

Italian Journal of Animal Science

ISSN: 1828-051X (Online) Journal homepage: www.tandfonline.com/journals/tjas20

Short communication: do milk purine derivatives reflect the urinary PDC index in dairy cows? Insights from integrative milk–urine targeted and untargeted screening

Michela Errico , Alessandro Catellani , Federico Froldi , Maurizio Moschini , Gabriele Rocchetti & Antonio Gallo

To cite this article: Michela Errico , Alessandro Catellani , Federico Froldi , Maurizio Moschini , Gabriele Rocchetti & Antonio Gallo (2026) Short communication: do milk purine derivatives reflect the urinary PDC index in dairy cows? Insights from integrative milk–urine targeted and untargeted screening, Italian Journal of Animal Science, 25:1, 1443-1450, DOI: 10.1080/1828051X.2026.2745959

To link to this article: https://doi.org/10.1080/1828051X.2026.2745959

© 2026 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group

Published online: 23 Oct 2026.

Submit your article to this journal

View related articles

View Crossmark data

Full Terms & Conditions of access and use can be found at https://www.tandfonline.com/action/journalInformation?journalCode=tjas20

ITALIAN JOURNAL OF ANIMAL SCIENCE
2026, VOL. 25, NO. 1, 1443–1450
https://doi.org/10.1080/1828051X.2026.2745959

Taylor & Francis
by informa

RAPID COMMUNICATION

OPEN ACCESS Check for updates

Short communication: do milk purine derivatives reflect the urinary PDC index in dairy cows? Insights from integrative milk–urine targeted and untargeted screening

Michela Errico, Alessandro Catellani, Federico Froldi, Maurizio Moschini, Gabriele Rocchetti and Antonio Gallo

Department of Animal Science, Food, and Nutrition (DIANA), Università Cattolica del Sacro Cuore, Piacenza, Italy

ABSTRACT

Urinary purine derivatives (PD) are widely used as non-invasive indicators of microbial protein synthesis in dairy cows, whereas their potential use in milk as an alternative biofluid remains poorly understood. This study investigated whether milk PD reflect urinary purine metabolism, assessed the effects of dietary protein degradability on PD profiles, and explored coordinated metabolic responses in milk and urine using an integrative targeted and untargeted approach. We hypothesised that differences in dietary protein degradability would induce coordinated metabolic changes detectable across both biofluids. Milk and urine samples from nine lactating Holstein cows fed diets differing in rumen-degradable (RDP) to rumen-undegradable (RUP) protein ratio, namely CTR (64:36 ratio), SOL (67:33 ratio), and BY (57:43 ratio), were analysed by targeted and untargeted liquid chromatography coupled with high-resolution mass spectrometry. Targeted quantification of allantoin, uric acid, and creatinine revealed no association between milk PD concentrations and the urinary purine derivatives-to-creatinine (PDC) index, indicating that milk PD do not directly reflect urinary purine metabolism. However, untargeted profiling identified diet-related metabolic differences in both biofluids, with changes involving pathways related to amino acid metabolism, particularly in cows receiving the high-rumen-undegradable-protein diet. Integration of milk and urine datasets using DIABLO further revealed coordinated metabolite patterns across biofluids and dietary groups. Overall, these findings indicate that milk PD alone may not be a suitable proxy for urinary PD-based assessment of microbial protein synthesis. Integrated profiling of multiple biofluids can reveal systemic metabolic responses to dietary protein degradability and provide complementary insights into nitrogen metabolism in dairy cows.

HIGHLIGHTS

- Milk purine derivatives did not reflect urinary Purine derivatives to Creatinine index.
- Urinary purine derivatives showed strong internal correlation.
- Data fusion improved interpretation of nitrogen metabolism

ARTICLE HISTORY
Received 18 August 2026
Revised 24 September 2026
Accepted 30 September 2026

KEYWORDS
Dairy cows; purine derivatives; nitrogen metabolism; untargeted screening; biofluids

Introduction

In ruminants, a substantial proportion of dietary nitrogen is transformed into ruminal ammonia which, when not efficiently incorporated into microbial protein, is absorbed, converted to urea in the liver, and largely excreted. This represents a nitrogen loss that could otherwise support productive processes such as milk protein synthesis and nitrogen use efficiency (Tamminga, 1992; Bach et al. 2005). Inefficient ruminal nitrogen utilisation is often associated with an imbalance between ruminally degradable nitrogen supply and microbial assimilation, resulting in increased nitrogen excretion (Hristov et al. 2019; Kand and Dickhoefer, 2021).

Purine derivatives (PD), mainly uric acid and allantoin, originate from microbial nucleic acid degradation and are widely used as indirect indicators of rumen microbial protein synthesis and nitrogen use efficiency (Giagnoni et al. 2025). Spot urine sampling combined with the PD-to-creatinine ratio (PDC index) provides a practical alternative to total urine collection for estimating PD excretion (Chen et al. 1992). Because

CONTACT Gabriele Rocchetti gabriele.rocchetti@unicatt.it

© 2026 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group
This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial License (http://creativecommons.org/licenses/by-nc/4.0/), which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited. The terms on which this article has been published allow the posting of the Accepted Manuscript in a repository by the author(s) or with their consent.

creatinine excretion is relatively stable and related to body protein mass, expressing PD relative to creatinine helps account for variations in urine dilution and provides an indicator of microbial protein synthesis (Pereira et al. 2022). Dietary protein degradability is an important determinant of nitrogen utilisation. Rumen degradable protein (RDP) supports microbial growth and protein synthesis, whereas rumen undegradable protein (RUP) escapes ruminal degradation and contributes to intestinal amino acid supply (Savari et al. 2018; Putri et al. 2021). Consequently, the dietary RDP balance can affect microbial protein synthesis, metabolisable protein supply, milk production, and nitrogen utilisation (Santos et al. 1998; Brito et al. 2007; Broderick and Reynal, 2009).

