

Research Article

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Self-control design reveals varied lactation and metabolic responses to rumen-protected methionine in dairy cows

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Abstract

One hundred and thirteen mid-lactation cows fed same diets and supplemented with 20 g/d rumen-protected methionine (RPM) for 8 weeks were used to investigate the individual responses of dairy cows to RPM in terms of lactation performance, amino acids (AA) metabolism, and milk metabolites. Among the cows, 10 cows exhibited positive responses (PR) and 10 cows showed limited responses (LR) in energy-corrected milk yield to RPM were used for further analysis. The lactation performance changed from gradual decline to steady increase in PR cows, while kept downward trend in LR cows following RPM supplementation. In PR cows, the AA metabolism was notably enhanced after RPM supplementation, evidenced by increased mammary blood flow (69.4%, $P = 0.05$), mammary uptake and clearance rate and uptake-to-output ratio (U:O) of essential AA. The improved AA metabolism could be attributed to the enrichment of pyrimidine ($P = 0.06$) and pyruvate ($P = 0.07$) metabolism pathways, which may have stimulated mammary cell proliferation and enhanced AA uptake. Additionally, the upregulation of milk biotin (fold change > 2, variable importance projection > 1), known to support milk yield, likely contributed to the PR observed in PR cows. Conversely, in LR cows, RPM supplementation did not improve AA metabolism, decrease was observed in mammary uptake, mammary clearance rate, and U:O of cysteine, potentially due to cysteine being irreversibly converted from methionine. Moreover, the enrichment of central carbon metabolism in cancer pathway ($P = 0.06$), which also utilize methionine, along with the lysine degradation pathway ($P = 0.04$), suggests that methionine in the mammary glands may have been diverted toward non-lactational metabolic processes, resulting in absence of PR in LR cows. Our results indicate that the responses to RPM in dairy cows are individualized, with variation in lactation performance likely driven by differential AA metabolism.

Introduction

Improving our ability to manipulate milk yields and milk protein content to increase profitability and nitrogen utilization efficiency is critical for human food supply security and dairy industry sustainability (Yoder et al. 2020). Amino acids (AA) are the key components in milk and milk protein synthesis, among which the first limiting AA are methionine (Met) and/or lysine (Lys) (NRC 2001). Although the effects of Met on lactation performance are well documented in lactating dairy cows, the results have been inconsistent, some researches show improved milk yield or improved milk protein content or milk fat content, while some other researches show little or no lactation performance responses of dairy cows to rumen-protected methionine (RPM) (Benefield et al. 2009; Davidson et al. 2008; Patton 2010; Rulquin and Delaby 1997; Socha et al. 2005).

Meta-analysis has shown that the factors that influence lactation performance responses to RPM supplementation include breeds, RPM product types, dietary AA levels, and lactation stages (Patton 2010; Zanton et al. 2014). Wang et al. (Wang et al. 2010) further speculated that the different responses of dairy cows to RPM in different trials may be caused by the proportions of other AA in the metabolizable protein (MP) and by varied experimental designs (Latin square or continuous lactation trial). However, the current meta-analysis studies only considered the differences among herds and paid less attention to individual variations. Base on some

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lactation performance responses of dairy cows to RPM from our previous studies, we found that about 62–75% of the cows on RPM supplementation showed improved milk yields and energy-corrected milk (ECM) yields, while the rest showed decreased when compared with control animals (Supplementary Table S1). This indicates high individual variance in response to RPM supplementation in dairy cows.

The randomized block-controlled experimental design, which may ignore individual differences, was the most common approach used to evaluate the effects of RPM on lactation performance in dairy cows. Self-control experimental designs that compare longitudinal changes in the same animal/human are widely used in veterinary and clinical medicine researches to avoid bias due to individual differences and dig out the precision effect of treatments (Hallas and Pottegård 2014; Knottnerus et al. 2002; Sun et al. 2020).

It is acknowledged that during the mid-lactation period milk yield is slowly decreasing, and dairy cows have a relatively stable physiological status and lactation performance (Fox et al. 2015; Silvestre et al. 2009). Therefore, it is feasible to use self-control experimental design to explore the changes in lactation performance before and after feeding RPM. As reported in human studies, different responses to the same drug between individuals were closely related to their own metabolism (Zeevi et al. 2015), and understanding the metabolic changes in dairy cows after RPM supplementation could provide valuable insights into individual responses to RPM. Feedomics including metabolomics offer important contributions on dairy cows feed and nutrition research (Sun et al. 2019). Many studies have explored metabolite changes and biomarkers in milk under different lactation stages or nutritional treatments using metabolomics (Gu et al. 2021; Rocchetti et al. 2020; Wang et al. 2020). These studies provide valuable insights into understanding the complexity of animal metabolism. The objectives of this study are to investigate the variations in lactation performance responses to RPM in mid-lactating dairy cows and to elucidate the potential mechanisms underlying these differences by analyzing AA metabolism and milk metabolome.

Materials and methods

The experiment was conducted at Hangjiang Dairy Farm (Hangzhou, China), and all procedures involving animals were approved by the Zhejiang University Institutional Animal Care and Use Committee.

Animals and experimental design

One hundred and thirteen healthy Holstein dairy cows (milk yield = 33.6 ± 6.50 kg/d; day in milk [DIM] = 111 ± 11.93 d; body weight [BW] = 692 ± 73.77 kg; parity = 1.6 ± 0.70 ; mean \pm SD) were selected. The experiment was designed as a before-after study where each experimental unit served as its own control; a separate untreated group is not included for comparison in current study. The experiment lasted 13 weeks, with the first 5 weeks serving as the baseline period during which cows were fed the same basal diet without RPM supplementation (Table 1). In the later 8-week experimental period, each cow was supplemented with 20 g/d RPM (Hangzhou King Techina Feed Co., Ltd., Hangzhou, China). This RPM is produced by a smart microencapsulation coating process, and the coating materials are carnauba wax, palm oil, and polyethylene glycol. The RPM used in current study contained a dextrorotatory and levorotatory (DL)-Met of $\sim 80\%$ based on our

Table 1. Ingredients and nutrient composition of the total mixed ration used in the experiment

| Items | Diet ingredients and nutrient composition |
|--|---|
| Dietary ingredient, g/kg of DM | |
| Alfalfa hay | 124 |
| Oat hay | 89.5 |
| Corn silage | 190 |
| Corn grain | 150 |
| Soybean meal | 178 |
| Steam-flaked corn | 125 |
| Sugar beet pulp | 72.4 |
| Beer grains | 20.6 |
| Premix ¹ | 50.7 |
| Nutrient composition, % of DM | |
| Crude protein | 17.6 |
| Neutral detergent fiber | 32.6 |
| Acid detergent fiber | 19.4 |
| Crude ash | 6.83 |
| NE _L , (Mcal/kg) ² | 1.78 |
| Lys, % of MP ² | 6.88 |
| Met, % of MP ² | 2.00 |
| Lys/Met (in basal diet) ² | 3.44:1 |
| Lys/Met (after added RPM) ³ | 2.97:1 |

Note: ¹Formulated to provide (per kilogram of DM): 18 g of yeast, 270 g of fatty powder, 90 g of salt, 180 g of NaHCO₃, 90 g of Ca(HCO₃)₂, 135 g of zeolite powder, 18 g of mold adsorbent (Solis Mos, Novus International Inc., St. Charles, Mo), 142,560 IU of vitamin A, 35,640 IU of vitamin D₃, 693 IU of vitamin E, 990 mg of nicotinamide, 20 mg of biotin, 4.75 mg of selenium yeast, 950.4 mg of Zn, 831.6 mg of Mn, 297 mg of Cu, 356.4 mg of Fe, 21.4 mg of I, 7.1 mg of Co, and 9.5 mg of Se.

²All values were estimated based on the Cornell Net Carbohydrate and Protein System model using CPM Dairy 3.0.

