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Original Research Article

Effect of red clover isoflavones on hormone, immune, inflammatory, and plasma biochemistry in lactating dairy cows

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ABSTRACT

This study was to conducted to investigate the effect of red clover isoflavones on the health indicated by immune status and blood biochemistry in dairy cows. Sixty-eight healthy Holstein lactating cows were randomly divided into four treatments ($n = 17$ per treatment) from 5 blocks according to milk yield using a randomized complete block design. No initial differences in parity (2.13 ± 1.21), days in milk (165 ± 21 d), and milk yield (33.93 ± 3.81 kg/d) between groups. Cows were fed the basal diet supplemented with 0, 2, 4, or 8 g/kg red clover extract (RCE) in diet (dry matter based). Feeding, refusal feed weights, and milk yield were recorded three consecutive days in weeks 0, 4, 8, and 12. Blood was collected from the tail vein of the cows on the last day of weeks 4, 8 and 12, 1 h after the morning feeding, and analyzed for hormones, immunoglobulins, inflammatory markers, and markers of liver and kidney activities. The dry matter intake was significantly decreased by 3.7% in the 8 g/kg group ($P < 0.05$). The fat-corrected milk yield was significantly higher in both of the 2 and 4 g/kg groups ($P < 0.01$). Plasma estradiol and prolactin showed a quadratic effect with increasing RCE levels, with the highest in the 4 g/kg group ($P < 0.05$). Plasma tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β levels decreased linearly with increasing dietary RCE levels. Plasma IL-18 levels showed a quadratic effect with increasing dietary RCE levels, with significantly lower levels in both of the 2 and 4 g/kg groups ($P < 0.05$). Plasma immunoglobulin A and D-lactic acid levels showed a quadratic effect with increasing dietary RCE levels, with significantly higher level in the 4 g/kg group ($P < 0.05$). The liver function and kidney activity makers were similar ($P > 0.05$). These results recommend the supplementation of RCE at a level from 2 to 4 g/kg DM.

1. Introduction

Red clover is a leguminous herb enriched with estrogenic isoflavones (phytoestrogens) such as formononetin and biochanin A (Yokoyama et al., 2020). In ruminants, isoflavones can improve growth performance and regulate microbial community structure parallel to hormone secretion. Biochanin A improves rumen fiber degradation and alleviates subacute rumen acidosis (Harlow et al., 2017). In particular, we found that biochanin A could hold rumen bacterial community structure to reduce urea degradation rate and improve nitrogen utilization in an in vitro fermentation test (Liu et al., 2020). The formononetin-rich clover improves ram weight gain and carcass meat quality (Pace et al., 2006).

The potential of isoflavones to alleviate problems such as low nitrogen utilization, resource wastage, water pollution (nitrogen in urine), and atmospheric pollution caused by an energy-N imbalance in ruminants is suggested (Hristov et al., 2012). The red clover silage isoenergetic and isonitrogenous replacement for alfalfa silage trials revealed a significant increase in dry matter digestibility and nitrogen utilization efficiency in the red clover group (Broderick et al., 2001; Halmemies-Beauchet-Filleau et al., 2014). We have demonstrated the effect of red clover extract (RCE) in enhancing

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nitrogen utilization and performance in dairy cows by supplementation with RCE in diets (Data unpublished).

Isoflavones can regulate the levels of estrogens (Wuttke et al., 2007), androgens (Sivoňová et al., 2019), growth hormones (Trifunović et al., 2016), and insulin (Oza and Kulkarni, 2018), as well as the immune response (Kanadys et al., 2020; Smeriglio et al., 2019) in animals or humans. Hence, isoflavones are an endocrine modulator due to their estrogenic effects in regulating endocrine homeostasis. Therefore, red clover and its extracts have been studied in the regulation of postmenopausal vascular inflammation (Wickham et al., 2022), diabetes control (Masuda et al., 2021), and cancer therapy (Akbaribazm et al., 2020) in women. Although isoflavone sources are present in dairy cows' diets, such as alfalfa hay, it is unknown whether exogenous supplementation influences hormone levels, immune status, and physical health in dairy cows. Based on the above isoflavone function of red clover, this study aimed to investigate the influence of RCE on the hormonal, immunological, and biochemical parameters in dairy cows.

