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Metabolomics and amino acid profiling of plasma reveals the metabolite profiles associated with nitrogen utilization efficiency in primiparous dairy cows

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Highlights

- The N utilization efficiency of dairy cows is excessively low;
- Determining blood metabolites related to N utilization efficiency is critical；
- Five key metabolites were identified, including glutamine and arginine；
- Arginine biosynthesis was the key biological pathway;
- The results provided information for optimization of nutritional strategies.

Abstract

Nitrogen (**N**) utilization efficiency (**NUE**, milk N yield [g/d]/N intake [g/d]) is an important performance indicator in dairy farming. Determining the NUE-associated blood metabolite profile will contribute to the optimization of nutritional strategies to further improve NUE among dairy cows. Here, 20 primiparous lactating cows with days in milk ranging from 95 to 115 days were selected from a total of 1 221 cows. Each cow's N intake and milk N yield were measured for 7 days. Subsequently, blood samples were collected before morning feeding. Based on analysis and calculations, cows were retrospectively classified into 2 groups based on their NUE values, namely, a low NUE group (LNUE, NUE = 24.8 ± 1.6%, n = 10, mean ± SD) and a high NUE group (HNUE, NUE = 35.2 ± 1.7%, n = 10, mean ± SD). Plasma samples were selected from 6 cows in each group for metabolomics and amino acid profiling. Among the 41 differential metabolites (**DMs**) identified in the metabolomic analysis, sucrose, MG(0:0/22:1(13Z)/0:0), 2-amino-6-hydroxyhexanoic acid, and L-glutamine exhibited significant correlations with NUE, milk yield, and body weight ($P < 0.05$). Moreover, the 5 differential amino acids and amino acid metabolites (**DAAs**) identified in the amino acid profiling and 5 of the 6 differential amino acids and amino acid conjugates identified by plasma metabolomics were found to be less abundant in the HNUE group ($P < 0.05$). Specifically, there was a 39.4% decrease in L-arginine content and a 29.2% decrease in L-glutamine content ($P < 0.05$). Pathway analysis indicated that the DMs and DAAs were mainly involved in arginine biosynthesis, glutathione metabolism, arginine and proline metabolism, and tryptophan metabolism (pathway impact > 0.1). These results provided new insights into the new blood metabolite profile associated with NUE in dairy cows. These new insights can provide foundational information for the formulation of new strategies to further enhance NUE in dairy cows.

Keywords: Nitrogen utilization; Blood; Amino acid; Metabolomic; Dairy cow.

Implications

Low nitrogen utilization efficiency in cows results in protein resources wastage and excessive nitrogen emissions. Determining the blood metabolites related

to nitrogen utilization efficiency is crucial for further improving nitrogen utilization efficiency. Our research identified 45 blood metabolites related to nitrogen utilization efficiency. Sucrose, MG(0:0/22:1(13Z)/0:0), 2-amino-6-hydroxyhexanoic acid, L-glutamine, and L-arginine were identified as key metabolites. Pathway analysis indicated that these metabolites were involved in arginine biosynthesis, glutathione metabolism, arginine and proline metabolism, and tryptophan metabolism. These results provided fundamental information for the optimization of nutritional strategies to enhance nitrogen utilization efficiency further.

Introduction

Nitrogen (**N**) is an essential nutrient for dairy cows; however, excessive N emissions have led to significant environmental problems in dairy systems (Hristov et al., 2019). Nitrogen utilization efficiency (**NUE**) refers to the ratio of milk N yield (g/d) to N intake (g/d) and is an important performance indicator in dairy farming (Powell et al., 2010; Lavery and Ferris, 2021). Holstein dairy cows exhibit a NUE ranging from 14% to 45%, with an average of 24.7% in North America (Huhtanen and Hristov, 2009) and only 17.0% in China (Zhang et al., 2017). Low NUE in dairy cows leads to wastage of protein resources and contributes to excessive N emissions (Lavery and Ferris, 2021). Accordingly, improving NUE in dairy cow production is critical for mitigating the negative impact of excessive N emissions during milk production on the environment. Numerous studies have explored measures for improving NUE in dairy cows, such as balancing diets for amino acids (Schwab and Broderick, 2017), synchronizing fermentable energy and N availability (Reynolds and Kristensen, 2008), and adding a variety of supplements and other ingredients to the diets of the animals. These approaches have been demonstrated to effectively enhance NUE, leading to a consistent improvement over time. In fact, the NUE of dairy cows in China increased from 10.0% in 1980 to 17.0% in 2010 (Zhang et al., 2017), primarily due to the increase in milk yield (**MY**). However, achieving a higher level of NUE in dairy cows, aiming for an average of 40%, presents considerable challenges, and requires the implementation of innovative strategies.

There are notable individual differences in NUE in lactating dairy cows, even under the same production conditions (Huhtanen and Hristov, 2009; Zhang et al., 2017). Xue et al., 2022 provided NUE data by violin plots for a total of 18 mid-lactating cows, with NUE ranging from around 22% to 33%. Li et al., 2022 investigated the NUE of 284 dairy cows based on differences in plasma ^{15}N and dietary ^{15}N, with the minimum value of the difference found to be no greater

than 1.5‰, while the maximum value exceeded 3.0‰. The individual differences in the NUE of cows have been recognized for a long time. As early as 2003, Chase, 2003 calculated the NUE of dairy cows from 62 research papers and reported a range of 16.2% to 45.2%. Despite these data, relatively little is known regarding inter-individual variation in NUE among dairy cows.

Milk protein production operates within a demand-driven system (Pszczolkowski and Arriola Apelo, 2020), meaning that differences in blood metabolite profiles, especially the amino acid profiles, are inevitable due to variations in MY or milk protein yield among dairy cows. When dairy cows exhibit higher MY or milk protein yield, they extract more amino acids from the blood for the synthesis of milk proteins. Xue et al., 2020 assessed the differences in blood metabolites in dairy cows with varying milk protein yields, and found that 8 out of 9 differential amino acid metabolites were more abundant in cows with high milk protein yields (Wu et al., 2018). In addition, Xue et al., 2020 highlighted the significance of blood metabolite profiles in the production of dairy cow milk protein, showing that the serum metabolome contributed 26.8% to the variation in milk protein yield. Previous studies have already demonstrated that plasma essential amino acid content and profile were associated with production performance, and have potential influences on NUE (Letelier et al., 2022). A recent study found that jugular arginine supplementation resulted in higher NUE in cows compared to alanine supplementation (28.20% vs. 24.69%) (Ding et al., 2019), supporting the idea that the blood amino acid (not just essential amino acid) content and profile can influence cow NUE. Based on the aforementioned findings, we infer that under similar production conditions, dairy cows with different NUE exhibit significant variations in blood metabolite profiles, especially amino acid profile. The aim of this study was to identify metabolite profiles associated with NUE in dairy cows through metabolomic analysis and amino acid profiling of blood.

