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Title	The MetaBó-Bainne Study – characterisation of the milk metabolome from a seasonal pasture-based dairy system using 1H-NMR spectroscopy
Authors	Rojas-Gómez, Paula;Pariyani, Raghunath;Dineen, Michael;Lynch, Denis;Bateman, Lorraine;Roche, Eoghan;Maguire, Anita R;McCarthy, Noel A;Murphy, Thomas Brendan;O'Mahony, James A;O'Callaghan, Tom F
Publication date	2026-07-30
Original Citation	Rojas-Gómez, P, Pariyani, R, Dineen, M, Lynch, D, Bateman, L, Roche, E, Maguire, A R, McCarthy, N A, Murphy, T B, O'Mahony, J A & O'Callaghan, T F 2026, 'The MetaBó-Bainne Study – characterisation of the milk metabolome from a seasonal pasture-based dairy system using 1H-NMR spectroscopy', Food Chemistry, vol. 518, 149492, pp. 1-13. https://doi.org/10.1016/j.foodchem.2026.149492
Type of publication	Article (peer-reviewed)
Link to publisher's version	https://www.scopus.com/pages/publications/105038839447 - 10.1016/j.foodchem.2026.149492
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Download date	2026-11-06 17:43:26
Item downloaded from	https://hdl.handle.net/10468/19562



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Food Chemistry 518 (2026) 149492

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

The MetaBó-Bainne Study – characterisation of the milk metabolome from a seasonal pasture-based dairy system using ^{1}H-NMR spectroscopy

Paula Rojas-Gómez [a], Raghunath Pariyani [a], Michael Dineen [d], Denis Lynch [b], Lorraine Bateman [b,c], Eoghan Roche [d], Anita R. Maguire [b,c], Noel A. McCarthy [e], Thomas Brendan Murphy [f], James A. O'Mahony [a], Tom F. O'Callaghan [a,*]

[a] School of Food and Nutritional Sciences, University College Cork, Cork, T12Y337, Ireland
[b] School of Chemistry, Analytical and Biological Chemistry Research Facility, University College Cork, Cork T12 K8AF, Ireland
[c] School of Pharmacy, University College Cork, Cork T12 K8AF, Ireland
[d] Teagasc Animal and Grassland Research and Innovation Centre, Moorepark, Fermoy, Co. Cork P61 P302, Ireland
[e] Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork P61 C996, Ireland
[f] School of Mathematics & Statistics, University College Dublin, D04 V1W8 Dublin, Ireland

ARTICLE INFO

Keywords:
milk
dairy
metabolomics
seasonality
lactation
biomarkers

ABSTRACT

This study aims to characterise the milk metabolome from a seasonal pasture-based dairy system using ^{1}H-NMR spectroscopy. Over 41 weeks, ten dairy farms were visited weekly for the collection of raw bulk tank milk samples (n = 410) and three commercial pasteurised skimmed milks were also purchased weekly (n = 123). In total, 38 milk metabolites were quantified, 30 of which exhibited significant seasonal variation. Multivariate analysis identified several key compounds associated with seasonal metabolic changes. Winter-Feb milk, corresponding to early-lactation period, was enriched in ketone bodies, O-phosphocholine, creatinine, and glucose-1-phosphate, reflecting increased metabolic stress and negative energy balance following parturition. In contrast, autumn milk, corresponding to late-lactation, contained higher concentrations of choline and urea, indicative of improved energy status but reduced nitrogen use efficiency. These findings highlight the potential of milk metabolomics as a valuable tool for monitoring physiological status and guiding interventions to enhance sustainability in dairy systems.

1. Introduction

Cow's milk is a nutritionally rich food composed of a complex mixture of macronutrients, micronutrients, and a wide array of low molecular weight compounds (Fox et al., 2015). Milk, consumed globally, forms the basis for a plethora of nutritional and functional ingredients and its composition is strongly influenced by various intrinsic and extrinsic factors that affect milk quality and processability, such as breed, genetics, diet, seasonality, age, number of calvings, and overall health status (Rojas-Gómez, Pariyani, McCarthy, et al., 2025). These factors collectively influence not only the nutritional profile of milk but also its techno-functional properties, such as gelation and heat stability (Zhu et al., 2021). Among these factors, seasonality, stage of lactation (SOL), and diet emerge as particularly important determinants of milk composition and overall productivity in pasture-based spring-calving dairy systems. In temperate regions such as Ireland and New Zealand, pasture-based spring-calving systems are widely adopted due to their favourable climatic conditions for pasture growth, including mild temperatures and abundant rainfall. Calving in spring ensures that lactation and dietary demands coincide with the availability of high-quality grazed pasture, enhancing the nutritional value of milk and improving the cost efficiency of production (Moscovici Joubran et al., 2021). Seasonal dairy systems, however, are characterised by substantial variation in feed composition throughout the year. Combined with physiological changes associated with the synchronized SOL at a national level, this variability induces pronounced shifts in milk composition throughout the year (Timlin et al., 2021). The seasonal modulation of macronutrients and micronutrients in milk, including lipids, amino acids, sugars, vitamins, and minerals, has been well documented (Li et al., 2019; Nogalska et al., 2020; Timlin et al., 2021). More recently, the focus has extended to the milk metabolome, which comprises low molecular weight compounds (<1500 Da) that are intermediates or by-

* Corresponding author
E-mail address: tom_ocallaghan@ucc.ie (T.F. O'Callaghan).

https://doi.org/10.1016/j.foodchem.2026.149492
Received 16 October 2025; Received in revised form 13 April 2026; Accepted 2 May 2026
Available online 3 May 2026
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products of metabolic pathways, including sugars, amino acids, lipids, vitamins, hormones, and other bioactive molecules (Wishart, 2019). Understanding how these metabolites vary across lactation is of growing interest, as they can serve as biomarkers of milk processability, sustainability and animal health, reflecting physiological states such as negative energy balance, ketosis, metabolic stress, and nitrogen utilisation efficiency (Chen et al., 2024; Klein et al., 2012; Pereira et al., 2025).

Advances in analytical technologies, particularly proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy, have enabled the targeted and untargeted quantification of a broad range of milk metabolites with minimal sample preparation (Sundekilde et al., 2013). Although less sensitive than mass spectrometry, NMR is highly reproducible, both qualitative and quantitative, and well-suited for comprehensive metabolomics in dairy research. However, its high cost and limited availability compared to other analytical techniques can restrict its application to some research settings, where it serves as an exploratory tool rather than routine diagnostics (Balthazar et al., 2021; Wishart et al., 2022).

Several studies have applied metabolomics approaches, including ^{1}H-NMR spectroscopy, to investigate seasonal fluctuations influencing the bovine milk metabolome. For example, Zhu et al. (2020a) used a combination of ^{1}H-NMR and ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC-QToF/MS) to study the milk metabolome, reporting variations throughout lactation. Similarly, Connolly et al. (2023) analysed the effect of SOL on the milk metabolome using ^{1}H-NMR and liquid chromatography–tandem mass spectrometry (LC-MS/MS), identifying numerous metabolites whose concentrations varied throughout lactation. These studies provide important insights into the milk metabolome; however, existing research is constrained in offering a robust and broad dataset by limited sample size, diversity of sources, and lactation timeline. To address these limitations, the MetaBó-Bainne study examined seasonal variations in the milk metabolome, generating a large and robust dataset from across the full lactation cycle and encompassing both bulk tank milk from spring-calving systems (Farm) and commercially source pasteurized milk (Shop).

The objectives of this study were to examine the milk metabolome as affected by month, season and milk source (Farm vs. Shop). This work aims to characterise the seasonality variations, which coincide with stage of lactation in spring calving dairy systems, and investigate metabolic pathways influenced by seasonal and physiological variation. In pasture-based spring calving systems, seasonal changes are closely aligned with progression through SOL (Timlin et al., 2021); therefore, seasonality differences in the milk metabolome were interpreted in the context of the physiological changes occurring across lactation. Furthermore, data generated in this study will form part of the larger MetaBó-Bainne database (www.metabobainne.ie), an open access database that explores the factors affecting and affected by the milk metabolome in pasture based dairy systems.

2. Materials & Methods

2.1. Experimental design

Ten commercial dairy farms in the south of Ireland were visited weekly for a period of 41 weeks from February to November across 2023. Farms selected for inclusion in this study were operating a spring-calving, pasture-based dairy system. Farmers, who voluntarily contributed to the study, routinely recorded production and performance metrics for their herd using PastureBase Ireland (Hanrahan et al., 2017), which included grass growth (kg DM/ha/day), milk yield (L/cow/day), stocking rate (cows/ha), concentrates fed (kg/cow), and silage intake (kg/cow). Milk yield data were reported less consistently, with records available for approximately 50% of farms in February and 70% during the rest of the year. Cow breeds used for this study consisted of both Holstein Friesian and Holstein Friesian × Jersey crossbred cows, typical of Irish farming systems. Three commercial brands of milk were also selected at the outset of the study that represented milk originating from a much larger regional supply across the country. Skimmed milk samples were purchased from these brands on a weekly basis for the duration of the study. All milk samples were collected on Mondays. During each visit, a bulk tank milk sample representing morning and evening milkings was collected. Prior to sampling, the milk was thoroughly agitated. The samples were transported on ice and subsequently stored at 4 °C until analysis. A total of 533 milk samples were analysed in this study, comprising 410 samples of Farm milk and 123 samples of Shop milk. For the purposes of this study, samples were grouped into seasonal categories based on the sampling calendar as follows: winter (February), spring (March–May), summer (June–August), and autumn (September–November). In Irish spring-calving pasture-based systems, these seasonal groupings broadly correspond to progression through lactation. Due to the use of bulk tank Farm and Shop milk samples, information on an individual cow basis such as days in milk (DIM) and parity was not available. Consequently, the metabolomic analysis reflects herd level variation rather than individual animal characteristics.

