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DART-HRMS reveals metabolic changes of whey through microparticulation and fermentations

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ABSTRACT

Despite native whey (hereafter termed WHEY) being a watery by-product rich in high-value nutritional components, it poses environmental concerns. Recently, the techniques of thermal-mechanical microparticulation to produce microparticulated whey (MPW) and a further process to ferment MPW (FMPW) were suggested, in order to recycle whey into a protein concentrated soft dairy cream that acquires also fat mimicking functionality. Aiming at monitoring the effects of this recycling process on the products' metabolic profiles, samples ($n = 8$) of WHEY, MPW and two sub-types of FMPW were analysed by combining direct analysis in real time coupled with high resolution mass spectrometry (DART-HRMS). The most informative ions were used to build a partial least squared discriminant analysis (PLS-DA) model to assess the biochemical compounds that characterize and differentiate WHEY from MPW and FMPW. The DART-HRMS fingerprints acquired in negative mode accurately differentiated samples throughout the steps of microparticulation and fermentation. Post-fermentation, greater relative abundances of lactic acid, glucosamine and histidyl-aspartic acid were detected in both subtypes of FMPW, which were moderately differentiated by DART-HRMS. Moreover, while WHEY and MPW contained high relative abundances of hydroxyglutaric and malic acids, the fermented derivates (FMPW) were characterised by elevated levels of volatile compounds (tetradecanal, hexadecene and tetradecene) and fermentative end-products (diethyltartrate and histidyl-aspartic acid). DART-HRMS successfully captured a pool of informative biomarkers useful to understand the chemical and metabolomic changes occurring in microparticulation and fermentation processes used to recycle whey. This analytical technique is an operative supporting step needed for comprehensive assessment of the nutritional and organoleptic properties of the proposed whey-based soft cream dairy food.

1. Introduction

The global dairy industry is experiencing significant growth due to the increased manufacturing of coagulated and fermented milk products, resulting in considerable production of whey, an aqueous by-product with little to no commercial value and that poses environmental concerns related to its dumping (Soares et al., 2023; Gantumur et al., 2023). Whey is an intricate blend of approximately 7.5 % dissolved particles, and the main components are lactose (4.8 %), protein (0.9 %) and minerals (0.6 %) with traces of fat (0.5 %) and lactic acid (0.2 %) (Bergamaschi & Bittante, 2018). Among these compounds, whey protein has great potential for versatile applications in the food industry due to its high biological value and excellent functional properties to enhance the viscosity, firmness and water binding capacity of many fermented milk, high-protein yogurt and other food products (Mahomud et al., 2017; Zhang et al., 2021). However, dumped whey is a serious pollutant due to its high biochemical oxygen demand, and consequently the dairy industry faces an important challenge when it comes to utilising wasted whey (Giulianetti de Almeida et al., 2023). In recent years, a more environmentally-friendly approach, and the diffusion of efficient biorefinery technologies have contributed to shift the dairy industry towards zero-waste processes and be more compliant with sustainable

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production models (Ipsen, 2017).

Therefore, the development of new techniques for properly recycling whey for use in nourishing fermented beverages or creamy sweets would be highly beneficial both for the environment and the dairy industry. These techniques intended for the complete valorisation of cheese whey and should help ensure the full transition of the dairy sector's whey stream into the circular economy, aiming at zero waste production from processing. Technologies like ultrafiltration (UF) and microparticulation (MP) of whey have been proposed to concentrate, split, transform and retrieve whey's high-value macro- and micronutrient content, improving the recycling value of this dairy by-product (Lai et al., 2020). Recent research has also demonstrated that homogenisation at high pressures (200–400 MPa) reduces the requirement for stabilisers and promotes stronger interaction between proteins and polysaccharides, resulting in a stable network structure suitable for the production of a large basket of lactose- or protein-enriched fermented, powder foods (Sato et al., 2021). MP is a method in which whey protein, after an UF step, is thermally and mechanically treated to induce controlled aggregation. Heat treatment modifies protein size and shape, and results in the exposure of hydrophobic sites and in aggregation and network formation. When a shear force is applied, bonds both within and between the network can break with cluster fragmentation. MP could result in functional characteristics of whey protein that are markedly different from whey's native characteristics (Dissanayake & Vasiljevic, 2009). Microparticulated whey (MPW) has a creamy texture and mimics the feel of milk fat (Ipsen, 2017). This functionality has been ascribed to the characteristics of particles; in fact, the spherical shape and the <5μm size are comparable to the characteristics of oil droplets in emulsions. In contrast, large particles are associated with roughness, dryness, grittiness and powdery attributes (Liu et al., 2016). The use of whey as an enriched protein ingredient or as a substrate to produce a variety of innovative, high-quality, value-added whey-based beverages or cream is bounded by the preliminary physical and/or chemical treatments (e.g., concentration and acidification), which are crucial in shaping the distinct characteristics of the derived fermented, microparticulated product. Indeed, in the food sector, MPW can be employed as a foaming, gelling, emulsifying and/or stabilising agent, improving the sensory attributes and texture of finished products; as a fat replacer, it is used to produce ice creams, yogurts, cheeses, mayonnaise and other foods (Melnikova et al., 2022).

Consequently, food quality and safety monitoring processes for recycled whey are of increasing concern, while the diversity and chemical complexity of miscellaneous analytes make their assessment in milk, whey and derived food matrices a great challenge in the modern dairy chain. In addition to wet-chemistry analyses, emerging multi-analytical techniques can ensure a detailed picture of food composition, allowing the simultaneous characterisation of a large number of compounds. Reliable assessment of the metabolomic fingerprint modifications from whey to a final, targeted food can be useful to hypothesise the changes to the chemical, functional and structural properties during the processing. The resultant data are useful to better understand the potential practical application of this innovative product within a more sustainable dairy chain (Lu et al., 2018; Lanza et al., 2021). Therefore, there is a need to accumulate a large amount of data useful in our understanding of the potential practical application of innovative whey-derived products in the dairy chain context.