Material and methods

Animals, Experimental Design, and Management

The experiment was conducted at the ZhongLi dairy farm in Ulanhot City, Inner Mongolia Autonomous Region, from October 2021 to December 2021. For the experiment, 20 primiparous Holstein cows with days in milk ranging between 95 and 115 days were selected from a group of 1 221 primiparous cows located in the same barn. These experimental animals had no history of disease treatment from birth until the beginning of the experiment, were raised under identical conditions, and were not pregnant. All animals were acclimated for 14 days after being transferred from a large herd to an individual pen measuring 2.4 m × 6 m. The diet and environmental conditions were not changed from

large herd to individual pen. The bedding in the animal pen was composed of dried feces and rice husks. The diets in this experiment were formulated based on the NRC, 2001 according to the actual production levels of the experimental cows. Staff members uniformly prepared the total mixed ration (**TMR**). Each cow was individually fed and allowed *ad libitum* access to feed to facilitate accurate calculation of DM intake (**DMI**). The ingredient and chemical composition of the experimental diet is shown in Table 1. After the adaptation period, an 8-day data recording and sampling period was conducted. During the initial 7 days, the daily DMI and MY of each cow were measured. Samples of the TMR and leftover feed were collected to calculate N intake while milk samples were taken to determine milk protein content. The NUE of each cow was calculated using the following formula: NUE (%) = milk N yield (g/d)/N intake (g/d) × 100% (Muller et al., 2021), where milk N yield (g/d) = MY (kg/d) × milk protein content (%)/6.38 × 1000 (Beltran et al., 2022). The 20 cows were retrospectively divided into 2 groups based on their NUE levels, namely, a low NUE group (**LNUE**) and a high NUE group (**HNUE**). The LNUE group consisted of 10 cows with an average NUE of 24.8 ± 1.6% (mean ± SD), while the HNUE group comprised 10 cows with an average NUE of 35.2 ± 1.7% (mean ± SD). Throughout the experiment, cows were milked 3 times daily at 0730 h, 1500 h, and 2230 h in the rotary milking parlor and provided with 60% TMR at 0730 h and 40% TMR at 1900 h. The cows were fed to *ad-libitum* intake and had unrestricted access to water. Barn cleaning and disinfection occurred when the cows were taken to the milk parlor for milking at 1500 h.

Data Recording and Sampling

Before the start of the data recording and sampling period, the BW of each cow was recorded for 2 d. An experienced evaluator recorded the body condition score for each cow using a 5-point scale (Wildman et al., 1982). During the initial 7 days of the data recording and sampling period, the daily feeding and leftover amounts were recorded for each cow, and samples of the TMR and leftovers were collected. Fecal samples were collected through the rectum 3 times a day (0800 h, 1600 h, and 2400 h). Each time, approximately 500 g of feces were collected, mixed, and 120 g of the samples were weighed and mixed with 30 mL of 10% tartaric acid. The TMR, leftover, and fecal samples collected over the 7 d were separately mixed at the end of the experiment for chemical analysis. In addition, the daily MY of each cow was recorded during the initial 7 days of the data recording and sampling period, and a 50-mL milk sample (collected in a 4:3:3 ratio for morning, afternoon, and evening) was mixed with potassium chromate preservative and stored at 4°C until analysis every day. Blood samples were taken from the caudal root vein before morning feeding on day 8 of the data recording and sampling period and collected in EDTA-containing 5 mL tubes (SX582, Sanxun Biotechnology Co., LTD, Guangzhou, China). After centrifugation at 3 000 × *g* for 15 min at 4°C, plasma samples

were collected, stored at −40°C, and used for metabolomics and amino acid profiling.

Chemical Analysis and Calculation

The contents of DM, ether extract, ADF, and acid-insoluble ash in feces and TMR samples were analyzed with the methods described in AOAC, 2004. The NDF content was determined following Van Soest et al., 1991. The CP and N content in fecal, TMR, and leftover samples was determined using an automatic Kjeldahl N analyzer (SKD-2000, Haineng Experimental Instrument Technology Co., Ltd, Shanghai, China). Acid insoluble ash was used as an endogenous indicator, according to the formula: the apparent digestibility of a nutrient (%) = 100 – 100 × the nutrient concentration in feces (%) × acid insoluble ash concentration in TMR (%) / the nutrient concentration in TMR (%) / acid insoluble ash concentration in feces (%), calculating the apparent digestibility of CP, ether extract, ADF, and NDF. Urinary N excretion and fecal N excretion were estimated based on the apparent digestibility of N to reflect the N balance of each cow, as follows:

Urinary N excretion (g/d) = 104 + 0.855 × N intake (g/d) − 13.2 × DMI (kg/d) − 6.8 × metabolizable energy (MJ/kg) (Johnson et al., 2016);

Fecal N excretion (g/d) = (1-N digestibility [%]) × N intake (g/d);

N balance (g/d) = N intake (g/d)– urinary N excretion (g/d) – fecal N excretion (g/d)–milk N yield (g/d) (Muller and Kuhla, 2021).

Milk samples were sent to the DHI Determination Center in Henan Province for analysis of fat, protein, lactose, total solids, milk urea nitrogen (**MUN**), and somatic cell counts using a MilkoScan FT+ analyzer (Foss Electric A/S, Denmark). The average values of these indicators over the 7-day digestive and metabolic analysis period were used for statistical analysis. According to the NRC, 2001 model, the requirements for metabolizable protein and energy for each cow were estimated using actual DMI, animal variables (days in milk, lactation number, and BW), MY and milk composition. The supply of metabolizable protein and energy for each cow was calculated using actual DMI and the nutrient composition of dietary ingredients, enabling the calculation of metabolizable protein and energy balance.

The levels of alanine transaminase and aspartate aminotransferase, as well as the content of glucose, triglycerides, total cholesterol, non-esterified fatty acids, albumin, total protein, and urea in plasma, were determined using a Chemray 240 automated blood biochemical analyzer (Shenzhen Raidu Life Sciences, Shenzhen, China). As described in the manual, total antioxidant capacity, glutathione peroxidase and superoxide dismutase activities, and malondialdehyde contents in plasma were assessed using a kit (Beijing

Solarbio Science & Technology Co., Ltd, Beijing, China) and were analyzed using a microplate reader (Synergy H1, Boten). Additionally, plasma levels of leptin, IGF-1, and insulin were determined using an ELISA kit (Jiangsu MEIMIAN Indus Experiment CO., Ltd, Yancheng, Jiangsu province, China) and analyzed using a microplate reader (Synergy H1, Boten). The sensitivity of ELISA kits of leptin, IGF-1, and insulin was typically less than 0.25 μg/L, 2.0 μg/L and 3.0 mIU/L, with tolerance within intra- and inter- CV < 10%.

Plasma Metabolic Profiling

Six cows from the HNUE group (cows 14, 15, 16, 17, 18, and 19; NUE = 35.5 ± 1.5%) (Figure. 1) and 6 cows from the LNUE group (cows 2, 3, 4, 5, 6, and 7; NUE = 24.3 ± 4.8%) (Figure. 1) were selected for metabolomic analysis. For metabolite extraction, 100 μL of plasma sample was combined with 400 μL of an extraction solution containing methanol and acetonitrile (1:1, *v*/*v*) and 0.02 mg/mL of L-2-chlorophenylalanine as an internal standard. After sonication at 40 kHz for 30 min at 5°C, the samples were placed at −20°C for 30 min to precipitate the protein. The resulting supernatant was then collected after centrifugation at 13 000 × *g* at 4°C for 15 min and evaporated under a gentle stream of N. Before UHPLC–MS/MS analysis, the extracted metabolites were reconstituted in a loading solution consisting of acetonitrile and water (1:1, *v*/*v*) by briefly sonicating in a 5°C water bath. Cleared supernatant was transferred to sample vials for LC–MS/MS analysis, while 20 μL of supernatant from each sample was mixed and used for quality control. Metabolite mass spectrometric data were collected using the UHPLC-Q Exactive system and analyzed with Majorbio (http://www.majorbio.com/). To ensure the stability of the entire assay, 1 quality control sample was inserted for every 4 analytical samples analyzed (3 in total) during instrumental analysis.

