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Research Paper

Primary metabolite profiling by NMR reveals the key role of sugars and organic acids in driving the domestication process in apple

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ABSTRACT

Primary metabolites are essential molecules playing a central role in plant biology and in the determination of fundamental fruit quality characteristics, such as sweetness and acidity. In this work, carbohydrates, organic and amino acids have been assessed with NMR for a large-scale screening in the apple flesh of an interspecific collection comprising 43 *Malus* species. Among the analyzed compounds, seven metabolites, with a Variable Importance in Projection score greater than 1, largely contributed to the distinction between wild *Malus* spp. and *M. domestica* accessions. The three most relevant compounds were sucrose, fructose and malic acid, each showing a distinct pattern. In general, sugars were more concentrated in the *M. domestica* group, while organic acids predominated in wild *Malus* species. Four amino acids were also detected, and although their overall contribution was smaller, they were more concentrated in *M. domestica* accessions. This metabolic survey elucidates the role of these specific primary metabolites in apple as domestication associated traits and provides a valuable resource for identifying promising accessions for modern breeding programs aimed at improving fruit quality.

1. Introduction

The domestication process in apples started in Central Asia, in the region of Tian Shan and progressed westward along the so-called Silk Route (Cornille et al., 2012). The domestication of apple initially recruited large mammal seed-dispersal and continued through the human-driven selection (Spengler, 2019). The domestication syndrome led to the fixation of phenotypes related to the quality features typical of the dessert apples, such as increased fruit size, texture and taste and a parallel decrease of acidity and astringency. These attributes are related to a myriad of different molecules, among which the most relevant are represented by primary metabolites (Cornille et al., 2014), a diverse group of essential molecules playing key roles in fundamental plant processes, including growth, development, and reproduction (Maeda et al., 2019). In this study, primary metabolites were defined as carbohydrates, organic acids, and amino acids, which are central to fruit development and quality. Carbohydrates, including sucrose and fructose, are produced via photosynthetic carbon fixation and can be stored as starch or transported to the vacuole, contributing to sweetness and turgidity (Stein and Granot, 2018; Dennis and Blakeley, 2000; Yamaki, 2010; Li et al., 2012; Maeda et al., 2019; Tijero et al., 2021). Organic acids, mainly malate and citrate, are synthesized through the TCA cycle and determine fruit acidity (Carrari et al., 2006; Mounet et al., 2009; Beauvoit et al., 2018; Etienne et al., 2013). Amino acids, derived from glycolysis and TCA cycle intermediates, form the building blocks of proteins essential for fruit growth (Stepansky and Laustek, 2006; Maeda and Dudareva, 2012; Forde and Lea, 2007; Pratelli and Pilot, 2014).

The quantification of these metabolites has already been investigated in apples due to their impact on fruit quality perception by consumers (Harker et al., 2006). In apples, the total sweetness is primarily determined by fructose and glucose, with fructose being the main compound (Begić-Akagić et al., 2014; Castel et al., 2020; Nookaraju et al., 2010), followed by sucrose and sorbitol (Amor et al., 1995; Buckeridge et al., 1999; Fan et al., 2009; Park et al., 2002; Pleyerová et al., 2022; Winter and Huber, 2000; Zhang et al., 2004). Sugar content shows substantial variation among apple cultivars and *Malus* species (Ma et al., 2015),

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reflecting both genetic background and domestication history. Similarly to sugars, organic acids also vary within the *Malus* genus, with malic and citric acids being the most abundant and contributing together to about 90 % of the total acidity of apple (Ma et al., 2018). Beyond these major primary metabolites, amino acids and secondary metabolites such as flavonoids play important roles in determining fruit quality, including flavor, nutritional value, pigmentation, and defense (Forde and Lea, 2007; Pratelli and Pilot, 2014; Stepansky and Laustek, 2006; Maeda and Dudareva, 2012). Their concentrations are also influenced by both genetic factors and domestication, highlighting that selection during apple domestication has acted on a broader network of metabolic pathways than sugars and acids alone. Due to the relevance of primary and secondary metabolites in the human diet, it is crucial to provide a comprehensive characterization of these compounds in apples, one of the most cultivated fruits worldwide.

Nowadays, wild apple species are considered as valuable genetic resources to improve agronomic traits such as disease resistance and environmental resilience (Bhora et al., 2022). Despite their recognized value for these traits, their potential contribution to variation in primary metabolites—compounds that directly influence fruit sweetness, acidity, and overall quality—has been largely overlooked. While modern breeding programs have focused on selecting *M. domestica* cultivars with favorable taste and size, wild *Malus* accessions may harbor allelic diversity capable of enhancing these primary metabolite profiles. Comprehensive metabolic profiling of both domesticated and wild apple accessions therefore provides a unique opportunity to identify promising wild alleles that could be integrated into breeding programs, ultimately improving fruit quality while retaining traits such as disease resistance and environmental adaptability.

In this work, the major primary metabolites, such as carbohydrates, organic acids and amino acids, have been investigated within an interspecific apple collection by employing Nuclear Magnetic Resonance (NMR) instead of classical chromatography strategies. NMR technique is in fact a valuable and more rapid approach for the determination of these molecules, especially for the reduced time needed for sample preparation, making this a suitable strategy to analyze different compounds in a large and diverse sample sets (Navarro et al., 2020; Biswas et al., 2025). This technique has been successfully applied to characterize the three categories of primary metabolites in different fruit matrices, confirming its applicability and efficiency (del Campo et al., 2006; Villa-Ruano et al., 2019; Botoran et al., 2019; Montgomery et al., 2024; Filho et al., 2025; Ma et al., 2025). In this study, we used NMR-based metabolomics to quantify the major primary metabolites in the fruit flesh of a diverse *Malus* collection and underlying the key domestication assessed metabolites shaped during the apple domestication.

2. Materials and methods

2.1. Plant material and sample preparation

In this work, an interspecific apple collection of 159 *Malus* spp. accessions representing 43 different species was employed (Supp. Table 1). Apple trees were grown in three replicates in two orchards with similar pedo-climatic conditions in the Trentino Alto-Adige region of northern Italy. The wild *Malus* spp. accessions were collected at the experimental orchard of the Fondazione Edmund Mach (San Michele all'Adige, Italy), whereas the *M. domestica* accessions belong to the RefPOP, a reference apple collection comprising the genetic diversity existing within *M. domestica* (Jung et al., 2020) grown and maintained at the Laimburg Research Centre (in Bolzano, Italy). Five apples were collected from each accession at horticultural maturity which was determined using standard management practices, including skin color evaluation, starch index and data from previous seasons. At harvest, also fruit diameter and weight were measured. The skin tissue of each fruit was initially removed from the fleshy part, which was then frozen in presence of liquid nitrogen and ground to a fine powder with an IKA analytical mill (IKA-Werke GmbH & Co. KG, Staufen, Germany). Samples were then stored in 15 ml falcon tubes at -80 °C upon further processing steps for metabolite screening. Each sample eventually underwent NMR analysis for the assessment of specific primary metabolites. For each accession, five fruits were collected and three biological replicates were prepared. Each biological replicate was analyzed independently for metabolite profiling.

