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A metabolic profiling approach to characterize and discriminate plant-based beverages and milk

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ABSTRACT

The rising demand for nondairy and nonanimal protein sources has increased plant-based beverages (PBB) consumption. However, research on their functional properties, metabolic profile, and discrimination potential is limited. This study evaluated the potential of proton nuclear magnetic resonance (^{1}H NMR) spectroscopy as an authentication method to discriminate milk (cow and goat) and PBB macro-groups, including soy-based, fruit-based (almond and coconut), and cereal-based (rice and oat) beverages, based on their metabolic profile. A total of 22 PBB (soy-, almond-, coconut-, rice-, and oat-based beverages), 4 cow milk, and 4 goat milk cartons were analyzed with ^{1}H NMR spectroscopy to obtain their metabolic profile. Relevant metabolites to discriminate PBB macro-groups and cow and goat milk were identified through the Mann-Whitney U test and partial least squares-discriminant analysis. Results revealed that uridine diphosphate glucose and adenosine were key metabolites for the identification of goat and cow milk. At the same time, choline and guanosine emerged as important markers for different PBB macro-group detection. In addition, lactose played a significant role in differentiating milk from PBB. In conclusion, these findings represent an initial step toward applying ^{1}H NMR spectroscopy for authentication and nutritional analysis of PBB, opening the door for further research into their authenticity and metabolic profiling.

Key words: authentication, NMR, nutrition, plant-based alternatives

INTRODUCTION

In recent years, the rising demand for nondairy and nonanimal protein sources, driven by lactose intolerance, allergies, and various lifestyle choices, has increased plant-based beverages (**PBB**) consumption. From 2004 to 2018, a negative correlation between milk and PBB consumption was assessed in the United States (Slade, 2023). For every 4 L of PBB sold, milk consumption decreased by approximately 2 L (Slade, 2023). Recent projections show that the PPB market is expected to grow more than 12% from 2019 to 2026, with incomes exceeding $34.8 billion by the end of 2026 (Sharma et al., 2024a).

These beverages are water-soluble extracts derived from legumes (e.g., soy), fruits (e.g., almond, coconut), cereal (e.g., oats), or a mix of them (Brooker et al., 2023). Due to the significant variation in PBB composition, the nutritional content depends on the ingredients and their quality during production (Rincon et al., 2020). Considering the variability of PBB and the rapidly growing industry, reliable classification and authentication methods are being developed to preserve consumers' interests. Most studies have focused on describing PBB lipid, AA, and mineral profile. However, research on their functional properties, metabolic profile, and discrimination potential is surprisingly limited (Brigante et al., 2024). Metabolites, defined as low-molecular-weight molecules (<1 kDa), represent cellular metabolism's intermediate and final products. This comprehensive analysis aids in understanding the quality of PBB, highlighting their nutritional value and potential health effects (Ferreira et al., 2024; Meoni et al., 2020).

Although methods for analyzing metabolites have been established since the early days of biochemistry, 2 dominant techniques have emerged for simultaneous metabolite analysis: MS and nuclear magnetic resonance (**NMR**) spectroscopy (Ghini et al., 2023). Nuclear magnetic resonance spectroscopy boasts several advantages compared with MS, making it well-suited for PBB and

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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-25. Nonstandard abbreviations are available in the Notes.

Table 1. Descriptive metabolite composition in both PBB and milk (cow and goat); concentrations expressed in arbitrary units

Metabolite	n[1]	Mean	Minimum	Maximum	CV, %
Acetate	30	14.3×10^{-4}	4.8×10^{-5}	53.6×10^{-4}	1.29
Adenosine	25	0.47×10^{-4}	0	3.2×10^{-4}	1.83
Alanine	30	3.71×10^{-4}	1.05×10^{-5}	20.1×10^{-4}	1.56
Choline	29	39×10^{-4}	0	179.8×10^{-4}	1.48
cis-Aconitate	12	0.14×10^{-4}	0	1.1×10^{-4}	1.8
Citrate	26	39.41×10^{-4}	0	184.3×10^{-4}	1.33
Ethanol	30	19.5×10^{-4}	26.4×10^{-5}	294×10^{-4}	3.08
Formate	29	0.44×10^{-4}	0	2.5×10^{-4}	1.13
Fumarate	29	0.43×10^{-4}	0	4.2×10^{-4}	1.84
Galactose	21	2.6×10^{-4}	0	30.9×10^{-4}	2.46
Glucose + G6P[2]	29	39×10^{-4}	0	278×10^{-4}	1.88
Histidine	27	1.5×10^{-4}	0	7.5×10^{-4}	1.32
Isoleucine	21	0.59×10^{-4}	0	4×10^{-4}	1.75
Lactate + threonine	18	3.19×10^{-4}	0	26.5×10^{-4}	2.09
Orotate	12	0.41×10^{-4}	0	1.9×10^{-4}	1.69
Tyrosine	24	0.48×10^{-4}	0	2.52×10^{-4}	1.33
Uridine	17	0.13×10^{-4}	0	0.65×10^{-4}	1.53
Valine	30	0.83×10^{-4}	1.83×10^{-6}	4.45×10^{-4}	1.32

[1]n = number of samples containing that specific metabolite.
[2]G6P = glucose-6-phosphate.

milk analysis. These include high reproducibility, non-destructive analysis, and the capability to identify and quantify diverse organic compounds, ultimately leading to unambiguous structural elucidation. Furthermore, NMR spectroscopy streamlines sample preparation and facilitates automated procedures, enhancing efficiency. However, limitations such as sensitivity constraints, potential spectral resolution issues, signal overlap, protocol reproducibility, and standardization challenges need consideration (Ghini et al., 2023). Metabolite fingerprinting employing NMR is a rapid, convenient, and efficient method for differentiating between groups of related samples, effectively serving as an authentication tool (Krishnan et al., 2005; Gottstein et al., 2024).

Nuclear magnetic resonance spectroscopy is widely used as an authentication method for milk and dairy products (Tenori et al., 2018), olive oil (Dais and Hatzakis, 2013; Mannina et al., 2016), coffee (Wei et al., 2012; Monakhova et al., 2015), honey (Spiteri et al., 2015, 2016), beer (Mannina et al., 2016), wine (Godelmann et al., 2013), and spices (Petrakis et al., 2015). Furthermore, in the dairy industry, NMR spectroscopy is a well-known technique allied to milk composition analysis (Niero et al., 2022), origin identification (Eltemur et al., 2023), adulteration (Lamanna et al., 2011; Rysova et al., 2021; Soyler et al., 2021), and dairy cow health status (Bobbo et al., 2022). However, NMR spectroscopy-based research on PBB remains scarce (Abadl et al., 2023), with no studies to date investigating its potential for authenticating PBB or comparing the metabolome across different types of PBB, as well as between PBB and cow and goat milk. Existing literature in this domain primarily focuses on a limited number of studies (Saha and Bhattacharya, 2010; Münger et al., 2017; Radziej et al., 2021).

For species identification in various contexts, such as detecting animal-derived contaminants in vegan products or identifying specific plant species in raw or processed materials, DNA-based techniques are the gold standard (Bruno et al., 2019; Cottenet and Blancpain, 2021). Although these methods offer high precision, NMR spectroscopy provides additional advantages. First, NMR spectroscopy can perform simultaneous analysis of the metabolic content, giving a comprehensive overview of the metabolites present, such as sugars, organic acids, AA, and other bioactive compounds. This holistic view can be crucial for assessing the quality and safety of PBB. Second, NMR spectroscopy allows for the quantification of individual components within the sample, essential for accurately determining nutrient concentrations and analyzing nutritional properties. Third, NMR spectroscopy requires a simpler sample preparation process than DNA-extraction techniques. Both techniques bring valuable insights into PBB and milk analysis, with NMR spectroscopy offering a more complete picture of the chemical makeup and nutritional profile.

The burgeoning popularity of PBB as a milk substitute requires the development of robust analytical techniques to ensure product quality, authenticity, and traceability. Therefore, NMR spectroscopy, with its inherent advantages, stands as a promising tool to address these challenges and drive innovation in the plant-based product sector. Thus, this study aimed to assess the potential of proton (^{1}**H**) NMR spectroscopy as an authentication method to discriminate PBB (soy-, fruit- [almond, coconut], cereal-

Table 2. Descriptive metabolite composition only present in PBB; concentrations expressed in arbitrary units

Metabolite	n[1]	Mean	Minimum	Maximum	CV, %
4-Aminobutyrate	19	0.17×10^{-4}	0	26.5×10^{-4}	1.88
Asparagine	22	4.27×10^{-4}	6.46×10^{-6}	278.1×10^{-4}	2.0
Aspartate	19	1.49×10^{-4}	0	179.8×10^{-4}	1.20
Guanosine	12	0.24×10^{-4}	0	1.9×10^{-4}	1.79
Leucine	19	0.43×10^{-4}	0	4.45×10^{-4}	1.02
Malate	19	7.78×10^{-4}	0	53.6×10^{-4}	1.27
Maltose	22	15.3×10^{-4}	6.70×10^{-6}	30.9×10^{-4}	1.56
Phenylalanine	19	0.43×10^{-4}	0	3.08×10^{-4}	1.69
Sucrose	16	186×10^{-4}	0	692×10^{-4}	1.25
Theophylline	9	0.38×10^{-4}	0	3.04×10^{-4}	2.08
trans-Aconitate	5	0.09×10^{-4}	0	0.75×10^{-4}	2.38
Trigonelline	9	0.25×10^{-4}	0	1.5×10^{-4}	1.93
Tryptophan	11	0.195×10^{-4}	0	1.1×10^{-4}	1.63

[1]n = number of samples containing that specific metabolite.

based beverages [rice, oat]), and milk (cow and goat milk) based on bucketed spectra and metabolite content.

MATERIALS AND METHODS

This study did not require approval from the Institutional Animal Care and Use Committee or the Institutional Review Board because the PBB and milk used were purchased directly from commercial channels.

Sampling

A total of 22 PBB, 4 UHT whole cow milk, and 4 UHT whole goat milk 1 L-cartons from different commercial brands and manufacturing plants were purchased in Northern Italy and subjected to analysis. The PBB included cartons of soy-based beverages (n = 5), fruit-based beverages (almond, n = 4; coconut, n = 6), and cereal-based beverages (rice, n = 3; oat, n = 4). To better capture the variability of products in the market, some of the PBB purchased in each category included added sugars and salt (Supplemental Table S1, see Notes), as this is a common practice during PBB processing to increase palatability.

NMR Spectroscopy and Metabolite Identification

Samples were mixed with CH_2Cl_2 at a 1:1 ratio (vol/vol), as described by Tenori et al. (2018). The resulting mixture was homogenized by vortexing and then incubated at 25°C for 10 min. Subsequently, the mixture underwent centrifugation at 5,000 × *g* at 4°C for 30 min. Following centrifugation, 350 µL of the supernatant was added to 350 µL of Na_3PO_4 buffer (70 m*M*; 20% [vol/vol] H_2O, 6.1 m*M* NaN_3; 4.6 m*M* sodium trimethylsilyl (2,2,3,3-H4)-propionate; pH 7.4). Then, 600 µL of the resulting mixture was transferred into a 5-mm NMR tube (Bruker BioSpin) and stored at −80°C for subsequent analysis at the CERM/CIRMMP Center (Sesto Fiorentino, Italy).

