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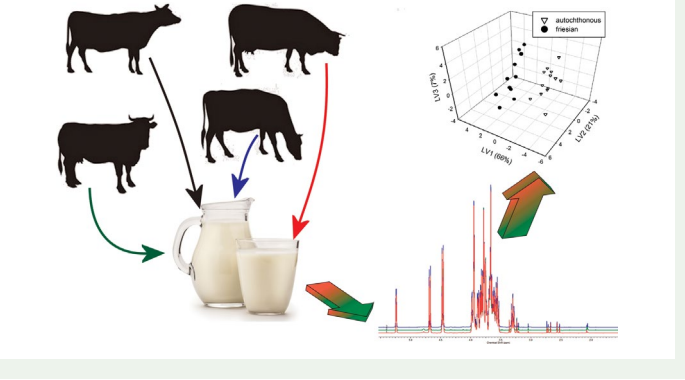
NMR-based metabolomics to evaluate the milk composition from Friesian and autochthonous cows of Northern Italy at different lactation times

A. Tomassini[a], G. Curone[b], M. Solè[b], G. Capuani[a], F. Sciubba[a], G. Conta[a], A. Miccheli[a] and D. Vigo[b]

[a]Department of Chemistry, University of Rome "La Sapienza", Rome, Italy; [b]Department of Veterinary Medicine, Università degli Studi di Milano, Milano, Italy

ABSTRACT

It is well established that different factors affect milk composition in cows and that milk composition, in turn, affect both technological and nutritional qualities. In this respect the comprehension of the metabolic variability of milk composition in relation to the lactation time as well as to the genetic background may be of paramount importance for the agri-food industries. In the present study we investigated the variations of the metabolic profiles during lactation in milks obtained from Friesian and autochthonous races from Northern Italy by ^{1}H NMR metabolomics. Furthermore, the external factors influencing the milk composition were minimized: the cows were breeded in the same farm, were fed with the same diet and were paired for the lactation interval and lactation stage. Our results showed a difference in milk composition between races and in relation to late lactation. The PLS-DA analysis permitted to distinguish the Friesian and autochthonous cow milks at the investigated different lactation times. Interestingly, the metabolites significantly involved into the discrimination between races appeared to be also technological property parameters, highlighting the importance of maintaining the biodiversity of cow breeds. Therefore, NMR-based metabolomics of milk could represent an informative tool to identify metabolites involved in milk quality both from a nutritional and industrial perspective.



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CONTACT A. Miccheli alfredo.miccheli@uniroma1.it

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1. Introduction

Milk has been described as an almost perfect food and cow milk represents a worldwide popular component in both infant and adult nutrition. From a chemical point of view, milk is a complex mixture of several substances ranging from oligosaccharides, proteins and lipids to vitamins and mineral salts, among others. (Solomons 2002), and is consumed both fresh and processed into dairy products. In this regard milk composition has been associated to both the nutritional quality and to the technological processing capabilities so that a profound characterization of milk metabolic profiles may represent a basis to improve its nutritional and technological quality.

However, it is known that milk composition varies in relation to many factors ranging from genetics to physiological (number and stages of lactation, cow health status, seasonal variations) and to zootechnical (feeding, climate, breeding techniques) ones so that a comprehensive knowledge of milk composition can be of paramount importance towards the optimisation of processing capabilities to dairy products (Lock and Garnsworthy 2003; McJarrow and van Amelsfort-Schoonbeek 2004; Tsioulpas, Grandison et al. 2007; Le Maréchal et al. 2011).

Up to now the breeding programs to improve milk production have been based on strategies that often favoured the economical parameters over milk quality, leading to an homogenisation of the cow's genetical pools and, consequently, to the risk of extinction of less producing autochthonous breeds and biodiversity loss.

The 'omics' technologies, namely genomics, proteomics, transcriptomics and metabolomics, have been applied in studies regarding lactation mainly in dairy cows (Hu et al. 2004; Lamanna et al. 2011; Li et al. 2017).

