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Original Research Article

Effects of biochanin A on lactational performance, nitrogen metabolism, and blood metabolites in dairy cows

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ABSTRACT

Optimizing nitrogen utilization efficiency and mitigating nitrogen losses in cows plays a pivotal role in fostering economic sustainability within contemporary agricultural systems. Biochanin A (BCA), a natural component in red clover, has the potential to improve nitrogen metabolism in dairy cows. The primary objective of this study was to probe the impact of biochanin A supplementation on lactational performance, nitrogen metabolism, and blood metabolites in dairy cows. A complete randomized block design experiment was conducted over 28 d, involving 36 multiparous Holstein cows (comparable milk yield = 37.1 ± 2.90 kg, BW = 642 ± 70.0 kg, days in milk = 92 ± 8.0 d, and parity = 2.4 ± 0.50), which were allocated to three treatment groups: the Control group (with 0 g/d BCA), the Low group (with 10 g/d per cow BCA), and the High group (with 40 g/d per cow BCA). Biochanin A supplementation improved the lactational performance of cows by increasing milk yield by 6.3% ($P = 0.007$) and feed efficiency by 12.7% ($P = 0.009$). Total intestinal apparent digestibility was unaffected by BCA supplementation ($P > 0.05$), but microbial nitrogen was increased by 30.0% ($P = 0.002$) for promoting nitrogen utilization efficiency by 20.7% ($P = 0.004$). Milk competent yields (protein, lactose, and non-fat milk solid) were increased with increasing BCA supplementation ($P < 0.05$). Urea nitrogen levels in plasma and milk were both decreased by BCA supplementation ($P < 0.05$). Blood routine parameters and plasma biochemical parameters both received no effect by BCA supplementation ($P > 0.05$). BCA did not affect body health of dairy cows. Additionally, none of the plasma endocrine hormones were affected ($P > 0.05$). A total of 95 significantly different metabolites were screened from the plasma metabolites of cows in the BCA-added and non-added groups. After performing an enrichment analysis of the metabolic pathways associated with the different metabolites, six specific pathways were identified: bile acid biosynthesis, aspartate metabolism, pyrimidine metabolism, arginine and proline metabolism, the urea cycle, and ammonia recycling. The inclusion of BCA is suggested to enhance milk yield and modulate nitrogen metabolism by influencing relevant metabolites within the metabolic pathways.

1. Introduction

In modern livestock farming, the digestive physiology of ruminants leads to inefficient conversion of nitrogen in their diets into nitrogen for livestock products (Nadeau et al., 2007). Approximately 57% of the nitrogen produced by rumen degradation of protein feeds and urea goes to the liver to be re-synthesized as urea, while only 38% of the synthesized urea is reintroduced into the gastrointestinal tract for metabolism (Recktenwald et al., 2014). Numerous studies have consistently demonstrated the nitrogen utilization rate is 23% to 30% in dairy goats and is 25% to 31% in dairy cows, which greatly contributes to the waste in protein feeds in the

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diet (Berthiaume et al., 2001; Lobley et al., 2000; Michalski et al., 2012; Patra and Aschenbach, 2018). After entering the rumen, dietary proteins are broken down by pepsin and extracellular proteases secreted by proteolytic bacteria, to produce peptides and amino acids (El-Shazly, 1952), after which they are further degraded to ammonia, which is utilized by rumen microorganisms to synthesize proteins and provide a high-quality nitrogen source for the animal. However, most of them enter the liver through blood circulation to synthesize endogenous urea (Alemneh, 2019; Jin et al., 2017). The nitrogen recycling mechanism in ruminants allows for the re-entry of endogenous urea synthesized by the liver into the gastrointestinal tract as a means of recycling nitrogen produced by amino acid metabolism (Lobley et al., 2000). After entering the rumen, urea is hydrolyzed to NH_3 to provide a nitrogen source for microbial protein synthesis (Jin et al., 2018). Nevertheless, the excessive rate of urea decomposition in ruminants diminishes the efficiency of animal's utilization of urea nitrogen, resulting in a considerable excretion of ammonia in feces and urine, causing nitrogen wastage and, in severe cases, ammonia toxicity (Alemneh, 2019; Jin et al., 2016; Patra, 2015).

Attention is being paid to ways of enhancing urea nitrogen utilization and reducing nitrogen emissions. Currently, acetohydroxamic acid can be used as a feed additive in livestock as a urease inhibitor, but its long-term effect is unstable (Ludden et al., 2000). Monensin, the most utilized antibiotic in ruminant diets, promotes nitrogen utilization within animals by inhibiting hyper-ammonia producing bacteria in the rumen resulting in increased bypass protein, but long-term use can lead to potential antimicrobial side effects (Ahmed et al., 2022; Beauchemin et al., 2008).

Biochanin A is one of the major components of isoflavonoid phytoestrogens in the legume family and was first discovered in the stems and leaves of red clover (Křížová et al., 2019). In addition to red clover, natural sources of biochanin A include alfalfa (Kagan et al., 2015), astragalus (Butkutė et al., 2018), peanut (Chukwumah et al., 2009), soybean (Hu et al., 2022), and chickpea (Gao et al., 2015). Studies have shown that the highest levels of biochanin A were found in red clover (Kim et al., 2020; Zgórka, 2009).

