Effects of Daidzein, Formononetin and Their Combination on Milk Production Performance and Hormone Contents in Plasma and Milk of Dairy Cows (Postprint)

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Abstract

This experiment aimed to investigate the effects of daidzein, formononetin, and their combination on milk production performance and hormone contents in plasma and milk of dairy cows. Forty healthy Holstein dairy cows with similar age and lactation stage were selected and randomly divided into 4 groups: control, I, II, and III, with 10 cows per group. All cows were fed a total mixed ration, with groups I, II, and III supplemented with 2.50 g daidzein, 35.0 g formononetin, and 1.25 g daidzein + 17.5 g formononetin, respectively. A 127-day feeding trial was conducted, including a 7-day preliminary period and a 120-day formal experimental period. The results showed: milk yield in groups I, II, and III was significantly different compared with the control group ($P<0.05$), increasing by 30.40%, 27.77%, and 28.37%, respectively; milk fat yield in the treatment groups was higher than that in the control group, but the difference was not significant ($P>0.05$); milk protein percentage and milk protein yield in the treatment groups were higher than those in the control group, with group III showing a significant difference compared with the control group ($P<0.05$); lactose yield in groups I, II, and III was significantly higher than that in the control group ($P<0.05$); milk urea nitrogen content in the control group was significantly higher than that in group II ($P<0.05$); estrone content in plasma of the treatment groups was lower than that in the control group, while estrone content in milk of the treatment groups was higher than that in the control group, but the differences were not significant ($P>0.05$); estradiol content in plasma and milk of the treatment groups was higher than that in the control group, with group III showing a significant difference compared with the control group ($P<0.05$); progesterone content in plasma and milk of the treatment groups was higher than that in the control group, with group II in plasma showing a significant difference compared with the control group ($P<0.05$), and groups I and III in milk showing significant differences compared with the control group (P<0.05); the differences in follicle-stimulating hormone and luteinizing hormone contents in plasma among all groups were not significant (P>0.05); prolactin content in plasma of all treatment groups was higher than that in the control group, with group II showing a significant difference compared with the control group (P<0.05); equol content in milk of the treatment groups was significantly higher than that in the control group (P<0.05). Therefore, supplementation with daidzein, formononetin, and their combination can increase milk yield, improve milk quality, significantly increase equol content in milk, and simultaneously increase estradiol and progesterone contents in plasma and milk of dairy cows.

Full Text

Abstract

This experiment was conducted to investigate the effects of daidzein, formononetin and their combination on milk performance, plasma and milk hormone contents of dairy cows. Forty healthy Holstein cows of similar age and lactation stage were randomly divided into 4 groups: control group, trial groups I, II, and III, with 10 cows per group. All cows were fed a total mixed ration (TMR), with trial groups I, II, and III receiving additional supplements of 2.50 g daidzein, 35.0 g formononetin, and 1.25 g daidzein + 17.5 g formononetin, respectively. The feeding trial lasted 127 days, including a 7-day adaptation period and a 120-day experimental period. The results showed that milk yield in trial groups I, II, and III was significantly higher than in the control group (P<0.05), with increases of 30.40%, 27.77%, and 28.37%, respectively. Milk fat yield in all trial groups was higher than in the control group, but the differences were not significant (P>0.05). Both milk protein percentage and yield were higher in trial groups than in the control group, with trial group III showing a significant difference (P<0.05). Lactose yield in trial groups I, II, and III was significantly higher than in the control group (P<0.05). Milk urea nitrogen content in the control group was significantly higher than in trial group II (P<0.05). Plasma estrone content in trial groups was lower than in the control group, while milk estrone content was higher, but these differences were not significant (P>0.05). Plasma and milk estradiol-17β content in trial groups was higher than in the control group, with trial group III showing a significant difference (P<0.05). Plasma and milk progesterone content in trial groups was higher than in the control group, with trial group II showing a significant difference in plasma (P<0.05) and trial groups I and III showing significant differences in milk (P<0.05). There were no significant differences in plasma follicle-stimulating hormone or luteinizing hormone content among groups (P>0.05). Plasma prolactin content in all trial groups was higher than in the control group, with trial group II showing a significant difference (P<0.05). Milk equol content in trial groups was significantly higher than in the control group (P<0.05). Therefore, supplementation with daidzein, formononetin and

their combination can increase milk yield, improve milk quality, significantly increase milk equol content, and elevate estradiol-17β and progesterone levels in both plasma and milk.

