Effects of Combined Supplementation of Rumen-Protected Methionine and Cinnamaldehyde on Milk Production Performance and Nitrogen Excretion in Dairy Cows: Postprint

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Abstract

This experiment was conducted to investigate the effects of combined supplementation of rumen-protected methionine (RPMet) and cinnamaldehyde (CA) on milk performance and nitrogen excretion in dairy cows. Forty Holstein dairy cows with similar age, body weight, parity, milk yield, milk composition, and lactation period [(90±15) d] were selected and divided into 10 groups with 4 cows per group. The control (C) group was fed a basal diet, while the experimental groups were supplemented with different level combinations of RPMet and CA. RPMet was set at three levels: 20 (L), 25 (M), and 30 (H) g/(d·head); CA was set at three levels: 15 (L), 18 (M), and 21 (H) g/(d·head), forming nine different level combinations: LL, ML, HL, LM, MM, HM, LH, MH, and HH (the first letter represents the RPMet supplementation level, the second letter represents the CA supplementation level). The pre-trial period was 15 d, and the formal trial period was 60 d. The results showed: 1) Except for the LH group, milk yield in all experimental groups was significantly or extremely significantly higher than that in the C group ($P<0.05$ or $P<0.01$), with the HL group being the highest. 2) Milk fat percentage and milk protein percentage in all experimental groups were higher than those in the C group, with the HL group being the highest, showing extremely significant differences from the C group ($P<0.01$); milk somatic cell count in all experimental groups was lower than that in the C group, with the HL group being the lowest, showing extremely significant differences from the C group ($P<0.01$). 3) Except for the LH group, nitrogen apparent digestibility and nitrogen lactation conversion efficiency in all experimental groups were significantly or extremely significantly higher than those in the C group ($P<0.05$ or $P<0.01$), with the HL group being the highest; total nitrogen excretion in the LL, ML, HL, LM, MM, HM, LH, MH, and HH groups was reduced by 17.45% ($P<0.01$), 18.79% ($P<0.01$),

20.80% (P<0.01), 10.41% (P<0.01), 12.49% (P<0.01), 15.22% (P<0.01), 3.37% (P>0.05), 5.12% (P<0.05), and 7.43% (P<0.05) compared with the C group, respectively, with the HL group being the lowest. The results suggest that supplementation of RPMet and CA in the diet of lactating dairy cows can improve milk performance and reduce nitrogen excretion; considering the above indicators comprehensively, the optimal combination is RPMet 30 g/(d · head) and CA 15 g/(d · head).

Full Text

Effects of Rumen-Protected Methionine and Cinnamic Aldehyde on Lactation Performance and Nitrogen Excretion of Dairy Cows

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Abstract

This experiment was conducted to investigate the effects of combined supplementation of rumen-protected methionine (RPMet) and cinnamic aldehyde (CA) on lactation performance and nitrogen excretion in dairy cows. Forty Holstein dairy cows with similar age, body weight, parity, milk yield, milk composition, and lactation stage [(90±15) days] were allocated into 10 groups with 4 cows per group. The control (C) group received a basal diet, while experimental groups were supplemented with different level combinations of RPMet and CA. RPMet was supplemented at three levels: 20 (L), 25 (M), and 30 (H) g/(d · head); CA was supplemented at three levels: 15 (L), 18 (M), and 21 (H) g/(d · head), forming nine different combinations: LL, ML, HL, LM, MM, HM, LH, MH, and HH (the first letter indicates RPMet level, the second indicates CA level). The pre-trial period lasted 15 days, followed by a 60-day formal experimental period. The results showed: (1) Except for the LH group, milk yield in all experimental groups was significantly or extremely significantly higher than in the C group (P<0.05 or P<0.01), with the HL group achieving the highest yield. (2) Milk fat percentage and milk protein percentage in all experimental groups exceeded those of the C group, with the HL group showing the highest values and extremely significant differences from the C group (P<0.01). Milk somatic cell count in all experimental groups was lower than in the C group, with the HL group having the lowest count and extremely significant differences from the C group (P<0.01). (3) Except for the LH group, nitrogen apparent digestibility and nitrogen conversion efficiency for lactation in experimental groups were significantly or extremely significantly higher than in the C group (P<0.05 or P<0.01), with the HL group showing the highest values.