NMR Spectra Acquisition

One-dimensional (**1D**) ^{1}H NMR spectra of the sample extracts were obtained using a Bruker spectrometer (Bruker BioSpin, Rheinstetten, Germany) operating at a proton Larmor frequency of 600.13 MHz and equipped with a 5-mm PATXI ^{1}H-^{13}C-^{15}N probe. The probe included a z-axis gradient coil, automatic tuning-matching, a BTO 2000 thermocouple to stabilize the acquisition temperature, and an automatic refrigerated sample changer (SampleJet, Bruker BioSpin; Rheinstetten, Germany). Before measurement, samples were allowed to equilibrate inside the NMR probe for at least 5 min, reaching a temperature of 37°C.

Each sample was analyzed in duplicate, obtaining 2 1D ^{1}H NMR spectra using the nuclear Overhauser effect spectroscopy (**NOESY**) sequence (noesygppr1d; Bruker BioSpin, Rheinstetten, Germany) and averaged to obtain a single spectrum per sample. Parameters for each spectrum collected included 64 scans, 98k data points, a spectral width of 18.028 Hz, an acquisition time of 2.7 s, a relaxation delay of 4 s, and a mixing time of 10 min. The resulting 1D NOESY spectra reveal both low-molecular-weight molecules and macromolecules.

Before applying the Fourier transformation of the NMR spectra, the free induction decay underwent multiplication by an exponential function corresponding to a 0.3-Hz line-broadening factor. Thereafter, the transformed spectra were automatically adjusted for phase and baseline distortions using TopSpin 4.1 software (Bruker BioSpin, Rheinstetten, Germany). Calibration of spectra was achieved using the α-lactose doublet (5.24 ppm for ^{1}H). For multivariate analysis, each 1D spec-

trum within the range of 0.02 to 10.00 ppm for ^{1}H was segmented into 0.02-ppm chemical shift bins (buckets). Regions corresponding to water (4.61–4.77 ppm for ^{1}H) and dichloromethane (5.30–5.33 and 5.42–5.65 ppm for ^{1}H) were excluded from the buckets of NOESY spectra.

Through the bucketing procedure, the whole sample spectrum was reduced to 328 variables, and a total of 43 metabolites were identified in the NMR spectra. Signal identification was carried out using a library of NMR spectra of pure organic compounds (Chenomx NMR Suite; https://www.chenomx.com/), along with public databases such as FoodDB (Food Database version 1.0, https://foodb.ca/) and the Milk Composition Database (MCDB; http://www.mcdb.ca/; Foroutan et al., 2019), supplemented by reference and literature data (Tenori et al., 2018; Meoni et al., 2020). From these signals, a total of 43 metabolites were identified across both milk and PBB samples.

Statistical Analysis

Data analyses were performed using Python version 3.9 (Van Rossum and Drake, 2009). For the statistical analysis, comparisons proposed were as follows:

1. Milk (n = 4) versus PBB (n = 22);
2. Soy- (n = 5) versus cereal- (n = 7) based beverages;
3. Soy- (n = 5) versus fruit- (n = 10) based beverages;
4. Monocotyledon- (n = 7; oat- and rice-based beverages) versus dicotyledon-based (n = 15; soy-, coconut-, and almond-based beverages) beverages;
5. Cereal- (n = 7) versus fruit- (n = 10) based beverages; and
6. Cow (n = 4) versus goat (n = 4) milk.

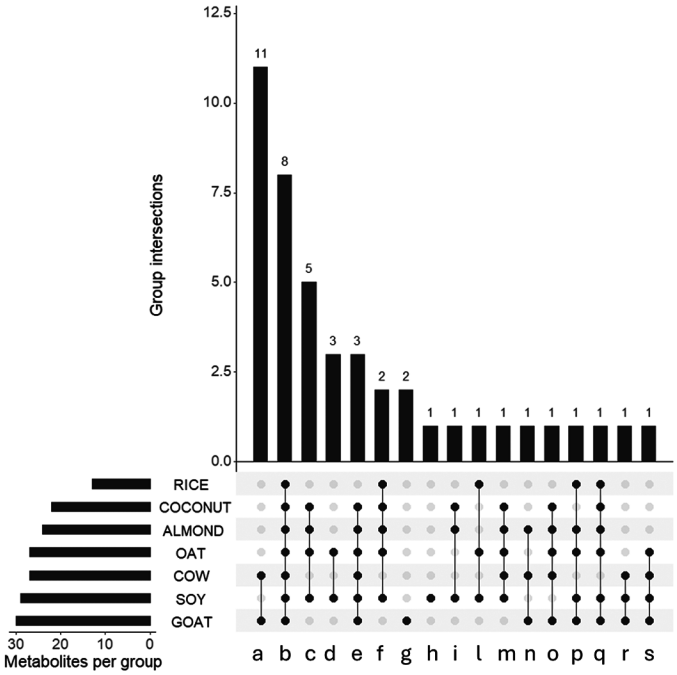


Figure 1. UpSet plot illustrating shared metabolites in all sample groups. An UpSet plot illustrates the intersections among multiple sets, allowing for the analysis of relationships between them. Bar identification: a, N-acetyl carbohydrates, O-acetyl carnitine, 2-oxoglutarate + carnitine, carnitine, 2-oxoglutarate, phosphocreatine + creatine, glycerophosphocholine, succinate, dimethylsulfone, lactose, and hippurate; b, valine, acetate, choline, glucose + glucose-6-phosphate, fumarate, formate, ethanol, alanine; c, leucine, 4-aminobutyrate; malate, aspartate, phenylalanine; d, trigonelline, tryptophan, theophylline; e, galactose; histidine, citrate; f, asparagine, maltose; g, uridine diphosphate-glucose, uridine diphosphate-galactose; h, *trans*-aconitate; i, sucrose; l, guanosine; m, isoleucine; n, orotate; o, lactate + threonine; p, adenosine; q, tyrosine; r, *cis*-aconitate; s, uridine.

The macro-groups were selected based on their distinct botanical origins, as suggested by Moore et al. (2023) because it influenced their nutritional content (Abdel-Aal, 2023). For instance, cereals typically have a greater carbohydrate content than fruits and legumes, but legumes boast the highest protein content, followed by cereals, with fruits having the lowest. These compositional differences are intrinsically linked to metabolic variations, given that metabolites play a critical role in the biosynthesis of these nutritional components (Roorkiwal et al., 2021).

First, the descriptive statistics of the area under the peak for each metabolite were represented graphically through box plots. Differences between groups were assessed using the Mann-Whitney U test because the data did not follow a normal distribution. The metabolite content variation across the previously defined macro-groups was presented as $\log_2$(fold change). Then, data normalization was conducted using probabilistic quotient normalization (Dieterle et al., 2006) to perform the principal component analysis (**PCA**) and partial least squares-discriminant analysis (**PLS-DA**). The PCA and PLS-DA models were developed on (1) the identified metabolites (matrix of ^{1}H peaks' area integrals in NMR spectra) and (2) the NMR spectra (bucket matrix). The PCA was used as the first exploratory unsupervised analysis (Chen et al., 2022). In contrast, the PLS-DA is a classification methodology that actively seeks to identify which metabolites differentiate the groups. Furthermore, it incorporates a multivariate perspective, enabling us to observe how metabolites behave collectively across the different classes through the weights. The model validation in the PLS-DA was done by k-fold cross-validation where "k" varied from 3 to 4 based on the number of samples in each group (Kombolo-Ngah et al., 2023). Moreover, the variable importance in projection (**VIP**) was also extracted for the PLS-DA models, and metabolites with VIP values >1 were considered relevant for

Table 3. Descriptive metabolite composition only present in goat and cow milk; concentrations expressed in arbitrary units

Metabolite[1]	n[2]	Mean	Minimum	Maximum	CV, %
2-Oxoglutarate	8	0.52×10^{-4}	0.38×10^{-4}	0.96×10^{-4}	0.37
Carnitine	8	0.17×10^{-4}	0.0053×10^{-4}	0.34×10^{-4}	0.64
Dimethyl sulfone	8	1.3	0.82	1.61	0.21
Glycerophosphocholine	8	26.8×10^{-4}	7.93×10^{-4}	38.6×10^{-4}	0.43
Hippurate	8	0.8	0.28	1.34	0.43
Lactose	8	634	584	665	0.051
N-Acetyl carbohydrates	8	17.8×10^{-4}	1.05×10^{-4}	28.8×10^{-4}	0.66
O-Acetylcarnitine	8	0.33×10^{-4}	0.18×10^{-4}	0.49×10^{-4}	0.35
Phosphocreatine + creatine	8	13.5×10^{-4}	10.7×10^{-4}	17.2×10^{-4}	0.20
Succinate	8	1.9	1.05	3.46	0.42
UDP-galactose	4	0.49×10^{-4}	0	1.32×10^{-4}	1.17
UDP-glucose	4	0.10×10^{-4}	0	0.35×10^{-4}	1.31

[1]UDP = uridine diphosphate.

[2]n = number of samples containing that specific metabolite.

the prediction model (Almeida et al., 2013). The performance of the PLS-DA model was assessed through the accuracy, F-score, precision, sensitivity, and specificity. The F-score is a metric that combines both recall and precision, with a perfect performance of the model with an F-score of 100% (Xu et al., 2024).

RESULTS

Descriptive Statistics

A total of 43 metabolites were identified in the analyzed PBB and milk samples: 18 were present in both PBB and milk (Table 1), 13 were detected in PBB (Table 2), and 12 were identified in milk (Table 3). Among all metabolites, 8 were shared by all milk and PBB types (i.e., valine, acetate, choline, glucose + glucose-6-phosphate [**G6P**], fumarate, formate, ethanol, and alanine; Figure 1). Acetate, alanine, ethanol, and valine were present in all samples, while choline, glucose + G6P, formate, and fumarate were identified in almost all samples (29 out of 30; Table 1).

Five metabolites were consistently identified in all samples across soy-, almond-, oat-, and coconut-based beverages (i.e., leucine, 4-aminobutyrate, malate, aspartate, and phenylalanine; Figure 1). Theophylline and trigonelline were found exclusively in soy- and oat-based beverages. Another shared metabolite between these 2 groups, tryptophan, was also identified in almond-based beverages. Rice-based beverages were the only ones lacking galactose, histidine, and citrate. Additionally, the metabolite sucrose was absent in cow and goat milk and rice-based beverages but was detected in almond-, coconut-, and soy-based beverages. Guanosine was detected exclusively in soy-, oat-, and rice-based beverages, and orotate was found in all cow and goat milk samples and almond-based beverages. Adenosine, present in all goat milk samples, was absent only from cow and coconut-based beverages. Conversely, tyrosine was found in all groups except cow milk. Additionally, uridine was present in cow and goat milk and in soy- and oat-based beverages. Therefore, soy-based beverages had the most diverse metabolic profile among PBB, and goat milk had the richest metabolic profile within the animal group (Figure 1).

