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Application of metabolomics to assess milk quality and traceability☆

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Milk is a foodstuff widely consumed around the world originating from a variety of different species, animal management and production systems. In recent years, consumers have placed a much greater emphasis on the authenticity and origin of some food products often willing to pay a premium price for such products that is, for example 'Grass-Fed Dairy'. Therefore, it is important to establish methods to assess both quality and authentication of milk and dairy products for increased food security and consumer protection. Accordingly, NMR-based, GC–MS-based, and LC–MS-based metabolomics have been established as useful tools in the analysis of dairy products, such as raw and processed milk. This short-review provides an updated and critical overview on the most useful metabolomics-based platforms and the most useful multivariate statistical tools available for metabolomic data interpretation.

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Introduction

Milk is a complex and highly variable, nutritious food item. There are a variety of factors that can affect the composition, quality, and with that the functionality, of milk and dairy products, namely cows' diet, breed, health, stage of lactation and parity [1]. While much research has been carried out to examine the impact of animal traits, farming practises and environmental factors on milk yield and production of macro nutrients in milk such as protein, fat and lactose, metabolomics has been gaining prominence for the characterisation and in some cases quantification of low molecular weight compounds, also referred to as metabolites, in milk [2].

Metabolomics is now an established but yet, ever evolving method of analysis widely applied to a variety of fields of study including medicine, environmental science, agriculture, crop and life sciences to mention a few. In recent years the application of metabolomics to the field of dairy science has grown considerably with important factors and biomarker compounds related to animal health, production, authentication and contributors to milk techno functional properties being reported. To date over two thousand metabolites have been identified in milk and a landmark study by Foroutan *et al.* [3••], resulted in the development of the milk composition database (MCDB); a freely available electronic database containing detailed information about small molecule metabolites found in cows' milk www.mcdb.ca. Similarly to the macro components, milk metabolites can be affected by a variety of factors including, complex matrix effects [4], animal species (e.g. cow, goat, and sheep) [5], thermal treatment [6], cow status [7], and farming systems [8]. With a particular focus on bovine milk, the purpose of this mini review is to provide an introduction to the field of milk metabolomics and highlight some insights on its application for monitoring herd health, analysis, prediction of techno-functional properties and authentication of milk and dairy products.

Tools for metabolomic analysis of milk and dairy products

The characterization of the milk metabolome is a promising approach for establishing its overall quality and authenticity [2]. The metabolomic fingerprint of milk and dairy products can be obtained using different tools, namely gas chromatography/mass spectrometry (GC–MS), NMR spectroscopy, high-resolution magic angle spinning (HRMAS) NMR spectroscopy, liquid chromatography/mass spectrometry (LC–MS), and liquid chromatography-tandem MS (LC–MS/MS) [2,3••,9]. These approaches are based on different analytical instruments, varying in terms of both sensitivity and metabolite coverage. In this regard, NMR remains the most commonly used analytical platform in milk metabolomics research [9]. It is often chosen for its reliability and utility in