³The amount of rumen-protected methionine (RPM) to be supplemented was 20 g/d/cow, equivalent to 8.4 g of metabolizable methionine.

measurement, its ruminal effective non-degradation was $\sim 70\%$ (*in vivo* nylon bag study) (Supplementary Table S2), and the intestinal digestibility of the RPM was $\sim 75\%$ determined from the residue of feedstuff incubated in the rumen for 16 h, according to the modified 3-step procedure (Gargallo et al. 2006). The amount of RPM to be supplemented (20 g/d RPM, equal to 8.4 g/d absorbable Met) was calculated based on the optimal ratio (3:1 final) of Lys to Met in MP estimated by the Cornell Net Carbohydrate and Protein System model using Cornell-Penn-Miner (CPM) Dairy 3.0. All cows were housed in a free-stall cowshed, had free access to water, and were fed and milked 3 times/day at 06:30, 14:00, and 19:30. total mixed ratio (TMR) was offered *ad libitum* to yield 5–10%orts after milking ($\sim 07:00$). The RPM was top-dressed onto TMR diets when the cows returned to the cowshed for feeding, and individual cows were fixed by a head lock to ensure complete consumption of the RPM. To avoid some confounding factors that could influence individual responses, all of the cows were under the same feeding and management process and were offered enough living and feeding space and lived in the same stall throughout the experiment. The BW was estimated for 3 consecutive days at week 0,

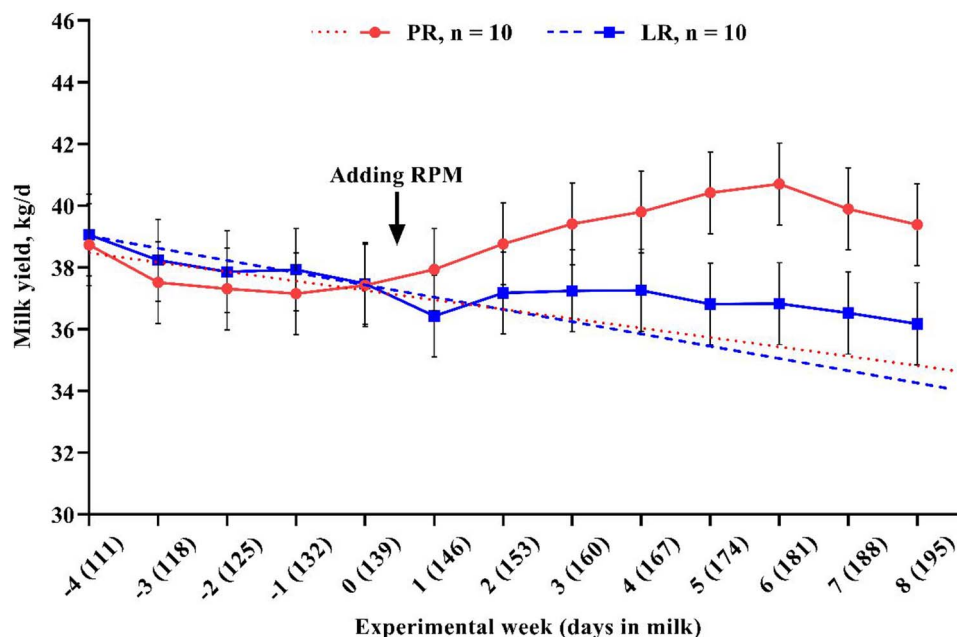


Figure 1. The milk yield curve of positive response cows (PR) and limited response cows (LR) throughout the experiment. The solid line represents the change in milk yield with the experimental week (days in milk), and the dotted line represents the trend line fitted based on the milk yield of 5 weeks before adding rumen-protected methionine (RPM).

2, 4, 6, and 8 based on the methods described by Yan et al. (Yan et al. 2009), the prediction equation was $BW \text{ (kg)} = 3.083 \times \text{heart girth} + 3.382 \times \text{body length} + 1.814 \times \text{belly girth} - 965.0$. Blood (2 ml) was collected from coccygeal vertebra vein for genotyping of dairy cows, the genotyping was performed using Bovine Geneseek Genomic Profiler - 100K Beadchip (Neogen Inc, Lincoln, NE) according to the Illumina Infinium Ultra manual (Illumina, San Diego, CA), and genotyping results are shown by principal component analysis (PCA) in Supplementary Figure S1.

Sampling and measurements

Milk sampling and analysis

Milk yield of the 113 cows were recorded daily throughout the experimental period. Milk samples were collected on day 7 (the last day of each experimental week) at week 0, 1, 2, 3, 4, 5, 6, 7, and 8, 50-mL of the composite milk samples were collected from each cow at a ratio of 4:3:3 following the milking time points (morning, afternoon, and evening) and were mixed with bronopol (milk preservative, D&F Control Systems, San Ramon, CA, USA) and stored at 4°C for further analysis of milk composition (fat, protein, lactose, milk urea nitrogen, total solid, and somatic cell counts) using an infrared analysis system with a 4-channel spectrophotometer (MilkoScan; Foss Electric A/S, Hillerod, Denmark). One set of 10-mL of the composite milk samples was collected from each cow based on the same ratio (4:3:3; morning, afternoon, and evening) on day 7 at week 0 and 8, and were stored at -20°C, for further analysis of milk AA content using an automatic AA analyzer (Hitachi High-Technologies Corporation, Tokyo, Japan) as previously described (Wang et al. 2016); Another 10-ml aliquot of milk sample was collected from each cow at each milk time point on day 7 at week 0 and 8, and immediately quenched in liquid nitrogen, and then the samples of each cow were thawed and mixed following a ratio of 4:3:3 (morning, afternoon, and evening), and then

preserved at -80°C for subsequent metabolome analysis with ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) as described below.

DMI calculation, income over feed cost calculation and TMR sample analysis

The dry matter intake (DMI) was measured for 2 consecutive days (days 6 and 7) every fortnight, and total feed intake was calculated following Liang et al. (Liang et al. 2021). In brief, DMI was measured within the first 2 hours after feeding (DMI-2 h) for each cow, and the total DMI was estimated with the forecast equation (total DMI (kg/d) = 8.499 + 0.2725 × DMI-2 h (kg/d) + 0.2132 × milk yield (kg/d) + 0.0095 × BW (kg/d)).

The income over feed cost (IOFC) of each cow was calculated on week 0 and 8 by subtracting feed costs from milk production income. The TMR samples were collected on days 6 and 7 every fortnight, dried at 65°C for 48 h, passed through a 1-mm screen in a horizontal hammer mill (ChangDing 15B, Hangzhou, China) and then used for the analysis of dry matter (DM) (method No. 934.01), CP (method No. 988.05), crude ash (method No. 942.05) and acid detergent fiber (ADF) (method No. 973.18) according to AOAC (Association of Official Analytical Chemists) methods (AOAC 2000). The neutral detergent fiber (NDF) content was analyzed using the methods of Van Soest et al. with the addition of sodium sulfite and amylase (Van Soest et al. 1991). An ANKOM2000 fiber analyzer (Ankom Technology Corp., Macedon, NY) was used to extract and filter NDF and ADF.

Blood sampling and analysis

Blood samples from the 113 cows were taken from the coccygeal artery and the subcutaneous mammary abdominal vein by venipuncture on day 7 of week 0 and week 8 at three time points viz 0630, 1400, and 1930. All blood samples were collected using lithium heparin-containing vacutainers (5 mL, Becton Dickinson, Franklin Lakes, NJ), centrifuged at 3,000 × g for 15 min at 4°C to