Biochanin A has antimicrobial activity mainly against high ammonia-producing bacteria of bovine and goat origin, reducing ammonia production from amino acid deamidation and improving nitrogen utilization efficiency (Flythe et al., 2013; Flythe and Kagan, 2010). It was shown that biochanin A can inhibit protein and urea decomposition by inhibiting proteolytic and ureolytic bacteria as well as urease activity and enhance microbial synthesis efficiency in the rumen (Liu et al., 2020). By feeding red clover or biochanin A, the bacteria were inhibited by biochanin A (BCA) resulting in improved weight gain performance (Harlow et al., 2020, 2017). It has been shown that red clover can increase dry matter intake as well as milk production and milk quality in cows (Halmemies-Beauchet-Filleau et al., 2014), and BCA has also been shown to improve production performance, milk quality and rumen fermentation in dairy goats and sheep (Li et al., 2023). However, the effects of biochanin A on dairy cows are not known. Hence, the aim of this study was to assess the influence of biochanin A on the lactational performance and nitrogen metabolism of dairy cows.

2. Materials and methods

2.1. Animal ethics statement

Experimental animals were treated in compliance with the ethical guidelines for animal welfare set forth by the Institute of Animal Science, Chinese Academy of Agriculture Sciences (Beijing, China; IAS2021-232).

2.2. Animals, experimental design, and treatments

The trial was carried out at Yangzhou University Experimental Farm (Yangzhou, China). A total of 36 Holstein cows (milk yield = 37.1 ± 2.90 kg; body weight = 642 ± 70.0 kg; days in milk = 92 ± 8.0 d; parity = 2.4 ± 0.50) were used for a randomized complete block design experiment of 28 d, with 21 d allocated for diet adaptation and 7 d for sample collection. Cows were divided into four blocks based on milk yield (9 cows per block). Cows within block were randomly assigned to three treatments (12 cows per treatment). The treatments were as follows: control (CON, basic diet without biochanin A), Low (basic diet with 10 g/d per cow BCA), and High (basic diet with 40 g/d per cow BCA). The doses were converted according to previous studies (Harlow et al., 2017). Biochanin A (purity 98%) was purchased from Xi'an Tianye Biotechnology Co., Ltd., China. The daily BCA allocation was distributed between 2 feedings at 08:00 and 15:00 (5 and 5 g for Low or 20 and 20 g for High). To guarantee that the treatment group obtained their required dosage of BCA, we blended it with a small quantity of basic diet and individually fed it to each cow before feeding total mixed ration (TMR) at 08:00 and 15:00. After the cows had consumed their basic diet with BCA, the remaining diet was subsequently offered to them. The basic diet composition and nutrient composition are shown in Table 1. The experimental diet was formulated and provided to meet the nutritional needs of NRC (2001).

Cows were individually housed. Components of feed were mixed as TMR by TMR SINO-GER mixer (Bvl Inc., Germany) and

Table 1
Diet composition and nutrient composition of basal diet in the experiment.

Item	Content
Ingredient, % of DM	
Corn silage	31.87
Alfalfa hay	21.02
Oat hay	3.75
Cottonseed	3.69
Corn grain, coarsely ground	17.43
Soybean meal	5.57
Urea	0.34
Wheat bran	3.74
DDGS	1.53
BSG	1.82
Malt hulls	1.49
Sprayed corn barn	1.87
Corn germ meal	3.76
Premix[1]	2.12
Total	100.00
Chemical composition[2], % of DM	
DM	48.63
CP	18.60
EE	5.96
NDF	31.15
ADF	12.03
NE_L, Mcal/kg	1.49
Isoflavone analysis, mg/kg	
Formononetin	Undetectable
Biochanin A	Undetectable
Genistein	166.13
Daidzein	75.00

DDGS = dried distiller's grains with solubles; BSG = brewer's spent grains; DM = dry matter; EE = ether extract; CP = crude protein; NDF = neutral detergent fiber; ADF = acid neutral detergent fiber; NE_L = net energy for lactating.

[1] Contained the following per kilogram of premix (DM basis): 4 g of Ca; 5.5 g of P; 2 g of Na; 4 g of Mg; 30 mg of Cu; 25 mg of Fe; 140 mg of Mn; 140 mg of Zn; 0.8 mg of Se; 40,000 IU of vitamin A; 37,000 IU of vitamin D; 500 IU of vitamin E.

[2] NE_L was calculated according to NRC (2001), the other indicators were measured values.

TMR were fed 3 times per day (at 08:00, 15:00 and 22:00). Daily feed supply was adjusted to accommodate 10% refusals and water was provided ad libitum. To keep track of cows' weight, a walk-through static scale (Pellon Group, Finland) was used every time they left the milking parlor. During the trial, veterinarians monitored and documented the daily mental and physical condition of the cows to ensure prompt treatment of any illnesses.

2.3. Feed sample collection and analysis

During d 26 to 28 of the experimental period, individual dry matter intake (DMI) was assessed using daily weight of TMR provided and refused for per cow, along with absolute DM percentages. Fresh TMR samples collected from different feeding time points (at 08:00, 15:00, and 22:00) were mixed daily in a 4:3:3 ratio. After 3 days of collection, fresh TMR samples from each day were thoroughly mixed and 250 g samples were taken by the quartering method for analysis. Refusals were collected and weighed all day before removing at 15:00. The feed samples were dried at 65 °C for a duration of 12 h in the drying oven (Thermo Fisher Scientific, China), followed by crushing using a Cyclotec 1093 Mill (Foss, Denmark) through a 1-mm screen using a Wiley mill (A. H. Thomas Co., USA) and were stored at room temperature until further general composition analysis.