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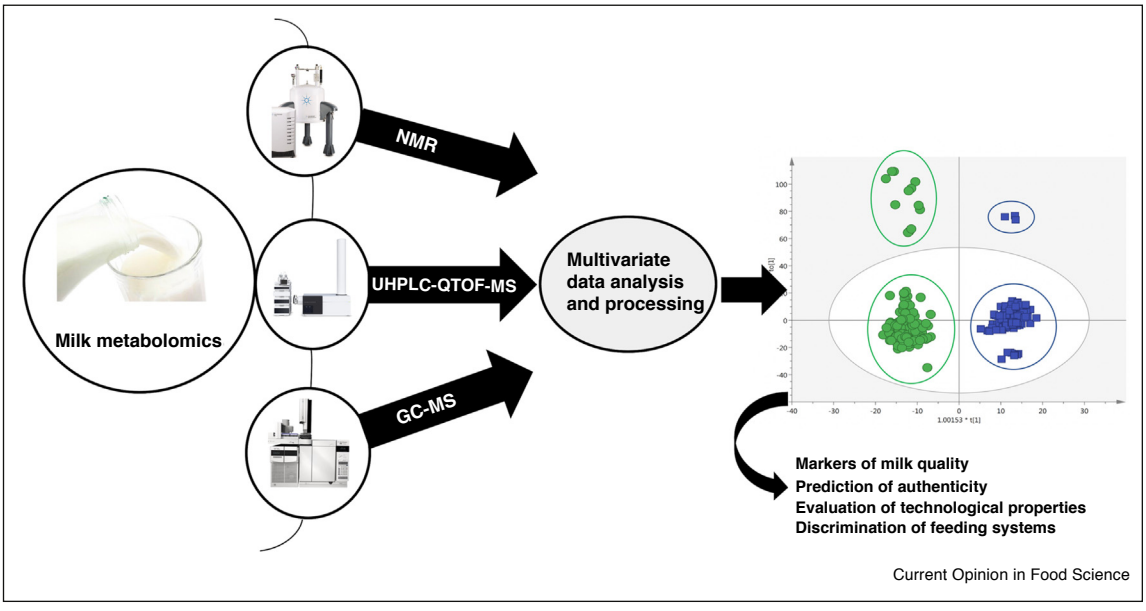
absolute quantitation; however, NMR is quite insensitive and limited to measuring substances in micromolar to millimolar concentrations [10]. On the other hand, MS-based platforms (such as LC–MS and LC–MS/MS) can identify metabolites at nanomolar to picomolar concentrations, allowing a much higher number of metabolites to be detected [10,11]. Also, GC–MS is less sensitive than LC–MS but is generally more robust and more reproducible. Therefore, GC–MS can be used to identify/quantify the milk metabolites with higher precision and reproducibility than either NMR or LC–MS [10,12]. LC–MS and GC–MS generate comprehensive metabolomics profiles, particularly for small metabolites (<1−2 kDa) in complex matrices. Obviously, each of the different methodologies has its advantages and disadvantages, and often the techniques complement each other. An overview on the most important platforms to carry out milk metabolomics is provided in Figure 1. However, a combined approach including more than one instrumental approach is the most advocated for to reach the greatest level of metabolomic coverage [13].

Metabolomic approaches based on GC-mass and LC-mass spectrometry

GC–MS represents one of the best platforms for metabolomic analysis (mainly for volatile compounds), which yields strong sensitivity and highly repeatable fragmentation [14]. The most used spectral libraries, such as NIST and AMDIS, allow the identification of several biomarkers and a better understanding of subsequent biological mechanisms or pathology alterations [15]. However, GC–MS usually requires difficult and tedious sample processing and derivatization when focused on non-volatile constituents [16]. To increase the volatility and thermal stability of the analytes, various derivatizations, such as alkylation, acylation, and silylation, can be employed to 'protect' functional groups. Among these derivatization methods, methoximation and trimethylsilylation are commonly used in large-scale metabolomics studies with GC–MS [16]. Therefore, basing on the different efficiency in the derivatization of each metabolite, the reproducibility of the overall analysis may be affected. Besides, it should be noted that the formation of byproducts resulting from the derivatization process may lead to a difficult comparison between the studies available in literature.

When dealing with metabolomic approaches based on GC–MS and LC–MS platforms, it is important to mention the differences existing between targeted and untargeted approaches. Overall, targeted approaches are able to identify and quantify a limited number (tens to hundreds) of known metabolites, such as those common marker compounds involved in clinical and/or technological analyses [17••]. On the other hand, untargeted approaches focus on acquiring the greatest amount of information, as annotating metabolites, and reviewing both known and unknown metabolic changes [18]. Data can be used for relative quantification across sample groups and to provide hypotheses that can be further studied and validated with targeted approaches. There are also two commonly used approaches for data acquisition in untargeted metabolomics studies; the first one is based on full scan MS-only acquisitions to generate accurate mass measurements for individual

Figure 1



Schematic overview on the most important instrumental platforms for milk metabolomics studies.

molecules (i.e. raw mass features), thus allowing multivariate statistical calculations, followed by a data dependent acquisition to drive identifications. In particular, this latter approach generates fragmentation patterns for metabolites exhibiting the highest signal intensity [18–20]. A second untargeted metabolomics approach is based on the so-called data independent acquisition, based on integrating a full scan MS-only acquisition with MS/MS fragmentation for all precursor ions either simultaneously or in specific mass ranges [18,21]. However, these latter methods produce complicated fragmentation spectra and the link between precursor and product can be difficult to decipher. In the following data analysis steps, fragment ions are matched with precursor ions based on retention time, mass, and drift time. Data independent acquisitions allow fragmentation data to be acquired regardless of metabolite signal intensity.