The metabolomics processing software Progenesis QI (Waters Corporation, Milford, CT, USA) was used for raw data baseline filtering, peak identification, integration, retention time correction, and peak alignment. The software generated a data matrix with retention time, mass-to-charge ratio, and peak intensity information. The same software was then employed to identify characteristic peaks and match the MS and MS/MS mass spectrometric information with information in the metabolite database. The MS mass error was set to less than 10 ppm and metabolites were identified based on the matching scores of the secondary mass spectra with the primary databases http://www.hmdb.ca/ and https://metlin.scripps.edu/. The data were then uploaded to the Majorbio cloud platform (https://cloud.majorbio.com) for analysis. Metabolic features detected in at least 80% of any set of samples were retained after filtering, and minimum metabolite values were imputed for samples with levels below the lower limit of quantitation; each metabolic feature was normalized by sum. To reduce errors due to sample preparation and instrument instability, the response intensity of sample mass spectrum peaks

was also sum-normalized. Variables with a relative standard deviation > 30% in quality control samples were removed, and the final data matrix was obtained by log10 transformation for subsequent analysis. After data pre-processing, a variance analysis was conducted on the matrix file. Principal component analysis and partial least squares discriminant analysis were performed using the R package ropls (Version 1.6.2). The stability of the model was evaluated using 7-fold cross-validation. Differential metabolites (**DMs**) were selected using variable importance in projection values obtained from the partial least squares discriminant analysis model and *P*-values of Student's *t*-tests. Metabolites with variable importance in the projection value > 1 and $P < 0.05$ were considered to be significantly differentially abundant.

Plasma Amino Acid Metabolic Profiling

The plasma samples used for metabolic profiling were also used for amino acid profiling. To save costs, the plasma samples were systematically pooled into composite samples by pairing every 2 samples sequentially according to the number of experimental animals. Thus, the values of NUE, DMI, MY, and BW were computed for each composite sample by averaging the respective values from the 2 individual samples. Three composite plasma samples were obtained for each group following the merging process, and these were subsequently subjected to amino acid profiling. After thawing, 50 μL of each plasma sample was mixed with 250 μL of 20% acetonitrile/methanol, vortexed for 3 min, and centrifuged at 12 000 rpm for 10 min at 4°C. The resulting supernatant was stored at −20°C for 30 min and then centrifuged again at 12 000 rpm for 10 min at 4°C. A total of 180 μL of the supernatant was transferred to a protein precipitation plate for further LC–MS analysis. In addition, 20 μL of the supernatant of each sample was mixed to create a quality control sample. MS data for amino acids and amino acid metabolites were obtained using the AB Sciex QTRAP 6500 LC–MS/MS platform and were analyzed with MetWare (http://www.metware.cn/). During instrumental analysis, 1 quality control sample was included for every 2 samples analyzed (3 in total) to evaluate the stability of the entire assay process.

Statistical Analysis

Throughout the entire experimental period, none of the test animals presented an abnormal condition, and no outliers (± 3 standard deviations from the mean) were detected in the obtained data; consequently, all the data were included in the statistical analysis. Animal characteristics, apparent digestibility, production performance, and blood parameters were measured before the grouping scheme was determined and were analyzed using SAS, 2002. The normality test and the variance homogeneity test were carried out through the PROC UNIVARIATE model and the PROC DISCRIM model, respectively. One-way ANOVA or nonparametric test was performed using the PROC MIXED model

according to the characteristics of the data, and the results were expressed as means. *P*-values > 0.05 and < 0.10 were considered a significant trend, while *P*-values < 0.05 were considered significant. To evaluate the Spearman association between NUE and the indicators of animal characteristics, production performance, and blood parameters, R 3.5.1 was used, and $P < 0.05$ was considered a significant association. Blood samples for metabolomics and targeted amino acid analysis were randomly selected by the experiment organizers, and the analysis were performed by experimenters blinded to the groupings. The correlation between differentially expressed metabolites and NUE, BW, DMI, and MY was analyzed using Pearson's correlation coefficient with the SciPy package in Python (version 1.0.0).

Results

Animal Characteristics

The NUE values for the 20 experimental cows were found to range from 11.9% to 39.2%, with the average being 30.0% (Figure. 1). The NUE of cows in the HNUE group ranged from 31.4% to 39.2%, with an average of 35.2%, whereas that of animals in the LNUE group ranged from 11.9% to 30.7%, with an average of 24.8%. The difference in NUE between the HNUE and LNUE groups was significant, with a difference of 10.4% units ($P < 0.001$; Figure. 1, Table 2). Milk N yield, N intake per day, N intake per unit of BW, and DMI were not significantly different between the 2 groups of cows ($P > 0.05$; Table 2). However, cows in the HNUE group consumed 9.5 g less dietary N per kg of milk produced than cows in the LNUE group ($P = 0.014$). Additionally, cows in the HNUE group had a negative N balance and metabolizable protein balance, with a mean of −29.30 g/d and −106 g/d, respectively, which was significantly lower than that in cows in the LNUE group ($P < 0.05$). The body condition scores and BWs of cows in the HNUE group were significantly lower than those in the LNUE group ($P < 0.05$), with a difference of 46.1 kg in BW. Spearman correlation analysis showed a significant correlation between N balance and BW with NUE ($P < 0.05$; Table 4). Both N balance and BW decreased with increasing NUE (Table 4 and Figure. S1).

Apparent Nutrient Digestibility, Milk Yield and Composition

Regarding apparent nutrient digestibility, cows in the HNUE group had higher CP digestibility than cows in the LNUE group ($P = 0.001$, Table 2), while ether extract, ADF, and NDF digestibility did not differ significantly between the 2 groups ($P > 0.05$). Cows in the HNUE group produced an average of 6.2 kg more milk ($P = 0.044$), 0.186 kg more milk protein ($P = 0.059$), and 0.335 kg more milk lactose ($P = 0.048$) per day than cows in the LNUE group (Table 2). Spearman correlation analysis identified a significant positive correlation

between MY and lactose yield with NUE ($P < 0.05$; Table 4 and Figure. S1). However, no significant differences in milk fat yield, or in the contents of protein, fat, lactose, total solids, MUN, and somatic cell counts in the milk were detected between the 2 groups of cows ($P > 0.05$).

Blood Parameters

The plasma contents of triglycerides, malondialdehyde, and insulin were significantly lower in cows of the HNUE group than in those of the LNUE group ($P < 0.05$; Table 3), whereas the total cholesterol content was significantly higher ($P = 0.034$) in cows of the HNUE group. However, no significant differences in other blood biochemical indicators were observed between these 2 groups ($P > 0.05$). Spearman correlation analysis identified 3 indicators—triglycerides, malondialdehyde, and insulin—that showed a significant correlation with NUE ($P < 0.05$; Table 4). Both plasma triglyceride, malondialdehyde, and insulin contents decreased with increasing NUE (Table 4 and Figure. S1).