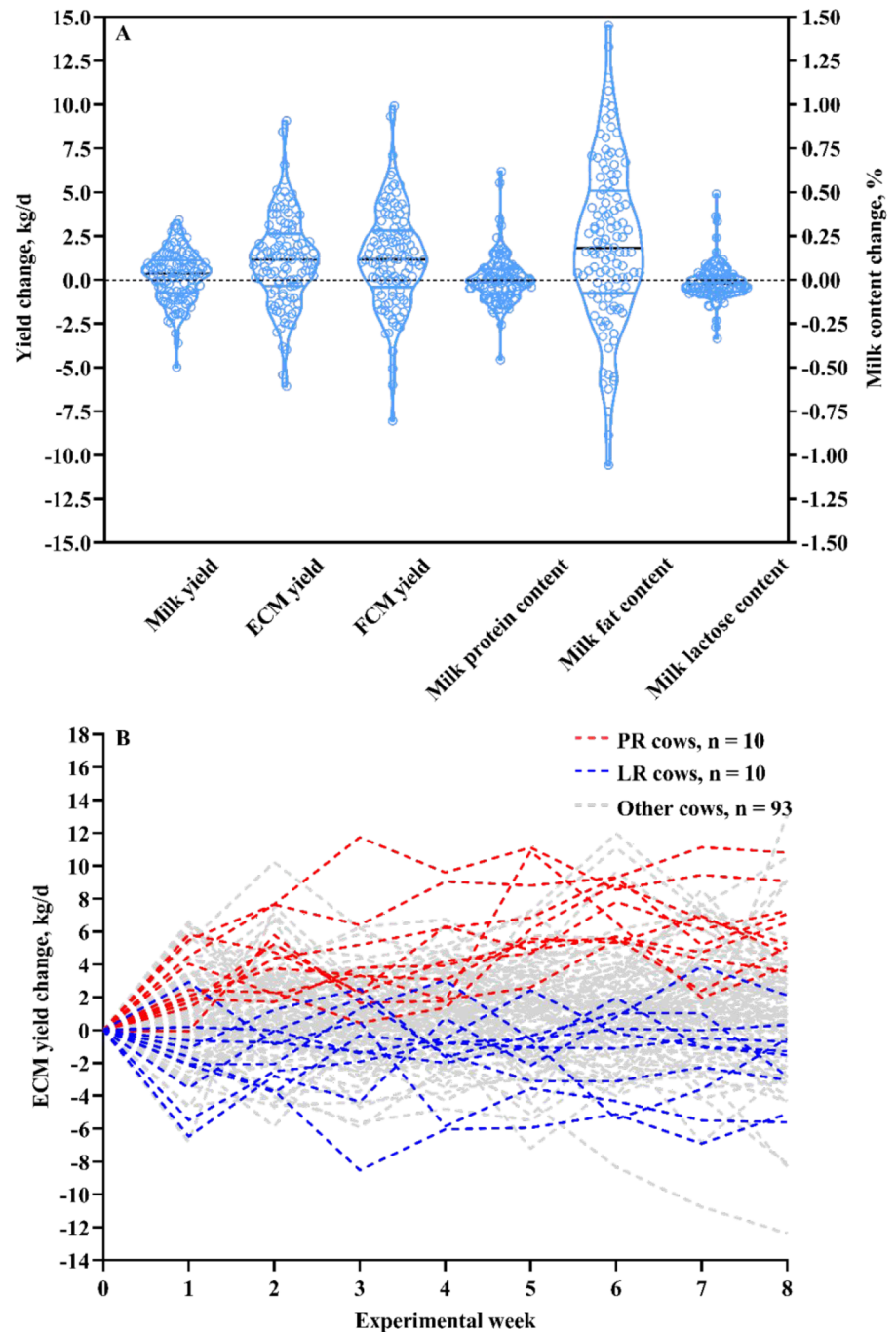


Figure 2. Interindividual variability of lactation performance responses to adding rumen-protect methionine in dairy cows. A: The change of milk yield, ECM yield, FCM yield, and milk content of dairy cows after adding RPM (mean of week 1–8 – mean of week 0). B: The change in ECM yield at every week of dairy cows after adding RPM, the red line and blue line represent positive responder cows (PR, $n = 10$) and limited responder cows (LR, $n = 10$) selected for downstream analysis. ECM: energy-corrected milk, FCM: fat-corrected milk.

collect the plasma, which was stored at -20°C until analysis. The three plasma samples from three time points on the sampling day of individual cows were mixed in equal proportions for analysis of circulating AA by an automatic AA analyzer (Hitachi High-Technologies Corporation) as previously described (Wang *et al.* 2016).

Milk metabolome analysis

Milk metabolites were analyzed using a high-performance liquid chromatography-electrospray Ionization (LC-ESI)-MS/MS system (UPLC, ExionLC AD; MS, QTRAP[®] 6500+ System, Sciex). The analytical procedures and conditions followed those previously reported by Gu *et al.* (Gu *et al.* 2021).

The mass spectrometry data were processed using Analyst 1.6.3 software. Qualitative analysis was conducted based on the retention time of the detected substances, ion pairs information, and secondary spectrum data from the Metware Database. Metabolites were quantified using the multiple reaction monitoring mode of triple quadrupole mass spectrometry. MultiQuant software was used to access the mass spectrometry files from the samples, integrate and correct the chromatographic peaks. The area under each chromatographic peak represents the relative content of the corresponding substance. Finally, all the integrated peak area data were exported and saved. To compare the differences in the content of each metabolite among different samples, chromatographic peaks detected for each metabolite in different samples were

Table 2. Difference of dry matter intake, lactation performance and efficiency between week 8 and week 0 of dairy cows

| Item | PR cows | | SEM | P-value | LR cows | | SEM | P-value |
|-------------------------------|---------|------|------|---------|---------|------|------|---------|
| | wk 0 | wk 8 | | | wk 0 | wk 8 | | |
| DMI, kg/d | 24.3 | 26.8 | 0.99 | 0.09 | 25.1 | 26.0 | 0.70 | 0.41 |
| Yield ¹ , kg/d | | | | | | | | |
| Milk | 37.4 | 39.4 | 1.59 | <0.01 | 37.5 | 36.2 | 0.89 | 0.10 |
| ECM | 40.9 | 47.2 | 1.38 | <0.01 | 41.7 | 39.9 | 1.33 | 0.04 |
| FCM | 39.1 | 46.2 | 1.37 | <0.01 | 40.2 | 38.7 | 1.57 | 0.10 |
| Protein | 1.30 | 1.35 | 0.04 | <0.01 | 1.29 | 1.21 | 0.02 | 0.01 |
| Fat | 1.42 | 1.80 | 0.06 | <0.01 | 1.48 | 1.42 | 0.08 | 0.26 |
| Lactose | 1.93 | 2.03 | 0.08 | <0.01 | 1.89 | 1.82 | 0.05 | 0.21 |
| Milk content ² , % | | | | | | | | |
| Protein | 3.48 | 3.46 | 0.07 | 0.54 | 3.45 | 3.35 | 0.07 | 0.06 |
| Fat | 3.83 | 4.61 | 0.19 | <0.01 | 3.95 | 3.92 | 0.18 | 0.85 |
| Lactose | 5.14 | 5.14 | 0.04 | 0.91 | 5.03 | 5.04 | 0.05 | 0.98 |
| Total solid | 12.7 | 13.5 | 0.21 | <0.01 | 12.5 | 12.6 | 0.21 | 0.54 |
| SCC, ×10 ³ /ml | 46.5 | 45.9 | 8.82 | 0.91 | 83.5 | 42.2 | 23.2 | 0.22 |
| MUN, mg/dL | 15.5 | 16.2 | 0.79 | 0.54 | 16.9 | 16.6 | 0.83 | 0.82 |
| IOFC, \$/cow/day ³ | 10.6 | 10.7 | 1.01 | 0.98 | 10.4 | 9.20 | 0.50 | 0.09 |
| Efficiency ⁴ | | | | | | | | |
| Feed efficiency | 1.70 | 1.81 | 0.10 | 0.47 | 1.69 | 1.53 | 0.06 | 0.14 |
| Nitrogen | 0.32 | 0.29 | 0.02 | 0.20 | 0.30 | 0.26 | 0.01 | 0.08 |
| BW, kg/d | 647 | 684 | 13.1 | <0.01 | 689 | 732 | 28.7 | <0.01 |

Note: ¹ECM: energy-corrected milk yield, $ECM = 0.3246 \times \text{milk yield} + 13.86 \times \text{milk fat yield} + 7.04 \times \text{milk protein yield}$; FCM: fat-corrected milk yield, $FCM = 0.432 \times \text{milk yield} + 16.216 \times \text{milk fat yield}$.

²SCC: somatic cell count; MUN: milk urea nitrogen.

³IOFC = income over feed cost. Calculated by subtracting feed costs from milk income.

⁴Feed efficiency calculated as ECM yield (kg/d)/DMI (kg/d), Nitrogen efficiency calculated as milk protein yield (kg/d)/total CP intake (kg/d).

corrected based on the metabolite retention time and peak shape information, ensuring the accuracy of qualitative and quantitative analysis.

Assessment of the response to RPM of individual dairy cows

Positive responses (PR) of cows to RPM normally reflected in increased average milk yield, ECM yield, fat-corrected milk (FCM) yield, and improved milk protein content and milk fat content based on previous studies (Broderick et al. 2008; NRC 2001; Osorio et al. 2013; Patton 2010; Wei et al. 2022). Since milk yield is a common parameter that is very important and easy to be measured, and as a comprehensive response index, ECM is a combinational indicator of milk yield, milk protein content and milk fat content: $ECM \text{ (kg/d)} = 0.3246 \times \text{milk yield (kg/d)} + 12.86 \times \text{milk fat yield (kg/d)} + 7.04 \times \text{milk protein yield (kg/d)}$ (Orth 1992), milk yield and ECM yield were used as criteria to assess the responses of individual dairy cows to RPM. First, cows with similar milk yield and lactation stages and milk yield trend during the first 5 weeks (week -4 to 0) before RPM supplementation were selected, and then a PR was recorded when the average ECM yield of week 1–8 after RPM supplementation were greater than week 0, otherwise, it was considered as a limited response (LR) (no response or negative

response). Based on the criteria, 10 PR cows and 10 LR cows were selected for further analysis of AA metabolism and other items. The milk yield of PR and LR cows were similar and kept similar slowly downtrend before RPM supplementation, but show increased of milk yield and ECM yield than week 0 in PR cows and decreased of milk yield and ECM yield than week 0 in LR cows after RPM supplementation (Figs. 1 and 2B). The lactation performance, BW, DIM, parity, genotypes, AA concentration in coccygeal vertebra artery, and AA concentration in subcutaneous mammary abdominal venous were similar between PR and LR cows prior to adding RPM. The number ($n = 10$) of dairy cows per group was determined based on the power analysis of the ECM response (power value > 0.95). Information on lactation performance, BW, DIM, and parity of the two groups before adding RPM are shown in Supplementary Table S3.

