increased rumen fluid pH from 28 to 80 days of age and microbial protein content at 56 and 80 days of age ($P<0.05$), though rumen ammonia nitrogen concentration was not significantly affected by any treatment ($P>0.05$). The MLF group showed significantly higher total volatile fatty acid concentration and butyrate content at 56 days of age compared with the CON group ($P<0.05$). There was a tendency for increased acetate-to-propionate ratio in the CT and MLF groups compared with the CON group at 42 days of age ($P=0.090$), while acetate and valerate contents

were not significantly affected among groups (P>0.05). In conclusion, dietary supplementation with CT helps improve rumen fluid pH and promotes rumen microbial protein synthesis in pre-weaning calves. The combination of CT and MLF enhances dietary metabolizable energy and nitrogen biological value in pre-weaning calves, improves gross energy metabolizability and nitrogen utilization while reducing fecal energy and total nitrogen excretion in post-weaning calves, and also improves rumen fermentation. The combined use of CT and MLF demonstrates superior effects compared with single supplementation.

Keywords: calf; *Candida tropicalis*; mulberry leaf flavonoids; microbial protein; nutrient metabolism; rumen fermentation

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Calves undergo dramatic changes in immune function and nutrient digestion and metabolism within a short period after birth and during weaning adaptation. Rumen health and development are crucial for nutrient absorption and utilization and for the expression of future production performance. Yeast probiotic substances serve as natural regulators of rumen fermentation in ruminants, promoting rumen development, maintaining stable rumen pH, improving the rumen environment, and enhancing nutrient utilization efficiency. *Candida tropicalis* (CT) shows great potential as a feed additive for ruminants in degrading dietary fiber by activating fiber-degrading bacteria in the rumen, increasing volatile fatty acid (VFA) content such as acetate, propionate, and butyrate to provide energy for ruminants, and improving in vitro dry matter (DM) digestibility. Flavonoids, as plant secondary metabolites widely present in the stems and leaves of most higher plants, regulate lipid and carbohydrate metabolism by modulating the activity and expression of key metabolic enzymes, thereby improving nutrient absorption and utilization efficiency. However, the application effects of single yeast preparations in ruminants have been inconsistent. Some studies have shown that active yeast or yeast culture improved animal performance, while others found no improvement or even reduced performance. Timmerman et al. reported that due to potential synergistic effects among microorganisms and their metabolites, combined use of probiotics from different genera or probiotics with natural plant extracts may be more effective than single-strain probiotics. Therefore, this experiment supplemented calf diets with CT and mulberry leaf flavonoids (MLF) to investigate the interactive effects of CT and MLF on energy and nitrogen metabolism, rumen microbial protein (MCP) synthesis, and rumen fermentation in pre- and post-weaning calves, providing theoretical support for the application of probiotics and plant extracts in calf rearing.

1.1 Experimental Time and Location

The experiment was conducted from August 2014 to November 2014 at Beijing Sanyuan Lvhe Xijiao Farm No. 1. The experimental period lasted 60 days, including a 7-day preliminary period and a 53-day formal experimental period.

1.2 Experimental Design and Diets

A single-factor randomized design was adopted with four groups. The control group (CON) received the basal diet; the CT group received the basal diet supplemented with CT; the MLF group received the basal diet supplemented with MLF; and the CM group received the basal diet supplemented with both CT and MLF. The CT preparation (viable count 5$×10^{9}$ CFU/g; produced by Beijing Huanong Bioengineering Co., Ltd.) was supplemented at 5$×10^{9}$ CFU per calf per day based on Chung et al. The MLF (flavonoid content 50 mg/g; extraction process: mulberry leaves were physically crushed, ultrasonically extracted, filtered, concentrated under reduced pressure, and vacuum-dried into powder; produced by Xi' an Feida Biotechnology Co., Ltd.) was supplemented at 3 g per calf per day based on Chen et al. The basal diet consisted of antibiotic-free and microbe-free milk replacer and starter, with the milk replacer produced by Beijing Precision Animal Nutrition Research Center according to the national invention patent CN 02128844.5. The nutrient levels of the basal diet are shown in Table 1 .

1.3 Experimental Animals and Management

Forty-eight Chinese Holstein bull calves born naturally with birth weight $(40\$\pm 2.5)kg, fedadequatecolostrumandfreshmilk, aged(20\pm\$2)$ days were selected and randomly divided into four groups (12 calves per group). The preliminary period (21–27 days of age) involved transition to milk replacer, with complete replacement by 27 days of age. The milk replacer was prepared with boiled water cooled to 50–60°C at a DM concentration of 12.5%, fed when temperature dropped to approximately 40°C, with two daily feedings (08:00 and 15:00) at 12% of body weight per day (adjusted every two weeks based on weight gain). Transition off milk replacer occurred from 50–56 days of age, with complete weaning at 56 days. Required additives were mixed into the milk replacer or starter during morning feeding. Starter was offered ad libitum throughout the trial with clean, abundant water available. Calves were individually housed in calf hutches (1.6 m × 3.6 m each).