The application of NMR spectroscopy to analyse milk metabolites has proven a valuable tool in studies ranging from mammary gland physiology to nutritional, quality and technological properties of milk (Cesare Marincola et al. 2012; Sundekilde et al. 2013; Praticò et al. 2014).

In the present study, the chemical composition of milk obtained from Friesian cows and different autochthonous breeds from Northern Italy has been analyzed by NMR spectroscopy. The matrices of the resulting data were analyzed by multivariate statistical methods in order to obtain a metabolic profile (metabotype) of each breed. Furthermore, the cows were breeded in the same farm, were identically fed with a standardized diet and milk was sampled at the same lactation times, in order to minimize the inter-individual variability.

2. Results and discussion

In the present study we first analysed the variation in milk composition in relation to the lactation stages that are commonly roughly divided in early (13 ± 1.8 days), mid (130 ± 4.6 days) and late (283 ± 3.4 days) lactation to investigate any possible influence of the genetical different breeds. We chose to analyze the milk samples collected in the mid and late lactation stages to avoid the possible metabolic stress or the negative energy balance that can affect individual animals in early lactation, introducing an additional source of variation (Kessel et al. 2008).

The ^{1}H NMR spectrum of the polar phase can be divided into three main spectral regions. The aliphatic region, from 0 up to 3.5 ppm, includes amino acids, tricarboxylic acid cycle

intermediates, short chain fatty acids, lactate, N-acetyl moieties, N-trimethyl moieties and oligosaccharides. The sugar region, between 3.5 and 5.5 ppm, includes a series of overlapped signals, due to the presence of simple and complex sugars. The most intense signals (d 5.22, 4.69 and 4.47) were assigned to the anomeric protons of free lactose, superimposed to the peaks of some lactosyl units of oligosaccharides. The aromatic region includes aromatic amino acids and phenolic compounds. A representative ^{1}H NMR spectrum of cow milk is shown in Figure S1; 50 metabolites were identified and quantified and their resonance assignements are reported in Table S1.

The DOSY experiment is reported in Figure S2. In this figure only the signals relative to TSP, lactose and of different N-acetyl groups were considered to better display their autodiffusion coefficients. It is interesting to observe that the most abundant N-acetyl groups show an autodiffusion coefficient about half the one exhibited by lactose, while the less abundant one has a coefficient of only one fifth of lactose. This means that all acetyl groups belongs to molecules heavier than a disaccharide, such as oligosaccharides or oligopeptides.

A further indication of the presence of N-acetyl groups with different mobility could be derived from the study of the slices of the DOSY experiment spectrum prior inverse Laplace transformation. Each slice corresponds to a specific gradient strength, and at progressively higher strength the resonances of fastest species can not refocus, and as such only the signals of the slowest molecules can be observed. In Figure S3, for example, it is possible to identify two N-acetyl groups belonging to relatively fast molecules, which are defocused at 75% gradient strength, and another group characterized by a lower translational mobility, the resonance of which is observable even at 100% gradient strength field. On the basis of the diffusion coefficients, we could disciminate the class of Nacetyl-groups (at 2.05–2.08 ppm) in N-acetyl-glucosamine or Nacetyl galactosamine-containing oligosaccharides and in N-acetylglutamine containing oligopeptides (at 2.04 ppm), having an higher hydrodinamic radius than oligosaccharides.

No significant variations were evidenced in the milk sampled between 100 and 200 days of lactation irrespective on the breeds by the PLS analysis, showing an almost steady composition in terms of the polar analyzed components ($R^2 = 0.48$; $Q^2 = 0.18$; data not shown).

On the contrary, a statistical significant variation is shown by the data-set including all the samples from every breed ranging from 200 to 300 days of lactation ($R^2 = 0.78$; $Q^2 = 0.42$) (Figure 1). Nine metabolites were responsible of the observed variation, namely 1,2-propandiol, 3 hydroxybutyrate, butyrate, N-acetyl-X4, galactose-1-P and glucose-1-P with decreased concentrations and N-acetyl-glucosamine and cytidine-X-P with increased levels.

