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Review

NMR metabolite profiles of dairy: A review

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A B S T R A C T

Nuclear magnetic resonance (NMR) spectroscopy, which is one of the most powerful "omics" analytical platforms, has been broadly adopted recently in foodomics. ^{1}H NMR has been applied to the study of the metabolite profile of dairy products throughout the supply chain, in relation to different aspects such as animal health, milk quality, geographical origin and cheese ripening process. This review reports and discusses the literature on the topic, also collecting the identified metabolites in a descriptive table and depicting them in a Venn diagram for both milk and cheese; moreover, experimental details of the reviewed papers have been reported. The present review provides an exhaustive state-of-the-art in the field of dairy products, addressing both NMR experts and non-experts to the still unexplored potential applications of NMR in dairy characterisation, and in general in foodomics.

Contents

1. Introduction

Milk is an extraordinary source of nutrients and a very flexible raw material, which can be processed into new unique foods. Among all foods, dairy products are probably the most diverse class. Research on milk and dairy products covers different topics, such as chemical characterisation, quality assessment, traceability, animal wellness, industrial processes, cheese making procedures, shelf-life, and fraud detection. Recently, to these goals, among the different molecular classes taken into consideration, hydrosoluble metabolites are gaining increased interest.

Metabolites are low-molecular mass molecules (<1500 Da), such as free amino acids, organic acids, sugars, polyols, nucleotides and others, detectable in a biological matrix (Cagliani, Scano, & Consonni, 2018). In the case of milk, they derive from the animal's metabolism, depend on the species, breeding conditions,

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stage of lactation, diet, and reflect the pathological status and technological aptitudes; while for cheese the metabolite profile strongly depends also on the processing technologies and the ripening process. The knowledge of the metabolite profiles can be of utmost importance to correlate the metabolic content to animal health status, milk quality, ripening of cheese and other aspects related to industrial processes. In this context, NMR spectroscopy has been demonstrated to be a powerful analytical tool for the study of the metabolite profiles of milk and dairy products and their modifications under different conditions.

NMR spectroscopy is theoretically complex, and the peculiarity of ^{1}H NMR, among other spectroscopic methods, relies in the capability to detect simultaneously, even in complex biological matrices, all the small molecules having a hydrogen atom and directly connecting the peak intensities to the number of hydrogens underlying the signal. The technical advantages and disadvantages of NMR over other analytical platforms, such as gas chromatography-mass spectrometry (GC–MS) have been amply described (Emwas, 2015). However, in our opinion, in dairy research these two techniques can be considered complementary, being each more sensitive to a different molecular class (Caboni et al., 2017, 2016; Consonni & Cagliani, 2008; Klein et al., 2010; Pisano, Scano, Murgia, Cosentino, & Caboni, 2016; Rodrigues et al., 2011; Scano, Murgia, Pirisi, & Caboni, 2014).

This review collates the NMR investigations present in the literature that focus on the metabolite content of milk and cheese; the collected data are discussed and the different applications highlighted.

2. Experimental conditions

A summarised description of the experimental conditions performed in the reviewed NMR studies is reported in Tables 1 and 2, for milk and cheese, respectively.

2.1. Sample preparation

Different sample preparation protocols were applied to obtain the aqueous extracts of milk and cheese. Milk can be skimmed as described by (Jensen, Holland, Poulsen, & Larsen, 2012), residual lipids and proteins could be removed by ultra-filtration using a 10 kDa cutoff spin filter at 10,000× *g* for 15 min (Buitenhuis et al., 2013; Klein et al., 2010, 2012; Sundekilde, Poulsen, Larsen, & Bertram, 2013; Sundekilde et al., 2014, 2015). Alternatively, stirring and centrifugation of milk with an aliquot of chloroform ($CHCl_3$) (Lamanna, Braca, Di Paolo, & Imparato, 2011) or with a methanol–chloroform (2:1) mixture (Tomassini et al., 2018) were applied to remove the lipid fraction. According to other protocols, freeze dried samples were placed in an ultrasonic bath and filtered (Sacco et al., 2009; Yang et al., 2016) to remove insoluble particles. Skimmed milk can also be obtained by centrifugation, proteins could be removed after denaturation obtained with ethanol addition, followed by centrifugation and drying process under vacuum condition of the supernatant (Hu, Furihata, Ito-Ishida, Kaminogawa, & Tanokura, 2004).

A mixture of solvents with different polarities has been used to obtain the water-soluble fraction of cheese (De Angelis Curtis et al., 2000; Gianferri, Maioli, Delfini, & Brosio, 2007; Mazzei & Piccolo, 2012; Piras et al., 2013) as described by Bligh and Dyer (1959) and Folch, Lees, and Sloane Stanley (1957). In other protocols, cheese is simply dissolved in deuterated water (D_2O) followed by centrifugation (Brescia, Monfreda, Buccolieri, & Carrino, 2005; Consonni & Cagliani, 2008; Lamanna, Piscioneri, Romanelli, & Sharma, 2008; Ruyssen et al., 2013). Cheese samples were also freeze-dried prior to solvent extraction to prevent degradation processes (Brescia et al., 2005; De Angelis Curtis et al., 2000; Piras et al., 2013; Rodrigues et al., 2011). An extract of Parmesan cheese was obtained as described by Ghandi, Cobra, Steele, Markley, and Rankin (2018), and used as a bacterial growth media for *Lactobacillus* strains; supernatants of

Table 1
Summary of main characteristics of ^{1}H NMR studies on milk metabolites.[a]

Goal	Animal species	Sample	pH	Other NMR experiments	Spectral data treatments	Other analysis	MVA
Characterisation (Hu et al., 2004)	cow	whole and extract	nc	HSQC, HMBC		–	–
Geographical origin (Sacco et al., 2009)	cow	whole and extract	nc	COSY, TOCSY		HPIC, IRMS, ICP-AES, FT-IR	PCA, DA
Technological properties (Klein et al., 2010)	cow	extract	buffer 7.4	HSQC	Integration	GC–MS, FT-IR, Flow cytometry	–
Technological properties (Sundekilde et al., 2011)	cow	extract	nc	^{13}C-NMR, HSQC	Binning, alignment	FT-IR, Flow cytometry, Rheometry	PCA
Fraud (Lamanna et al., 2011)	cow and sheep	extract	nc	HSQC, HMBC		–	ANN, DA, PCA
Animal health (Klein et al., 2012)	cow	extract	buffer 7.4	HSQC, NOESY, HMBC	Integration	energy balance and fat-to-protein ratio	–
Genotyping (Buitenhuis et al., 2013)	cow	extract	nc	–	Integration	FT-IR, Flow cytometry, Genotyping	–
Animal health and technological properties (Sundekilde et al., 2013)	cow	extract	nc	2D	Binning, alignment	FT-IR, Flow cytometry	PCA, PLS, OPLS-DA
Technological properties (Sundekilde et al., 2014)	cow	extract	nc	2D	Binning, alignment	FT-IR, Flow cytometry, Rheometry	PCA, PLS, OPLS-DA
Diet (Sundekilde et al., 2015)	cow	extract	nc	2D	Binning, alignment	FT-IR	PCA, OPLS-DA
Characterisation (Yang et al., 2016)	cow and others	extract	buffer 7.4	–	Binning	LC-MS	PCA, PLS-DA, OPLS-DA
Animal health and technological properties (Tomassini et al., 2018)	cow	extract	buffer 7.4	TOCSY, HSQC, HMBC, DOSY	Integration	–	PLS-DA, PLS

