Effects of Dietary Calcium and Phosphorus Levels on Milk Composition and Milk Fat Fatty Acid Composition of Lactating Yili Mares and on Growth, Development, and Plasma Physiological and Biochemical Indices of Foals: Postprint

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Abstract

This study aimed to investigate the effects of feeding diets with different calcium and phosphorus levels on milk composition, milk fat fatty acid profile, foal growth and development, and plasma physiological and biochemical parameters in lactating Yili horses, thereby providing a reference for further exploration of calcium and phosphorus nutrition in this breed. Twenty-five Yili horses at the end of the second month of lactation, with similar age, body weight, and parity, were selected and randomly allocated into five groups (n=5 per group) based on mare and foal body weight. The dietary calcium and phosphorus levels for the five groups were set at 45.0, 30.0 g/d (Group), 48.5, 32.0 g/d (Group), 52.0, 34.0 g/d (Group), 55.5, 36.0 g/d (Group), and 59.0, 38.0 g/d (Group), respectively. The experiment lasted for 90 days, with each 30-day period constituting one experimental cycle. The results showed that dietary calcium and phosphorus levels significantly affected average milk yield per milking and estimated daily milk yield ($P<0.05$), but had no significant effect on milk fat percentage, milk protein percentage, lactose percentage, or total milk solids content ($P>0.05$). The average milk yield per milking in Group was 20.65% ($P<0.05$) and 15.22% ($P<0.05$) higher than that in Groups and , respectively, while the calcium content in milk from Group was 21.54% lower than that in Group ($P<0.05$). Diets with different calcium and phosphorus levels had significant effects on the contents of myristoleic acid (C14:1), palmitoleic acid (C16:1), linoleic acid (C18:2n6c), arachidic acid (C20:0), cis-11-eicosenoic acid (C20:1n9), and -linolenic acid (C18:3n3) in milk fat ($P<0.05$); as dietary calcium and phosphorus levels increased, the content of saturated fatty acids in milk fat

increased while that of unsaturated fatty acids decreased. Feeding mares diets with different calcium and phosphorus levels had no significant effects on foal growth and development indices such as body weight, body length, withers height, and cannon circumference, nor on plasma calcium, phosphorus, parathyroid hormone, or osteocalcin contents (P>0.05), whereas chest circumference decreased to varying degrees with increasing dietary calcium and phosphorus levels. Plasma calcitonin content in foals from Group was significantly higher than that in Groups , , and (P<0.05), with increases of 34.24%, 24.89%, and 23.64%, respectively. It was concluded that increasing dietary calcium and phosphorus levels could improve milk yield in Yili horses but decreased milk calcium content; dietary calcium and phosphorus levels did not affect milk composition but increased saturated fatty acid content and decreased unsaturated fatty acid content in milk fat; due to the decreased milk calcium content, plasma calcitonin content in foals decreased and chest circumference development was slower.

Full Text

Effects of Feeding Different Dietary Calcium and Phosphorus Levels on Milk Composition, Milk Fat Fatty Acid Composition, and Foal Growth, Development, and Plasma Physiological-Biochemical Indices in Lactating Yili Mares

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Abstract: This study investigated the effects of feeding different dietary calcium (Ca) and phosphorus (P) levels to lactating Yili mares on milk composition, milk fat fatty acid composition, and foal growth, development, and plasma physiological-biochemical indices, providing a reference for further research on Ca and P nutrition in Yili horses. Twenty-five Yili mares at the end of their second lactation month, with similar age, body weight, and parity, were randomly divided into five groups (n=5) based on mare and foal body weight. The five groups received diets with Ca and P levels of 45.0 and 30.0 g/d (Group I), 48.5 and 32.0 g/d (Group II), 52.0 and 34.0 g/d (Group III), 55.5 and 36.0 g/d (Group IV), and 59.0 and 38.0 g/d (Group V), respectively. The 90-day trial consisted of three 30-day cycles. Results showed that dietary Ca and P levels significantly affected average milking volume per session and estimated daily milk yield (P<0.05) but had no significant effects on milk fat percentage, protein percentage, lactose percentage, or total solids content (P>0.05). Group