Ethical approval was not required for this study, as Farm milk samples were collected directly from farmers' bulk tanks through routine collection procedures that did not involve any form of interaction or intervention with animals beyond standard and existing normal routine farming and milking practices.

2.2. Sample preparation and analysis of metabolome via proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy

Sample preparation was performed in accordance with protocols previously followed by Rojas-Gómez, Pariyani, Bateman, et al. (2025). The procedure utilised 3 kDa filters (Amicon Ultra-0.5 centrifugal filters, Millipore, Ireland), as recommended by the Chenomx guidelines (Chenomx, 2021). The samples were processed using a Micro Star 30R centrifuge (VWR International, USA). First, the 3 kDa filters were thoroughly washed by passing 0.5 mL of ultrapure water (18.2 MΩ cm) through the filters at 9,610 × g for 10 minutes at room temperature, which was repeated five times. Milk samples were processed by centrifugation of 2 mL aliquots at 2,400 × g for 15 minutes at 4 °C to separate the cream from the skimmed milk. A volume of 500 µL of skimmed milk was transferred into pre-washed centrifugal filters and centrifuged at 11,630 × g for 35 minutes at 4 °C to eliminate macromolecules that could interfere with low molecular weight metabolite signals in the NMR spectra. The resulting permeate was collected in 1.5 mL microcentrifuge tubes. The filtration step was repeated to obtain a sufficient volume of permeate for ^{1}H-NMR analysis. A volume of 630 µl of permeate was combined with 70 µl of Chenomx Internal Standard solution (Chenomx, Edmonton, Alberta, Canada), containing 5 mM of 2,2-dimethyl-2-silapentane-5-sulfonate-d6sodium salt (DSS-d_6), and transferred into 5 mm Ø NMR tubes. Spectra were acquired using a Bruker AVANCE III 600 MHz spectrometer equipped with a 5 mm Triple Channel Inverse (TCI) CryoProbe, with samples maintained at 298 K. One-dimensional ^{1}H-NMR spectra were obtained using the noesypr1d pulse sequence, with the following acquisition parameters: spectral width of 14 ppm, 64,000 data points, a relaxation delay (D1) of 5 seconds, and 128 scans.

2.3. ^{1}H-NMR spectral preprocessing and metabolite quantification

Chenomx NMR Suite V10.1 (Chenomx Inc., Edmonton, AB, Canada) was used for detailed spectral analysis, encompassing two main stages: processing and profiling, which involved the identification and

quantification of metabolites. The software enabled essential spectral processing, including adjustments for phase, baseline, shim, and line broadening, with the line broadening factor set at 0.30 Hz. The methyl singlet of DSS-d_6 was calibrated to 0 ppm, serving both as the internal reference for chemical shift calibration and as a standard for quantification. Following spectral processing, metabolite profiling was performed to identify and quantify compounds based on their unique spectral signatures and chemical shifts, expressed in parts per million (ppm). The profiling was conducted using the Profiler module in conjunction with the Chenomx compound library. To ensure accuracy and reduce the potential for misidentification or quantification errors, each spectrum was independently reviewed by two scientists experienced in NMR spectroscopy.

2.4. Statistical analysis and representation of data

Statistical analyses and data visualization were conducted using R (version 4.3.3) within the RStudio environment (version 2023.12.1+402). To evaluate the influence of seasonality on the milk metabolome, a linear mixed effects model was developed using the lme4 package. For each metabolite, a model was constructed "Season", "Milk Source", and their interaction ("Season*Milk Source") specified as fixed effects. For “Season”, months were grouped as follows: winter = Feb; spring = March, April, May; Summer = June, July, August; autumn = September, October, November. Only February samples were collected to represent the winter period, as is normal practise in these spring-calving dairy systems, where cows are dried off and maintained indoors in the latter part of the year prior to calving. For clarity, this is denoted as “winter-Feb” throughout. To account for potential variability across time and farm origin, "Calendar Week" and "Farm/Company" were included as random effects. The distribution of model residuals was evaluated through visual inspection of Q-Qplots. Log transformation was applied when deviations from normality were evident in the Q-Q plots. Post hoc pairwise comparisons for significant interactions ("Season", "Milk Source ", "Season*Milk Source") were performed using the emmeans package. Differences were considered statistically significant at $p < 0.05$. Visual representations, including seasonal and Milk Source line graphs, were generated using ggplot2. Both seasonal and monthly interpretation of the trends observed in this data are deemed important; however, it was not possible to combine both into a single model due to collinearity. Therefore, an equivalent modelling approach was applied to evaluate the effect of Month, substituting "Season" with "Month" as fixed effect. For each metabolite model, "Calendar Week" was specified as a random effect nested within Month (expressed as `1|Month:Calendar Week`), and primary "Source” (Farm/Company) was included as a crossed random intercept to account for between-source variability and similar post hoc analysis was carried out. Due to the exploratory and characterisation nature of this study, multiple test procedures were not performed beyond the use of Tukey’s honestly significant difference (HSD) within each metabolite profile. In models, only random intercepts were fitted; random slopes were not included due to limitations in data size and to maintain model parsimony and avoid convergence issues given the data structure. Model assumptions were checked through visual inspection of Q-Q plots. Log transformation was applied when deviations from normality were evident. Multivariate analysis was conducted using MetaboAnalyst 6.0 (www.metaboanalyst.ca) (Pang et al., 2024). Data were imported into the program, log-transformed, and auto-scaled. Subsequent analyses included partial least squares discriminant analysis (PLS-DA), variable importance in projection (VIP) scores, and biomarker evaluation using receiver operating characteristic (ROC) curves. Validation of PLS-DA was carried out by assessing the permutation test (2000 permutations), along with R^2 and Q^2 values. For ROC analysis, metabolites were classified according to their area under the ROC curve (AUC) values: 0.9–1.0 as excellent, 0.8–0.9 as good, 0.7–0.8 as moderate, 0.6–0.7 as poor, and 0.5–0.6 as very poor (Sun et al., 2020). For the ROC analysis, only farm samples were included to minimise the influence of extrinsic factors representing raw milk samples and ensure that observed differences reflected true seasonal variation under farm conditions. Therefore, this approach aided in excluding potential changes introduced by dairy processing such as pasteurisation. Furthermore, as anticipated, information on primary farm management practices (e.g. grass growth, milk yield, stocking rate, silage and concentrate intake, and calving system) was unavailable for shop milk, excluding these samples prevented the introduction of uncontrolled variability that could confound the biomarker analysis.

3. Results & Discussion

3.1. Grazing and feeding management practices

Farm records demonstrated clear variation across seasons in grass growth, milk yield, and feeding strategies, as shown in Table 1. Grass growth was lowest in winter-Feb and peaked in summer, in agreement with typical grass growth patterns for this region and dairy system. These findings align with those reported by Wingler and Hennessy (2016), who explained that low temperatures and limited light availability during winter and early spring limit photosynthesis and reduce grass growth. Milk yield peaked in spring, coinciding with early lactation and increased availability of high-quality pasture, and declined progressively into autumn, likely influenced by both advanced stage of lactation and changes in pasture quality. In spring-calving pasture-based systems, typically SOL of cows evolves in tandem with seasonal changes, therefore these variations reflect the combined influence of season, lactation, and feeding changes. This seasonal trend is well documented in pasture-based dairy herds, particularly in systems where calving is synchronized with the onset of spring grass growth to optimize milk production (O’Brien et al., 2015). Stocking rate increased from winter-Feb to spring and summer, in line with greater pasture growth and milder climatic conditions. Feeding strategies also varied across seasons. The use of supplemental feed concentrates was highest in winter-Feb and declined as the year progressed, reflecting the reduced reliance on supplementary feeds when pasture quality and availability improve. Silage supplementation followed a similar trend, with higher usage in winter when pasture is limited, minimal use in summer, and increased reliance again in autumn as pasture growth declines. These adjustments are essential for maintaining nutritional adequacy and supporting milk

Table 1
Seasonal variation in grass growth, milk yield, stocking rate, and concentrates and silage intake (mean ± standard deviation) across seasons.

	Winter-Feb[¥]	Spring[¥]	Summer[¥]	Autumn[¥]
Grass growth (kg DM/ha/day)	9.6 ± 5.8	47.7 ± 27.3	61.3 ± 19.3	38.8 ± 18.8
Milk yield (L/cow/day)	21.1 ± 6.3	25.4 ± 3.3	20.5 ± 2.4	13.7 ± 2.8
Stocking rate (cows/ha)	1.9 ± 0.8	3.4 ± 0.7	3.3 ± 0.4	2.6 ± 0.7
Concentrates (kg/cow)	3.5 ± 1.3	3.1 ± 1.3	2.6 ± 1.4	2.4 ± 0.8
Silage (kg/cow)	4.6 ± 3.1	1.2 ± 2.1	0.4 ± 1.4	3.7 ± 4.4

[¥] Lactation = Sample collection from February to November
Calendar Season criteria: Winter = February; Spring = March, April, May; Summer = June, July, August; Autumn = September, October, November

Table 2
Effect of seasonality on the milk metabolome concentrations (mean ± standard deviation (mM)) (n = 533) collected from farm (n = 10) and shop (n = 3) samples weekly (n = 41) across calendar seasons throughout lactation.