Plasma Metabolic Profile

A total of 439 metabolites were identified but none were specific to either group. The partial least squares discriminant analysis score plot showed a clear separation between HNUE and LNUE samples in both positive and negative ion modes, with R^2 and Q^2 values after 200 permutation tests indicating that the model was useful for downstream analysis (Figure. S2). A total of 41 DMs were identified in the metabolomics analysis, including 7 glycerophosphocholines, 6 amino acids and amino acid conjugates, 5 fatty acids and fatty acid conjugates, and 23 other compounds; 12 of the DMs displayed higher abundance in the HNUE group while 29 were more abundant in the LNUE group (Figure. S3). To evaluate the individual differences in NUE in dairy cows from the perspective of the blood metabolite profile, a Pearson correlation analysis was performed among the 41 DMs as well as between the DMs and NUE, DMI, BW, and MY. A total of 360 significant correlations were found among the 41 DMs (Figure. S4); additionally, 15 DMs were significantly correlated with NUE, while 4 were significantly correlated with both MY and BW. 2-Amino-6-hydroxyhexanoic acid was significantly and positively correlated with NUE and MY and significantly and negatively correlated with BW. Sucrose, L-glutamine, and MG(0:0/22:1(13Z)/0:0) showed significant negative correlations with NUE and MY but were significantly and positively correlated with BW (Figure. 2). The above 4 metabolites all had variable importance in the projection values greater than 1.51 and were among the 20 most differentially abundant metabolites in terms of variable importance in the projection value; they also exhibited strong correlations with each other, except for 2-amino-6-hydroxyhexanoic acid and MG(0:0/22:1(13Z)/0:0) (Figure. S4).

Plasma Amino Acid Metabolic Profile

A total of 67 amino acids and amino acid metabolites were identified among 94 compounds. The results of Pearson's correlation analysis performed on the quality control samples indicated that the entire assay was highly stable. Principal component analysis and orthogonal partial least squares discriminant analysis score plots showed that there was a clear separation between the HNUE and LNUE samples. Permutation testing confirmed the usability of the partial least squares discriminant analysis model for downstream analysis (Figure. S5). The average total contents of the 67 identified amino acids and amino acid metabolites in the LNUE and HNUE groups were 1 902 576.0 and 1 663 178.8 ng/mL, respectively, and were 12.6% lower in the HNUE group than in the LNUE group. The 3 most abundant amino acids and amino acid metabolites in both groups were L-glutamine, urea, and L-tryptophan (Figure. 3a).

Of the 67 amino acids and amino acid metabolites, 5 (L-glutamine, L-lysine, L-arginine, L-ornithine, and L-leucine) were found to be differentially abundant (differential amino acids and amino acid metabolites [**DAAs**]) between the HNUE and LNUE samples, and all were present at higher levels in samples of the LNUE group (Table 5). These 5 DAAs had variable importance in projection values ranging from 1.62 to 1.93 and $\log_2$(Fold change) ranging from −0.499 to −0.976. To understand the role of the DAAs in NUE differences in dairy cows, correlation analysis was performed between these 5 DAAs and NUE, DMI, BW, and MY. Of the 5 DAAs, only L-lysine showed a significant negative correlation with NUE (Figure. 3b).

Pathway Analysis

Pathway analysis was performed by combining the results of the plasma metabolic profile analysis, which identified 41 DMs, and the plasma amino acid metabolic profiling, which identified 5 DAAs. We found that 7 pathways were significantly altered in the HNUE group relative to the LNUE group—arginine biosynthesis, aminoacyl-tRNA biosynthesis, tryptophan metabolism, arginine and proline metabolism, glutathione metabolism, D-glutamine and D-glutamate metabolism, and linoleic acid metabolism (Figure. 4a). Notably, the abundance of several metabolites involved in arginine biosynthesis, including L-glutamine, N-acetylornithine, L-ornithine, and L-arginine, was lower in the HNUE group than in the LNUE group (Figure. 4b).

Discussion

Low NUE contributes to high N emissions in dairy farming and is well known to be influenced by a variety of factors, including physiological status, nutritional

level, feeding environment, and genetic predisposition. Due to differences in the utilization and allocation of absorbed N between primiparous and mature lactating cows, with primiparous cows allocating a portion of absorbed N for weight gain, it is essential to distinguish between these two groups. This study exclusively focused on identifying metabolite profiles associated with NUE in the blood of primiparous cows. Previous studies have already demonstrated that individual differences in NUE can be significant even under the same feeding conditions (Huhtanen and Hristov, 2009; Zhang et al., 2017). One study sought to determine differences in NUE among 284 cows (2 parities, days in milk = 48 ± 1 d) by calculating the difference between plasma ^{15}N and dietary ^{15}N (Li et al., 2022); however, plasma N isotope fractionation may not be a suitable method for estimating NUE in dairy cows during early lactation (Correa-Luna et al., 2022). In our study, primiparous cows with similar days in milk, BW, and feeding environments were selected to allow the identification of individual differences in NUE among milk cows. This approach facilitated the exploration of blood NUE-associated metabolite profiles. The NUE of cows was found to range from 11.9% to 39.2% and was 10.4 percentage points higher in cows of the HNUE group than in those of the LNUE group.

No significant difference in N intake or DMI was found between cows of the 2 groups, and no significant correlation was identified between DMI and NUE; this was surprising as N intake and DMI are believed to be negatively correlated with NUE (Huhtanen et al., 2009), and can potentially be used to predict NUE in cows (Lavery and Ferris, 2021). Concurrently, Beltran et al., 2022 also reported that there was no correlation between DMI and NUE when evaluating the factors that influence NUE in Chilean dairy cows. However, as expected, the results of the current study showed that cows in the HNUE group were 8.0% lighter (532 *vs*. 578 kg), had 31.6% higher MY (25.8 *vs*. 19.6 kg/d), and required 38.5% less dietary N to produce one unit of milk (15.2 *vs*. 24.7 g/kg MY) compared with cows in the LNUE group. Notably, although the N and metabolizable protein balance status of the cows in this study was calculated based on a formula, it was evident that cows in the HNUE group displayed a significantly negative N and metabolizable protein balance. Dietary N levels might limit the productive performance of cows in the HNUE group. Correspondingly, the level of L-lysine, which is typically a limiting amino acid (Schwab and Broderick, 2017), was lower in the blood of cows in the HNUE group than in that of animals in the LNUE group. These results suggested that the main reasons for the different NUEs in this study were the differences in BW and MY, resulting in a negative N balance in animals of the HNUE group at identical DMI. This also indicated that cows in the HNUE group performed better at the expense of their own body mass, which was corroborated by the results of the correlation analysis, namely, that NUE decreases with increasing BW but increases with increasing MY. Li et al., 2022 also observed that cows with higher MY had smaller difference between plasma ^{15}N and dietary ^{15}N.

Furthermore, the results of the current study showed that 4 of the 5 identified DAAs were significantly correlated with BW, indicating that this parameter plays a major role in determining individual differences in NUE.

The individual differences in NUE in dairy cows were not reflected in milk composition as no significant differences in milk protein, fat, lactose, total solids, or MUN contents were found between cows of the two groups. The increased milk protein and lactose production recorded in the HNUE group relative to that in the LNUE group can be attributed to variations in MY, which was found to be the most significant factor affecting NUE. Plasma urea and MUN contents, regarded as reliable predictors of NUE, were inversely correlated with NUE (Gulinski et al., 2016; Beltran et al., 2022), as were DMI and N intake. However, we did not find an association between NUE and MUN or plasma urea contents (Muller et al., 2021; Li et al., 2022). Most studies that have investigated the correlation between NUE and MUN content have been based on higher dietary protein levels (Lavery and Ferris, 2021); in this study, however, the negative N balance state of cows in the HNUE group suggested that the total N intake was inadequate for the cows in this group. The limited correlation between NUE and milk composition, especially the milk protein and MUN content, means that protein resource waste resulting from individual differences in NUE is often overlooked.