2. Materials and methods

2.1. Animal ethics statement

All procedures were approved by the Animal Care and Use Committee of the Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing, China (Protocol Number: IAS 2022-107).

2.2. Experimental materials

The RCE was purchased from Hunan Phyto-way Plant Resource Co., Ltd., Hunan, China. The active ingredient of RCE is red clover isoflavone. Its composition is 20% red clover isoflavones (formononetin and biochanin A) and 80% carrier. The red clover isoflavones supplemented in each group were 0, 0.46 g/kg (formononetin at 0.31 g/kg and biochanin A at 0.15 g/kg), 0.93 g/kg (formononetin at 0.67 g/kg and biochanin A at 0.26 g/kg), and 1.83 g/kg (formononetin at 1.32 g/kg and biochanin A at 0.52 g/kg) with the increase of RCE level.

2.3. Experimental design

Sixty-eight healthy Holstein lactating cows were randomly divided into four treatments ($n = 17$ per treatment) from 5 blocks ($n = 8$, 16, 12, 16, and 16, respectively) according to milk yield using a randomized complete block design. No initial differences in parity (2.13 ± 1.21; $P = 0.618$), days in milk (165 ± 21 d; $P = 0.586$), and milk yield (33.93 ± 3.81 kg/d; $P = 0.757$) between groups. Cows were fed the basal diet (Table 1) supplemented with 0, 2, 4, or 8 g/kg RCE in total mixed ration (TMR) (DM based). The doses were converted according to previous studies (Pace et al., 2006; Weinert-Nelson et al., 2023). RCE was fed to cows by mixing it into the premix.

2.4. Feeding management

Cows were given the diet ad libitum with a target refusal rate of 10%. Cows were milked triple daily at 08:00, 15:00, and 22:00 by a rotary milking machine (AutoRotor PerFormer Plus, GEM Inc., Munich, Germany). Water was available to cows ad libitum. The barn had been renovated and cows were kept in a single pen per group. Humane endpoints are established to monitor daily pain or illness.

Table 1
Composition and nutrient level of the basal diet.

Item	Content[1]
Feed ingredients, % of DM	
Corn silage	30.0
Alfalfa hay	13.5
Whole cottonseed	5.68
Corn	7.32
Soybean meal (46% CP)	10.9
Cooked wheat hulls	3.72
Urea	0.54
Flaked corn	19.4
Puffed soybeans	3.01
Fat powder	0.82
Potassium bicarbonate	0.32
$NaHCO_3$	0.33
Rumen-protected lysine	0.17
Rumen-protected methionine	0.08
Molasses	0.99
Premix[2]	3.25
Total	100
Nutrient level, % of DM	
CP	18.3
Starch	24.1
NDF	26.1
ADF	19.3
NE_L[3], MCal/kg	1.69

DM = dry matter; CP = crude protein; NDF = neutral detergent fiber; ADF = acid detergent fiber; NE_L = net energy for lactation.
[1] The basal diet was formulated according to NRC (2001) for dairy cows.
[2] Concentration per kilogram of premix DM: 4 g of calcium, 5.5 g of phosphorus, 2 g of sodium, 4 g of magnesium, 40,000 IU of vitamin A, 37,000 IU of vitamin D, 500 IU of vitamin E, 30 mg of copper, 25 mg of iron, 140 mg of manganese, 140 mg of zinc, 0.8 mg of selenium.
[3] Net energy concentration of the diets was estimated using CPM CNCPS v3.0.8.1 (Cornell University, Ithaca, NY).