Metabolites*	Winter-Feb[¥]	Spring[¥]	Summer[¥]	Autumn[¥]	Mean lactation	*p*-value Season	*p*-value Milk Source	*p*-value Season-Milk Source
2-Oxoglutarate	0.153 ± 0.071[a]	0.145 ± 0.072[a]	0.149 ± 0.053[a]	0.173 ± 0.085[a]	0.154 ± 0.072	0.143	0.162	<0.001
3-Hydroxybutyrate	0.047 ± 0.024[b]	0.033 ± 0.018[a]	0.030 ± 0.010[a]	0.034 ± 0.016[a]	0.034 ± 0.017	0.003	0.297	0.901
Acetate	0.067 ± 0.043[b]	0.053 ± 0.037[ab]	0.048 ± 0.028[a]	0.062 ± 0.040[b]	0.055 ± 0.036	0.006	<0.001	0.599
Acetoacetate	0.025 ± 0.015[d]	0.016 ± 0.009[c]	0.011 ± 0.006[d]	0.009 ± 0.005[a]	0.014 ± 0.009	<0.001	0.754	0.165
Acetone	0.126 ± 0.110[c]	0.064 ± 0.059[b]	0.037 ± 0.020[a]	0.050 ± 0.060[a]	0.058 ± 0.063	<0.001	0.654	0.075
Alanine	0.049 ± 0.022[b]	0.041 ± 0.016[ab]	0.039 ± 0.013[ab]	0.037 ± 0.011[a]	0.040 ± 0.015	0.030	0.962	0.917
Betaine	0.180 ± 0.128[b]	0.088 ± 0.045[a]	0.082 ± 0.058[a]	0.128 ± 0.060[b]	0.106 ± 0.072	<0.001	0.718	0.021
Butyrate	0.081 ± 0.048[a]	0.067 ± 0.045[a]	0.078 ± 0.059[a]	0.076 ± 0.069[a]	0.074 ± 0.057	0.105	0.797	0.758
Caprate+Caprylate	0.033 ± 0.017[b]	0.024 ± 0.012[a]	0.028 ± 0.014[ab]	0.033 ± 0.022[b]	0.029 ± 0.017	0.002	0.597	0.024
Carnitine	0.085 ± 0.026[b]	0.067 ± 0.028[a]	0.066 ± 0.021[a]	0.088 ± 0.041[b]	0.074 ± 0.032	<0.001	0.419	0.138
Choline	0.255 ± 0.273[a]	0.368 ± 0.182[a]	0.606 ± 0.214[b]	0.742 ± 0.332[b]	0.533 ± 0.302	<0.001	0.023	<0.001
Citrate	10.24 ± 4.067[b]	7.38 ± 2.48[a]	7.344 ± 1.811[a]	6.628 ± 1.544[a]	7.445 ± 2.486	<0.001	0.004	0.326
Creatine	0.858 ± 0.372[a]	0.730 ± 0.326[a]	0.826 ± 0.299[a]	0.819 ± 0.282[a]	0.797 ± 0.314	0.063	0.716	0.092
Creatinine	0.239 ± 0.104[c]	0.142 ± 0.063[b]	0.092 ± 0.039[a]	0.086 ± 0.033[a]	0.120 ± 0.072	<0.001	0.724	0.362
Dimethyl sulfone	0.034 ± 0.020[a]	0.050 ± 0.036[a]	0.070 ± 0.040[b]	0.046 ± 0.026[a]	0.054 ± 0.036	<0.001	0.047	0.050
Formate	0.017 ± 0.009[a]	0.013 ± 0.008[a]	0.012 ± 0.005[a]	0.012 ± 0.006[a]	0.013 ± 0.007	0.071	0.022	0.862
Fucose	0.035 ± 0.017[b]	0.030 ± 0.015[b]	0.022 ± 0.008[a]	0.034 ± 0.015[b]	0.029 ± 0.015	<0.001	0.020	<0.001
Fumarate	0.040 ± 0.019[b]	0.033 ± 0.019[a]	0.032 ± 0.014[ab]	0.032 ± 0.018[a]	0.033 ± 0.017	0.040	0.355	0.203
Glucose-1-phosphate	0.262 ± 0.182[c]	0.069 ± 0.050[b]	0.028 ± 0.034[a]	0.033 ± 0.027[a]	0.067 ± 0.098	<0.001	0.387	<0.001
Glutamate	0.294 ± 0.127[c]	0.255 ± 0.102[bc]	0.224 ± 0.076[b]	0.159 ± 0.070[a]	0.223 ± 0.099	<0.001	0.523	0.118
Hippurate	0.221 ± 0.076[ab]	0.262 ± 0.097[b]	0.262 ± 0.081[b]	0.194 ± 0.071[a]	0.240 ± 0.089	<0.001	<0.001	0.001
Isoleucine	0.007 ± 0.005[b]	0.005 ± 0.002[a]	0.005 ± 0.002[ab]	0.005 ± 0.002[a]	0.005 ± 0.003	0.003	0.208	0.162
Lactate	0.071 ± 0.047[b]	0.049 ± 0.026[a]	0.054 ± 0.024[a]	0.069 ± 0.029[b]	0.058 ± 0.030	<0.001	0.006	<0.001
Lactose	138.6 ± 48.73[b]	129.3 ± 48.31[ab]	122.7 ± 39.99[ab]	110.2 ± 31.96[a]	122.9 ± 42.78	0.013	0.210	0.657
Malonate	0.069 ± 0.030[b]	0.039 ± 0.014[a]	0.038 ± 0.011[a]	0.051 ± 0.019[b]	0.045 ± 0.019	<0.001	0.839	0.046
N-Acetylglucosamine	1.067 ± 0.591[b]	0.601 ± 0.322[b]	0.344 ± 0.202[a]	0.255 ± 0.170[a]	0.472 ± 0.381	<0.001	0.142	<0.001
O-Acetylcarnitine	0.106 ± 0.055[c]	0.055 ± 0.028[b]	0.035 ± 0.017[a]	0.062 ± 0.029[bc]	0.056 ± 0.035	<0.001	0.125	<0.001
O-Phosphocholine	0.772 ± 0.487[b]	0.346 ± 0.215[b]	0.070 ± 0.098[a]	0.055 ± 0.082[a]	0.223 ± 0.304	<0.001	0.034	<0.001
Orotic acid	4.283 ± 1.593[a]	5.022 ± 1.793[a]	6.112 ± 1.951[b]	5.259 ± 1.359[ab]	5.359 ± 1.815	0.001	<0.001	<0.001
Phenylalanine	0.015 ± 0.006[a]	0.012 ± 0.006[a]	0.012 ± 0.005[a]	0.013 ± 0.005[a]	0.013 ± 0.005	0.531	0.001	<0.001
Proline	0.068 ± 0.030[b]	0.042 ± 0.015[a]	0.038 ± 0.013[a]	0.040 ± 0.016[a]	0.043 ± 0.019	<0.001	0.011	0.304
Pyruvate	0.028 ± 0.041[a]	0.027 ± 0.028[a]	0.023 ± 0.021[a]	0.021 ± 0.019[a]	0.024 ± 0.026	0.078	<0.001	<0.001
Succinate	0.026 ± 0.014[a]	0.026 ± 0.014[a]	0.028 ± 0.017[a]	0.035 ± 0.031[a]	0.029 ± 0.021	0.311	0.480	0.003
Trimethylamine	0.004 ± 0.002[c]	0.002 ± 0.001[b]	0.002 ± 0.001[a]	0.002 ± 0.001[a]	0.002 ± 0.001	<0.001	0.015	0.022
Urea	2.802 ± 0.840[a]	4.284 ± 2.128[b]	4.127 ± 1.910[b]	5.878 ± 1.833[c]	4.517 ± 2.099	<0.001	0.337	0.945
Valerate	0.020 ± 0.009[a]	0.019 ± 0.009[a]	0.018 ± 0.015[a]	0.019 ± 0.016[a]	0.019 ± 0.013	0.122	0.880	0.761

(*continued on next page*)

Table 2 (*continued*)

Metabolites*	Winter-Feb[¥]	Spring[¥]	Summer[¥]	Autumn[¥]	Mean lactation	*p*-value Season	*p*-value Milk Source	*p*-value Season-Milk Source
Valine	0.015 ± 0.007[a]	0.013 ± 0.006[a]	0.014 ± 0.005[a]	0.013 ± 0.005[a]	0.014 ± 0.006	0.421	0.893	0.094
sn-Glycero-3-phosphocholine	0.985 ± 0.406[a]	0.890 ± 0.401[a]	0.864 ± 0.348[a]	0.753 ± 0.242[a]	0.854 ± 0.355	0.048	0.868	0.824

*Total samples = 533

[¥]Lactation = Sample collection from February to November

[¥]Calendar Season criteria: Winter = February; Spring = March, April, May; Summer = June, July, August; Autumn = September, October, November

production throughout the year (Walsh et al., 2023).