We also did not find a significant association between the individual differences of NUE in dairy cows and plasma total protein contents, which is a protein metabolism marker (Cozzi et al., 2011), or non-esterified fatty acids and β-hydroxybutyrate levels, which are indicative of the energy status (Puppel and Kuczynska, 2016). In contrast, plasma triglyceride and insulin contents showed a significant and negative correlation with NUE, potentially associated with differences in metabolizable energy and protein balance among cows with different NUE. Cows in the HNUE group had a negative N balance and metabolizable protein balance, which was significantly lower than that in cows in the LNUE group. Meanwhile, cows in the HNUE group exhibited a trend of lower metabolizable energy balance compared to cows in the LNUE group. Previous studies have indicated that when dietary energy level was decreased (reflecting reduced energy balance), insulin content in the blood was decreased, while non-esterified fatty acid content was increased (Janovick et al., 2011; Zhang et al., 2015). The insufficiency of metabolizable energy intake for cows in the HNUE group could be reflected by an increase in cholesterol content, which is associated with lipid mobilization (Puppel and Kuczynska, 2016). Cows in the HNUE group may have required greater tissue mobilization to provide energy to meet milk production needs. Wu et al., 2018 found that the serum cholesterol content of cows with high milk and milk protein yields was significantly higher than that of cows with low milk and milk protein yields. Malondialdehyde is one product of lipid peroxidation, and elevated blood malondialdehyde content indicates lipid peroxidation (Ma et al., 2021). Blood

malondialdehyde content showed a negative correlation with NUE, suggesting that cows experience less oxidative stress with increasing NUE under the same diet. The results of the plasma metabolomic analysis also supported that the energy levels in the diets of HNUE cows were relatively inadequate, as evidenced by the higher levels of all 7 differentially abundant glycerophosphorylcholines and the lower levels of all 5 differentially abundant fatty acids and fatty acid conjugates in the HNUE group relative to that seen in the LNUE group. Glycerophosphorylcholine is a putative acetylcholine precursor that contributes significantly to circulating choline levels (Al-Sulaiti et al., 2019) and promotes fat oxidation for energy (Yang et al., 2018; Coleman et al., 2019), thereby supporting the high MY of cows in the HNUE group. To sum up, when feed and management conditions were constant, cows exhibiting high NUE tended to have high MYs but were prone to experiencing severe negative N balances and comparatively inadequate intake of dietary energy, which raises concerns regarding the sustainability of milk production in such cows. Conversely, cows displaying low NUE consumed an equivalent amount of feed as HNUE cows, but channeled a larger portion of dietary N and energy toward augmenting BW instead of milk production.

Among the DMs identified by plasma metabolomics, 6 were amino acids and amino acid conjugates; of these, 5 were less abundant in the HNUE group than in the LNUE group. Similarly, all 5 DAAs identified in the amino acid metabolic profiling were less abundant in the HNUE group. In addition, total plasma amino acids and amino acid metabolites were 12.8% less abundant in cows of the HNUE group than in animals of the LNUE group. These results are consistent with those of Pszczolkowski and Arriola Apelo, 2020, who reported that the supply of amino acids for milk protein synthesis is regulated based on a demand-driven system. This means that, given their relatively higher MY, cows in HNUE group uptake more amino acids from the blood for milk protein synthesis than cows in LNUE group given the same DMI and N intake. These amino acids, especially leucine (Wolfson et al., 2016) and arginine (Chantranupong et al., 2016), along with insulin, the contents of which were also low in the plasma of cows of the HNUE, are absorbed into the mammary tissue, where they activate mTORC1 and thereby stimulate milk protein synthesis (Lei et al., 2012; Pszczolkowski and Arriola Apelo, 2020).

Plasma metabolomics and targeted amino acid profiling led to the identification of 5 key differential metabolites—sucrose, MG(0:0/22:1(13Z)/0:0), 2-amino-6-hydroxyhexanoic acid, L-glutamine, and L-arginine—that associated with NUE in dairy cows. Additionally, the first 4 were found to be significantly correlated with NUE, BW, and MY. The lower abundance of sucrose and MG(0:0/22:1(13Z)/0:0) in the HNUE group of cows was further evidence of the relative lack of energy levels in the diets of cows in the HNUE group. MG(0:0/22:1(13Z)/0:0), a 2-monoacylglycerol, plays a crucial role in converting dietary fat into triglycerides in animal intestines (Yang and Kuksis, 1991). The

decreased MG(0:0/22:1(13Z)/0:0) abundance observed in the plasma of cows in the HNUE group corresponds to a lower triglyceride content. The correlation between 2-amino-6-hydroxyhexanoic acid and NUE may potentially be influenced by BW. 2-Amino-6-hydroxyhexanoic acid is a chiral intermediate used for synthesizing a vasopeptidase inhibitor that has shown potential in reducing hypertension caused by obesity in humans (Hanson et al., 1999; Molinaro et al., 2002). L-glutamine was also identified as a DAA in the plasma amino acid metabolic profiling. However, likely owing to the small sample size, no correlation was found between L-glutamine and NUE, BW, or MY. In contrast, pathway analysis identified significant differences in glutamate metabolism between cows of the different NUE groups. The role of glutamine in lactating cows was extensively reviewed by Lobley et al., 2001. Glutamine represents a source of carbon and nitrogen for the biosynthesis of non-essential amino acids in the mammary gland and is also a regulator of cellular activity. Notably, L-glutamine can serve as a precursor for the synthesis of arginine (Wu et al., 2022), another DAA identified in the plasma amino acid metabolic profiling. Pathway analysis revealed that there were significant differences in arginine biosynthesis and metabolism between cows with different NUEs. Despite being considered a non-limiting nutrient in diets, arginine plays a critical role in regulating nitrogen metabolism, amino acid uptake in the mammary gland (Pszczolkowski and Arriola Apelo, 2020), and nutrient utilization. Furthermore, the positive effect of arginine on the production performance of dairy cows is well-documented (Wu et al., 2022). Future research should prioritize the investigation of metabolites associated with NUE, particularly focusing on L-glutamine and L-arginine. Understanding the roles of these metabolites in NUE of dairy cows will provide foundational knowledge for developing novel nutritional strategies aimed at further enhancing NUE in the future.

Conclusions

In summary, in the present study, we found that there were significant variations in NUE among primiparous cows with similar days in milk, which were directly related to MY and BW. Cows with higher NUE mobilised more body mass, and the resulting potential negative N balance and inadequate intake of dietary energy raises about the sustainability of this productivity. In addition, our research has identified 5 key differential metabolites—sucrose, MG(0:0/22:1(13Z)/0:0), 2-amino-6-hydroxyhexanoic acid, L-glutamine, and L-arginine—that were associated with NUE. Meanwhile, pathway analysis indicated that the DMs and DAAs were mainly involved in arginine biosynthesis, glutathione metabolism, arginine and proline metabolism, and tryptophan metabolism (pathway impact > 0.1). These results provided new insights into the new blood metabolite profile associated with NUE and fundamental information for the optimization of nutritional strategies to further enhance NUE.

Ethics approval

The animal study protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Henan Agriculture University (Zhengzhou, China) (HNND2021072798).

Data and model availability statement

None of the data were deposited in an official repository. Data are available upon request.

Declaration of Generative AI and AI-assisted technologies in the writing process

While preparing this work, the author utilized the ChatGPT to address potential grammatical errors, and improve the quality of the written sentences. After using ChatGPT, the authors have reviewed and edited the content and take full responsibility for the content of the publication.