Although urinary PD are widely recognised indicators of microbial protein synthesis, their responses may vary according to dietary conditions and individual differences in urine output, endogenous purine metabolism, and renal handling and excretion of purine derivatives (Dórea et al. 2017). Moreover, nitrogen partitioning involves coordinated processes across the rumen, liver, and mammary gland, suggesting that biomarkers measured in a single biological matrix, such as urine or milk, may not fully capture the systemic metabolic response to dietary protein. In this context, F3-metabolomics (Feed, Fluid, and Food metabolomics) has been proposed as a multi-matrix omics framework that connects the metabolic characterisation of feed with that of animal biofluids and animal-derived food products, allowing dietary inputs to be investigated in relation to their downstream metabolic signatures across interconnected biological compartments (Rocchetti and Gallo, 2026). Rather than focusing on individual biomarkers or a single biological matrix, this approach emphasises the integration of complementary metabolomic information across matrices to provide a more comprehensive representation of metabolic responses to nutritional factors. Applying this framework together with targeted biomarkers may therefore help elucidate how dietary protein degradability is reflected across different biological compartments and provide broader insights into nitrogen metabolism in dairy cows. In this study, we hypothesised that differences in dietary protein degradability would induce coordinated metabolic changes detectable across biofluids; therefore, nitrogen metabolism was investigated in lactating dairy cows fed diets differing in RDP and RUP. Targeted quantification of PD and creatinine in milk and urine was used to calculate the PDC index and evaluate relationships between biological matrices, while untargeted ultra-high performance liquid chromatography (UHPLC) coupled with high-resolution mass spectrometry (HRMS) was applied to identify coordinated metabolic responses and features associated with dietary protein degradability.

Materials and methods

This study was conducted in accordance with Italian legislation on animal experimentation (Legislative Decree No. 116, 27/01/1992). The study was exempted from authorisation and ethical approval by the Ethical Animal Care and Use Committee (OPBA) of the Università Cattolica del Sacro Cuore (Piacenza, Italy), as the procedures were deemed non-invasive.

Experimental trial and diets

The trial was conducted at the experimental dairy farm of the Romeo and Enrica Invernizzi Research Centre (CREI) and CERZOO Ltd. (San Bonico, Piacenza, Italy) between February 18 and April 21, 2024. Nine multiparous Holstein-Friesian dairy cows (3 ± 1 lactations, 153 ± 37 DIM, 44 ± 5 kg milk yield, and 656 ± 41 kg BW across experimental periods) were included in the present sub-study from a larger replicated 3×3 Latin-square feeding trial involving 18 cows and based on the collection of both urine and milk samples. However, due to sampling on spontaneous urination, complete urine sample sets could not be consistently obtained across all cows and experimental periods. Also, the available urine and milk samples were subsequently assessed for their completeness and analytical suitability for the targeted and untargeted UHPLC-HRMS analyses, and nine cows out of the original 18 with sufficiently complete and analytically suitable sample sets were retained for the final analyses. Consequently, the nine cows included in this sub-study did not represent complete Latin-square sequences from the original feeding trial. Each period lasted 21 days (7 days of adaptation and 14 days of data collection; 63 days in total). Cows were housed in a free-stall barn, milked twice daily (04:00 and 16:00), and fed ad libitum using an automated roughage intake control system (RIC, Hokofarm, Netherlands) to record individual intake. The three experimental diets were formulated to be isoenergetic using the CNCPS 6.5 model (Van Amburgh et al. 2015) implemented in NDS Professional software (Rumen S.r.l., Reggio Emilia, Italy), and differed mainly in CP concentration and ruminal protein degradability. A total of 54 biological samples were analysed, comprising 27 urine and 27 milk samples collected from the 9

cows across 3 experimental periods. Each cow received all three dietary treatments: Control (CTR), high-soluble protein (SOL), and high-bypass protein (BY), resulting in 9 observations per treatment for each biological matrix. CTR and SOL contained 16.47 and 16.49% CP, respectively, whereas BY contained 14.32% CP. Dietary OM, NDF, starch, and EE ranged from 91.9–92.3, 31.5–36.2, 24.7–25.2, and 4.4–4.7% of DM (dry matter), respectively. Predicted RDP concentrations were 10.48, 11.05, and 8.14% of DM for CTR, SOL, and BY, respectively, while RUP concentrations were 5.95, 5.31, and 6.22% DM. Model outputs indicated similar metabolisable energy (63.2–65.5 Mcal/d), metabolisable protein (MP) (2,437–2,665 g/d), and microbial metabolisable protein production (1,303–1,374 g/d) among treatments. Considering an estimated DMI of 28 kg/cow/d, BY provided 110 g MP/kg DM for 45.2 kg MY (4.0% fat, 3.4% protein), with lysine and methionine accounting for 8.12 and 2.71% of MP, respectively. CTR provided 107.50 g MP/kg DM for 44.6 kg MY, with lysine and methionine at 6.70 and 2.09% of MP, respectively. SOL provided 100.60 g MP/kg DM for 39 kg MY, with lysine and methionine at 6.21 and 2.21% of MP, respectively.