Calculations and statistical analysis

The parameters related to AA utilization by the mammary gland were calculated as below according to Cant et al (Cant et al. 1993):

Mammary blood flow (MBF, L/d) = $(\text{Milk [Phe + Tyr] [mg/d]} \times 0.965) / \text{Arterial and venous (AV) difference of (Phe + Tyr) (mg/L)}$.

Table 3. Difference of free amino acid concentration in coccygeal arterial between week 8 and week 0 of dairy cows

| Item, mg/L | PR cows | | SEM | P-value | LR cows | | SEM | P-value |
|--------------------|---------|------|------|---------|---------|------|------|---------|
| | wk 0 | wk 8 | | | wk 0 | wk 8 | | |
| Arg | 18.8 | 16.4 | 0.67 | 0.02 | 18.9 | 17.4 | 1.10 | 0.30 |
| His | 9.96 | 9.81 | 0.46 | 0.77 | 9.79 | 9.67 | 0.51 | 0.76 |
| Ile | 20.5 | 19.6 | 0.69 | 0.28 | 21.0 | 19.9 | 1.20 | 0.42 |
| Leu | 28.9 | 27.2 | 1.05 | 0.17 | 30.5 | 29.2 | 1.80 | 0.50 |
| Lys | 17.2 | 16.5 | 0.61 | 0.46 | 18.3 | 17.8 | 0.85 | 0.58 |
| Met | 3.83 | 3.95 | 0.19 | 0.70 | 3.94 | 4.07 | 0.21 | 0.41 |
| Phe | 18.6 | 10.1 | 1.41 | <0.01 | 15.5 | 10.5 | 0.99 | 0.03 |
| Thr | 33.1 | 34.8 | 1.44 | 0.38 | 31.3 | 34.8 | 1.41 | 0.04 |
| Val | 43.9 | 40.1 | 1.39 | 0.03 | 44.3 | 40.6 | 2.59 | 0.22 |
| Ala | 24.3 | 23.8 | 1.25 | 0.63 | 23.4 | 23.6 | 1.13 | 0.88 |
| Asp | 3.02 | 2.63 | 0.21 | 0.20 | 2.81 | 2.39 | 0.19 | 0.02 |
| Glu | 25.2 | 22.8 | 0.83 | 0.01 | 25.0 | 22.5 | 1.13 | 0.03 |
| Gly | 19.1 | 19.9 | 1.41 | 0.42 | 17.9 | 18.1 | 0.91 | 0.80 |
| Pro | 13.2 | 11.1 | 0.90 | 0.03 | 13.7 | 12.0 | 0.95 | 0.11 |
| Ser | 11.2 | 9.04 | 0.75 | 0.02 | 10.6 | 8.91 | 0.55 | < 0.01 |
| Tyr | 13.1 | 11.9 | 0.59 | 0.18 | 13.5 | 12.4 | 0.81 | 0.23 |
| Cys | 9.39 | 8.42 | 0.72 | 0.26 | 9.24 | 7.44 | 0.91 | 0.09 |
| BCAA ¹ | 93.3 | 86.9 | 3.04 | 0.07 | 95.8 | 89.8 | 5.54 | 0.33 |
| TEAA ² | 195 | 178 | 5.56 | 0.03 | 193 | 184 | 8.73 | 0.35 |
| TNEAA ³ | 119 | 110 | 5.12 | 0.08 | 116 | 107 | 4.34 | 0.12 |
| TAA ⁴ | 313 | 288 | 9.66 | 0.02 | 309 | 291 | 12.6 | 0.23 |

Note: ¹BCAA = branched-chain amino acids (Val + Ile + Leu).

²TEAA = total essential amino acids (Arg + His + Ile + Leu + Lys + Met + Phe + Thr + Val).

³TNEAA = total non-essential amino acids (Ala + Asp + Glu + Gly + Pro + Ser + Tyr + Cys).

⁴TAA = total amino acids (TEAA + TNEAA).

Mammary uptake of AA (mg/d) = AV difference of AA (mg/L) × MBF (L/d).

Clearance rate of AA in the mammary gland was calculated using the following model of Hanigan et al (Hanigan et al. 2002):

Clearance rate (L/h) = MBF (L/h) × AV difference of AA (mg/L)/Venous concentration of AA (mg/L).

Uptake-to-output ratio (U:O) = AA uptake in the mammary gland (mg/d)/AA output in milk (mg/d).

The statistical analysis and visualization of all data were performed in GraphPad Prism software version 8.0.1 (GraphPad Software, San Diego, CA 92108). The paired T-test was used to compare the differences in lactation performance and AA metabolism between before (week 0) and after (week 8) RPM supplementation in PR and LR cows. All analysis results with $P \leq 0.05$ were defined as statistically significance, and $0.05 < P \leq 0.10$ was defined as a statistical trend.

The metabolite content data was normalized using unit variance scaling method in R (www.r-project.org) and then analyzed using MetaboAnalystR 5.0 (<https://www.metaboanalyst.ca/MetaboAnalyst/home.xhtml>). PCA was performed using the built-in statistical “prcomp” function in R. Hierarchical cluster analysis and orthogonal partial least squares discriminant analysis (OPLS-DA) were conducted to analyze metabolite accumulation patterns among different samples using R. Based on the OPLS-DA

results, the variable importance projection (VIP) scores from the OPLS-DA model were obtained to preliminarily screen for differential metabolites between groups. Additionally, FC values were used to identify differential metabolites, with the criteria being: metabolites with $VIP \geq 1$ and $0.5 \geq FC \geq 2$ were considered significantly different between the two groups.

Upon identification of differential metabolites, the KEGG (Kyoto Encyclopedia of Genes and Genomes) database (<http://www.kegg.jp/kegg/Compound/>) was used for the functional annotation of these metabolites. Subsequently, the annotated metabolites were mapped to the KEGG pathway database (<http://www.kegg.jp/KEGG/pathway.html>). Metabolite set enrichment analysis was conducted by incorporating pathways containing significantly regulated metabolites, with statistical significance determined by the P -values derived from hypergeometric testing.

Results

Changes in lactation performance of PR and LR cows after adding RPM

The results showed high interindividual variability in lactation performance responses to supplemented RPM in dairy cows (Fig. 2, Supplementary Table S4). After RPM supplementation, the

Table 4. Difference of mammary blood flow (MBF) and mammary uptake of amino acid between week 8 and week 0 of dairy cows

| Item: g/d | PR cows | | SEM | P-value | LR cows | | SEM | P-value |
|---------------------|---------|--------|------|---------|---------|--------|-------|---------|
| | wk 0 | wk 8 | | | wk 0 | wk 8 | | |
| MBF, L/d | 13,759 | 23,305 | 2706 | 0.05 | 17,449 | 20,606 | 2251 | 0.38 |
| Mammary uptake, g/d | | | | | | | | |
| Arg | 83.3 | 149 | 19.0 | 0.05 | 88.5 | 115 | 20.1 | 0.40 |
| His | 29.0 | 63.7 | 8.00 | 0.01 | 28.0 | 48.5 | 7.03 | 0.11 |
| Ile | 87.5 | 153 | 18.8 | 0.05 | 123 | 130 | 19.1 | 0.84 |
| Leu | 133 | 237 | 28.7 | 0.04 | 184 | 211 | 27.6 | 0.60 |
| Lys | 104 | 186 | 22.3 | 0.05 | 125 | 158 | 20.9 | 0.39 |
| Met | 26.6 | 49.4 | 5.57 | 0.04 | 32.9 | 41.7 | 5.32 | 0.37 |
| Phe | 98.1 | 29.0 | 13.4 | 0.01 | 91.1 | 39.5 | 12.2 | 0.02 |
| Thr | 78.5 | 302 | 33.4 | <0.01 | 84.2 | 253 | 32.6 | 0.02 |
| Val | 84.0 | 143 | 22.2 | 0.13 | 140 | 116 | 20.2 | 0.50 |
| Ala | 51.2 | 71.2 | 14.8 | 0.41 | 55.1 | 41.8 | 18.1 | 0.65 |
| Asp | 20.0 | 21.8 | 4.70 | 0.83 | 25.9 | 14.3 | 4.89 | 0.16 |
| Glu | 106 | 96.1 | 15.9 | 0.70 | 124 | 74.3 | 13.0 | 0.05 |
| Gly | 13.8 | 50.3 | 14.0 | 0.16 | 27.3 | 22.9 | 9.70 | 0.77 |
| Pro | 13.8 | 10.9 | 6.75 | 0.81 | 23.1 | 9.17 | 7.43 | 0.19 |
| Ser | 39.7 | 78.2 | 12.0 | 0.04 | 48.4 | 60.7 | 7.89 | 0.34 |
| Tyr | 16.8 | 84.4 | 12.9 | 0.01 | 29.3 | 66.9 | 11.5 | 0.06 |
| Cys | -11.3 | -20.2 | 5.84 | 0.39 | 16.0 | -14.3 | 8.37 | 0.06 |
| BCAA ¹ | 305 | 533 | 68.7 | 0.06 | 447 | 456 | 65.5 | 0.94 |
| TEAA ² | 724 | 1312 | 142 | 0.03 | 897 | 1111 | 132 | 0.38 |
| TNEAA ³ | 250 | 393 | 69.9 | 0.26 | 349 | 276 | 61.6 | 0.49 |
| TAA ⁴ | 974 | 1704 | 207 | 0.06 | 1246 | 1387 | 186.9 | 0.68 |