Absolute dry matter (DM) percentages were determined using the method 930.15 of AOAC (2016). Crude protein (CP, N × 6.25) was measured by the Kjeldahl method (Mckenzie and Wallace, 1954) with Kjeltec 8400 (Foss, Denmark). Ether extract (EE) percentages was measured by method 920.39 of AOAC (2016). The concentrations of NDF and ADF were measured by the protocol of Van Soest et al. (1991) and Goering and Van Soest (1970) with Ankom 220 fiber analyzer (ANKOM Technology, USA). Ash contents were measured through the ignition of samples at a temperature of 550 °C for a period of 8 h, utilizing the AOAC method 942.05, with the aid of a chamber muffle furnace (Thermo Fisher Scientific, China). The net energy (NE) of the diet was calculated by multiplying the net energy for lactation (NE_L-3X) density of each feed ingredient by its proportion in the diet, as outlined by the NRC (2001).

2.4. Milk sample collection and analysis

The dairy farm used a rotary milking unit (AutoRotor Performer Plus, GEM Inc., Munich, Germany) to milk cows three times a day at 08:30, 15:30, and 22:30. Milk yield was recorded per milking. Milk samples were collected using milk splitters at the sampling period, which included d 21 to 23. The milk samples were mixed thoroughly following the ratio of 4:3:3 for morning, afternoon, and night milking. Furthermore, duplicate samples were collected from each cow. One sample was preserved with Broad Spectrum Microtabs II (D&F Control System Inc., Canada) and stored at 4 °C for further milk composition analysis. The other duplicate sample was stored at −20 °C for milk urea nitrogen and milk total nitrogen measurement.

Milk urea nitrogen was detected using the diacetyl monoxime method. Milk total nitrogen was measured with Kjeltec 8400 (Foss, Denmark), as described by (Mckenzie and Wallace, 1954). The milk composition was analyzed using the MilkoScan FT3 milk analyzer (Foss Food Technology Corp, US) and included fat, protein, lactose, non-fat milk solid, and total milk solid.

2.5. Blood sample collection and analysis

Blood samples were collected 2 h after morning feeding from the tail root vein using vacutainers to demonstrate the metabolic changes in cows after intake of biochanin A on d 24 of the experimental period. The collection tube contained Venoject II VP-H100K with heparin sodium (Terumo Corporation, Japan) and Venoject II VP-H100K with EDTA-2K (Terumo Corporation, Japan).

Blood samples containing EDTA-2K were stored at 4 °C until shipped on same day in cold ice packs to the laboratory be analyzed. White blood cell count, red blood cell count, hemoglobin, platelet count and plateletcrit were analyzed by the Automatic Hematology Analyzer (Mindray BC-5100CRP, China).

Blood samples containing heparin sodium were centrifuged at 3000 × *g* for 15 min at 4 °C. The supernatant was taken for plasma preparation and stored at −20 °C for the next plasma biochemistry analyses. Total protein, albumin, globulin, albumin/globulin ratio, creatinine, uric acid, glucose, total amino acids, ammonia, β-hydroxybutyrate, alanine aminotransferase and aspartate aminotransferase were analyzed by the Catalyst One Chemistry Analyzer (IDEXX Laboratories Inc., China). Plasma endocrine hormone indicators included growth hormone, prolactin, insulin, estradiol and insulin-like growth factor 1 and were analyzed by a radiation immunoassay analyzer (XH6080, Xi'an CNNC Nuclear Instrument Co., Ltd., China). Plasma urea nitrogen was detected by a reagent kit (Beijing North Institute of Biotechnology, China).

2.6. Urine sample collection and analysis

Urine samples were collected across nine times over three consecutive days (d 26-28) from 10 randomly selected cows per group, with spot samples collected by stimulating the nerves in the pubic area by massaging the underside of the vulva. The duration of each urine sampling interval was 8 h (at 08:30, 16:30 and 00:30). For each sample, 5 mL of urine (total 45 mL) was transferred to the 50-mL test tube containing 5 mL of 10% dilute sulfuric acid and refrigerated at 4 °C until the sampling process was finished. Samples were stored frozen for total nitrogen, allantoin, uric acid, and creatinine analyses.

Microbial nitrogen flow in the rumen was calculated based on purine derivatives following the protocol by Chen and Gomes (1992). In brief, microbial nitrogen (g/d) = $X \times 70/(0.116 \times 0.83 \times 1000)$, where X is microbial purine uptake at duodenum (mmol/d), 70 is the N content of purine derivatives, 0.116 is ratio of purine N to total N in rumen microbiome, 0.83 is the average digestibility of microbial purines at duodenum; $X = (Y - 0.385 \times BW^{0.75})/0.85$, where Y is urinary excretion of purine derivatives (mmol/d), $0.385 \times BW^{0.75}$ is the endogenous excretion of purine derivatives (mmol/d), 0.85 is the recovery ratio of absorbed purines as purine derivatives in urine. The urinary excretion of purine derivatives was calculated from the daily urine output and the determined concentration of allantoin and uric acid. Daily urine output was estimated based on creatinine excretion of 29 mg/kg BW (Valadares et al., 1999). Urine creatinine and uric acid concentrations were determined using analytical kits (Nanjing Jiancheng Bioengineering Inc., Nanjing, China). Allantoin concentration was measured by a colorimetric method according to the protocol by Chen and Gomes (1992).

Urea nitrogen concentration was determined using analytical kits (Nanjing Jiancheng Bioengineering Inc., Nanjing, China). Nitrogen concentration in urine was determined in the same way as in milk with the Kjeltec 8400 (Foss, Denmark). Total urinary nitrogen excretion was calculated by multiplying nitrogen concentration by daily urine output.