1.4 Sample Collection and Analysis

1.4.1 Feed Samples

Representative samples of milk replacer and starter were collected during the experiment. Nutrient composition was determined according to AOAC (2000) methods: gross energy (GE) using a PARR-6400 automatic oxygen bomb calorimeter; crude protein (CP) using a KDY-9830 automatic Kjeldahl nitrogen analyzer; ether extract (EE) using an ANKOM-XT15i automatic fat analyzer; and organic matter (OM), neutral detergent fiber (NDF), acid detergent fiber (ADF), calcium (Ca), and phosphorus (P) contents.

1.4.2 Digestion-Metabolism Trial Samples

Four healthy calves near the average body weight were selected from each group for total collection of feces and urine using metabolism cages (Patent No. ZL 201420358189.7) at 35 and 63 days of age to estimate pre- and post-weaning nutrient metabolism. Each trial lasted 7 days (3-day preliminary period, 4-day collection period). Daily feed intake, fecal output, and urine volume were recorded. During the collection period, 10% of daily total feces was continuously collected as a composite sample, with 10 mL of 10% dilute hydrochloric acid added per 100 g fresh feces for nitrogen fixation. One percent of daily total urine was continuously collected as a composite sample, adjusted to pH $\leq$ 3 with 10% dilute hydrochloric acid. Representative samples of milk replacer and starter were collected daily. All feed, fecal, and urine samples were stored at -20°C pending analysis. DM, GE, and CP contents in milk replacer, starter, and feces, as well as urine energy and nitrogen, were determined according to AOAC (2000) methods using the aforementioned instruments. Dietary digestible energy, metabolizable energy, apparent GE digestibility, GE metabolizability, and digestible energy metabolizability were calculated as follows:

Digestible energy = Intake GE - Fecal energy
Metabolizable energy = Intake GE - Fecal energy - Urine energy - Methane energy
Apparent GE digestibility = Digestible energy / Intake GE
GE metabolizability = Metabolizable energy / Intake GE
Digestible energy metabolizability = Metabolizable energy / Digestible energy

Methane energy was calculated as 8% of GE.

1.4.3 Rumen Fluid Samples

Four calves near the group average weight were selected from each group. Rumen content (100 mL) was collected using a sterilized oral tube before morning feeding at 28, 42, 56, and 80 days of age, filtered through four layers of gauze, and immediately measured for pH using a portable pH meter (testo-206-pH2). Samples were aliquoted into 10 mL sterilized centrifuge tubes, transported in liquid nitrogen, and stored at -80°C pending analysis. Rumen fluid was thawed at 4°C, and 1 mL supernatant was mixed with 0.3 mL of 25% metaphosphoric acid, vortexed for 3–5 seconds, left to stand for 30 minutes, then centrifuged at 15,000×g for 15 minutes. The supernatant was aliquoted into 0.5 mL portions. VFA content was determined according to Cao et al., ammonia nitrogen (NH_3-N) concentration was measured by the indophenol method, and MCP content was determined according to Makkar et al.

1.5 Statistical Analysis

Data were analyzed using SAS 9.2 software. Except for energy and nitrogen data from digestion-metabolism trials analyzed using one-way ANOVA, other data were analyzed using the MIXED model. When significant differences were detected ($P<0.05$), the least significant difference (LSD) method was used for comparison.

One-way ANOVA model:
Yij = μ + Ti + ij
where μ is the mean, T is treatment (i=1,2,3,4) as a fixed effect, and is the residual (j=1···16).

MIXED model:
Yijk = μ + Ti + Dj + TDij + C(T)ik + ijk
where μ is the mean, T is treatment (i=1,2,3,4) as a fixed effect, D is day of age (j=28,42,56,80) as a fixed effect, C is calf (k=1···48) as a random effect, and is the residual.

2.1 Effects of CT and MLF on Feed Intake of Calves

As shown in Table 2 , dietary supplementation with CT and MLF tended to increase total DM intake before weaning (P=0.086), with starter intake in the CM group significantly higher than in the CON group (P<0.05). No significant differences were observed among groups in milk replacer intake or post-weaning starter intake (P>0.05).