[a] All ^{1}H NMR techniques were high resolution. Abbreviations are: HSQC, heteronuclear single quantum coherence spectroscopy; HMBC, heteronuclear multiple bond correlation spectroscopy; COSY, correlation spectroscopy; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; DOSY, diffusion-ordered spectroscopy; MVA, multivariate statistical data analysis; PCA, principal component analysis; DA, discriminant analysis; ANN, artificial neural network; PLS-DA, PLS-discriminant analysis; OPLS-DA, orthogonal PLS-DA; HPIC, high pressure ion chromatography; IRMS, isotope ratio mass spectrometry; ICP-AES, inductively coupled plasma-atomic emission spectrometry; FT-IR, Fourier transform infrared spectroscopy; GC–MS, gas chromatography-mass spectrometry; LC-MS, liquid chromatography-mass spectrometry; nc, not controlled.

Table 2
Summary of main characteristics of ^{1}H NMR studies on cheese metabolites.[a]

Goal	Animal species	Sample	pH	^{1}H NMR techniques	Other NMR experiments	Spectral data pretreaments	Other analysis	MVA
Fraud (Brescia et al., 2005)	buffalo	extract	nc	HR	–		FT-IR, HPIC, IRMS, ICP-AES, ICP-OES	HCA, PCA, DA
Characterisation (Gianferri et al., 2007)	buffalo	intact, extract	buffer 6.5	LR, HR	CPMG, COSY, TOCSY		–	–
Characterisation, geographical origin, ageing (Mazzei & Piccolo, 2012)	buffalo	intact, extract	nc	HR-MAS	CPMG, TOCSY, HSQC		–	HCA, PCA, DA
Aminoacid profile (De Angelis Curtis et al., 2000)	cow	extract	nc	HR	COSY, CPMG	Integration	TG, DTG	–
Characterisation (Shintu et al., 2004)	cow	intact	corrected 6.70	HR-MAS	TOCSY, HMQC, HMBC		–	–
Ripening (Shintu & Caldarelli, 2005)	cow	intact	buffer 7.0	HR-MAS	–	Binning	–	PCA, DA
Geographical origin (Shintu & Caldarelli, 2006)	cow	intact	buffer 7.0	HR-MAS	TOCSY, HMQC, HMBC		–	PCA, DA
Shelf-life related to packaging (Lamanna et al., 2008)	cow	intact, extract	nc	HR, HR-MAS	COSY, TOCSY, HSQC, HMBC	Integration	–	PCA
Ripening and geographical origin (Consonni & Cagliani, 2008)	cow	extract	checked 5.48–5.73	HR	TOCSY, HSQC, HMBC	Binning	–	PCA, PLS-DA
Effects of probiotics and prebiotics (Rodrigues et al., 2011)	cow	extract	checked	HR	NOESY	Integration and binning	Microbiological and chemical analyses	PCA, OPLS
Ripening in function of starters (Piras et al., 2013)	sheep	extract	corrected 4.14–4.52	HR	COSY, TOCSY	Binning, alignment	Microbiological and chemical analyses	PCA, PLS
Molecular fingerprint and quality parameters (Ruyssen et al., 2013)	cow	extract	checked 6.7	HR	–	Binning	Sensory test, microbiological analysis, HPAEC-PAD, HPLC/ESI/MS, SPME/GC–MS	PCA
Browning (Gandhi et al., 2018)	cow	extract	nc	HR	–	Integration	Microbiological analysis, SPME/GC–MS, GC–MS	

[a] Abbreviations are: CPMG, Carr-Purcell-Meiboom-Gill; COSY, correlation spectroscopy; TOCSY, total correlation spectroscopy; HSQC, heteronuclear single quantum coherence spectroscopy; HMQC, heteronuclear multiple quantum coherence spectroscopy; HMBC, heteronuclear multiple bond correlation spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; MVA, multivariate statistical data analysis; HCA, hierarchical cluster analysis; PCA, principal component analysis; DA, discriminant analysis; FT-IR, Fourier transform infrared spectroscopy; HPIC, high pressure ion chromatography; IRMS, isotope ratio mass spectrometry; ICP-AES, inductively coupled plasma–atomic emission spectrometry; ICP-OES, ICP–optical emission spectrometry; TG, thermogravimetry; DTG, derived thermogravimetry; HPAEC-PAD, high performance anion exchange chromatography with pulsed amperometric detection; HPLC/ESI/MS, high pressure liquid chromatography/electrospray ionisation/tandem mass spectroscopy; SPME/GC–MS, solid-phase microextraction/gas chromatography-mass spectrometry; nc, not controlled.

cultures were obtained by centrifugation and filtration (Ghandi et al., 2018). Typically, the aqueous extracts obtained (containing a few milligrams per 400 μL) were diluted in D_2O containing sodium 3-trimethylsilyl-(2,2,3,3-d_4)-1-propionate (TSP) as internal reference standard; alternatively, sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was used (Ghandi et al., 2018; Ruyssen et al., 2013). Intact cheese matrices in phosphate buffer were analysed by solid state NMR (Shintu & Caldarelli, 2005, 2006; Shintu, Ziarelli, & Caldarelli, 2004).

2.2. NMR experiments

High-resolution (HR) one-dimensional (1D) ^{1}H NMR spectra were recorded for liquid extracts. An issue in the analysis of the aqueous fraction was the presence of the huge resonance peak due to pure water (H_2O) or the residual proton of deuterated water (HDO) occurring at about 4.7 ppm at room temperature; therefore, a presaturation scheme was applied to remove the residual solvent signal and increase the dynamic range. The scheme was also inserted in all NMR pulse sequences for two-dimensional (2D) experiments. Typically, the 1D proton spectrum contains several overlapped signals due to the complexity of the investigated matrix. Therefore, the use of "multidimensional" and/or "multinuclear" experiments can afford spectral complexity reduction. As reported in Tables 1 and 2, the 2D homonuclear experiments applied to the study of dairy products were correlation spectroscopy (COSY) and total correlation spectroscopy (TOCSY); multinuclear experiments were heteronuclear multiple quantum coherence spectroscopy (HMQC), heteronuclear single quantum coherence spectroscopy (HSQC), heteronuclear multiple bond correlation spectroscopy (HMBC). In addition, the 1D version of the nuclear Overhauser effect spectroscopy (NOESY), Carr-Purcell-Meiboom-Gill (CPMG), also known as "spin-echo" experiment and J-resolved experiments, were adopted. Moreover, taking the advantage of different diffusion properties of small molecules dissolved in solvent, diffusion-ordered spectroscopy (DOSY) provided a measure of diffusion coefficients, allowing metabolites separation according to these values, similarly to a thin layer chromatography. High resolution magic angle spinning (HR-MAS) is a valid tool in NMR spectroscopy to study intact biological matrices, suspended or swollen in a deuterated solvent. The main advantages of HR-MAS are that no sample preparation is needed and that it allows the simultaneous detection of polar and nonpolar metabolites within a single experiment (Cagliani et al., 2018).