Total nitrogen excretion in the LL, ML, HL, LM, MM, HM, LH, MH, and HH groups decreased by 17.45% ($P<0.01$), 18.79% ($P<0.01$), 20.80% ($P<0.01$), 10.41% ($P<0.01$), 12.49% ($P<0.01$), 15.22% ($P<0.01$), 3.37% ($P>0.05$), 5.12% ($P<0.05$), and 7.43% ($P<0.05$) respectively compared with the C group, with the HL group showing the lowest excretion. These results indicate that dietary supplementation with RPMet and CA can improve lactation performance and reduce nitrogen excretion in dairy cows. Considering all these indices, the optimal combination is 30 g/(d · head) RPMet and 15 g/(d · head) CA.

Keywords: rumen-protected methionine; cinnamic aldehyde; lactation performance; nitrogen excretion

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In recent years, the rapid development of China' s dairy industry, particularly the increasing intensification and scale of operations, has effectively alleviated the supply-demand contradiction in the dairy market. However, dairy manure has simultaneously caused severe environmental pollution, with nitrogen pollution recognized as a major environmental threat. The rumen of ruminants represents a relatively stable anaerobic fermentation system characterized by dynamic equilibrium relationships among host microorganisms and between different microbial populations [1]. Due to rumen microbial degradation of dietary protein and deamination of amino acids, dietary protein cannot efficiently provide amino acids for rumen microbes and the host. Excessive ammonia nitrogen (NH -N) also exceeds microbial utilization capacity, resulting in waste of dietary protein. Therefore, employing nutritional regulation techniques to improve dietary protein utilization and reduce nitrogen excretion without affecting lactation performance holds significant importance for addressing nitrogen pollution in dairy production.

Methionine serves as the first or second limiting amino acid for ruminants, particularly crucial for high-yielding dairy cows and known as a "life amino acid" [2]. Zou et al. [3] reported that supplementing early-lactation Holstein cows with rumen-protected methionine (RPMet) significantly increased milk yield, milk protein percentage, and milk specific gravity, while also improving milk fat percentage and non-fat solids content. Yang [4] found that intraruminal infusion of methionine coated with animal oil significantly increased digestible nitrogen and retained nitrogen in beef cattle. Cinnamic aldehyde (CA), also known as cinnamaldehyde or phenylacrolein, is a yellow liquid that can be extracted from cinnamon plants or synthesized artificially [5]. Cardozo et al. [6] discovered that low levels of cinnamon oil could reduce milk urea nitrogen content and somatic cell count. Cao [7] observed a significant linear increase in feed conversion efficiency when supplementing beef cattle diets with 300, 600, or 900 mg/(d · head) CA. Our research group previously investigated the effects of RPMet and CA separately on dairy cow lactation performance and nitrogen excretion, determining the optimal supplemental levels to be 25 and 18 g/(d · head), respectively [8-9]. Currently, few studies have reported on the combined use of RPMet and CA in dairy cows, and the optimal combination of supple-

mental levels remains unclear. Based on our previous findings, this experiment established three levels of RPMet (optimal level 25±5 g) and three levels of CA (optimal level 18±3 g), creating nine different combinations to explore the optimal RPMet and CA combination. The objective was to improve dietary protein utilization and lactation performance while reducing nitrogen excretion, thereby providing a theoretical basis for the combined application of RPMet and CA in dairy production.

1.1 Experimental Design

This study utilized 40 Holstein dairy cows from Qingdao Aote Dairy Farm with similar age, body weight, parity, milk yield, milk composition, and lactation stage [(90±15) days]. The cows were randomly divided into 10 groups with 4 cows per group. The control (C) group received a basal diet, while experimental groups were supplemented with different combinations of RPMet and CA. RPMet was supplemented at three levels: 20, 25, and 30 g/(d · head); CA was supplemented at three levels: 15, 18, and 21 g/(d · head), forming nine different combinations. The experimental design is presented in Table 1 .

