Food Research International 113 (2018) 131–139

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

# NMR metabolomic fingerprinting distinguishes milk from different farms

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ARTICLE INFO

*Keywords:*
Metabolomics
Nuclear magnetic resonance spectroscopy
Milk analysis
Origin and traceability
Silages

ABSTRACT

A fast and reproducible protocol for milk Nuclear Magnetic Resonance (NMR) metabolomic fingerprinting was developed, allowing for an accurate discrimination among milk samples from large-scale distribution, as well as among milk sample from different farms located in the same restricted geographical area. Seasonal variations in milk composition and correlations with cows' nutritional patterns are also assessed, underlining relationships between feeding and metabolites. The most important difference was related to the use of silage feeding. This finding is relevant to assess the suitability of milk for different dairy products. A prominent example is parmesan cheese, the preparation protocol of which excludes milk from silage-fed cows.

## 1. Introduction

Bovine milk is an important diet constituent. Lipids and lactose are the two main nutrients, but milk also contains a wide range of bioactive compounds such as immunoglobulins and other immune proteins, peptides and nucleotides (Ulrik K. Sundekilde, Larsen, & Bertram, 2013). Being a biological fluid, the composition of milk is influenced by several factors such as breed, individual metabolism of the animals, season, health status, nutrition and milking protocols (Lamanna, Braca, Di Paolo, & Imparato, 2011; Tian et al., 2016). All these factors contribute to the variability of milk metabolite profiles; in fact, milk metabolites can originate from different metabolic pathways in the animal organism. The chemical composition of milk is important to understand its nutritional value, including bioactive compounds, its technological properties, and the potential use of biomarkers in milk as diagnostic tools for cows health (Sundekilde, Poulsen, Larsen, & Bertram, 2013). Metabolomics allows for the simultaneous characterization of large amounts of compounds in biological matrices and can provide a more detailed molecular picture of food composition, food consumption or about the consequences of diets (Wishart, 2008). Milk metabolome has been studied using different metabolomics approaches in order to extrapolate information on fat composition (Kalo, Kemppinen, & Kilpeläinen, 1996) or structural changes in caseins and other proteins (Leslie, Irons, & Chapman, 1969). Through metabolomics it was possible to find biomarkers that are correlated with the health status of bovines: for example β-hydroxybutyrate, phosphocholine and glycerophosphocholine could be used as biomarkers for ketosis (M. S. Klein et al., 2010). The composition of milk is also important for the production of dairy products because many factors are known to influence the coagulation properties of milk (Bittante, Penasa, & Cecchinato, 2012). Two additional aspects are also related to consumers' expectation: the first concerns the nutritional value of milk, and $^{1}$H NMR spectroscopy has also been used to validate the labelling in relation to particular nutritional features (Monakhova, Kuballa, Leitz, Andlauer, & Lachenmeier, 2011); the second is related to the authentication and the control of geographical origin. In fact, the commercial value of many dairy products is closely associated with the origin and composition of milk. A few cases have been reported about the use of NMR spectroscopy for traceability of geographical origin of cow and buffalo milk (Brescia, Monfreda, Buccolieri, & Carrino, 2005)(Sacco et al., 2009). However, they were focused on the identification of provenance from a large territorial basis (e.g. different provinces or countries), relied on a limited number of samples, and did not address the relation between milk composition and cows' feedings.

In the present study, to provide a proof of concept for the ability of NMR-based metabolomics to distinguish different milk samples, we first determined the metabolic profiles of three different milk brands obtained from large-scale distribution. In doing so we developed an

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https://doi.org/10.1016/j.foodres.2018.06.066
Received 6 April 2018; Received in revised form 27 June 2018; Accepted 28 June 2018
Available online 30 June 2018
0963-9969/ 

original protocol for metabolites extraction from milk to allow for a fast and reproducible acquisition of NMR spectra and compared our procedure with the most common methods described in the literature. Then we applied the same analytical protocol to monitor the origin of milk samples collected in ten farms located in a relatively small valley in the north of Tuscany (Italy), called Mugello. The rationale for this design was to prove that NMR-metabolomics is sensitive enough to accurately assess the provenance of milk even at the scale of each individual farm in a small territory. We analysed also cow feeding data in order to understand the contribution of different nutritional profiles on the changes of the milk metabolic profile.