2.7. Fecal sample collection and analysis

Fecal samples were collected directly from the rectum of each cow during conscious defecation 9 times over three consecutive

days (d 26-28) from 10 randomly selected cows per group with spot samples. The duration of each sampling interval was 8 h (at 08:30, 16:30 and 00:30). For each sample, 50 g of feces (total 450 g) was transferred to a self-sealing bag refrigerated at −20 °C until the sampling process was finished, for further chemical analysis.

Fecal samples were thawed at 25 °C on metal trays and dried for approximately 72 h at 65 °C in a drying cabinet (Thermo Fisher Scientific, CN). The samples were turned several times during drying to accelerate the rate of moisture evaporation and were ground using a Cyclotec 1093 Mill (Foss, Denmark) before being passed through a 1-mm screen (A. H. Thomas Co., US). Total nitrogen contents were measured following the Kjeldahl method (Mckenzie and Wallace, 1954) with a Kjeltec 8400 (Foss, Denmark). The concentrations of NDF and ADF were determined according to the protocol of (Van Soest et al., 1991) using an Ankom 220 fiber analyzer (ANKOM Technology, USA). Total intestinal apparent digestibility was calculated employing acid insoluble ash (AIA) as the internal standard by measuring AIA content in feed samples and manure (Van Keulen and Young, 1977).

2.8. Plasma metabolome and analysis

Methanol with the addition of phenylhydrazine was used for the extraction of polar metabolites. Subsequently, the samples underwent vortexing and were then kept at −20 °C for 30 min to facilitate the derivatization of alpha-keto acids (Shen et al., 2021). Following the derivatization process, the samples were incubated at 4 °C and were centrifuged at 1500 × *g* for 30 min. After incubation, centrifugation was performed at 4 °C and 12,000 × *g* for 10 min to separate the components effectively. The obtained clean supernatant was moved to a new tube and dried by a SpeedVac concentrator under H_2O mode in preparation for further LC-MS analysis. The dried extract was reconstituted in a solution of 5% acetonitrile in water. The LC-MS analysis was performed by the Agilent 1290 II UPLC, in connection with the Sciex 5600+ quadrupole-TOF MS. Separation of the polar metabolites involved using a Waters ACQUITY HSS-T3 column (3.0 × 100 mm, 1.8 μm) for reverse phase chromatography and a Waters ACQUITY BEH Amide column (2.1 × 100 mm, 1.7 μm) for normal phase chromatographic separation. MS parameters included positive ion mode with an electron spray ionization voltage of 5.5 kV and negative ion mode with -4.5 kV. The vaporizer temperature was 500 °C, while the drying gas (N_2) pressure, nebulizer gas (N_2) pressure, and curtain gas (N_2) pressure were set at 50, 50, and 35 psi, respectively. The mass spectrometry scan range was 60 to 800 *m*/*z* (Tian et al., 2022). Metabolites were subjected to MS/MS analyses using an information-dependent acquisition mode. The collision energy was configured at (±) 35 ± 15 eV during the analyses. Data collection and evaluation were carried out utilizing Analyst TF 1.7.1 Software (AB Sciex, Concord, ON, Canada). All identified ions were exported from MarkerView 1.3 (AB Sciex, Concord, ON, Canada) to Excel, presenting a two-dimensional matrix containing mass to charge ratio (*m*/*z*), retention time, and peak areas. Subsequently, PeakView 2.2 (AB Sciex, Concord, ON, Canada) was employed to extract MS/MS data and perform comparisons with the Metabolites database (AB Sciex, Concord, ON, Canada), HMDB, and standard references for ion annotation (Tian et al., 2022).

The metabolite data were normalized on the MetaboAnalyst 5.0 (https://www.metaboanalyst.ca) and then were clustered and analyzed in conjunction with enriched metabolic pathways. The processed data information was subjected to partial least squares discriminant analysis (PLS-DA) and required permutation tests as well as the derivation of the variable importance in projection (VIP). The VIP values, derived from the PLS-DA model with a screening condition of VIP > 1, were utilized to identify significant differential metabolites between groups. Subsequently, precise comparison based on mass-to-charge ratios, matching to the database using secondary mass spectrometry plots, was employed to finally identify the differential metabolites.

2.9. Data analyses

All data (n = 12 per group) were analyzed using the MIXED model of SAS version 9.4 (SAS Institute Inc., USA). Fixed effect in the model was *T* with the following model:

$$Y_{ijk} = \mu + C_i + T_j + B_{k(i)} + e_{ijk}$$

where Y_{ijk} = dependent variable; μ = overall mean; C_i = random effect of block *i* (*i* = 1–5); T_j = fixed effect of BCA treatment *j* (*j* = 0, 10, 40); $B_{k(i)}$ = random effect of cows *k* (*k* = 1–36) within block *i*; e_{ijk} = residual error. Differences between treatment groups were compared using the least squares means and Tukey–Kramer's least significant difference method for multiple comparisons where significance was at $P < 0.05$.

3. Results

3.1. Lactational performance

DMI, milk yield, ECM, feed efficiency, and total intestinal apparent digestibility are presented in Table 2. DMI was similar ($P = 0.114$) between treatments. No statistically significant differences were found in milk yield, ECM, and feed efficiency between the Low and Control groups. However, the High group increased by 6.3% ($P = 0.007$) in milk yield, increased by 7.3% in ECM ($P = 0.042$), and increased by 12.7% in feed efficiency ($P = 0.009$). Dietary BCA supplementation did not have a significant effect on the total intestinal apparent digestibility of crude protein (CP), ether extract (EE), and neutral detergent fiber (NDF) ($P > 0.05$).

