Effects of Small Peptides on Rumen Microbial Protein Production, Milk Production Performance, and Nitrogen Excretion in Dairy Cows: Postprint

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Abstract

The present experiment was conducted to investigate the effects of small peptides (SP) on rumen microbial protein production, milk performance, and nitrogen excretion in dairy cows. Forty Holstein cows with similar age, body weight, milk yield, milk composition, and lactation period [(45$±$15) d] were selected and allocated to 4 groups (n=10 per group). The control group and experimental groups 1, 2, and 3 were supplemented with 0, 50, 100, and 150 g/(d · head) SP, respectively. The pre-trial period was 15 d, and the formal trial period was 60 d. The results showed that: 1) Rumen microbial protein production in the experimental groups was significantly higher than that in the control group (P<0.05), with experimental groups 1, 2, and 3 increasing by 17.38%, 22.94%, and 12.22% compared with the control group, respectively. 2) Milk yield in the experimental groups was significantly higher than that in the control group (P<0.05), increasing by 9.93%, 12.64%, and 7.53% compared with the control group, respectively; SP significantly increased milk fat percentage and milk protein percentage (P<0.05), and significantly decreased milk somatic cell count (P<0.05) (with experimental group 2 being the lowest). 3) In terms of total nitrogen excretion, the experimental groups were significantly lower than the control group (P<0.05), with experimental groups 1, 2, and 3 decreasing by 13.31%, 15.01%, and 9.43%, respectively. Under the conditions of this experiment, considering rumen microbial protein production, milk yield, milk composition, and nitrogen excretion, SP supplementation at 100 g/(d · head) was the most beneficial.

Full Text

Effects of Small Peptides on Ruminal Microbial Protein Production, Milk Performance and Nitrogen Excretion in Dairy Cows

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Abstract: This experiment was conducted to investigate the effects of small peptides (SP) on ruminal microbial protein production, milk performance, and nitrogen excretion in dairy cows. Forty Holstein cows with similar age, body weight, milk yield, milk composition, and lactation stage [(45$±$15) days in milk] were allocated to four groups of ten cows each. The control group and test groups 1, 2, and 3 were supplemented with 0, 50, 100, and 150 g/(d · head) of SP, respectively. The experiment consisted of a 15-day preliminary period followed by a 60-day formal trial period. The results showed: (1) Ruminal microbial protein production in the test groups was significantly higher than in the control group ($P<0.05$), with increases of 17.38%, 22.94%, and 12.22% for test groups 1, 2, and 3, respectively. (2) Milk yield in the test groups was significantly higher than in the control group ($P<0.05$), with increases of 9.93%, 12.64%, and 7.53% for test groups 1, 2, and 3, respectively. SP supplementation significantly increased milk fat percentage and milk protein percentage ($P<0.05$) and significantly reduced milk somatic cell count ($P<0.05$), with test group 2 showing the lowest values. (3) Total nitrogen excretion in the test groups was significantly lower than in the control group ($P<0.05$), with reductions of 13.31%, 15.01%, and 9.43% for test groups 1, 2, and 3, respectively. Under the conditions of this experiment and considering ruminal microbial protein production, milk yield, milk composition, and nitrogen excretion comprehensively, supplementation with 100 g/(d · head) of SP was most beneficial.

Keywords: small peptides; ruminal microbial protein; milk performance; nitrogen excretion

Introduction

In recent years, the intensification and scaling-up of dairy farming in China have effectively alleviated the supply-demand contradiction in the dairy market, but have also generated substantial cow manure that causes serious environmental pollution, with nitrogen being particularly problematic. Although harmless treatments such as solid-liquid separation, anaerobic fermentation, and wastewater purification can mitigate nitrogen pollution, their high costs make widespread implementation difficult in small and medium-sized dairy farms. Nutritional regulation technology offers a more cost-effective alternative that

can also generate economic benefits, making it more acceptable to small and medium-sized operations. Developing nutritional strategies that improve dairy cow performance while enhancing nitrogen utilization and reducing nitrogen excretion represents an important approach to addressing nitrogen pollution and accelerating sustainable development in dairy farming.