2.5. Sample collection and processing

2.5.1. Feed samples

The weights of feed consumed and refused were recorded on the last 3 days of weeks 4, 8 and 12, and fresh TMR and refusal feed samples were collected. Samples were oven-dried at 65 °C for 72 h. The dried sample was crushed using a Wiley mill (Thomas Scientific, USA) and filtered through a 1-mm sieve. The DM was determined by further drying at 105 °C for 4 h, and chemical analyses were based on the final absolute DM. The nitrogen content was determined using a nitrogen analyzer (Model CNS-2000; LECO Company, St. 1990) by the combustion method (Method 988.05.; AOAC, 1990), and CP was calculated as N × 6.25. The neutral detergent fiber and acid detergent fiber contents were determined as described by Van Soest et al. (1991) and Goering et al. (1970). Starch was determined by an enzymatic degradation (Amyloglucosidase, Novozymes, Curitiba, PR, Brazil) method according to Hendrix (1993). Net energy concentration of the diet was estimated using CPM CNCPS v3.0.8.1 (Cornell University, Ithaca, NY). Ingredients and chemical composition of the basal diet are listed in Table 1.

All dietary isoflavone levels per group were determined by reference to Krenn et al. (2002). The TMR sample (2 g, crushed) was mixed into 400 mL of 1 mol/L HCl–MeOH (4.17 mL of 12 mol/L hydrochloric acid fixed with 50 mL of methanol), and the mixture was sonicated for 10 min followed by a water bath at 85 °C for 30 min. Then, it was mixed well and cooled. The liquid was filtered through a 0.02-μm membrane. The filtrate (10 μL) was analyzed by HPLC (LC-2695, Waters, Milford, MA, USA) with a XBridge C18 column (250 mm × 4.6 mm, 5 μm). The column temperature was 40 °C, the flow rate was 1.0 mL/min, and the detection wavelength was 270 nm. The flow phase comprised acetonitrile (eluent A) and a

water/acetonitrile (75/25, vol/vol) mixture (eluent B). The gradient curves were as follows: 10% A/90% B (start), 10% A to 45% A (0 to 14 min), 45% A to 100% A (14 to 25 min), and 100% A to 0% A (25 to 27.5 min).

2.5.2. Milk samples

The milk yield was recorded on the last 3 d in weeks 4, 8, and 12. Milk samples (50 mL) were collected from each cow at 08:30, 15:30, and 22:30 and mixed well in the ratio of 4:3:3. The samples were added to a lyophilized tube containing bromo-2-nitropropane-1,3-diol. Milk composition was determined on the day of sampling using a milk composition instrument (EKOMILK, SPECTRA, Bulgaria). The yield of fat-corrected milk (FCM) was calculated by reference to Mjoun et al. (2010): FCM (kg/d) = [0.4 + 15 × (milk fat, %)] × (milk yield, kg/d).

2.5.3. Blood sample

On the last day of weeks 4, 8, and 12 of the experiment, blood samples were collected from the tail vein of the cow to demonstrate the metabolic changes in cows after intake of red clover isoflavones. Blood samples was chosen to be collected 1 h after morning feeding using a blood collection tube containing sodium heparin or ethylenediaminetetraacetic acid (EDTA, BD Biosciences, USA).

EDTA-containing blood samples were stored at 4 °C and then analyzed on the same day. Analyses included red blood cell count, hemoglobin, hematocrit, and total white blood cell count using an automated hematology analyzer (HemaVet, Drew Scientific, USA).

Blood with sodium heparin was centrifuged at 3,000 × *g* for 15 min, and the supernatant was taken for plasma preparation and frozen at −20 °C in a refrigerator. Plasma biochemical indexes (total protein, albumin, globulin, uric acid, creatinine, aspartate aminotransferase, alkaline phosphatase, and alanine aminotransferase) were analyzed using the Catalyst One Chemistry Analyzer (Idexx Laboratories Inc., China). Plasma estradiol, pituitary lactogen (prolactin), growth hormone, insulin, insulin-like growth factor-I (IGF-I), tumor necrosis factor (TNF)-α, interleukin-6 (IL-6), and interleukin-1β (IL-1β), were determined using a radioimmunoassay kit (Beijing Huaying Institute of Biotechnology, China). Plasma immunoglobulin G (IgG), immunoglobulin A (IgA), immunoglobulin M (IgM), interleukin-18 (IL-18), D-lactate, and diamine oxidase were measured using ELISA kits (Beijing Welab Science and Technology Co., Ltd., China).