2.2 Effects of CT and MLF on Energy Digestion and Metabolism of Calves

Table 3 shows that dietary supplementation with CT and MLF tended to increase GE intake (P=0.067) and digestible energy (P=0.089) in pre-weaning calves, while metabolizable energy in the CM group was significantly higher than in the CON group (P<0.05). Compared with the CON group, the CM group significantly improved GE metabolizability (P<0.05) by reducing fecal energy (P<0.05) after weaning, while no significant differences in GE metabolizability were observed between the CT and MLF groups (P>0.05). Dietary supplementation with CT and MLF had no significant effect on urine energy throughout the trial period (P>0.05).

2.3 Effects of CT and MLF on Nitrogen Digestion and Metabolism of Calves

As shown in Table 4 , dietary supplementation with CT and MLF tended to increase retained nitrogen (P=0.058) and nitrogen utilization (P=0.062) in pre-weaning calves, with the MLF group showing significantly higher nitrogen biological value than the CON and CT groups (P<0.05). After weaning, the CT and CM groups significantly improved nitrogen utilization compared with other groups (P<0.05) by reducing urinary nitrogen and total nitrogen excretion. Dietary supplementation with CT and MLF also tended to increase retained nitrogen (P=0.076) and nitrogen biological value (P=0.068) post-weaning. However, CT and MLF supplementation had no significant effect on nitrogen intake or fecal nitrogen excretion before or after weaning (P>0.05).

2.4 Effects of CT and MLF on Rumen Fluid pH, NH_3-N Concentration, and MCP Content

Table 5 shows that dietary supplementation with CT increased rumen fluid pH, with the CT group significantly higher than the CM and MLF groups at 28 and 42 days of age ($P<0.05$). No significant effects of CT and MLF supplementation on rumen NH_3-N concentration were observed before or after weaning ($P>0.05$). Rumen MCP content increased significantly with calf age ($P<0.05$), with differences among groups after weaning: the CT group at 56 and 80 days and the MLF group at 80 days were significantly higher than the CON group ($P<0.05$).

2.5 Effects of CT and MLF on VFA Contents in Rumen Fluid of Calves

As shown in Table 6 , supplementation with CT and MLF influenced rumen VFA profiles, with total volatile fatty acid (TVFA) concentration and butyrate content increasing significantly with calf age ($P<0.05$). The MLF group had significantly higher TVFA concentration than the CON group at 56 days of age ($P<0.05$), while no significant differences were observed among groups at other ages ($P>0.05$). The CT group significantly reduced propionate content at 42 days of age ($P<0.05$), while the MLF group significantly increased butyrate content at 56 days of age ($P<0.05$). Additionally, the CT and MLF groups tended to increase the acetate-to-propionate ratio compared with the CON group at 42 days of age ($P=0.090$), though acetate and valerate contents were not significantly affected among groups ($P>0.05$).

3.1 Effects of CT and MLF on Energy and Nitrogen Digestion and Metabolism of Calves

Gastrointestinal development directly affects nutrient digestion and utilization. Zhang et al. and Sun et al. reported that dietary supplementation with yeast and flavonoids improved nutrient apparent digestibility in sheep and broilers, respectively. In this study, the CT and MLF combination increased metabolizable energy by improving starter intake and GE intake in pre-weaning calves, while MLF alone and its combination with CT improved apparent GE digestibility and GE metabolizability while reducing fecal energy excretion post-weaning. This suggests that CT and MLF supplementation enhances appetite and GE intake in suckling calves. The primary carbohydrates in milk replacer are lactose, glucose, and galactose, while starter contains mainly starch. Post-weaning supplementation with MLF and its combination with CT may have improved carbohydrate digestion and absorption by stimulating gastrointestinal digestive enzyme secretion or increasing amylase activity, thereby reducing fecal energy.

Regarding nitrogen utilization, MLF and its combination with CT significantly improved nitrogen biological value in pre-weaning calves and nitrogen utilization post-weaning. This may be because CT stimulates various proteolytic bacteria

in the rumen to degrade more dietary CP into MCP, while MLF enhances gastrointestinal digestive enzyme activity, with their interaction synergistically improving nitrogen utilization, consistent with Lee et al. This study also showed that MLF and its combination with CT reduced total nitrogen excretion (23.49–24.71 g/d) post-weaning (63 days of age), lower than the 19.3–34.9 g/d reported by Hill et al. for 8-week-old calves. This may be due to interactions between various metabolites such as small peptides and amino acids produced by MLF and CT, which improved dietary amino acid balance, increased nitrogen utilization, and reduced nitrogen excretion in feces and urine. Combined results for energy and nitrogen metabolism indicate that CT and MLF combination shows clear advantages over single supplementation, possibly because organic acids and vitamins produced by yeast interact with flavonoids to jointly promote energy and nitrogen utilization.