III exhibited the highest average milking volume per session, which was 20.65% higher than Group I (P<0.05) and 15.22% higher than Group IV (P<0.05). However, milk Ca content in Group III was 21.54% lower than in Group I (P<0.05). Dietary Ca and P levels significantly influenced the contents of myristoleic acid (C14:1), palmitoleic acid (C16:1), linoleic acid (C18:2n6c), arachidic acid (C20:0), cis-11-eicosenoic acid (C20:1n9), and -linolenic acid (C18:3n3) in milk fat (P<0.05). As dietary Ca and P levels increased, saturated fatty acid content increased while unsaturated fatty acid content decreased. Mare dietary Ca and P levels had no significant effects on foal body weight, body length, body height, cannon circumference, or plasma Ca, P, parathyroid hormone (PTH), and osteocalcin levels (P>0.05), though chest circumference decreased with increasing dietary Ca and P levels. Plasma calcitonin (CT) concentration in Group I foals was significantly higher than in Groups II, III, and IV (P<0.05), increasing by 34.24%, 24.89%, and 23.64%, respectively. In conclusion, increasing dietary Ca and P levels improved milk yield but reduced milk Ca content in Yili mares. While dietary Ca and P levels did not affect milk composition, they increased saturated fatty acids and decreased unsaturated fatty acids in milk fat. Reduced milk Ca content led to decreased plasma CT levels and slower chest girth development in foals.

Keywords: mares; calcium; phosphorus; milk composition; fatty acids; foals

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Calcium (Ca) and phosphorus (P) are essential macro-mineral elements that play crucial roles in maintaining normal physiological functions and health. Late gestation and lactation represent physiological stages with the greatest Ca and P requirements. In dairy cows, Ca and P deficiency can cause metabolic disorders, leading to nutritional diseases such as milk fever, decreased milk fat percentage, and reduced feed utilization efficiency. Supplementing dairy cows with calcium boluses twice within 0–2 h and 8–35 h postpartum significantly increased milk yield. In early lactation cows, dry matter intake and milk yield peaked at dietary P levels of 0.40%–0.42% without further increase at higher P levels. The effects of dietary P on milk composition remain inconclusive, with some studies reporting increased milk protein and fat percentages while others found contradictory results. The impact of dietary Ca and P levels on milk yield and composition in lactating Yili mares remains unclear. Additionally, human studies demonstrated that daily supplementation with 1,250 mg Ca for 8 weeks significantly reduced serum total cholesterol, low-density lipoprotein

cholesterol, and apolipoprotein B levels, while higher Ca intake in preschool children correlated with lower body fat content. These findings suggest that dietary Ca regulates lipid metabolism. However, whether dietary Ca and P levels affect fatty acid composition in mare milk fat has not been reported. Therefore, this study aimed to investigate the effects of different dietary Ca and P levels on milk yield, milk composition, milk fat fatty acid composition, and foal growth, development, and plasma physiological-biochemical indices in lactating Yili mares.

1.1 Experimental Animals and Diets

Twenty-five unrelated Yili mares aged 11–14 years, weighing 371±21 kg, and at the end of their second lactation month were randomly divided into five groups (n=5) based on mare and foal body weight. The 90-day trial included a 12-day preliminary period, with the last 12 days of each lactation month as the sampling period, comprising three 30-day cycles. Five diets with different Ca and P levels were formulated according to NRC (2007) standards for 400 kg mature lactating mares. Based on the nutritional requirements of lactating mares, concentrate feed was provided at 2,700 g/d during the third lactation month. From the fourth month onward, forage intake remained constant while concentrate was gradually reduced by approximately 500 g/d until the end of the fifth lactation month to control nutritional intake levels. Diet composition and nutrient levels for lactating mares are presented in Table 1 . Foal supplementary concentrate was formulated according to Jin Weixing et al.