Unfortunately, chromatography-based analytical techniques are generally accompanied by a series of tedious and time-consuming procedures along with high analytical costs. For this reason, alternative approaches can ensure the suitable, near real-time, direct and high-throughput analysis of dairy foods to speed up research and industrial investigations. In this context, ambient ionisation techniques in mass spectrometry (AIMS) is a family of powerful analytical tools in which samples are quickly analysed under ambient conditions (i.e., atmospheric pressure, room temperature) with little or no sample preparation, allowing high-throughput extraction of chemical information useful for rapid acquisition of informative fingerprints (Augusti et al., 2024). The AIMS techniques are capable of determining the metabolic content of samples and, upon integration with statistics, discovering whether these metabolites might be correlated with authenticity, botanical source or storage and processing factors, especially in uncontrolled, real manufacturing conditions (Tata et al., 2021; Goryainov et al., 2021). Among the plethora of existing AIMS techniques, the following ones stand out for the number of reported applications and extensive commercialization (Augusti et al., 2024): (1) desorption electrospray ionization (DESI); (2) direct analysis in real time (DART); (3) easy ambient sonic-spray ionization (EASI); (4) paper spray ionization (PS); (5) laser ablation electrospray ionization (LAESI); (6) rapid evaporative ionization mass spectrometry (REIMS). Among these techniques, the utility of REIMS and DART-MS have been extensively demonstrated in food analysis and authentication, as well as their current commercialization by well-known mass spectrometry companies (Guo et al., 2017; Wang, 2024). Specifically, DART coupled with high resolution mass spectrometry (DART-HRMS) has already been utilised in different dairy food authentication tasks, such as ascertaining the main dietary forage source in the milk production chain (Riuzzi et al., 2021) or the geographical origin of milk (Tata et al., 2022). DART-HRMS operates under atmospheric conditions, eliminating the need for extensive sample preparation. It allows to analyse liquid samples directly, only mixing a very small amount of solvent to the sample (Hrbek et al., 2014). However, to the best of our knowledge, no explorative study has been performed on the performance of DART-HRMS to assess the metabolic profile of whey and derived fermented and concentrated whey, and to shortlist, upon integration with chemometrics, the biomarkers that can potentially distinguish regular whey and whey products from whey subjected to the different processing treatments that are used to obtain an edible, high-quality whey-based food.

Therefore, the main purpose of this research was to identify the chemical markers of whey that differentiate it from microparticulate and fermented microparticulated whey derivates using an AIMS-based non-targeted approach in positive and negative ion modes. In detail, univariate analysis followed by partial least square discriminant analysis (PLS-DA) were applied in a modelling approach to assess whether the solid concentration of MPW or following different acidification patterns produced via two kinds of starter bacteria cultures significantly influence the biochemical composition of the resultant fermented soft cream dairy foods.

2. Materials and methods

2.1. Experimental design and microparticulation system

As shown in Fig. 1, eight bulk milk samples were collected on intensive lowland dairy farms located in the Veneto Region (Italy) and rearing high genetic merit dairy cows (i.e., Italian Friesian) fed total mixed rations mainly based on maize silage. The bulk milk samples were collected at regular intervals over a period of 45 days between the end of May and beginning of July 2023. Each sample was then promptly transported to the same dairy processing plant (Tomasoni, Breda di Piave, Italy), where milk was used to produce fresh soft cheese according to the following cheesemaking procedure. Briefly, milk was filtered with a 3-mm membrane, centrifuged, pasteurised (73 °C for 20 s), and a thermophilic starter and rennet were added. After coagulation and curd cutting, the whey (hereafter called WHEY) was maintained at 37 °C for 12 h, then fat and residual casein were removed by centrifugation at approximately 13,950 *g* and 4 bar via a GEA Westfalia MSB 60-01-076 milk separator (Machinery World, Wolvey, England), after which the WHEY was subjected to bactofugation with a stated centrifugal force of 10,000 *g*, 0.5 bar inlet and 3.5 bar outlet via a RE120B bacteria separator (Reda, Isola Vicentina, Italy) and heated at 70 °C for 15 s.

After the previous preliminary treatments, whey was ultrafiltered at

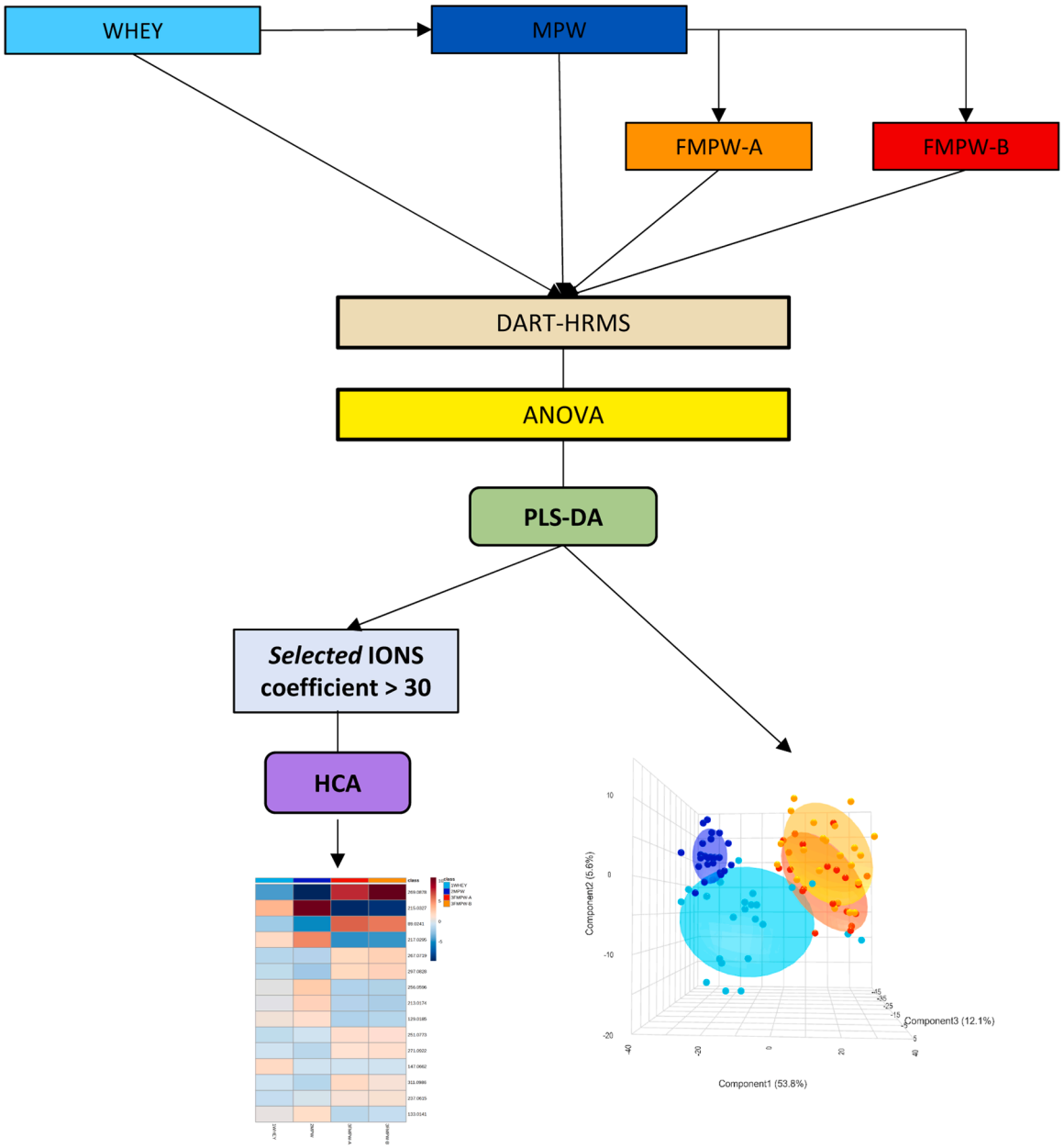


Fig. 1. Experimental design and flow chart of the non-targeted approach. Metabolic fingerprints of whey (WHEY), microparticulated whey (MPW) and fermented MPW (FMPW-A and FMPW-B) were acquired by direct analysis in real time high resolution mass spectrometry (DART-HRMS) in positive and negative ion modes. After DART-HRMS analysis, the data were submitted to non-parametric ANOVA, and on the resultant most informative ions ($P_{adj} < 0.05$), a partial least squared discriminant analysis (PLS-DA) was performed, and the outcoming scores were plotted in a scatter gram (Fig. 2 and 3, panel A). A hierarchical cluster analysis (HCA) was carried out on the 15 most informative ions (coefficient > 30; Tables 1 and 2) by Euclidean distance and Ward's linkage, and a heat map was generated (Fig. 2 and 3, panel B).