2.6. Statistical analysis

All data analyses were conducted by the MIXED program of SAS (Version 9.4; SAS Inst. Inc., USA). The CS covariance structure was used as a repeated measure for the analysis. A Tukey–Kramer test was used for multiple comparisons of differences. The milk yield and dry matter intake (DMI) data were analyzed using the following model:

$$Y_{ijkl} = \mu + \mathrm{Cov}_i + \mathrm{Block}_j + \mathrm{RCE}_k + \mathrm{Cow}_{i:k} + \mathrm{Week}_l + \mathrm{RCE} \times \mathrm{Week}_{kl} + e_{ijkl}$$

The blood indicator data were analyzed using the following model:

$$Y_{ijkl} = \mu + \mathrm{Block}_j + \mathrm{RCE}_k + \mathrm{Cow}_{i:k} + \mathrm{Week}_l + \mathrm{RCE} \times \mathrm{Week}_{kl} + e_{ijkl}$$

In these two models, Y_{ijkl} was the dependent variable, μ was the least squares mean, Cov_i was the covariate effect (data for week 0 are covariates), Block_j was the random effect of the jth block ($j = 1$ to 5), RCE_k was the fixed effect of the kth treatment ($k = 0, 2, 4, 8$). $\mathrm{Cow}_{i:k}$ was the random effect of the ith cow ($i = 1$ to 68) in the kth diet treatment. Week_l was the effect of repeated measurements at week l ($l = 4, 8, 12$), RCE × Week_{kl} was the interaction effect between the kth diet treatment and week l. The e_{ijkl} is the random error associated with ijk^{th} data value assuming that e_{ijkl} is independently identically N(0, σ^2). Linear and quadratic effects are identified by using orthogonal polynomial comparison coefficients. The results were presented as means and standard error of the mean (SEM). The effect is significant at $P < 0.05$, while a tendency was declared at $0.05 \leq P \leq 0.10$.

3. Results

3.1. Feed intake and milk yield

Table 2 demonstrates the effect of RCE on cow DMI and the yield of FCM. Throughout the trial period, as the RCE level in the diet increased, the DMI of cows had a linear decreasing trend (Linear, $P = 0.05$) and was significantly lower in the 8 g/kg group ($P < 0.01$). As the level of RCE in the diet increased, there is a quadratic effect on FCM (Quadratic, $P < 0.01$), with the 2 g/kg group being the highest. The effect of RCE on DMI and FCM of cows had a significant interaction at different times.

3.2. Blood hormone concentration

As shown in Table 3, the plasma estradiol concentrations showed a quadratic effect with increasing dietary RCE content (Quadratic, $P = 0.01$), with the highest estradiol concentrations in the 4 g/kg group. The plasma growth hormone levels varied linearly with increasing dietary RCE levels (Linear, $P < 0.01$), with the highest growth hormone levels in the 8 g/kg group. The plasma prolactin levels showed a quadratic effect (Quadratic, $P < 0.01$) with increasing levels of dietary RCE, with the highest prolactin levels in the 4 g/kg group. Plasma insulin decreased linearly and IGF-I concentration increased linearly with increasing dietary RCE levels (Linear, $P < 0.01$). As the RCE dose increased, plasma insulin levels significantly decreased by 6.76%, 12.84%, and 20.95% than 0 g/kg group, respectively. Plasma IGF-I levels were significantly increased by 12.04%, 19.89%, and 32.46% in the 2, 4, and 8 g/kg groups ($P < 0.01$), respectively, compared to the 0 g/kg group. The regulation of RCE on cow estradiol, growth hormone, prolactin, and IGF-I interacted with time ($P < 0.05$).

Table 2
Effect of dietary red clover extract (RCE) on dry matter intake (DMI), 4% fat-corrected milk (FCM) yield in dairy cows.