3.2 Effects of CT and MLF on Rumen Fermentation of Calves

Rumen fluid pH, NH_3-N, and VFA are important indicators of fermentation that reflect rumen function and environmental stability. This study showed that CT supplementation increased rumen fluid pH in pre-weaning calves, consistent with Bayatkouhsar et al.' s findings with lactobacilli. Rumen fluid pH significantly affects rumen microorganisms, particularly fiber-degrading bacteria, whose activity increases with higher pH, which is important for establishing beneficial rumen microflora in calves. Rumen NH_3-N concentration is dynamic, reflecting the balance between protein degradation and MCP synthesis, with 6–30 mg/dL being optimal for microbial growth. Anderson et al. found that NH_3-N concentration was significantly lower post-weaning than pre-weaning, which was also observed in this study. This may be because the rumen is functionally immature in newborn calves, and as age increases and microbial flora become established, more NH_3-N is converted to MCP during microbial proliferation, reducing NH_3-N concentration and explaining the age-related increase in rumen MCP. Rumen MCP provides 50–80% of small intestine absorbable protein for ruminants, and its synthesis is primarily related to microbially available energy and protein. The increased MCP synthesis with CT and MLF supplementation indicates that these additives promote rumen microbial utilization of dietary energy and protein, which is consistent with the improved nutrient digestion and utilization observed in the metabolism trials. Pang et al. also reported that dietary supplementation with *Candida utilis* improved rumen microbial utilization efficiency of feed protein and promoted microbial growth without affecting NH_3-N concentration.

3.3 Effects of CT and MLF on Rumen Fermentation Products of Calves

VFA, as important products of rumen fermentation, provide 70–80% of metabolizable energy for ruminants. Acetate, primarily from roughage fermentation, generates ATP for cell maintenance and tissue synthesis through the tricar-

boxylic acid cycle, while propionate, mainly from soluble carbohydrate fermentation, is the primary precursor for blood glucose synthesis after absorption through the rumen wall. This study found that TVFA concentration increased significantly with calf age, indicating gradual development and maturation of rumen function. Supplementation with CT, MLF, and their combination enhanced rumen fermentation, with TVFA concentrations at 56 days of age increasing by 11.83%, 73.98%, and 15.64% in the CT, MLF, and CM groups compared with the CON group, respectively, and TVFA reaching 111.59 mmol/L with MLF supplementation. This is consistent with Sun et al.' s findings with *Bacillus subtilis* natto and Newbold et al.' s report that plant essential oils significantly increased TVFA concentration in sheep, though Spanghero et al. observed decreased VFA with plant extracts. These discrepancies may be related to plant extract type, dosage, and animal species. CT improved rumen fermentation pattern by reducing propionate and increasing the acetate-to-propionate ratio, possibly because CT interacts with other microorganisms in the rumen to stimulate rapid microbial proliferation that utilizes large amounts of propionate, which also explains the enhanced MCP synthesis observed with CT supplementation. MLF significantly increased butyrate content, which is crucial for rumen development in young ruminants as it promotes rumen epithelial cell proliferation and differentiation, enhances gastrointestinal sensitivity, and stimulates intestinal motility, suggesting that MLF supplementation supports rumen development around weaning. However, CT supplementation did not significantly affect butyrate content, similar to findings by Sun et al. with *Bacillus subtilis* natto and Ding et al. with *Candida utilis*.

In summary, dietary supplementation with both CT and MLF positively affected energy and protein utilization in calves. CT primarily stimulates massive rumen microbial growth and proliferation through its various metabolites, increasing rumen MCP content, reducing propionate content, and improving rumen fluid pH and energy and nitrogen utilization. MLF, as an estradiol analog, promotes gastrointestinal digestive enzyme secretion and improves dietary energy and protein digestibility by regulating key metabolic enzyme activity and expression. The combined use of CT and MLF demonstrates advantages in improving nutrient digestion and utilization, promoting MCP synthesis, and enhancing rumen fermentation.

1. Dietary supplementation with CT helps improve rumen fluid pH and promotes rumen microbial protein synthesis in pre-weaning calves.
2. Supplementation with CT and MLF helps increase dietary metabolizable energy and nitrogen biological value in pre-weaning calves, improves gross energy metabolizability and nitrogen utilization while reducing fecal energy and total nitrogen excretion in post-weaning calves, and improves rumen fermentation. The combined use of CT and MLF is superior to single supplementation.

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Note: Figure translations are in progress. See original paper for figures.