3.2. Milk metabolome

Throughout the lactation period, a total of 38 metabolites from diverse chemical classes, including amino acids, nitrogenous compounds, carbohydrates, volatile fatty acids, organic acids, and alcohols, were identified and quantified using ^{1}H-NMR spectroscopy. As presented in Table 2, 30 of these metabolites showed significant variation across seasons, indicating a strong seasonal influence on the milk metabolome, while 14 metabolites exhibited significant differences based on milk source. Monthly variations are further detailed in Supplementary Table 1. Characteristic chemical shift positions (ppm) of the 38 identified metabolites are illustrated in Figure 1, which displays a representative annotated ^{1}H-NMR spectrum of a milk sample collected in February 2023. The reliability of metabolite detection and quantification in this study was supported by the sample preparation approach employed. The use of 3 kDa ultrafiltration for sample preparation is supported by previous methodological studies in NMR metabolomics. Emwas et al. (2025) provides recommendations for sample preparation for NMR metabolomics and specifically suggests the use of 3 kDa filters for ultrafiltration. Similarly, House et al. (2024) recommends 3 kDa ultrafiltration to remove proteins, preventing enzymatic activity that can alter the metabolome after extraction. In addition, Chollangi and Hossain (2007) evaluated the use of 3, 5, and 10 kDa ultrafiltration membranes for the separation of components in dairy, highlighting that smaller cut-offs retain more macromolecules, which supports choosing 3 kDa instead of 10 kDa as they provide stronger protein and lipid removal.

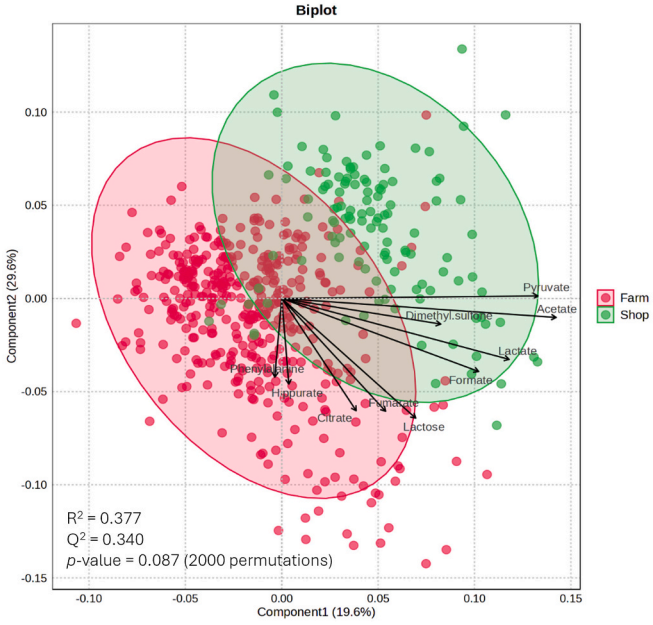


Figure 2. Partial Least Squares Discriminant Analysis (PLS-DA) score plots differentiating between farm and shop milk metabolome profiles. PLS-DA accounts for a total of 49.2% of variation (Component 1: 19.6%, Component 2: 29.6%). Metabolites contributing most to the separation are indicated as loading vectors. The permutation test (n= 2000) resulted in a *p*-value of 0.087, indicating that the observed separation between groups is not statistically significant.

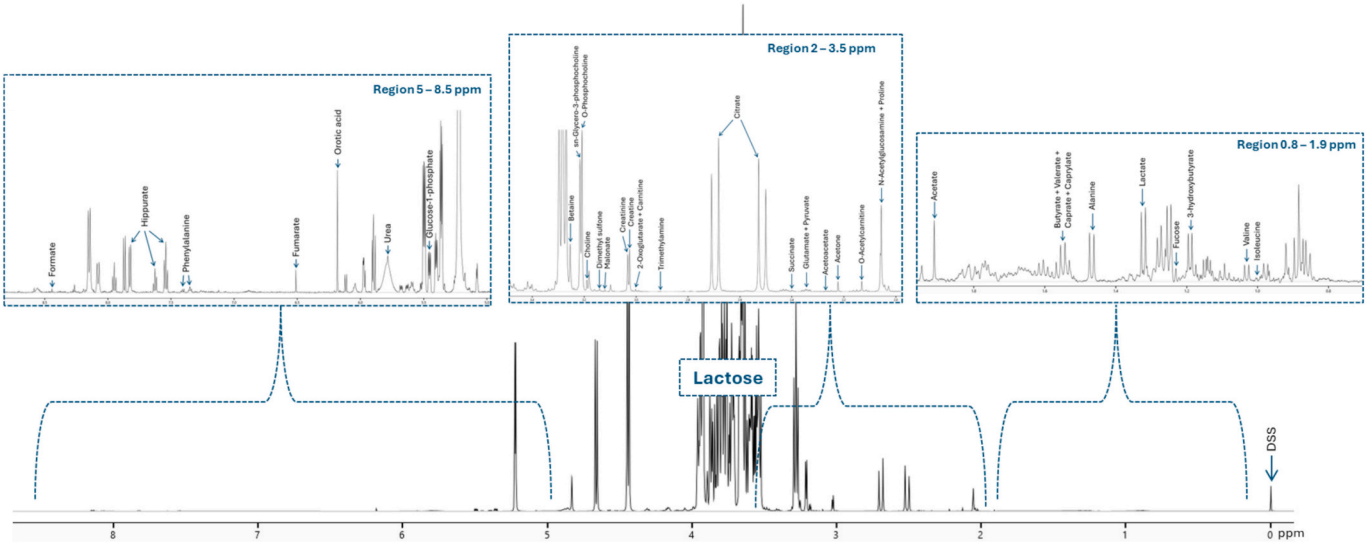


Figure 1. Representative annotated ^{1}H-NMR spectra (600 MHz) of a milk sample collected in February, showing compounds identified and quantified.

3.3. Influence of milk source on the milk metabolome

Milk source exhibited only a limited effect on the overall milk metabolome, as illustrated by the partial least squares discriminant analysis (PLS-DA) (Figure 2), which shows partial separation between Farm and Shop milk samples. The permutation test (2,000 permutations) yielded a *p*-value of 0.087, suggesting no significant difference overall in the metabolic profiles between farm and shop milk. This is a notable result, as it highlights the similarity between Farm and Shop milk samples. However, univariate analysis revealed statistically significant differences in the concentrations of some specific metabolites (Table 2).

Acetate, pyruvate, lactate, formate, proline, and dimethyl sulfone concentrations were higher in Shop milk compared to Farm milk ($p <$ 0.001, $<$ 0.001, $<$ 0.001, 0.006, 0.011, and 0.047, respectively). Lactate concentrations showed a notable Season–Milk Source interaction ($p <$ 0.001). Shop milk exhibited elevated lactate concentrations during winter-Feb, spring, and summer, while concentrations were similar between Shop and Farm milk in autumn. Fucose also exhibited a significant Season–Milk source interaction ($p <$ 0.001), with higher concentrations in Shop milk in winter-Feb and spring, similar concentrations in summer, and higher concentrations in Farm milk in autumn.

Hippurate, phenylalanine, citrate, trimethylamine, and choline concentrations were higher in Farm milk compared to Shop milk ($p <$ 0.001, 0.001, 0.004, 0.015, and 0.023, respectively). Hippurate exhibited a significant Season–Milk Source interaction ($p = 0.001$), with higher concentrations in Farm milk in winter-Feb, spring, and summer and similar concentrations in autumn. Similarly, phenylalanine showed a significant Season-Milk Source interaction ($p < 0.001$), being higher in Farm milk during winter-Feb and having similar concentrations between groups throughout the rest of the lactation period. Choline exhibited a significant Season–Milk Source interaction ($p <$ 0.001). Choline concentrations were higher in Farm milk during summer and autumn, similar in both groups in spring, and higher in Shop milk in winter-Feb. O-Phosphocholine had a significant Season–Milk Source interaction ($p <$ 0.001). O-Phosphocholine concentrations were higher in farm milk during winter-Feb and similar between groups in the other seasons. Orotic acid was significantly higher in shop milk in winter ($p < 0.001$), with no differences in other seasons.

The overall similarity in the milk metabolome between Farm and Shop milk suggests that milk processing and supply-chain factors may have only a moderate impact on the milk metabolome. The differences observed in specific metabolites may result from pasteurisation, homogenisation, and compositional standardisation typically applied to Shop milk. These findings align with those of Zhu et al. (2020b), who investigated how low-temperature long-time (LTLT) pasteurisation (63°C for 30 min) affects the milk metabolome, showing only minor changes in the metabolome. In their study, Zhu et al. (2020b) described reductions in 2-butanone, adenine, deoxyguanosine, and taurocholic acid, while concentrations of 2-methylbutyrylcarnitine, 2-nonanone, arginylproline, lysophosphatidylcholine (lysoPC), and N-acetyl-D-glucosamine 1-phosphate increased. However, the changes to the milk metabolome caused by high-temperature short-time (HTST) pasteurisation, currently used for commercial milk, have received limited attention. Research comparing the metabolome of Farm and Shop milk remains limited, therefore further targeted studies are required to elucidate the specific effects of these treatments on the milk metabolome.