Collection and processing of milk and urine samples

Milk samples were collected during the morning and afternoon milkings on the last day of each experimental period. Urine was collected once per cow by spot sampling during spontaneous urination in the early morning before feeding, directly into sterile 50–mL Falcon tubes, without acidification. Samples were immediately stored at −20 °C until analysis. BW was recorded twice daily after morning and afternoon milking throughout the experimental period. Milk extraction followed Zuo et al. (2015) with minor modifications. Briefly, thawed milk (4 mL) was mixed with 2.5 mL of 3% acetic acid, vortexed, diluted with ultrapure water, centrifuged (4,227 × g, 10 min, 4 °C), and filtered (0.2 μm) into UHPLC vials. Urine samples were processed according to Kwon et al. (2012), with minor modifications, by centrifugation (14,000 × g, 5 min, 4 °C), dilution with ultrapure water, and mixing with acetonitrile before UHPLC analysis.

Chromatographic separation was performed by UHPLC coupled to an Orbitrap Q-Exactive Focus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany; 70,000 FWHM at m/z 200) using an ACQUITY UPLC BEH C18 column (2.1 × 100 mm, 1.7 μm; Waters) at 35 °C. Water (A) and acetonitrile (B), both containing 0.1% formic acid, were delivered at 0.2 mL/min. Full-scan data were acquired over m/z 50–250 with a 10 μL injection volume. Uric acid, allantoin, and creatinine were quantified by accurate mass and MS/MS fragmentation (10 eV), monitoring transitions of 167.01 → 124.01 Da (RT 1.25 min), 157.03 → 114.03 Da (RT 1.18 min), and 114.06 → 86.07 Da (RT 1.10 min), respectively. Creatinine was analysed in ESI positive mode, whereas uric acid and allantoin were analysed in ESI negative mode. The method showed linearity over 25–1000 μg/L ($R^2 > 0.99$). Recovery was assessed by pre- and post-extraction spiking at 250 ppb (Matuszewski et al. 2003), yielding 93%, 108%, and 118% in milk and 80%, 99%, and 73% in urine for uric acid, allantoin, and creatinine, respectively. Additionally, for untargeted screening of small metabolites, full-scan data were processed using MS-DIAL v4.90 for peak detection, deconvolution, alignment, and annotation. Metabolites were putatively identified by matching their accurate-mass spectra against the Bovine Metabolome Database ESI-positive spectral library, using an identification score ≥60%.

Statistical analyses

Statistical analyses were performed using R (version 4.4.1; R Foundation for Statistical Computing, Vienna, Austria). Linear mixed-effects models were used to analyse the following phenotypes as dependent variables (Y): DMI, milk yield, BW, urinary creatinine, uric acid, allantoin, and PDC index, as well as milk creatinine, uric acid, and allantoin. Dietary treatment and experimental period were included as fixed effects, whereas cow was included as a random effect:

$$Y_{ijk} = \mu + Treatment_i + Period_j + Cow_k + \varepsilon_{ijk}$$

where ε represents the residual error. Estimated marginal means were obtained using the emmeans package, and pairwise comparisons were performed using Tukey's adjustment. Statistical significance was set at $p < 0.05$, whereas $0.05 \leq p < 0.10$ was considered a tendency. The PDC index was calculated according to Chen et al. (2004) by adjusting the urinary PD-to-creatinine ratio for metabolic body weight ($BW^{0.75}$):

$$PDC\ index = \frac{PD}{Creatinine} \times BW^{0.75}$$

Pearson correlations with 95% confidence intervals were calculated in RStudio. Untargeted UHPLC-HRMS data were $\log_{10}$-transformed, median-normalized, and Pareto-scaled. Unsupervised hierarchical clustering analysis (HCA) was performed using MetaboAnalyst 6.0 to assess metabolic similarities between milk and urine. Coordinated metabolic responses were

investigated through DIABLO multiblock analysis implemented in the mixOmics package. DIABLO integrates datasets by identifying latent components that maximise covariance between datasets and discrimination among experimental groups. Integration strength was assessed from correlations between latent components, while variable importance (VIP) scores were used to identify discriminant metabolites. Pearson correlation networks ($|r| \geq 0.7$) were generated to visualise strong associations between milk and urine metabolites.