## 2. Materials and methods

### 2.1. Sample collection

Whole pasteurized milk was collected from the large-scale distribution in three different periods: Autumn 2013 (September/October), Spring 2014 (April/May) and Autumn 2014 (September/October). Packaged milk of three major Italian brands ("Latte Coop", "Granarolo" and "Mukki Mugello") was collected (twenty milk samples for each brand in each period).

Samples coming from ten different farms located in the Mugello valley were collected in two different periods, Autumn 2013 and Spring 2014, in parallel with large-scale distribution (supermarket) milk. For each period, twenty raw milk samples were collected from each farm in twenty different days. Each sample consisted of a mixture of the milk produced in the same day by all bovines in a single farm. A total of 40 samples per farm were thus collected in the two collection periods (200 samples in Autumn 2013 and 200 samples in Spring 2014) and analysed.

The ten Mugello farms belongs to two categories, "organic" and "non-organic". The organic farms meet the requirements stated in the Europeans Regulations (N. 1804/1999, 834/2007, 889/2008), about organic productions and labelling of organic products. The organic group is composed by three farms, whereas the remaining seven farms compose the non-organic group. Once the milk coming from these farms arrives at the Milk Factory of Florence, Italy, it is pooled in two distinct tanks, one for the organic and one for non-organic milk. This bulk raw milk is then pasteurized and packaged. In addition to the collections at the individual farms, bulk raw milk samples (before pasteurization and packaging) coming from non-organic and organic farms were collected in Autumn 2013 and Spring 2014 directly from the Milk Factory; in both collections, 20 samples from each of the two kinds of bulk milk were analysed. Finally, eight samples coming from eight randomly selected Mugello farms were collected in 2015 (2nd March) as a blind dataset to be used to test the predictive ability of the milk fingerprints obtained in the two previous collections. A summary diagram of collected samples is reported in Supplementary Fig. 1.

### 2.2. Cow feeding data

Regarding raw milk samples from farms, for each day of collection we recorded also the cow feeding rations, reported as kilograms of food per bovine. In particular, we divided the nutritional rations in eight categories: silages, wrapped hays, dry hays, legumes flours and proteic foods, cereal flours and energetic foods, protein supplements, energy supplements, mineral salts and vitamin supplements.

### 2.3. Sample preparation for NMR analysis

All milk samples were processed in the same way in order to minimize errors due to the pre-analytical phase. All samples were stored at 4 °C for at most 12 h before sample preparation. Freezing was avoided to prevent breaking of the somatic cells and the leaking of cell metabolites in the milk.

In order to evaluate the best preparation procedure, we compared a method developed in our laboratory with respect to other methods described in literature. The metabolite profiling following our own method consists of mixing 700 µl of milk with 700 µl of dichloromethane ($CH_2Cl_2$). The mixture is homogenized by vortexing and then is incubated for 10 min at room temperature. The mixture is then centrifuged at 14000 rpm for 30 min and the supernatant is recovered.

For comparison, we acquired also NMR spectra after using other published extraction procedures. We followed four different protocols described in literature, in particular: chloroform and deuterated chloroform instead of dichloromethane was used in 1:1 ratio with the sample (Lamanna et al., 2011); filtration of milk using a 10 kDa cut-off was performed in order to remove high-molecular weight molecules (Matthias S. Klein et al., 2012; U. K. Sundekilde, Poulsen, et al., 2013); a 75 min ultracentrifugation followed by a centrifugation of the obtained supernatant was also tested (Lu et al., 2013); the analysis of the whole milk without any manipulation was also finally performed (Ulrik Kræmer Sundekilde, Frederiksen, Clausen, Larsen, & Bertram, 2011).

In all cases 300 µl of the samples obtained by the various methods were mixed with 300 µl of a sodium phosphate buffer (70 mM $Na_2HPO_4$; 20% (*v*/v) $^2H_2O$; 0.025% (*w*/*v*) $NaN_3$; 0.8% (w/v) sodium trimethylsilyl [2,2,3,3-$^2H_4$]propionate (TSP); pH 7.4). A total of 450 µl of this mixture was transferred into a 4.25 mm NMR tube (Bruker BioSpin srl) for analysis.