Each cow was reserved 0.5 kg of concentrate daily as a carrier to mix with RPMet and CA. The remaining concentrate was mixed with roughage to form a total mixed ration (TMR). RPMet and CA were mixed with the concentrate and then fed together with the TMR. The entire experimental period lasted 75 days, including a 15-day pre-trial period and a 60-day formal experimental period. The RPMet (rumen bypass rate 85%) and CA used in this experiment were purchased from Qingdao Runbot Biological Technology Co., Ltd. The RPMet was a white granular material composed of DL-methionine and silicon dioxide, with DL-methionine 60% and moisture 12%. The CA was a white powder composed of CA, silicon dioxide, and starch, with CA 5% and moisture 12%.

1.2 Feeding Management

Cows were milked twice daily (04:00 and 16:00) using a DeLaval milking machine and fed TMR twice daily (04:30 and 16:30), ensuring cows had access to TMR for over 20 hours per day. After feeding, cows had free access to water and exercise in the exercise yard. Routine deworming, lighting, and management practices were implemented according to standard procedures. The composition and nutrient levels of the TMR are shown in Table 2 .

1.3.1 Feed Intake Measurement

Cows were fed in separate pens, with individual feed intake recorded for each cow. During the pre-trial period, feed offered and refused was recorded on days 1-3, 5-7, 9-11, and 13-15 to calculate individual feed intake. After the pre-trial period, average feed intake during this period was calculated. During the formal experimental period, feed intake was recorded every 10 days for a total of 6 recordings, with each recording lasting 3 consecutive days. Average feed

intake was calculated based on these 3-day periods to adjust TMR allocation for the subsequent stage. After the formal experimental period, average feed intake during the entire period was calculated from the 6 recordings to determine intake of major nutrients.

1.3.2 TMR and Fecal Sample Collection and Analysis

TMR samples were collected using the quartering method, dried at 65°C in an oven to produce air-dried samples, and then ground for analysis [11]. Fecal samples were collected three times during the pre-trial period (days 1-3) and formal experimental period (days 28-30 and 58-60) using the total collection method, with fecal samples collected from all 4 cows in each group. Before collection, cow beds were thoroughly cleaned. All fecal samples were collected promptly each day, mixed thoroughly, and weighed. Daily fecal samples were collected using the quartering method, with 25 mL of 10% sulfuric acid added per 100 g of feces for nitrogen fixation before storage at -20°C. After the 3-day collection period, the preserved fecal samples were mixed proportionally by weight, dried at 65°C to constant weight, and stored.

TMR samples were analyzed for moisture content using GB/T6435-2006 to calculate dry matter (DM) content; crude protein (CP) content using the Kjeldahl method (GB/T 6432-1994); acid detergent fiber (ADF) content using NY/T 1459-2007; neutral detergent fiber (NDF) content using GB/T20806-2006; calcium (Ca) content using the potassium permanganate method (GB/T6436-2002); and phosphorus (P) content using spectrophotometry (GB/T6437-2002). Fecal crude protein content was determined using the same method as for TMR samples.

1.3.3 Urine Sample Collection and Analysis

Urine samples were collected three times during the pre-trial period (days 1-3) and formal experimental period (days 28-30 and 58-60) using the spot urine collection method described by Zhu [12]. Samples were collected using a combination of manual collection and bladder catheterization. Cows were restrained using neck clamps, and a catheter was inserted into the bladder to collect urine from each cow sequentially. If a cow exhibited spontaneous urination during collection, a dedicated personnel collected the urine. Urine was collected twice daily at 12-hour intervals for 3 consecutive days, with collection times delayed by 4 hours each day relative to the previous day. Collected urine was acidified with concentrated sulfuric acid (98%) to adjust pH (pH<3) and stored at -20°C.

1.3.4 Milk Sample Collection and Analysis

Cows were milked twice daily (04:00 and 16:00) using a DeLaval herringbone milking system, with milk yield displayed automatically. Milk yield was recorded every 5 days during both pre-trial and formal experimental periods, with each recording lasting 3 consecutive days and the average calculated.