20 °C and 0.7 bar through a semi-permeable polyether sulfone filter (6338 HFK-328 fluid system element) with approximately 29-mm-diameter pores (Koch Membrane System-Lenntech, Delfgauw, The Netherlands) to obtain a residual concentration corresponding to 18-19 °Bx. The final step of microparticulation was carried out with a CreamoProt® plants instrument (ALPMA, Dresden, Germany) through homogenisation at 1.7 bar and the inlet cylinder set at 83 °C with a frequency of 45 Hz, the outlet cylinder operating at 60 °C with a frequency of 18 Hz to produce microparticulated WHEY (MPW). The day after microparticulation, each MPW sample was split into aliquots. One MPW aliquot was fermented at 37 °C until pH 4.5 by a direct-to-vat freeze-dried starter culture (Lyofast MOS 062 C) of *Lactococcus lactis* ssp. *lactis* and *Streptococcus thermophilus* (Sacco, Cadorago, Italy), which is hereafter identified as fermented microparticulated whey-A (FMPW-A). The second MPW aliquot was fermented at 42 °C until pH 4.5 by a direct-to-vat freeze-dried starter culture (Lyofast BLC 1) of *Bifidobacterium animalis* ssp. *lactis* (Sacco, Cadorago, Italy), identified hereafter as fermented microparticulated whey-B (FMPW-B). When the pH of 4.5 was achieved, the fermentation processes were quickly stopped by blast chilling the products until they were frozen (Tecnodom, Vigodarzere, Italy).

2.2. DART-HRMS analysis

The sample preparation ($n = 8$ per whey type) was performed as described in a previous research trial (Tata et al., 2022). Briefly, a 1−mL

volume of each WHEY, MPW, FMPW-A and FMPW-B sample was suspended in 1 mL of water and methanol (H_2O:MeOH; 80:20 v/v) solution (MilliQ water and methanol HPLC-grade with 99.9 % purity, from Sigma Aldrich, Darmstadt, Germany), vortexed for 30 s, sonicated for 15 min and centrifuged for 5 min at 12,000 *g* to extract the polar metabolites. Five microliters of each extract were transferred onto a glass capillary rod which was then placed in front of the mass spectrometer to acquire the metabolic profile. To this aim, an Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA), equipped with a DART SVP 100 ion source (IonSense, Saugus, MA, USA) and a Dip-it(R) autosampler (IonSense, Saugus, MA, USA) was employed for full scan mass measurements. The instrument operated in positive and negative ion mode between 75 and 1125 Da. The resolving power of the spectrometer was 70,000 FWHM (full width at half maximum) at 200 *m/z*. The system parameter settings are reported in the supplementary material (**Tables S1 and S2**). An aqueous solution of 30 % ammonia was placed below the DART gun exit to facilitate the formation of $[M + NH_4]^+$ ions. For each extract, triplicate analyses were performed, and Xcalibur QualBrowser software (Thermo Fisher Scientific, Waltham, MA, USA) was used to open and visualize the .raw file. These were converted to mzML files using Proteowizard and then opened with mMass software (http://www.mmass.org/) and finally converted into. csv files by an internally developed R code using R-software (v4.0.2; R Core Team 2022, Vienna, Austria). We verified the reliability of the results by acquiring the spectra of the pooled quality control (QC) samples. As suggested in the literature (Dunn et al., 2011), we performed the DART-HRMS analysis of the same pool of samples (split in several aliquots) at the beginning and the end of each day of analysis.

2.3. Statistical analysis

The spectral data were statistically analysed by using the R 4.0.2 software *maldiquant* package and the MetaboAnalyst 5.0 web portal (www.metaboanalyst.ca). The isotopes were removed, and then the signals aligned using internally developed codes. As described previously (Massaro et al., 2021) for all the samples, the ion signals with more than 75 % of missing ion intensities were removed. The ions with less than 75 % of missing ion intensity were replaced with 1/5 of the lowest intensity registered for that specific ion. Afterwards, the data were normalised by sum and scaled by Pareto scaling. To verify the reproducibility of these pooled QC samples over time, a partial least squared discriminant analysis (PLS-DA) model was performed. Since the same sample is injected repeatedly, pooled QC samples must be grouped (Woolman et al., 2021). **Fig. S1** (supplementary materials) shows both the pooled QC and experimental samples arranged in a PLS-DA plot, indicating highly reproducible of the QC data.

The pre-processed data (without the QC data) were then submitted to a non-parametric ANOVA to retrieve the most informative variables. The *p-values* were adjusted by false discovery rate (FDR) ($P_{adj} \leq 0.05$). As shown in Fig. 1, the selected variables, with $P_{adj} < 0.05$, were used to build up a partial least squared discriminant analysis (PLS-DA) model for supervised visualization of the discrimination capability of DART-HRMS. The top 15 most informative molecular features (*m/z* values) were submitted to hierarchical clustering analysis (HCA) with Euclidean distance and Ward's linkage. Consequently, a heatmap was built to visualise the similarity between selected informative molecular features and the four groups of samples. The informative *m/z* values were tentatively assigned by consulting the FOODB library (www.foodb.ca). The database assignment was verified by literature search.

3. Results

The PLS-DA score plot, built-up with the most informative DART-HRMS *m/z* values teased out by non-parametric ANOVA, provided a graphical representation that allowed the discovery of patterns, clusters and outliers among the four investigated whey matrices (WHEY, MPW, FMPW-A and FMPW-B). Specifically, the PLS-DA score plot of the fingerprints acquired in negative ion mode accurately differentiated WHEY, MPW and FMPW samples, while a spatial overlap was observed between the fermented FMPW-A and FMPW-B products (Fig. 2**A**). The PLS-DA score plot of the spectral data acquired in positive ion mode showed a slightly spatial overlap of the metabolic profiles of WHEY and MPW samples, which were still clearly separated from the fermented products (Fig. 3**A**).