Small peptides (SP) typically refer to oligopeptides composed of 2-3 amino acids. They are important products of dietary protein degradation by digestive enzymes and can be absorbed intact into the circulatory system, often more readily than free amino acids [1]. Wang et al. [2] found that peptide supplementation significantly improved milk yield, milk protein percentage, and milk fat percentage in dairy cows. Wang et al. [3] demonstrated through ruminal infusion experiments that soybean SP improved nutrient digestibility and increased nitrogen retention in beef cattle. Current research on SP in dairy production has focused primarily on milk yield, with limited reports on whether dietary SP supplementation can increase microbial crude protein (MCP) production and reduce nitrogen excretion. This experiment investigated the effects of different dietary SP levels on ruminal MCP production, milk performance, and nitrogen excretion in dairy cows, aiming to improve milk production, conserve protein feed resources, and reduce nitrogen excretion to provide a reference for healthy and sustainable dairy industry development.

Materials and Methods

1.1 Small Peptide Product

The SP product was purchased from a company in Harbin as a brown powder. The main raw material was soybean, with specifications of crude protein (CP) $ \$50\%$, peptide content $ \$40\%$, moisture $ \$8\%$, and ash $ \$8\%$ (dry matter basis). The carrier concentrate was calf starter provided by Qingdao Aote Dairy Farm.

1.2 Experimental Design

A single-factor randomized block design was employed. Forty healthy Holstein cows from Qingdao Aote Dairy Farm with similar body condition, age, body weight, milk yield, milk composition, and lactation stage [(45$\pm$\$15) days in milk] were divided into four groups of ten cows each. The control group and test groups 1, 2, and 3 received SP supplementation at 0, 50, 100, and 150 g/(d $\cdot$ head), respectively. The SP was mixed with 0.25 kg of calf starter carrier, divided into two equal portions, and fed twice daily with the total mixed ration (TMR). The composition and nutrient levels of the TMR are shown in Table 1 .

The entire trial lasted 75 days, including a 15-day preliminary period and a 60-day formal trial period. Cows were housed in a barn, milked twice daily (03:30 and 15:30) using Lely milking equipment, and fed TMR twice daily (04:00 and 16:00), ensuring cows had access to TMR for at least 20 hours per day. After

feeding, cows had free access to exercise areas and water, with routine lighting, deworming, and management practices.

1.3 Sample Collection

1.3.1 Feed Samples TMR and carrier calf starter samples were collected using the quartering method, dried in a 65°C oven to produce air-dried samples, and ground for subsequent analysis.

1.3.2 Fecal Samples Fecal samples were collected on days 1-3 of the preliminary period, days 28-30 of the formal period, and days 58-60 of the formal period using the total collection method. Feces from all ten cows in each group were collected continuously for three days during each collection period. Barn floors were cleaned before collection, and feces were collected in buckets immediately after defecation. Daily fecal collections were mixed and weighed, and samples were taken using the quartering method. For every 100 g of fecal sample, 25 mL of 10% sulfuric acid was added for nitrogen fixation before storage at -20°C.

1.3.3 Urine Samples Urine samples were collected on the same schedule as fecal samples using the spot urine collection method described by Zhu [6], combining manual collection with bladder catheterization. During each sampling, cows were restrained with neck clamps and catheterized to collect bladder urine. If cows urinated spontaneously during the procedure, personnel collected the urine. Samples were collected from all ten cows in each group for three consecutive days, twice daily at 12-hour intervals, with collection times delayed by 4 hours each day. Concentrated sulfuric acid (98%) was added to adjust urine pH below 3, and samples were stored at -20°C.