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Declaration of 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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Tables

Table 1

Ingredient and chemical composition of diets for experimental cows.

Item	Content
Ingredients (% DM)	
Corn silage	32.56
Alfalfa hay	11.65
Molasses cane	3.38
Urea	0.21
Soybean meal	13.12
Extruded soybean	1.93
Corn slice	15.27
Corn grain, ground	13.06
Cottonseed	5.00
Palmit[1]	1.29
Mycotex[2]	0.08
Premix[3]	2.45
Chemical composition (% DM)	

Organic matter	91.37
CP	17.25
Ether extract	4.9
NDF	30.19
ADF	16.4
Calcium	0.76
Phosphorus	0.39
Metabolizable protein (% DM)	11.21
Lysine (% of metabolizable protein)	6.56
Methionine (% of metabolizable protein)	2.09
Metabolic energy (MJ/kg of DM)	10.1
Net energy for lactating cow (MJ/kg of DM)	6.1

[1] Palmit contains 92.1% palmitic acid, 4.6% oleic acid, 1.3% stearic acid and 1.0% myristic acid, produced by Wilmar Oil Technology (Tianjin) Co., Ltd, Tianjin, China.

[2] Produced by Shanghai Comiy Biotechnology Ltd, Shanghai, China.

[3] Contained per kg premix DM: 31.3 mg Co, 343.5 mg Cu, 2 258 mg Fe, 1 160 mg Mn, 1 534 mg Zn, 40.3 mg I, 17.7 mg Se, 317.4 KIU vitamin A, 80.8 KIU vitamin D, and 3 030 IU vitamin E. Produced by ZhongLi dairy farm, Ulanhot City, Inner Mongolia Autonomous Region, China.

Table 2

Animal characteristics, apparent nutrient digestibility and production performence in cows of the low and high nitrogen utilization efficiency groups.

Item	Group[1]		SEM	*P*-value
	LNUE	HNUE		
NUE (%)	24.8[b]	35.2[a]	1.17	<0.001
Days in milk (d)	100.3	101.9	1.33	0.558
BW (kg)	578[a]	532[b]	9.7	0.027
Body condition score	3.08[a]	2.92[b]	0.028	0.012
DMI (kg/d)	16.9	15.3	0.70	0.262
Nitrogen intake (g/d)	431.2	390.4	18.2	0.256
Nitrogen intake (g/kg BW)	0.744	0.728	0.027	0.774
Nitrogen intake (g/kg MY)	24.7[a]	15.2[b]	1.72	0.014
MY (kg/d)	19.6[b]	25.8[a]	1.43	0.044
Milk protein yield (kg/d)	0.669	0.855	0.046	0.059
Milk nitrogen yield (g/d)	107.1	136.7	7.32	0.059
Milk fat yield (kg/d)	0.823	0.974	0.066	0.267
Milk lactose yield (kg/d)	1.025[b]	1.360[a]	0.079	0.048

Milk composition (%)				
Protein	3.47	3.33	0.060	0.692
Fat	4.21	3.80	0.187	0.290
Lactose	5.19	5.27	0.048	0.395
Total solids	13.3	12.8	0.27	0.419
MUN (mg/dL)	14.9	14.7	0.35	0.789
Milk somatic cell counts ($\times 10^{-3}$/ml)	141.0	126.8	31.95	0.827
Nitrogen balance (g/d)	6.46[a]	-29.30[b]	5.910	0.008
Metabolizable protein requirement[2] (g/d)	1 519	1 820	33.9	0.071
Metabolizable protein supply (g/d)	1 894	1 714	21.5	0.239
Metabolizable protein balance (g/d)	375	-106	20.6	<0.001
Metabolic energy requirement (MJ/d)	142.2	155.6	2.02	0.374
Metabolic energy supply (MJ/d)	170.7	154.5	3.13	0.479
Metabolic energy balance (MJ/d)	28.5	-1.1	0.913	0.068
Apparent nutrient digestibility (%)				
CP	67.9[b]	71.1[a]	0.36	0.001
Ether extract	73.1	75.1	0.82	0.257

NDF	45.8	49.3	0.85	0.075
ADF	43.0	46.0	0.98	0.172

Abbreviations: NUE = Nitrogen utilization efficiency; DMI = DM intake; MY = Milk yield; MUN = Milk urea nitrogen.

[1] LNUE = lower nitrogen utilization efficiency (NUE = 24.8 ± 1.6%, n = 10, mean ± SD); HNUE = higher nitrogen utilization efficiency (NUE = 35.2 ± 1.7%, n = 10, mean ± SD).

[2] Metabolizable protein and energy requirements were estimated using the NRC (2001) model with actual DMI, animal variables (days in milk, lactation number, and BW), and MY and milk composition. Metabolizable protein and energy supply were calculated with actual DMI and nutrient composition of dietary ingredients.

[a, b] Means with different superscripts in each row differ significantly ($P<0.05$).

Table 3

Blood parameters in cows of the low and high nitrogen utilization efficiency groups.

Item	Group[1]		SEM	*P*-value
	LNUE	HNUE		
Urea (mmol/L)	4.44	3.91	0.188	0.178
Glucose (mmol/L)	4.23	4.07	0.077	0.330
Total protein (g/L)	76.2	77.2	1.74	0.773
Albumin (g/L)	35.8	35.8	0.60	0.992
Triglyceride (mmol/L)	0.184[a]	0.141[b]	0.008	0.018
Non-esterified fatty acid (μmol/L)	88.0	92.2	6.57	0.752
Cholesterol (mmol/L)	4.76[b]	5.50[a]	0.159	0.034
β-hydroxybutyrate (mmol/L)	0.638	0.584	0.020	0.375
Alanine transaminases (U/L)	27.9	25.3	1.18	0.293
Aspartate aminotransferase (U/L)	86.7	88.2	7.62	0.922
Total antioxidant capacity (μmol/L)	0.470	0.448	0.012	0.387
Glutathione peroxidase (U/mL)	30.1	27.9	1.62	0.491
Malondialdehyde (nmol/L)	4.03[a]	2.42[b]	0.251	0.005

Superoxide dismutase (U/mL)	113.3	105.7	2.82	0.196
Insulin (mIU/L)	7.75[a]	6.23[b]	0.181	0.001
IGF-1 (μg/L)	5.15	4.95	0.074	0.214
Leptin (μg/L)	0.511	0.525	0.025	0.798

[1] LNUE = lower nitrogen utilization efficiency (NUE = 24.8 ± 1.6%, n = 10, mean ± SD); HNUE = higher nitrogen utilization efficiency (NUE = 35.2 ± 1.7%, n = 10, mean ± SD).

[a, b] Means with different superscripts in each row differ significantly ($P<0.05$).

Table 4

Results of the correlation analysis between the nitrogen utilization efficiency and animal characteristics, production performance, and blood parameters in experimental cows.

Item	r[1]	*P*-value
N balance (g/d)	-0.847	<0.001
BW(kg)	-0.592	0.008
Milk yield (kg/d)	0.530	0.020
Lactose yield (kg/d)	0.496	0.031
Plasma triglyceride content (mmol/L)	-0.663	0.002
Plasma malondialdehyde content (nmol/L)	-0.605	0.006
Plasma insulin content (mIU/L)	-0.812	<0.001

[1] r = correlation coefficient.

Table 5

Detailed information on plasma differential amino acids and amino acid metabolites between cows of the low and high nitrogen utilization efficiency groups.