On days 15, 30, 45, and 60 of the formal experimental period, 65 mL of milk samples were collected proportionally to morning and evening milk yields. Of this, 50 mL was preserved with potassium dichromate (0.6 mg/mL), mixed thoroughly, and stored at 4°C for milk composition analysis. The remaining 15 mL was centrifuged to remove milk fat and protein, and 1.5 mL of the processed sample was stored at -20°C for milk urea nitrogen determination. Milk fat percentage, milk protein percentage, milk lactose percentage, and somatic cell count were measured using an automatic milk composition and somatic cell analyzer (CombiFoss FT+, Foss, Denmark) at the Dairy Performance Testing Laboratory of Shandong Academy of Agricultural Sciences. Weighted averages were used to calculate milk composition during the formal experimental period.

1.3.5 Nitrogen Metabolism Indices

Urine nitrogen content was determined using a Foss KjeltecTM 8200 Kjeldahl nitrogen analyzer (Foss, Denmark). Urine creatinine content was measured using the picric acid colorimetric method [13] with a UV-1800PC spectrophotometer (Shanghai Mapada Instruments Co., Ltd.). Reagent kits were purchased from Nanjing Jiancheng Bioengineering Institute. Following the method of Valadares et al. [13], urinary creatinine (approximately 29 mg per kg body weight per day) was used as a marker to estimate daily urine volume.

Nitrogen metabolism was calculated using the following formulas: - Fecal nitrogen (g/d) = daily fecal output × fecal crude protein content × 0.16 - Total nitrogen excretion (g/d) = fecal nitrogen + urinary nitrogen - Nitrogen apparent digestibility (%) = [(dietary nitrogen intake - fecal nitrogen) / dietary nitrogen intake] × 100 - Nitrogen conversion efficiency for lactation (%) = (milk nitrogen / dietary nitrogen intake) × 100

1.4 Data Processing and Analysis

Experimental data were initially processed using Excel 2016 software. One-way ANOVA was performed using SPSS 20.0 software, with Duncan' s multiple comparison test used to examine significant differences between groups. $P<0.05$ and $P<0.01$ were considered statistically significant and extremely significant, respectively. Results are expressed as means ± standard error.

2.1 Effects of Different RPMet and CA Combinations on Major Nutrient Intake in Dairy Cows

As shown in Table 3 , dietary supplementation with different combinations of RPMet and CA tended to increase major nutrient intake in dairy cows, though no significant differences were observed between experimental and C groups ($P>0.05$).

2.2 Effects of Different RPMet and CA Combinations on Milk Yield of Dairy Cows

Table 4 shows that milk yield in the LL, ML, HL, LM, MM, HM, LH, MH, and HH groups increased by 16.07% ($P<0.01$), 19.40% ($P<0.01$), 22.17% ($P<0.01$), 12.49% ($P<0.01$), 13.46% ($P<0.01$), 15.27% ($P<0.01$), 3.94% ($P>0.05$), 6.60% ($P<0.05$), and 9.30% ($P<0.01$), respectively, compared with the C group, with the HL group achieving the highest yield.

2.3 Effects of Different RPMet and CA Combinations on Milk Composition of Dairy Cows

Table 5 demonstrates that milk fat percentage was highest in the HL group, which was extremely significantly higher than the C, LM, LH, MH, and HH groups ($P<0.01$) and significantly higher than the MM group ($P<0.05$). Milk protein percentage was also highest in the HL group, being extremely significantly higher than the C, LM, LH, MH, and HH groups ($P<0.01$) and significantly higher than the MM and HM groups ($P<0.05$). Milk somatic cell count decreased with RPMet and CA supplementation, with the HL group showing the lowest count and an extremely significant difference from the C group ($P<0.01$). No significant differences in milk lactose percentage were observed among groups ($P>0.05$).