1.3.4 Milk Samples Milk samples were collected on the first day of the preliminary period and every 15 days during the formal period. Morning and evening milk samples were pooled proportionally to milk yield to obtain 65 mL total. Of this, 50 mL was mixed with potassium dichromate preservative (0.6 mg/mL) and refrigerated at 4°C for milk composition analysis. The remaining 15 mL was centrifuged at 1,500×g for 10 minutes; 4 mL of the centrifuged milk was mixed with an equal volume of 25% trichloroacetic acid (TCA), left to stand for 5 minutes, then centrifuged at 3,500×g for 20 minutes to remove proteins. The processed milk sample (1.5 mL) was stored at -20°C for milk urea nitrogen determination.

1.4 Measurements and Calculations

1.4.1 Feed Intake During the preliminary period, refusals were weighed every two days and feed amounts were recorded (from electronic display of TMR mixer when stationary). Refusals were collected and weighed before each feeding. Average feed intake per cow was calculated from feed offered and refused, with six records total. The average intake during the preliminary period was

calculated from these six records. During the formal period, intake was recorded and calculated every ten days (six records total), with the average calculated similarly. TMR amounts were adjusted for each subsequent period based on previous intake measurements. Nutrient intake was calculated from average intake and TMR nutrient content.

1.4.2 Ruminal MCP Production Purine derivatives (PD) excreted in urine originate primarily from ruminal microbial purines, allowing estimation of MCP production from PD excretion. Colorimetric methods were used to determine uric acid and allantoin concentrations in urine, with the sum representing total urinary PD content [7].

The amount of exogenous purines absorbed in the small intestine (X, mmol/d) was calculated using the formula:

$$Y = 0.85X + 0.385BW^{0.75}$$

where Y is urinary PD excretion (mmol/d), 0.85 is the recovery rate of absorbed purines converted to urinary PD in cattle, 0.385 is endogenous PD excretion when purine absorption is zero, and BW^{0.75} is metabolic body weight (kg).

MCP production (g/d) was calculated as:

$$\text{MCP (g/d)} = 6.25 \times \frac{70 \times X}{0.83 \times 0.116 \times 1000} = 4.54X$$

where X is the amount of exogenous purines absorbed in the small intestine (mmol/d), 70 is the nitrogen content per mole of purine (mg/mol), 0.83 is the digestibility of microbial nucleic acid purines, 0.116 is the proportion of purine nitrogen in total ruminal microbial nitrogen, and 6.25 is the average coefficient for converting nitrogen to protein. Ruminal MCP production during the formal period was calculated as the average of values from day 30 and the end of the formal period.

1.4.3 Milk Yield and Composition Milk yield was measured using a Lely herringbone milking machine with electronic display. Milk yield was recorded every five days during both preliminary and formal periods, with three consecutive days recorded each time and averaged. Milk protein percentage, milk fat percentage, lactose percentage, and somatic cell count were determined 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 period.

1.4.4 Nitrogen Metabolism Indices Urinary nitrogen content was analyzed using the Kjeldahl method [5], urea nitrogen by the urease method [8], and urinary creatinine by the picric acid colorimetric method [9]; all kits were

purchased from Nanjing Jiancheng Bioengineering Institute. Urine volume was estimated using creatinine as a marker, assuming approximately 29 mg creatinine excreted per kg body weight per day [9]. Dietary and fecal CP contents were determined according to methods described by Zhang [5].

Nitrogen metabolism indices were calculated as: - Fecal nitrogen (g/d) = daily fecal output × fecal CP content × 0.16 - Milk nitrogen (g/d) = milk yield × milk protein percentage × 0.16 - Digestible nitrogen (g/d) = nitrogen intake - fecal nitrogen - Total nitrogen excretion (g/d) = fecal nitrogen + urinary nitrogen - Nitrogen apparent digestibility (%) = [(nitrogen intake - fecal nitrogen) / nitrogen intake] × 100

1.5 Data Processing and Analysis

Experimental data were processed using Excel 2010. One-way ANOVA was performed using SPSS 17.0 software, and Duncan's multiple comparison test was used for intergroup difference significance testing. Differences were considered significant at $P<0.05$ and highly significant at $P<0.01$. Results are expressed as means ± standard error.