The negative and positive ions that mostly contributed to the discrimination of the processed samples are listed in Table 1 and Table 2, respectively. The statistical relation between these most informative ions and the four whey types are shown in the heatmaps generated by HCA (Fig. 2**B** and Fig. 3**B**). As regards to the (−) DART-HRMS fingerprinting, the discrimination between the four types of whey was correlated to galactose (*m/z* 215.0327), lactic acid (the $[M-H]^-$ of *m/z* 89.0244 and the trimer $[3M-H]^-$ of *m/z* 269.0878), and the unknown signal of *m/z* 217.0295. The heatmap generated with the informative (+) DART-HRMS metabolites displayed a wide pattern of discrimination among whey and its derivates (MPW and FMPW) as well as the high number of ions that strongly contributed to the differentiation across these whey types. In Fig. 4 and Fig. 5, the box plots summarise the effects of the whey processing on the metabolomics of the resultant products; the descriptive statistics depict the most significant ($P_{adj} < 0.05$) changes of the ions within the four investigated whey groups through non-parametric ANOVA providing a degree of discrimination for these products. The P_{adj} values of each metabolite are listed in Tables 1 and 2. These differences of each biomarker ion, which are reported as normalized abundance and are caused by whey microparticulation and its following fermentation, were consistent with the results showcased in the heatmaps (Fig. 2 and 3). As regards to the DART-HRMS analysis performed in negative ion mode (Fig. 4), the discrimination between WHEY and MPW can be ascribed to galactose (*m/z* 215.0327), N-acetylglucosamine (*m/z* 256.0596), ketogalactose (*m/z* 213.0174) and malic acid (*m/z* 133.0141). The ions (Z)-3-(1-formyl-1-propenyl) pentanedioic acid (*m/z* 201.0757) and tetradecanal (*m/z* 230.2478) were also detected by (+) DART-HRMS analysis in FMPW-B. Relevant changes in biosignatures detected in positive ion mode were associated with the variation of normalized abundances of hydroxymandelic acid (*m/z* 186.0758), creatinine (*m/z* 114.0663) and phenylglycine (*m/z* 152.0703) (Fig. 5). Moreover, significant differences were detected in ketoisovalerate (*m/z* 134.0810) and diethyltartrate (*m/z* 224.1123) that discriminated between non-fermented (lower abundance) and fermented (higher normalized abundance) samples, being especially discriminative for MPW (Fig. 5).

4. Discussion

The dairy supply chain has been focused on the improvement of its sustainability and the related bioeconomic policy that is intended to deliver a strong podium for dairy waste utilization by reusing and valorising whey for the development of a circular economy. Converting food waste, such as whey, into value-added products is a robust tool to answer the demanding challenges of the dairy system, and is a full strength operative tool in a sector that is providing daring solutions to food sustainability and security worldwide. Henceforth, the search for new whey-derived food products that promote consumer health, reduce waste, and help mitigate the demanding economic costs for whey disposal is of paramount importance. For this purpose, microparticulation and fermentation technologies are strategies for attaining comprehensive whey recycling while concurrently generating refrigeration-stable and biochemically enhanced, high-protein soft cream products. The utilization of microparticulation technology enables aggregation of whey proteins into particles, hence improving product stability during subsequent processing procedures. The microbial activity from fermenting microparticulated whey resulted in the occurrence of additional biochemical modification and the development

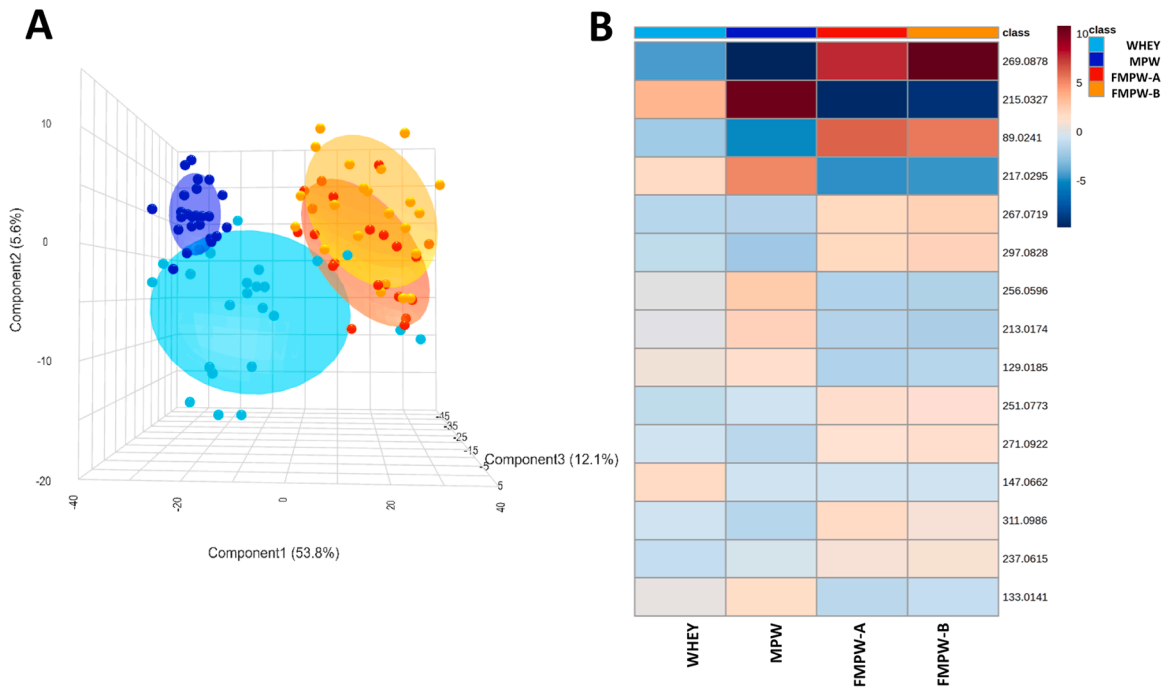


Fig. 2. Partial least squared discriminant analysis (PLS-DA) score plot of whey (WHEY, light blue), microparticulated whey (MPW, purple) and fermented microparticulated whey (FMPW-A, red; FMPW-B, orange) extracted in methanol:water (MeOH:H_2O) and acquired by direct analysis in real time high resolution mass spectrometry (DART-HRMS) in negative ion mode; ninety-five percent ellipses confidence intervals (0.95-CI) are drawn around each centroid of grouping (panel **A**). Heatmap obtained by hierarchical cluster analysis (HCA) of the 15 most informative metabolites (*m/z* values) selected by PLS-DA; the red (positive) and blue (negative) colour scales indicate the degree of similarity between metabolic ion and whey groups (panel **B**).