3.2. Milk composition

Milk composition contents of protein, fat, lactose, non-fat milk solid, and total milk solid were not affected significantly by BCA supplementation, as shown in Table 3 ($P > 0.05$). However, yields of protein, lactose, and non-fat milk solid in milk of the high group

Table 2
Effect of different levels of biochanin A on the lactational performance of cows.

Item	Treatment[1]			SEM	*P*-value
	Control	Low	High		
DMI, kg/d	24.63	23.22	22.72	0.375	0.114
Milk yield, kg/d	35.39[b]	35.27[b]	37.62[a]	0.545	0.007
ECM[2], kg/d	39.54[b]	39.83[b]	42.41[a]	0.558	0.042
Feed efficiency[3]	1.42[b]	1.52[ab]	1.60[a]	0.026	0.009
Total intestinal apparent digestibility[4], %					
CP	76.36	77.63	76.25	0.569	0.534
EE	39.43	44.19	38.95	1.511	0.263
NDF	76.10	77.52	75.92	0.672	0.599

DMI = dry matter in take; ECM = energy-corrected milk; CP = crude protein; EE= ether extract; NDF = neutral detergent fiber.
[a,b] Means within a row with different superscripts differ significantly ($P < 0.05$).
[1] Control, basic diet without biochanin A; Low, basic diet with biochanin A at 10 g/d per cow; High, basic diet with biochanin A at 40 g/d per cow.
[2] ECM was calculated according to NRC (2001): ECM (kg/d) = 12.55 × fat yield (kg/d) + 7.39 × protein yield (kg/d) + 5.34 × lactose yield (kg/d).
[3] Feed efficiency was calculated as the rate of ECM (kg/d) divided by DMI (kg/d).
[4] Total intestinal apparent digestibility of nutrients was calculated using acid insoluble ash as an internal standard (Keulen and Young, 1977).

were significantly increased by 9.2% ($P = 0.017$), 5.5% ($P = 0.017$), and 5.2% ($P = 0.015$), respectively. The high group showed no significant effect on the yield of milk fat and total milk solids ($P > 0.05$).

3.3. Nitrogen metabolism

As shown in Table 4, no significant difference was observed between the control and BCA treatment groups in nitrogen intake, fecal nitrogen excretion, urinary urea nitrogen, or urinary nitrogen excretion ($P > 0.05$). However, urea nitrogen concentration in the milk and in the plasma were decreased significantly with High BCA supplementation ($P < 0.05$). When compared with Control group, the milk nitrogen excretion, microbial nitrogen, and nitrogen utilization efficiency in High group were increased by 10.4% ($P = 0.002$), 30.0% ($P = 0.002$), and 20.7% ($P = 0.004$), respectively. In contrast, none of the differences in the Low group were significant.

3.4. Body health

Body health indicators included blood routine parameters and plasma biochemical parameters as presented in Table S1. Blood routine parameters of white blood cell count, red blood cell count, hemoglobin, platelet count, and plateletcrit were not affected by the dietary BCA ($P > 0.05$). The same is true for plasma biochemical parameters like total protein, albumin, globulin, albumin/globulin ratio, creatinine, uric acid, glucose, total amino acids, ammonia, β-hydroxybutyrate, alanine aminotransferase and aspartate aminotransferase, which also were not significantly different in the BCA treated and control groups ($P > 0.05$).

3.5. Plasma endocrine hormones

Plasma endocrine hormone indicators included growth hormone, prolactin, insulin, estradiol, and insulin-like growth factor 1 and the results are shown in Table S2. We observed no difference in plasma endocrine hormone indicators with BCA treatment ($P > 0.05$).

3.6. Plasma metabolites

Fig. 1 shows a plot of the PLS-DA analysis scores, indicating the significant differences in the differential metabolites among the groups. The VIP data obtained from the PLS-DA model analysis were used to screen significantly different metabolites using VIP > 1 as the criterion. A total of 98 significantly different metabolites were screened in this experiment, of which Fig. 2 shows only the top 20 in VIP score ranking, of which 18 were down-regulated and 2 were up-regulated. The differential metabolites were 4-ethylphenylsulfate, gamma-glutamylalanine, 5′-diphospho-N-acetyl-D-glucosamine (UDP-GlcNAc), 3-oxodecanoic acid, 2-methyl-tridecanoic acid, 4-pyrimidineethanesulfonic acid, N-acetyl-L-proline, hydrocinnamic acid, leucyl-aspartate, 3-hydroxycapric acid, cinnamic acid, gamma-glutamyl-leucine, indole-3-lactic acid, L-valine, dodecanedioic acid, thymidine, glycochenodeoxycholic acid, 11-HpODE, cholic acid, and paracetamol sulfate.

Metabolic pathways involved in differential metabolites were analyzed using the MetaboAnalyst 5.0 online analysis platform in combination with KEGG. The metabolic pathways with *P*-values < 0.05 were screened. The results are shown in Fig. 3. A total of six metabolic pathways were screened as follows: bile acid biosynthesis; aspartate metabolism; pyrimidine metabolism; arginine and proline metabolism; urea cycle; ammonia recycling. Each metabolic pathway is represented by a circle. Circle color depth indicates the degree of metabolite change in each metabolite pathway. The size of circle indicates the degree of the pathway's influence on the organism.

Table 3
Effect of different levels of biochanin A on the milk composition of cows.