Note: ¹BCAA = branched-chain amino acids (Val + Ile + Leu).

²TEAA = total essential amino acids (Arg + His + Ile + Leu + Lys + Met + Phe + Thr + Val).

³TNEAA = total non-essential amino acids (Ala + Asp + Glu + Gly + Pro + Ser + Tyr + Cys).

⁴TAA = total amino acids (TEAA + TNEAA).

milk yield change ranged from -4.98 to 3.41 kg/d, and the coefficient of variation (CV) was 840%; 68 cows showed an increase and 45 cows presented a decrease (Supplementary Table S4). The ECM yield changes ranged from -6.08 to 9.08 kg/d, with a CV of 231% and 79 cows presented an increase and 34 cows showing a decrease (Supplementary Table S4), among them, 10 PR and 10 LR cows selected were used for downstream analysis (Fig. 2B). The milk yield curve of PR and LR cows throughout the experiment is displayed in Fig. 1. The milk yield of the two groups were similar and kept similar slowly downtrend before RPM supplementation. After RPM supplementation, the milk yield of PR cows showed an uptrend, while the milk production of LR cows maintained a downward trend (Fig. 1). In PR cows, compared with week 0, the milk yield, ECM yield, FCM yield, milk fat content and total solids at week 8 were higher ($P < 0.01$), the DMI at week 8 tended to be higher ($P = 0.09$) (Table 2). In contrast, in LR cows, the milk yield ($P = 0.10$), FCM yield ($P = 0.10$), and milk protein content ($P = 0.07$) and IOFC ($P = 0.09$) at week 8 tended to be lower than those at week 0, and the ECM yield at week 8 was lower than that at week 0 ($P = 0.04$) (Table 2).

AA metabolism and milk metabolome between week 0 and week 8 in PR and LR cows

Arterial plasma AA

The difference of arterial plasma AA concentration between week 0 and week 8 in PR and LR cows is shown in Table 3. In PR cows, the concentration of Arg ($P = 0.02$), Phe ($P < 0.01$), Val ($P = 0.03$), Glu ($P = 0.01$), Pro ($P = 0.03$), Ser ($P = 0.02$), total essential AA (TEAA) ($P = 0.03$), and total AA ($P = 0.02$) decreased significantly after adding RPM; the concentration of branched-chain AA (BCAA) ($P = 0.07$) and total non-essential AA (TNEAA) ($P = 0.08$) tended to decrease. In LR cows, the concentration of Phe ($P = 0.03$), Asp ($P = 0.02$), Glu ($P = 0.03$), Ser ($P < 0.01$) decreased significantly and that of cysteine (Cys) ($P = 0.09$) had a decrease trend, while Thr concentration ($P = 0.04$) increased significantly.

Mammary uptake of AA

Difference of MBF and AA uptake by the mammary between week 0 and week 8 are shown in Table 4. The data of AA concentration in abdominal subcutaneous vein and AV difference is

Table 5. Difference of mammary clearance rate of amino acid between week 8 and week 0 of dairy cows

| Item | PR cows | | | <i>P</i> -value | LR cows | | | <i>P</i> -value |
|--------------------|---------|-------|------|-----------------|---------|-------|------|-----------------|
| | wk 0 | wk 8 | SEM | | wk 0 | wk 8 | SEM | |
| Arg | 321 | 661 | 95.9 | 0.03 | 327 | 453 | 90.1 | 0.34 |
| His | 161 | 382 | 45.9 | 0.01 | 147 | 294 | 42.3 | 0.09 |
| Ile | 258 | 521 | 69.0 | 0.04 | 386 | 425 | 65.3 | 0.74 |
| Leu | 291 | 617 | 79.3 | 0.03 | 404 | 506 | 72.7 | 0.46 |
| Lys | 465 | 934 | 111 | 0.03 | 509 | 707 | 109 | 0.32 |
| Met | 684 | 1231 | 166 | 0.08 | 735 | 920 | 138 | 0.45 |
| Phe | 377 | 185 | 63.8 | 0.09 | 375 | 209 | 54.4 | 0.06 |
| Thr | 127 | 596 | 70.7 | <0.01 | 134 | 469 | 60.7 | 0.01 |
| Val | 93.8 | 181 | 28.8 | 0.10 | 171 | 148 | 26.9 | 0.60 |
| Ala | 115 | 154 | 36.9 | 0.50 | 117 | 83.2 | 39.4 | 0.60 |
| Asp | 712 | 721 | 207 | 0.98 | 922 | 456 | 202 | 0.16 |
| Glu | 256 | 226 | 42.4 | 0.64 | 308 | 172 | 33.8 | 0.03 |
| Gly | 42.4 | 115 | 39.5 | 0.25 | 73.4 | 57.1 | 27.1 | 0.70 |
| Pro | 55.5 | 42.8 | 27.4 | 0.79 | 74.4 | 40.8 | 34.0 | 0.41 |
| Ser | 213 | 556 | 59.1 | <0.01 | 271 | 466 | 63.4 | 0.09 |
| Tyr | 75.3 | 446 | 60.2 | <0.01 | 108 | 333 | 62.1 | 0.05 |
| Cys | -35.9 | -88.3 | 20.8 | 0.13 | 53.0 | -67.8 | 33.7 | 0.06 |
| BCAA ¹ | 179 | 361 | 48.2 | 0.04 | 279 | 301 | 45.5 | 0.78 |
| TEAA ² | 215 | 464 | 50.9 | 0.02 | 272 | 377 | 49.0 | 0.25 |
| TNEAA ³ | 110 | 180 | 34.3 | 0.25 | 149 | 127 | 28.9 | 0.66 |
| TAA ⁴ | 171 | 339 | 43.0 | 0.05 | 220 | 267 | 37.1 | 0.50 |

Note: ¹BCAA = branched-chain amino acids (Val + Ile + Leu).

²TEAA = total essential amino acids (Arg + His + Ile + Leu + Lys + Met + Phe + Thr + Val).

³TNEAA = total non-essential amino acids (Ala + Asp + Glu + Gly + Pro + Ser + Tyr + Cys).

⁴TAA = total amino acids (TEAA + TNEAA).

shown in Supplementary Table S5 and S6. MBF increased by 69.4% ($P = 0.05$) in response to RPM for PR cows, whereas no significant MBF increasing were observed in LR cows ($P = 0.38$). Net uptake of all other essential AA (EAA) significantly increased in response to RPM in PR cows ($P \leq 0.05$), except for Phe ($P = 0.01$) which was significantly decreased and Val ($P = 0.13$) which had no significant change. The uptake of Met increased by 85.7% ($P = 0.04$), and the uptake of Ser ($P = 0.04$), Tyr ($P = 0.01$), BCAA ($P = 0.06$), TEAA ($P = 0.03$), and total AA ($P = 0.06$) significantly increased or tended to increase in PR cows. In LR cows, only the uptake of Thr ($P = 0.02$) show significantly increased in response to RPM; while the uptake of Phe ($P = 0.02$), Glu ($P = 0.05$), and Cys ($P = 0.06$, changing from 16.0 g/d to -14.3 g/d) show significantly decreased or tended to decrease in response to RPM supplementation.