Item	Group[1]		VIP	Log_2 (Fold Change)	*P*-value
	LNUE	HNUE			
L-Arginine (ng/mL)	24 723.7[a]	14 973.0[b]	1.764	-0.723	0.025
L-Glutamine (ng/mL)	380 606.2[a]	269 313.6[b]	1.930	-0.499	0.007
L-Leucine (ng/mL)	41 681.8[a]	27 647.1[b]	1.624	-0.592	0.043
L-Lysine (ng/mL)	26 642.3[a]	13 547.8[b]	1.925	-0.976	0.001
L-Ornithine (ng/mL)	42 911.6[a]	26 423.6[b]	1.631	-0.700	0.041

Abbreviations: VIP = Variable importance in projection.

[1] LNUE = lower nitrogen utilization efficiency (NUE = 24.3 ± 4.8%, n = 3, mean ± SD); HNUE = higher nitrogen utilization efficiency (NUE = 35.5 ± 1.5%, n = 3, mean ± SD).

[a, b] Means with different superscripts in each row differ significantly ($P<0.05$).

Figures

Figure. 1. The levels of nitrogen utilization efficiency in the experimental cows.

Abbreviations: NUE = Nitrogen utilization efficiency.

The x-axis indicates the animal number, while the y-axis represents the level of NUE. Red bars represent cows with low NUE (LNUE), while blue bars indicate cows with high NUE (HNUE). From top to bottom, the 3 dashed lines represent the average NUE level for 10 cows with HNUE, 20 experimental animals, and 10 cows with LNUE, respectively.

Figure. 2. A heat map showing the correlation between the differential metabolites and nitrogen utilization efficiency, DM intake, BW, and milk yield in experimental cows.

Abbreviations: DMI = DM intake; MY = Milk yield; NUE = Nitrogen utilization efficiency.

In the figure, the increasing red color indicates higher positive correlation coefficients, while the deepening blue color indicates higher negative correlation coefficients.

Figure. 3. Overview of the plasma amino acid metabolic profiling analysis in experimental cows.

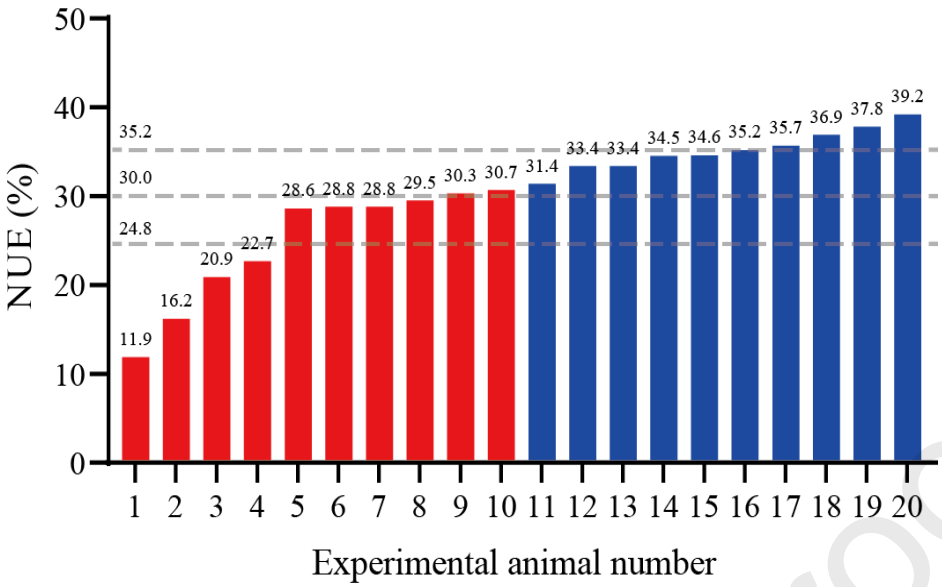
Abbreviations: Arg = L-Arginine; DMI = DM intake; Gln = L-Glutamine; HNUE = Higher nitrogen utilization efficiency (NUE = 35.5 ± 1.5%, n = 3, mean ± SD); Leu = L-Leucine; LNUE = Ligher nitrogen utilization efficiency (NUE = 24.3 ± 4.8%, n = 3, mean ± SD); Lys = L-Lysine; MY = Milk yield; NUE = Nitrogen utilization efficiency.

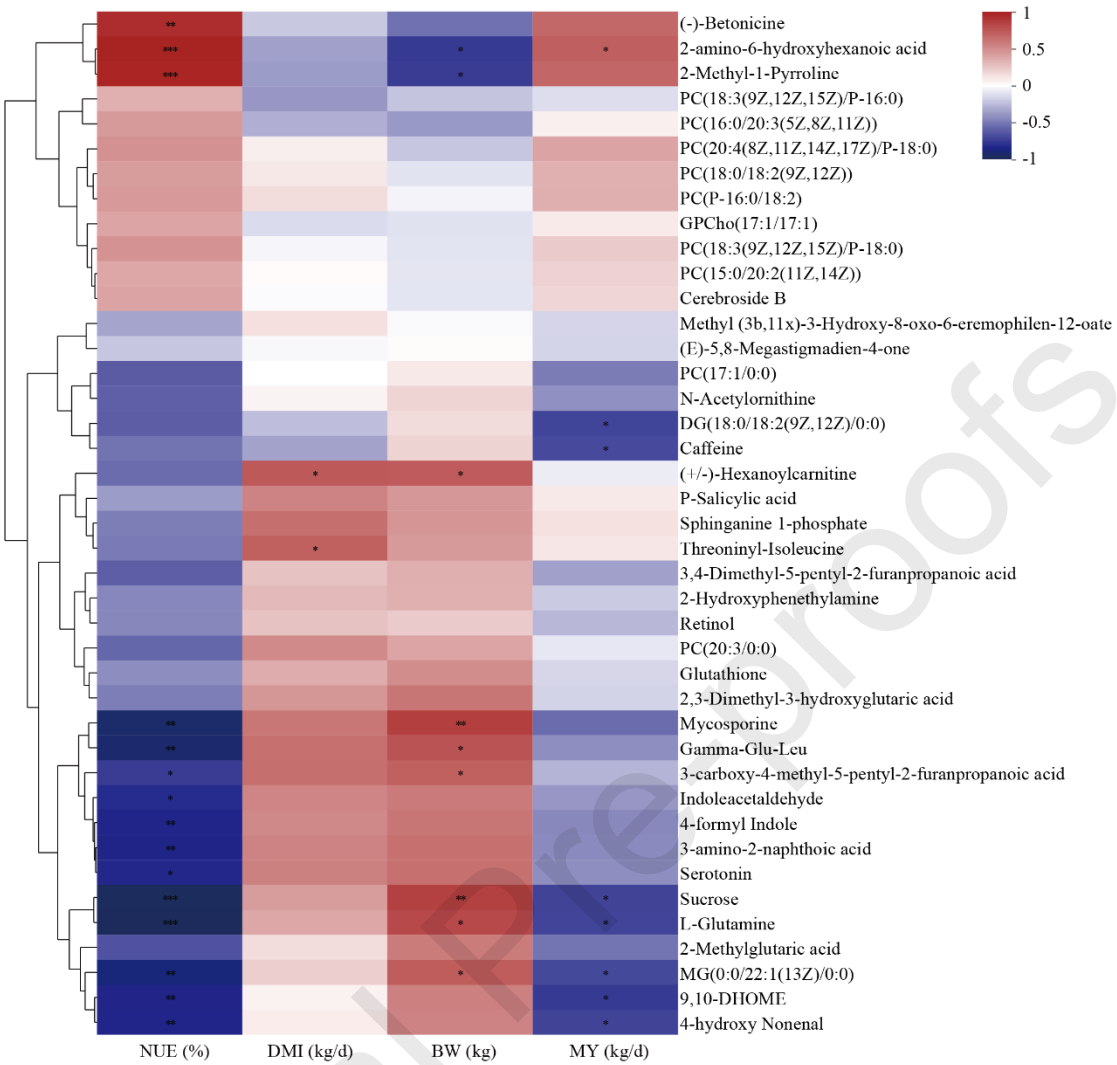
a: The amino acid and amino acid metabolite contents in the plasma of cows in the HNUE and LNUE groups.