Item	RCE, g/kg DM				SEM[1]	*P*-value				
	0	2	4	8		Treatment	Time	Interaction	Linear	Quadratic
DMI, kg/day	21.1 [a]	20.9 [a]	21.2[a]	20.4[b]	0.14	<0.01	<0.01	<0.01	0.05	0.44
FCM (4%) yield, kg/day	29.1[b]	32.3[a]	31.6[a]	29.8[b]	0.34	<0.01	<0.01	<0.01	0.89	<0.01

[a,b]Mean values within a row with different superscripts differed ($P < 0.05$).
[1] SEM = standard error of the mean; $n = 17$.

Table 3
Effect of dietary red clover extract (RCE) on plasma hormones in lactating dairy cows.

Item	RCE, g/kg DM				SEM[1]	*P*-value				
	0	2	4	8		Treatment	Time	Interaction	Linear	Quadratic
Estradiol, pg/mL	252[b]	308[a]	328[a]	295[a]	18.1	0.03	<0.01	0.02	0.07	0.01
Growth hormone, ng/mL	5.48[b]	6.67[a]	5.42[b]	7.17[a]	0.275	<0.01	0.27	<0.01	<0.01	0.31
Prolactin, μIU/mL	209[bc]	225[b]	318[a]	162[c]	12.5	<0.01	0.12	<0.01	0.38	<0.01
Insulin, μIU/mL	14.8[a]	13.8[b]	12.9[c]	11.7[d]	0.16	<0.01	<0.01	0.57	<0.01	0.77
Insulin-like growth factor-I, ng/mL	191[c]	214[b]	229[b]	253[a]	3.2	<0.01	<0.01	0.01	<0.01	0.79

[a to d]Mean values within a row with different superscripts differed ($P < 0.05$).
[1] SEM = standard error of the mean; $n = 17$.

3.3. Blood immunity levels

Table 4 demonstrates the effect of dietary RCE on blood inflammatory and immune factors in dairy cows. Plasma TNF-α, IL-6, and IL-1β levels decreased linearly with increasing dietary RCE levels (Linear, $P < 0.01$). As the RCE dose increased, plasma TNF-α significantly decreased by 8.28%, 15.94%, and 23.44%, respectively ($P < 0.01$). Plasma IL-6 levels were significantly reduced by 9.35%, 15.83%, and 23.74%, respectively ($P < 0.01$). Plasma IL-1β levels were reduced by 8.86%, 20.57%, and 30.69%, respectively ($P < 0.01$). Plasma IL-18 levels showed a quadratic effect with increasing dietary RCE levels (Quadratic, $P < 0.01$), with both the 2 and 4 g/kg groups being significantly lower at 12.45% and 7.59%, respectively ($P < 0.01$). Plasma IgA levels showed a quadratic effect (Quadratic, $P = 0.04$) with increasing dietary RCE levels, with the highest in the 4 g/kg group. The IgM and IgG were similar among the groups ($P > 0.05$).

3.4. Blood intestinal barrier indicators

As shown in Table 5, plasma D-lactate levels showed a quadratic effect (Quadratic, $P = 0.01$) with increasing dietary RCE levels, with the lowest level in the 4 g/kg group. The RCE regulation of plasma D-lactate levels in cows interacts with time ($P < 0.01$). The diamine oxidase was similar among the groups ($P > 0.05$).

3.5. Blood hematological parameters

The effect of dietary RCE on hematological parameters in cows is shown in Table 6. As dietary RCE levels increased, hemoglobin (Linear, $P = 0.02$) and platelets (Linear, $P < 0.01$) decreased linearly. The hemoglobin at RCE 8 g/kg group and platelets at RCE 4 and 8 g/kg groups are significantly lower ($P = 0.04$ and $P < 0.01$, respectively). Blood total protein increased linearly with increasing dietary RCE levels (Linear, $P = 0.01$), with the highest in the 8 g/kg group.

4. Discussion

This paper investigated the effect of RCE on the health status of lactating dairy cows in terms of hormone and immune-inflammatory factor levels and liver and kidney function activity. We found that the dietary RCE increased estrogen, prolactin, immune factor levels, and anti-inflammatory capacity in lactating dairy cows. In particular, the dairy supplementation of RCE at 2 and 4 g/kg DM improved milk production without affecting the liver, kidney marker activity, and DMI.