Results and discussion

Animal performance was evaluated in terms of DMI, milk yield, and BW. Average DMI was 28.9 ± 5, 28.7 ± 5, and 28.9 ± 4 kg/d for BY, CTR, and SOL, respectively, while mean milk yield was 45.8 ± 4, 44.0 ± 8, and 43.2 ± 5 kg/d. Average BW was 659 ± 38, 652 ± 42, and 657 ± 45 kg, respectively. The quantitative results (Table 1) are consistent with previous chromatographic studies validating urinary PD determination in dairy cows, including the RP-HPLC method developed by Visentin et al. (2026). The UHPLC-HRMS Orbitrap platform enabled accurate PD quantification and simultaneous targeted and untargeted profiling. Urinary creatinine, uric acid, and allantoin were numerically highest in BY, reaching 196.3, 507.3, and 125.6 mg/L, respectively. The urinary PDC index ranged from approximately 800 to 1400, with values of 1375, 973, and 845 for CTR, BY, and SOL, respectively (Table 1). However, urinary creatinine, uric acid, allantoin, and PDC index were not affected by dietary treatment or experimental period ($p > 0.05$). This agrees with previous evidence showing that changes in the RUP ratio do not necessarily affect urinary PDC index or microbial protein synthesis (Kand and Dickhoefer, 2021). Moreover, endogenous purine metabolism, incomplete urinary recovery, and animal variability may obscure dietary effects when differences among diets are moderate (Firkins et al. 2007; Del Valle et al. 2023). Compared with urine, milk showed lower variability in PD concentrations. When expressed as daily secretion, milk creatinine and uric acid were unaffected by treatment ($p > 0.05$) but differed among experimental periods ($p < 0.05$), whereas milk allantoin secretion was not affected by diet (Table 1). Conversely, milk allantoin concentration (mg/L) differed among treatments ($p = 0.029$), with higher values in SOL than CTR ($p = 0.036$), while BY did not differ from either treatment. This discrepancy may reflect differences in milk yield rather than total allantoin output. The increase in milk allantoin concentration under SOL may therefore indicate changes in purine metabolism or mammary allantoin handling rather than increased microbial protein synthesis. Milk PD account for less than 2% of total purine derivative excretion (Gonda and Lindberg, 1997); therefore, their response should be interpreted cautiously as an indicator of microbial protein supply (Carro et al. 2012; Errico et al. 2026). In addition, milk uric acid may partly originate from purine catabolism within mammary tissues rather than directly reflecting systemic purine metabolism (Stentoft et al. 2015). The absence of correlation between milk and urinary PD further suggests that the two biofluids capture distinct aspects of nitrogen metabolism. Urinary allantoin and uric acid were strongly correlated within all dietary groups: CTR ($r = 0.78$; $p = 0.013$), SOL ($r = 0.97$; $p < 0.001$), and BY ($r = 0.78$; $p = 0.014$) (Figure 1). These associations confirm the coordinated urinary excretion of purine degradation products originating from microbial nucleic acids. Conversely, no significant relationships were observed among milk PD or between milk and urinary PD. Milk allantoin and uric acid were weakly correlated ($r = -0.17$, $p = 0.404$), while milk allantoin and urinary allantoin were unrelated ($r = -0.04$, $p = 0.850$). The urinary PDC index was not affected by diet ($p = 0.327$) or period ($p = 0.676$), and linear mixed-effects models showed no significant association between urinary PDC index and milk PD.

Untargeted UHPLC-HRMS analysis identified 198 urinary and 147 milk metabolites, with the complete dataset reported in the supplemental material. The two metabolomes were mainly characterised by amino

Table 1. Quantitative analysis of creatinine, uric acid, and allantoin in urine and milk.

Urine	CTR[1]	BY[2]	SOL[3]
Creatinine, mg/L	156 ± 93	196 ± 62	168 ± 62
Uric acid, mg/L	459 ± 252	507 ± 125	358 ± 203
Allantoin, mg/L	1374 ± 487	1398 ± 239	1205 ± 559
PDC index	1375 ± 1065	973 ± 352	845 ± 250
Milk	**CTR**	**BY**	**SOL**
Creatinine, mg/d*	18.8 ± 6.3	19.6 ± 4.6	17.0 ± 5.3
Uric acid, mg/d*	56.7 ± 34.4	55.5 ± 25.4	55.2 ± 31.8
Allantoin, mg/d	27.4 ± 3.3	29.8 ± 3.1	31.1 ± 5.2

Results are reported as mean ± standard deviation ($n = 9$). Milk metabolites are expressed as daily excretion (mg/d), whereas urinary metabolites are expressed as concentrations (mg/L), as urine samples were collected as spot samples. Urinary PDC index was calculated from as per Chen et al. (2004).

[1]The CTR diet consisted of a standard TMR supplemented with soybean meal, providing 16.5% CP and an RDP:RUP ratio of (64:36).

[2]The BY diet consisted of a TMR supplemented with soybean meal and conventional soybean meal, providing 14.3% CP and a lower RDP:RUP ratio (57:43).

[3]The SOL diet included rapidly degradable protein sources, mainly decorticated sunflower meal, and was formulated to be isonitrogenous with CTR but characterised by a higher RDP:RUP ratio (67:33).

* = significantly ($p < 0.05$) differing as a function of period.