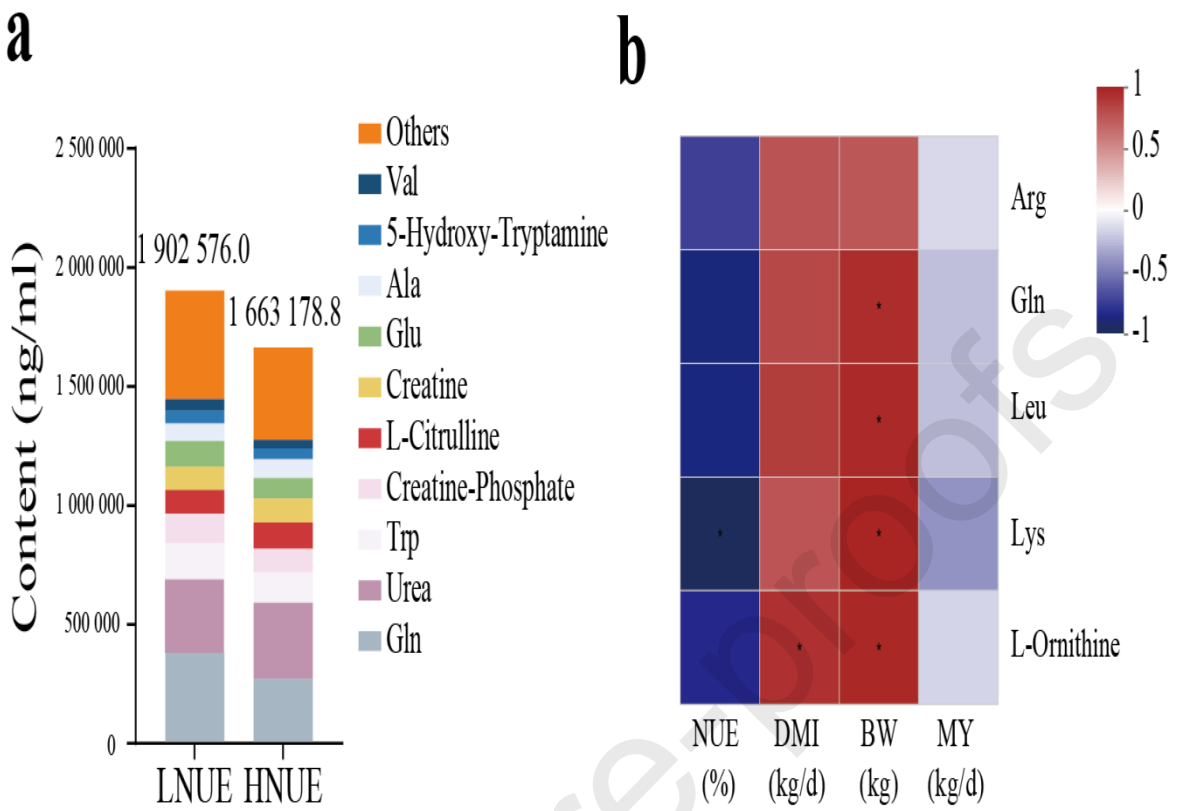
b: A heat map displaying the correlation between differential amino acids and amino acid metabolites and NUE, DMI, BW, and MY. In the figure, the increasing red color indicates higher positive correlation coefficients, while the deepening blue color indicates higher negative correlation coefficients.

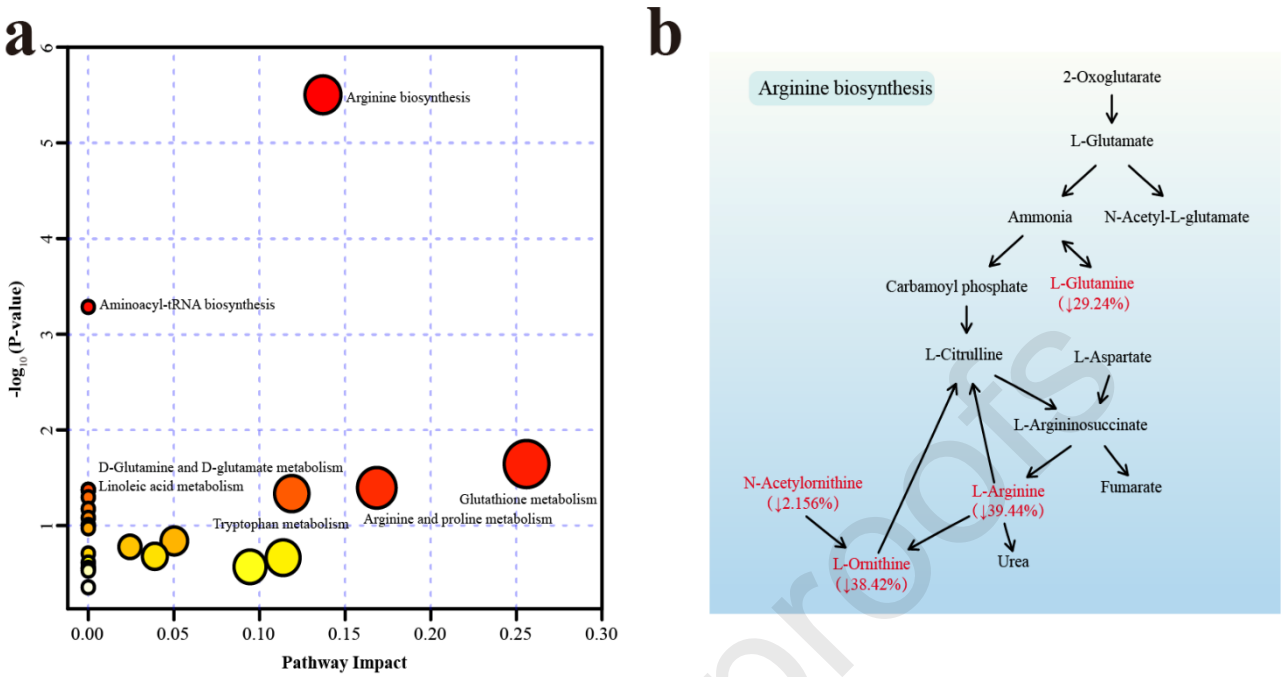
Figure. 4. Pathway analysis of the differential metabolites and differential amino acid and amino acid metabolites between cows of the low and high nitrogen utilization efficiency groups.

a: The pathway analysis results are presented in a graph, where the x-axis represents the pathway impact value and the y-axis represents pathway enrichment. Each dot in the graph represents a Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, with larger sizes and darker colors indicating higher pathway impact values and greater pathway enrichment, respectively.
b: The arginine biosynthesis pathway is highlighted, where the red font indicates metabolites that are downregulated in the plasma of cows of the higher nitrogen utilization efficiency group compared with that in cows of the lower nitrogen utilization efficiency group.









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