As already mentioned, the ^{1}H NMR spectrum contains signals due to protons of the molecules in solution. Under conditions of full relaxation, i.e., when full recovery conditions are satisfied for the all spin systems, the area of the NMR signal is directly proportional to the number of protons that contribute to the peak. The control of pH is mandatory in case of further use of NMR data in multivariate statistical analysis, even though nowadays some tools for spectral alignment are available (Savorani, Tomasi, & Engelsen, 2010). In case of signal overlapping, the use of multidimensional/multinuclear contour plots facilitates the resonance assignment of metabolites. The metabolite identification is one of the strong points of the NMR technique. This is normally achieved by the aid of the existing knowledge in the literature and online databases (www.hmdb.ca, mmcd.nmrfam.wisc.edu, foodb.ca, www.bmrb.wisc.edu, etc.). However, a definitive metabolite identification can be obtained by spiking the sample with standard compounds (Cagliani et al., 2018).

2.3. Spectral data pretreatment and multivariate statistical analysis

The ^{1}H NMR spectra of milk and cheese are quite complex, and the information suitable for quali-quantitative studies or to be submitted to multivariate statistical data analysis (MVA) can be obtained by different methods. Spectral data can be obtained by peak integration of selected resonances (Buitenhuis et al., 2013; De Angelis Curtis et al., 2000; Klein et al., 2012, 2010; Tomassini et al., 2018) or by integrating spectral intervals with a fixed or a variable width (spectral binning or bucketing) (Consonni & Cagliani, 2008; Lamanna et al., 2008; Sundekilde et al., 2013, 2014, 2015). Moreover, row-wise normalisation to 100 or to an internal standard (often the TSP signal) can be performed. Among column-wise scaling, Pareto is the most used (Piras et al., 2013; Sundekilde et al., 2013). As reported in Tables 1 and 2, the most used MVA were the untargeted principal component analysis (PCA) and hierarchical cluster analysis (HCA), the targeted partial least square (PLS), PLS-discriminant analysis (DA), and their orthogonal variants (OPLS and OPLS-DA). PCA is an explorative approach, while DA helps in finding discriminant metabolites between predefined classes assigned to samples. PLS is a calibration method adopted to correlate spectral data to measured characteristics. To this latter goal, the predictive potential of a trained artificial neural network (ANN) was also adopted.

3. Results

The NMR metabolites detected are reported in Tables 3 and 4, for milk and cheese, respectively, while for the corresponding NMR assignments we refer to the original studies. A qualitative comparison of the metabolite content between milk and cheese is depicted in the Venn diagram of Fig. 1. A total of 89 metabolites were identified, 34 of them were found solely in milk, and 24 solely in cheese. Compared with cheese, milk had more carboxylic acids and other compounds such as choline, carnitine, and ethanolamine, conversely, cheese had more amino acids and alcohols.

3.1. Milk metabolites

3.1.1. ^{1}H NMR spectral features

The analysis of the ^{1}H NMR spectra of whole milk is quite challenging due to the presence of compounds with different concentrations and polarity, i.e., lipids, sugars and small metabolites, together with proteins and protein aggregates. The ^{1}H NMR spectra of milk exhibit high intensity resonances assigned to the functional groups of fatty acids and lactose, and broad signals, detectable especially in the aromatic region, most likely due to proteins. In ruminant's milk, caseins are by far the most abundant proteins, organised in micelles with high molecular weight. Moreover, non-homogenised milk contains very large fat globules that broaden the NMR signals (Hu et al., 2004). Broad resonances of high molecular weight molecules can hide other sharp resonances due to smaller molecules, especially when in lower abundance, which are identifiable only by performing 2D NMR experiments (Hu et al., 2004). Conversely, ^{1}H NMR spectra of the aqueous fraction of milk show a number of sharp peaks assigned to lactose, organic acids, amino acids and others; an example of the aqueous extract of cows' milk is shown in Fig. 2.

3.1.2. Literature

It is well known that milk composition depends on different factors, such as the stage of lactation, diet, genetic variability, seasonality, and animal health, as well as heat treatments. The possible influence on milk metabolite profile of the addition of oregano (*Origanum vulgare*) and caraway (*Carum carvi*) essential oils to cows' feed was investigated (Sundekilde et al., 2015). These essential oils are known to introduce differences in the volatile fraction of milk. PCA of ^{1}H NMR data of milk hydrosoluble extracts was not able to highlight variability in the milk metabolite profiles upon the addition of essential oils to the feed. However, by performing OPLS-DA, a metabolite profile modification was evident, and several metabolites were found involved (Sundekilde et al., 2015).

To find a general procedure for milk traceability, raw milk from autochthonous cows bred in Southern Italy (individual morning milk) were compared with foreign milk samples (bought at local market in central-east Europe) (Sacco et al., 2009). The comparison between the ^{1}H NMR spectra of the aqueous extracts showed some differences in the metabolite content. Foreign milk had a higher content of sugar, free amino acids (particularly tyrosine, lysine and glutamic acid) and glycerol. Interestingly, ^{1}H NMR spectra of Southern Italy milk showed resonances of lactate, absent in the foreign milk samples. However, it was pointed out that the commercial foreign milk samples were obviously submitted to some technological processes (e.g., pasteurisation) and possible differences in the metabolite profiles could be also due to these treatments (Sacco et al., 2009).

Measurement of selected milk constituents has attracted much attention in dairy research to monitor the udder health or the metabolic status of cows and it is widely used on dairy farms. However, the detection of prognostic metabolic biomarkers is the main goal of modern industry. Within this aim, correlations between the ^{1}H NMR profile and other cows' milk characteristics were investigated (Sundekilde et al., 2013, 2014, 2011; Klein et al., 2012, 2010; Tomassini et al., 2018). Comprehension of the metabolic variability of milk composition in relation to the lactation time may be of paramount importance for the breeders and dairy industries. The first part of lactation in cow lasts 100 days, the mid 150 days and the last until the dry period that varies greatly among cows depending on persistence on milk production. Metabolic stress or negative energy balance can affect individual animals in the early stage of lactation (Kessel et al., 2008). On the other hand, late lactation stage is characterised by an involution of the mammary gland with a loss of tight junction integrity and thus resulting in a variation of the metabolite fluxes via the paracellular pathway (Stelwagen & Singh, 2014).