2.2. NMR spectroscopy: apple sample preparation and analysis

Two grams of frozen apple flesh tissue were weighed (± 1.5 - 3 %) and defrosted at +4°C in a 2 ml centrifuge plastic vial. It was then centrifuged at 14,000 RPM for 20 min, frozen at -20 °C and defrosted prior to analysis. 100 µl of phosphate D_2O juice buffer (Schievano et al., 2017) were added to 900 µl of the previously prepared supernatant juice. The mixture was shaken for 20 min at 1000 RPM, after which 600 µl were inserted into a 5 mm NMR tube (2001,745, Hilgenberg GmbH, Malsfeld, Germany). In the presence of visible particles, a 0.22 µm 13-mm PVDF syringe filter (Merck Millipore, USA), was used to improve the clarity of the solution. The spectra were generally recorded within 24 h from sample preparation.

2.3. Acquisition of the spectra

A Bruker Avance Neo 400 spectrometer was utilized to record NMR spectra, equipped with a broadband Z-gradient probe for 5 mm sample tubes and 60-position fast SampleXpress autosampler (Bruker BioSpin GmbH, Rheinstetten, Germany), operating at a base frequency of 400.13 MHz for proton (^{1}H) nuclei measurement. Acquired spectra were automatically processed through a Topspin 4.3.9 with Icon NMR 6.1.0 and apk0.noe as phase correction program. The 9:1 mixture of H_2O and D_2O (v/v) was used to optimize the deuterium lock signal. The experimental parameters of SGF profiling routine (Spraul et al., 2009) were followed to record proton NMR spectra. For each pulse a power level of 51.16 dB (25 Hz suppression window) with a *noesygppr1d* pulse sequence was utilized, the size of the spectrum window (sweep width, SW) was 20.83 ppm, time domain (TD) consisted of 131,072 (128 K) data points, number of scans (NS) was 64 and the number of dummy scans (DS) was 4, time for relaxation delay (D1) was 10 sec, receiver gain (RG) for all spectra was fixed at 16, and *baseopt* digitization mode was used. Automatic adjustment of the probe (ATMA routine) and automatic shimming (TOPSHIM) were performed before each spectrum acquisition.

2.4. NMR data processing

To perform the quantitative analysis, an Assure NMR software (Colson et al., 2012) coupled with an external standard method known as ERETIC technique (electronic reference to access in vivo concentrations, (Akoka et al., 1999; Hong et al., 2013)) was utilized. The external standard method was selected over the external standard such as TMSP-d4 (3-(trimethylsilyl)-2,2,3,3-tetradeuteropropionic acid) or DSS (2,2-Dimethyl-2-silapentane-5-sulfonate sodium salt) due to the well-known possibility of interaction of such molecules with complex plant matrices (Beckonet et al., 2007, Gowda et al., 2021).

A solvent, containing a 9:1 mixture of H_2O and D_2O (v/v) was used to dissolve a sample of interest, and a second sample of 2 mmol sucrose in the same solvent in a flame-sealed tube (Z157174, Bruker BioSpin GmbH) was recorded following the same experimental parameters as described in the previous section with respect to the sample of interest. The signals of glucopyranosil-α-C1 proton (5.40 ppm, d), fructofuranosyl-ß-C3 proton (4.2 ppm, B part of the AB system), and fructopyranosyl-C2 methylene protons (3.67 ppm, s) were used as ERETIC reference signals. The 90° pulse was calibrated by the *pulsecal* automatic sub-routine executed during acquisition of all spectra. A periodic validation was performed measuring the sucrose standard against

another one, in a flame-sealed tube (20 mmol sucrose and hippuric acid in water each, Z157762, Bruker BioSpin GmbH), following the same experimental parameters as above. For the analysis, the accuracy with the external standard is reported to be at 95 % (Cullen et al., 2013). Individual compounds were identified either in automation mode, with AssureNMR utilizing the Human Metabolome Database (HMDB) (Wishart et al., 2022) and the BBIOREFCODE database of NMR metabolites (v.2.01, Bruker BioSpin GmbH, Rheinstetten, Germany) or manually using data reported in literature (Di Matteo et al., 2021; Eisenmann et al., 2016; Tomita et al., 2015). Signal integration was performed on manually validated, well-resolved resonances using AssureNMR, ensuring the absence of overlap and baseline distortion. For compound identification, automated matching against HMDB and BBIOREFCODE databases was complemented by manual inspection of chemical shifts, multiplicity patterns and literature references. Metabolites presenting severe spectral overlap in crowded carbohydrate regions were carefully evaluated. Compounds whose proton resonances overlapped extensively with other abundant sugars were excluded from quantitative analysis when reliable deconvolution was not achievable.

2.5. Data analysis

Multivariate statistical analyses were performed through the utilization of the software R 4.3.2 and the external packages “ggplot2” for boxplots, whereas “mixOmics” was used for the generation of PCAs on the Log10 transformed data. Statistical significance for this study was defined with a *t*-test considering *P-values* adjusted of 0.05 corrected with Benjamini-Hockberg procedure (FDR for multiple comparison). Fold change was based on arithmetic means. The Pearson based correlation and visualization was obtained with the "Corrplot" R package.

3. Results

3.1. Distribution of primary metabolites within the interspecific apple collection

The concentration of the three categories of primary metabolites assessed in this work by NMR (carbohydrate, organic and amino acids) were profiled in the flesh tissue of the apples collected from each of the 159 accessions. The variability of each metabolite for each accession was initially analyzed through the multivariate statistical analysis of principal component analysis (PCA). The PCA-2D plot was defined by the