For the identification step, precursor ions and corresponding fragment ions (when available) are searched against databases for metabolite assignments. Looking at available database, we can assert that Milk Composition Database (MCDB) [3••] and Bovine Metabolome Database (BMDB) [22••] represent two of the most comprehensive databases to work on milk metabolomics and to provide a better understanding of bovine biology, the micronutrients found in other bovine tissues and biofluids, as well as improving veterinary care for dairy cattle. However, it is essential to report the confidence level in metabolite assignments, and therefore metabolite annotation represents the crucial link between acquired data and meaningful biological information [23,24]. To date, five levels of confidence in identification have been established; in particular, the highest confidence of validated identification (Level 1) confirms a structure with a minimum of two independent and orthogonal data from a pure standard compound analysed under identical analytical conditions. A lack of reference standard acquisition but predictive or externally acquired structure evidence, namely MS/MS data, exhibiting specific fragments or neutral losses consistent with a specific structure would be considered a putative identification (Level 2). Preliminary identifications (Level 3) arise when accurate mass and isotopic profiles produce tentative structures from database searches. Therefore, it is important to consider that a single molecular formula typically corresponds to multiple candidate structures (e.g. isomeric compounds, such as lipids). Finally, the molecular formula candidates (Level 4) and de-convoluted experimental m/z features (Level 5) characterize the less confident annotation levels.

Metabolomic approaches based on NMR spectroscopy

Regarding non MS-based metabolomic studies, it is important to cite the role of ^{1}H NMR in dairy research. ^{1}H NMR requires minimal sample preparation and enables the detection of mobile hydrogen-containing molecules [9,25]. Compared with LC–MS and GC–MS, one of the principal advantages of NMR spectroscopy is the direct and quantitative relationship between molar concentration and the intensity of the NMR resonances. Moreover, it is a non-destructive technique; consequently, the sample can be analyzed in multiple consecutive experiments, or additionally be analyzed by other analytical techniques after the NMR experiments are performed. Besides the initial high capital costs (an NMR spectrometer is a quite expensive instrument), the running costs are lower compared with other techniques when considering the minimal sample preparation required [26]. Besides, in the case of milk metabolomic applications, difficulties associated with sample size (typical of NMR applications) are rarely a problem. Therefore, high-field ^{1}H-NMR spectroscopy allows detection with great efficiency of several compounds in milk, including sugars, small organic acids, vitamins, nucleotides, and aromatic compounds, thus representing one of the best tools for milk metabolomics research [9].

Data interpretation and multivariate statistics

It is important to cite the importance of data processing when dealing with milk metabolomics data [27]. To date, there are various open access and commercial softwares available for MS data processing and analysis. These tools involve peak alignment, peak extraction, metabolite identification, data normalization and scaling, and metabolic pathway analysis by searching metabolomic databases. However, there is no currently available standardized software/website for MS and NMR data processing and analysis on milk metabolomic data. In fact, as is reviewed in literature [14], using different software, the results can be very different, thus revealing that these tools have a great influence on data processing and final outcomes.

Furthermore, the already described diversity of instrumental approaches makes multivariate analysis techniques as an essential element to analyze the collected data and to reduce their complexity [28]. In multivariate statistics, a data matrix X, containing n observation row vectors of k variables each, is quite common and very few mathematical constraints can be found to its application. Therefore, the spectral data collected from NMR, MS, or any other source can be used as input into the data matrix X. The data matrix X can be immediately decomposed using unsupervised dimensionality reduction methods, such as principal component analysis (PCA), or it can be paired with a matrix Y of n corresponding m-dimensional outputs to be used in supervised dimensionality reduction, in the case of partial least squares (PLS) and orthogonal projections to latent structures (OPLS) regressions [28].

Therefore, one should choose the workflow to statistically process and analyze data according to the matrix of interest (i.e. milk or cheese), to maximize the biological significance.