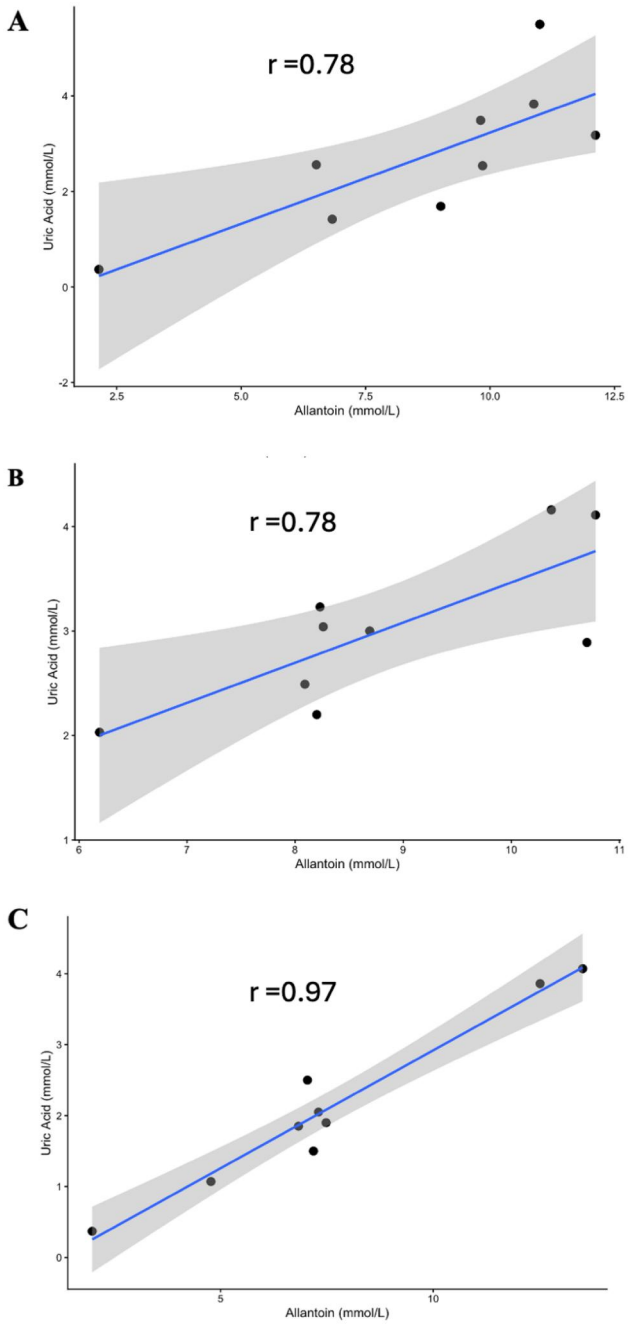


Figure 1. Correlations between urinary allantoin and uric acid (expressed as millimolar concentrations) under different treatments, namely (A) CTR, (B) BY, and (C) SOL. The blue lines show the linear regression with 95% confidence interval.

acid derivatives, peptides, carboxylic acids, fatty acids, and nucleotide-related metabolites. DIABLO data fusion revealed a 69% correlation between the first latent components of milk and urine (Figure 2(A)), indicating partially coordinated metabolic responses while retaining matrix-specific information (Figure 2(B)). The integrated analysis therefore provided additional information beyond conventional PDC biomarkers. In milk, the features contributing most to the discrimination among dietary treatment groups were cinnamic acid (VIP = 7.543), N,N-dimethylformamide (VIP = 2.329), malic acid (VIP = 1.929), urea (VIP = 1.010), and uracil (VIP = 0.760). Their log_2FC values (SOL vs CTR; BY vs CTR) were −0.459/1.429, 0.402/−0.349, −0.071/−0.440, 0.431/−0.516, and 0.055/−0.307, respectively. Cinnamic acid is a phenylpropanoid derived from plant secondary metabolites and microbial transformation of dietary phenolics in the rumen (Rocchetti et al. 2022a; Ahsin et al. 2025). Since corn meal and corn silage were supplied at identical amounts among treatments, the greater contribution of cinnamic acid to the discrimination among dietary treatment groups in the BY diet may indicate effects of dietary protein sources on ruminal microbial metabolism and phenolic intermediate release. Malic acid, a TCA-cycle intermediate involved in energy metabolism and ruminal fermentation, may reflect changes in microbial or host metabolic fluxes associated with nitrogen availability (Bach et al. 2005; Zhou et al. 2024). Milk urea contributed more strongly to the discrimination of the SOL diet, consistent with its role in systemic nitrogen metabolism and nitrogen utilisation and with the greater RDP supply. (Zhao et al. 2025). Uracil may reflect microbial nucleic acid turnover (Rocchetti et al. 2022b), whereas N,N-dimethylformamide may indicate microbial or host transformations of nitrogen-containing compounds associated with dietary protein sources (Eom et al. 2021).

In urine, the main features contributing to the discrimination among dietary treatment groups were bis-(acetyl)diaminopentane (VIP = 4.871), N6-benzyl-7H-purine-2,6–diamine (VIP = 4.588), N-acetyl-leucine (VIP = 3.868), N-acetyl-L-tryptophan (VIP = 2.958), and sebacic acid (VIP = 0.737), with log_2FC values (SOL vs CTR; BY vs CTR) of −0.853/1.380, −0.494/2.816, −0.034/2.539, −1.246/0.603, and −0.873/1.665, respectively. Several of these metabolites are related to amino acid metabolism and nitrogen turnover. N-acetylated amino acids have been implicated in primary and alternative metabolic pathways and urea-cycle regulation (Jacobs et al. 2023), and their higher abundance in BY may indicate increased amino acid catabolism and hepatic processing associated with greater RUP supply. Bis-(acetyl)diaminopentane, related to polyamine and lysine degradation, may reflect altered microbial growth and nitrogen recycling (Krysenko and Wohlleben 2022), while N6-benzyl-7H-purine-2,6–diamine suggests a contribution of nucleotide metabolism to nitrogen handling beyond conventional PD (Guna et al. 2024). Sebacic acid, a product of fatty acid ω-oxidation, may reflect interactions between energy