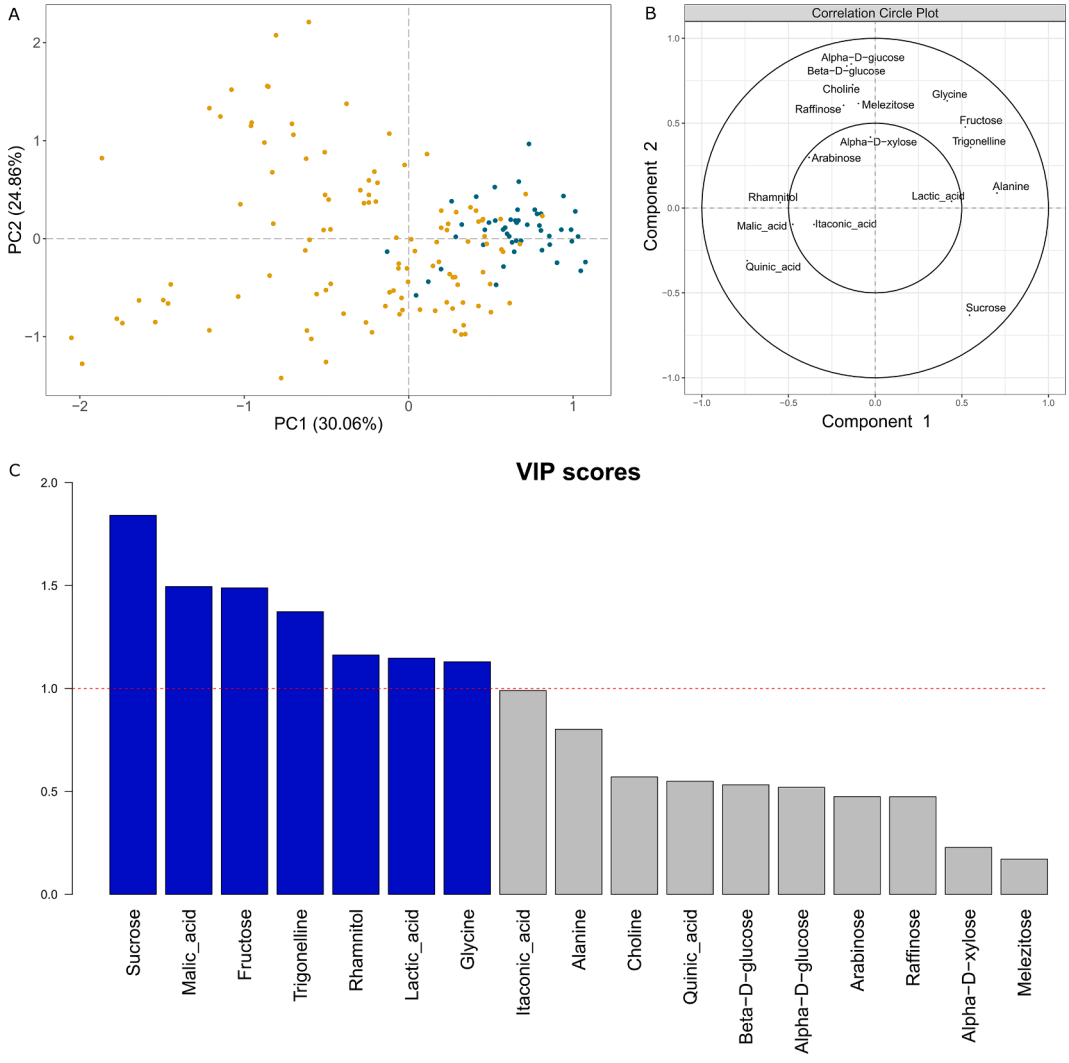

Fig. 1. Variability and VIP values of the three categories of primary metabolites assessed by principal component analysis in panel A of the PCA plot is illustrated the distribution of each sample highlighted in blue color for *M. domestica* accessions and yellow color for *Malus* spp. accessions. The contribution of each variable in the distribution is reported in the correlation circle variable plot illustrated in panel B. The variable importance in projection (VIP) for each primary metabolite is reported in panel C. The horizontal red line indicates VIP=1 and blue bars refer to the compounds with a VIP value >1.

first two principal components (PCs) accounting together for 54.92 % of the total metabolite variability (PC1: 30.06 %; PC2: 24.86 %). PC1 substantially distinguished *M. domestica* from *Malus* spp. accessions (Fig. 1A). The samples collected from the individuals of the *M. domestica* group were in fact located in the PC1 positive area of the plot, while those of the *Malus* spp. group were, for the most, located in the PC1 negative area. The sample distribution on the 2D-PCA plot was based on the collective analysis of 17 molecules from three categories: i) 9 carbohydrates: alpha-D-glucose, beta-D-glucose, alpha-D-xylose, arabinose, fructose, raffinose, rhamnitol, sucrose and melezitose; ii) 4 organic acids: itaconic acid, lactic acid, malic acid, quinic acid; iii) 4 amino acids: alanine, choline, glycine and trigonelline. The projection of each of these primary metabolites in defining the sample distribution illustrated in the PCA plot was highlighted in the PCA-based correlation circle variable plot (Fig. 1B). In this plot, defined by the implementation of the first two PCA components, glycine, fructose, trigonelline, alanine and sucrose were mainly projected in the positive area of the component 1, comprehended by the two radii, and characterizing the distinction of the *M. domestica* accessions. The other compounds were instead projected in the opposite area of the variable plot, defined by the negative value of the component 1, and more associated to the distribution of the *Malus* spp. species. While PCA provided an unsupervised overview of the natural structure of the metabolomic dataset and revealed a clear separation between *M. domestica* and *Malus* spp., it does not allow a formal quantification of the contribution of individual metabolites to this class discrimination. Therefore, to identify and rank the variables responsible for the group separation observed in the PCA, a supervised orthogonal partial least squares discriminant analysis (OPLS-DA) was performed and variable importance in projection (VIP) scores were calculated. The VIP score reflects the contribution of each metabolite to the predictive ability of the OPLS-DA model and thus to the discrimination between the two *Malus* groups. Metabolites with VIP ≥ 1 were considered as the most relevant contributors to the separation. The OPLS-DA model showed a good explanatory and predictive performance (R2Y = 0.68, Q2 = 0.638), which was statistically validated by permutation testing (pQ2 = 0.05). The unsupervised PCA (Fig. 1A–B) reveals, therefore, the global pattern of variation and group separation, whereas the supervised OPLS-DA and the associated VIP scores (Fig. 1C) identify the specific metabolites driving this separation. Seven primary metabolites, among the 17 investigated here, showed a VIP > 1 (Fig. 1C), and among them, it is worth highlighting the contribution of the first three compounds: sucrose, malic acid and fructose.