Results

2.1 Effects of SP Supplementation Level on Main Nutrient Intakes of Dairy Cows

As shown in Table 2 , dietary SP supplementation had minimal effects on dry matter and other nutrient intakes.

2.2 Effects of SP Supplementation Level on Ruminal MCP Production of Dairy Cows

Table 3 shows that urinary uric acid, allantoin, PD excretion, and MCP production in all test groups were significantly higher than in the control group ($P<0.05$). Test group 2 was significantly higher than test group 3 ($P<0.05$), while no significant differences were observed between groups 1 and 2 or between groups 1 and 3 ($P>0.05$). MCP production in test groups 1, 2, and 3 increased by 17.38%, 22.94%, and 12.22% compared with the control group, respectively.

2.3 Effects of SP Supplementation Level on Milk Yield and Milk Composition of Dairy Cows

Table 4 shows that milk yield in test groups 1, 2, and 3 was significantly higher than in the control group ($P<0.05$), with increases of 9.93%, 12.64%, and 7.53%, respectively. No significant difference was observed between groups 1 and 3 ($P>0.05$). Milk fat percentage in test groups 1 and 2 was significantly higher than in the control group ($P<0.05$), with no significant difference between groups 1 and 2 ($P>0.05$); test group 3 did not differ significantly from

the control group (P>0.05). All test groups showed significantly higher milk protein percentage than the control group (P<0.05), with test groups 1 and 2 significantly higher than test group 3 (P<0.05) but not different from each other (P>0.05). Milk somatic cell count in all test groups was significantly lower than in the control group (P<0.05), with test groups 1 and 2 significantly lower than test group 3 (P<0.05) but not different from each other (P>0.05).

2.4 Effects of SP Supplementation Level on Nitrogen Apparent Digestibility and Excretion of Dairy Cows

Table 5 shows that fecal nitrogen excretion in all test groups was significantly lower than in the control group (P<0.05), with test groups 1 and 2 significantly lower than test group 3 (P<0.05) but not different from each other (P>0.05). Urinary nitrogen excretion in all test groups was significantly lower than in the control group (P<0.05), with test group 2 significantly lower than test group 3 (P<0.05); no significant differences were observed between groups 1 and 2 or between groups 1 and 3 (P>0.05). Milk urea nitrogen excretion was significantly lower in all test groups compared with the control group (P<0.05). Total nitrogen excretion was reduced by 13.31%, 15.01%, and 9.43% in test groups 1, 2, and 3, respectively, with all test groups significantly lower than the control group (P<0.05); test groups 1 and 2 were significantly lower than test group 3 (P<0.05) but not different from each other (P>0.05). Digestible nitrogen in test groups 1 and 2 was significantly higher than in the control group (P<0.05), with no significant difference between groups 1 and 2 (P>0.05); test group 3 did not differ significantly from the control group (P>0.05). Nitrogen apparent digestibility was significantly higher in all test groups compared with the control group (P<0.05). These results indicate that dietary SP supplementation can significantly improve nitrogen digestibility and utilization while reducing nitrogen emissions.

Discussion

3.1 Effects of SP Supplementation Level on Main Nutrient Intakes of Dairy Cows

Zhou [10] reported that plant SP had no significant effect on average feed intake of lactating sows or finishing pigs. Chen [11] found that neither feeding nor infusing SP significantly affected main nutrient intakes in goats. The present results similarly showed that SP supplementation did not significantly improve feed intake in dairy cows. However, Wang et al. [3] demonstrated that ruminal infusion of soybean SP significantly improved nutrient digestibility and nitrogen apparent digestibility while increasing nitrogen retention. The improved nitrogen apparent digestibility observed with SP supplementation may be related to SP' s ability to regulate ruminal fermentation, reducing activity of certain proteolytic bacteria, increasing rumen bypass protein utilization, stimulating intestinal digestive enzyme activity, prolonging digesta retention time in the intestine, and enhancing gastrointestinal motility and digestive enzyme secretion.