To date, there are three notable softwares and websites allowing comprehensive metabolomics data analysis, namely Mass Profiler Professional (from Agilent Technologies), MetaboAnalyst (https://www.metaboanalyst.ca/) [29•], and Maven (https://maven.princeton.edu) [30]. Accordingly, these tools accept all data file formats from different instruments (such as. d or. raw), allowing users to perform multivariate statistical analyses (such as unsupervised and supervised), together with pathway analysis and data visualization.

Selection of the best multivariate statistical workflow

The selection of the best multivariate statistical workflow is usually driven by the experimental goal and the quality of the collected data. The initial application of unbiased unsupervised statistics, such as hierarchical cluster analysis or PCA, provides a first look of the similarities and differences between the observation groups. Therefore, the results of unsupervised statistical data analyses are useful to formulate an initial biological conclusion, which PLS or OPLS can then verify and validate in more detail, by maximising the so-called within-group variability [27,28].

However, before producing a reliable dataset to be further statistically elaborated, some data pre-processing is required, including binning, alignment, data normalization, data scaling, and noise removal. The binning procedure is particularly useful to elaborate ^{1}H NMR data, considering that chemical shifts are particularly sensitive on temperature, pH, ionic strength, and other factors influencing their electronic environment [28]. The imprecisions in chemical shifts measurements might affect the X variables of the further statistical model, thus determining a scarce separation into the PCA or PLS/OPLS score plot. One mitigation strategy is to divide each spectrum into bins having a lower spectral width (e.g. 0.04 ppm), then integrating signal intensities within each bin to produce a smaller set of variables. On the other hand, this method can hide potentially significant changes of low-intensity peaks nearby strong signals, thus loosing possible relevant information [28]. Therefore, in the recent years, several alternative binning strategies have been developed, as widely reviewed in literature [28]. Also, full-resolution spectral signals may be computationally aligned within a dataset to remove chemical shift variability. It has been demonstrated that OPLS-DA more effectively copes with chemical shift variation in full-resolution ^{1}H NMR datasets without requiring binning or alignment steps [28]. Similar problems can be found for GC–MS and LC–MS data, when considering retention time and accurate mass alignments to extrapolate those mass features to be retained for multivariate data analysis. However, to date, several algorithms performing alignments have been introduced, thus reducing the amount of operator intervention required for large metabolomic datasets [31].

Data normalization and data scaling are two other important operations to produce reliable metabolomic datasets. Data normalization ensures that all observations are directly comparable. It can be internally realised by using internal standards (e.g. trimethylsilylpropanoic acid in NMR) or using a constant-sum normalization, where each spectrum is normalized such that its integral is 1 [28,32]. Another important approach is represented by data scaling options; the standard approach is usually based on autoscaling, which positions all variables on a comparable scale to achieve separation of two or more groups. According to literature, the most commonly used data scaling methods in metabolomics research are UV, Range, Pareto, Vast, and Level scaling options [28]. On the other hand, some previous works demonstrated that data pretreatment is context-dependent and that no single superior method actually exists, although Vast-scaling showed the highest robustness and stability when considering NMR and GC–MS data [33].

Finally, regarding the multivariate statistical tools commonly used in metabolomics research, it is important to mention three major techniques, namely unsupervised methods, supervised methods, and pathway analysis [31,34]. Unsupervised methods help to discover the most important data trends, considering that the data are not labelled under any class, with no or little information/assumption about the data. Being the first step in the analysis process, unsupervised statistical approaches assist in visualizing the data. The most common unsupervised tools are represented by PCA, clustering analyses, and self-organizing maps (SOM) [34]. PCA algorithm can help to visualize the total variation in the original dataset, and it is particularly useful in dimension reduction. Most information about the dataset is retained by the principal components (usually the first two components) that actually replace all the correlated variables. A score plot is used to find the groups, while a loading plot indicates those variables that separate the groups from each other. Similarly, clustering methods help to group data that are similar, so that the data in one cluster are relatable when compared with the data in another cluster. The two most widely used clustering techniques in metabolomics research are k-means clustering and hierarchical clustering. In k-means clustering, the data are divided into k-clusters that do not overlap. Unlike k-means clustering, hierarchical clustering continues to split all the data until a hierarchy of clusters is formed. It is often combined with a heat map for data matrix visualization where the different colours represent the fold-change of each metabolite across the sample groups. Finally, self-organizing map (SOM) is a visualization tool that assists in visual discovery of the clusters present in data [35].