1.2 Feeding Management

Experimental mares were housed individually in separate stalls and fed concentrate twice daily at 11:30 and 20:00 (half at each feeding). Concentrate amounts for each lactation month are shown in Table 1. Alfalfa hay and wheat straw were mixed in equal proportions using a mixer for 30 minutes to prepare the forage component. Forage was fed four times daily at 09:30, 13:30, and 18:00 (3.00 kg each) and at 02:00 (5.00 kg). Water was available ad libitum. During the preliminary period, foals had free access to mare' s milk, water, and activity, and received 0.30 kg concentrate supplement at 10:00 and 18:00 daily in feed buckets.

1.3.1 Mare Milk Sample Collection

During the trial, foals were tethered and isolated during milking to prevent milk consumption. Milking began 1.5 h after feeding and was performed manually by skilled personnel every 2 h, four times daily, until the udder was emptied. Milk volume was recorded electronically each session. Each milking cycle consisted of 2 consecutive sampling days followed by a 2-day interval, totaling three cycles. Collected milk was filtered through two layers of gauze to remove impurities, and 50 mL was accurately measured and stored at -20°C. Samples from the three milking cycles at different time points were pooled into one sample per mare for

milk composition and fatty acid analysis. Daily milk yield was calculated using the method of Jin Weixing et al.

1.3.2 Foal Blood Sample Collection

On the second day following each experimental period, before morning feeding, 10 mL of fasting blood was collected from the jugular vein using heparinized vacuum tubes. Tubes were inverted several times, then centrifuged at 3,500 r/min for 10 min to separate plasma, which was stored at -20°C until analysis.

1.4.1 Milk Composition Determination

A 50 mL milk sample was thoroughly mixed and analyzed using a Foss 4000 milk composition analyzer for milk protein percentage, fat percentage, total solids content, and lactose percentage. Somatic cell count was determined using a Foss 5000 somatic cell counter.

1.4.2 Milk Fat Fatty Acid Composition Determination

Fatty acid composition in mare milk fat was determined by gas chromatography using an SP2560 column (100 m×0.25 mm×0.20 m) with area normalization. Crude fat extraction involved mixing 1 mL milk with 2 mL n-hexane/isopropanol (3:2 v/v), vortexing for 2 min, adding 1 mL 66.70 g/L Na SO solution, vortexing again for 2 min, and centrifuging at 3,000 r/min for 10 min. For saponification and esterification, 1 mL 2% NaOH methanol solution was added to the n-hexane phase, saponified at 50°C for 15 min, cooled, then 1 mL 10% HCl methanol solution was added and esterified at 80°C for 1 h. For analytical sample preparation, after cooling, 3 mL deionized water and 3 mL n-hexane were added, mixed, left to stand for 20 min, and the supernatant was diluted to 10 mL with n-hexane. Approximately 0.5 g anhydrous Na SO was added, shaken for 30 s, left for 10–20 min, and 1 mL was taken for analysis. GC conditions: injector temperature 250°C, column flow 1.5 mL/min, injection volume 1.0 L, split ratio 10:1; oven temperature 100°C held for 5 min, increased to 150°C at 6°C/min, then to 240°C at 2°C/min and held for 8 min; FID temperature 260°C, makeup flow 20 mL/min, air flow 400 mL/min, hydrogen flow 30 mL/min.

1.4.3 Foal Body Weight and Body Measurement Determination

Foal body weight, body length, body height, chest girth, and cannon circumference were measured at the end of month 2 (pre-trial), month 3, month 4, and month 5.

1.4.4 Foal Plasma Physiological-Biochemical Indices Determination

Plasma Ca content was determined by methylxylenol blue colorimetry at 610 nm, plasma P by molybdenum blue colorimetry at 340 nm, and Ca^2 metabolism-

related hormones including parathyroid hormone (PTH), calcitonin (CT), and bone Gla protein (BGP) by radioimmunoassay. All assay kits were provided by Beijing Huaying Biotechnology Research Institute.