In NMR-based metabolomics studies of bovine milk, Klein et al. (2010; 2012) focused their attention on lactating cows' health, correlating NMR profiles of milk to lactation stages for different breeds. Metabolites related to energy metabolism in early and late lactation stages were identified (Klein et al., 2010). Elevated levels of ketone bodies were proposed as possible biomarkers for hyperketonaemia, since correlations of acetone and β-hydroxybutyrate (BHBA) with the metabolic status of cows were identified by the authors (Klein et al., 2010). While these latter metabolites describe an acute disease status, in a subsequent work (Klein et al., 2012) the same authors suggested that glycerophosphocholine and phosphocholine can have a predictive capability in identifying those cows less prone to undergo metabolic stress. As possible explanation for the role of these metabolites is that glycerophosphocholine and phosphocholine are indices of phospholipids break down and release of fatty acids, an energy source to satisfy the enhanced requirements of early lactation (Klein et al., 2012).

In a recent work, Tomassini et al. (2018) investigated the differences in the metabolic profiles of milk from Friesian and autochthonous cows from Northern Italy, at different lactation stages. To highlight differences in milk composition due to either different cow populations or in relation to late lactation, the external factors influencing the milk composition were minimised, like feeding diets and pairing cows for the lactation interval and

Table 3
Metabolites identified in the ^{1}H NMR spectra of milk.

	References											
	Hu et al. (2004)	Sacco et al. (2009)	Klein et al. (2010)	Sundekilde et al. (2011)	Lamanna et al. (2011)	Klein et al. (2012)	Buitenhuis et al. (2013)	Sundekilde et al. (2013)	Sundekilde et al. (2014)	Sundekilde et al. (2015)	Yang et al. (2016)	Tomassini et al. (2018)
Sugars												
Fucose							x		x	x		x
Galactose			x				x		x	x		
Galactose-1-phosphate							x		x	x		x
Glucose							x		x	x		
Glucose-1-phosphate							x		x	x		x
Lactose	x		x	x	x		x	x	x	x	x	x
Amino acids and analogues												
Alanine			x		x	x	x		x	x		x
Glutamine											x	
Glutamic acid		x				x	x		x	x		x
Glycine			x			x						
Isoleucine							x	x	x	x		
Leucine											x	
Lysine		x										
Methionine							x		x	x		x
Ornithine							x		x	x		
Tyrosine		x										
Valine							x		x	x	x	x
Taurine			x									
3-Methyl-histidine			x									
Carboxylic acids												
Acetate			x		x	x	x	x	x	x	x	x
Acetoacetate						x						
Butyrate							x	x	x	x		x
Caproate												x
cis-Aconitate							x					
Citrate	x			x	x	x	x	x	x	x	x	x
Formate									x	x	x	x
Fumarate						x	x	x	x	x		x
Hippurate							x	x	x	x		x
Isobutyrate							x					
Lactate		x				x	x	x	x	x		x
Malate											x	x
Malonate							x		x	x		x
Oxaloacetate						x						
Orotate						x	x	x	x	x		x
Pyruvate											x	x
Succinate											x	x
β-Hydroxy-butyrate (BHBA)			x			x	x	x			x	x
2-Oxoglutarate							x					x
Nucleotides												
Adenine								x	x	x		
Others												
Acetamide					x							
Acetone			x			x			x	x		
Acetylcarnitine												x
Acetylcholine												x
Allantoine												x
Betaine			x			x						
Carnitine			x	x			x		x	x		x
Choline			x	x		x	x	x	x	x	x	x
Creatinine			x			x	x	x	x	x	x	x
Creatine	x				x	x						x
Dimethylamine (DMA)												x
Ethanol									x	x		x
Ethanolamine			x									x
Glycero-phosphocholine			x			x	x		x	x	x	x
Glycerol		x										
Lecithin	x											
Methanol												x
Methylamine												x

Table 3 (*continued*)

	References											
	Hu et al. (2004)	Sacco et al. (2009)	Klein et al. (2010)	Sundekilde et al. (2011)	Lamanna et al. (2011)	Klein et al. (2012)	Buitenhuis et al. (2013)	Sundekilde et al. (2013)	Sundekilde et al. (2014)	Sundekilde et al. (2015)	Yang et al. (2016)	Tomassini et al. (2018)
N-Acetyl-carbohydrates	x		x				x	x				
N-Acetyl-lactosamine					x							
N-Acetyl-hexosamines									x	x		
Phosphocholine			x			x		x	x	x	x	x
Phosphocreatine			x			x						x
Trimethylamine (TMA)												x
Trimethylamine N-oxide (TMAO)			x								x	
Urea							x					

lactation stage. It was found that *N*-acetyl-groups, probably part of oligosaccharides as evidenced by DOSY experiments (Tomassini et al., 2018), decreased in late lactation, together with cytidine-X-P, possibly associated with the decline of RNA synthesis showed in prolonged lactation (Hadsell, George, & Torres, 2007). Other metabolites, such as citrate, phosphocholine, carnitine, acetate and hippurate, associated to milk technological quality and processing capabilities (Harzia et al., 2012; Sundekilde, Frederiksen, Clausen, Larsen, & Bertram, 2011) were found statistically different in the two populations (Tomassini et al., 2018). Moreover, singlets assigned to the methyl groups of different off-flavour methylamines (methylamine, dimethylamine and trimethylamine) were detected in the ^{1}H NMR spectra of milk (Tomassini et al., 2018). In our opinion, these assignments should be further and carefully confirmed.

To obtain high-quality dairy products, the technological and nutritional properties of milk are of primary importance. One of the factors that mostly affect cheese making performance is the presence of mastitis which is accompanied by a high count of somatic cells (SCC) in milk. Mastitis is the costliest disease of the dairy industry, being normally associated with clinical signs in response to invading microorganisms. Subclinical mastitis is not easily detected, as no visual indicators of the inflammation exist, however, SCC is elevated, and milk quality is affected. Sundekilde et al. (2013) correlated the ^{1}H NMR metabolite profile of milk from two dairy breeds to SCC values. Milk samples were divided in two groups, with very high and low SCC. Relative quantification of the discriminant metabolites revealed that in milk with high SCC, lactate, butyrate, isoleucine, acetate, and BHBA were increased, whereas hippurate and fumarate were decreased.

The protein content and the rennet coagulation properties of the milk are key aspects in cheese production, influencing cheese yield and quality. Associations between the bovine milk metabolites, protein content and rennet-induced coagulation properties of milk were found by Sundekilde et al. (2011, 2014), suggesting that the involved metabolites may act as milk quality markers for cheese production. In the first work, 14 individual milk samples from the two dairy breeds, Danish Jersey and Danish Holstein-Friesian, were collected (Sundekilde et al., 2011). Measurements of rennet coagulation time, curd firming rate, and curd firmness were performed on samples and scored into a composite coagulation value describing how well the milk coagulates. PCA of ^{1}H NMR data showed that along the PC1 milk samples clustered depending on the breed, and lactose was the main discriminant. Along PC2, samples clustered for their coagulation properties, summarised in two classes, endowed with good and poor coagulation properties. In summary, it was found a tendency of citrate concentration to increase in samples with poor coagulation properties. Furthermore, in good-coagulating milk samples choline concentration was found higher while carnitine lower.