Milk production during lactation is a multiphasic process and, furthermore, milk metabolites have different sources, being synthesized by multiple cell types in the mammary gland or by different metabolisms in the whole organism contributing to the variability of milk metabolic profiles (McManaman and Neville 2003). Late lactation is characterized by an involution of the mammary gland with a loss of tight junction integrity and the resulting variation of metabolite fluxes via the paracellular pathway (Stelwagen and Singh 2014). A loss of function and a diminished milk yield linked to increased secretory cell apoptosis and diminished secretory cell biosynthetic capacity in the mammary gland with late lactation have also been shown (Hadsell et al. 2007).

The observed significant decrease of N-acetyl-X2, glucose-1P and galactose-1-P with the concurrent increase of N-acetyl-X4 in milk sampled from 200 to 300 days of lactation

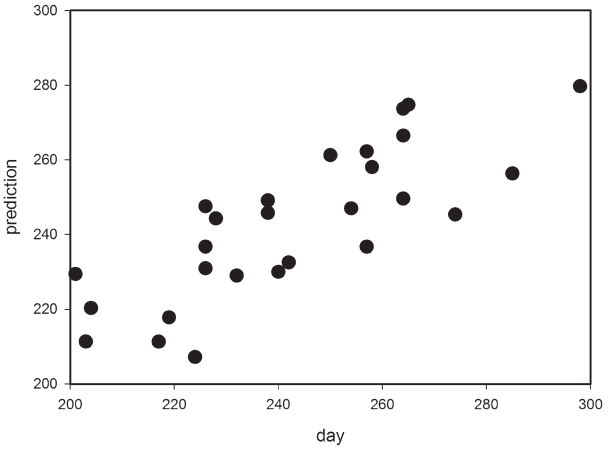

Figure 1. PLS model for the lactation time (200–300 days of lactation).

suggested significant qualitative and quantitative variations in the oligosaccharides synthesis and secretion in milk as a function of lactation time.

It has been suggested that the levels of β-hydroxybutyrate in milk could be used as biomarkers of subclinical and/or acute ketosis in cows, and high levels of β-hydroxybutyrate and butyrate have been linked to elevated numbers of somatic cells counts (Klein et al. 2012; Sundekilde et al. 2012). Our results showed a decrease of the levels of these two metabolites in late lactation. As we did not observe any case of ketosis that, incidentally, is more frequent in early lactation, and although we did not perform somatic cell counts, we suggest that our data are more likely associated to the energy status in dairy cows in late lactation.

Furthermore, an oxidative damage within mammary cells in late lactation could be suggested by the increase in the levels of cytidine-X-P in milk possibly associated with the RNA synthesis decline showed in prolonged lactation (Hadsell et al. 2007).

On the basis of these results, we applied a PLS-DA analysis on the NMR data matrices to disentangle potential differences of milk composition among the breeds while keeping the lactation stages between 100–200 and 200–300 days.

At 100–200 days of lactation, significant differences between Friesian and autochthonous breeds were shown (Figure 2; $R^2 = 0.93$; $Q^2 = 0.57$). Nine metabolites were involved, namely valine, acetate, phosphorylcholine and glucose-1P and citrate, carnitine, fumarate, hippurate and fucose with higher and lower levels in the Friesian cows, respectively. Acetate, citrate, carnitine, fumarate, hippurate and fucose were found to be statistically different at the univariate analysis, too (Table S2).

The PLS-DA analysis could also distinguish the Friesian and autochthonous milks after 200–300 days of lactation (Figure 3; $R^2 = 0.99$; $Q^2 = 0.65$), with lower levels of acetylcarnitine, carnitine and fumarate and higher levels of ribosyl and cytidineXP in the Friesian and autochthonous breeds, respectively. Carnitine, fumarate and ribosyl were also statistically different at the univariate analysis (Table S2).

Citrate, phosphocholine, carnitine, acetate and hippurate have been associated with milk coagulation properties, thus being metabolites of paramount importance in the evaluation of milk technological quality and processing capabilities (Sundekilde et al. 2011; Harzia et al. 2012).