Regarding supervised tools, these are widely used for biomarker discovery, categorization, and prediction.

These methods deal with datasets having response variables that are either continuous or discrete, finding associations between covariates and response variables, and providing accurate degree of predictions [31,34]. In particular, PLS-DA and OPLS-DA are widely used in metabolomics research for identifying biomarkers by extrapolating the so-called variables of importance in projection (VIP), that is, those compounds maximizing group separations.

Finally, pathway analysis helps to find the biological mechanisms hidden within the dataset. The two most common methods are represented by overrepresentation analysis (ORA) and functional class scoring (FCS) [36]. ORA is performed when the pathways differ considerably among the two study groups. However, the limitations of ORA are addressed by the FCS method. In particular, single metabolite statistics are obtained first and these are aggregated to evaluate a pathway-level statistic, either univariate or multivariate. Most often enrichment score, mean, and median are used for univariate pathway-level statistics, while Hotelling's T2 statistics is widely used for multivariate statistics [36].

Milk metabolites for prediction of herd health

In recent years there has been an increased emphasis on the development of methods to quickly and non-invasively predict animals' health and welfare status, which can have important implications for production and quality of milk and dairy products. A variety of approaches have been suggested in the past such as rapid mid-infrared spectrometry analysis of milk samples for prediction of energy balance [37] and the rapid detection of somatic cell counts (SCC) in milk has been widely used for many years as a predictor of the presence of mastitis in the cow's udder. Sundekilde *et al.* [38] reported a significant correlation between the SCC of milk and its metabolite profile, whereby levels of lactate, butyrate, isoleucine, acetate and ß-hydroxybutyrate increased and levels of hippurate and fumarate decreased with increasing SCC in milk. A number of studies have also identified a series of milk metabolites that could potentially be used as biomarkers of animal's health. For example, the ratio of metabolites glycerophosphocholine and phosphocholine detected in milk by NMR has been highlighted as a biomarker for risk of ketosis [39]. Furthermore, links between the milk metabolome and energy metabolism of cows was investigated by Klein *et al.* [40]. The authors reported biomarkers such as acetone and ß-hydroxybutyrate were highly correlated with metabolic status of cows in early lactation. Xu *et al.* [41] examined the milk metabolome of cows in a status of negative energy balance and found that the animals' energy balance was highly corelated to several milk metabolites including glycine, choline and carnitine. Tian *et al.* [42] also highlighted a number of milk metabolites that could act as biomarkers of cows suffering from heat stress, with significant differences in the milk metabolome of heat stressed cows through changes in animal metabolism. Such studies would suggest milk metabolomics could be a useful tool for the continuous monitoring of herd health.

Milk metabolites for prediction of techno-functionality

Milk metabolomics can be very useful to assess not only milk quality, but also to predict the response of milk to different processing strategies. An overview of the most comprehensive works dealing with milk metabolomics for both authenticity and quality assessment can be found in Table 1.

The technological properties of milk are an important and constant consideration for dairy manufacturers, in particular with seasonal milking systems, where the composition, and with that the functionality of milk, can change as cows progress through early, mid and late stages of lactation. These properties will impact how milk reacts during the different stages of processing and with that ultimately affect the efficiency of the process and quality of the final product. Such properties can include, but are not limited to, the heat stability of milk, acid coagulation, and rennet coagulation properties. Sundekilde *et al.* [44] on examination of the impact of the milk metabolome on the technological properties of milk from two dairy herds, demonstrated that the acquired metabolite profiles could be correlated with the coagulation properties of the milks, in particular with variation in the concentrations of citrate, choline, carnitine and lactose. In another study, several milk metabolites detected using NMR were correlated with the protein content of milk and the rennet-induced coagulation properties [49]. Namely, lactate, acetate, glutamate, creatinine, choline, carnitine, galactose 1-phosphate, and glycerophosphocolines were highlighted to be different between non coagulating and well coagulating milks, and these, according to the authors, could act as quality markers for cheese milk. Similarly, Harzia *et al.* [50] found that levels of the metabolites lactate, acetate, glutamate, creatinine, choline, carnitine, and glycerophosphocolines were found to be associated with non-coagulating milk properties and were significantly different between non coagulating and well coagulating milks. Recently, Salzano *et al.* [58] established the utility of GC–MS-based metabolomics coupled with mass spectral libraries as a powerful technology platform to determine the authenticity, thus creating a market protection, for Mozzarella di Bufala Campana (i.e. a Protected Designation of Origin, PDO, Italian cheese). In particular, the authors reported that both PDO milk and mozzarella cheese were characterized by exclusive metabolites (when compared with non-PDO products), namely talopyranose, panthothenic acid, mannobiose, and 2,3-dihydroxypropyl icosanoate, highlighting also the impact of PDO on the final quality of the product. Rocchetti *et al.* [46••] demonstrated that the feeding system could