Keywords: daidzein; formononetin; dairy cow; milk performance; hormone

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Daidzein and formononetin are aromatic-ring non-steroidal phenolic compounds with diverse natural biological activities. Their structure and function are similar to mammalian estradiol and its metabolites, and they can selectively bind to estrogen receptors, exerting estrogen-like or anti-estrogenic activity. Studies have shown that feeding daidzein and formononetin to dairy cows can stimulate mammary gland cell development and increase milk yield. Additionally, daidzein and formononetin can be converted to equol by gastrointestinal bacteria in animals. Equol promotes animal growth and reproductive system development, reduces the risk of breast cancer in women and prostate cancer in men associated with estrogen imbalance, prevents cardiovascular disease, alleviates menopausal symptoms, and prevents osteoporosis. Since equol is stable in the body and can be secreted into the milk of lactating animals, producing milk products containing equol offers promising economic value. Hao et al. fed Chinese Holstein cows diets containing 10, 20, and 30 mg/kg daidzein, resulting in milk yield increases of 7.29%, 12.66%, and 10.13%, respectively. Höjer et al. fed lactating dairy cows red clover containing formononetin and observed a significant increase in milk equol content. Previous studies have confirmed that daidzein and formononetin can increase dairy cow milk yield and milk equol content, but their effects on reproduction-related hormones in blood and milk remain unclear. Therefore, this study used lactating Holstein cows to investigate the effects of daidzein, formononetin and their combination on milk performance, further confirm their role in increasing milk equol content, and determine whether they affect hormone levels in blood and milk, providing a scientific basis for producing equol-enriched milk.

1.1 Experimental Time and Location

The experiment was conducted from June 2015 to November 2015 at Xinjiang Tianshan Animal Husbandry Bio-Engineering Co., Ltd.

1.2 Experimental Animals and Design

Forty healthy Holstein cows of similar age and lactation stage were randomly divided into 4 groups: control group and trial groups I, II, and III, with 10 cows per group. The control and trial groups were all fed a total mixed ration (TMR). The supplementation levels of daidzein and formononetin in the trial groups were based on previous studies by Hao et al. and Mustonen et al. Trial group I received 2.50 g daidzein (purchased from Zhengzhou Saina Kang Chemical Products Co., Ltd., purity 99%), trial group II received 35.0 g formononetin (purchased from Shaanxi Lüqing Bio-Engineering Co., Ltd., purity 99%), and trial group III received 1.25 g daidzein + 17.5 g formononetin. The feeding trial lasted 127 days, including a 7-day adaptation period and a 120-day experimental period. Milk yield was recorded and milk and plasma samples were collected on days 1, 30, 60, 90, and 120 of the experimental period. During the adaptation period, cow health status, mastitis incidence, and acceptance of supplements were monitored.

1.3 Feeding Management

The control and trial groups were kept under the same environmental conditions. Based on cow feeding habits, TMR was fed at 10:00 and 17:00 daily. Daidzein and formononetin were mixed uniformly with a small amount of TMR for individual trough feeding. Cows had free access to water throughout the trial period. Manure was removed regularly to maintain pen cleanliness, and cows were milked three times daily. Diet composition and nutrient levels are shown in Table 1 .

Table 1 Composition and nutrient levels of the TMR (air-dry basis) %

Items	Content
Ingredients	
Corn silage	
Alfalfa hay	
Tomato skin	
Soybean meal	
Limestone	
Cottonseed meal	
Corn gluten meal	
Premix[1]	
NaCl	
$Ca(HCO_3)_2$	
$KHCO_3$	
$NaHCO_3$	
MgO	
Total	
Nutrient levels[2]	
NEL/(MJ/kg)	
EE	
CP	
ADF	
NDF	
Ca	
Mg	

[1] Each kilogram of premix contained: Cu 3,230 mg, Zn 5,950 mg, Mn 4,850 mg, I 120 mg, Se 150 mg, Co 90 mg, VA 804,800 IU, VD_3 188,800 IU, VE 4,600 IU, nicotinic acid 800 mg.

[2] Nutrient levels were measured values.

1.4 Sample Collection

Milk samples were collected on days 1, 30, 60, 90, and 120 of the experimental period. Based on lactation patterns, samples were collected during milking at 04:00, 11:00, and 17:00 using mechanical milking. The three samples were mixed, and 150 mL was placed in clean plastic bottles.