3.2 Effects of SP Supplementation Level on Ruminal MCP Production of Dairy Cows

MCP is the most important nitrogen source for ruminants, supplying 60-70% of their protein requirement. MCP production reflects microbial nitrogen utilization and indirectly indicates ruminal microbial population size. Griswold et al. [12] found in vitro that peptide-supplied amino nitrogen significantly increased MCP production. Wang et al. [13] reported that oligopeptides significantly increased MCP synthesis compared with ammonium chloride in culture medium. The present study demonstrated that dietary SP supplementation significantly increased ruminal MCP production in dairy cows, consistent with these previous findings. MCP production primarily depends on whether the degradation rates of carbohydrates and nitrogen sources match, i.e., the energy-nitrogen balance. Wang et al. [14] showed that SP infusion increased total volatile fatty acid (TVFA) concentration in rumen fluid. Volatile fatty acids (VFA) are metabolic products of ruminal microbial carbohydrate fermentation and represent the main energy source for ruminants. Hoover [15] reported that ruminal microbes such as *Lactobacillus*, *Escherichia coli*, and *Streptococcus faecalis* can directly utilize certain SP in the rumen to enhance their activity. Enhanced microbial activity improves nitrogen metabolism, while suppressed urease activity reduces nitrogen degradation rate, creating better energy-nitrogen balance that favors MCP synthesis.

3.3 Effects of SP Supplementation Level on Milk Yield and Milk Composition of Dairy Cows

Ma and Chen [16] reported that dietary soybean protein peptide supplementation significantly increased milk yield in dairy cows, consistent with the present results. Kung and Huber [17] noted that reducing dietary protein degradation rate in the rumen to increase amino acid supply to the small intestine is a common practice to improve milk yield. Taylor et al. [18] demonstrated that increasing dietary rumen bypass protein rate could improve milk yield and increase milk fat and lactose percentages. Jiang et al. [19] found that dietary SP supplementation increased rumen bypass protein digestion and absorption in the small intestine, increasing serum amino acid concentrations and subsequently insulin (INS) concentration. INS promotes insulin-like growth factor-I (IGF-I), which stimulates mammary gland development and mammary cell proliferation, indirectly regulating lactation function. Additionally, certain concentrations of peptides or free amino acids can reduce proteolytic activity of some bacteria, decreasing ruminal protein degradation rate, increasing rumen bypass protein utilization, and increasing digestible protein and amino acid quantities in the small intestine, thereby providing more digestible protein for milk synthesis and positively affecting milk yield. Wang et al. [2] observed significantly increased milk yield with SP supplementation, though milk fat and protein percentages were not significantly affected. Huang et al. [20] reported that SP supplementation significantly improved milk yield and increased milk fat, protein, and

lactose percentages. Research findings on SP effects on milk quality have been inconsistent.