Table 1

Studies summarising the application of metabolomics for both authenticity and quality assessment of milk and cheese

Food matrix	Metabolomic platform	Statistical analysis	Main findings	Reference
Bovine milk	^{1}H-NMR	PLS-DA	Discrimination of Friesian and autochthonous cow milks. The discriminant metabolites were correlated to technological property parameters.	[43]
Bovine milk	LC–QQQ–MS and ^{1}H NMR	Pearson's correlations between NMR and LC–MS data.	Cows in negative energy balance produced more milk with increased milk fat yield but lower concentrations of choline, ethanolamine, fucose, *N*-acetyl-neuraminic acid, i-acetyl-glucosamine, and *N*-acetyl-galactosamine.	[13]
Bovine milk	^{1}H-NMR	PCA	The milk metabolite profiles obtained could be correlated to breed and also with the coagulation profile.	[44]
Bovine milk	^{1}H-NMR	PCA	Biologically fed buffaloes were characterized by larger content of unsaturated lipids and phosphatidylcholines.	[45•]
Bovine milk	^{1}H-NMR	PCA, PLS-DA, and OPLS-DA	Strong association between milk metabolites and somatic cell count in bovine milk.	[38]
Bovine milk	UHPLC-QTOF-MS	Hierarchical clustering and OPLS-DA.	Untargeted metabolomics followed by multivariate statistics discriminated bulk milk from dairy cows following different feeding regimens.	[46••]
Raw milk from commercial dairy plant	UHPLC-QTOF-MS	PCA	Metabolomics allowed to classify the oxylipids as adequate markers for distinguishing UHT milk from raw and pasteurized milk samples.	[47•]
Ovine milk	GC–MS	PCA and OPLS-DA	Different grazing system significantly affected the ovine milk metabolome.	[48]
Bovine milk	Ultra-fast LC-Triple TOF-MS and ^{1}H-NMR	Hierarchical clustering, PCA, and OPLS-DA	A total of 53 discriminating metabolites were significantly upregulated or downregulated in the heat stress (HS) dairy cow group compared with the HS-free group.	[42]
Bovine milk	^{1}H-NMR	PCA and OPLS-DA	Novel correlations of milk metabolites with protein content and rennet-induced coagulation properties were demonstrated.	[49]
Bovine milk	LC-QTRAP-MS/MS	PCA and Volcano plots	Milk metabolome and its coagulation potential are affected by the lactation period of dairy cows.	[50]
Bovine milk	GC-TOF-MS	OPLS-DA and pathway analysis	Understanding of the metabolic mechanisms affecting milk production as resulted by forage quality.	[51]
Bovine milk	^{1}H-NMR	Hierarchical clustering and PLS-DA	Discrimination of milk derived from cows following different feeding systems, specifically between indoor total mixed ration and pasture-based diets.	[52••]
Bovine milk	^{1}H-NMR	PLS-DA	Evaluation of the bovine colostrum and milk metabolome at the onset of lactation.	[53]
Bovine milk	LC-QTRAP-MS/MS	Hierarchical clustering and PLS-DA	The type of bovine feeding system is able to affect the amino acid composition and metabolome of skim milk and whey powders and may aid in the selection of raw materials for a better product manufacturing.	[54]

Table 1 (*Continued*)