As represented in Supplemental Table S2 (see Notes), a total of 36 metabolites discriminate between PBB and milk ($P < 0.05$). Among these metabolites, isoleucine, ethanol, adenosine, alanine, glucose + G6P, acetate, tyrosine, and formate were higher in PBB, while *cis*-aconitate, uridine, galactose, and orotate were lower (Figure 2). Twelve metabolites were statistically significant for milk identification ($P < 0.05$; i.e., adenosine, alanine, galactose, glycerophosphocholine, histidine, N-acetyl carbohydrates, orotate, phosphocreatine + creatine, uridine diphosphate (**UDP**) galactose, UDP-glucose, uridine, and valine; Supplemental Table S2). In cow milk, galactose, N-acetyl carbohydrates, and glycerophosphocholine were higher, whereas phosphocreatine + creatine, alanine, valine, and uridine levels were lower than in goat milk (Figure 3).

Among the analyzed macro-groups, 14 metabolites were significant in differentiating between monocotyledon- and dicotyledon-based beverages (i.e., acetate, adenosine, asparagine, aspartate, choline, *cis*-aconitate, citrate, histidine, isoleucine, leucine, orotate, phenylalanine, *trans*-aconitate, and tryptophan; $P < 0.05$). Citrate, tryptophan, adenosine, asparagine, choline, histidine, aspartate, phenylalanine, acetate, isoleucine, and leucine were higher in dicotyledons (Figures 4A and 4B). Between soy- and fruit-based beverages, 14 metabolites (i.e., adenosine, *cis*-aconitate, citrate, fumarate, guano-

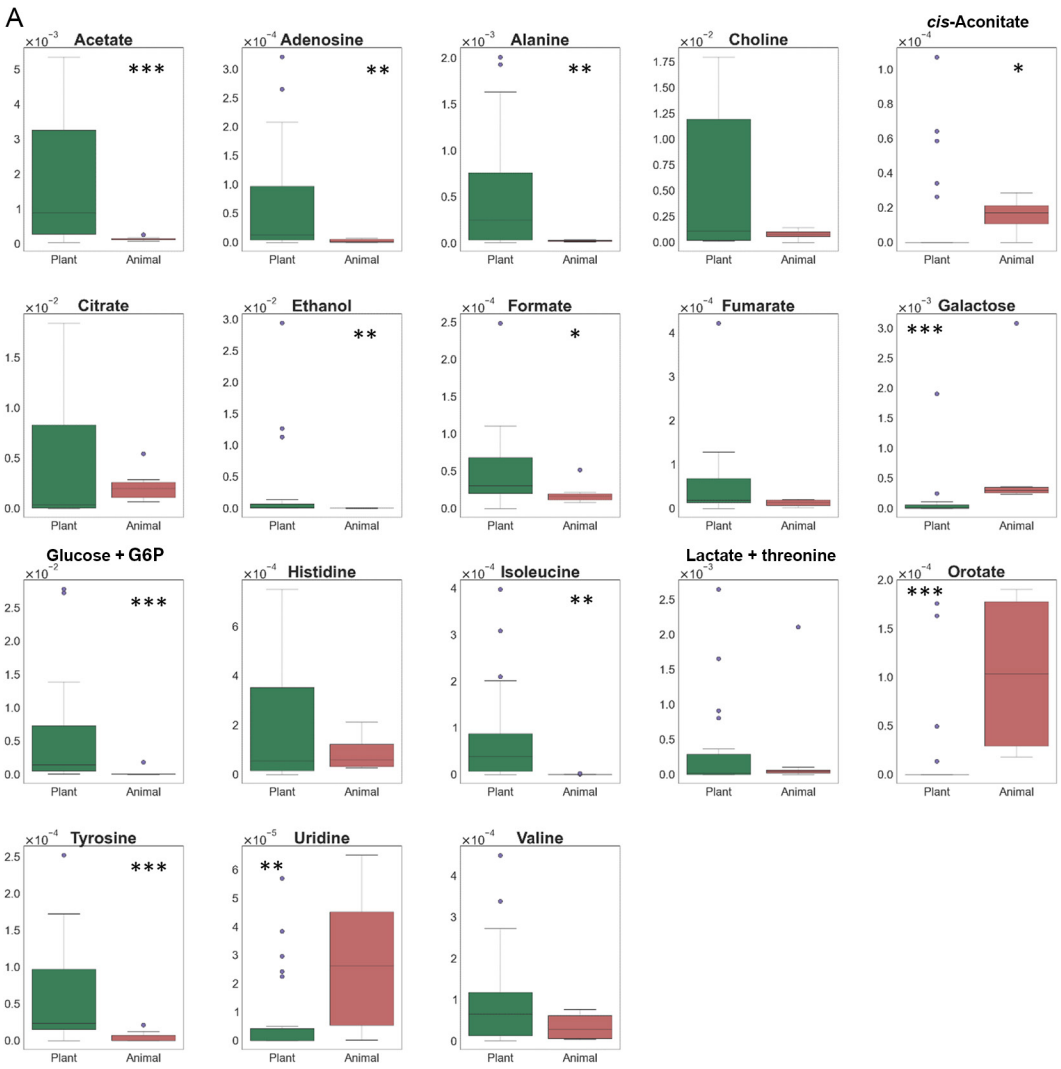


Figure 2. (A) Box plots of the area under the peaks of the identified metabolites in PBB (n = 22) and animal (n = 8) milk samples (cow and goat) represented as boxes for the interquartile range (IQR), with lines in boxes for the median. Samples within whiskers are in 1.5 × IQR. Dots refer to potential outliers. The y-axis concentrations are expressed in arbitrary units. PCr = phosphocreatine; G6P = glucose-6-phosphate; ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. (B) Logarithmic fold change, Log_2(FC), of the area under the peaks of the identified metabolites. Positive Log_2(FC) bars indicate higher metabolite levels in milk samples than in PBB, and negative Log_2(FC) bars indicate lower metabolite levels in milk samples than in PBB samples. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

sine, histidine, lactate + threonine, sucrose, theophylline, *trans*-aconitate, trigonelline, tryptophan, tyrosine, and uridine) differentiated both groups ($P < 0.05$). Adenosine, citrate, histidine, tryptophan, tyrosine, and fumarate were higher in soy, and sucrose and lactate + threonine were lower (Figures 5A and 5B).

Between soy- and cereal-based beverages, 27 metabolites differentiated both groups ($P < 0.05$). Soy presented lower content in maltose (Figures 5A and 5C). Between cereal- and fruit-based beverages, 20 metabolites (i.e., 4-aminobutyrate, acetate, alanine, aspartate, choline, citrate, ethanol, glucose + G6P, guanosine, isoleucine, lactate + threonine, leucine, malate, maltose, phenylalanine, sucrose, theophylline, trigonelline, uridine, and valine) differentiated both groups ($P < 0.05$). Sucrose, ethanol, 4-aminobutyrate, lactate + threonine, isoleucine, malate, alanine, choline, citrate, leucine, valine, phenylalanine, aspartate, and acetate were higher in fruit-based beverages, and glucose + G6P and maltose were lower (Figures 5A and 5D).

Despite aspartate, leucine, and phenylalanine being common metabolites to discriminate among monocotyledon- versus dicotyledon-, fruit- versus cereal-, and cereal- versus soy-based beverages, these metabolites were unable to discriminate between soy- versus fruit-based beverages.

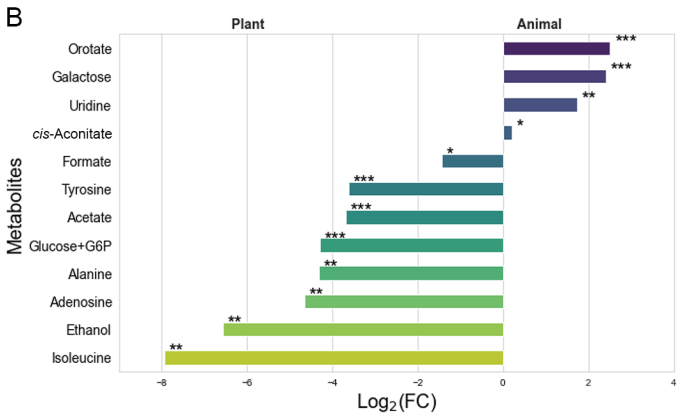


Figure 2 (Continued). (A) Box plots of the area under the peaks of the identified metabolites in PBB (n = 22) and animal (n = 8) milk samples (cow and goat) represented as boxes for the interquartile range (IQR), with lines in boxes for the median. Samples within whiskers are in 1.5 × IQR. Dots refer to potential outliers. The y-axis concentrations are expressed in arbitrary units. PCr = phosphocreatine; G6P = glucose-6-phosphate; ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. (B) Logarithmic fold change, Log_2(FC), of the area under the peaks of the identified metabolites. Positive Log_2(FC) bars indicate higher metabolite levels in milk samples than in PBB, and negative Log_2(FC) bars indicate lower metabolite levels in milk samples than in PBB samples. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

Principal Component Analysis

The PCA, performed on both NMR bucketed spectra and metabolite data, did not reveal outliers (Figure 6). Based on the NMR spectra (Figure 6A), the PCA showed an overlap between the coconut- and almond-based beverage groups, while clearly distinguishing the other groups. The first principal component (PC1) explained 39.51% of the variance and differentiated soy-based beverages and goat milk from the other categories. The second principal component (PC2) explained 13.67% of the variance and mostly differentiated milk from PBB (Figure 6A). Based on the metabolite matrix (Figure 6B), the PCA indicated limited clustering among the coconut-, oat-, and almond-based beverage samples. In contrast, soy- and rice-based beverages were clearly distinguished from the other PBB classes. Although cow and goat milk appeared closer to one another in the PCA plot, they formed distinct clusters, highlighting a clear differentiation between the 2 groups. The PC1 accounted for 51.92% of the variance, showing a clear separation between most PBB and milk and rice-based beverages samples, and PC2 (15.94% of variance) successfully discriminated between milk and soy-based beverages and rice- and oat-based beverages. Overall, the PCA of NMR bucketed spectra (Figure 6A) demonstrated greater discriminatory power than the results based on the metabolic profile (Figure 6B).

Partial Least Squares-Discriminant Analysis

The key metabolites for sample classification determined by the VIP values from the PLS-DA are presented in Supplemental Table S3 (see Notes). Consistent with the Mann-Whitney U test, which identified 12 significant metabolites for discriminating between cow and goat milk, the PLS-DA VIP analysis was based on 10 of those 12 metabolites identified in the Mann-Whitney U test (adenosine, alanine, glycerophosphocholine, histidine, N-acetyl carbohydrates, orotate, UDP-galactose, UDP-glucose, uridine, and valine). Additionally, in agreement with the Mann-Whitney U test, which identified 36 significant metabolites to discriminate PBB from milk, the PLS-DA was based on 18 metabolites, all included in the Mann-Whitney U list ($P < 0.001$).