4.1. Feed intake and milk yield

There are few studies on the effect of red clover isoflavones on the performance of dairy cows. This study showed a linear decrease

Table 4
Effect of dietary red clover extract (RCE) on blood inflammatory and immune factor levels in lactating dairy cows.

Item	RCE, g/kg DM				SEM[1]	*P*-value				
	0	2	4	8		Treatment	Time	Interaction	Linear	Quadratic
Inflammatory factors										
TNF-α, pg/mL	64.0[a]	58.7[b]	53.8[c]	49.0[d]	0.75	<0.01	<0.01	0.27	<0.01	0.79
IL-6, pg/mL	139[a]	126[b]	117[c]	106[d]	1.0	<0.01	<0.01	0.33	<0.01	0.29
IL-1β, pg/mL	31.6[a]	28.8[b]	25.1[c]	21.9[d]	0.44	<0.01	<0.01	0.79	<0.01	0.72
IL-18, pg/mL	514[a]	450[b]	475[b]	488[ab]	10.9	<0.01	<0.01	0.29	0.25	<0.01
Immune factors										
IgA, μg/mL	97.6[b]	103.2[b]	106.8[a]	102.7[b]	2.56	0.05	0.01	0.39	0.07	0.04
IgG, μg/mL	945	965	972	933	31.3	0.37	<0.01	0.81	0.69	0.10
IgM, μg/mL	70.6	69.5	70.2	66.7	1.36	0.07	<0.01	0.38	0.03	0.30

TNF-α = tumor necrosis factor-α; IL = interleukin; Ig = immunoglobulin.
[a to d]Mean values within a row with different superscripts differed ($P < 0.05$).
[1] SEM = standard error of the mean; $n = 17$.

Table 5
Effect of dietary red clover extract (RCE) on blood intestinal barrier indicators in lactating dairy cows.

Item	RCE, g/kg DM				SEM[1]	*P*-value				
	0	2	4	8		Treatment	Time	Interaction	Linear	Quadratic
D-Lactic acid, μmol/L	15.0[a]	14.0[a]	13.6[b]	14.2[a]	0.30	<0.01	<0.01	<0.01	0.03	0.01
Diamine oxidase, IU/L	70.4	68.9	68.3	68.8	1.68	0.32	<0.01	0.74	0.15	0.23

[a,b]Mean values within a row with different superscripts differed ($P < 0.05$).
[1] SEM = standard error of the mean; $n = 17$.

Table 6
Effect of dietary red clover extract (RCE) on blood hematological parameters and plasma markers of liver and kidney activity of lactating dairy cows.

Item	RCE, g/kg DM				SEM[1]	*P*-value				
	0	2	4	8		Treatment	Time	Interaction	Linear	Quadratic
Blood hematological parameter										
White cells, $\times10^9$/L	10.4	10.4	11.0	9.6	0.78	0.67	0.13	0.77	0.61	0.39
Red blood cells, $\times10^{12}$/L	6.12	6.14	6.24	6.03	0.127	0.69	0.09	0.68	0.79	0.35
Hemoglobin, g/L	103^{ab}	106^{a}	102^{ab}	97^{b}	2.2	0.04	<0.01	0.08	0.02	0.06
Blood platelets, $\times10^9$/L	242^{a}	223^{a}	176^{b}	181^{b}	13.9	<0.01	0.30	0.83	<0.01	0.60
Hematocrit, %	28.0	28.3	27.9	27.8	0.44	0.93	<0.01	0.64	0.73	0.71
Plasma metabolite										
Total protein, g/L	65.8^{b}	65.4^{ab}	67.5^{ab}	70.6^{a}	1.45	0.05	0.10	0.07	0.01	0.23
Albumin, g/L	28.4	28.9	29.2	30.0	0.64	0.30	<0.01	020	0.06	0.80
Globulin, g/L	37.37	36.50	38.30	40.66	1.590	0.28	0.42	0.11	0.10	0.31
A:G ratio[2]	0.79	0.82	0.79	0.77	0.041	0.80	<0.01	0.40	0.58	0.46
Creatinine, μmol/L	80.6^{ab}	80.8^{ab}	76.5^{b}	84.4^{a}	1.78	0.01	<0.01	0.31	0.34	0.02
Uric acid, μmol/L	29.0	29.0	28.4	32.2	1.53	0.31	<0.01	0.07	0.20	0.23
Aspartate aminotransferase, U/L	85.4	87.8	76.0	76.8	9.31	0.66	<0.01	0.76	0.31	0.92
Alanine aminotransferase, U/L	25.8	25.1	24.8	24.0	0.86	0.60	<0.01	0.14	0.18	0.98
Alkaline phosphatase, U/L	31.3	37.2	32.1	31.2	2.15	0.37	<0.01	0.39	0.68	0.23
Total bilirubin, μmol/L	2.03	1.93	1.96	1.62	0.148	0.15	<0.01	0.16	0.05	0.37