### 2.4. NMR analysis

One-dimensional $^1$H NMR spectra of milk extracts were measured on a Bruker spectrometer operating at 600.13 MHz proton Larmor frequency and equipped with a 5 mm CPTCI 1H-13C/31P-2H cryo-probe including z-axis gradient coil, an automatic tuning-matching (ATM) and an automatic sample changer. A PT 100 thermocouple provided temperature stabilization at the level of approximately 0.1 K at the sample. Before measurement, samples were kept for at least 3 min inside the NMR probe for temperature equilibration (310*K*)·One-dimensional NMR spectrua were acquired with water peak suppression adopting a standard pulse sequence (NOESYpresat, Bruker), using 64 free induction decays (FIDs), 64 k data point, a spectral width of 12,019 Hz, an acquisition time of 2.7 s, a relaxation delay of 4 s and a mixing time of 100 ms. 2D $^1$H−$^1$H COSY with presaturation during relaxation delay using gradient pulses for selection (cosygpprqf, Bruker) was acquired using 2 k to 512 data points, 8 scans with a relaxation delay of 4 s, acquisition time of direct and inverse dimensions of 0.15 s and 0.038 s respectively. 2D $^1$H−$^{13}$C HSQC via double inept transfer using sensitivity improvement, phase sensitive using Echo/Antiecho-TPPI gradient selection with decoupling during acquisition using trim pulses in inept transfer with gradients in back-inept (hsqcetgpsi2, Bruker) was acquired using 1 k to 256 data points, 32 scans with a relaxation delay of 2 s, acquisition time of direct and inverse dimensions are 0.77 s and 0.005 s respectively.

### 2.5. Spectral processing

Free induction decays were multiplied by an exponential function equivalent to a 1.0 Hz line-broadening factor before applying Fourier transform. Transformed spectra were automatically corrected for phase and baseline distortions and calibrated (TSP peak at 0.00 ppm) using TopSpin (Bruker). For multivariate analysis, each 1D spectrum in the range between 0.02 and 10.00 ppm was segmented into 0.02-ppm chemical shifts bins, and the corresponding areas were integrated using AMIX software (Bruker BioSpin). The dichloromethane and water regions (between 4.5 and 5.65 ppm) were removed. After having excluded that signals of lactose, that account for a major part of the total area, vary too much between farms/seasons etc. (Supplementary Fig. 2), the total spectral area was calculated on the remaining bins and was used for the normalization of the data prior to pattern recognition.

Normalization to the external reference (TSP) has been avoided in this study since TSP levels are not stable, suggesting the interaction with some proteins or peptides in the samples (Supplementary Fig. 3). Normalization using probabilistic quotient normalization (Dieterle, Ross, Schlotterbeck, & Senn, 2006) (PQN) was also used for comparison (see section 3.3).

### 2.6. Statistical analysis

Data reduction was carried out by means of projection into a Partial Least Square (PLS) subspace, and the canonical analysis (CA), as a method to post-process the PLS results (Ergon, n.d.; Ghadiri, Rezaei, Tabatabaei, Shahsavari, & Shahsavari, 2016), was applied to enhance the supervised separation of the analysed groups (Yu & MacGregor, 2004),. Accuracy, sensitivity and specificity for the different classifications were assessed by means of 100 cycles of a Monte Carlo cross-validation scheme (MCCV, R script in-house developed). Briefly, 90% of the data were randomly chosen at each iteration as a training set to build the PLS-CA model. Then, the PLS-CA scores for the remaining 10% (test set) were obtained by projecting the test spectra into the training PLS-CA space. For classification, k-Nearest Neighbours (k-NN) method (k = 5) was applied (Cover & Hart, 1967) using the PLS-CA scores of the training set to drive the classification of the calculated PLS-CA scores of the test set. Test samples were predicted according to the position of the respective PLS-CA scores in the PLS-CA space calculated using the training set. Sensitivity, specificity and accuracy for the classification were assessed. The procedure was randomly repeated 100 times to derive an average discrimination accuracy for each group of samples.

Principal Component Analysis (PCA) was used to analyse cow feedings data obtained for each farm in order to highlight common nutritional profiles.

For the analysis of metabolite content, all resonances of interest were manually checked, and signals were assigned on template one-dimensional NMR profiles by using matching routines of AMIX 7.3.2 (Bruker BioSpin) in combination with the BBIOREFCODE (Version 2–0-0; Bruker BioSpin), reference database and published literature when available (Sundekilde, Larsen, & Bertram, 2013). The relative concentrations of each metabolite were calculated by integrating the corresponding signals in the spectra. Variations were reported as $\log_2$(Fold Change) and significant differences were assessed using the Wilcoxon test. *P*-values were adjusted for multiple comparisons using Bonferroni correction(Bonferroni, 1935) in which the *P*-values are multiplied by the number of comparisons.

All data analysis were performed using the R statistical environment ("R Core Team (2014). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.," n.d.).