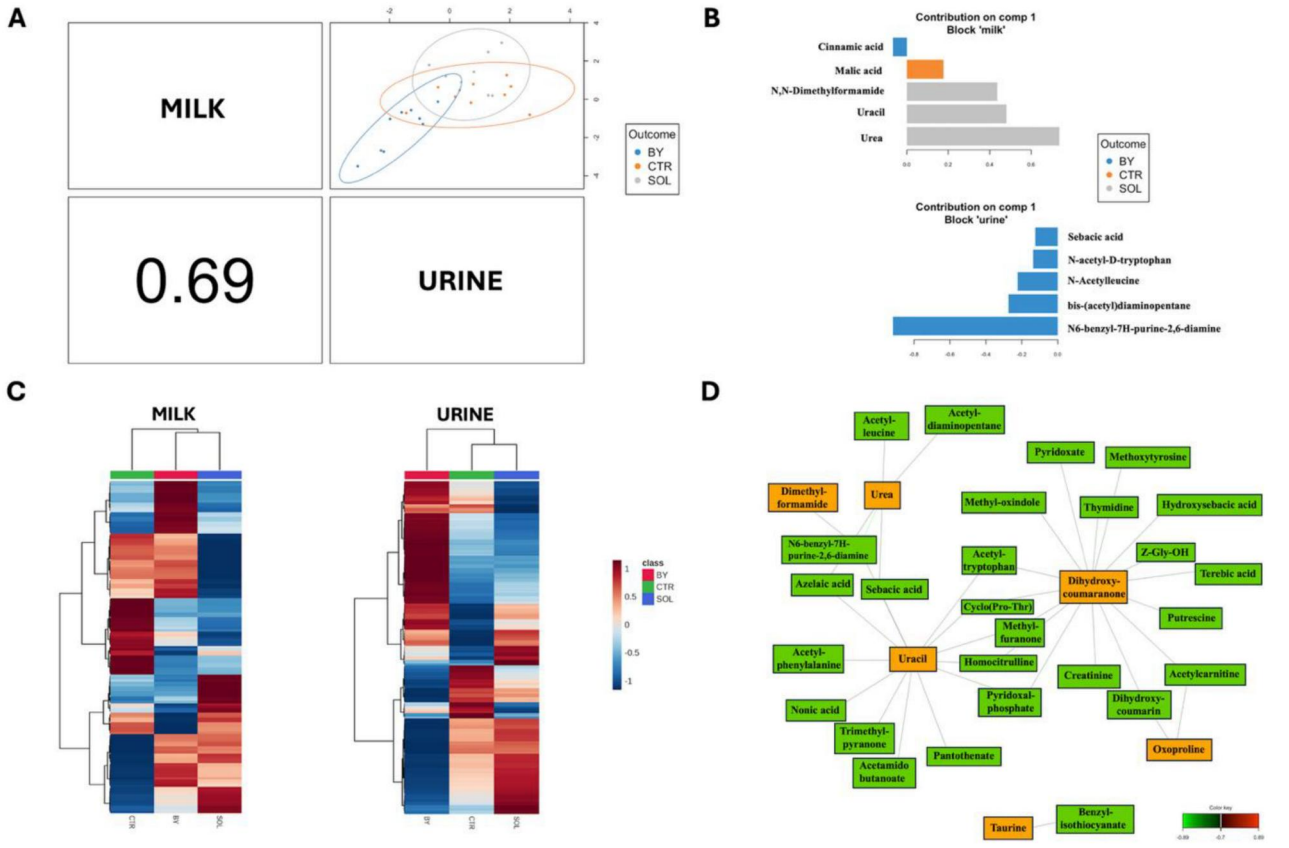


Figure 2. Results of the DIABLO multiblock integration analysis. (A) Correlation between the first latent components extracted from milk and urine UHPLC-HRMS datasets. (B) Loading plots showing the variables with the highest contribution to component 1 in each block; variables with larger absolute loading values contribute more strongly to treatment discrimination. (C) Hierarchical clustering heatmaps of milk and urine chemical profiles. (D) Correlation network generated from variables selected by the DIABLO model, displaying associations with pearson correlation coefficients greater than |0.7|. orange nodes represent milk metabolites and green nodes represent urine metabolites.

metabolism and amino acid utilisation (Artegoitia et al. 2017).

Hierarchical clustering supported these patterns (Figure 2(C)). CTR milk samples showed a distinct metabolic profile compared with SOL and BY, whereas BY samples were more clearly differentiated in urine, suggesting stronger metabolic signatures related to amino acid and nitrogen metabolism under higher RUP supply. The DIABLO correlation network also revealed several cross-matrix associations, with uracil and dihydroxy-coumaranone showing the strongest relationships with urinary metabolites (Pearson $r < -0.7$; Figure 2(D)). Uracil is linked to nucleotide metabolism and microbial nucleic acid turnover, whereas benzofuran derivatives may derive from microbial degradation of plant phenolics. Milk amino acid profiles (supplemental material) showed a slight increase in BY compared with CTR (cumulative $\log_2FC = 0.07$), whereas SOL showed a decreasing trend (cumulative $\log_2FC = -0.06$). Although these differences were not significant ($p > 0.05$), BY showed higher abundance of several NEAA, including glycine, glutamic acid, arginine, and proline, which are involved in nitrogen metabolism and transamination pathways (Phang et al. 2013). Their enrichment may reflect increased mammary amino acid turnover and utilisation following greater post-ruminal amino acid supply from RUP. However, targeted and quantitative analyses on amino acids are required to confirm these trends.

Overall, urinary PD maintained the expected physiological relationships but did not respond to dietary treatment, and the PDC index was not associated with milk PD under the present experimental conditions. In contrast, multi-matrix profiling based on UHPLC-HRMS revealed coordinated metabolic responses associated with dietary protein degradability and highlighted metabolites related to amino acid and nitrogen metabolism. Combining targeted biomarkers with untargeted screening may therefore improve the characterisation of nitrogen partitioning and metabolic adaptation in dairy cows, although larger studies are needed to validate potential indicators of nitrogen use efficiency.