Mammary AA clearance rates

Change in mammary AA clearance rates from week 0 to week 8 in PR and LR cows are listed in Table 5. The Phe clearance rate tended to decrease ($P = 0.09$), while the clearance rate of Arg, His, Ile, Leu, Lys, Thr, Ser, Tyr, total EAA, BCAA, and total AA increased ($P \leq 0.05$) in response to RPM supplementation in PR cows, clearance rate of Met ($P = 0.08$, increased by 80%) and Val ($P = 0.10$) tended to increase. In LR cows, the clearance rate of His ($P = 0.09$), Thr ($P = 0.01$), Ser ($P = 0.09$), and Tyr ($P = 0.05$) increased or

tended to increase after RPM supplementation, whereas that of Phe ($P = 0.06$), Glu ($P = 0.03$), and Cys ($P = 0.06$), changed from 53.0 L/h to -67.8 L/h decreased or tended to decrease after RPM supplementation.

Ratio of mammary AA uptake to milk AA output

Difference in U:O of AA between week 0 and week 8 in PR and LR cows are shown in Table 6. In PR cows, the U:O of Arg ($P = 0.08$), His ($P = 0.03$), Ile ($P = 0.08$), Leu ($P = 0.06$), Lys ($P = 0.07$), Met ($P = 0.06$, increased by 92.6%), Thr ($P < 0.01$), Ser ($P = 0.05$), Tyr ($P = 0.01$), BCAA ($P = 0.09$), total EAA ($P = 0.05$), and total AA ($P = 0.08$) increased or tended to increase, whereas that of Phe ($P < 0.01$) decreased after RPM supplementation. In LR cows, the U:O of Thr ($P = 0.01$), Tyr ($P = 0.04$), and His ($P = 0.08$) increased or had increase trend, while that of Phe ($P = 0.05$), Cys ($P = 0.02$, changed from 1.11 to -1.57), and Glu ($P = 0.09$) decreased or tended to decrease after adding RPM.

Milk metabolome

The OPLS-DA analysis revealed a distinct clustering pattern of metabolites in PR cows at week 8 in comparison to week 0 (Fig. 3A). Differential analysis of relative concentrations of metabolites identified 36 differential metabolites between the 8th

Table 6. Difference of amino acids uptake (g/d) to output (g/d) ratios (U:O) across the mammary gland between week 8 and week 0 in dairy cows

| Item ¹ | PR cows | | | P-value | LR cows | | | P-value |
|--------------------|---------|-------|------|---------|---------|-------|------|---------|
| | wk 0 | wk 8 | SEM | | wk 0 | wk 8 | SEM | |
| Arg | 1.99 | 3.72 | 0.53 | 0.08 | 2.04 | 2.87 | 0.47 | 0.27 |
| His | 0.88 | 2.02 | 0.26 | 0.03 | 0.83 | 1.56 | 0.21 | 0.08 |
| Ile | 1.45 | 2.61 | 0.34 | 0.08 | 1.89 | 2.22 | 0.32 | 0.57 |
| Leu | 1.06 | 2.02 | 0.26 | 0.06 | 1.43 | 1.79 | 0.22 | 0.38 |
| Lys | 1.02 | 1.90 | 0.25 | 0.07 | 1.18 | 1.63 | 0.21 | 0.25 |
| Met | 0.81 | 1.56 | 0.19 | 0.06 | 0.96 | 1.32 | 0.16 | 0.24 |
| Phe | 1.59 | 0.48 | 0.20 | <0.01 | 1.41 | 0.71 | 0.19 | 0.05 |
| Thr | 1.53 | 6.23 | 0.75 | <0.01 | 1.54 | 5.08 | 0.62 | 0.01 |
| Val | 1.15 | 2.05 | 0.34 | 0.17 | 1.80 | 1.64 | 0.28 | 0.73 |
| Ala | 1.28 | 1.96 | 0.42 | 0.35 | 1.32 | 1.08 | 0.44 | 0.74 |
| Asp | 0.22 | 0.28 | 0.07 | 0.58 | 0.28 | 0.17 | 0.05 | 0.24 |
| Glu | 0.42 | 0.47 | 0.09 | 0.76 | 0.50 | 0.32 | 0.05 | 0.09 |
| Gly | 0.63 | 2.35 | 0.63 | 0.14 | 1.12 | 1.02 | 0.40 | 0.89 |
| Pro | 0.14 | 0.13 | 0.07 | 0.92 | 0.21 | 0.09 | 0.07 | 0.25 |
| Ser | 0.59 | 1.32 | 0.20 | 0.05 | 0.72 | 0.98 | 0.12 | 0.23 |
| Tyr | 0.31 | 1.47 | 0.21 | 0.01 | 0.50 | 1.24 | 0.20 | 0.04 |
| Cys | -1.22 | -1.84 | 0.59 | 0.53 | 1.11 | -1.57 | 0.63 | 0.02 |
| BCAA ¹ | 1.18 | 2.17 | 0.30 | 0.09 | 1.65 | 1.85 | 0.25 | 0.66 |
| TEAA ² | 1.25 | 2.37 | 0.28 | 0.05 | 1.47 | 2.01 | 0.23 | 0.22 |
| TNEAA ³ | 0.39 | 0.72 | 0.14 | 0.22 | 0.52 | 0.47 | 0.10 | 0.73 |
| TAA ⁴ | 0.80 | 1.51 | 0.20 | 0.08 | 0.98 | 1.20 | 0.15 | 0.44 |

Note: ¹BCAA = branched-chain amino acids (Val + Ile + Leu).

²TEAA = total essential amino acids (Arg + His + Ile + Leu + Lys + Met + Phe + Thr + Val).

³TNEAA = total non-essential amino acids (Ala + Asp + Glu + Gly + Pro + Ser + Tyr + Cys).

⁴TAA = total amino acids (TEAA + TNEAA).

and 0th weeks in PR cows, including 6 AA and their derivatives, 2 benzene and substituted derivatives, 3 amines, 2 coenzymes and vitamins, 2 glycerophospholipids, 9 nucleotides and their derivatives, 11 organic acids and their derivatives, and 1 fatty acyl compound (Fig. 3B). At week 8, the relative concentrations of 8 metabolites significantly increased (FC > 2, VIP > 1), one of which was biotin. Conversely, the relative concentrations of 28 metabolites significantly decreased (FC < 0.5, VIP > 1) (Fig. 3B). The 36 significantly differential milk metabolites underwent KEGG functional annotation and pathway enrichment analysis (Fig. 3C), identifying propionate metabolism as significantly differential ($P < 0.01$). Pathways with tendency of significance included glucagon signaling ($P = 0.06$), pyrimidine metabolism ($P = 0.06$), pyruvate metabolism ($P = 0.07$), HIF-1 (Hypoxia-inducible factor 1) signaling ($P = 0.07$), purine metabolism ($P = 0.09$), oxidative phosphorylation ($P = 0.09$), and GABAergic synapse ($P = 0.10$). Other enriched pathways did not reach statistical significance ($P > 0.10$) (Fig. 3C).

In LR cows, OPLS-DA results demonstrated a clear clustering trend of metabolites at week 8 compared to week 0 (Fig. 4A). A total of 36 significantly differential metabolites were detected between the 8th and 0th week in LR cows, including 10 AA and their derivatives, 3 benzene and substituted derivatives, 1 amine, 6 glycerophospholipids, 1 glycerolipid, 3 nucleotides and their

derivatives, 11 organic acids and their derivatives, and 1 fatty acyl compound (Fig. 4B). At week 8, the relative concentrations of 7 metabolites significantly increased (FC > 2, VIP > 1); the relative concentrations of 29 metabolites significantly decreased (FC < 0.5, VIP > 1), including L-Met (Fig. 4B). The KEGG pathway enrichment analysis revealed significant pathways such as propionate metabolism ($P < 0.01$), glucagon signaling ($P = 0.03$), lysine degradation ($P = 0.04$), pyruvate metabolism ($P = 0.04$), and HIF-1 signaling ($P = 0.04$). Additional pathways exhibiting tendency of significance included GABAergic synapse ($P = 0.06$), oxidative phosphorylation ($P = 0.07$), butyrate metabolism ($P = 0.08$), tricarboxylic acid cycle ($P = 0.08$), and central carbon metabolism (CCM) in cancer ($P = 0.09$). Remaining enriched metabolic pathways did not achieve statistical significance ($P > 0.10$, Fig. 4C).