1.5 Data Processing and Statistical Analysis

Data were organized using Excel 2010. Statistical analysis was performed using multifactor single-response variable analysis of variance in SPSS 18.0. The model was Xij = + i + j + i× j + ij, where Xij represents observed values, is the overall mean, i (i=1,2,3,4,5) is diet effect, j (j=1,2,3) is lactation month (or foal month age) effect, i× j is the interaction effect, and ij is error. Duncan' s multiple comparison was applied when factor levels were significant. Data are expressed as mean ± SD, with significance level at P 0.05 and trend at 0.05<P 0.10.

2.1 Effects of Different Dietary Calcium and Phosphorus Levels on Milk Yield and Composition in Lactating Yili Mares

As shown in Table 2 , dietary Ca and P levels significantly affected average milking volume per session, estimated daily milk yield, and milk Ca content (P<0.05), but had no significant effects on milk fat percentage, protein percentage, lactose percentage, or total solids content (P>0.05). No significant diet × lactation month interactions were observed for these parameters (P>0.05). Group III achieved the highest average milking volume per session, which was 20.65% higher than Group I (P<0.05) and 15.22% higher than Group IV (P<0.05). Estimated daily milk yield followed the same pattern. Milk Ca content did not increase linearly with dietary Ca levels; instead, Groups II, III, and IV had significantly lower milk Ca content than Group I (P<0.05), decreasing by 15.38%, 21.54%, and 16.92%, respectively. Milk P content tended to increase with dietary P levels (P<0.10). Lactation month significantly affected all milk components (P<0.05). As lactation progressed, average milking volume per session, estimated daily milk yield, somatic cell count, and milk Ca and P content decreased to varying degrees, while other milk composition parameters increased.

2.2 Effects of Different Dietary Calcium and Phosphorus Levels on Milk Fat Fatty Acid Composition in Lactating Yili Mares

Table 3 shows that 27 fatty acids were detected in mare milk fat, with capric acid (C10:0), lauric acid (C12:0), myristic acid (C14:0), palmitoleic acid (C16:1), and -linolenic acid (C18:3n3) each exceeding 5% of total fatty acids, while palmitic acid (C16:0), oleic acid (C18:1n9c), and linoleic acid (C18:2n6c) each exceeded 10%. Dietary Ca and P levels significantly affected myristoleic acid (C14:1), palmitoleic acid, linoleic acid, arachidic acid (C20:0), cis-11-eicosenoic acid (C20:1n9), and -linolenic acid contents (P<0.05), but not the remaining 21 fatty acids including butyric acid (C4:0) (P>0.05). Myristoleic and palmitoleic acid contents decreased gradually with increasing dietary Ca and P levels,

reaching minimum values in Group III before increasing again. Group I had significantly higher linoleic acid content than other groups (P<0.05), but significantly lower arachidic and cis-11-eicosenoic acid contents (P<0.05). Group V had significantly lower -linolenic acid content than Groups I and III (P<0.05). As dietary Ca and P levels increased, saturated fatty acid (SFA) content increased while unsaturated fatty acid (USFA) content decreased. Group III had significantly lower monounsaturated fatty acid (MUFA) content, and Group V had significantly lower polyunsaturated fatty acid (PUFA) content compared with other groups (P<0.05). Regarding lactation month, butyric acid (C4:0), capric acid, undecanoic acid (C11:0), lauric acid, tridecanoic acid (C13:0), cis-8,11,14-eicosatrienoic acid (C20:3n6), and lignoceric acid (C24:0) contents decreased with advancing lactation. Myristic acid, myristoleic acid, pentadecanoic acid (C15:0), and palmitic acid showed a pattern of initial decrease followed by increase, while oleic and -linolenic acids increased initially then decreased. Linoleic acid content increased progressively with lactation month, and cis-11-eicosenoic acid was lowest in the third lactation month. Significant diet × lactation month interactions were observed for linoleic acid, cis-11,14-eicosadienoic acid (C20:2), lignoceric acid, and nervonic acid (C24:1) contents (P<0.05).