For monocotyledon- versus dicotyledon-based beverages identification, 11 metabolites contributed to the PLS-DA model (orotate, asparagine, choline, *trans*-aconitate, *cis*-aconitate, adenosine, citrate, sucrose, tryptophan, 4-aminobutyrate, and histidine). Among these, only 4-aminobutyrate and sucrose were not deemed significant with the Mann-Whitney U test. At the same time, acetate, adenosine, aspartate, isoleucine, leucine, and phenylalanine were considered important solely in the Mann-Whitney test. For the classification of soy- and fruit-based beverages, all identified metabolites in the PLSA-DA, except choline, were also recognized by the Mann-Whitney U test. To differentiate cereal- from fruit-based beverages, 15 relevant metabolites in the PLS-DA model were identified, in agreement with the Mann-Whitney U results. The same metabolites were identified in the PLS-DA and the Mann-Whitney U test to discriminate cereal from soy-based beverages. Moreover, guanosine and choline allowed discrimination among soy-, cereal-, and fruit-based beverages groups in the PLS-DA model. Whereas choline and guanosine can be considered important for the identification of all PBB

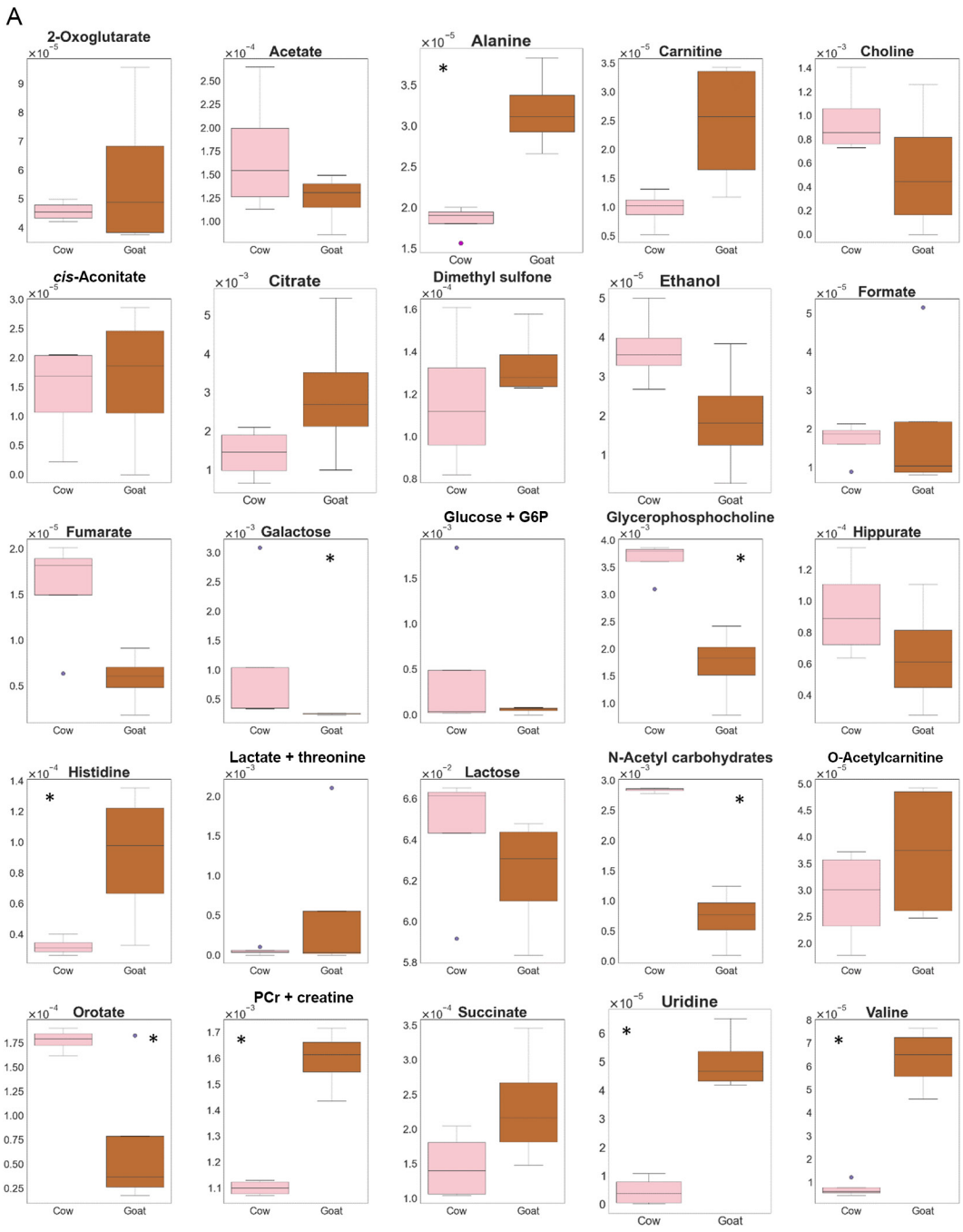


Figure 3. (A) Box plots of the area under the peaks of the identified metabolites in cow (n = 4) and goat (n = 4) milk samples represented as boxes for the interquartile range (IQR), with lines in boxes for the median. Samples within whiskers are in 1.5 × IQR. Dots refer to potential outliers. The y-axis concentrations are expressed in arbitrary units. PCr = phosphocreatine; G6P = glucose-6-phosphate; *$P < 0.05$. (B) Logarithmic fold change, Log_2(FC), of the area under the peaks of the identified metabolites. Positive Log_2(FC) bars indicate higher metabolite levels in cow than in goat milk samples, and negative Log_2(FC) bars indicate lower metabolite levels in cow than in goat milk samples. *$P < 0.05$.

macro-groups, guanosine did not discriminate between monocotyledon- and dicotyledon-based beverages.

The PLS-DA built on the bucketed spectra (Figure 7) and metabolites (Figure 8) accurately predicted all groups with a 100% F1 score, except for monocotyledon- and dicotyledon-based beverages. The F1 scores were 83% for the model built on the spectra and 92% for the model built on the metabolites (Supplemental Table S4, see Notes).

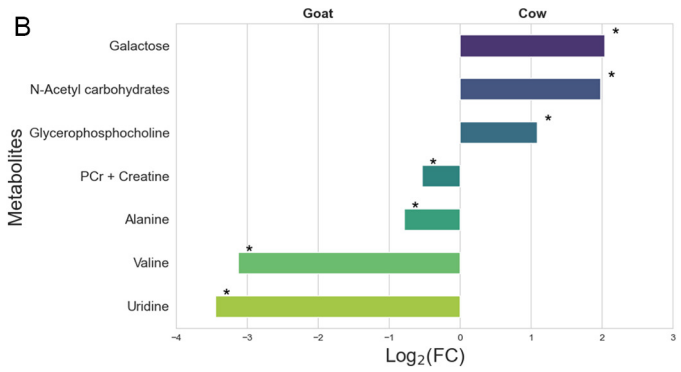


Figure 3 (Continued). (A) Box plots of the area under the peaks of the identified metabolites in cow (n = 4) and goat (n = 4) milk samples represented as boxes for the interquartile range (IQR), with lines in boxes for the median. Samples within whiskers are in 1.5 × IQR. Dots refer to potential outliers. The y-axis concentrations are expressed in arbitrary units. PCr = phosphocreatine; G6P = glucose-6-phosphate; *$P < 0.05$. (B) Logarithmic fold change, Log_2(FC), of the area under the peaks of the identified metabolites. Positive Log_2(FC) bars indicate higher metabolite levels in cow than in goat milk samples, and negative Log_2(FC) bars indicate lower metabolite levels in cow than in goat milk samples. *$P < 0.05$.

DISCUSSION

To our knowledge, this is the first study to use NMR data with both univariate and multivariate approaches to classify PBB, cow, and goat milk. It focused on the analysis of ^{1}H NMR bucketed spectra and the metabolic composition, emphasizing the key metabolites significant for differentiation and their varying abundance within the analyzed macro-groups.

Clustering Analysis of PBB and Milk Based on Metabolic and Spectral Data

The PCA distinctly revealed significant differences among the ^{1}H NMR spectra of 5 (soy-, oat-, and rice-based beverages, and cow and goat milk) out of the 7 analyzed groups. The 2 groups that were not discriminated were fruit-based beverages, i.e., coconut- and almond-based beverages. Moreover, the PCA we performed on the 7 groups based on metabolic content revealed an overlap between almond-, coconut-, and oat-based beverages. This distribution aligns closely with the findings of Moore et al. (2023), where a linear discriminant analysis based on AA and fatty acid content from the same 7 groups we investigated revealed a comparable distribution. In their study, soy-based beverages, cow, and goat milk were distinctly separated, but oat-, coconut-, and almond-based beverages clustered closely together. These results demonstrate that a single NMR spectra-based analysis can achieve a distribution comparable to that obtained from AA and fatty acid analyses, showing its high informative capacity.

The reduced discriminative power we observed between metabolic and spectral data is likely due to the loss of information inherent in spectral data, as well as the nondetection of specific metabolites. This suggests that spectral data, when considered as a whole, provide a more comprehensive view than metabolic data alone. However, when we limited the PCA just to the metabolic profile of almond- and coconut-based beverages, a partial separation was observed, with the corresponding ellipses showing only minor overlap (Supplemental Figure S1a; see Notes), which was not seen in spectra-based PCA performed with these 2 groups (Supplemental Figure S1b). This result aligns with the reported differences in protein, fat, and carbohydrate content, as well as the overall caloric value of almond- and coconut-based beverages reported by Xie et al. (2023). Although spectra information is more complete, in cases of uncertainty, metabolic content can provide additional confirmation, as we observed for almond- and coconut-based beverages.