3.2. Variability of carbohydrates within the interspecific apple collection

Among the three categories of primary metabolites, the carbohydrate is the most relevant, being represented in this work by 9 molecules out of 17 (Supp. Table 1, Supp. Figure 1 and Supp. Figure 2). The highest

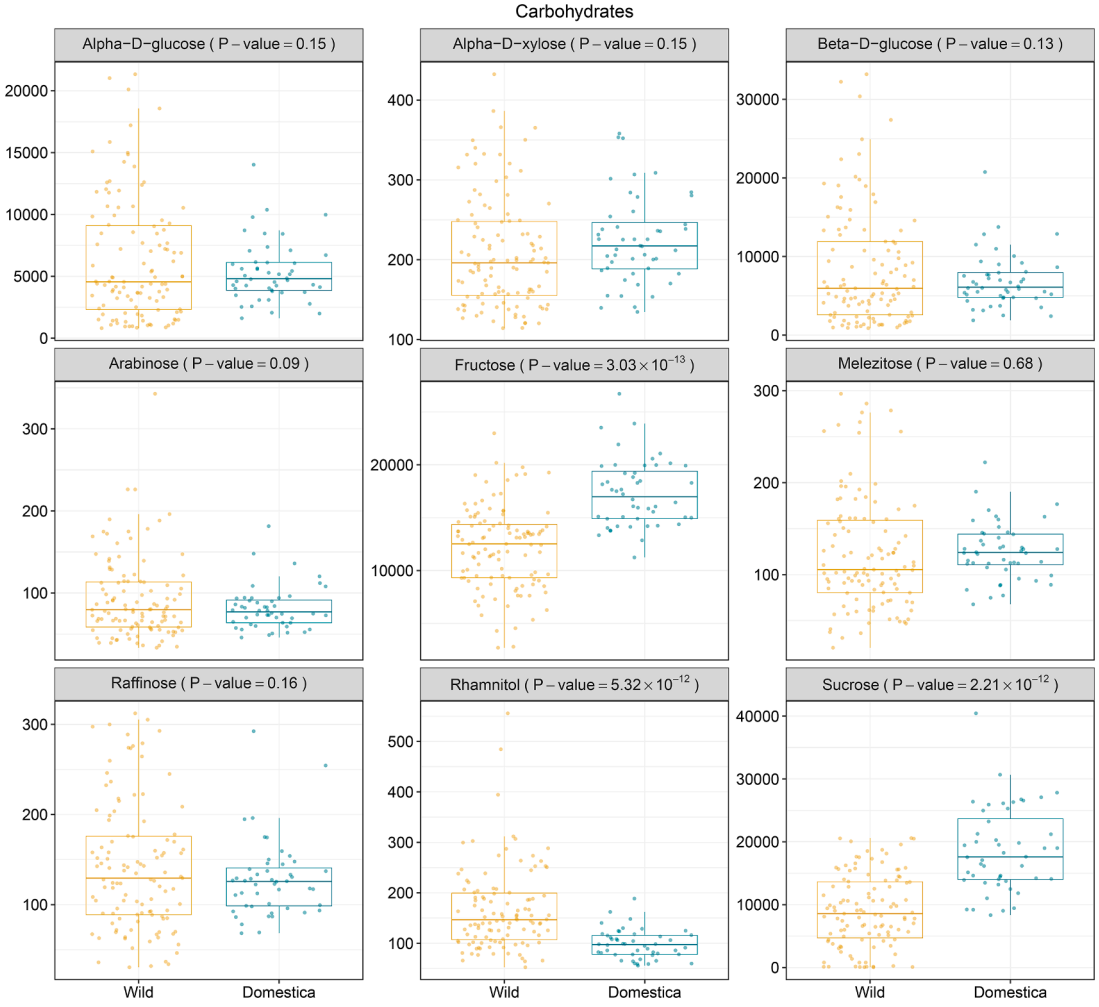

Fig. 2. Pattern of carbohydrate concentration. Boxplot reporting the concentration pattern of the nine carbohydrates detected for the accessions distinguished in the two *Malus* categories: *M. domestica* and *Malus* spp. In each box corresponding to a specific carbohydrate the adjusted *P-value* is also reported. *Malus* spp. accessions are visualized in yellow (Wild), while *M. domestica* accessions are visualized in blue (Domestica). Values are reported in mg/l. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

concentrated compound was sucrose in the *M. domestica* accession 'Ard Cairn Russet' (with 4.04 × 10^4 mg/l), while melezitose showed the lowest concentration, measured in the accession '1–15–3' of *M. sieversii* (20.63 mg/l). Sucrose and fructose are the two sugars showing the highest VIP score, with a value of 1.84 for sucrose and 1.48 for fructose. Sucrose and fructose were, moreover, significantly more concentrated in *M. domestica* compared to wild *Malus* spp. (Fig. 2). Sucrose had a *P-value* of $2.21{\cdot}10^{-12}$ with a 2.07 fold change, while fructose had a *P-value* of $3.03{\cdot}10^{-13}$, with a 1.42 fold change. In the group of *Malus* spp., specific species showed however a level of sucrose and fructose comparable to *M. domestica* (Fig. 3). Species like *M. sylvestris, M. sieversii, M. robusta* and *M. paradisiaca* showed in fact concentration values for these two carbohydrates comparable to *M. domestica.* Other species were instead characterized by a more sugar-specific concentration. *M. floribunda, M. baccata* and *M. prunifolia* showed, for instance, a concentration comparable to *M. domestica* accessions only for fructose. Within the group of carbohydrates, also rhamnitol showed a statistically significant difference (*P-value* of $5.32{\cdot}10^{-12}$, and a VIP of 1.16), but with a concentration higher in the group of *Malus* spp. accession than *M. domestica*, with a fold change of 1.69. The other six sugars, instead, did not show any difference between the two groups and did not result statistically significant. In this group it is worth noting the low VIP value of the two isomeric forms of glucose detected in this work, alpha-D-glucose and beta-D-glucose, showing a VIP score of 0.51 and 0.53, respectively.

3.3. Organic acid variability within the interspecific apple collection

Another important group of primary metabolites is represented by organic acids, comprehending four different compounds (Supp. Table 1, Supp. Figure 1 and Supp. Figure 2). The four organic acids detected showed a statistically significant difference (*P-values*< 0.05) when comparing the concentration between the two groups of *M. domestica* and wild *Malus* spp. accessions. On average, the most abundant compound was malic acid in the wild *Malus* spp. group (1.03 × 10^4 mg/l), with a mean concentration nearly double compared to *M. domestica* accessions (5.26 × 10^3 mg/l), with a fold change of 1.97 and a *P-value* of $4.96{\cdot}10^{-14}$ (Fig. 4). The role of malic acid in the distinction between the two groups of *Malus* was also supported by the VIP score of 1.49. Although predominant in the category of wild accessions, specific *Malus* spp. also showed lower concentrations comparable to *M. domestica*, such as: *M. spectabilis, M. pumila, M. paradisiaca, M. keepsake* and *M. britegold.* All the detected organic acids except for lactic acid were more concentrated in *Malus* spp. than in the *M. domestica* accessions. It is also worth noting that together with malic acid, lactic acid is another organic acid with a relevant VIP score, showing a value of 1.14. Quinic acid was the compound with the highest concentration per single accession, with a value of 6.20 × 10^4 mg/l in the *M. Coronaria* '1–23–2′ accession. The lowest concentrated organic acid was instead lactic acid, with a value of 4.08 mg/l, detected in the *hybrid* accession '1–20–3' (Supp. Table 1).