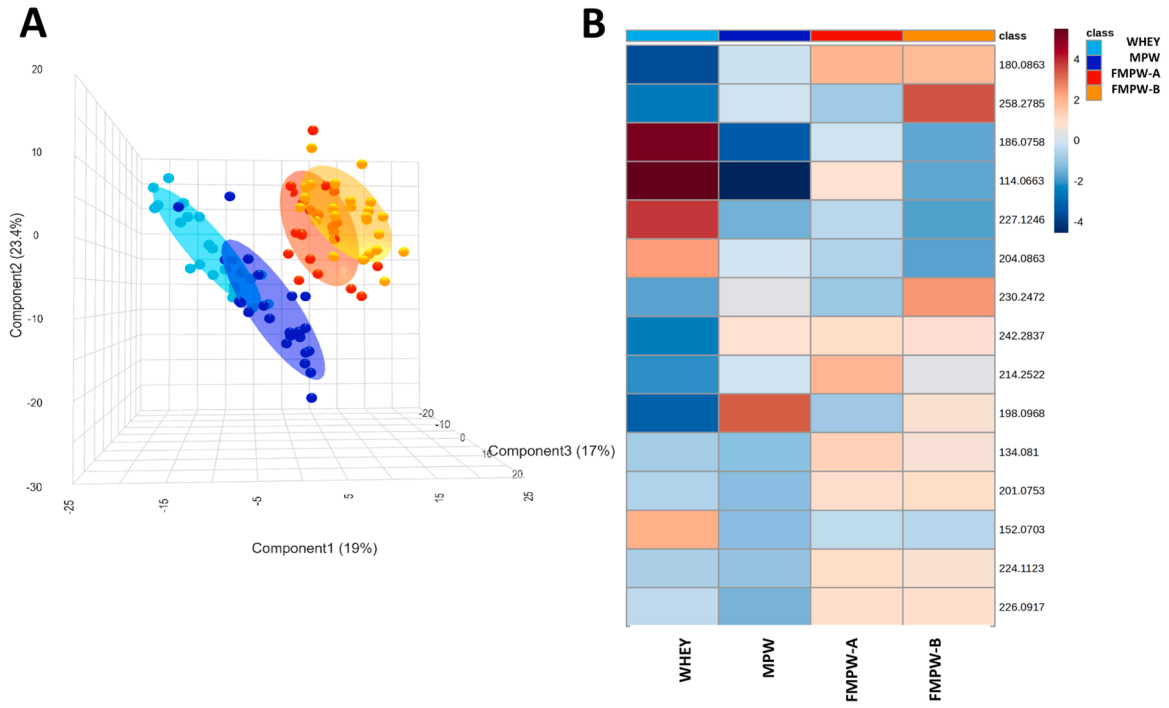


Fig. 3. Partial least squared discriminant analysis (PLS-DA) score plot of whey (WHEY, light blue), microparticulated whey (MPW, purple) and fermented microparticulated whey (FMPW-A, red; FMPW-B, orange) extracted in methanol:water (MeOH:H_2O) and acquired by direct analysis in real time high resolution mass spectrometry (DART-HRMS) in positive ion mode; ninety-five percent ellipses confidence intervals (0.95-CI) are drawn around each centroid of grouping (panel **A**). Heatmap obtained by hierarchical cluster analysis (HCA) of the 15 most informative metabolites (*m/z* values) selected by PLS-DA; the red (positive) and blue (negative) colour scales indicate the degree of similarity between metabolic ion and whey groups (panel **B**).

Table 1
Discriminant compounds that enable differentiation of whey (WHEY), microparticulated whey (MPW) and fermented microparticulated whey (FMPW-A and FMPW-B) in negative ion mode. The observed *m/z*, theoretical *m/z*, error (ppm), elemental formula, type of ion, adjusted *P*-values (P_{adj}), and tentative assignment are listed.

Whey type	*m/z*	*m/z* theoretical mass	Error (ppm)	Elemental formula	Type of ion	P_{adj}	Tentative assignment
WHEY	147.0662	147.0663	-0.67	$C_6H_{12}O_4$	$[M-H]^-$	2.17e-07	Fatty alcohol
MPW	129.0185	129.0188	-2.33	$C_5H_8O_5$	$[M-H-H_2O]^-$	1.76e-04	Hydroxyglutaric acid
	133.0141	133.0142	-0.75	$C_4H_6O_5$	$[M-H]^-$	8.01e-11	Malic acid
	213.0174	213.0170	1.88	$C_6H_{10}O_6$	$[M-Cl]^-$	2.54e-09	Ketogalactose
	215.0327	215.0328	-0.47	$C_6H_{12}O_6$	$[M+Cl]^-$	3.60e-08	Galactose
	217.0295	–	–	–	–	7.81e-08	–
	256.0596	256.0593	1.17	$C_8H_{15}NO_6$	$[M+Cl]^-$	1.70e-10	N-acetyl-glucosamine
FMPW-A	89.0241	89.0244	-3.36	$C_3H_6O_3$	$[M-H]^-$	5.52e-03	Lactic acid
	311.0986	311.0984	0.64	$C_{11}H_{20}O_{10}$	$[M-H]^-$	4.29e-11	O-glycosyl compound
FMPW-B	237.0615	237.0616	-0.42	$C_8H_{14}O_8$	$[M-H]^-$	1.66e-06	–
	251.0773	251.0780	-2.79	$C_{10}H_{14}N_4O_5$	$[M-H-H_2O]^-$	2.07e-05	Histidyl-aspartic acid
	267.0719	267.0722	-1.12	$C_9H_{16}O_9$	$[M-H]^-$	5.87e-07	3-deoxy-D-glycero-D-galacto-2-nonulosonic acid
	269.0878	269.0878	-0.04	$C_{10}H_{14}N_4O_5$	$[3M-H]^-$	1.30e-09	Lactic acid
	271.0922	271.0930	-2.95	$C_{11}H_{18}N_2O_7$	$[M-H-H_2O]^-$	1.09e-09	–
	297.0828	–	–	–	–	2.30e-11	–

WHEY: whey; MPW: microparticulated whey; FMPW-A and FMPW-B fermented microparticulated whey via starter A (mix of *L. lactis* and *S. thermophilus*) or starter B (*B. animalis*). Histidyl-aspartic acid is also termed aspartyl-histidine acid.

Table 2
Discriminant compounds that enable differentiation of whey (WHEY), microparticulated whey (MPW) and fermented microparticulated whey (FMPW-A and FMPW-B) in positive ion mode. The observed *m/z*, theoretical *m/z*, error (ppm), elemental formula, type of ion, adjusted *P*-values (P_{adj}), and tentative assignment are listed.