3.4. Seasonal variations on the milk metabolome

Seasonality was shown to significantly impact the milk metabolome. Multivariate analysis using PLS-DA demonstrated clear discrimination of milk samples by Season, as illustrated in Figure 3A. The seasonal evolution in the metabolome showed a progressive metabolic shift from winter to autumn, coinciding with transition through early-, mid- and late-stages of lactation. The robustness of the PLS-DA was confirmed by a permutation test based on 2,000 permutations ($p < 0.001$), indicating that the observed separations were statistically significant. To identify the main metabolites driving this seasonal variation, variable importance in projection (VIP) scores were calculated (Figure 3B). Metabolites with VIP scores > 1 were considered relevant for discrimination, with 13

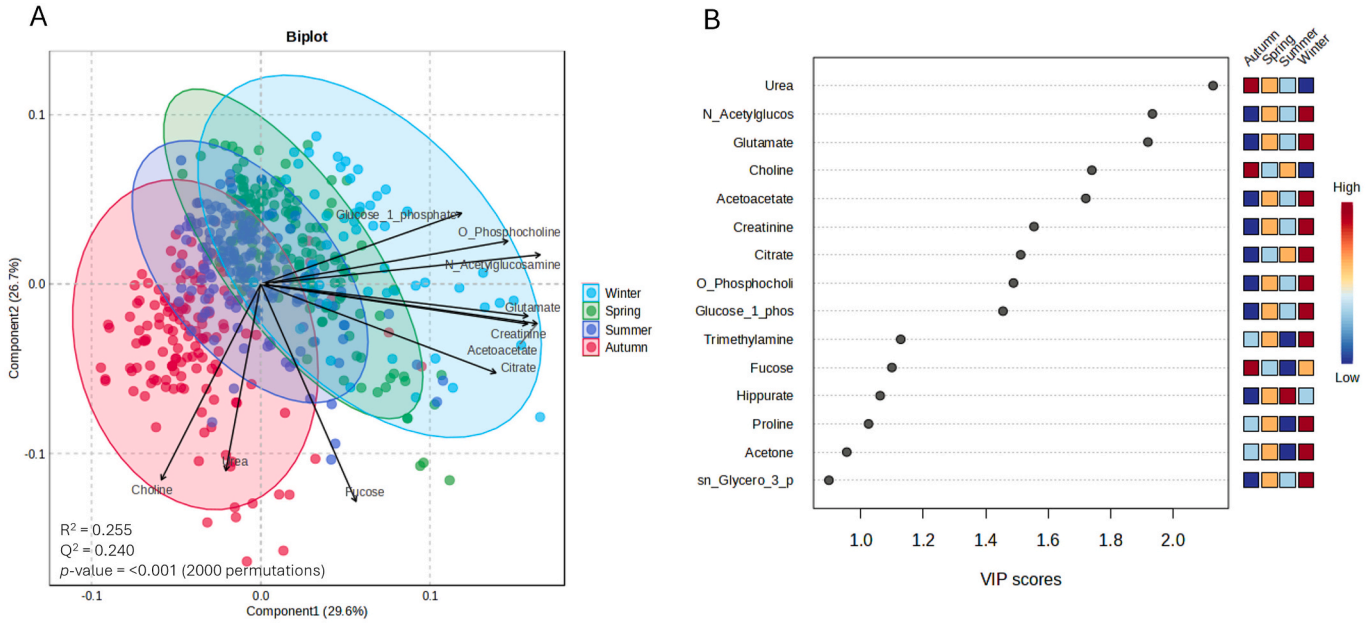


Figure 3. Partial Least Squares Discriminant Analysis (PLS-DA) score plots differentiating milk metabolome profiles by season (3A). PLS-DA accounts for a total of 56.3% of variation (Component 1: 29.6%, Component 2: 26.7%). Metabolites contributing most to the separation are indicated as loading vectors. To characterise these discriminant metabolites, variable importance in projection (VIP) scores differentiating milk metabolome profiles by season (3B) were calculated. Metabolites with VIP scores >1 are considered relevant for discrimination. Heatmap colours in the VIP indicate metabolite concentrations, where red indicates higher concentrations and blue indicates lower concentrations across seasons. Calendar Season criteria: Winter = February; Spring = March, April, May; Summer = June, July, August; Autumn = September, October, November.

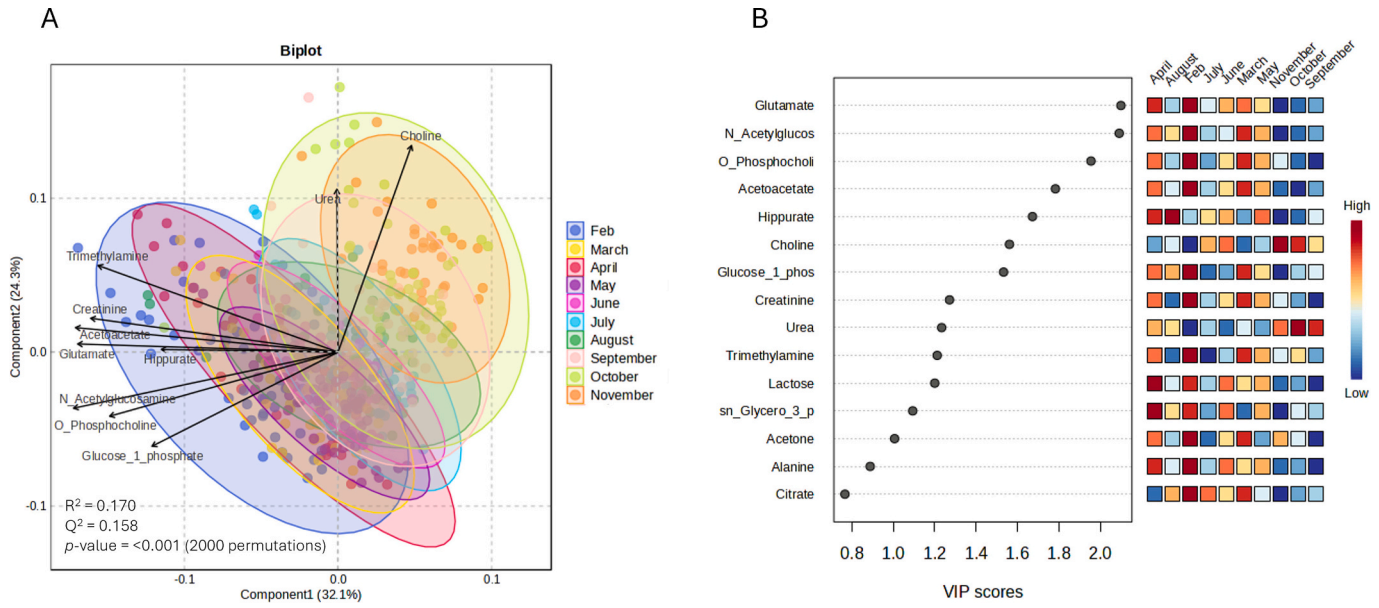


Figure 4. Partial Least Squares Discriminant Analysis (PLS-DA) score plots differentiating milk metabolome profiles by month (4A). PLS-DA accounts for a total of 56.4% of variation (Component 1: 32.1%, Component 2: 24.3%). Metabolites contributing most to the separation are indicated as loading vectors. To characterise these discriminant metabolites, variable importance in projection (VIP) scores differentiating milk metabolome profiles by month (4B) were calculated. Metabolites with VIP scores >1 are considered relevant for discrimination. Heatmap colours in the VIP indicate metabolite concentrations, where red indicates higher concentrations and blue indicates lower concentrations across months.

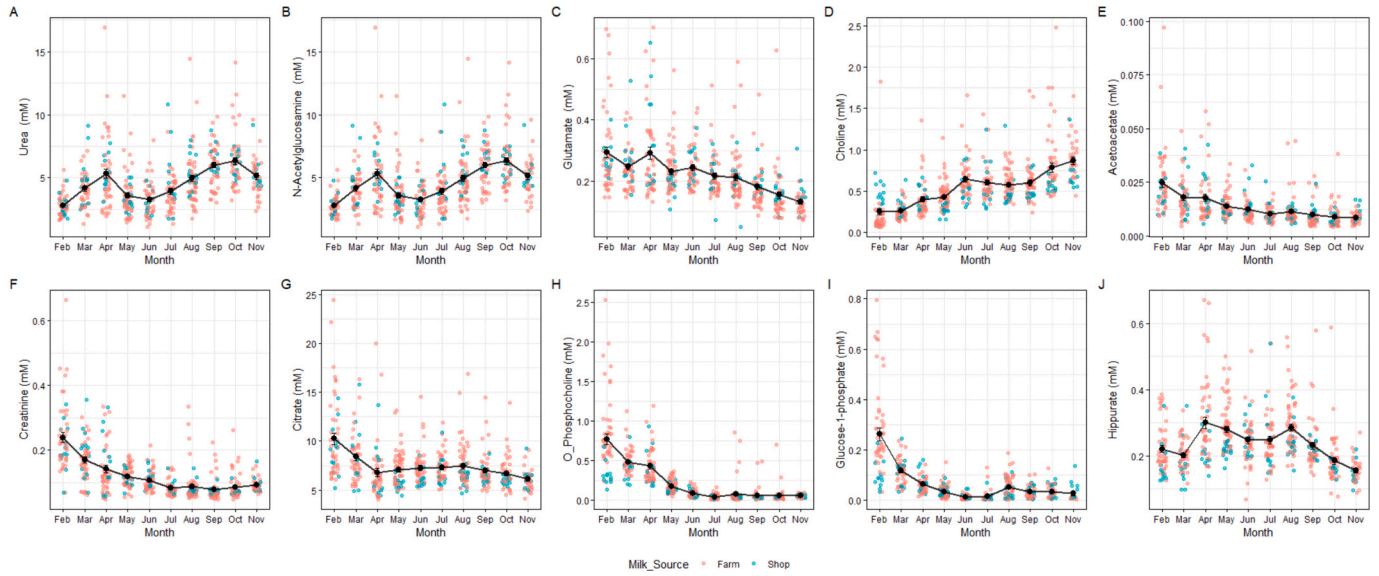


Figure 5. Seasonal variation of selected milk metabolites across calendar months. Concentrations (mM) of urea (5A), N-acetylglucosamine (5B), glutamate (5C), choline (5D), acetoacetate (5E), creatinine (5F), citrate (5G), O-Phosphocholine (5H), glucose-1-phosphate (5I), and hippurate (5J) are shown. The black line indicates the monthly mean concentrations with standard error. Each point represents an individual milk sample, with red dots corresponding to farm milk and blue dots to shop milk.

metabolites contributing to the separation by season (VIP > 1). Similarly, the PLS-DA by month (Figure 4A) revealed a consistent evolution from February to November. The corresponding VIP analysis highlighted also 13 metabolites contributing to monthly variation (VIP > 1) (Figure 4B). The concentrations of the top metabolites contributing most to the seasonal differentiation are illustrated in Figure 5.