This work was later extended by the same research group (Sundekilde et al., 2014) who, using MVA, related the milk metabolome of 407 Swedish Red dairy cows to the protein content and to the rheological measurements on rennet-induced coagulation. Metabolites associated with the prediction of total protein content were mainly choline, *N*-acetyl hexosamines, creatinine, glycerophosphocholine, glutamate. Moreover, levels of lactate, acetate, glutamate, creatinine, choline, carnitine, galactose-1-phosphate, and glycerophosphocholine were significantly different when comparing non-coagulating and well-coagulating milks (Sundekilde et al., 2014).

Given the involvement of metabolites in disease predisposition and milk quality, genetic parameters and detection of quantitative trait loci responsible for milk metabolite production were investigated (Buitenhuis et al., 2013). Out of 31 metabolites studied, 5 were found to have low heritability (lactate, isobutyrate, acetate, fumarate and galactose), while orotate and BHBA had the highest. A single SNP association analysis revealed 7 genome-wide significant quantitative trait loci for 6 metabolites (malonate, galactose-1-phosphate, *cis*-aconitate, urea, carnitine, and glycerophosphocholine; Buitenhuis et al., 2013). These results demonstrated that selection for milk metabolites in cows may be possible.

Concerning animal milk for human consumption, bovine milk has the largest production and is the most studied by ^{1}H NMR; far less research has been dedicated to the metabolite profile of milks from other animal species. The combined approach of NMR profiling and ANN was tested by Lamanna et al. (2011) to detect milk adulteration due to addition of cheaper milk from other animal species. In this work, the aqueous fractions of cow and sheep milk mixtures, at different mixing ratio, were analysed by ^{1}H NMR. A model of prediction of mixture content was built using selected variables of the NMR spectra. The correspondence between the mixture content and the ANN predicted value was very high (Lamanna et al., 2011). Yang et al. (2016) compared the NMR metabolite profiles of milk for human consumption from different animal species, ruminants and non-ruminants. Pair-wise OPLS-DA were applied to ^{1}H NMR spectral data, obtaining a good classification of samples based on their animal species. Different pathways involved in the milk biosynthesis were found to differ between cows and the other animals (Yang et al., 2016).

Table 4
Metabolites identified in the ^{1}H NMR spectra of cheese.

Metabolite	References												
	Brescia et al. (2005)	Gianferri et al. (2007)	Mazzei and Piccolo (2012)	De Angelis Curtis et al. (2000)	Shintu et al. (2004)	Shintu & Caldarelli (2005)	Shintu & Caldarelli (2006)	Lamanna et al. (2008)	Consonni & Cagliani (2008)	Rodriguez et al. (2011)	Piras et al. (2013)	Ruyssen et al. (2013)	Gandhi et al. (2018)
Sugars													
Galactose	x	x	x								x		
Glucose	x	x						x			x		
Lactose	x	x	x							x			
Amino acids and analogues													
Alanine	x	x		x	x	x	x	x	x	x	x	x	
Arginine		x			x		x		x		x	x	
Asparagine				x		x	x		x		x	x	
Aspartic acid				x		x			x		x		
Citrulline					x	x			x				
γ-Amino-butyric acid (GABA)								x			x		
Glutamine					x						x	x	
Glutamic acid	x	x		x	x	x	x		x		x	x	
Glycine				x	x	x	x		x		x	x	
Histamine											x		
Histidine				x						x	x		
Isoleucine	x	x		x	x	x	x	x	x	x	x	x	
Leucine	x	x		x	x	x	x		x	x	x	x	
Lysine	x			x	x	x	x		x	x	x	x	
Methionine				x	x	x	x		x		x	x	
Ornithine												x	
Phenylalanine		x		x	x	x	x		x	x	x	x	
Proline				x	x	x	x	x	x		x		
Pyroglutamic acid					x	x	x		x				
Serine				x	x	x	x		x		x		
Threonine	x	x		x	x	x	x		x		x		
Tyramine											x		
Tyrosine	x	x		x	x	x	x	x	x	x	x	x	
Tryptophan										x	x	x	
Valine	x	x		x	x	x	x	x	x	x	x	x	
Carboxylic acids													
Acetate	x	x	x					x	x	x	x	x	
Acetoacetate									x	x			
Butyrate									x				
Citrate	x	x						x		x	x	x	
Diacetyl (2,3 butanedione)												x	
Formate	x	x			x	x		x	x		x	x	
Lactate	x	x			x	x	x	x	x	x	x	x	
Malate								x					
Proprionate							x						
Pyruvate		x									x		
Succinate	x	x					x				x		
Nucleotides and analogues													
Uracil											x		
Uridine diphosphate (UDP)-hexoses											x		
Others													
Acetoin												x	
Acetol													x
Carnitine											x		
Choline								x		x	x		
Creatinine								x					
Creatine											x		
Ethanol	x	x						x		x		x	
Glycerol	x	x						x		x			
Isobutanol	x	x	x										
Isopropanol											x		
Methanol	x	x						x		x			
Phosphocholine								x			x		
1,2-Propanediol													x
Pyridine					x	x							

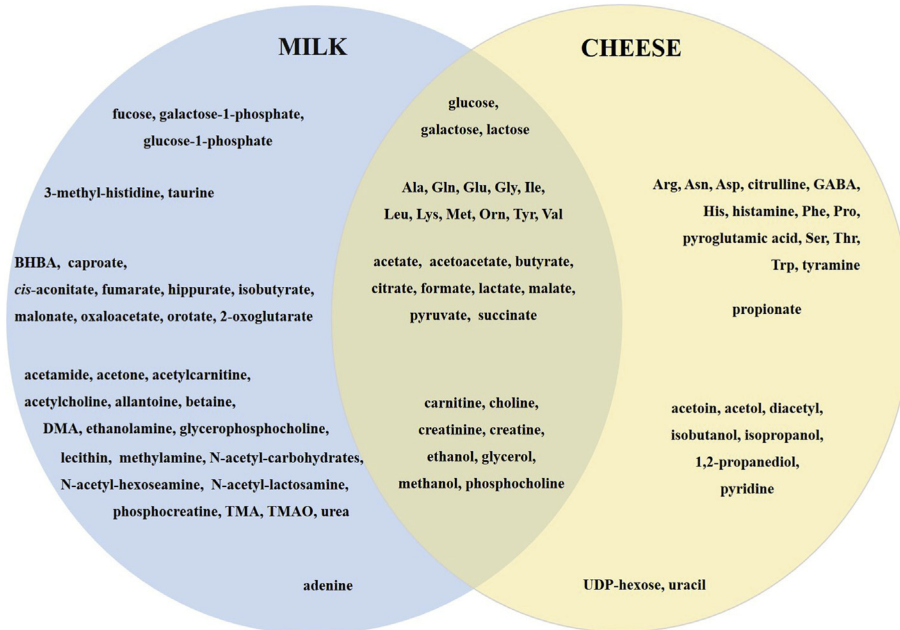

Fig. 1. Venn diagram of metabolites in milk and cheese; the three-letter system is used for amino acids, other abbreviations are: BHBA, β-hydroxy-butyrate; DMA, dimethylamine; TMA, trimethylamine; TMAO, trimethylamine N-oxide; GABA, γ-amino-butyric acid; UDP, uridine diphosphate.