The present study demonstrated that SP supplementation improved milk fat percentage, milk protein percentage, and lactose percentage while reducing milk somatic cell count. Guo et al. [21] found that mammary tissue is highly active in milk protein synthesis, with over 90% of milk proteins synthesized from absorbed amino acids in the mammary gland. Dietary SP supplementation can increase ruminal MCP production, increasing digestible protein and amino acid quantities in the small intestine and thereby increasing available amino acids for milk protein synthesis, which explains the improved milk protein percentage. Glucose, as the main nutrient for metabolic activity and the most effective energy source, is the only carbohydrate that circulates systemically through blood plasma and cells. Increased blood glucose content in dairy cows provides more precursors for lactose synthesis and necessary substrates for milk fat synthesis. Liu et al. [22] demonstrated that duodenal infusion of soybean SP increased serum growth hormone (GH) and INS concentrations. Molento et al. [23] found that interactive effects of INS and recombinant bovine somatotropin significantly improved milk yield and milk protein production in early lactation cows. Chaiyabutr et al. [24] reported that GH significantly increased milk fat percentage in early and mid-lactation cows, with increasing trends in late lactation. Staples et al. [25] and Johnson et al. [26] found that bovine recombinant GH administration significantly increased milk fat percentage by elevating GH concentration, which can increase synthesis of acetyl-CoA carboxylase, fatty acid synthase, and lipoprotein lipase, with acetyl-CoA carboxylase being the rate-limiting enzyme for fatty acid synthesis. SP can increase serum GH concentration, promoting enzyme synthesis and thereby increasing milk fat percentage. Increased INS enhances IGF-I secretion, raising serum IGF-I levels, which promotes mammary cell proliferation, nutrient uptake from circulation, and synthesis of milk protein, lactose, and milk fat, while stimulating the milk ejection reflex to improve milk yield.

Milk somatic cell count is an indicator of udder health, affecting milk yield, milk quality, and shelf life; higher counts indicate poorer udder health and higher mastitis incidence. The significant reduction in somatic cell count observed with SP supplementation in this study indicates improved mammary gland development and udder health.

3.4 Effects of SP Supplementation Level on Nitrogen Apparent Digestibility and Excretion of Dairy Cows

SP can promote amino acid absorption by reducing antagonism from competition among free amino acids for common absorption sites, accelerate protein synthesis, and enhance ruminal bacterial growth by accelerating bacterial reproduction and shortening cell division cycles, thereby improving nitrogen utilization and increasing nitrogen retention while reducing nitrogen excretion. Yin et al. [27] reported that dietary dipeptide supplementation promoted carbohydrate

fermentation, increased VFA and MCP production, reduced ammonia nitrogen concentration, and improved energy utilization efficiency. Li et al. [28] found that duodenal SP infusion in goats significantly improved nitrogen retention compared with other treatments. Jiang et al. [19] reported that dietary SP supplementation enhanced small intestinal digestion and absorption of rumen bypass protein. In the present study, fecal nitrogen, urinary nitrogen, and milk urea nitrogen excretion in test groups were significantly lower than in the control group, with test group 2 showing the lowest values. SP supplementation significantly improved nitrogen digestibility and utilization efficiency and enhanced nitrogen deposition efficiency.

The reduced nitrogen excretion, improved nitrogen deposition efficiency, and enhanced nitrogen digestibility can be attributed to several mechanisms. First, SP limits the rate at which dietary protein is degraded to ammonia in the rumen; certain SP concentrations can significantly reduce proteolytic activity of some bacteria, decreasing ruminal protein degradation rate, increasing rumen bypass protein utilization, reducing nitrogen loss from ammonia release exceeding microbial utilization efficiency, and improving ruminal nitrogen utilization. Second, SP can stimulate activities of duodenal maltase, amylase, and trypsin; increased intestinal digestive enzyme activity improves utilization of rumen bypass protein and MCP, enhancing protein digestion and nitrogen utilization in the small intestine. Additionally, SP can improve nitrogen utilization by increasing IGF-I concentration, which acts on target cells via IGF-I receptors to stimulate glucose and amino acid uptake, promote protein synthesis, and inhibit protein degradation. Furthermore, INS promotes amino acid uptake into cells, increasing intracellular available amino acids and stimulating activity of RNA polymerase involved in amino acid synthesis, thereby promoting amino acid synthesis and providing adequate precursors for protein synthesis.

Conclusion

Dietary SP supplementation in dairy cows can significantly increase MCP production, reduce nitrogen excretion, and improve production performance. Considering these indices comprehensively, supplementation with 100 g/(d · head) of SP was optimal under the conditions of this experiment.

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