Blood samples were collected via jugular vein on days 1, 30, 60, 90, and 120 of the experimental period. Fifteen milliliters of blood was collected from each cow and placed in 5 mL sodium heparin anticoagulant tubes. All blood samples were centrifuged at 3,500 r/min for 15 minutes. Plasma was collected and aliquoted into 2 mL Eppendorf tubes (1.5 mL per tube) and stored at -20°C until analysis.

1.5 Sample Analysis

Milk samples were immediately analyzed for composition using a MilkoScan FT 120 milk composition analyzer [FOSS Group (China) Co., Ltd., Denmark] after mixing.

Plasma and milk contents of estrone (E), estradiol-17β (E2-17β), estriol (E3), progesterone (P), follicle-stimulating hormone (FSH), luteinizing hormone (LH), and prolactin (PRL) were determined at Beijing Huaying Biotechnology Research Institute.

Milk equol content was determined by high-performance liquid chromatography according to the methods of Hoikkala et al. and Li et al.

1.6 Data Processing and Analysis

Experimental data were organized using Excel and analyzed using the Mixed procedure in SAS 8.0. Fixed effects included group, time, and their interaction. The variance structure used was CS. Data are presented as least squares means. Significance was determined at $P<0.05$, and multiple comparisons were performed using the Lsmeans method.

2.1 Effects of Daidzein, Formononetin and Their Combination on Milk Performance

The effects of daidzein, formononetin and their combination on milk performance are shown in Table 2 . Daidzein, formononetin and their combination increased milk yield, with trial groups I, II, and III showing significant differences compared to the control group ($P<0.05$), increasing by 30.40%, 27.77%, and 28.37%, respectively. Milk fat percentage in trial groups I, II, and III was lower than in the control group, with a significant difference between group I and the control (P<0.05). However, milk fat yield in trial groups I, II, and III was higher than in the control group, though differences were not significant (P>0.05). Milk protein percentage and yield in trial groups I, II, and III were higher than in the control group, with trial group III showing a significant difference (P<0.05). Lactose percentage showed no significant differences between trial groups and the control (P>0.05), while lactose yield in trial groups I, II, and III was significantly higher than in the control group (P<0.05). Milk urea nitrogen content in the control and trial group III was significantly higher than in trial group II (P<0.05), with no significant differences among the control and trial groups I and III (P>0.05).

Table 2 Effects of daidzein, formononetin and their combination on milk performance of dairy cows (n=10)

Items	Control	Group I	Group II	Group III	P-value	Group×time
Milk yield/(kg/d)	25.57b	31.40a	28.77a	29.37a	<0.0001	<0.0001
Milk fat percentage/%	5.00a	4.39b	4.60ab	4.84ab	<0.0001	<0.0001
Milk fat yield/(g/d)	872.83b	1,008.92a	944.75ab	1,005.10a	<0.0001	<0.0001
Milk protein percentage/%	3.24b	3.24b	3.28ab	3.44a	<0.0001	<0.0001
Milk protein yield/(g/d)	1,265.27b	1,547.94a	1,435.38a	1,455.80a	<0.0001	<0.0001
Lactose percentage/%	15.66a	15.27ab	14.66b	16.09a	<0.0001	<0.0001
Lactose yield/(g/d)	-	-	-	-	-	-

Items	Control	Group I	Group II	Group III	P-value	Group×time
Milk urea nitrogen/(mg/dL)	-	-	-	-	-	-

In the same column, values with no letters or the same letter superscripts mean no significant difference (P>0.05), while different small letter superscripts mean significant difference (P<0.05). The same as below.

2.2 Effects of Daidzein, Formononetin and Their Combination on Plasma and Milk Hormone Contents

The effects of daidzein, formononetin and their combination on plasma and milk hormone contents are shown in Table 3 . Plasma estrone content in trial groups I, II, and III was lower than in the control group, while milk estrone content was higher, but differences were not significant (P>0.05). Plasma and milk estradiol-17β content in trial groups I, II, and III was higher than in the control group, with trial group III showing a significant difference (P<0.05). Plasma and milk estriol content in trial groups was higher than in the control group, but differences were not significant (P>0.05). Plasma and milk progesterone content in all trial groups was higher than in the control group, with trial group II showing a significant difference in plasma (P<0.05) and trial groups I and III showing significant differences in milk (P<0.05). There were no significant differences in plasma follicle-stimulating hormone or luteinizing hormone content among groups (P>0.05). Plasma prolactin content in all trial groups was higher than in the control group, with trial group II showing a significant difference (P<0.05). Milk equol content in trial groups I, II, and III was significantly higher than in the control group (P<0.05).