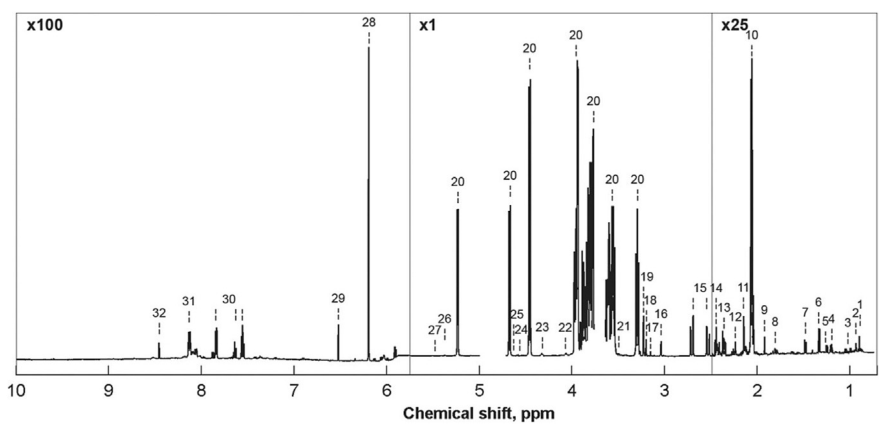

Fig. 2. Median spectrum of milk samples (n = 392) including assignments. The region 10–5.8 ppm is enlarged 100 times and the region 2.48–0.8 ppm is enlarged 25 times compared with the central region. Peak labels are: 1, butyrate; 2, isoleucine; 3, valine; 4, ethanol; 5, fucose; 6, lactate; 7, alanine; 8, ornithine; 9, acetate; 10, *N*-acetyl-hexosamines; 11, methionine; 12, acetone; 13, glutamate; 14, carnitine; 15, citrate; 16, creatinine; 17, malonic acid; 18, choline; 19, carnitine; 20, lactose; 21, phosphocholine; 22, phosphocholine; 23, glycerophosphocholine; 24, galactose; 25, glucose; 26, galactose-1-phosphate; 27, glucose- 1-phosphate; 28, orotate; 29, fumarate; 30, hippurate; 31, adenine; 32, formate. Reprinted with permission from Sundekilde et al. (2014).

3.2. Cheese metabolites

Many different typologies of cheeses exist worldwide. Some of them, being strongly linked to the culture and traditions of the region where they are produced, have been endorsed with a Protected Designation of Origin (PDO) certification under European Union regulations.

3.2.1. ¹H NMR spectral features

During cheese manufacture, milk whey is let to drain, and many milk metabolites are drained as well; therefore, most of the detectable metabolites in cheese are the product of enzymes and/or microorganism activity during manufacturing. As shown in Fig. 1, several compounds present in the NMR spectra of milk were not detected in cheese; on the other hand, mature cheese spectra were rich in proteic and non-proteic amino acid spin systems, while fresh cheese exhibited resonances of alcohols. During cheese ripening, biochemical reactions such as glycolysis, proteolysis and lipolysis take place, leading to the production of a wide range of metabolic compounds responsible for the organoleptic characteristics of cheese. As an example of NMR metabolite profile of cheese, the ^{1}H NMR spectrum of the aqueous extract of Parmigiano Reggiano cheese is shown in Fig. 3; as can be seen the lactate and amino acids spin systems dominate the spectrum.

3.2.2. Literature

In this paragraph, discussion on the NMR studies will be ordered by the cheese classification based on the cheese consistency (soft, medium, hard), which depends mainly on moisture content. Other works addressing different aspects of cheese industry will be illustrated at the end of the paragraph.

Among soft cheese, buffalo mozzarella was the most studied by NMR, so far (Brescia et al., 2005; Gianferri et al., 2007; Mazzei & Piccolo, 2012). The buffalo mozzarella is a fresh ‘pasta filata’, or stretched curd cheese, produced in southern Italy, from Mediterranean buffalo (*Bubalus bubalis*) milk, which in 1996 gained PDO recognition. Buffalo mozzarella is traditionally manufactured from raw milk employing natural whey starter cultures. Lactic acid bacteria (LAB) and yeasts are the major components of natural whey starter cultures and have great importance in driving the fermentation and in determining the rheological and sensory characteristics of this traditional cheese (Coppola, Villani, Coppola, & Parente, 1990). Moreover, it was found that the metabolite fraction, assessed by GC–MS, reflected the microbial complexity of buffalo mozzarella (Pisano et al., 2016). The ^{1}H NMR spectra of the aqueous extract of buffalo mozzarella were dominated by the lactate resonances; moreover, glucose, galactose, lactate, ethanol, and others small molecules were detected (Brescia et al., 2005; Gianferri et al., 2007; Mazzei & Piccolo, 2012). The main biochemical pathway for lactate production in mozzarella is due to the glycolysis of lactose into monosaccharides such as glucose or galactose. Lactose is also degraded to ethanol by lactobacilli with ethanol fermenting capabilities, commonly present in the mozzarella microflora. Ethanol is further converted into acetic acid by acetic fermentation. Moreover, isobutyl alcohol was recognised as a minor component in the ^{1}H NMR metabolite profiles of buffalo mozzarella (Brescia et al., 2005; Gianferri et al., 2007; Mazzei & Piccolo, 2012), and the presence of this alcohol was attributed to progressive valine degradation promoted by enzymes released from *Saccharomyces cerevisiae* yeast, usually present in buffalo mozzarella (Pisano et al., 2016). In agreement with these observations, investigating the ageing process of buffalo mozzarella, Mazzei and Piccolo (2012) observed that isobutyl alcohol, lactate and acetate increased with time.

There have been many attempts to misappropriately and unlawfully produce buffalo mozzarella from cows' milk copying and counterfeiting the Italian PDO mark of quality. To protect buffalo mozzarella uniqueness, Brescia et al. (2005) carried out a study to link the NMR metabolite profile to the geographical origin of this product. They were able to distinguish the different production sites of buffalo mozzarella by means of analytical and spectroscopic determinations. Acetate and tyrosine were found discriminant for two different buffalo mozzarella producing areas.

With the same aim, Mazzei and Piccolo (2012) demonstrated that HR-MAS spectroscopy can rapidly characterise the metabolic profile of buffalo mozzarella cheese. They compared certified (P) with non-certified (C) commercial samples. In comparison with C samples, P samples had a larger content of β-lactose and β-galactose, as well as a smaller amount of acetate (Mazzei & Piccolo, 2012). The assessed differences in the fatty acid content were ascribed to the original milk employed; on the contrary, differences in the levels of lactose and galactose were ascribed to enzymatic processes and ageing.