Item	Treatment[1]			SEM	*P*-value
	Control	Low	High		
Milk composition, %					
Protein	3.71	3.65	3.67	0.044	0.816
Fat	4.18	4.29	4.13	0.094	0.759
Lactose	5.26	5.18	5.23	0.027	0.513
Non-fat milk solid	9.75	9.66	9.67	0.059	0.726
Total milk solid	13.93	13.94	13.81	0.138	0.889
Milk component yield, g/d					
Protein	1300[b]	1271[b]	1420[a]	19.0	0.017
Fat	1473	1512	1563	31.6	0.521
Lactose	1862[b]	1824[b]	1965[a]	28.7	0.017
Non-fat milk solid	3451[b]	3401[b]	3630[a]	44.7	0.015
Total milk solid	4924	4913	5173	64.3	0.108

[a,b] Means within a row with different superscripts differ significantly ($P < 0.05$).
[1] Control, basic diet without biochanin A; Low, basic diet with biochanin A at 10 g/d per cow; High, basic diet with biochanin A at 40 g/d per cow.

4. Discussion

Due to the scarcity of studies investigating the impact of BCA on production performance of dairy cows, the discussion cites the findings of trials related to red clover that contains large quantities of BCA.

4.1. Lactational performance

It has been shown that feeding red clover silage (converted BCA supplement of 41.4–43.6 g/d per cow) in comparison to alfalfa silage showed a significant decrease in daily dry matter intake (DMI) in cows by 2.3 to 2.5 kg (Broderick, 2018; Broderick et al., 2001). In the ram lamb study, where DMI was reduced and increased feed efficiency in levels of red clover hay inclusion at reported BCA concentrations (converted BCA supplement 0.6 g/d per lamb) compared to orchardgrass hay (Jr et al., 2023). However, the results indicated BCA had no effect on the DMI in a trial of feeding 6 g/d per goat to dairy goats (Li et al., 2023). In this study, we obtained results that 10 or 40 g/d per cow dose BCA had no effect on the DMI of dairy cows. Forage forms of BCA such as red clover silage and red clover hay may impact on rumen fullness leading to a decrease in DMI. We hypothesize that this may be different in the results about DMI due to different forms of BCA supplementation. It was found that 6 g/d per goat high dose BCA significantly increased milk yield by 30.71% but the 2 g/d per goat low dose had no significant effect when applied to dairy goat feeding (Li et al., 2023). Our results are that the dietary supplementation of 40 g/d per cow BCA had improved milk yield and feed efficiency in the study; however, the 10 g/d per cow BCA dose did not have an advantage. Therefore, consistent with the previous report in dairy goats, our results indicate that high doses of BCA had a significant effect on milk yield in ruminants.

Several studies have reported that the replacement of grass silage by red clover silage (converted BCA supplement 33.9–48.9 g/d per cow) in the diet does not affect the total intestinal OM and NDF nutrient digestibility (Bertilsson and Murphy, 2003; Kuoppala et al., 2009; Vanhatalo et al., 2007). In this study, we also found that the dietary BCA supplementation had no significant effect on the apparent digestibility of NDF. In addition, CP and EE are not significantly affected either.

Table 4
Effect of different levels of biochanin A on the nitrogen metabolism of cows.

Item	Treatment[1]			SEM	*P*-value
	Control	Low	High		
Nitrogen intake, g/d	733.5	693.6	679.4	10.61	0.114
Feces					
Nitrogen excretion, g/d	247.4	221.6	230.4	6.75	0.306
Urine					
Urea nitrogen, mg/dL	472.8	513.6	501.6	21.16	0.731
Nitrogen excretion, g/d	243.5	245.5	225.1	9.70	0.392
Milk					
Urea nitrogen, mg/dL	13.88^{a}	14.10^{a}	11.18^{b}	0.469	0.017
Nitrogen excretion, g/d	202.9^{b}	199.2^{b}	224.1^{a}	2.89	0.002
Plasma					
Urea nitrogen, mg/dL	18.32^{a}	17.64^{a}	12.86^{b}	0.936	0.037
Rumen					
Microbial nitrogen, g/d	2069^{b}	2267^{b}	2689^{a}	76.6	0.002
Nitrogen utilization efficiency, %	27.90^{b}	28.81^{b}	33.67^{a}	0.649	0.004

^{a,b} Means within a row with different superscripts differ significantly ($P < 0.05$).
[1] Control, basic diet without biochanin A; Low, basic diet with biochanin A at 10 g/d per cow; High, basic diet with biochanin A at 40 g/d per cow.

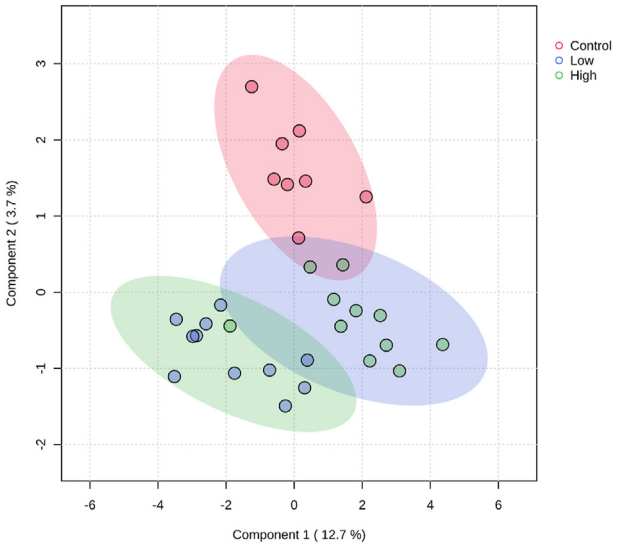

Fig. 1. Partial least squares discriminant analysis (PLS-DA) score chart of plasma metabolites. Control, basic diet without biochanin A; Low, basic diet with 10 g/d per cow biochanin A; High, basic diet with 40 g/d per cow biochanin A.