Food matrix	Metabolomic platform	Statistical analysis	Main findings	Reference
Bovine milk	^{1}H-NMR	PCA and PLS-Canonical Analysis	Seasonal variations in milk composition and correlations with cows' nutritional patterns were observed, showing underlining relationships between feeding and metabolites.	[55••]
Bovine milk	UHPLC-QTOF-MS	PCA and OPLS-DA	The metagenomic profile of milk was found to be significantly correlated to some lysophospholipids, with *Staphylococcaceae*, *Pseudomonadaceae*, and *Dermabacteraceae* establishing the highest number of correlations.	[56••]
Ovine milk and 'Fiore Sardo' cheese	GC–MS	PLS-DA and OPLS-DA	The metabolites that mostly changed due to the thermization process belonged to the classes of free amino acids and saccharides.	[57]
Buffalo milk and mozzarella cheese	GC–MS	Hierarchical clustering, PCA, and PLS-DA	Development of a powerful technology platform to determine the authenticity and create market protection for 'Mozzarella di Bufala Campana'.	[58]
Mozzarella cheese	^{1}H-HRMAS-NMR	Hierarchical clustering, PCA, and Discriminant Analysis	This work shows that ^{1}H HRMAS-NMR spectroscopy can rapidly characterise the metabolic profile of intact 'Mozzarella di Bufala Campana' samples and statistically distinguish the geographical origin of buffalo milk mozzarella and its freshness.	[59]
PDO-Grana Padano cheese	UHPLC-QTOF-MS	Hierarchical clustering, PCA, and OPLS-DA	The untargeted metabolomic profiles of PDO and non-PDO Grana Padano were successfully discriminated.	[60]
Milk and yogurt samples	UHPLC-QTOF-MS	Hierarchical clustering and Principal Coordinates Analysis (PCoA).	Untargeted metabolomics was successfully used to track the molecular changes in milk and yogurt samples, as resulting by processing technology.	[61]
Traditional and commercial dairy products	UHPLC-QTOF-MS	PCA, PLS-DA and Pathway Analysis	The greatest difference between commercially available and traditional cheese was in the short peptide composition.	[62]
Milk and mozzarella cheese	^{1}H-NMR	PCA and PLS-DA	Pasture-based dairying may be differentiated in terms of the provenance of milk produced along with the accrual of additional benefits during ripening of the resulting mozzarella cheeses.	[63]
Milk and mozzarella cheese	GC–MS	PCA, PLS-DA, and OPLS-DA	Evaluation of the metabolomic differences between buffalo and cow mozzarella cheese. Possible correlations between orotic acid and animal origin of milk.	[64]

represent one of the main factors driving raw milk composition, thus determining differences in both its nutritional value and technological properties. In particular, using an untargeted metabolomic approach based on UHPLC-QTOF mass spectrometry coupled with both unsupervised and supervised multivariate statistics, the authors discriminated the chemical profile of bulk milk for the production of hard cheese, collected from 103 dairy cows following different feeding regimens, including corn silage, hay, and a mixed ration based on fresh forage and hay. This comprehensive approach highlighted the importance of both feed-derived (such as phenolic metabolites likely related to forage) and animal-derived compounds (such as fatty acids) for discrimination purposes. In a recent work, Bellassi *et al.* [56••] observed intriguing correlations between some lysophospholipids (such as lysophosphatidylethanolamines), discriminating raw milk from cows following a fresh forage/hay-based versus a hay-based feeding system, and its metagenomic profile (mainly when considering *Staphylococcaceae*, *Pseudomonadaceae*, and *Dermabacteraceae*). The potential and positive correlations observed could affect milk in the subsequent processing stages; therefore, future research is advisable in this field in order to better understand the chemical behaviour of cheese during the long ripening stage.