Among medium-hard cheese, Emmenthal cheese is very popular, and in Europe, Switzerland, France, and Germany are the most important producers. Emmenthal is a yellowish cheese, characterised by the presence of large holes, due to the activity, in the late stage of cheese production, of *Propionibacterium freudenreichii*, which transforms the lactic acid and releases carbon dioxide forming bubbles that make holes. MVA of HR-MAS NMR data were able to differentiate the geographic origin of the 20 Emmenthal cheese samples examined from 5 different European countries (Shintu & Caldarelli, 2006). Besides signals due to fatty acids, serine, aspartic acid and asparagine were the mostly involved metabolites. Interestingly, since Emmenthal can be produced either from raw or heat-treated milk, untargeted classification of samples was mainly driven by the heat treatment of the milk (Shintu & Caldarelli, 2006), thus confirming the strong effects of milk thermisation on cheese characteristics. Samples of cheese from heated milk had higher relative content of serine and lower relative contents of unsaturated fatty acids and aspartic acid (Shintu & Caldarelli, 2006).

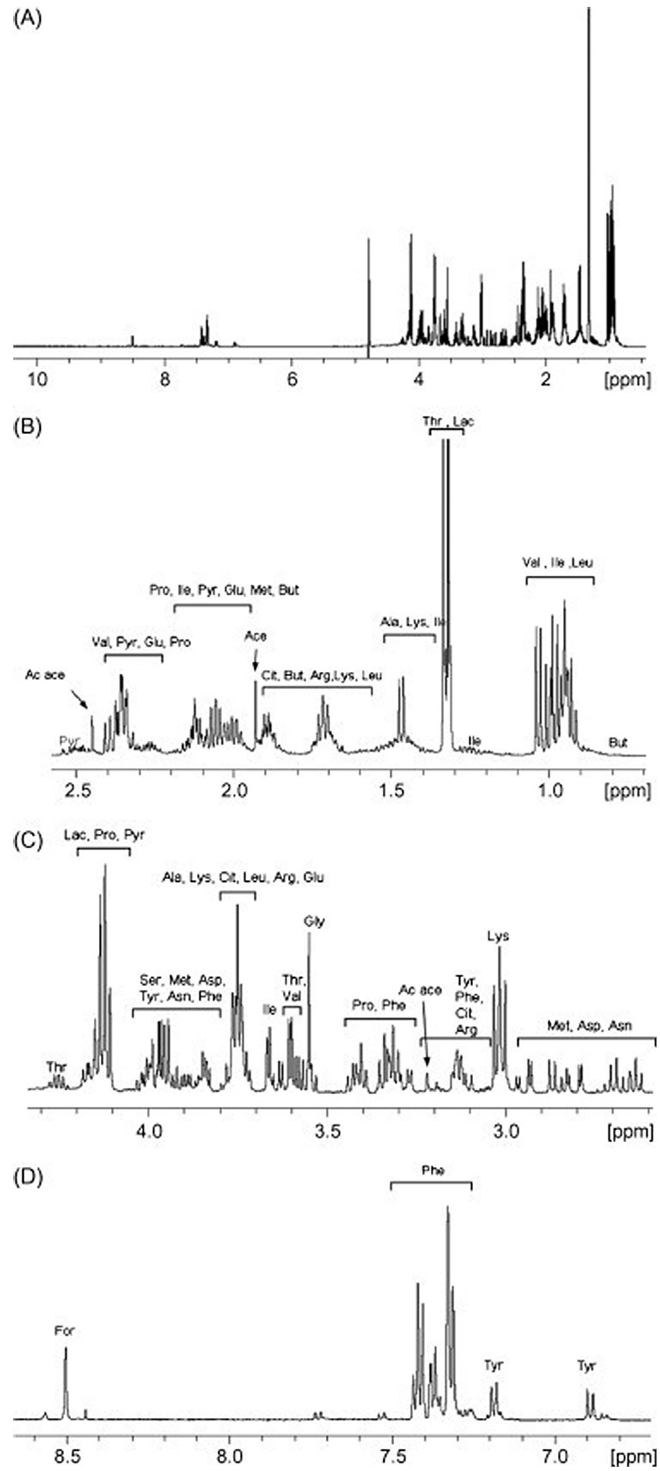

Fig. 3. ^{1}H NMR spectrum of “Parmigiano Reggiano” aqueous extract sample. Complete spectrum (A) and expanded regions (B, C, D) with principal spin system assignments indicated. Reprinted with permission from Consonni and Cagliani (2008).

Gouda is a semi-hard cheese produced worldwide. Brining of Gouda cheese is a basic treatment, whereby salt is used to control the maturation process and flavouring of cheese. Because of health concerns associated with high levels of sodium consumption, a number of organisations and governments are forcing cheese manufacturers to reduce salt and sodium level in cheese. The study of Ruyssen et al. (2013) aimed at the interception of flavour deviations of salt-reduced Gouda-type cheeses, salted in sodium-reduced brines (NaCl + KCl brine), compared with a reference brine (NaCl brine). Moreover, the effect of adjunct strains of *Lactobacillus helveticus* and/or *Lactobacillus paracasei* was investigated. Cheese samples at different ripening time (1, 28 and 56 days) were analysed. PCA of NMR spectral data indicated that samples mainly clustered along PC1 (70% of variance explained) according to their ripening stage, and samples at 28 and 56 days were characterised by a higher content of free amino acids, irrespective of the brine treatments.

Different typologies of hard cheeses were also studied by NMR. The reported ^{1}H NMR spectra were dominated by amino acids, together with organic acids and other minor compounds. In addition to the standard amino acids found in proteins, others, such as γ−aminobutyric acid (GABA) (Lammanna et al., 2008; Piras et al., 2013) and pyroglutamate (Consonni & Cagliani, 2008; Shintu & Caldarelli, 2005, 2006; Shintu et al., 2004), were observed. Moreover, citrulline (Consonni & Cagliani, 2008; Shintu et al., 2004) and ornithine (Ruyssen et al., 2013), derived from arginine, were detected. At very low intensities, the biogenic amines tyramine and histamine were identified as well (Piras et al., 2013; Schievano, Guardini, & Mammi, 2009).

Worldwide, Parmigiano Reggiano is probably the most appreciated hard cheese, it is produced from raw cows' milk under rigorous PDO discipline. HR-MAS studies on Parmigiano Reggiano were carried out by Shintu et al. (2004) and Shintu and Caldarelli (2005). They investigated the HR-MAS metabolic profile of grated Parmigiano Reggiano, and assigned more than 30 molecular species, mostly amino acids and fatty acids (Shintu et al., 2004); this was used for elucidating the ageing process of Parmigiano Reggiano (Shintu & Caldarelli, 2005). To this aim, changes in the metabolite profile of 4–24 month old cheese were studied. By PCA and DA, correct classification of samples was gained.

Despite the exploratory character of this investigation, it was found that some amino acids, namely aspartate, citrulline, valine, methionine, tyrosine, threonine and serine, and some unassigned peaks accounted for the ripening stage (Shintu & Caldarelli, 2005). Consonni and Cagliani (2008) studied the metabolite profile of the aqueous extract of Parmigiano Reggiano at different ripening stages (14, 24 and 30 months). A careful spectral assignment followed by a bucketing procedure, allowed extraction of information from MVA. Besides an overall expected increase of amino acids during ripening, a decrease of leucine and an increase of proline, glutamic acid and threonine were observed. In the same work, the ^{1}H NMR profile of other analogue “Grana type” cheeses from east Europe countries present in the Italian market was studied. Comparison with Italian Parmigiano Reggiano, demonstrated that pair-wise DA correctly classified samples from the different countries (Consonni & Cagliani, 2008).