Comparative Analysis of Metabolic Profiles Between PBB and Milk

Both PBB and milk include metabolites directly involved in AA metabolism. The PBB (e.g., 4-aminobutyrate, asparagine, aspartate, leucine, phenylalanine, and tryptophan) features a strong representation of EAA, such as leucine, phenylalanine, and tryptophan, that are critical for protein synthesis and cellular function (Lopez and Mohiuddin, 2024). In addition to these, 4-aminobutyrate (**GABA**), an inhibitory neurotransmitter derived from glutamate (de Leon and Tadi, 2023), and asparagine and aspartate, both involved in nitrogen metabolism, reflect the dynamic nature of AA pathways in maintaining cellular balance (Brouquisse et al., 1992; Schubert et al., 2021). In contrast, cow and goat milk (2-oxoglutarate, phosphocreatine + creatine) include metabolites acting

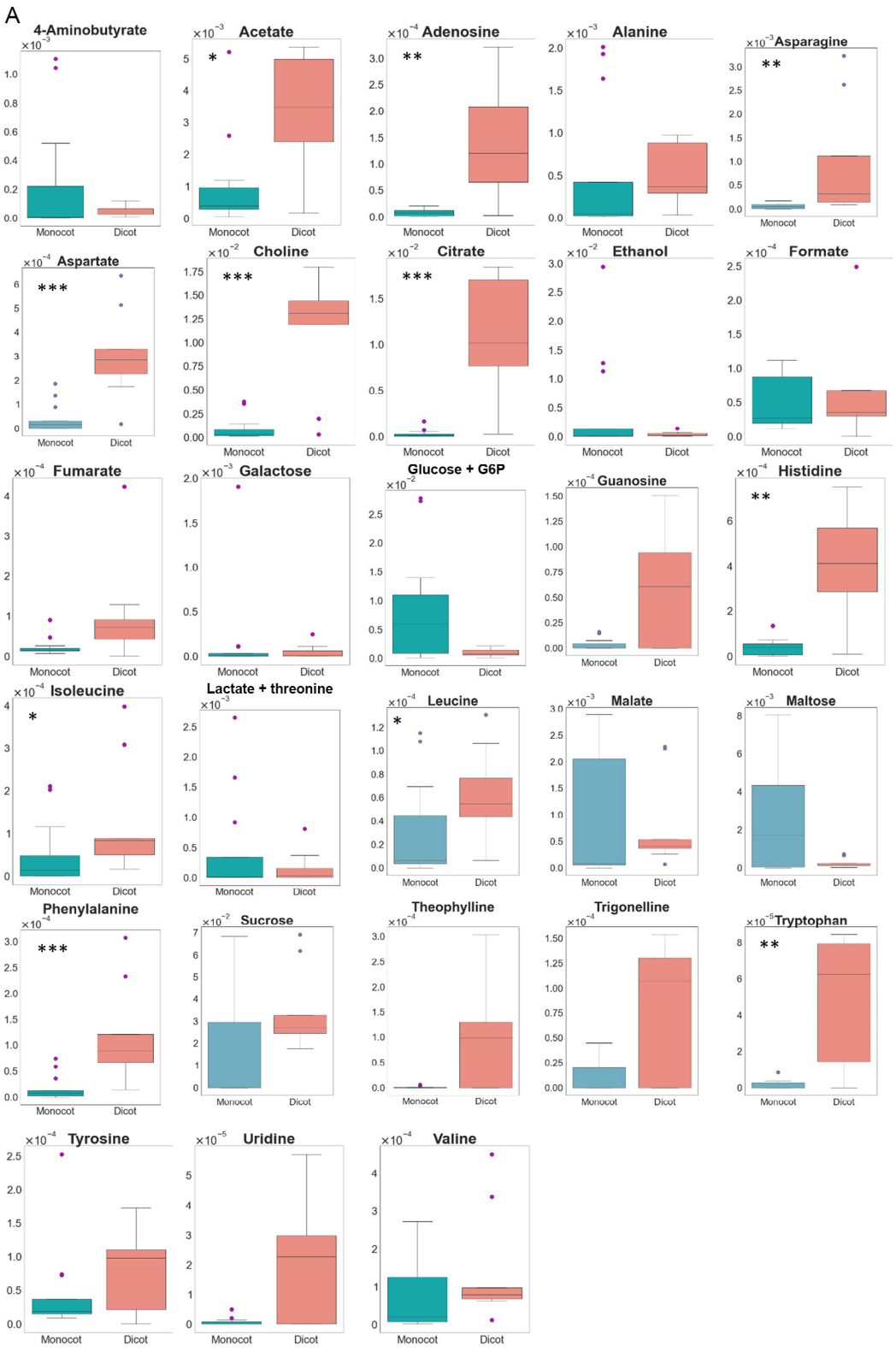


Figure 4. (A) Box plots of the area under the peaks of the identified metabolites in monocotyledon- (Monocot; n = 13) and dicotyledon- (Dicot; n = 9) based beverages samples represented as boxes for the interquartile range (IQR), with lines in the boxes for the median. Samples within whiskers are in 1.5 × IQR. Dots refer to potential outliers. The y-axis concentrations are expressed in arbitrary units. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. (B) Logarithmic fold change, Log_2(FC), of the area under the peaks of the identified metabolites. Positive Log_2(FC) bars indicate higher metabolite levels in dicotyledon-based beverage samples than in monocotyledon-based beverage samples. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

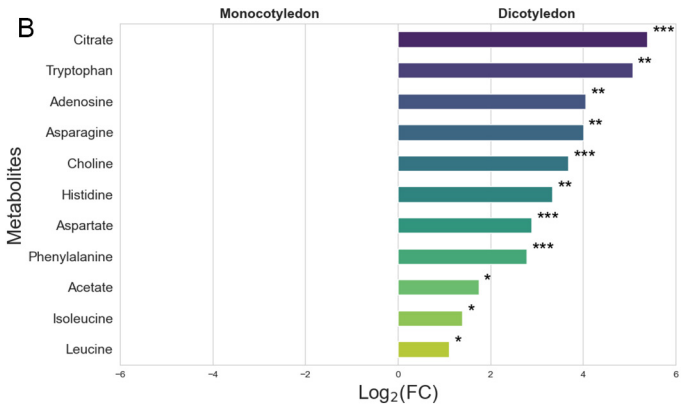


Figure 4 (Continued). (A) Box plots of the area under the peaks of the identified metabolites in monocotyledon- (Monocot; n = 13) and dicotyledon- (Dicot; n = 9) based beverages samples represented as boxes for the interquartile range (IQR), with lines in the boxes for the median. Samples within whiskers are in 1.5 × IQR. Dots refer to potential outliers. The y-axis concentrations are expressed in arbitrary units. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. (B) Logarithmic fold change, Log_2(FC), of the area under the peaks of the identified metabolites. Positive Log_2(FC) bars indicate higher metabolite levels in dicotyledon-based beverage samples than in monocotyledon-based beverage samples. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

as intermediates in AA metabolism or energy-production processes. 2-Oxoglutarate is a key intermediate in the tricarboxylic acid (**TCA**) cycle and plays a pivotal role in AA synthesis, particularly in the formation of glutamate and other AA (Araújo et al., 2014). Phosphocreatine + creatine derivatives from the AA glycine, arginine, and methionine, which are involved in the storage and transfer of high-energy phosphate groups, are crucial for rapid energy regeneration in muscle tissues (Clark, 1997). These compounds highlight the interconnectedness of AA metabolism and energy production, particularly in tissues with high metabolic demands.

The PBB also includes intermediates of carbohydrate metabolism such as malate and *trans*-aconitate, which are critical components of the TCA cycle (Igamberdiev and Eprintsev, 2016), and maltose and sucrose as disaccharides. In contrast, milk metabolites focus more strongly on nucleotide sugars involved in glycosylation and other cellular processes. The UDP-galactose and UDP-glucose contribute to lactose synthesis and overall metabolic functions within the mammary gland (Sadovnikova et al., 2021). Including lactose, a disaccharide, aligns with the metabolic processing of sugars.

Both PBB and cow and goat milk contain metabolites related to energy production. The PBB includes malate and *trans*-aconitate, both of which play pivotal roles in the TCA cycle, a central hub for energy production (Igamberdiev and Eprintsev, 2016). However, milk introduces more specialized compounds related to lipid metabolism and energy storage than PBB. Carnitine, glycerophosphocholine, and O-acetylcarnitine are key players in the transport of fatty acids into mitochondria for β-oxidation, a process central to cellular energy production (Schulz, 1991; Modre-Osprian et al., 2009).

Finally, regarding metabolic diversity and functional complexity, cow and goat milk can be considered richer than PBB. Cow and goat milk represents a broader range of biochemical pathways, covering not only basic processes like AA metabolism and energy production but also specialized pathways such as lipid metabolism. The presence of nucleotide sugars (e.g., UDP-glucose), compounds involved in membrane synthesis (e.g., glycerophosphocholine), and high-energy phosphates (e.g., phosphocreatine) expands the role of cow and goat milk in essential cellular functions beyond simple energy production and AA synthesis. Moreover, cow and goat milk include metabolites involved in critical processes for maintaining cellular integrity, metabolism, and stress responses. In contrast, PBB are more focused on AA metabolism and basic energy-production pathways, which, although essential, represent a narrower spectrum of metabolic functions compared with the broader array of processes found in milk.

Metabolite Characterization in PBB and Milk

Valine, alanine, and acetate were detected across all samples. These metabolites play pivotal roles in cellular metabolism, a common feature being their involvement in the TCA cycle. They either directly participate or contribute with key intermediates essential for energy production and biosynthetic processes (Murín et al., 2009). Valine is an EAA that is not only important for metabolic processes but also for the growth and reproduction of both plants and animals (Wang et al., 2023). Furthermore, research has demonstrated its regulatory effects on gut microbiota in animals (Wang et al., 2023). Walther et al. (2022) reported the presence of valine in bovine milk and various