Whey type	*m/z*	*m/z* theoretical mass	Error (ppm)	Elemental formula	Type of ion	P_{adj}	Tentative assignment
WHEY	114.0663	114.0662	0.88	$C_4H_7N_3O$	$[M+H]^+$	1.58e-12	Creatinine
	152.0703	152.0706	-1.97	$C_8H_9NO_2$	$[M+H]^+$	3.12e-09	Phenylglycine
	186.0758	186.0761	-1.61	$C_8H_8O_4$	$[M+NH_4]^+$	2.36e-11	Hydroxymandelic acid
	204.0863	204.0872	-4.42	$C_8H_{15}NO_6$	$[M+H-H_2O]^+$	1.38e-08	N-acetyl-glucosamine
MPW	198.0968	198.0972	-2.02	$C_6H_{12}O_6$	$[M+NH_4]^+$	3.04e-03	Galactose
	242.2837	242.2842	-2.06	$C_{16}H_{32}$	$[M+NH_4]^+$	1.08e-10	Hexadecene
FMPW-A	134.081	134.0812	1.49	$C_5H_8O_3$	$[M+NH_4]^+$	1.47e-05	Ketoisovalerate
	214.2522	214.2529	-3.3	$C_{14}H_{28}$	$[M+H]^+$	3.82e-12	Tetradecene
	224.1123	224.1129	-2.7	$C_8H_{14}O_6$	$[M+NH_4]^+$	2.25e-10	Diethyltartrate
FMPW-B	180.0863	180.0861	1.11	$C_6H_{10}O_5$	$[M+NH_4-H_2O]^+$	5.30e-04	Glucosamine
	201.0753	201.0757	-1.98	$C_9H_{12}O_5$	$[M+H]^+$	3.82e-12	(Z)-3-(1-Formyl-1-propenyl) pentanedioic acid
	226.0917	226.0921	-1.77	$C_7H_{12}O_7$	$[M+NH_4]^+$	5.71e-10	O-methylglucoronic acid
	230.2472	230.2478	-2.61	$C_{14}H_{28}O$	$[M+H]^+$	6.49e-09	Tetradecanal
	258.2785	258.2791	-2.32	$C_{16}H_{32}O$	$[M+NH_4]^+$	1.26e-08	1-hexadecanal

WHEY: whey; MPW: microparticulated whey; FMPW-A and FMPW-B fermented microparticulated whey via starter A (mix of *L. lactis* and *S. thermophilus*) or starter B (*B. animalis*).

of specific metabolic profiles (Buchanan et al., 2023; Vieira et al., 2023). For this reason, the primary goals of this study were to examine the effectiveness of multi-modal DART-HRMS to assess the subtle metabolic differences among whey and derived, processed types of whey (MPW, FMPW-A and FMPW-B) and to unveil their distinctive metabolic fingerprinting. For this purpose, the ability of DART-HRMS to capture the metabolic variability among whey and processed whey samples was investigated.

4.1. Discriminant analysis

An effective predictive capacity, which solved the discriminant challenge related to whey's transformation into a fermented microparticulated derived food, was demonstrated by DART-HRMS assessment integrated with chemometrics. This combined approach highlighted the accurate spatial separation among our WHEY, MPW, and FMPW samples, especially based on the restricted molecular features acquired in negative ion mode. However, the powerful capacity of AIMS to differentiate between WHEY and MPW was not confirmed in the comparison between the two FMPW subtypes (i.e., FMPW-A vs. FMPW-B), which indicates these value-added dairy by-products have similar metabolic fingerprints. Probably the lactic acid bacteria associated with the related starter cultures (i.e., a mix of *L. lactis* and *S. thermophilus* vs. *B. animalis*) resulted in a similar biochemistry pattern composed of lactic acid, alcohols, peptides and volatile fatty acids via enzymatic degradation of lactose, proteins and fatty acids. These compounds contribute to the distinct flavours, aromas and nutritional properties of fermented whey and are associated with the relatively long shelf life (Chen et al., 2017). Notably, the 0.95 ellipses confidence intervals (0.95-CI) of the PLS-DA score plot highlighted the wide experimental variability of the WHEY samples, which was substantially reduced after the microparticulation process; this indicates that this nutrient concentration treatment decreased the metabolic variability of the MPW in relation to WHEY. The statistical spatial resolution of the FMPW samples indicates that fermentation of MPW led to wide metabolic variability in the fermented products, likely due to the large diversity in the release of key differential compounds during the repeated experimental fermentation processes (Melnikova et al., 2015).

4.2. Metabolic profiles of whey (WHEY) and microparticulated whey (MPW)

In the investigated whey and whey-derived products, the identified classes of compounds detected by DART-HRMS analysis included a wide range of carbohydrates, hydroxy acids, carbohydrate derivates, keto acids, fatty acids, aldehydes and sugar acids.