3.4. Amino acid variability within the interspecific apple collection

The group of amino acids assessed in this work showed instead an opposite concentration pattern with regards to the organic acids (Supp. Table 1; Supp. Figure 1 and Supp. Figure 2). Among the four compounds assessed in the framework of this study, three of them (alanine, glycine and trigonelline) were statistically more concentrated (*P-values* < 0.05) in the *M. domestica* accessions (Fig. 5). Only choline resulted not statistically different between the two groups (*P-value* of 0.17), although it showed a higher concentration in wild *Malus* spp. accessions, with a mean value of 9.66 mg/l compared to the 8.56 mg/l in *M. domestica* accessions. The highest concentrated compound was glycine, which despite being on average more concentrated in the group of *M. domestica* accessions, showed the highest absolute value in the *M. Hartwigii* '3–13–1′ accession (1.68 × 10^3 mg/l). Trigonelline and glycine are moreover the two amino acids with a VIP score >1. Trigonelline has a VIP of 1.37, while glycine showed a VIP of 1.29.

3.5. Correlation analysis between primary metabolites and fruit size/weight

The concentration value detected for the 17 primary metabolites assessed by NMR was finally correlated with fruit size and weight measured for the apples harvested by each accession included in the interspecific collection (Fig. 6). Among carbohydrates, fructose and sucrose showed a positive correlation with fruit size and weight.

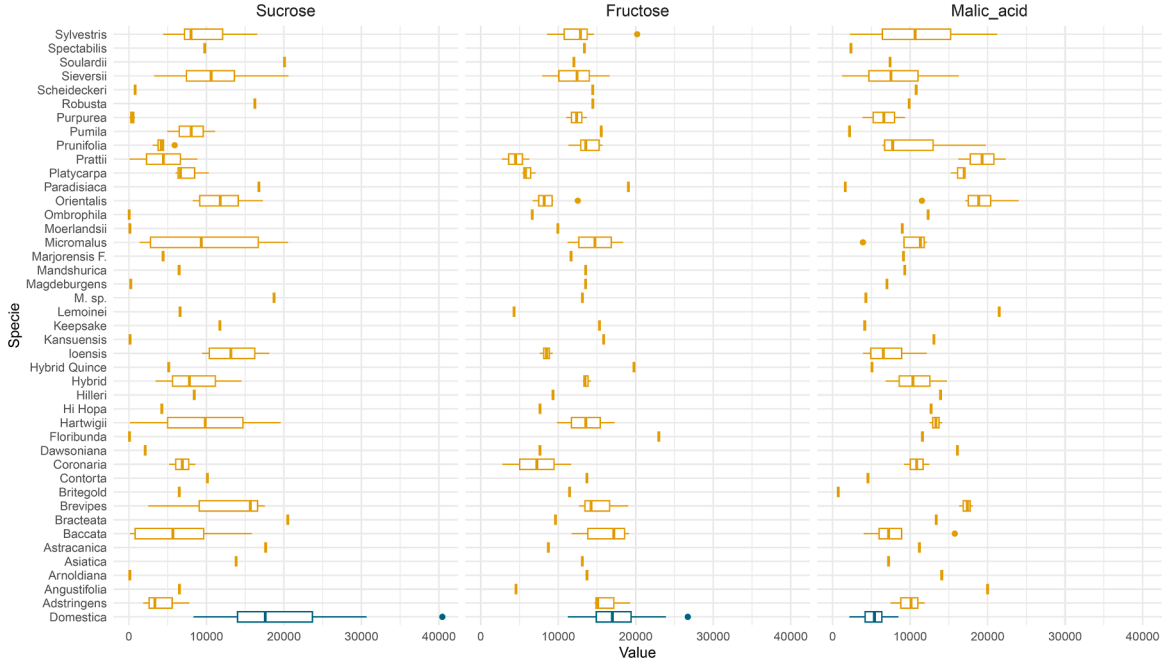

Fig. 3. Concentration patter for sucrose, fructose and malic acid across the 43 *Malus* species. Boxplot illustrating the concentration pattern of sucrose, fructose and malic acid in the 43 *Malus* species. *Malus* spp. and *M. domestica* are highlighted in yellow and blue colors, respectively. Values are in mg/l.

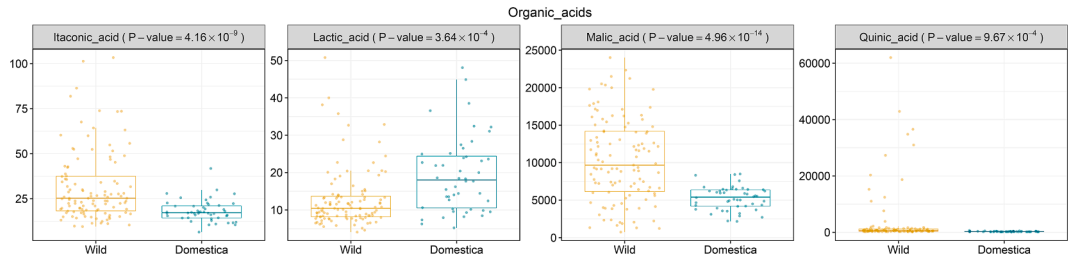

Fig. 4. Pattern of organic acid concentration. Boxplot reporting the concentration pattern of the four organic acids detected for the accessions distinguished in the two *Malus* categories: *M. domestica* and *Malus* spp. In each box corresponding to a specific organic acid the *P-value* is also reported. *Malus* spp. accessions are visualized in yellow (Wild), while *M. domestica* accessions are visualized in blue (Domestica). Values are reported in mg/l. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