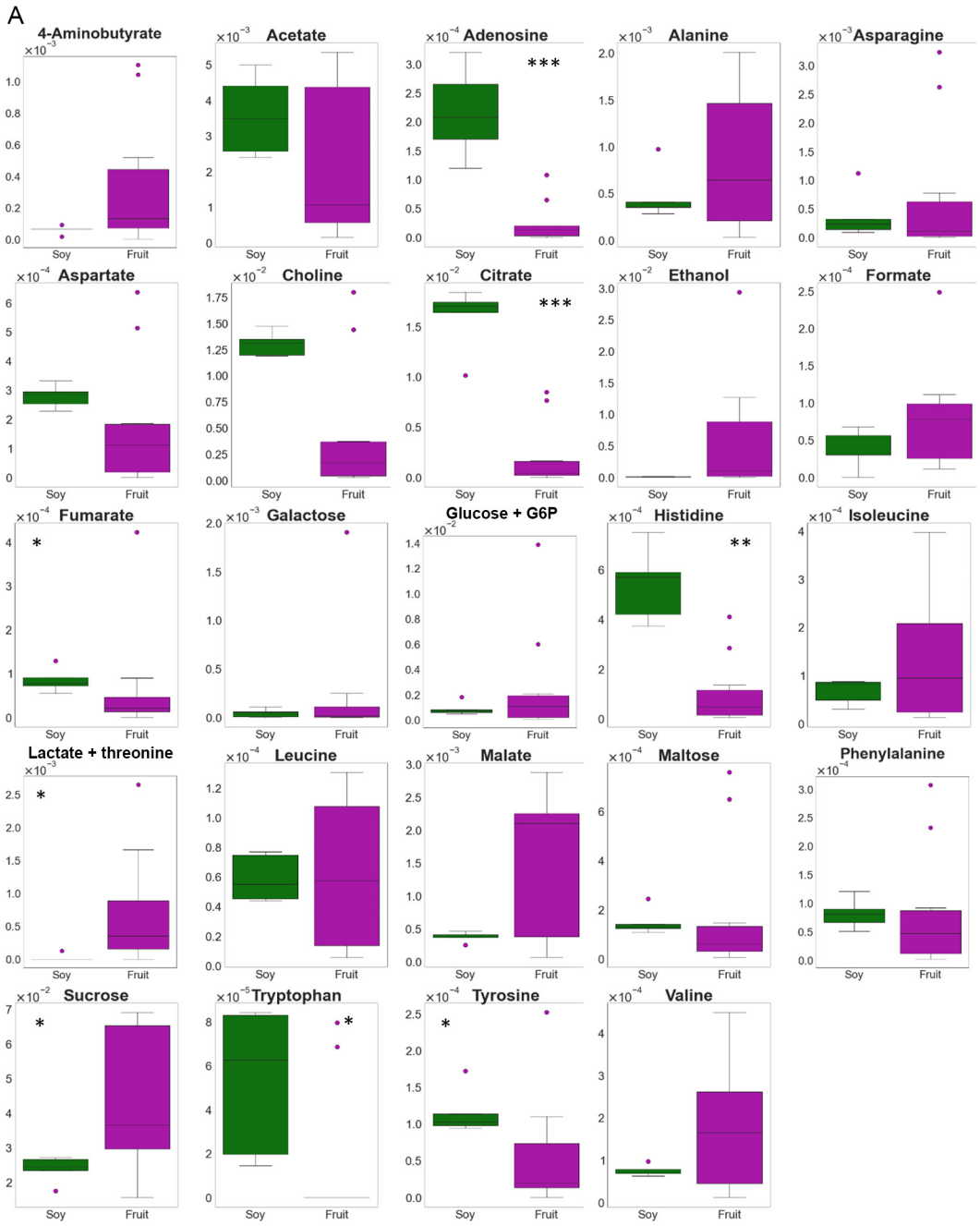


Figure 5. (A) Box plots of the area under the peaks of the identified metabolites in cereal- (n = 7; blue), fruit- (n = 10; purple), and soy- (n = 5; green) based beverages samples represented as boxes for the interquartile range (IQR), with lines in the boxes for the median. Samples within whiskers are in 1.5 × IQR. Dots refer to potential outliers. The y-axis concentrations are expressed in arbitrary units. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. (B) Logarithmic fold change, Log_2(FC), of the area under the peaks of the identified metabolites. Positive Log_2(FC) bars indicate higher metabolite levels in soy-than in fruit-based beverage samples, and negative Log_2(FC) bars indicate lower metabolite levels in soy- than in fruit-based beverage samples. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. (C) Logarithmic fold change [Log_2(FC)] of the area under the peaks of the identified metabolites. Positive Log_2(FC) bars indicate higher metabolite levels in soy- than in cereal-based beverage samples, and negative Log_2(FC) bars indicate lower metabolite levels in soy- than in cereal-based beverage samples. **$P < 0.01$; *$P < 0.05$. (D) Logarithmic fold change, Log_2(FC), of the area under the peaks of the identified metabolites. Positive Log_2(FC) bars indicate higher metabolite levels in fruit-based beverage samples than in cereal-based beverage samples, and negative Log_2(FC) bars indicate lower metabolite levels in fruit-based beverage samples than in cereal-based beverage samples. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

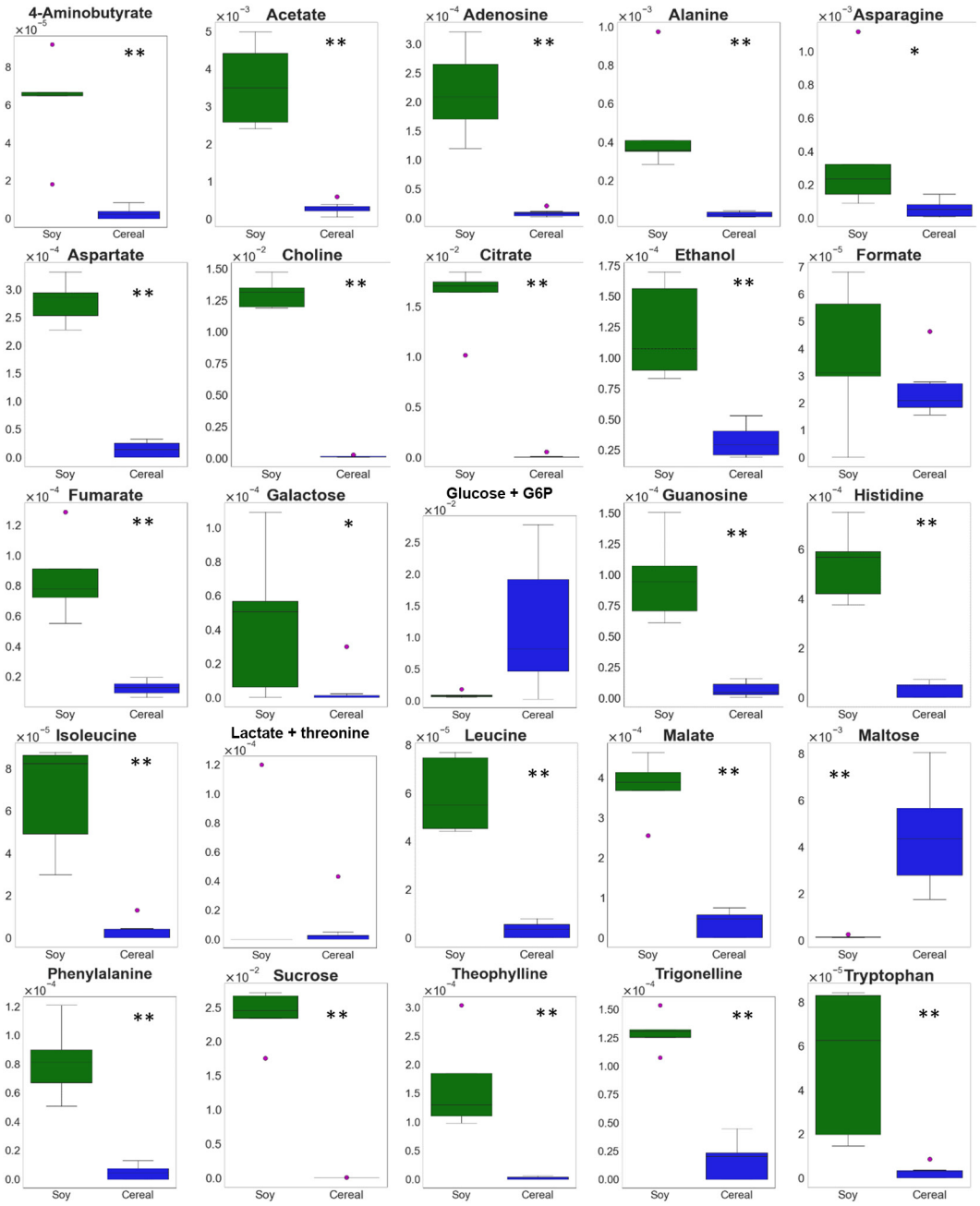
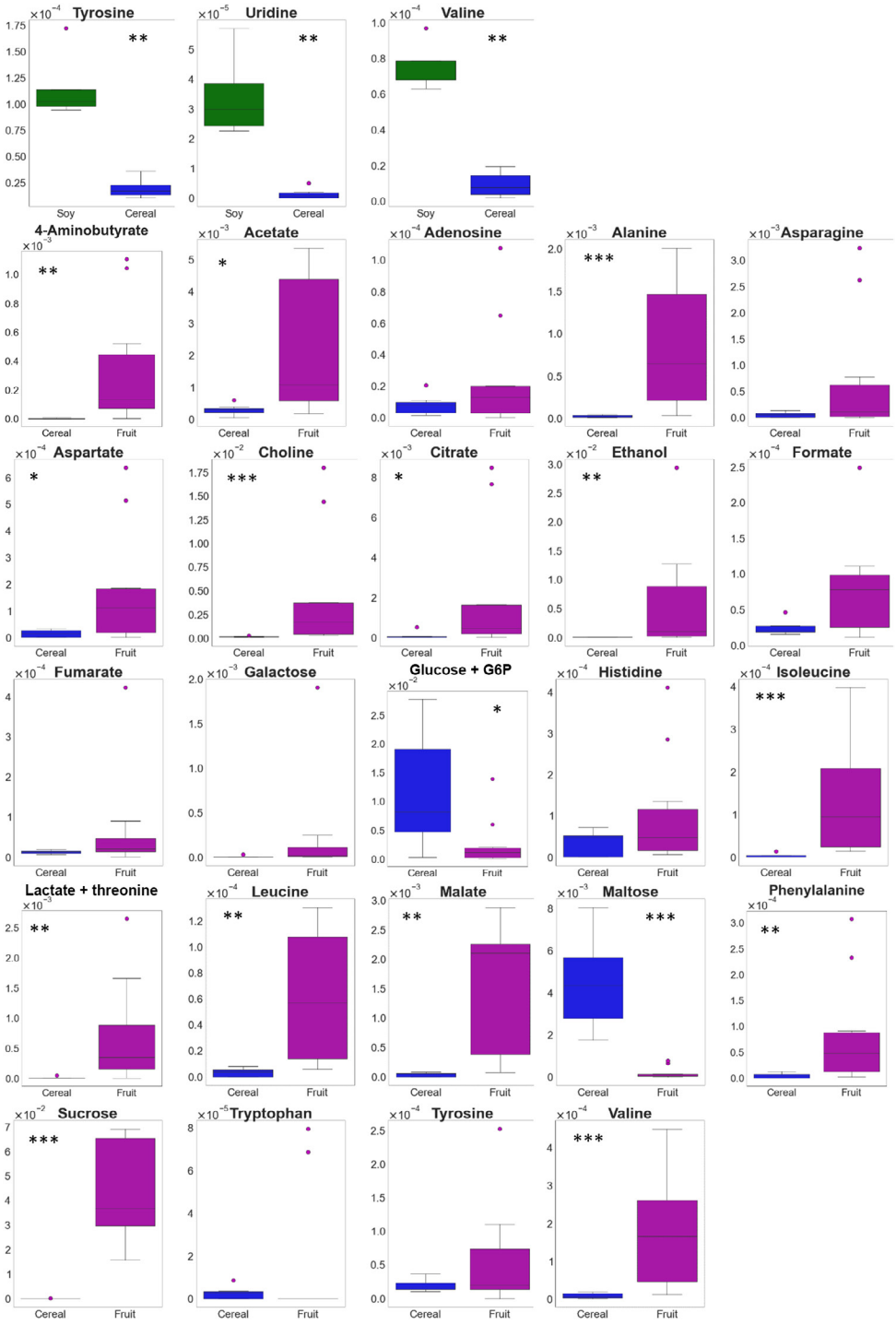


Figure 5 (Continued). (A) Box plots of the area under the peaks of the identified metabolites in cereal- (n = 7; blue), fruit- (n = 10; purple), and soy- (n = 5; green) based beverages samples represented as boxes for the interquartile range (IQR), with lines in the boxes for the median. Samples within whiskers are in 1.5 × IQR. Dots refer to potential outliers. The y-axis concentrations are expressed in arbitrary units. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. (B) Logarithmic fold change, Log_2(FC), of the area under the peaks of the identified metabolites. Positive Log_2(FC) bars indicate higher metabolite levels in soy-than in fruit-based beverage samples, and negative Log_2(FC) bars indicate lower metabolite levels in soy- than in fruit-based beverage samples. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. (C) Logarithmic fold change [Log_2(FC)] of the area under the peaks of the identified metabolites. Positive Log_2(FC) bars indicate higher metabolite levels in soy- than in cereal-based beverage samples, and negative Log_2(FC) bars indicate lower metabolite levels in soy- than in cereal-based beverage samples. **$P < 0.01$; *$P < 0.05$. (D) Logarithmic fold change, Log_2(FC), of the area under the peaks of the identified metabolites. Positive Log_2(FC) bars indicate higher metabolite levels in fruit-based beverage samples than in cereal-based beverage samples, and negative Log_2(FC) bars indicate lower metabolite levels in fruit-based beverage samples than in cereal-based beverage samples. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.