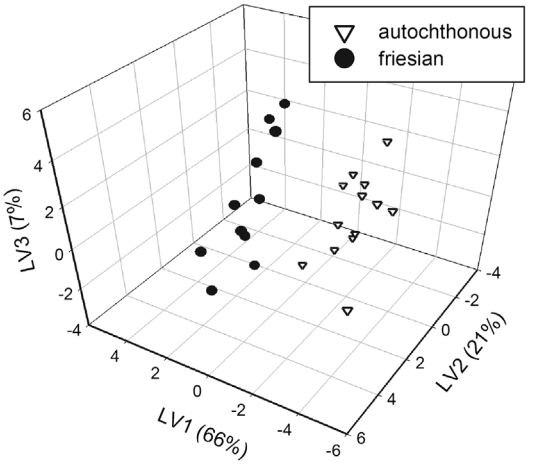

Figure 2 PLS-DA 3D score plot of autochthonous and Friesian cow milk at 100–200 days of lactation (LV = latent variables).

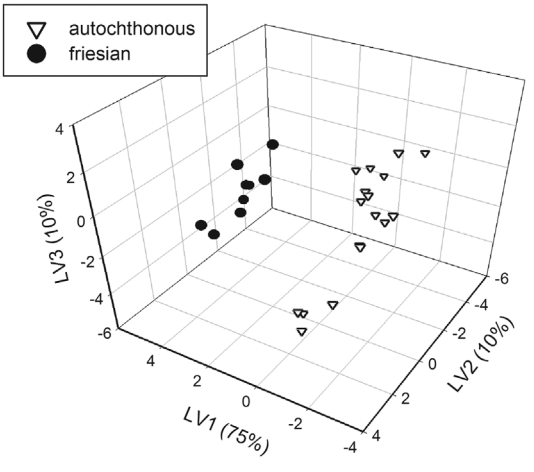

Figure 3 PLS-DA 3D score plot of autochthonous and Friesian cow milk at 200–300 days of lactation (LV = latent variables).

In agreement with our results, good coagulating milks have been characterized by increased levels of choline and acetate and decreased levels of citrate and carnitine, and different milk metabolite profiles characterized by differences in the relative abundance of these metabolites have been demonstrated in milk between two cow breeds (Sundekilde et al. 2011; Harzia et al. 2012). In this regard, our results suggested a better technological power of Friesian milk than autochthonous breeds milk from 100 days of lactation.

In particular citrate, that is associated with the whey fraction, is the most abundant organic acid in milk either complexed with calcium and magnesium ions or in free form. Changes in its concentration have important effects on the physico-chemical properties of milk. High levels of citrate are capable of disrupting the casein micelle structure thus affecting the coagulation parameters. It is well known that the coagulation properties are also influenced

by other factors like pH, ions availability, as well as lactation time, climate, and cow's nutritional and health status (Salaün et al. 2005; Skeie 2007; Tsioulpas, Lewis et al. 2007). However, in our experimental set both Friesian and autochthonous cows were breeded in the same farm and consequently in the same climate, were equally fed and their milks were sampled at equivalent lactation stages, thus ruling out these external sources of variation. Furthermore, our data did not show variations of citrate levels in relation to lactation stages, in agreement with previous results (Klein et al. 2010), suggesting that the observed variations could be ascribed to genetical differences of the studied breeds.

Interestingly, our results could be evaluated from a nutritional point of view beside the processing capabilities into dairy products. In fact choline, that is crucial in human development as well in adult life, and carnitine, whose deficiency has been associated to systemic effects on the infants, were among the metabolites discriminating the Friesian and autochthonous breeds. Therefore, NMR-based metabolomics of milk could represent an informative tool from a nutritional perspective when selecting milks for premature children or to be used in infant formulas.

Supplementary material

Supplementary material relating to this paper is available online: Experimental section; A representative ^{1}H NMR spectrum of cow milk (Figure S1); DOSY experiment spectrum (Figures S2–S3); Table of assignements (Table S1); Univariate analysis (Table S2).

Disclosure statement

No potential conflict of interest was reported by the authors.

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