[a,b]Mean values within a row with different superscripts differed ($P < 0.05$).
[1] SEM = standard error of the mean; $n = 17$.
[2] A:G ratio = albumin-to-globulin ratio.

in DMI as the dietary RCE content increased. The results are consistent with the findings that red clover silage decreased the DMI of dairy cows and growing lambs (Broderick et al., 2001). Studies with the feeding of red clover may have differed in DMI due to differences in nutrient intake and digestibility (Brito et al., 2007). In contrast, the results of this study may be due to a linear reduction in plasma insulin levels with increasing dietary RCE. Red clover isoflavones can improve insulin sensitivity (Faria et al., 2018), allowing for lower levels of secreted insulin. Reduced insulin levels can regulate the expression of agouti-related protein/neuropeptide Y in the hypothalamus to regulate the desire to feed (Mitchell and Begg, 2021).

Dietary supplementation of 2 to 4 g/kg RCE increased milk yield in dairy cows. The same results were obtained for feeding red clover silage to improve milk yield as in trials comparing different forage silage fed to lactating cows (Johansen et al., 2017). Increased plasma IGF-I and prolactin levels in cows by increasing dietary RCE may be one of the reasons for increased milk yield. As reported, prolactin (Toledo et al., 2020) and IGF-I (Marshman and Streuli, 2002) positively impact mammary gland development and milk production.

Overall, there was no negative impact of dietary supplementation with 2 to 4 g/kg RCE on DMI and milk yield of lactating cows. Increased milk production is inextricably linked with hormone level changes.

4.2. Plasma hormones

Isoflavones are derivatives of heterocyclic phenols with a structure similar to estrogen and are endocrine disruptors. Isoflavones act through multiple mechanisms, including hormone-mimicking and interaction with downstream signals that can modulate regulators of growth and cell proliferation pathways and affect various hormones or endocrine homeostasis (Sivoňová et al., 2019).

Dietary RCE increased plasma IGF-I, estrogen, and prolactin and decreased insulin levels in dairy cows. The structural similarities between estradiol and formononetin (and many other phytoestrogens) explain the estrogenic effects of isoflavones. Estrogens and phytoestrogens have similar structures allowing them to be identical in the estrogen receptor binding region (Vaya and Tamir, 2004). Estradiol regulates hypothalamic dopamine levels to stimulate prolactin secretion and increase prolactin receptors, which may be why dietary RCE increases plasma prolactin. Biochanin A increased pancreatic *SIRT1* mRNA expression and improved pancreatic energy supply and insulin sensitivity (Oza and Kulkarni, 2018) and formononetin with α-glucosidase inhibitory function (Masuda et al., 2021), which may explain the reduced insulin secretion in cows with reduced dietary RCE. Some phytoestrogens can regulate the intensity of growth hormone releasing hormone and somatostatin neuronal responses in the growth hormone response (Trifunović et al., 2016), which may be responsible for the growth hormone and IGF-I increased by dietary RCE. Similar results were achieved in calves fed soy isoflavones (Zhao et al., 2017).