4.2. Milk composition

It has been shown that replacing part of the corn silage with red clover silage increased milk yield in dairy cows, and that the concentration of lactose and milk urea nitrogen increased linearly with increasing red clover silage, while milk protein content and yield decreased linearly (Halmemies-Beauchet-Filleau et al., 2014). Additionally, it has been stated that with increasing addition of isoflavone, milk protein and milk fat content gradually decreased and lactose content gradually increased. However, the difference was not significant, and the content of total milk solids showed a linear decrease (Zhan et al., 2017). A trend of decreasing milk yield was also observed after supplementing cows with 12.5 g of 40% isoflavone (Kasparovska et al., 2016). The composition of isoflavones may be the main reason for decreasing milk yield since the effect of isoflavones on animal performance depends on the relative effect between the components (Harlow et al., 2020). In our trial, we found that the dietary supplement BCA significantly increased the yield of all milk components except milk fat and total solids, in cows; however, the observed differences in milk components were not significant, perhaps due to the dilution effect accompanying increased milk yield (Johansen et al., 2017).

4.3. Nitrogen metabolism

In this trial, although the addition of BCA did not have a significant effect on urinary nitrogen concentration or urinary urea nitrogen excretion, the microbial nitrogen was increased by 30%, indicating that BCA could improve protein utilization. This may be attributed to the inhibition of urease activity by BCA, which slowed down the hydrolysis of urea in the rumen, reducing the concentration of ammonia (Liu et al., 2020). Because only a small portion of ammonia in the rumen is absorbed by rumen microorganisms, most of it is transported through the blood to the liver to synthesize endogenous urea, which is excreted with urine (Flythe and Kagan, 2010). On the other hand, the addition of BCA reduced protein and amino acid degradation and increased bypass protein and digestible amino acids by inhibiting the activity of protein-degrading and super ammonia-producing bacteria in the rumen (Harlow et al., 2017; Liu et al., 2020). The higher the utilization of protein by rumen microorganisms, the lower the blood urea nitrogen and blood ammonia levels (Nicholson et al., 1992) and correspondingly significantly lower plasma levels of urea nitrogen with BCA supplementation were observed in our experiment.

4.4. Body health

The health and nutritional status of animals was assessed using hematological analyses, which encompassed parameters such as hemoglobin, hematocrit, red blood cells, and white blood cells (Gunun et al., 2022). Plasma biochemical indicators are one of the most important indicators of health status and include total protein, albumin and globulin (Wang et al., 2011). In our trials, we found that dietary BCA supplementation had no significant effect on any of the cow hematological parameters and plasma biochemical parameters within normal expected levels, so BCA had no effect on the health of the cows.

4.5. Plasma endocrine hormones

As an isoflavonoid phytoestrogen, BCA mainly regulates the reproductive and nutritional processes of the body through neuroendocrine regulation, inducing changes in the levels of endogenous hormones such as prolactin and insulin-like growth

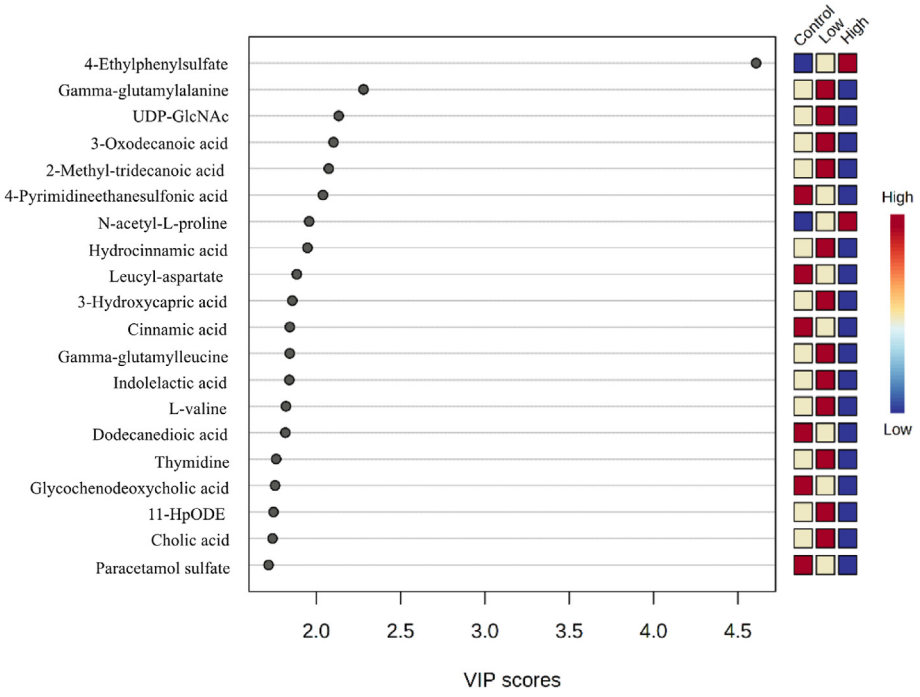

Fig. 2. Top 20 significantly different plasma metabolites identified in the biochanin A (BCA) groups and the control group. Control, basic diet without biochanin A; Low, basic diet with 10 g/d per cow biochanin A; High, basic diet with 40 g/d per cow biochanin A. VIP = variable importance in projection.