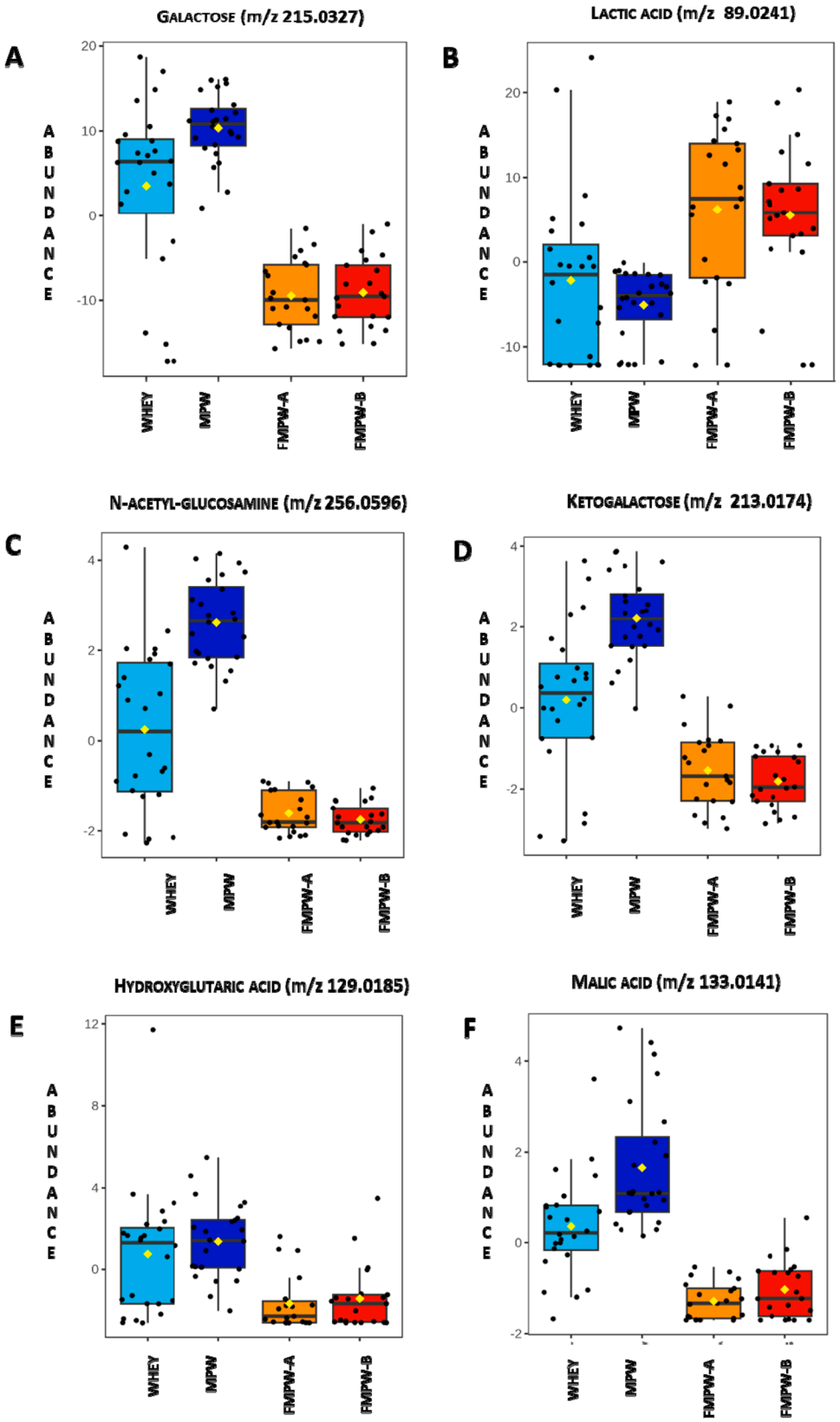
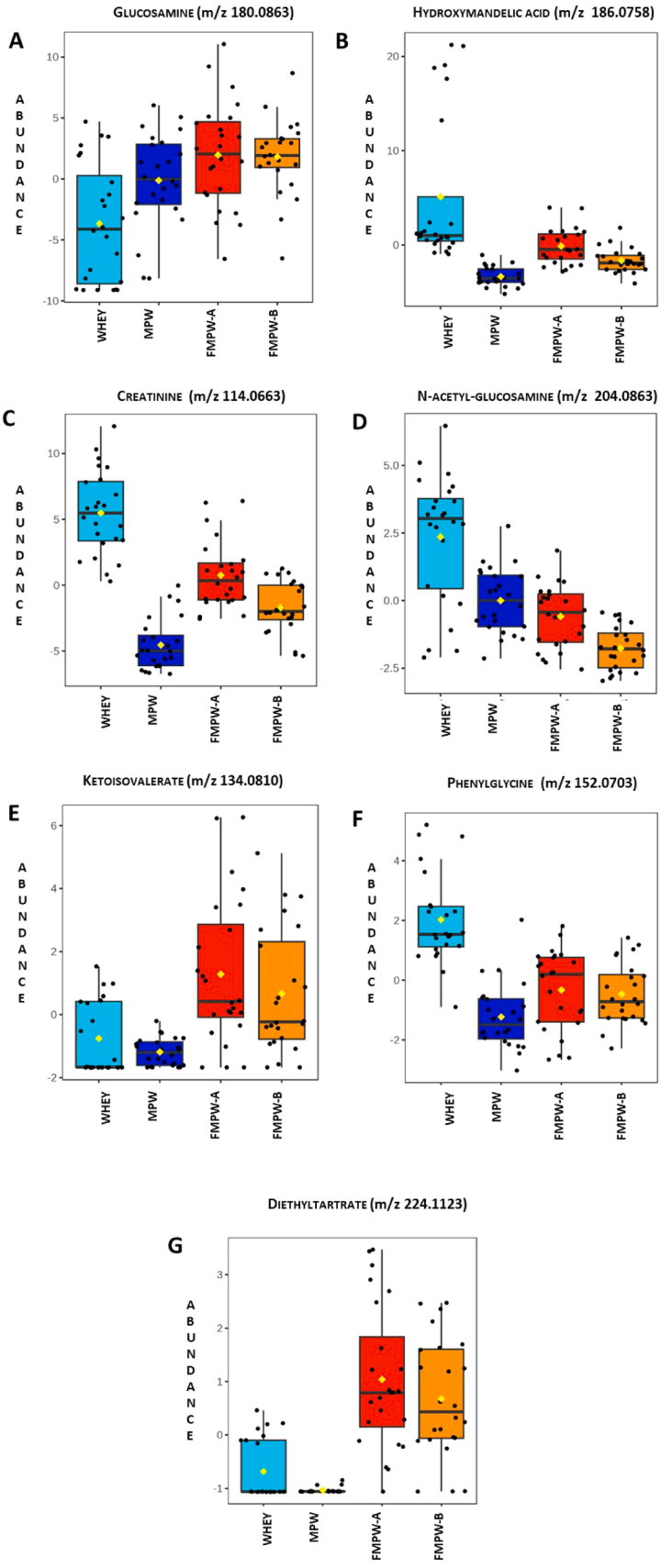


Fig. 4. Box plots of the highest statistical relevant ($P_{adj} < 0.05$) metabolites acquired by direct analysis in real time high resolution mass spectrometry (DART-HRMS) in negative ion mode. WHEY: whey; MPW: microparticulated whey; FMPW-A and FMPW-B fermented microparticulated whey via starter A (mix of *L. lactis* and *S. thermophilus*) or starter B (*B. animalis*). The bottom and top of each box represent the 25th and 75th percentiles, respectively, the mid-line indicates the 50th percentile or the median. The yellow square indicates the mean. The black circles represent the entire data range, including the extreme value outliers not taken into consideration.



(caption on next page)

Fig. 5. Box plots of the highest statistical relevant ($P_{adj} < 0.05$) metabolites acquired by direct analysis in real time high resolution mass spectrometry (DART-HRMS) in positive ion mode. WHEY: whey; MPW: microparticulated whey; FMPW-A and FMPW-B fermented microparticulated whey via starter A (mix of *L. lactis* and *S. thermophilus*) or starter B (*B. animalis*). The bottom and top of each box represent the 25th and 75th percentiles, respectively, the mid-line indicates the 50th percentile or the median. The yellow square indicates the mean. The black circles represent the entire data range, including the extreme value outliers not taken into consideration.

The heatmap analysis clearly demonstrated that galactose (*m/z* 215.0328) ion was one of the most relevant informative biomarkers of MPW compared to WHEY. This outcome underlines the role of the whey microparticulation process that influences the distinctive chemical profile of MPW, leading to significant increases of the galactose and lactic acid concentrations over those of WHEY, likely due to growth of *S. thermophilus* in these lactose-containing substrates. It has been already reported how galactose and lactic acid concentrations change when whey is maintained at over 30°C for several hours, a storage condition promoting the metabolic activity of residual starter culture (Rao et al., 2004). Additionally, it is well know that the microparticulation steps led to a selective increase of sugars, especially galactose (Buchanan et al., 2023).