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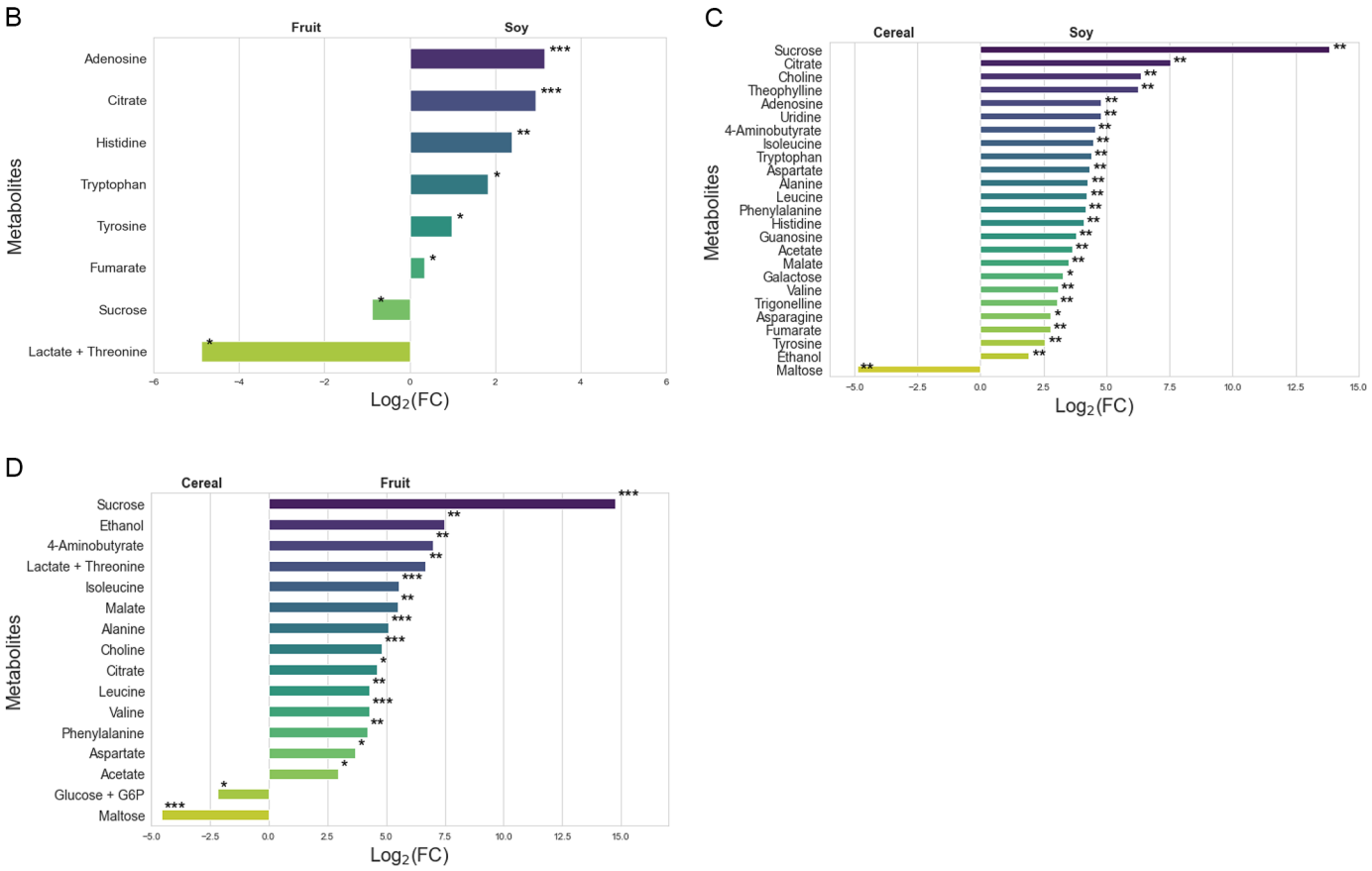

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PBB such as soy-, almond-, and coconut-based varieties, corroborating the findings of our study. Moreover, our study found similar valine content in PBB and milk.

Alanine, an NEAA, was also present in all samples; other than being present in dairy milk (Klein et al., 2013), it has been identified in almonds (House et al., 2019), rice (Jayaprakash et al., 2022), soy (Dudásová and Grancicova, 1992), oat (Vilmane et al., 2015), and coconut (Magnaval et al., 1995). Moreover, alanine can be used when evaluating Amarone wine based on ^{1}H NMR spectral peaks (Consonni and Cagliani, 2019). Additionally, alanine levels are dynamically regulated in plants under stress conditions. Those levels increase in *Arabidopsis thaliana* during cadmium exposure (Sun et al., 2010) and remain unaffected by nitrogen deficiency in *Lotus japonicas* (Rocha et al., 2010). This underscores alanine's role as a biomarker and a participant in stress response. In our study, we observed a significantly higher alanine content in PBB than in milk. Furthermore, alanine levels were higher in soy- and fruit-based beverages when compared with cereal-based beverages, with no differences between soy- and fruit-based beverages. A study by Dandare et al. (2021) in rats demonstrated that L-alanine supplementation significantly reduced weight and blood glucose levels, and Brennan et al. (2002) reported a correlation between higher L-alanine content and enhanced glucose metabolism and pancreatic β-cell function. These findings highlight the importance of

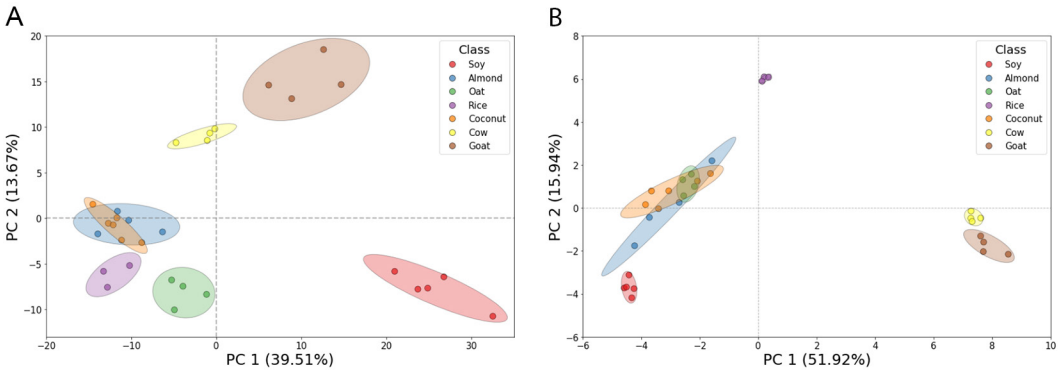

Figure 6. Score plot of first (PC1) and second (PC2) principal components of the principal component analysis (PCA). (A) The PCA of NMR bucketed spectra; (B) the PCA of NMR metabolites. The ellipse represents the 95% CI. Each dot in the plots represents a sample, and different colors represent the different categories of samples.

understanding L-alanine levels in various food sources, enabling informed dietary choices.

Acetate is critical in cow and goat milk production, serving as a key precursor for fat synthesis (Urrutia and Harvatine, 2017). Its concentration is influenced by dietary factors and species-specific metabolic pathways, reflecting the nuanced metabolic adaptations of milk-producing animals. Beyond its involvement in milk production, acetate is indispensable for numerous cellular processes in plants and animals. In animals, acetate has a role in modulating immune responses and maintaining cellular homeostasis (Che-Othman et al., 2020; Choi et al., 2021). In this study, acetate content was higher in PBB than in milk, but similar between cow and goat milk, whereas Li et al. (2017) reported a greater content of acetate in goat than in cow milk.

Ethanol, which was also identified in all samples, may be present in PBB as a result of industrial processing, particularly during flavor extraction (Plaskova and Mlcek, 2023). In dairy milk, ethanol is found in normal conditions at about 0.8 μ*M*, but during storage, ethanol levels can rise nonlinearly alongside other metabolites like formic acid and lactic acid due to temperature and duration of storage, positioning ethanol as a potential indicator for assessing milk spoilage (Edwards et al., 2021). In the present study, ethanol content was higher in PBB, indicating its possible addition during production, and no significant difference was observed between milk and cow goat. In normal food applications, ethanol is used in minimal amounts, making it unlikely to pose a health hazard unless consumed in large quantities.

We exclusively identified trigonelline and theophylline in soy- and oat-based beverages, highlighting their potential as biomarkers for distinguishing these products. Trigonelline identification in soy-based beverages aligns with the findings in Brigante et al. (2024), who identified trigonelline in human urine as a reliable biomarker for soy intake. Trigonelline, a plant-derived alkaloid, is also found in coffee beans, garden peas, hemp seeds, barley, corn, tomato, onion, cantaloupe, and Tung-kua-jen (Gupta et al., 2021). Furthermore, trigonelline is suggested to have therapeutic properties, particularly in managing diabetes and central nervous system disorders (Zhou et al., 2012). In our study, trigonelline content was higher in soy- compared with cereal-based beverages. Theophylline is an alkaloid that is also naturally present in coffee, black tea, chocolate, and dried mate (Montaño et al., 2022). It is employed in managing respiratory conditions characterized by airway obstruction, including asthma and chronic bronchitis (Montaño et al., 2022). The presence and concentration of theophylline in PBB remain largely unexplored, with no comprehensive studies currently available to evaluate its role or variability within these products.

We exclusively detected the EAA tryptophan in soy-, almond-, and oat-based beverages, which partially agrees with Walther et al. (2022). These authors identified tryptophan in soy- and almond-based beverages through ultra-performance liquid chromatography and confirmed its absence in both bovine milk and rice-based beverages. However, they also identified tryptophan in coconut-based beverages. Our results showed that tryptophan content was higher in soy- than in fruit- and cereal-based beverages, with a similar level between cereal- and fruit-based beverages. Tryptophan is essential for brain function, and because serotonin is produced by this metabolite its nutritional imbalance can influence serotonin brain levels, which is correlated with cognitive performance in stress-vulnerable people (Markus et al., 2002).