Acknowledgments

The authors acknowledge the NODES ('Nord Ovest Digitale e Sostenibile') project, which has received funding from the MUR–M4C2 1.5 of The National Recovery and Resilience Plan with grant agreement n. ECS00000036. This study was supported by the PhD in Agro-Food System (Agrisystem) and by Portus project funded by Romeo ed Enrica Invernizzi Foundation.

Author contributions

CRediT: **Michela Errico**: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft; **Alessandro Catellani**: Conceptualization, Investigation, Methodology, Writing – original draft; **Federico Froldi**: Investigation, Supervision, Writing – review & editing; **Maurizio Moschini**: Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing; **Gabriele Rocchetti**: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing; **Antonio Gallo**: Conceptualization, Investigation, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Data availability statement

The supplementary data that support the findings of this study are openly available in Digital Commons Data at reserved doi: 10.17632/3hsf54z8xs.1.

Supplemental material

Excel file containing the following sheets: a) urine metabolites annotated through untargeted UHPLC-HRMS approach; b) milk metabolites annotated through untargeted UHPLC-HRMS approach; c) relationship between urinary allantoin and uric acid concentrations (mmol/L) independent from Treatment. The supplementary material for this publication has been deposited on a public repository to the following DOI:10.17632/3hsf54z8xs.1.

References

Artegoitia VM et al. 2017. Rumen fluid metabolomics analysis associated with feed efficiency on crossbred steers. Sci Rep. 7(1):2864. https://doi.org/10.1038/s41598-017-02856-0

Ahsin M et al. 2025. Phenolic compounds and derivatives in ruminant meat and milk: a systematic review. J Agric Food Chem. 73(47):29961–29982. https://doi.org/10.1021/acs.jafc.5c06118

Bach A, Calsamiglia S, Stern MD. 2005. Nitrogen metabolism in the rumen. J Dairy Sci. 88(Suppl 1S):E9–21. https://doi.org/10.3168/JDS.S0022-0302(05)73133-7

Brito AF, Broderick GA, Reynal SM. 2007. Effects of different protein supplements on omasal nutrient flow and microbial protein synthesis in lactating dairy cows. J Dairy Sci. 90(4):1828–1841. https://doi.org/10.3168/jds.2006-559

Broderick GA, Reynal SM. 2009. Effect of source of rumen-degraded protein on production and ruminal metabolism in lactating dairy cows. J Dairy Sci. 92(6):2822–2834. https://doi.org/10.3168/jds.2008-1865

Carro MD, Cantalapiedra-Hijar G, Ranilla MJ, Molina-Alcaide E. 2012. Urinary excretion of purine derivatives, microbial protein synthesis, nitrogen use, and ruminal fermentation in sheep and goats fed diets of different quality. J Anim Sci. 90(11):3963–3972. https://doi.org/10.2527/jas.2011-4577

Chen XB, Chen YK, Franklin MF, Orskov ER, Shand WJ. 1992. The effect of feed intake and body weight on purine derivative excretion and microbial protein supply in sheep. J Anim Sci. 70(5):1534–1542. https://doi.org/10.2527/1992.7051534x

Chen XB, Jayasuriya MCN, Makkar HPS. 2004. Measurement and application of purine derivatives:creatinine ratio in spot urine samples of ruminants. In: Makkar, H.P.S., Chen, X.B. (eds), Estimation of microbial protein supply in ruminants using urinary purine derivatives. Springer. https://doi.org/10.1007/978-1-4020-2844-1_20

Del Valle TA et al. 2023. Purine derivatives and creatinine urine excretion as a tool to estimate sheep feed intake. Anim Feed Sci Technol. 301:115666. https://doi.org/10.1016/j.anifeedsci.2023.115666

Dórea JRR, Danés MAC, Zanton MGI, Armentano LE. 2017. Urinary purine derivatives as a tool to estimate dry matter intake in cattle: a meta-analysis. J Dairy Sci. 100(11):8977–8994. https://doi.org/10.3168/jds.2017-12908

Eom J-S et al. 2021. Metabolic profiling of rumen fluid and milk in lactating dairy cattle influenced by subclinical ketosis using proton nuclear magnetic resonance spectroscopy. Animals (Basel). 11(9):2526. https://doi.org/10.3390/ani11092526

Errico M et al. 2026. UHPLC-HRMS-based screening of purine and pyrimidine metabolites in cow milk collected across four different seasons. JDS Comm. 7(4):446–451. https://doi.org/10.3168/JDSC.2025-0924

Firkins JL, Yu Z, Morrison M. 2007. Ruminal nitrogen metabolism: perspectives for integration of microbiology and nutrition for dairy. J Dairy Sci. 90(Suppl 1S):E1–16. https://doi.org/10.3168/jds.2006-518

Giagnoni G et al. 2025. Relationship between pyrimidines, purines, and fatty acids in milk of dairy cows fed distinct carbohydrate types: a metabolomic approach. JDS Commun. 6(1):24–28. https://doi.org/10.3168/JDSC.2024-0612

Gonda HL, Lindberg JE. 1997. Effect of diet on milk allantoin and its relationship with urinary allantoin in dairy cows. J Dairy Sci. 80(2):364–373. https://doi.org/10.3168/jds.S0022-0302(97)75946-0