N-acetyl-glucosamine ion was previously found in whey ultrafiltration permeate samples as a non-protein-nitrogen compound (Tsermoula et al., 2023). In accordance with literature (Allen et al., 2021), creatinine (*m/z* 114.0662) and n-acetyl-glucosamine (*m/z* 204.0872) in our WHEY samples were previously found in acid whey derived from Greek yogurt, and the same authors observed that the metabolite profile of whey was influenced by the milk source used in the yogurt production process. Phenylglycine (*m/z* 152.0706) is a glycine carrying an N-phenyl substituent and belongs to the class of non-proteinogenic amino acids, organic compound knowns as an α-amino acid (Al Toma et al., 2015). Hydroxymandelic acid is a metabolite formed from the bacterial degradation of tyramine or polyphenols (Tabasco et al., 2011). Tyramine, a biogenic amine, is synthesized from tyrosine that can be found in fresh whey (Pu et al., 2021) and whey products (Mirzaei et al., 2022). The high amount of hydroxymandelic acid in our WHEY could be ascribed to the metabolic activity of residual LAB during the step before microparticulation. The lower amounts of this acid in MPW could depend on the microbial (e.g., *Pseudomonas*, yeast, fungi) metabolism of mandelate and related compounds (Fewson, 1988).

4.3. Metabolic profiles of FMPW

In FMPW-A and FMPW-B, the identified classes of compounds detected by DART-HRMS analysis were hydroxy acids, carbohydrate derivates, keto acids, fatty acids, aldehydes and sugar acids. As expected, a key discriminative role in fermented MPW was linked to the relative high ion abundances of the metabolites *m/z* 89.0241 and *m/z* 269.0878, which were both tentatively assigned as lactic acid. These metabolites were produced during fermentation processes when lactose was enzymatically broken down by the microbial starters using the main biochemical pathway (Bandara et al., 2023). As already stated above, the (−) DART-HRMS captured a decrease in the relative abundances of galactose and ketogalactose with the fermentations of the MPW. While *L. lactis* and *B. animalis* subsp. *lactis* fully metabolise galactose, *S. thermophilus* and *Lactobacillus bulgaricus* seem to not completely metabolise galactose during fermentation (Wu et al., 2015; Egan et al., 2018). Ketogalactose is an intermediate in galactose metabolism, but it could also originate from the oxidation of galactose throughout the thermal and mechanical processes that led to our MPW product. Note that galactose accumulation can have detrimental effects both on dairy product quality and on individuals suffering from galactosemia. The starter culture employed in fermentation to produce MPW-A and MPW-B can convert galactose in exopolysaccharides through the Leloir pathway (Wu et al., 2015). Reduced relative abundances of hydroxyglutaric acid and malic acid were also detected after MPW fermentation. Both *Lactobacillus* spp. and *Bifidobacterium* spp. utilise malic acid as a carbon source for bacterial growth and secondary fermentation (malolactic fermentation), by decarboxylating malic acid to lactic acid via the malolactic enzyme (Guo et al., 2022). It is well known that the amounts of free amino acids change with fermentation, and different amounts of histidine and arginine were already encountered in fermented acidophilus dairy products (Ivanov et al., 2021). A high relative abundance of ketoisovalerate ion (*m/z* 134.0812) was also detected by (+) DART-HRMS analysis in FMPW-A samples. Ketoisovalerate is a ketoacid that probably arises from the incomplete deamination of branched-chain amino acids in whey proteins (e.g. leucine, isoleucine and valine), which are broken down through enzymatic processes aided by proteolytic enzymes mainly derived from *L. lactis* and *B. animalis* (Wang et al., 2018). Additionally, α-ketoisovaleric acid (derived from valine) is the precursor of 2-methylpropanal, a volatile organic compound (VOC) that is essential for providing dairy products with their distinct odour and flavour (Yavuz et al., 2021; Zacometti et al., 2023). Post-fermentation changes in volatile compounds (tetradecanal, hexadecane and tetradecene) were also observed in our MPW. Tetradecanal (*m/z* 230.2478) is a long chain aldehyde, and the presence of fat in whey allows the bacteria lipases to hydrolyse triacylglycerides effectively. It is known that aldehydes can be derived from the decomposition of hydroperoxides (octanoic acid, 9-decenoic acid and tetradecanoic acid) of unsaturated fatty acids (Bergamaschi & Bittante, 2018). These aldehydes can be further reduced, which explains the presence of the fatty alcohol at *m/z* 147.0662 (Zacometti et al., 2023). It is worth remembering that the whey in the present study come from whole milk cheese processing, which concentrates the free fatty acids within whey, and subsequently, they become substrates for lipolysis and oxidation reactions (Zareba et al., 2012; Risner et al., 2019).

5. Conclusions

The study highlights microparticulation technology with subsequent fermentation as an effective process to transform native whey into an innovative soft cream dairy product. The application of DART-HRMS coupled with supervised multivariate modelling provided insights into the intricate metabolic differences between whey and its microparticulated and fermented derivatives, thus shedding light on the specific metabolic fingerprints of various subtypes of fermented whey concentrate. The specific DART-HRMS biosignatures reveal a set of low-molecular-weight biomolecules that together could be an effective metabolic benchmark useful for monitoring the adoption of this technological approach in the whey recycling sector and for further studies in dairy foodomics fields. However, the different fermentative bacteria strains tested in this study did not produce significant differences across the complex biochemical pathways involved mainly in the breakdown of lactose and serum proteins into a wide range of metabolites, categorised as carbohydrate derivates, amino acid derivates, sugar acids, hydroxy acids, keto acids, aldehydes and alcohols. Importantly, as the dairy supply chain continues to address challenges and push the boundaries of whey recycling via valorisation of whey's nutritional and protein-based organoleptic properties, further investigations are necessary for better understanding the chemical and metabolomic characteristics of microparticulated and fermented whey-derived products. For this purpose, DART-HRMS or other AIMS techniques are poised to play a central role in the analytical toolbox for unlocking the comprehensive understanding of complex food dairy production systems at the screening level. Note that one of the reported caveats of AIMS is the strong matrix effect that results in low recoveries (Tata et al., 2022), which can lead to

fluctuations in relative intensity of the metabolites. This strong matrix effects results in limited quantification abilities (Gross, 2013), which issue can be disregarded when AIMS are meant for screening applications.

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Ethical statement – Studies in humans and animals

The authors declare that this study did not involve neither humans nor animals.

CRediT authorship contribution statement

Carmela Zacometti: Investigation, Methodology, Writing – original draft. **Sara Khazzar:** Investigation, Methodology, Software, Writing – original draft. **Andrea Massaro:** Software, Data curation. **Alessandra Tata:** Formal analysis, Data curation, Writing – review & editing, Writing – original draft. **Giorgia Riuzzi:** Formal analysis, Writing – original draft. **Roberto Piro:** Formal analysis, Funding acquisition, Project administration. **Enrico Novelli:** Conceptualization, Writing – original draft, Writing – review & editing, Funding acquisition, Project administration. **Severino Segato:** Validation, Writing – review & editing, Writing – original draft, Funding acquisition, Supervision. **Stefania Balzan:** Conceptualization, Investigation, Writing – review & editing, Writing – original draft.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.afres.2024.100443.

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