Urea was one of the most discriminative metabolites across seasons ($p < 0.001$, VIP > 2), with the highest concentrations observed in autumn milk ($p < 0.01$), followed by spring and summer, and lowest in winter-Feb milk. Urea is an amide, nitrogen containing compound, formed in the liver through urea cycle from ammonia and carbon dioxide (Spek et al., 2013). The mean urea concentration across lactation was 4.5 ± 2.1 mM. Urea followed a biphasic trend (Figure 5A), increasing from February to April, decreasing until June, rising again until September, and declining from September to November. The described trend for urea aligns with the findings of Henao-Velásquez et al. (2014), who reported that urea concentrations increased during early lactation, declined slightly during mid-lactation, and increased again after day 195. However, the lactational pattern of urea remains insufficiently studied, and contradictory results have been reported in the literature. For instance, Rzewuska and Strabel (2013) observed the lowest urea concentrations during the first month of lactation, with its

peak in the fifth month but did not report a decline in concentrations between April and June. Similarly, Fatehi et al. (2012) found that urea concentrations were lowest in the first month of lactation, gradually increased to a peak in the sixth month, and declined thereafter. Urea has been described as an indicator of protein nutrition and nitrogen use efficiency (NUE) in dairy cows, as reported by Zhao et al. (2025), positioning it as a key compound for strategies to focus on more sustainable and efficient dairy production systems by greater dietary management of protein that would result in reduced urea losses to land and environment. As such, urea measurement in milk serves as a valuable parameter for improving NUE and optimizing feeding strategies in dairy herds. Elevated urea concentrations have been associated with increased urinary nitrogen excretion and reduced NUE. Most recently, Heffernan et al. (2025) described the seasonal variation in pasture crude protein (CP) content from March to early October, reporting the lowest CP concentrations in June and July, followed by an increase from September onwards. Zhao et al. (2025) described an optimal urea range of 2.9–5.7 mM for balanced dietary protein intake, with deviations above or below this range indicating nutritional imbalances. In our study, winter-Feb milk urea average was 2.8 ± 0.8 mM, being on the lower boundary of the recommended range. Spring and summer were in the optimal window, with values of 4.3 ± 2.1 in spring and 4.1 ± 1.9 mM in summer. Autumn milk rose above the upper boundary at 5.9 ± 1.8 mM, indicating a reduced NUE.

N-Acetylglucosamine (GlcNAc) was identified as a discriminative metabolite across seasons ($p < 0.001$, VIP > 1.8), present in higher concentrations in winter-Feb and declining progressively through the year, reaching the lowest concentrations in summer and autumn. N-Acetylglucosamine is an N-acetylated amino sugar that serves as a precursor and a structural component of N-glycans. In milk, these N-glycans contribute to immune protection (Parc et al., 2015). Conversely to our results, Connolly et al. (2023) did not observe changes in GlcNAc across seasons, being in similar concentrations in early-, mid- and late-lactation. Although Rehan et al. (2015) did not investigate the milk metabolome, their study demonstrated that environmental stress alters N-glycan composition of bovine serum. The elevated GlcNAc concentrations observed during winter-Feb may reflect a seasonal shift in glycan metabolism, possibly driven by increased energy demands associated with colder temperatures and the onset of early lactation.

Glutamate exhibited significant seasonal variations ($p < 0.001$, VIP > 1.8), present in higher concentrations in winter-Feb and decreasing gradually over lactation, reaching the lowest concentrations in autumn. Glutamate concentrations in winter-Feb were higher compared to autumn ($p < 0.001$) and summer ($p = 0.048$). Concentrations in spring were significantly higher than in autumn ($p < 0.001$) but not different from summer. Glutamate is a non-essential amino acid that has been associated with non-coagulating properties of milk (Sundekilde et al., 2014). The link between elevated glutamate concentrations and poor coagulation properties is of particular importance for the dairy industry, as poor coagulation properties directly affect the yield of rennet products such as cheese (Horne & Lucey, 2017). From an industrial perspective, monitoring glutamate concentrations could therefore be useful to support processability predictions, contributing to a greater efficiency and consistency in the dairy sector.

Choline was a distinguishing metabolite across seasons ($p < 0.001$, VIP > 1.6), present at lower concentrations in winter-Feb and increasing over the course of lactation, reaching the highest concentrations in autumn. Choline concentrations were significantly higher in summer and autumn compared to winter-Feb and spring (both $p < 0.001$). This seasonal trend aligns with findings by Zhu et al. (2020a) and Klein et al. (2010), who reported higher choline concentrations in milk during late lactation compared to early lactation. Choline is involved in phospholipid synthesis, acetylcholine production, and methyl-group transfer and plays an important role in lipid transport and cell membrane integrity (Fischer et al., 2010). The milk metabolite choline was negatively correlated with milk yield (r = – 0.506), with its concentrations increasing as milk yield decreased throughout the year. Similarly, Suthar et al. (2025) observed that choline in rumen was negatively correlated with milk yield, highlighting a correlation between milk yield and both milk and rumen choline metabolite concentrations. Additionally, choline has been previously associated with good coagulating properties of milk (Sundekilde et al., 2011, 2014).

Acetoacetate displayed significant seasonal variation ($p < 0.001$, VIP > 1.6), being in higher concentrations in winter-Feb, and decreasing progressively over the course of lactation, reaching the lowest concentrations in autumn. Concentrations were significantly higher in winter-Feb compared to spring ($p = 0.007$), summer ($p < 0.001$), and autumn ($p < 0.001$). Concentrations in spring were also significantly higher than those in summer ($p < 0.001$) and autumn ($p < 0.001$), while summer concentrations were higher than those in autumn ($p = 0.021$). Acetoacetate is a ketone body formed in the liver during fatty acid oxidation, especially under negative energy balance (NEB). High concentrations of acetoacetate in milk are related with subclinical and clinical ketosis, making it a valuable metabolite to monitor metabolic health, especially during early lactation (Huang et al., 2024).

Creatinine exhibited seasonal fluctuation ($p < 0.001$, VIP > 1.4), with the highest concentrations in winter-Feb, that then decreased progressively over the course of lactation, reaching the lowest concentrations in summer and autumn. Concentrations in winter-Feb were significantly higher than those in spring ($p = 0.001$), summer ($p < 0.001$), and autumn ($p < 0.001$). Similarly, concentrations in spring were significantly higher than those in summer and autumn (both $p < 0.001$), with no significant differences between summer and autumn. Creatinine is an imidazole derivative formed from creatine in the muscle tissue and elevated concentrations have been linked to NEB in cows (Xu, Van Knegsel, et al., 2020). Creatinine has also been associated with heat stress, as reported by Jorge-Smeding et al. (2024). In their paper, heat stress significantly increased creatinine concentrations in milk by 22%, reflecting the enhanced muscle catabolism during heat stress. The same study showed that dietary supplementation with vitamin E and selenium reduced muscle protein breakdown, as indicated by lower milk creatinine concentrations. Hanno et al. (2025) further demonstrated that skeletal muscle reserves in dairy cows fluctuate across lactation, with greater muscle loss occurring in early lactation and partial restoration in late lactation. Blood creatinine concentrations, as reported by Hanno et al. (2025), peaked in early lactation and declined thereafter, closely mirroring the seasonal patterns we observed in milk. Given the relationship between creatinine and muscle protein turnover, the quantification of creatinine could be of further interest for improved dietary strategies that could facilitate interventions to adapt better to the cattle metabolic needs.

Citrate ($p < 0.001$, VIP > 1.4) followed the same seasonal trend as creatinine, displaying higher concentrations in winter-Feb and a progressive decline throughout lactation, reaching the lowest concentrations in summer and autumn. Winter-Feb concentrations were significantly higher than those in spring, summer, and autumn (all $p < 0.001$), with no significant differences among spring, summer, and autumn. This citrate trend aligns with the findings of Garnsworthy et al. (2006), who reported a decrease in citrate concentrations from early to late lactation. Citrate is an intermediate of the tricarboxylic acid (TCA) cycle that plays an important role in fatty acid synthesis and calcium regulation in milk (Garnsworthy et al., 2006). Priyashantha et al. (2019) studied the effect of calcium and citrate on the coagulation properties of milk. In their study, adding calcium promoted faster coagulation and stronger gel formation, as calcium strengthens micelle interactions, decreasing micelle size and facilitating bridging between casein particles. By contrast, high concentrations of citrate in milk had the opposite effect, extending coagulation time and weakening gel structure, due to its ability to bind with calcium and reducing micelle interactions.