2.4 Effects of Different RPMet and CA Combinations on Nitrogen Apparent Digestibility and Excretion in Dairy Cows

Table 6 reveals that RPMet and CA supplementation reduced both fecal and urinary nitrogen excretion. Total nitrogen excretion in the LL, ML, HL, LM, MM, HM, LH, MH, and HH groups decreased by 17.45% ($P<0.01$), 18.79% ($P<0.01$), 20.80% ($P<0.01$), 10.41% ($P<0.01$), 12.49% ($P<0.01$), 15.22% ($P<0.01$), 3.37% ($P>0.05$), 5.12% ($P<0.05$), and 7.43% ($P<0.05$), respectively, compared with the C group, with the HL group showing the lowest excretion. Nitrogen apparent digestibility and nitrogen conversion efficiency for lactation increased with RPMet and CA supplementation, with all experimental groups except LH showing significant or extremely significant differences from the C group ($P<0.05$ or $P<0.01$).

3.1 Effects of Different RPMet and CA Combinations on Major Nutrient Intake in Dairy Cows

Dry matter intake (DMI) is a crucial factor affecting dairy cow performance, and increasing DMI provides more nutrients and energy for lactation. In this experiment, experimental groups receiving lower CA levels showed relatively higher DMI, suggesting that low-level CA tended to increase DMI, while RPMet had minimal effect on DMI. Wu et al. [14] observed a tendency for increased DMI in sheep fed N-acetyl-DL-methionine. Zhang et al. [15] also reported that feeding a combination of garlic oil and CA tended to increase DMI in dairy cows. The

increased DMI following RPMet and CA supplementation may be attributed to improved rumen environment and enhanced nutrient digestion and absorption in the rumen and intestine. Dietary CA supplementation can increase saliva secretion [5], and the alkaline nature of saliva helps maintain stable rumen pH. Additionally, small amounts of methionine released from RPMet in the rumen can improve the rumen nutritional environment and promote microbial growth and reproduction [16], accelerating feed degradation rate. Furthermore, CA can promote digestive juice secretion, enhance digestive function, and relieve gastrointestinal smooth muscle spasms and cramping pain, which also contributes to increased DMI [5].

3.2 Effects of Different RPMet and CA Combinations on Milk Yield of Dairy Cows

Milk yield is a key indicator of lactation performance. Under the conditions of this experiment, groups receiving lower CA levels showed significantly increased milk yield. When CA supplementation level was constant, groups with higher RPMet levels showed greater improvements in milk yield, with the HL group performing best. Han et al. [17] and Zhang et al. [15] reported increased milk yield in dairy cows supplemented with RPMet and a combination of garlic oil and CA, respectively. Rhoads et al. [18] found that the lactation system in dairy cows is primarily regulated by the growth hormone (GH) axis, with GH at its core. Macrina et al. [19] observed that GH treatment in early-lactation cows increased milk yield by 36% compared with the control group. The interaction between GH and insulin (INS) can accelerate mammary gland metabolism and promote nutrient transport to the mammary gland, providing more precursors for milk synthesis [20]. Dietary RPMet and CA supplementation can increase serum GH and insulin-like growth factor I (IGF-I) concentrations [21-22], with IGF-I promoting mammary gland development and mammary cell proliferation, thereby indirectly stimulating lactation [23]. CA can reduce dietary protein degradation rate in the rumen and increase the amount of amino acids reaching the small intestine [24], positively contributing to increased milk yield. Additionally, both RPMet and CA can improve the rumen environment, promote microbial growth and reproduction, and enhance nutrient digestion and absorption, which also benefits milk yield improvement.

3.3 Effects of Different RPMet and CA Combinations on Milk Composition of Dairy Cows

Milk fat and protein percentages are important indicators of milk quality. In this experiment, RPMet and CA supplementation increased both milk fat and protein percentages. Han et al. [17] reported that supplementing 12 g/d RPMet to dairy cows during summer increased milk fat and protein percentages while reducing somatic cell count. Zhang et al. [15] found that supplementing a combination of garlic oil and CA to early-lactation cows significantly increased milk yield and reduced somatic cell count. During peak lactation, milk pro-