2.3 Effects of Feeding Different Dietary Calcium and Phosphorus Levels to Lactating Yili Mares on Foal Growth and Development

As shown in Table 4 , mare dietary Ca and P levels had no significant effects on foal body weight, body length, body height, or cannon circumference (P>0.05). However, increasing dietary Ca and P levels significantly reduced chest circumference, except in Group III (P<0.05). All growth parameters increased significantly with age (P<0.05). No significant diet × age interactions were observed for any growth indices (P>0.05).

2.4 Effects of Feeding Different Dietary Calcium and Phosphorus Levels to Lactating Yili Mares on Foal Plasma Physiological-Biochemical Indices

Table 5 indicates that mare dietary Ca and P levels had no significant effects on foal plasma Ca, P, PTH, or BGP contents (P>0.05), but significantly affected plasma CT content (P<0.05). Specifically, Group I had significantly higher plasma CT than Groups II, III, and IV (P<0.05). Age significantly affected all plasma indices (P<0.05). Plasma Ca, P, CT, and BGP contents increased initially then decreased with age, peaking at 4 months, while plasma PTH decreased initially then increased, reaching its lowest point at 4 months. Significant diet × age interactions were observed for plasma Ca, PTH, and CT contents (P<0.05).

3.1 Effects of Different Dietary Calcium and Phosphorus Levels on Milk Yield and Composition in Yili Mares

Milk yield and composition in livestock are influenced by breed, genetics, physiology, management, diet, and environment. Under consistent genetic, physiological, and environmental conditions, management and diet play dominant roles. Calcium and phosphorus are widely distributed in bone, muscle, and teeth. Calcium is involved in nerve transmission, cell permeability, enzyme activity, and blood coagulation, while phosphorus is essential for bone formation, bioenergy, and microbial digestion. Postpartum livestock maintain constant blood and milk Ca through dietary intake, bone mobilization, and renal reabsorption. Inadequate Ca supply reduces blood Ca and affects milk yield. Supplementing lactating dairy cows with 8.5 g/d ionic calcium for 3 days during peak lactation increased milk yield by 1.6 kg/d and prolonged the peak lactation period. Calcium bolus supplementation twice within 0–2 h and 8–35 h postpartum increased milk yield by 7.2%–7.7% in high-producing cows. Lopez et al. reported that dietary P levels of 0.37% or 0.57% had no significant effects on milk yield, fat percentage, or protein percentage in Holstein cows. Zhao et al. also found no significant effect of dietary P levels (0.32%, 0.44%, and 0.56%) on milk yield in dairy cows. These findings suggest that Ca supplementation improves milk yield, while P supplementation has minimal effect. The effects of dietary Ca and P levels on mare milk yield and composition have not been reported. Studies show that blood PTH increases while total and ionic Ca decrease between 3 days prepartum and 2 days postpartum in mares, necessitating increased Ca supply during lactation. The Ca and P requirements for 400–500 kg mares in the second lactation month are 47.1–58.9 g/d and 30.5–38.1 g/d, respectively. In this study, Yili mares in the second lactation month received 45.0–58.9 g/d Ca and 30.0–38.0 g/d P, with maximum milk yield observed at 51.96 g/d Ca and 34.01 g/d P, beyond which no further benefit was observed. Physiological stage is another important factor affecting milk yield. Unlike dairy cows, mares lactate for approximately 180 days. Yili foals are typically weaned at 5 months, after which milk yield drops sharply. Yao reported that Xinji mares peaked at 19.13 kg/d on day 30 postpartum, decreasing to 14.05 kg/d on day 110 and 10.51 kg/d on day 150. Yili mares maintained relatively stable production during the first 110 days, peaking at 12.78 kg/d on day 60, decreasing to 10.75 kg/d on day 110 and 7.51 kg/d on day 140. Lusitano mares peaked at 14.0 kg/d on day 31, gradually decreasing to 7.57 kg/d by day 180. In this study, estimated milk yield was 10.99 kg/d at the end of the third lactation month, decreasing to 8.54 kg/d by the end of the fifth month. Thus, appropriate dietary Ca and P levels increase milk yield, but production declines with advancing lactation, with notable differences among breeds.