O-Phosphocholine (PC) showed significant variation across seasons ($p < 0.001$, VIP > 1.4), with higher concentrations in winter-Feb and spring, decreasing progressively across lactation and reaching the lowest

concentrations in summer and autumn (all $p < 0.001$). Concentrations did not differ between winter-Feb and spring or summer and autumn. The same trends were observed by Artegoitia et al. (2014) and Connolly et al. (2023), who reported higher concentrations of PC in early lactation compared to mid and late lactation. O-phosphocholine is involved in the metabolism and turnover of membrane phospholipids in the milk fat globule membrane. Xu, Van Knegsel, et al. (2020) demonstrated that elevated concentrations of PC in milk are associated with NEB. That could explain why the concentrations of PC are higher in early lactation, reflecting the higher demand for cell membrane formation in the mammary gland.

Glucose-1-phosphate concentrations varied markedly with season ($p < 0.001$, VIP > 1.4), with higher concentrations in winter-Feb compared to spring ($p = 0.029$), summer ($p < 0.001$), and autumn ($p < 0.001$). Concentrations in spring were also significantly higher than those in summer ($p < 0.001$) and autumn ($p = 0.017$), with no significant differences observed between summer and autumn. This trend aligns with the findings reported by Connolly et al. (2023), who reported higher concentrations of glucose-1-phosphate in early lactation compared to mid and late lactation. Glucose-1-phosphate is a phosphorylated monosaccharide produced during glycogenolysis and is linked to the synthesis of lactose in the mammary gland (Sadovnikova et al., 2021). Glucose-1-phosphate was moderately negatively linked to grass growth (r = - 0.505), indicating that periods of greater pasture availability and higher glucogenic supply reduce the concentrations of glucose-1-phosphate in milk.

Hippurate showed significant variation across seasons ($p < 0.001$, VIP>1), with the highest concentrations observed in spring and summer, followed by winter-Feb, and then autumn. Concentrations in summer and spring were higher than in autumn ($p < 0.001$ and $p = 0.008$), with no statistical differences between spring and summer. These results are consistent with the findings of Connolly et al. (2023) and Zhu et al. (2020a), who reported elevated hippurate concentrations during mid-lactation compared to early and late lactation. One likely explanation for the elevated concentrations of hippurate in spring and summer is the increased availability and nutritional quality of pasture. The strong correlation between higher concentrations of hippurate and higher proportions of pasture has been previously reported by Rojas-Gómez et al., (2025a).

Overall, the seasonal trends observed in the present study show points of convergence and divergence with previous metabolomic studies. Several metabolites associated with early lactation and negative energy balance, including acetoacetate, creatinine, and O-phosphocholine, followed patterns consistent with previous papers (Zhu et al., 2020a), supporting their role as indicators of metabolic stress during this period. Similarly, the progressive increase in choline concentrations towards late lactation aligns with previous findings linking choline with lipid metabolism (Artegoitia et al., 2014). However, some discrepancies were identified; for instance, GlcNAc exhibited clear seasonal variation in the present study, whereas Connolly et al. (2023) reported no significant changes across lactation. These differences may reflect variations in experimental design, including sampling frequency, sample size and different farm management conditions.

3.5. Biomarker metabolites for seasonality

For biomarker analysis, ROC analysis was employed to evaluate the discriminatory power of specific metabolites as candidate biomarkers for seasonal variation. The analysis encompassed six pairwise seasonal comparisons: winter-Feb vs. summer, winter-Feb vs. autumn, winter-Feb vs. spring, spring vs. summer, spring vs. autumn, and autumn vs. summer. Across these comparisons, several metabolites demonstrated excellent or good AUC values, underscoring their robustness as candidate biomarkers capable of distinguishing milk according to season. The metabolites identified, along with their corresponding AUC values and $\log_2$ fold-change values, are presented in Table 3. The metabolites identified in the biomarker analysis were consistent with those highlighted in the PLS-DA, reinforcing their role as key drivers of seasonal

Table 3
Area under the curve values from Receiver Operating Characteristic (ROC) analysis for identification of potential metabolite biomarkers of seasonality comparing Spring vs. Winter, Spring vs. Summer, Spring vs. Autumn, Summer vs. Autumn, Summer vs. Winter, and Autumn vs. Winter in farm milks. Metabolites highlighted exhibited good (0.8-0.9) and excellent (0.9-1.0) biomarker potential.

ROC analysis for biomarkers of seasonality in farm milk								
Winter-Feb vs. Summer[¥]			Winter-Feb vs. Autumn[¥]			Winter-Feb vs. Spring[¥]		
Metabolite	AUC	Log2Fold-change	Metabolite	AUC	Log2Fold-change	Metabolite	AUC	Log2Fold-change
Glucose-1-phosphate	0.99	3.48	O-Phosphocholine	1.00	3.98	Glucose-1-phosphate	0.96	2.21
O-Phosphocholine	0.99	3.61	Glucose-1-phosphate	0.99	3.90	O-Phosphocholine	0.91	1.33
O-Acetylcarnitine	0.98	1.81	N-Acetylglucosamine	0.98	2.26	Malonate	0.90	0.90
Choline	0.98	-1.72	Choline	0.97	-2.05	Choline	0.90	-0.99
N-Acetylglucosamine	0.97	1.78	Creatinine	0.96	1.51	O-Acetylcarnitine	0.88	1.11
Trimethylamine	0.96	1.42	Trimethylamine	0.95	1.30	Betaine	0.87	1.21
Creatinine	0.95	1.42	Urea	0.95	-1.06	Trimethylamine	0.87	0.88
Acetone	0.93	1.82	Acetoacetate	0.94	1.55	N-Acetylglucosamine	0.86	0.97
Malonate	0.93	0.91	Glutamate	0.90	0.96	Creatinine	0.85	0.84
Acetoacetate	0.91	1.23	Acetone	0.86	1.30	Acetone	0.82	1.14
Betaine	0.90	1.33	O-Acetylcarnitine	0.85	0.88			
Orotic acid	0.89	-0.70	Citrate	0.85	0.65			
Dimethyl sulfone	0.86	-1.13	Orotic acid	0.82	-0.48			
Proline	0.86	0.79	Proline	0.81	0.69			

Spring vs. Summer[¥]			**Spring vs. Autumn[¥]**			**Autumn vs. Summer[¥]**		
Metabolite	**AUC**	**Log2Fold-change**	**Metabolite**	**AUC**	**Log2Fold-change**	**Metabolite**	**AUC**	**Log2Fold-change**
O-Phosphocholine	0.95	2.28	O-Phosphocholine	0.97	2.65	O-Acetylcarnitine	0.86	0.93
Choline	0.86	-0.73	N-Acetylglucosamine	0.91	1.29	Glutamate	0.83	-0.55
N-Acetylglucosamine	0.82	0.81	Choline	0.90	-1.07	Fucose	0.82	0.70
Glucose-1-phosphate	0.80	1.27	Glutamate	0.88	0.71	Betaine	0.80	0.75
			Acetoacetate	0.85	0.87			
			Glucose-1-phosphate	0.84	1.69			

[¥]Lactation = Sample collection from February to November
[¥]Calendar Season criteria: Winter = February; Spring = March, April, May; Summer = June, July, August; Autumn = September, October, November

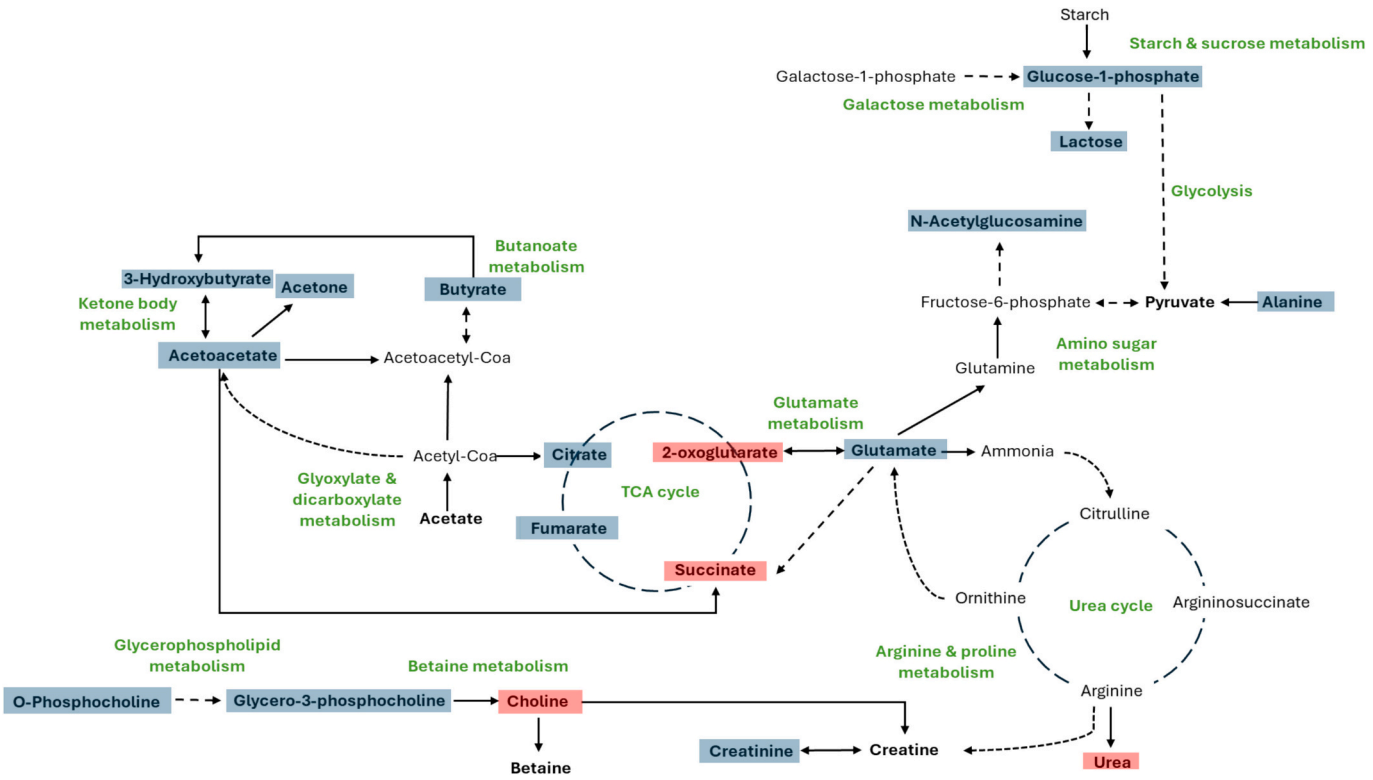


Figure 6. Integrated representation of key metabolic pathways influenced by seasonality, comparing milk metabolomes from winter and autumn. Metabolites identified in this study are shown in bold. Solid arrows indicate direct biochemical reactions, whereas dashed arrows represent indirect pathways involving intermediates not explicitly depicted. Metabolites highlighted in blue were enhanced in winter, while those in red were enhanced in autumn.

variation in the milk metabolome.