tein percentage is generally low because the increase in DMI lags behind the increase in milk yield, resulting in negative energy balance. Therefore, RPMet supplementation is necessary during early lactation to ensure milk quality [25]. Direct methionine supplementation would be largely degraded by rumen microbes, with insufficient methionine reaching the small intestine for absorption, negating the supplementation effect. This study demonstrated that when CA level was constant, higher RPMet levels were more effective in increasing milk fat and protein percentages, with the HL group showing the best results. The increased milk fat and protein percentages may be attributed to elevated GH and INS concentrations following RPMet and CA supplementation. GH can promote synthesis of acetyl-CoA carboxylase, fatty acid synthase, and lipoprotein lipase (with acetyl-CoA carboxylase being the rate-limiting enzyme for fatty acid synthesis) [23], and increased synthesis of these three enzymes facilitates fatty acid synthesis, providing more precursors and energy for lactation. INS is an essential hormone for mammary gland development and function, directly regulating protein synthesis in mammary tissue [26]. The interaction between GH and INS can significantly increase milk yield and milk protein production in early-lactation cows [27]. Additionally, RPMet and CA can improve the rumen nutritional environment and promote microbial protein synthesis, providing more precursors for milk protein synthesis. The small amount of rumen-protected fat in RPMet can provide raw materials for milk fat synthesis [28], and CA has blood glucose and lipid-regulating effects [5], both contributing to increased milk fat percentage.

Milk somatic cell count is an important indicator of milk quality and udder health; lower somatic cell counts indicate better udder health and lower incidence of subclinical mastitis. Under the conditions of this experiment, combined RPMet and CA supplementation reduced somatic cell count, indicating improved udder health. Both RPMet and CA can enhance immune function: RPMet can scavenge free radicals, reduce lymphocyte apoptosis, and improve immunity and antioxidant capacity [17], while CA can significantly increase lymphocyte proliferation and dramatically activate macrophage phagocytosis [29]. Additionally, CA exhibits strong antibacterial properties; its aldehyde group is hydrophilic and can be adsorbed by hydrophilic groups on fungal surfaces, disrupting cell wall polysaccharide structures and penetrating cell walls [30]. These antibacterial effects and immune-enhancing functions contribute to reduced somatic cell count and improved udder health.

3.4 Effects of Different RPMet and CA Combinations on Nitrogen Apparent Digestibility and Excretion in Dairy Cows

NH -N loss in the rumen is a major factor contributing to low dietary protein utilization efficiency in dairy cows. Therefore, employing nutritional regulation techniques to improve NH -N utilization is crucial for enhancing nitrogen efficiency and reducing nitrogen excretion. Methionine, as the first or second limiting amino acid for ruminants [2], not only affects amino acid balance but

also limits the utilization of other amino acids when deficient. Unutilized amino acids are converted to urea via the ornithine cycle and excreted in urine. This experiment demonstrated that groups receiving lower CA levels showed extremely significant reductions in total nitrogen excretion. When CA supplementation level was constant, groups with higher RPMet levels showed the greatest reductions in nitrogen excretion, with the HL group performing best. CA can reduce protein degradation rate in the rumen [24], decreasing nitrogen loss from rapid protein decomposition. Small amounts of methionine released from RPMet in the rumen can improve the rumen nutritional environment and promote microbial growth and reproduction [16], accelerating microbial utilization of NH -N and reducing NH -N loss. The regulatory effects of RPMet and CA on NH -N production and utilization rates help improve their balance, enhance nitrogen utilization, and reduce nitrogen excretion. Effective release of methionine from RPMet in the post-ruminal digestive tract increases methionine availability in the small intestine, promoting a more balanced amino acid profile and improving small intestine amino acid utilization [31], thereby reducing nitrogen excretion. CA can promote butyrate secretion in the intestine, which not only stimulates proliferation of digestive tract cells but also induces pancreatic secretion of large amounts of digestive enzymes, improving nutrient absorption [32-33] and contributing to reduced nitrogen excretion.

Combined supplementation of RPMet and CA in dairy cow diets can improve lactation performance and reduce nitrogen excretion. Considering all these indices, the optimal combination is 30 g/(d · head) RPMet and 15 g/(d · head) CA.

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