Few studies have reported the effects of dietary Ca and P levels on mare milk composition. Cui found no significant effects on milk composition in lactating water buffalo, consistent with our results. Zhang et al. reported significant negative correlations between milk yield and milk fat percentage, somatic cell count,

and dry matter content in dairy cows. In contrast, equine studies showed strong positive correlation between milk yield and fat percentage, no correlation with protein percentage, and strong negative correlation with total solids content. However, we found no significant correlations between milk yield and composition, possibly because our trial was limited to months 3–5 of lactation.

3.2 Effects of Different Dietary Calcium and Phosphorus Levels on Milk Fat Fatty Acid Composition in Yili Mares

Dietary Ca^2 significantly affects lipid digestion and metabolism. Ca^2 forms calcium soaps with lipids in the digestive tract, affecting absorption, and binds bile acids, increasing excretion and reducing cholesterol formation. High dietary Ca^2 increased fat breakdown by 5.2-fold in rats. Low-Ca diets increase synthesis of Ca^2 -related hormones (PTH and 1,25-(OH) -vitamin D), promoting Ca^2 influx into cells, elevating intracellular Ca^2 in adipocytes, enhancing fatty acid synthase expression and activity, and increasing lipid storage. High-Ca diets suppress cellular responses to these hormones. Increased dietary Ca reduces intracellular Ca^2 , enhancing lipolysis and decreasing triglyceride accumulation. The effects of dietary Ca on mare milk fat percentage have not been reported. In this study, no significant differences in milk fat percentage were observed among groups, possibly due to short-term homeostatic mechanisms maintaining milk composition. From months 3 to 5, dietary Ca levels decreased while milk fat percentage increased, possibly related to reduced milk yield and increased lipid synthesis triggered by low Ca^2 . The effects of dietary Ca and P on milk fat fatty acid composition have not been reported. Our study found significant effects on myristoleic acid, palmitoleic acid, linoleic acid, arachidic acid, cis-11-eicosenoic acid, -linolenic acid, and total saturated and unsaturated fatty acids. Whether these changes result from altered lipid metabolism due to dietary Ca^2 requires further investigation.

3.3 Effects of Feeding Different Dietary Calcium and Phosphorus Levels to Lactating Yili Mares on Foal Growth, Development, and Plasma Physiological-Biochemical Indices

Body weight and measurements are important indicators of animal growth and development. Foals are typically weaned at 6 months, and the period from birth to 6 months represents the fastest growth phase, accounting for over 50% of postnatal total growth. Feeding high-protein and high-energy diets to late-lactation Yili mares significantly improved foal body length development, likely due to increased milk production. In this trial, all foals received identical concentrate supplements at the same rate, so foal growth was related to mare milk yield. Although milk yield differed significantly among groups, no significant differences in foal growth indices were observed except for chest circumference, possibly due to small sample size. Studies on Guanzhong donkeys showed rapid growth during fetal and postnatal periods, with chest girth growth intensity greater than body height and cannon circumference, indicating that chest girth

is more sensitive to nutritional intake.

Serum Ca and P metabolism is regulated by PTH, CT, and 1,25-(OH) -vitamin D to maintain relatively constant levels. When dietary Ca^{2} decreases, the body increases PTH and 1,25-(OH) -vitamin D release while decreasing CT release. In this study, although plasma Ca and P contents did not differ significantly among groups, milk Ca content in Groups II, III, and IV was significantly lower than in Group I, resulting in lower Ca intake from milk for foals in these groups. This may explain why plasma CT content in Groups II, III, and IV was significantly lower than in Group I.

In conclusion, increasing dietary Ca and P levels improved milk yield but reduced milk Ca content in Yili mares. While dietary Ca and P levels did not affect milk composition, they increased saturated fatty acids and decreased unsaturated fatty acids in milk fat. Reduced milk Ca content slowed chest girth development and decreased plasma CT levels in foals.

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