Discussion

Despite previous studies reporting inconsistencies in lactation performance in dairy cows supplemented with RPM (Patton 2010; Zanton et al. 2014), these studies primarily focused on population-level differences and overlooked intra-herd variability. As evidenced by studies showing significant interindividual variability in drug response and pharmacokinetics of patients (Hanna et al. 2005; Turner et al. 2015) or human blood glycemic response to

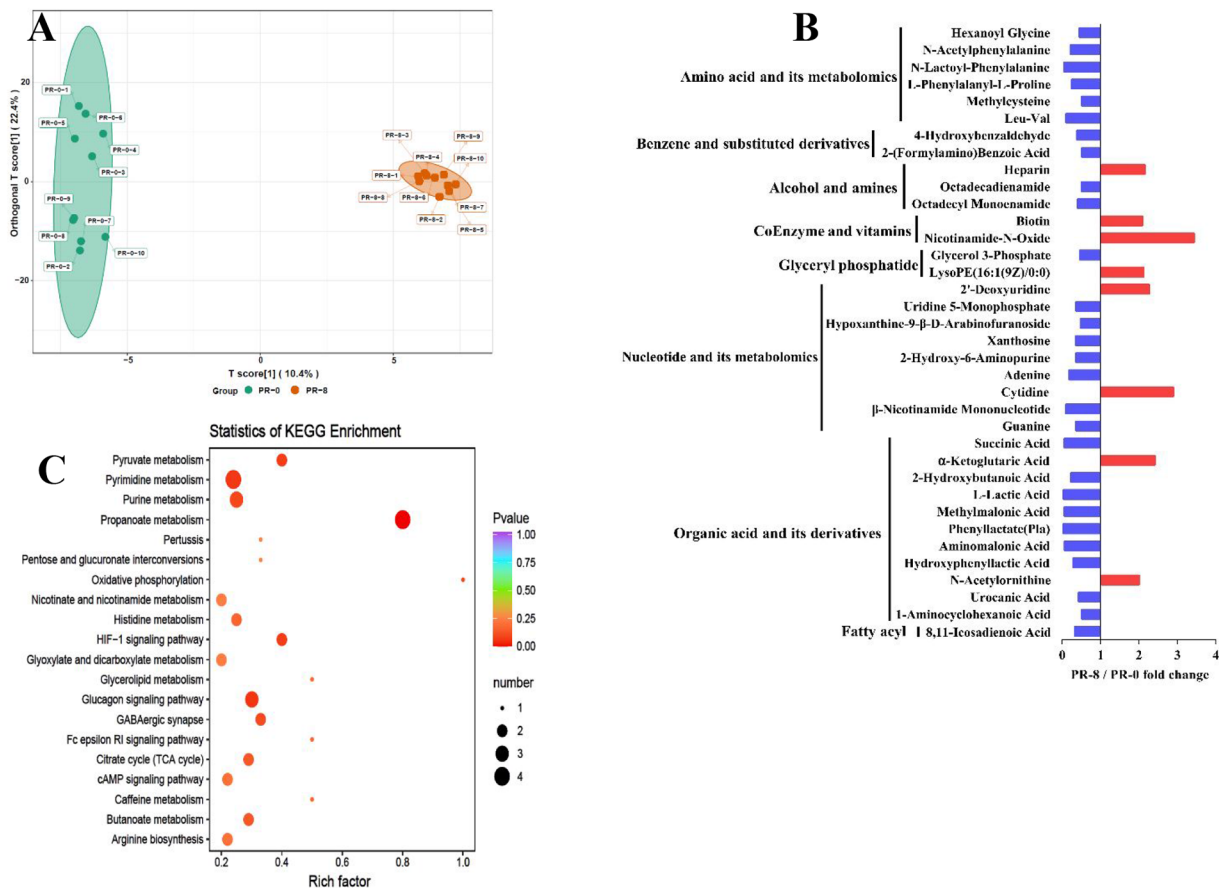


Figure 3. Difference of milk metabolome between week 8 and week 0 in PR cows. A: OPLS-DA analysis of the milk metabolome at the 8th week and the 0th week. B: The relative concentration ratios of significantly differential milk metabolites between the 8th week and the 0th week (PR-8, PR cows at the 8th week; PR-0, PR cows at the 0th week). C: Results of metabolic pathway enrichment based on significantly differential milk metabolites, where the x-axis represents the rich factor for each pathway (the ratio of the number of differential metabolites in the corresponding pathway to the total number of metabolites detected and annotated in that pathway, with a higher value indicating a greater degree of enrichment). The y-axis represents the pathway names, the color intensity of the bubbles represents the *P*-value size, with deeper red indicating more significant enrichment, and the size of the bubbles represents the number of differential metabolites enriched.

the same diet (Zeevi *et al.* 2015), the individual responses of cows to RPM were observed in current study. In clinical research, self-controlled experimental designs that compare the longitudinal changes within the same subjects are often employed to mitigate individual differences and ascertain the precise effects of treatments (Knottnerus *et al.* 2002). Inspired by this, our experiment was conducted using a self-control design in mid-lactation dairy cows, with each cow serving as its own control. Consistent with previous researches that dairy cows have a relatively stable physiological status and lactation performance during the mid-lactation period (Fox *et al.* 2015; Silvestre *et al.* 2009), no differences were observed in LR cows for the average milk yield (36.8 kg/d vs 36.4 kg/d, $P = 0.36$) and ECM yield (39.9 kg/d vs 40.0 kg/d, $P = 0.96$) of the first 2 weeks (weeks 1 and 2) after RPM supplementation compared to the last 2 weeks (weeks 7 and 8) after RPM supplementation, which indicate that little variation in LR cows were caused by time throughout the 8 weeks of RPM supplementation period. The milk yield kept similar slowly downtrend after RPM supplementation as before RPM supplementation, and lower milk yield ($P = 0.10$) and ECM yield ($P = 0.04$) at week 8 compared to week 0 were observed in LR cows. Whereas, the milk yield of the 10 PR cows kept similar slowly downtrend as the LR cows before RPM supplementation, but changed to uptrend after

RPM supplementation (Fig. 1), and show significantly improved milk yield ($P < 0.01$) and ECM yield ($P < 0.01$) at week 8 compared to week 0, indicating that the PR of lactation performance in PR cows were mainly caused by RPM.

Increases in plasma Met or other AA are commonly reported post-RPM supplementation (Fagundes *et al.* 2018; Overton *et al.* 1998; Wang *et al.* 2010). However, no significant difference was observed for Met concentrations in both PR and LR cows in our experiments. This might mainly due to the increased MBF, which indicated that promoted total blood circulations after RPM supplementation in PR cows, and therefore decreased the blood AA absorbed from the intestine during each circulation, finally reflected in no difference was observed in Met concentrations.

The significant increase in MBF in PR cows may stem from enhanced nitric oxide synthesis from arginine, regulating MBF positively (Cieslar *et al.* 2014; Wu and Morris 1998). Decreased venous plasma arginine (Supplementary Table S5) and increased mammary arginine uptake suggest arginine utilization for nitric oxide synthesis, and promoting MBF. AA substrates for increased yield originate from reduced catabolism, protein accretion in the mammary gland, or increased arterial uptake (Yoder *et al.* 2020). For instance, threonine catabolism to α -ketobutyrate for energy production via the tricarboxylic acid cycle (House *et al.* 2001)