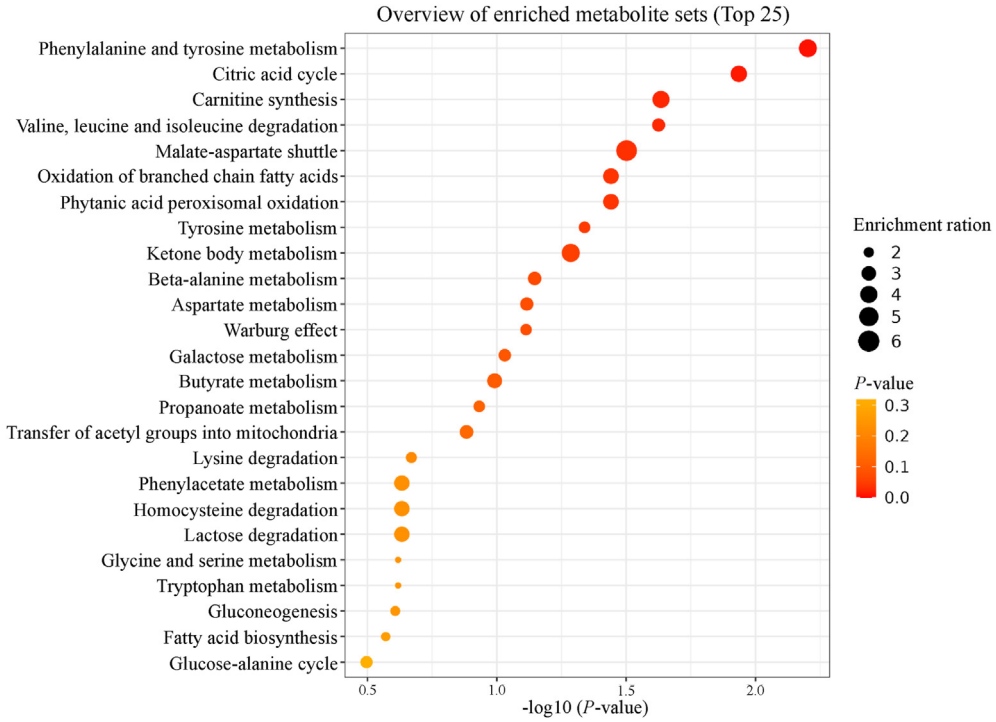

Fig. 3. Analysis of significantly different metabolite enrichment pathways.

factor-**1** in the blood of animals, promoting mammary gland development, improving milk production, and promoting the growth of dairy goats (Li et al., 2023). In our research, we found no significant differences in plasma endocrine hormone levels, which may be due to an insufficient amount of BCA additives, or the difference in animal species, which warrants further study in this area.

4.6. Plasma metabolites

The metabolic process of arginine involves organs such as the liver, kidney, and intestinal mucosa. Both citrulline and ornithine are precursors for endogenous arginine synthesis. In mitochondria, acetyl ornithine is produced by ornithine acetyltransferase, and

guanine, produced from ornithine, is catalyzed by argininosuccinic acid synthase in the cytosol to react with aspartic acid to produce argininosuccinic acid, which is cleaved to arginine by the argininosuccinic acid cleavage enzyme (Wu and Meininger, 2008). The process of L-arginine metabolism is involved in nitrogen metabolism, hormone secretion, immune response, and other biochemical reactions, and plays a pivotal role in protein synthesis and growth of the body. The small intestine is the main site of arginine degradation and there are two main metabolic pathways for arginine in the body: first, it is deguanylated by arginase I to produce urea and ornithine. Secondly, L-arginine can produce nitric oxide) and citrulline under the action of nitric oxide synthase. Putrescine and other polyamines produced from arginine in the body are essential for protein synthesis, and the maintenance of normal growth and development in living organisms (Gogoi et al., 2016). In this study, significant differences were observed in the metabolome, indicating that arginine and aspartate metabolism affect urea cycling and ammonia recycling. This suggests that postpartum supplementation of BCA in cows can promote increased endogenous synthesis of arginine and aspartate, thereby enhancing the rate of urea cycling and ammonia recycling efficiency. Consequently, this leads to higher nitrogen utilization and protein synthesis in the cow organism.

5. Conclusion

In this study lactational performance, milk production, and feed efficiency of cows was shown to be improved with BCA supplementation. Total intestinal apparent digestibilities (CP, EE, and NDF) were not affected by BCA supplementation. Milk competent yields (fat, protein, lactose, non-fat milk solid, and total milk solid) were increased with increasing BCA supplementation. Supplementation with BCA decreased urea nitrogen levels in both plasma and milk, while increasing microbial nitrogen, thereby enhancing nitrogen utilization efficiency. Routine blood parameters and plasma biochemical parameters both showed no significant changes with BCA supplementation, and it is shown that BCA did not affect body health of dairy cows. Additionally, none of the plasma endocrine hormones were affected. A total of 95 significantly different metabolites were screened from the plasma metabolites of cows in the BCA-added and non-added groups, and after enrichment analysis of the metabolic pathways involving the different metabolites, a total of six metabolic pathways were screened: bile acid biosynthesis, aspartate metabolism, pyrimidine metabolism, arginine and proline metabolism, the urea cycle, and ammonia recycling. It is suggested that the addition of BCA may improve milk production and regulate the function of nitrogen metabolism by altering related metabolites in the above metabolic pathways.

Author contributions

Zhanbo Xiong: Writing – original draft, Investigation, Formal analysis. **Yanjun Li:** Investigation, Resources, Validation. **Xiaoyin Zhang:** Visualization. **Shiqi Zhang:** Data curation. **Kexin Li:** Investigation and Data Curation. **Nan Zheng:** Supervision. **Shengguo Zhao:** Conceptualization, Methodology, Writing – review & editing. **Jiaqi Wang:** Project administration, Funding acquisition.

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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Appendix Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2024.05.004.

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