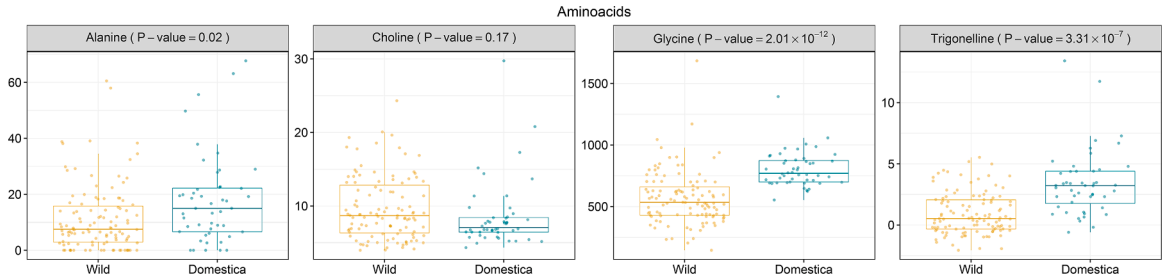

Fig. 5. Pattern of amino acids concentration. Boxplot reporting the concentration pattern of the four amino acids detected for the accessions distinguished in the two *Malus* categories: *M. domestica* and *Malus* spp. In each box corresponding to a specific amino acid the adjusted *P-value* is also reported. *Malus* spp. accessions are visualized in yellow (Wild), while *M. domestica* accessions are visualized in blue (Domestica). Values are reported in mg/l. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fructose had correlation values (*r*) of 0.51 and 0.53 for size and weight, respectively, while sucrose had values of 0.55 and 0.52. The remaining seven compounds were instead negatively correlated with the two fruit characteristics (size and weight), with rhamnitol showing the highest negative correlation, with an average value of r: -0.53. Among the organic acids, itaconic, malic and quinic acids resulted to be negatively correlated with fruit size and weight, with malic acid having the highest negative correlation, with r values of -0.59 and -0.54 for fruit size and weight respectively. Only lactic acid resulted positively correlated with fruit size and weight, with r values of 0.26 and 0.18, respectively. Among the four amino acids, three exhibited positive correlations with fruit size and weight, with trigonelline showing the highest correlation (r: 0.40 for size and r: 0.45 for weight). Choline showed instead negative correlation values for fruit size and weight, with r values of -0.31 and -0.18 for the two properties respectively.

4. Discussion

4.1. Fructose, sucrose and malic acid as key contributors to apple domestication

Apple domestication is a two-phase selective process, initially driven by megafaunal frugivores favoring large and palatable fruits, and subsequently reshaped by human-mediated selection targeting sweetness, reduced acidity and increased fruit size. This transition implies a shift from natural selection acting on dispersal efficiency to artificial selection optimizing sensory quality and agronomic traits. Primary metabolites are fundamental molecules supporting both basic physiological processes and fruit quality, contributing to sweetness and acidity. Carbohydrates and organic acids showed the highest variability within the genus *Malus*. Besides the analysis of the variability existing within the group of *M. domestica*, the species cultivated for human consumption, it is nowadays compelling to comprehensively investigate these metabolites also in wild *Malus* species, given their potential contribution to breeding programs focused on disease resistance and resilience to new climatic and environmental conditions. In this work, the employment of a NMR enabled an efficient and exhaustive characterization of major primary metabolites, including carbohydrates, organic and amino acids in the flesh of fruit collected from an interspecific collection of apple accessions. The metabolic profiling highlighted substantial variation among metabolites, and the VIP analysis underlined the prevalent role of sucrose, malic acid and fructose (Fig. 1C), distinguishing the group of accessions belonging to *M. domestica* from the other *Malus* spp. accessions (Fig. 1A). The circular correlation variable plot (Fig. 1B) showed that sucrose and fructose were associated with the distribution of the *M. domestica* accessions over the plot, while malic acid resulted, on the contrary, more associated with the *Malus* spp. accessions. The opposite projection of these two compounds finds consistency with the evolutionary history of apples. Our results suggest that not all domestication-associated metabolic changes reflect direct adaptive selection. The increased accumulation of fructose and sucrose, positively correlated with fruit size and weight, is consistent with an adaptive process driven by selection for sweetness. In contrast, the reduced concentration of malic acid and rhamnitol in domesticated accessions may partly reflect non-adaptive processes, such as metabolic dilution associated with fruit enlargement and increased water content, rather than active negative selection on these compounds. This interpretation aligns with the early phase of apple domestication, during which megafaunal frugivores preferentially selected larger and sweeter fruits. Compounds with higher perceived sweetness, such as fructose and sucrose, would have been disproportionately favored, whereas metabolites primarily contributing to acidity may have been indirectly reduced because of fruit expansion. The balance between sweetness and acidity was altered by a significant metabolic reprogramming during apple domestication. This pattern originated from an initial phase of animal-mediated selection favoring larger and sweeter fruit, was later shaped through the human-driven artificial selection (Spengler, 2019). Domesticated apples are generally sweeter and less acidic than wild species, and although these changes have determined a considerable metabolic effort for sugar production, it turned out successful for the recruitment of seed dispersing animals.