Table 3 Effects of daidzein, formononetin and their combination on plasma and milk hormone contents of dairy cows (n=10)

Items	Control	Group I	Group II	Group III	P-value	Group×time
Plasma						
Estrone/(pmol/L)	13.51b	14.37ab	16.47a	16.34a	<0.0001	<0.0001
Estradiol-17β/(pg/mL)	1.09b	1.21ab	1.32a	1.28ab	<0.0001	<0.0001
Estriol/(ng/mL)	-	-	-	-	-	-
Progesterone/(ng/mL)	5.49c	8.32b	7.01b	6.50bc	<0.0001	<0.0001
FSH/(mIU/mL)	7.86a	9.38a	9.81ab	9.96a	<0.0001	<0.0001
LH/(mIU/mL)	-	-	-	-	-	-
Prolactin/(mIU/mL)	240.97b	250.75ab	253.85a	246.85ab	<0.0001	<0.0001
Milk						

Items	Control	Group I	Group II	Group III	P-value	Group×time
Estrone/(pmol/L)	-	-	-	-	-	-
Estradiol-17β/(pg/mL)	-	-	-	-	-	-
Estriol/(ng/mL)	-	-	-	-	-	-
Progesterone/(ng/mL)	-	-	-	-	-	-
Equol/(g/L)	<0.10c	166.91b	517.99a	402.59a	52.274	<0.0001

3.1 Effects of Daidzein, Formononetin and Their Combination on Milk Performance

Milk yield is a crucial economic indicator for lactating dairy cows, influenced by genetic level, nutritional status, and physiological state, which directly affect endocrine function and ultimately milk production. Some studies have shown that low doses of phytoestrogens can increase milk yield in female livestock. Liu et al. fed 24 Holstein cows diets containing 2.8 and 3.3 g/d daidzein, increasing daily milk yield by 11.8% and 3.4%, respectively. Yang et al. added 1 g/d daidzein to Holstein cows and improved milk yield in mid-lactation cows. Lundh reported that daidzein supplementation in dairy cow diets increased daily and total lactation milk yield. Lu et al. co-cultured bovine mammary epithelial cells with different concentrations of daidzein (10, 100, and 1,000 mg/L) for 72 hours, resulting in increased β-casein, lactose, and triglyceride secretion.

In this experiment, all trial groups had higher milk yield than the control group, indicating that daidzein, formononetin and their combination can significantly increase milk yield, consistent with previous studies. This may be because daidzein and formononetin exert weak estrogenic effects, increasing blood prolactin levels, which acts on mammary tissue to enhance milk secretion. After feeding daidzein, formononetin and their combination, milk fat and lactose percentages decreased, possibly related to the increased milk yield. However, milk protein percentage and yield increased, which may be due to two mechanisms: first, daidzein and formononetin increase blood prolactin, which binds to its receptors, increases ribosomal RNA, and accelerates casein mRNA translation and transcription, thereby increasing milk protein content; second, the estrogen-like substances metabolized from daidzein and formononetin can regulate nitrogen metabolism, reduce urea nitrogen production, increase nitrogen retention, promote protein synthesis, decrease protein degradation, and improve feed conversion efficiency, ultimately increasing milk protein content.

Milk urea nitrogen is an important indicator of dietary protein utilization and the balance between dietary protein and energy in dairy cows. Hwang et al. suggested that when milk urea nitrogen is 12-19 mg/dL and milk protein percentage is $ $3%, dietary crude protein and energy are relatively balanced. In this experiment, milk urea nitrogen ranged from 14.66 to 16.09 mg/dL and milk protein percentage ranged from 3.24% to 3.44%, indicating that the dietary protein and energy were balanced. Feeding daidzein and formononetin decreased milk urea nitrogen content, suggesting they may improve the utilization of dietary crude protein, which is consistent with the increased milk protein percentage and yield.