NMR investigations of Parmigiano Reggiano (Consonni & Cagliani, 2008; Shintu & Caldarelli, 2005; Shintu et al., 2004) indicated the presence of citrulline and of pyroglutamate, which are the result of enzymatic processes on amino acids. Citrulline derives from the arginine-deaminase pathway, which has ornithine as the final product. The results of the studies on Parmigiano Reggiano suggested that the arginine-deaminase pathway was only partially adopted since only citrulline was found, lacking the ^{1}H NMR spectra of assigned signals of ornithine. Pyroglutamate, produced by thermophilic lactic acid bacteria used as starters (Mucchetti et al., 2000), is present in many cheese varieties and particularly in high amounts (0.5 g 100 g^{-1} cheese) in ripened Italian hard cheese like Grana Padano and Parmigiano Reggiano (Mucchetti et al., 2000).

Concerning hard cheese, Fiore Sardo PDO is produced from raw ewes' milk without the use of exogenous starter cultures or with the addition of exclusively autochthonous LAB. To study the effects of different LAB cultures on cheese characteristics, Piras et al. (2013) monitored the modifications of the ^{1}H NMR metabolite profile at different ripening stages in Fiore Sardo produced with different starter cultures; five samplings were performed (2, 6, 15, 28, 90 days). Evolution of the metabolite profiles during ripening and in regard to the different starters used was analysed together with microbiological information. As previously observed with other analytical techniques (Pisano et al., 2016), the microorganisms differently influence the metabolite profiles. Piras et al. (2013) assessed that the main carbohydrate sources (lactate, glucose and galactose) were consumed within the first 15 days of ripening by the starter and non-starter LAB. Lactate, after an initial increase that lowered the pH, declined; a decrease of citric acid was also observed, while acetic acid increased. During ripening, due to the proteolytic activity of microorganisms, an increase of free amino acids was detectable. A concomitant decrease of glutamic acid and increase of GABA was also observed.

GABA is endowed with interesting health promoting characteristics (Aryana & Olson, 2017) and is produced by the decarboxylation of glutamate, catalysed by L-glutamic acid decarboxylase enzyme (GAD). This enzyme is present in different species of microorganisms (Wang, Dong, Chen, Cui, & Zhang, 2010), some of which are also involved in the production of biogenic ammines (Manca et al., 2015), indeed, tyramine and histamine were found in the ^{1}H NMR spectra of Fiore Sardo extracts (Piras et al., 2013). Finally, the effects of the different starter cultures, either autochthonous or commercial strains, in ripening of Fiore Sardo were investigated by MVA of spectral data (Piras et al., 2013).

Concerning functional foods, specific new formulations were adopted for innovative cheese products. In fact, cheese is considered a good carrier of probiotics (Sanders, 2003), enabling their passage as viable cells through the gastrointestinal tract. In this regard, ripening of cheese from pasteurised cows' milk manufactured with the addition of probiotics and prebiotics was studied by ^{1}H NMR (Rodrigues et al., 2011). Two strains of probiotics, namely *Lactobacillus casei*-01 (LCS) and *Bifidobacterium lactis*-B94 (BLC) were tested. Prebiotics consisted in fructo-oligosaccharides (FOS) or in a FOS/inulin mixture (50:50). Probiotic and symbiotic (containing both prebiotics and probiotics) cheeses were sampled at 6 ripening stages (0, 7, 14, 30, 45, 70 days). NMR spectra of the aqueous extracts of cheese at early ripening time were characterised by higher amounts of lactose and ethanol as well as by the presence of some organic acids, such as lactate, acetate, and citrate, due to the fermentation process via probiotic bacteria cells. At 60 days of ripening, ^{1}H NMR spectra showed a lower amount of lactose, ethanol, and organic acids, except lactate, while free amino acids increased. It was observed that the pH falls during ripening had lower rate in cheese with LCS compared with that with BLC, and that BLC had less proteolytic potential than LCS. The addition of FOS or FOS/inulin resulted in a further reduction in proteolysis since the probiotics can use these polysaccharides as an alternative source of energy. The decrease of the broad peak in the 7.50–6.75 ppm region of the NMR spectra was used to monitor the proteolytic activity in the cheese. MVA applied to the NMR data of the metabolic profiles allowed discrimination between the cheese samples according to ripening stage, added probiotics and prebiotics.

Food packaging is a growing research field involving different aspects of food supply chain. The primary task of a package is to preserve the quality and safety of a foodstuff over a period of time. There are different ways to evaluate the quality of food and then to assess its package performance. A large number of properties that characterise the food (chemical composition, organoleptic and nutritional properties, microbiological contamination, colour, etc.) has to be monitored. In this context, Lamanna et al. (2008) studied the variations of the levels of the hydrosoluble metabolites in Robiola cheese under different packaging conditions. Robiola cheese is made with cows' milk and is sold in a composite paper foil package under controlled atmosphere. The NMR metabolite profiles of cheese samples exposed to air for 2, 4 and 7 days were compared with that of control samples. In exposed samples, the metabolites with increasing concentration with time were glucose, lactate, phosphorylcholine, glycophosphorylcholine, and creatine. On the other hand, both choline and acetate exhibited a very small dependency on time, while GABA, alanine, valine and isoleucine decreased in their concentrations with time (Lamanna et al., 2008).

In a recent work, Gandhi, Cobra, Steele, Markley, and Rankin (2018) studied the molecular mechanisms of the development of brown pigmentation in Parmesan cheese during ageing, an issue that can cause economic depreciation of affected cheese. Methylglyoxal (MG) of microbial production was indicated as one of the key steps of this mechanism. In this work, the ability of some culture adjuncts to reduce the level of MG in parmesan cheese extracts was tested (Gandhi et al., 2018). Microbial reduction of MG to acetol and to 1,2-propanediol was monitored in the ^{1}H NMR spectra of aqueous extracts of Parmesan cheese inoculated with different *Lactobacillus* strains. ^{1}H NMR detection of these two molecules, which has never been performed before in dairy products, was probably possible only because MG was abundantly added to Parmesan cheese extracts.

4. Conclusion and future directions

As reported in this review, different aspects of the dairy industry have been successfully addressed by the application of the ^{1}H NMR spectroscopy at the metabolite level, demonstrating not only the power of this technique but also the valuable contribute of the metabolite profiling in unravelling complicate issues, as those inherent the dairy products. Further applications of this approach in the dairy field are strongly suggested, such as the study of the impact of the different milk heat treatments on the metabolite profile of cheese for food fraud detection in Parmigiano Reggiano PDO, Fiore Sardo PDO and other high-quality dairy products. The application of the NMR metabolite profile as predictor of milk traits, animal diseases, cheese making milk performance, and other aspects of the dairy sector, has great, still unexploited, potential.

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