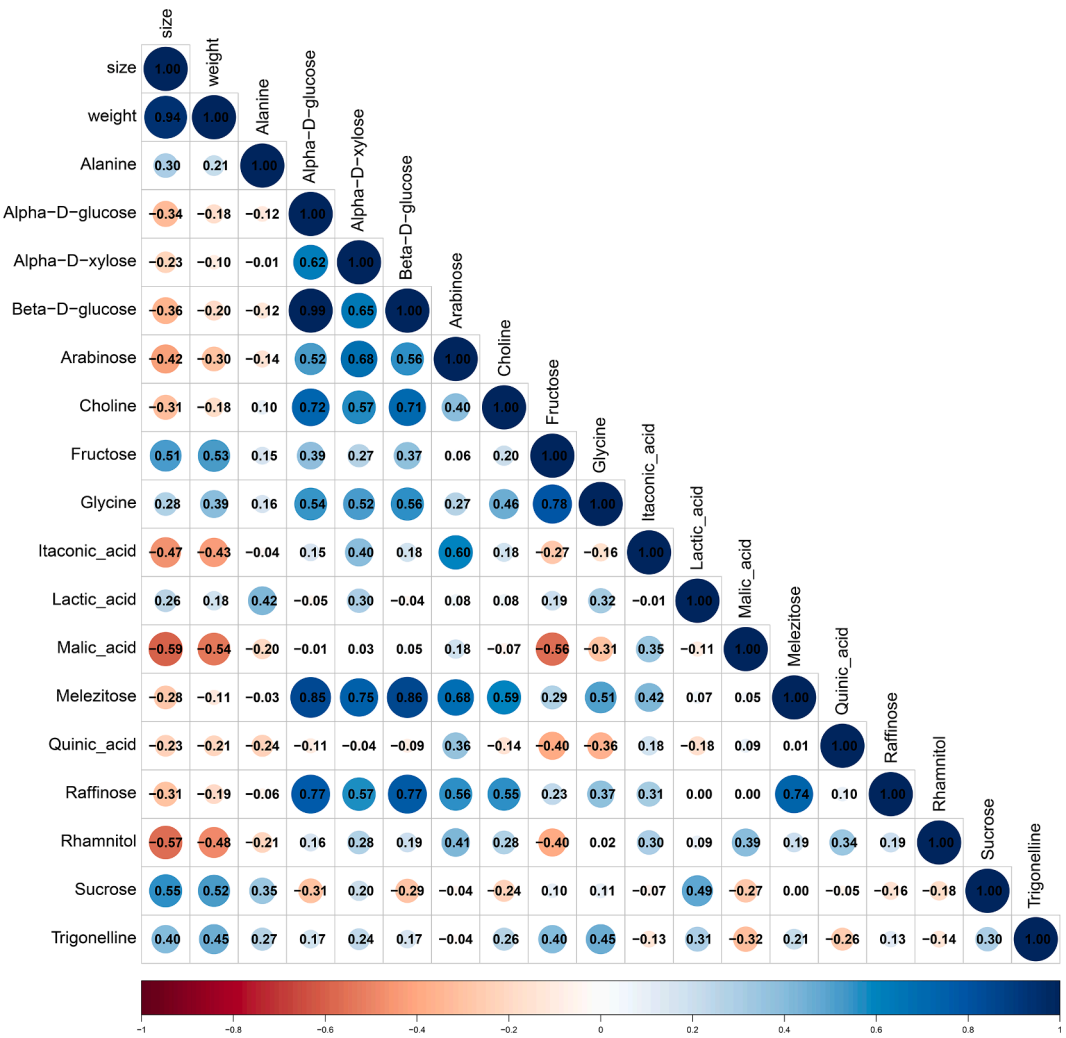

Fig. 6. Analysis of correlation. Correlation plot indicating the Pearson correlation value between each metabolite with size and weight. The correlation value range is illustrated with a red-blue color scale, with red color for negative correlation values and blue for positive correlation values.

Whole-genome comparative investigation has also revealed selective sweep regions enriched in genes associated with sugar content in *M. domestica* and acidity in *M. sylvestris* (Cornille et al., 2014; Duan et al., 2017 and Spengler, 2019). In the work of Duan et al. (2017), through the resequencing of 117 diverse accessions, several selective sweep regions characterizing the domestication syndrome were identified and associated with these traits. These regions were moreover interested by the presence of candidate genes involved in these processes, such as *sucrose phosphate synthase, sucrose synthase, sugar transporter* and *sorbitol 6-phosphate dehydrogenase*. On chromosome 12 was also identified a QTL for sorbitol that was interested by an intense selection. Other selective sweep regions were associated with fruit size, validating the observation that flavor and size were two major domestication associated traits. In particular, two selective regions co-located with QTLs for fruit weight, and mapped on chromosomes 15 (*fw1*) and 8 (*fw2*). Interestingly, *fw1* was characterized by the presence of candidate genes involved in the control of fruit weight, such as *cell division regulatory* and *β-galactosidase*. In addition, it is also interesting to highlight the MetaQTL analysis carried out in apple that revealed the presence of QTL regions associated to fruit size on chromosomes 8 and 15. In chromosome 8 were moreover mapped other regions associated to sugar and acid content (Costa, 2015), which underline the genetic architecture of these traits. In the most large and comprehensive genetic analysis carried out resequencing 497 *Malus* accessions, several selection signatures related to domestication and improvement were specifically detected for fruit size and acidity. In this work, acidity was therefore recognized as the main key factor in the evolution of the fruit taste during domestication and breeding. Single mutation in *Ma1, MdTDT* and *MdSOT2* genes, was pointed as a relevant domestication event, resulting in an important decrease in malate, citrate and sorbitol. Moreover, gene homologs to *fw2.2*, the main locus controlling the fruit size in tomato (Frary et al., 2000), was located close to *Ma1*, one of the major gene for fruit acidity in apple (Liao et al., 2021). In this work it was also reported as other genes than *Ma1* can control the concentration of malate in apple, such as *MdPP2CH,* mapped on chromosome 8 and consistent with the genomic regions aforementioned reported.

Among the main sugars produced during the photosynthetic carbon fixation, only fructose and sucrose with VIP values higher than 1 were significantly different between the two groups of accessions, while glucose, the main sugar produced during the Calvin cycle, did not. Both the two isomers of glucose showed a VIP lower than 1, and this difference can be attributed to the level of sweetness proper of each sugar component. Fructose is in fact characterized by a higher sweetness than sucrose, which in turn is sweeter than glucose (Colonna et al., 2006). This different concentration and values are consistent with the initial phase of the apple domestication process guided by frugivores, mainly attracted by sweet and large fruits. This observation is supported by our data, which show a positive correlation between fruit size/weight and fructose and sucrose concentrations, and a negative correlation with malic acid. Despite the essential role of glucose, domestication and

breeding have primarily enhanced the accumulation of fructose and sucrose. Within the category of sugar, in addition to fructose and sucrose, also rhamnitol showed a VIP score higher than 1 (VIP: 1.16), but contrary to the other two, it was more concentrated in the group of *Malus* spp. accessions. This sugar was already found and quantified via NMR in apples, with different concentrations associated with different factors, such as origin and cultivars (Tomita et al., 2015; Sobolev et al., 2015; di Matteo et al., 2021). The lower level of rhamnitol observed in domesticated accessions suggests that selective or evolutionary pressure may have acted to reduce the accumulation of this sugar. The different correlation value of fructose, sucrose, malic acid and rhamnitol, although all distinguished by a VIP>1, hypothesized therefore a specific metabolic reprogramming. Fructose and sucrose, being positively correlated with fruit size and weight, were characterized by an active reprogramming, with a metabolic increase that paralleled the fruit enlargement. Malic acid and rhamnitol, which are instead defined by a negative correlation with fruit size and weight underwent a different metabolic process. The lower concentration of these metabolites in *M. domestica* accessions may be due to a lack of an active accumulation or a potential dilution effect resulting from the increase in fruit size, a phenomenon also observed for polyphenols (Busatto et al., 2019; Zambiasi et al., 2025). The domestication process that led to an increase in sugar concentration was also paralleled by an increased fruit size, which was accompanied by a higher amount of internal water. The augmented water content might have represented a non-adaptive mechanism leading to a decreased concentration of organic acid, mainly malic acid in apples, contrasting the adaptive increase of sucrose and fructose.

In this metabolic profiling, it is important to underline the lack of reliable quantification of the alcohol sugar sorbitol. Although sorbitol is a sugar typically accumulated in the *Rosaceae* family, especially in apples (Yamaki, 2010), its absence in our findings can be attributed to two main factors. First, NMR is not highly efficient at detecting sorbitol due to its resonance signals being located between 3.86 and 3.58 ppm and heavily overlapping with framework signals of other carbohydrates that prevent a proper integration. Secondly, sorbitol is translocated within the fruit and converted into other carbohydrates (Yamaki, 2010), which makes the detection in ripe apples difficult. The work of Aprea and colleagues (2017) has moreover underlined that sorbitol contributes however for less than 10 % to the total amount of carbohydrate, with a lower sweetness compared to fructose, sorbitol and glucose (Wrolstadt, 2012). The variation in the content of sorbitol was moreover assigned as a main event during the apple domestication. In the work of Liao and colleagues (2021) it was also reported that both the homozygous and heterozygous allelic conformation of *MdSOT2* (a gene encoding for a sorbitol transporter) found in 86 % in cultivated apple accessions was associated with an almost absence of sorbitol concentration in the fruits (Liao et al., 2021), supporting our results.