3.6. Pathway analysis

To explore the metabolic pathways most impacted by seasonality in this study, a comparative pathway analysis was performed using the metabolites that showed significant differences between winter-Feb and autumn, as shown in Figure 6. This provides information about the physiological adaptations occurring in cattle across seasons.

Glycerophospholipid metabolism was significantly enriched in the pathway analysis. O-phosphocholine (PC), glycero-3-phosphocholine (GPC), and choline were identified as key metabolites. Concentrations of PC and GPC were higher in winter-Feb, whereas choline concentrations were elevated in autumn. Klein et al. (2012) observed that the GPC/PC ratio in bovine milk is a predictor of ketosis risk. In their study, a ratio above 2.5 in the first month of lactation was associated with a low probability of developing ketosis and better ability to cope with metabolic stress due to more efficient breakdown and use of phospholipids. In our study, the GPC/PC ratio in the first month of lactation was 1.27, indicating risk of ketosis. Understanding these changes in the metabolic profile provides valuable information about the metabolic status and risk of disease in cows, information that can be used to optimize feeding strategies and improve management practices. In contrast, the higher choline concentrations observed in autumn may reflect improved energy balance, associated with reduced energy demands during late lactation. Supporting this, Xu et al., (2020) reported a negative correlation between choline concentrations and NEB in bovine milk. These findings suggest that elevated choline concentrations may serve as an indicator of improved metabolic status. Furthermore, McGuffey (2017) emphasized the role of rumen-protected choline (RPC) supplementation in reducing the risk of fatty liver and ketosis, further linking choline metabolism to energy homeostasis in dairy cows and highlighting RPC as a potential strategy to enhance metabolic health in dairy cows.

Betaine concentrations were higher in winter-Feb and autumn ($p < 0.05$), which may reflect its role in helping cows cope with elevated energy demands during early lactation. As a methyl donor, betaine supports the export of very low-density lipoproteins (VLDL), reducing the risk of fatty liver, and its antioxidant properties help alleviate oxidative stress, both of which contribute to maintain metabolic stability during this critical period (Coleman et al., 2021). Starch and sucrose metabolism and galactose metabolism were enriched in winter-Feb, marked by higher concentrations of glucose-1-phosphate and lactose. This pattern coincides with higher levels of concentrate feed supplementation during this period (Table 1), since concentrates can constitute a major source of starch in pasture-based diets, as reported by Tahir et al. (2013).

Citrate, fumarate, succinate, and 2-oxoglutarate are key TCA intermediates. Citrate was elevated in winter-Feb, likely in response to energy stress during this period, which activates mitochondrial oxidative metabolism (Chen et al., 2024; Xu, Van Knegsel, et al., 2020). Chen et al. (2024) investigated milk citrate as a biomarker for NEB during early lactation. Similarly, Xu et al., (2020) used NMR and LC-MS to identify milk metabolites correlated with energy balance, also highlighting citrate as a potential biomarker for NEB. Consistent with our findings, Xu et al., (2020) further reported positive associations between NEB and elevated concentrations of creatinine, glucose-1-phosphate, and phosphocholine, supporting the utilisation of these metabolites in monitoring metabolic status. On the other side of the TCA cycle, succinate and 2-oxoglutarate were higher in autumn, reflecting adaptations to reduced milk production and increased tissue catabolism. This pattern is further supported by Sun et al. (2017), who conducted a comprehensive metabolomic analysis of mammary gland tissue, milk, serum, urine, and rumen fluid in lactating and non-lactating cows. Their results showed enhanced oxidative metabolism in early lactation and increased glutamate-related metabolism in late lactation, linked to 2-oxoglutarate, supporting the seasonal changes observed in our data. Taken together,

our results and those of Sun et al. (2017) suggest that 2-oxoglutarate may serve as a potential indicator for low risk of NEB.

The ketone body metabolism pathway was also more active in winter-Feb, as evidenced by elevated concentrations of 3-hydroxybutyrate (BHB), acetoacetate, and acetone, metabolites indicative of NEB. This is consistent with the findings by Xu, Vervoort, et al. (2020), who studied how the milk and the plasma metabolome changes during early lactation, identifying glycine, BHB and acetone in milk strongly linked with mobilization of fat and ketosis. Acetone showed a strong positive correlation with BHB ($r = 0.81$) and acetoacetate ($r = 0.72$), suggesting that acetone alone may provide a reliable indication of ketone body metabolism and reinforce its potential as a candidate biomarker of NEB. Similarly, Santschi et al. (2016) used Fourier-transform infrared (FTIR) spectroscopy to quantify milk BHB, as an indicator of ketosis. Cows with elevated milk BHB had lower milk yield and milk protein content but had higher fat content and somatic cell count. Concurrently, creatinine was also elevated in winter-Feb. Pires et al. (2022) reported that higher milk creatinine concentrations in early lactation are correlated with NEB, in parallel with other energy-related metabolites such as BHB, indicating enhanced muscle breakdown to support milk production. These metabolites can serve as candidate biomarkers for NEB, particularly during early lactation when energy demands are highest, enabling interventions to improve cow health and productivity.

The urea cycle was highly impacted by season, with higher urea concentrations in late lactation, reflecting a decline in NUE. As milk yield decreases in late lactation, the demand for nitrogen in milk protein synthesis is reduced. Consequently, excess dietary nitrogen is catabolized, leading to increased urea production (Pereira et al., 2025). In their study, Pereira et al. (2025) also observed that cows with lower milk urea concentrations exhibited greater NUE, suggesting that phenotyping for low urea could be a valuable strategy for breeding more sustainable and efficient dairy herds.

4. Conclusion

This study demonstrates variation in the milk metabolome across the production year in pasture-based spring-calving systems, with 30 out of 38 metabolites showing significant variation throughout lactation. Multivariate and biomarker analyses identified several metabolites, including urea, GlcNAc, glutamate, choline, acetoacetate, creatinine, citrate, O-phosphocholine, and glucose-1-phosphate that were associated with seasonal changes in milk metabolite profiles, many of which are linked to energy balance, physiological stress, and nitrogen use efficiency. While overall differences between Farm and Shop milk were limited, some metabolites, such as acetate and pyruvate, differed by Milk Source. Pathway analysis revealed that winter-Feb milk, representative of early lactation, was marked by elevated ketone bodies, O-phosphocholine, creatinine, and glucose-1-phosphate, suggesting increased metabolic stress and negative energy balance. Autumn milk was marked by elevated choline, indicative of improved energy status, and elevated urea, suggesting reduced NUE towards late lactation. This study provides one of the first large-scale longitudinal assessments of the milk metabolome across both Farm and Shop milks, offering insight into metabolomic variation under pasture-based production conditions and identifies a series of candidate biomarker compounds for further validation in future studies. Furthermore, greater understanding of the milk metabolome could be beneficial in developing strategies to improve feeding, farm management, breeding for enhanced metabolic resilience and sustainability in dairy production.

CRediT authorship contribution statement

Paula Rojas-Gómez: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Raghunath Pariyani:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Michael Dineen:** Writing – review & editing, Project administration, Methodology, Investigation, Data curation. **Denis Lynch:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Lorraine Bateman:** Writing – review & editing, Supervision, Project administration, Investigation, Formal analysis, Data curation. **Eoghan Roche:** Writing – review & editing, Investigation, Data curation. **Anita R. Maguire:** Writing – review & editing, Methodology, Investigation. **Noel A. McCarthy:** Writing – review & editing, Methodology, Investigation. **Thomas Brendan Murphy:** Writing – review & editing, Methodology, Formal analysis, Data curation. **James A. O'Mahony:** Writing – review & editing, Supervision, Investigation. **Tom F. O'Callaghan:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to graciously acknowledge the farmers who contributed milk samples to the MetaBó-Bainne study. This publication emanated from research funded by the Irish Government Department of Agriculture, Food and the Marine (Dublin, Ireland; DAFM) through the MetaBó-Bainne (Cork, Ireland) project reference 2021R568; and in part supported by grants from the Science Foundation Ireland Research Infrastructure Programme (Dublin, Ireland) under grant numbers 15/RI/3221 and 21/RI/9705, and from Research Ireland under Grant Number [21/RC/10303_P2] - VistaMilk".

The authors do not report any conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2026.149492.

Data availability

Data will be made available on request.

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