Guna K et al. 2024. An experimental and computational analysis on 2,6-diamine-7H-purine ligand with spectroscopic, AIM, NLO and biological activity. Opt Laser Technol. 168:109872. https://doi.org/10.1016/j.optlastec.2023.109872

Hristov AN et al. 2019. Invited review: nitrogen in ruminant nutrition: a review of measurement techniques. J Dairy Sci. 102(7):5811–5852. https://doi.org/10.3168/jds.2018-15829

Jacobs J et al. 2023. Non-enzymatic formation of N-acetylated amino acid conjugates in urine. Appl Sci. 13(18): 10002. https://doi.org/10.3390/app131810002

Kand D, Dickhoefer U. 2021. The effects of rumen nitrogen balance on nutrient intake, nitrogen partitioning, and microbial protein synthesis in lactating dairy cows offered different dietary protein sources. J Dairy Sci. 104(4):4223–4235. https://doi.org/10.3168/JDS.2019-18005

Krysenko S, Wohlleben W. 2022. Polyamine and ethanolamine metabolism in bacteria as an important component of nitrogen assimilation for survival and pathogenicity. Med Sci (Basel). 10(3):40. https://doi.org/10.3390/medsci10030040

Kwon W, Kim JY, Suh SI, In MK. 2012. Simultaneous determination of creatinine and uric acid in urine by liquid chromatography-tandem mass spectrometry with polarity switching electrospray ionization. Forensic Sci Int. 221(1-3):57–64. https://doi.org/10.1016/j.forsciint.2012.03.025

Matuszewski BK, Constanzer ML, Chavez-Eng CM. 2003. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. Anal Chem. 75(13):3019–3030. https://doi.org/10.1021/ac020361s

Phang JM, Liu W, Hancock C. 2013. Bridging epigenetics and metabolism: role of non-essential amino acids. Epigenetics. 8(3):231–236. https://doi.org/10.4161/epi.24042

Pereira TCdeJ et al. 2022. Creatinine as a urinary marker of the purine derivatives excretion in urine spot samples of lambs fed peach palm meal. Animals (Basel). 12(9):1195. https://doi.org/10.3390/ani12091195

Putri EM, Zain M, Warly L, Hermon H. 2021. Effects of rumen-degradable-to-undegradable protein ratio in ruminant diet on in vitro digestibility, rumen fermentation, and microbial protein synthesis. Vet World. 14(3):640–648. https://doi.org/10.14202/VETWORLD.2021.640-648

Rocchetti G, Ghilardelli F, Mosconi M, Masoero F, Gallo A. 2022a. Occurrence of polyphenols, isoflavonoids, and their metabolites in milk samples from different cow feeding regimens. Dairy. 3(2):314–325. https://doi.org/10.3390/dairy3020024

Rocchetti G et al. 2022b. Milk metabolome reveals pyrimidine and its degradation products as the discriminant markers of different corn silage-based nutritional strategies. J Dairy Sci. 105(11):8650–8663. https://doi.org/10.3168/jds.2022-21903

Rocchetti G, Gallo A. 2026. F3-metabolomics: integrating feed, fluid, and food metabolomics in dairy production. Anim Feed Sci Technol. 334:116653. https://doi.org/10.1016/j.anifeedsci.2026.116653

Santos FA, Santos JE, Theurer CB, Huber JT. 1998. Effects of rumen-undegradable protein on dairy cow performance: a 12-year literature review. J Dairy Sci. 81(12):3182–3213. https://doi.org/10.3168/jds.S0022-0302(98)75884-9

Savari M et al. 2018. Effects of rumen-degradable protein: rumen-undegradable protein ratio and corn processing on production performance, nitrogen efficiency, and feeding behavior of Holstein dairy cows. J Dairy Sci. 101(2):1111–1122. https://doi.org/10.3168/JDS.2017-12776

Stentoft C et al. 2015. Absorption and intermediary metabolism of purines and pyrimidines in lactating dairy cows. Br J Nutr. 113(4):560–573. https://doi.org/10.1017/S0007114514004000

Tamminga S. 1992. Nutrition management of dairy cows as a contribution to pollution control. J Dairy Sci. 75(1):345–357. https://doi.org/10.3168/JDS.S0022-0302(92)77770-4

Van Amburgh ME et al. 2015. The cornell net carbohydrate and protein system: updates to the model and evaluation of version 6.5. J Dairy Sci. 98(9):6361–6380. https://doi.org/10.3168/jds.2015-9378

Visentin E et al. 2026. Validation of a reversed-phase high-performance liquid chromatography method for quantification of allantoin, creatinine, and uric acid in individual spot cow urine samples. JDS Commun. 7(1):113–118. https://doi.org/10.3168/jdsc.2025-0855

Zhao X et al. 2025. Assessing milk urea nitrogen as an indicator of protein nutrition and nitrogen utilization efficiency: a meta-analysis. J Dairy Sci. 108(5):4851–4862. https://doi.org/10.3168/jds.2024-25656

Zhou X et al. 2024. Enteric methane emissions, rumen fermentation, and milk composition of dairy cows fed 3-nitrooxypropanol and L-malate supplements. Front Vet Sci. 11: 1479535. https://doi.org/10.3389/fvets.2024.1479535

Zuo R, Zhou S, Zuo Y, Deng Y. 2015. Determination of creatinine, uric and ascorbic acid in bovine milk and orange juice by hydrophilic interaction HPLC. Food Chem. 182: 242–245. https://doi.org/10.1016/J.FOODCHEM.2015.02