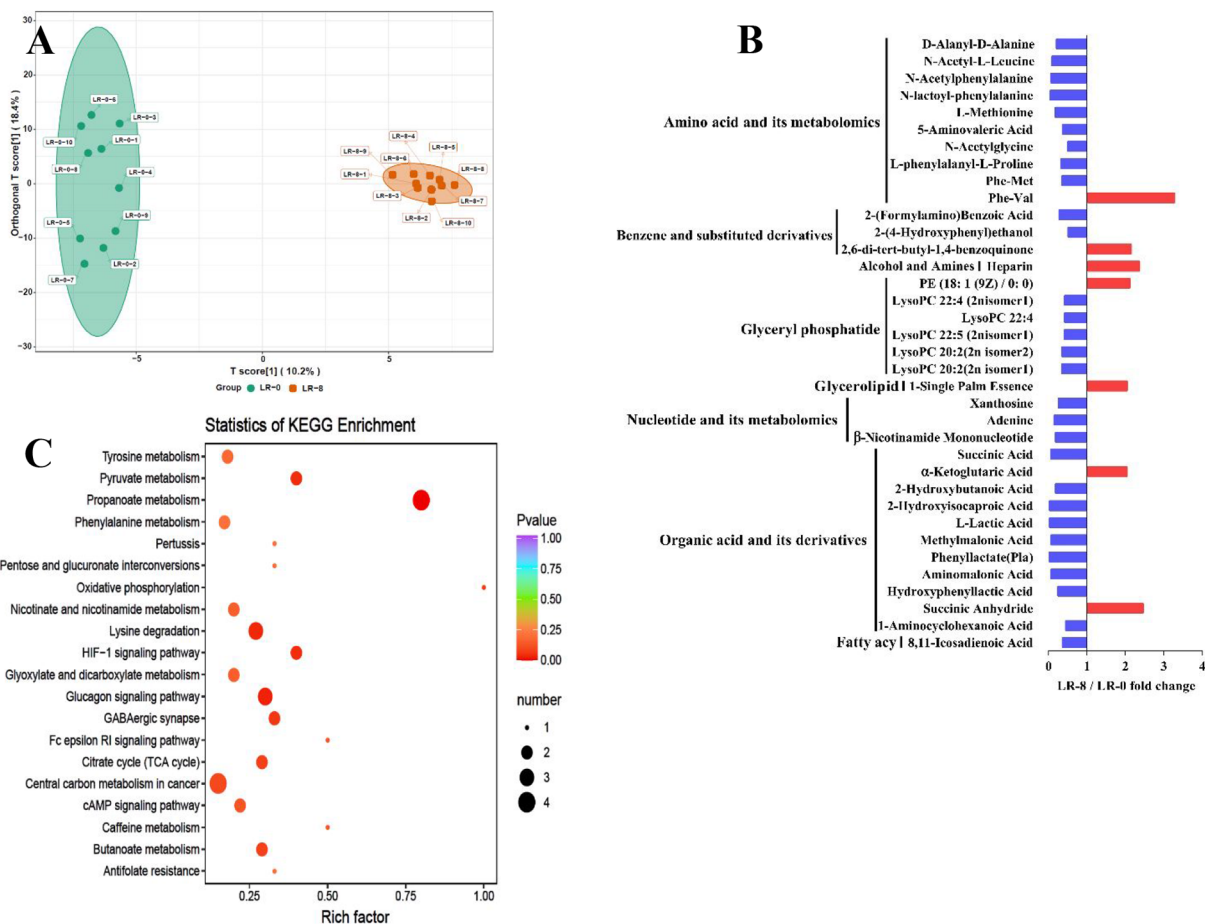


Figure 4. Difference of milk metabolome between week 8 and week 0 in LR cows. A: OPLS-DA analysis of the milk metabolome between the 8th week and the 0th week. B: The relative concentration ratios of significantly differential milk metabolites between the 8th week and the 0th week (LR-8, LR cows at the 8th week; LR-0, LR cows at the 0th week). C: Results of the metabolic pathway enrichment based on significantly differential milk metabolites, where the x-axis represents the rich factor for each pathway (the ratio of the number of differential metabolites in the corresponding pathway to the total number of metabolites detected and annotated in that pathway, with a higher value indicating a greater degree of enrichment), the y-axis denotes the pathway names, the color intensity of the bubbles represents the *P*-value size, with deeper red indicating more significant enrichment, and the size of the bubbles represents the number of differential metabolites enriched.

and BCAA catabolism providing carbon skeletons for the same cycle (Coleman et al. 2020a). Increased mammary EAA uptake directly contributes to significant increases in milk protein and milk yield in PR cows. Increased milk fat content may also benefit from increased mammary EAA uptake, as AA participate in milk fat synthesis and secretion in mammary epithelial cells (Qi et al. 2018). LR cows, however, did not show significant lactation performance improvements, consistent with the lack of significant mammary AA uptake increases.

Mammary AA clearance rates reflect mammary gland affinity for AA (Apelo et al. 2014). An increase in clearance rate indicates higher affinity for extracting AA from extracellular space, reducing availability for splanchnic catabolism (Yoder et al. 2020). EAA are generally not taken up in excess in the mammary gland when their supplies increase over mammary demand for milk synthesis (Lapierre et al. 2012), and the mammary gland can enhance affinity for AA to improve AA uptake in the gland based on the requirement for lactation (Pszczolkowski and Apelo 2020). Thus, a dramatic increase in clearance rate suggests improved EAA requirement and mammary gland affinity in PR cows. The unchanged clearance rate of EAA in LR cows indicates that no improved EAA requirement and mammary gland affinity occur.

The U:O reflects whether these AA are involved in anabolism or catabolism in the mammary gland, in which the U:O > 1 means more catabolism or transamination and <1 means more anabolism (Ivanisevic et al. 2015; Lapierre et al. 2012). In PR cows, the U:O of most EAA, TEAA, BCAA, and TAA increased after RPM supplementation, which likely means these AA are more utilized for catabolism in the udder. The catabolism of AA in the mammary gland can provide energy for milk synthesis, catabolism of BCAA and some EAA can finally enter the citric acid cycle to provide energy (Coleman et al. 2020a). This might explain the elevation in milk yield (especially ECM) because of an increment in cellular energy status.

Interestingly, we found that the Cys metabolism changed most between PR and LR cows among all AA, no significant difference of arterial plasma Cys concentration, mammary uptake of Cys, Cys clearance rate and U:O of Cys were observed in PR cows; whereas, all these parameters of Cys were decreased in PR cows ($P < 0.10$). Met can be converted to Cys by transsulfation, which is irreversible and requires Met consumption (McFadden et al. 2020). In LR cows, the value of mammary uptake, mammary clearance rate and U:O of Cys were positive before RPM supplementation, which means that udder does not synthesize sufficient Cys and therefore need

to uptake adequate amount of free Cys from arterial blood. After RPM supplementation, the mammary uptake, mammary clearance rate and U:O of Cys in LR cows were decreased and the values changed from positive to negative. Therefore, we speculated that the RPM reached the mammary gland was more involved in the metabolism of Cys synthesis rather than milk synthesis metabolism in LR cows, and the insufficient AA metabolic response lead to the limited lactation performance response.

The milk metabolome results of PR cows showed that biotin was significantly upregulated at the 8th week, which is one of the B vitamins and is an essential nutrient for the body to maintain normal growth and health and metabolism (Zempleni and Kuroishi 2012; Zempleni *et al.* 2009). Supplementation of biotin improved blood and milk biotin content and increased 320 kg of milk yield for 305-day calculated milk production (Midla *et al.* 1998), and many other researches also show significant improved milk yield with biotin supplementation (Chen *et al.* 2011; Zimmerly and Weiss 2001). These results collectively suggest that the significant increase in biotin concentration in the milk of PR cows at the 8th week may also be one of the reasons for the significant improvement in milk yield and milk fat content.

Additionally, the enriched metabolic pathway results showed that the metabolism of pyrimidines and purines in PR cow has a tendency of significant change. Pyrimidine and purine substances are essential for cell proliferation (Coleman *et al.* 2020a), so it is possible that the significant downregulation of the relative concentrations of nucleotides and their metabolites is a result of increased proliferation of mammary cells in PR cows. The proliferation of mammary cells enhances the uptake capacity of AA, which is consistent with the observed significant increase in mammary AA clearance rate and uptake. In contrast, in LR cows, the majority of the significantly downregulated differential metabolites were AA and their metabolites, with a significant downregulation in L-Met concentration. Meanwhile, unlike PR cows, the lysine degradation and CCM in cancer pathways were significantly enriched in LR cows. Lysine is the second limiting AA for dairy cows and is essential for milk protein synthesis and milk yield. Therefore, the enrichment of the lysine degradation pathway suggests that there may be more utilization of lysine in no-lactation metabolism in LR cows, and affected the positive lactation performance responses to RPM. Moreover, as a key nutrient molecule in one-carbon metabolism in cancer metabolism circle (Locasale 2013; Newman and Maddocks 2017), Met may also be consumed in large amounts by this metabolic pathway. The results indicate that Met in the mammary glands of LR cows may be utilized more by metabolic pathways unrelated to lactation and milk protein synthesis, leading to a LR in lactation performance.

Conclusions

The responses of dairy cows to supplemental RPM under similar conditions exhibited significant individual variability. The differential changes in lactation performance between PR and LR cows following RPM supplementation may be attributed to the distinct alterations in AA metabolism, along with the enrichment of pyrimidine and pyruvate metabolism pathways and upregulated milk biotin, likely contributed to the positive lactation responses in PR cows. Conversely, the limited AA metabolic response and the enrichment of non-lactational metabolic pathways that potentially consume Met may explain the lack of improvement in lactation performance in LR cows. These results underscore the role of

AA metabolism in influencing lactation outcomes and offer novel insights for advancing precision nutrition and developing potential targeted nutritional strategies in dairy production.

Supplementary material. The supplementary material for this article can be found at <https://doi.org/10.1017/anr.2024.25>.

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Author contributions. ZHW and SLL, performing experiment, data curation, original draft preparation; FFG, methodology, manuscript revision; JW, manuscript revision; HZS, experiment design, manuscript revision. All the authors reviewed and approved the manuscript.

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Conflicts of interest. The authors declare that the research was conducted with no commercial or financial relationships that could be construed as a potential conflict of interest.

Ethical standards. The procedures of this study were approved by the Animal Care and Use Committee of Zhejiang University (Hangzhou, China) and were in accordance with the university's guidelines for animal research.

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