3.2 Effects of Daidzein, Formononetin and Their Combination on Plasma and Milk Hormone Contents

Milk secretion involves both initiation and maintenance of lactation. During puberty and pregnancy, estrone and progesterone jointly promote alveolar development, while growth hormone and prolactin synergistically promote mammary gland development, increasing mammary cell numbers to prepare for lactation initiation. Maintenance of lactation requires preserving alveolar cell numbers and maintaining metabolic activity and milk ejection function in each mammary cell. Hormones comprehensively control the lactation process, with growth hormone, prolactin, glucocorticoids, thyroid hormones, insulin, and parathyroid hormone involved in maintaining lactation. Daidzein and formononetin can be metabolized into estrogen-like substances that competitively bind to cytoplasmic estradiol receptors in mammary glands, pituitary, and hypothalamus, inducing increased blood prolactin, growth hormone, and insulin levels. Liu et al. fed 15 mid-lactation Holstein cows 200 mg/d daidzein for 60 days and observed increased serum prolactin and estradiol-17β. Yang et al. added 20 mg/kg daidzein to 15 late-lactation Holstein cows, increasing serum and milk prolactin by 13.89% and 17.53%, respectively.

In this experiment, daidzein, formononetin and their combination increased plasma estradiol-17β, progesterone, and prolactin levels, similar to previous findings. This occurs because the metabolites have estrogen-like effects that competitively bind to estrogen receptors. Since estrogen receptors are limited in plasma, endogenous estrogen cannot bind and remains free, increasing estradiol-17β content. Dairy cow lactation is regulated by hormones including progesterone and prolactin, and the increased plasma progesterone and prolactin in this experiment corresponded with increased milk yield. Under normal physiological conditions, follicle-stimulating hormone and luteinizing hormone have synergistic effects: FSH promotes follicular growth and development, while LH induces follicular maturation and ovulation, stimulates granulosa cells to produce aromatase converting testosterone to estradiol-17β, and promotes uterine development. In this experiment, feeding daidzein, formononetin and their combination did not significantly affect plasma FSH and LH content, possibly because the cows were pregnant with high progesterone levels that inhibited FSH and LH secretion. Therefore, short-term feeding of daidzein, formononetin and their combination to lactating cows increased milk yield without significantly affecting reproduction-related hormones, possibly because excess hormones were secreted into milk, maintaining hormonal balance. The effects of long-term feeding warrant further investigation.

The main steroid hormones in mammalian milk include estrone, progesterone, and testosterone, with levels affected by animal breed, dietary protein level, and physiological state. Feeding high animal protein diets may increase milk yield and milk estrogen content. Campbell et al. reported that plasma and milk estrogen content significantly increased in late pregnancy. Zhu found that changes in milk estradiol-17β, growth hormone, and prolactin content paralleled changes in plasma. Batra and Abeyawardene found that milk estradiol-17β content correlated with plasma estradiol-17β content, and milk estriol content increased with gestation length.

In this experiment, changes in milk estrone, estradiol-17β, estriol, and progesterone content paralleled changes in plasma, indicating positive correlations between milk and plasma levels. Malekinejad et al. reported average milk estrone and estradiol-17β contents of 63 pg/mL throughout gestation, while this experiment showed estrone and estradiol-17β contents of 58.87-65.34 pg/mL and 5.49-7.86 pg/mL, respectively, lower than previous reports. This may be related to breed, physiological state, genetic factors, and milk sample extraction and separation methods. Yuan et al. measured milk progesterone content at 10-20 ng/mL, higher than in this experiment. Feeding daidzein, formononetin and their combination did not significantly affect milk estriol content.

Daidzein and formononetin are isoflavone phytoestrogens that can be converted to equol by gastrointestinal bacteria. Antignac et al. measured equol content in conventional milk at 36 g/L. Eeva et al. found that feeding cows red clover silage rich in daidzein increased milk equol content to 458-643 g/L. This experiment confirmed that feeding daidzein, formononetin and their combination significantly increased milk equol content, demonstrating the feasibility of producing equol-enriched milk. Therefore, feeding daidzein, formononetin and their combination significantly increases milk equol content while maintaining estrone, estradiol-17β, estriol, and progesterone levels within normal ranges.

Conclusion

Supplementation with daidzein, formononetin and their combination can increase dairy cow milk yield, improve milk quality, significantly increase milk equol content, and elevate estradiol-17β and progesterone levels in both plasma and milk.

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