By disentangling adaptive and non-adaptive components of metabolic variation, this study provides a framework for interpreting domestication-related metabolomic patterns beyond descriptive comparisons. Our results position primary metabolites as integrative traits shaped by both evolutionary history and breeding practices, offering a conceptual bridge between evolutionary biology and applied apple improvement.

4.2. Wild Malus species accumulate higher level of organic acids

Apples are known to accumulate high concentrations of malic acid (Wu et al., 2007; Ma et al., 2018). In addition to this, we identified itaconic, quinic and lactic acids. The first two, similarly to malic acid, were more abundant in the group of *Malus* spp. compared to the domesticated accessions. Quinic acid was already discovered in apple (Hulme, 1956) and kiwifruit (Marsh et al., 2009), while itaconic acid has been associated with fermentation processes in several fruits (Saeed et al., 2024 and 2025). In this regard, it is also interesting to note the higher concentration of lactic acid in the group of *M. domestica* accession, with a VIP>1. This organic acid, typically present in several vegetable and fruit species, also derived by fermentation processes generated by lactic acid bacteria, part of the autochthonous microbiota of these species (Savino et al., 2012; Di Cagno et al., 2013). The presence of lactic acid in wild species and old *M. domestica* accessions may indicate early fermentation process already at the time of sampling. *M. domestica* accessions, like 'Discovery', 'Duchess of Oldenburg' and 'Golden Russet of Western New York' are in fact distinguished by higher value of lactic acid compared to modern types of apple cultivars, like 'MC38 Crimson Snow', 'Rosy Glow', 'Golden Delicious' and 'Honeycrisp'. This can be explained by the fact that these accessions are characterized by an early drop phenomenon, associated with an early ripening process that can have promoted an early fermentation. It is also worth to underline that although all the accessions were harvested at horticultural maturity, the wild accessions are normally characterized by an early drop phenomenon forcing an anticipated harvesting for some individuals. This, together with the complex heritability of these traits, might have in some way impacted the level of these metabolites.

4.3. The role of amino acids in the differentiation between wild and domesticated apple accessions

The level of amino acidic compounds generally present in apples is very limited, accounting for less than 1 g over 100 g of fresh weight (Feliciano et al., 2010; Di Matteo et al., 2021). Among the four compounds, glycine, trigonelline and alanine are oriented in the positive area of component 1 of the correlation circle variable plot, in the same direction of fructose, known to play a central role in the contribution to sweetness. This similar orientation can be hypothesized by the fact that certain amino acids, especially alanine, are implicated in the production of volatile compounds (Sobolev et al., 2015; Wu et al., 2007). In the work of Zambiasi et al. (2025) carried out on the same set of samples, it was indeed observed that apples collected from the group of *M. domestica* accessions are characterized by a higher production of volatile aromatic compounds (VOCs) compared to the fruit harvested from the *Malus* spp. accessions. From the dataset reported in Zambiasi et al. (2025) two VOCs were extracted: ester (*m/z* 43.0183) and alcohol (*m/z* 43.0547), and their concentrations were compared with the four amino acids in a reduced set of samples, composed by 48 *M. domestica* accessions and 50 wild accessions (represented by *M. baccata, M. orientalis, M. sieversii* and *M. sylvestris*). In this subset, all these compounds were generally more concentrated in the group of *M. domestica* than *Malus* spp. Alanine and choline, although more concentrated in *M. domestica* (17.86 and 8.56 mg/l) than *Malus* spp. (14.41 and 7.78 mg/l), did not show a statistically significant difference. Glycine and trigonelline, distinguished by a VIP score >1 in the original dataset, showed instead a statistically significant difference (*P-value* adj $2e^{-07}$), with an average concentration of 803.13 mg/l (*M. domestica*) and 511.17 (*Malus* spp.) for glycine and 3.42 mg/l (*M. domestica*) and 1.02 mg/l (*Malus* spp.) for trigonelline. The possible connection with the volatile was proposed by the higher concentration of the ester and alcohol VOCs, the two main compounds determining the aroma in apple, in *M. domestica* accessions (1890.11 and 350.80 μ g m^{-3}, for the two VOCs respectively), than in *Malus* spp. (988.48 and 240.91 μg m^{-3}, for the two VOCs respectively). Glycine showed, moreover, the highest concentration in contrast with other studies that reported alanine as the most abundant compound (Wu, J., et al. 2006). This difference may be attributed to the analytical methods used, as previous studies often employed gas chromatography (Wu et al., 2007), whereas NMR-based quantification may have different sensitivities for specific compounds. Glycine also plays a beneficial role in the postharvest quality and cell wall metabolism in apples. Application of glycine betaine increased in fact specific quality parameters, such as soluble solids and fruit firmness, by impacting for instance the activity of particular cell wall modifying enzymes (Gao et al., 2025). Trigonelline, known for its antioxidant and antimutagenic activities (Mohamadi et al., 2017), was significantly

more abundant in *M. domestica* accessions supporting its association with apples domestication. Choline, although not significantly different between the two groups is recognized as a vital dietary amine involved in protein biosynthesis (Blusztajn, 1998).

5. Conclusions

Apples are one of the most consumed fruits worldwide and they have been largely characterized in terms of primary metabolite production. By using NMR technique, we rapidly and cost-effectively profiled three classes of primary metabolites—carbohydrates, organic acids, and amino acids—in an interspecific apple collection. The comparative analysis of wild and domesticated *Malus* accessions supports the hypothesis about the role of sugars and organic acids as key metabolic signatures in the evolutionary process and domestication of apples. Specifically, fructose and sucrose increased more in *M. domestica* accessions whereas the most important organic acid, malic acid, remained highly predominant in wild *Malus* spp. Nevertheless, certain wild accessions showed sugar and acid profiles comparable to *M. domestica*, suggesting unexploited potential for breeding. Given the growing interest in using wild crop relatives to reintroduce traits for disease and environmental resistance that were lost during domestication, these findings provide valuable insight for integrating wild germplasm into modern breeding programs aimed at improving both fruit quality and nutritional value.

CRediT authorship contribution statement

Genny Zambiasi: Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Formal analysis, Data curation. **Pavel Solovyev:** Writing – review & editing, Writing – original draft, Software, Methodology, Formal analysis, Data curation. **Marta Degasperi:** Software, Formal analysis. **Nicola Busatto:** Writing – review & editing, Writing – original draft, Validation, Resources. **Walter Guerra:** Writing – review & editing, Writing – original draft, Supervision, Resources, Investigation, Conceptualization. **Luana Bontempo:** Writing – review & editing, Methodology, Formal analysis. **Michela Troggio:** Writing – review & editing, Supervision, Funding acquisition. **Brian Farneti:** Writing – review & editing, Writing – original draft, Supervision, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Fabrizio Costa:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, 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.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scienta.2026.114681.

Data availability

Data will be made available on request.

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