Asparagine and maltose, which we identified exclusively in the PBB, are naturally present in plants and play critical roles in metabolic pathways, contributing to

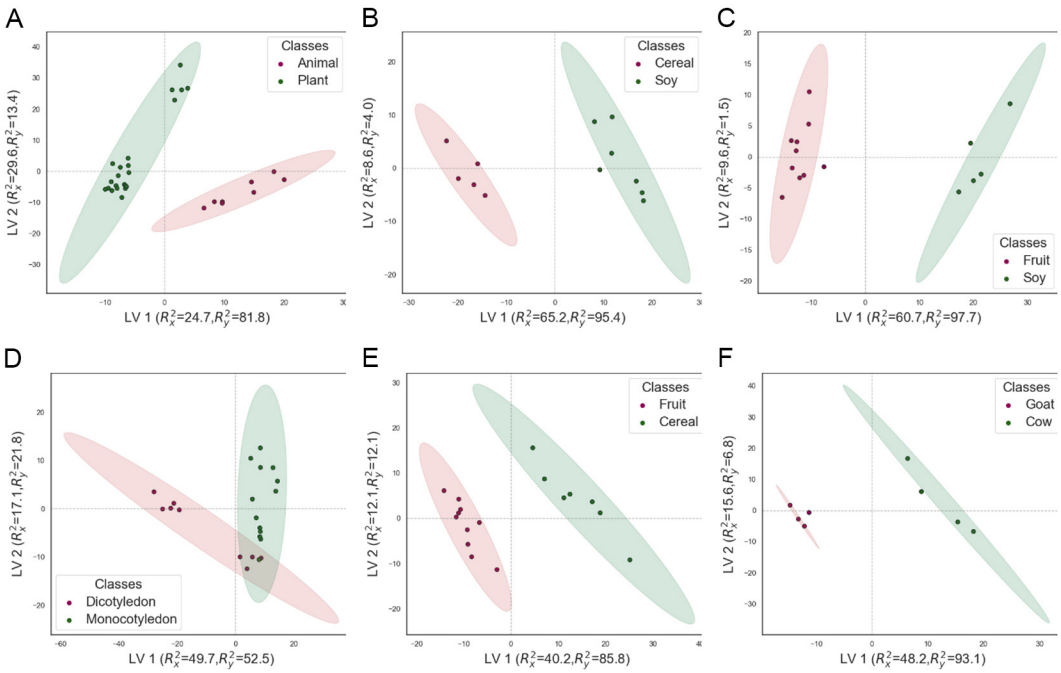

Figure 7. Score plot of partial least square-discriminant analysis from NMR bucketed spectra. (A) Milk versus PBB; (B) soy- versus cereal-based beverages; (C) soy- versus fruit-based beverages; (D) monocotyledon- versus dicotyledon-based beverages; (E) cereal- versus fruit-based beverages; (F) cow versus goat milk. LV = latent variable. R_x^2 = fraction of variance in X. R_y^2 = fraction of variance in Y. Each dot in the plots represents a sample; different colors represent the different categories of samples.

growth, development, and stress responses (Oddy et al., 2020). However, our finding contrasts with the results of Kang et al. (2022), who detected asparagine in bovine milk using HPLC and emphasized its significance in determining the geographical origin of milk. The findings regarding maltose are consistent with those of Jeske et al. (2018), who identified this disaccharide as commonly present in PBB but absent in milk, where lactose and galactose are the predominant sugars.

Discrimination Among PBB and Milk Macro-Groups

In our study, lactose emerged as the most significant metabolite for distinguishing between PBB and milk, with a strong association with several metabolites. Particularly, orotate, which was more abundant in cow and goat milk compared with PBB in the present study, has been linked to SCC, providing valuable insight into the health status of the cow (Bobbo et al., 2022). However, the presence and concentration of orotate in PBB remain unexamined, highlighting a significant gap in understanding its potential role and implications in PBB.

We identified adenosine, alanine, glycerophosphocholine, histidine, N-acetylcarbohydrates, orotate, UDP-galactose, UDP-glucose, uridine, and valine as key metabolites distinguishing cow and goat milk. Although UDP-glucose and UDP-galactose are necessary for lactose synthesis and are usually present in both cow and goat milk, in our study, these metabolites were not detected in cow milk. The greater levels of adenosine in goat than cow milk agreed with Zulak et al. (1976), who described a lower concentration of adenosine in cow and human milk than in goat milk. In addition to adenosine, glycerophosphocholine, histidine, orotate, and valine are typically present in both goat and cow milk. However, specific concentrations for these metabolites are not well-documented in the literature. These metabolites play important roles in milk nutritional profiles. Histidine and valine are EAA that are crucial for protein synthesis and various metabolic functions (Brosnan and Brosnan, 2020; Sharma et al., 2024b). In our study glycerophosphocholine was more abundant in cow than in goat milk, while valine was greater in goat milk, which was in line with Landi et al. (2021), who reported a greater content of valine in goat and sheep than in cow raw milk.

Choline demonstrated considerable effectiveness as a marker for identifying PBB macro-groups because plant sources contribute significantly to the overall free choline content (Van Parys et al., 2021). In our study, choline content was greater in dicotyledon- than in monocotyledon-based beverages, greater in soy- than in cereal-based beverages, and greater in fruit- than in cereal-based

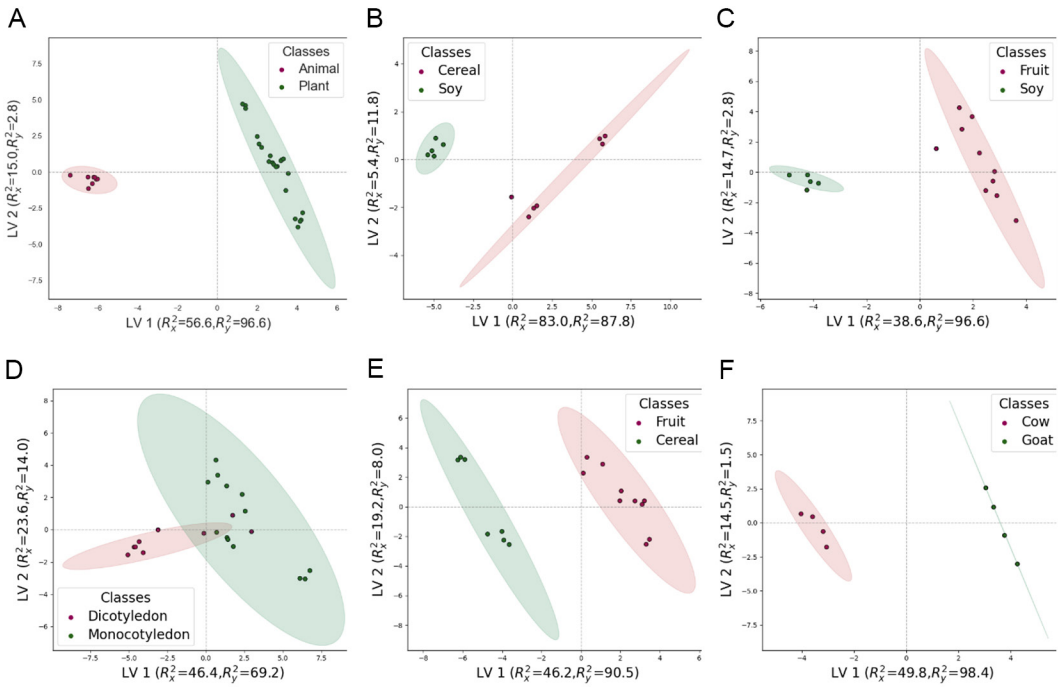

Figure 8. Score plot of partial least square-discriminant analysis from the area under the peaks of the identified metabolites. (A) Milk versus PBB; (B) soy- versus cereal-based beverages; (C) soy- versus fruit-based beverages; (D) monocotyledon- versus dicotyledon-based beverages; (E) cereal- versus fruit-based beverages; (F) cow versus goat milk. LV = latent variable. R_x^2 = fraction of variance in X. R_y^2 = fraction of variance in Y. Each dot in the plots represents a sample; different colors represent the different categories of samples.

beverages. Insufficient choline intake has been linked to an increased risk of cancer and neurodegenerative disorders (Zeisel et al., 2018). The information provided in the present study on the level of choline content among different PBB can guide the selection of the most suitable beverage to meet individual needs.

We identified the AA aspartate, leucine, and phenylalanine as key metabolites for distinguishing monocotyledon- versus dicotyledon-, fruit- versus cereal-, and cereal- versus soy-based beverages. Leucine, phenylalanine, and aspartate content were greater in soy- than cereal-based beverages, suggesting a higher content of these metabolites in legumes than in cereals, as reported by Bonke et al. (2020), who observed a greater content in lentils and peas than in oats. Moreover, we observed a greater content of aspartate, leucine, and phenylalanine in fruit- than in cereal-based beverages. In contrast, our data showed that these same metabolites did not discriminate between soy- and fruit-based beverages. However, leucine, phenylalanine, and aspartate concentrations are expected to be higher in soy- than in fruit-based beverages due to their relatively greater protein content (Moore et al., 2023). Nevertheless, the discrepancy could be related to the production of the drinks, because the industrial process is based on the use of different technologies, such as heating and the addition of stabilizers, which may reduce the solubility and bioavailability of the proteins (Poliseli-Scopel et al., 2014).

The 100% accuracy achieved for all classificatory groups except for monocotyledons and dicotyledons indicates the strong predictive capabilities for classifying PBB macro-groups and milk.

CONCLUSIONS

The PCA applied to NMR bucketed spectra was highly effective, providing an overall clear separation between the different classes of PBB and milk. Moreover, metabolite fingerprinting proved to be an effective tool to discriminate among different PBB, cow, and goat milk. Furthermore, the combination of the Mann-Whitney U test and VIP scores from PLS-DA proved highly effective in identifying key metabolites that discriminate between PBB and milk, providing valuable insights into their metabolic profiles. Adenosine, alanine, glycerophosphocholine, histidine, N-acetylcarbohydrates, orotate, UDP-galactose, UDP-glucose, uridine, and valine were identified as key metabolites for the classification of goat and cow milk. Choline and guanosine emerged as important markers for PBB macro-group detection.

Additionally, lactose was a crucial factor in distinguishing animal milk from PBB. These findings highlight metabolite fingerprinting as a reliable method for classification, product authentication, and quality control in the dairy, goat milk, and PBB industries.

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Nonstandard abbreviations used: 1D = one-dimensional; FC = fold change; ^{1}H = hydrogen-1 isotope (proton); IQR = interquartile range; G6P = glucose-6-phosphate; GABA = 4-aminobutyrate; LV = latent variable; NMR = nuclear magnetic resonance; NOESY = nuclear Overhauser effect spectroscopy; PBB = plant-based beverages; PCA = principal component analysis; PC1 = first principal component; PC2 = second principal component; PLS-DA = partial least squares-discriminant analysis; TCA = tricarboxylic acid; UDP = uridine diphosphate; VIP = variable importance in projection.

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