Milk metabolites for product authentication

Metabolomics has been applied to milk to examine its suitability for authentication of origin of source, milk species and farming methods. O'Callaghan *et al.* [52••] demonstrated the ability of ^{1}H-NMR metabolomics coupled with PLS-DA to distinguish milks from pasture and indoor total mixed ration (TMR) based diets. A series of metabolites were correlated with pasture derived milk as opposed to TMR milk, including dimethyl sulfone and hippuric acid. The authors concluded that NMR metabolomics could be a useful tool in the future for verification purposes of 'Grass-Fed' milk. For the purpose of preventing food fraud, Scano *et al.* [12] examined the ability of GC–MS based metabolomics to distinguish between caprine and bovine milk. The authors reported significant difference between the species' milk metabolomes, whereby in particular valine and glycine concentrations were more correlated with caprine milk, while talose and malic acid were associated with bovine milk. The authors also reported the method was capable of distinguishing caprine milk which had been mixed with different proportions of bovine milk, however, the high levels of variability in the composition of milk from both species present challenges to the robustness of the methods. Therefore, the high variability of milk composition from both species need to be still elucidated to better develop a more robust method of verification. ^{1}H-NMR has also be used to distinguish between sheep and cows' milk, and mixtures of each at different levels [65]. The authors concluded that this method, when combined with multilinear regression and a trained artificial neural network, could be a potential tool for the evaluation of the composition of milk mixtures from different species. Yang *et al.* [66] using an untargeted metabolomics approach with NMR and LC–MS, demonstrated the differences in metabolite profiles of Chinese Holstein, jersey, yak, buffalo, goat camel and horse milk. The authors reported that some traits were shared across some ruminant animals such as glycerophospholipid metabolism and valine, leucine and isoleucine biosynthesis, while biosynthesis of unsaturated fatty acids was comparable across the non-ruminant animals. Cross contamination of milk with colostrum has previously been reported to cause issues with the processability of milks, as such, O'Callaghan *et al.* [53] demonstrated the ability of NMR metabolomics to distinguish between colostrum and milk from subsequent days at the onset of lactation.

On examination of other forms of metabolites, the fatty acid profile and levels of the pigment beta-carotene in milk and dairy samples has been utilised for the authentication of 'Grass-Fed' milk and dairy products. Fatty acid profiling coupled with multivariate analysis was demonstrated to clearly distinguish between products from pasture and TMR feeding systems in milk [67,68], butter [68] and cheese [69]. Magan *et al.* [54] used LC–MS based metabolomics to examine the impact of cows' diet on skim milk and whey protein ingredients. The authors reported significantly higher concentrations of some metabolites, such as glutamine, valine, and phosphocreatine in each ingredient type derived from TMR than those from pasture.

The available data would suggest that metabolomics has the potential to be used as a tool for dairy product authentication and verification. The high variability of milk composition resulting from a variety of factors as previously mentioned, makes it challenging to produce a universal authentication method. However, an increased understanding of the factors affecting the milk metabolome, namely the variability of certain metabolites throughout early, mid and late lactation periods, should enhance our ability to create robust testing methods and data analysis models.

Conclusions and future perspectives

The field of metabolomic research and its application to the agri-food sector is continuously evolving, yielding important insights into traits of animal and food production such as health, welfare, quality and authenticity. This article provides an overview of the application of metabolomic technologies to the field of dairy science with important discoveries and implications for animal health, milk quality, and authentication. A major challenge for dairy processors today is understanding the performance of milk throughout the processing stages and supply chain over the production season. To date, several studies have highlighted the presence of important biomarker

compounds of milk techno-functionality. Therefore, further research could offer prediction tools for decision making around suitability of milks for certain processing conditions, increasing its processability and efficiency, and sustaining the quality of the final product. Furthermore, metabolomics has been demonstrated as a potentially powerful tool for authentication of milk and dairy products for a variety of traits including species, PDO and 'Grass-Fed'. As more and more commodities like dairy products appear on the market demanding a premium price, the development of robust methods to authenticate and validate product source and claims will be important for the future.

Finally, herein we have provided an overview of the most widely applied technologies for investigation of milk metabolomics ranging from LC–MS, GC–MS and NMR based systems. While each has their specific advantages and disadvantages in terms of coverage, sensitivity and sample preparation, a combined approach has been advised to offer the greatest coverage and with that, more comprehensive overview of a sample's metabolome. The field of milk metabolomics is still at an early stage with important steps already taken to characterise the different metabolites present in milk. Future research is now required to understand the factors that affect their relative concentrations in milk and understand what implications certain metabolites can have on the processability and nutritional value of milk and quality of final products. Furthermore the validation of functional biomarker compounds in milk and tools and programs for their rapid and accurate detection and measurement will be important for the future.

Conflict of interest statement

Nothing declared.

References and recommended reading

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