Growth hormone, IGF-I, prolactin, and estrogen are closely associated with milk yield. Insulin-like growth factor-I is mediated by IGF-I receptors (IGF-IRs), which also activate IGF-IRs and exert biological effects to regulate mammary gland growth and development, promote mammary cell growth and mammary epithelial cell proliferation. IGF-I is an important cellular factor for increasing milk yield, especially milk fat (Akers et al., 2005; Dehnhard et al., 2000). Estradiol regulates milk production, reduces the secretion of lactose and milk fat, and increases milk protein production (Delbecchi et al., 2005). Prolactin stimulates mammary gland growth and development, initiates milk synthesis, and maintains lactation (Kobayashi et al., 2017). Improving plasma levels of IGF-I, prolactin and estrogen is one of the most critical factors in increasing milk production with the dietary supplement RCE.

4.3. Immune and anti-inflammatory

Decreased plasma TNF-α, IL-6, and IL-1β levels and increased IgA levels indicate that dietary RCE has anti-inflammatory effects. Isoflavones have been shown to benefit the restoration of the epithelial barrier, enhance the secretion of immune factors (Abdou and Abd Elkader, 2022) and to inhibit protein kinases and lymphocyte factors from acting as immune agents (Akiyama et al., 1987). The elevated IgA levels in this study may be related to the ability of isoflavones to promote the interaction between natural killer cells and B cells (Wei, 2011). Ferguson et al. (2014) also reported that dietary intake of isoflavones in healthy volunteers improved the immune response. Improved immunity of lactating

cows is essential for stress resistance and maintenance of production performance.

4.4. Intestinal barrier

Formononetin is proved to repair the epithelial barrier (Li et al., 2018); therefore, we explored the RCE influence on D-lactate and diamine oxidase, blood markers of the intestinal barrier in cows, and found that RCE had a beneficial role on the intestinal barrier in cows. Inflammation is the main factor causing damage to the intestinal barrier (Jeong et al., 2014). Phytoestrogens protect the intestinal barrier by increasing immune factors, decreasing the levels of inflammatory factors, and inhibiting the NF-κB pathway (Chacko et al., 2005). Furthermore, isoflavones enhance intestinal activity by stimulating the PI3K/Akt/mTOR signaling pathway, estrogen receptors, and 5-AMP-activated protein kinases to promote the synthesis of intestinal tight junction proteins (McCarty and Lerner, 2021). Dietary RCE could protect the intestinal mucosa of cows by increasing immune factors and decreasing inflammatory factor levels, and the exact mechanism needs further investigation.

4.5. Blood hematological parameters and plasma markers of liver and kidney activity

The dietary supplementation of 2 to 4 g/kg RCE had no significant effect on cows' plasma liver and kidney activity markers and blood parameters. Total protein and creatinine levels were significantly higher in the 8 g/kg group in cows, but still within the healthy range values (60 to 80 g/L and 45 to 195 μmol/L, respectively) (Kahn and Line, 2016). Moreover, the 8 g/kg group did not have an advantage over the 2 and 4 g/kg groups regarding estrogen, prolactin, and D-lactate levels and reduced DMI. We speculate that adding 8 g/kg RCE to the diet may increase intestinal, liver, and kidney loads.

5. Conclusion

Dietary supplementation with 2 to 4 g/kg of RCE did not modify feed intake, liver activity marker, and kidney activity marker levels. It increased immunity, anti-inflammatory capacity, improved lactation performance endocrine hormone levels, and improved intestinal barrier function to increase milk production in dairy cows. These results recommend the supplementation of RCE at a level from 2 to 4 g/kg DM.

Author contributions

Shiqi Zhang: Investigation, Writing-Original Draft, Visualization. **Xiaoyin Zhang:** Investigation. **Zhanbo Xiong:** Writing-Review and Editing. **Kexin Li:** Conceptualization, Methodology, Writing-Reviewing and Editing. **Yuan Gao:** Conceptualization, Methodology. **Ying Bu:** Conceptualization, Methodology. **Nan Zheng:** Conceptualization, Methodology. **Shengguo Zhao:** Conceptualization, Methodology, Data curation, Writing-Reviewing and Editing. **Jiaqi Wang:** Conceptualization, Methodology, Supervision.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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