## 3. Results

### 3.1. A novel preanalytical protocol for NMR analysis of milk

All the procedures described in Materials and Methods section can be considered equally effective for the analysis of milk samples. In Fig. 1, a comparison among spectra obtained from the same milk sample using different extraction methods is reported. In the case of the use of whole milk without any pre-processing (Ulrik Kræmer Sundekilde et al., 2011) (spectrum 6), signals derived from high-molecular weight molecules and lipids are clearly visible and they tend to cover signals derived from metabolites. To remove macromolecules, filtration with 10 kDa cut-off (Matthias S. Klein et al., 2012; Sundekilde, Larsen, & Bertram, 2013) (spectrum 4) or ultracentrifugation (Lu et al., 2013) (spectrum 5) permit the obtainment of good quality spectra, but they are time consuming procedures and, in the case of filtration, they are also expensive when a large number of samples need to be processed. Using a 10 kDa cut-off it is possible to eliminate the macromolecules and the obtained spectrum shows well resolved signals and no baseline distortion: the effectiveness of this method was also demonstrated in (M. S. Klein et al., 2010), where the authors were able to assign a total of 44 metabolites in filtered milk using both GC–MS and NMR (25 and 23 metabolites, respectively). With ultracentrifugation, followed by another centrifugation of the supernatant, the quality of the spectrum is lower with respect to filtration because some broader signals due to lipids and proteins are still present.

The use of an organic solvent (Lamanna et al., 2011) is the fastest way for the preparation of milk samples. Chloroform stabilized with 1% of ethanol has been used in several cases, therefore ethanol peaks are present in the NMR spectrum and cover important spectral regions (spectrum 1). Conversely dichloromethane has only a singlet peak near the water peak, which can be easily removed before pattern recognition analysis. Significantly, the comparison of spectra obtained after extraction with deuterated chloroform (spectrum 2) and dichloromethane (spectrum 3) demonstrates that β-hydroxybutyrate and creatine signals can be better detected when dichloromethane is used. Moreover, considering also that non-deuterated solvents are cheaper, we eventually decided to proceed using dichloromethane extraction. Last but not least, dichloromethane is the least toxic of the chlorohydrocarbons (Brown-Woodman et al., 1998; Honma & Suda, 1997).

### 3.2. Metabolomic fingerprinting of large-scale distribution milk

According to the experimental scheme reported in Supplementary Figs. 1, 60 samples from three Italians milk brands (20 samples each) were collected in a supermarket during three periods: Autumn 2013, Spring 2014, and Autumn 2014, for a total of 180 milk samples retrieved from the large-scale distribution. Our first aim was to test our system and to evaluate whether it was possible to discriminate each single brand.

The preanalytical and analytical approaches chosen proved to be particularly efficient for the NMR analysis of milk samples. The acquired spectra resulted of high quality, well resolved and with many visible peaks. Supplementary Fig. 4 shows a superimposition of the 60 spectra from the Autumn 2013 collection. From the figure, the high reproducibility of the spectra can be inferred. Supplementary Fig. 5 and Supplementary Table 1 show in detail the assigned peaks. Supplementary Figs. 6 and Supplementary Fig. 7 show the 2D spectra, $^{1}H-^{1}H$ COSY and $^{1}H-^{13}C$ HSQC respectively, used to confirm 1D assignments; Supplementary Fig. 8 shows a zoomed region of the HSQC spectra.

The analysis of the metabolic fingerprints of milk samples was performed using PLS-CA and Monte Carlo cross-validation: for all the three collection periods, it was possible to clearly distinguish the three brands (Fig. 2A, B and C), with a mean cross-validated accuracy over 95% in all the three cases (Supplementary Table 2), demonstrating the efficacy of both the chosen extraction protocol and the statistical strategy.

### 3.3. Metabolomic fingerprinting of milk from specific farms

To assess whether NMR-metabolomics, coupled with suitable preanalytical and statistical approaches, is sensitive enough to accurately identify the origin of milk samples even from individual farms in a limited geographic area, twenty samples from ten different farms located in the Mugello valley (about 1100 $Km^2$) were collected in Autumn 2013 and Spring 2014, for a total of 400 milk samples (Supplementary Fig. 1).

Using PLS-CA on the spectral data (Autumn 2013 collection), the ten farms were well-discriminated, except two of them (3 and 4) that were not distinguishable (Fig. 3A). In this case, the mean cross-validated accuracy was 82%. It later turned out that farms 3 and 4 indeed were a single farm, represented by two different company names used

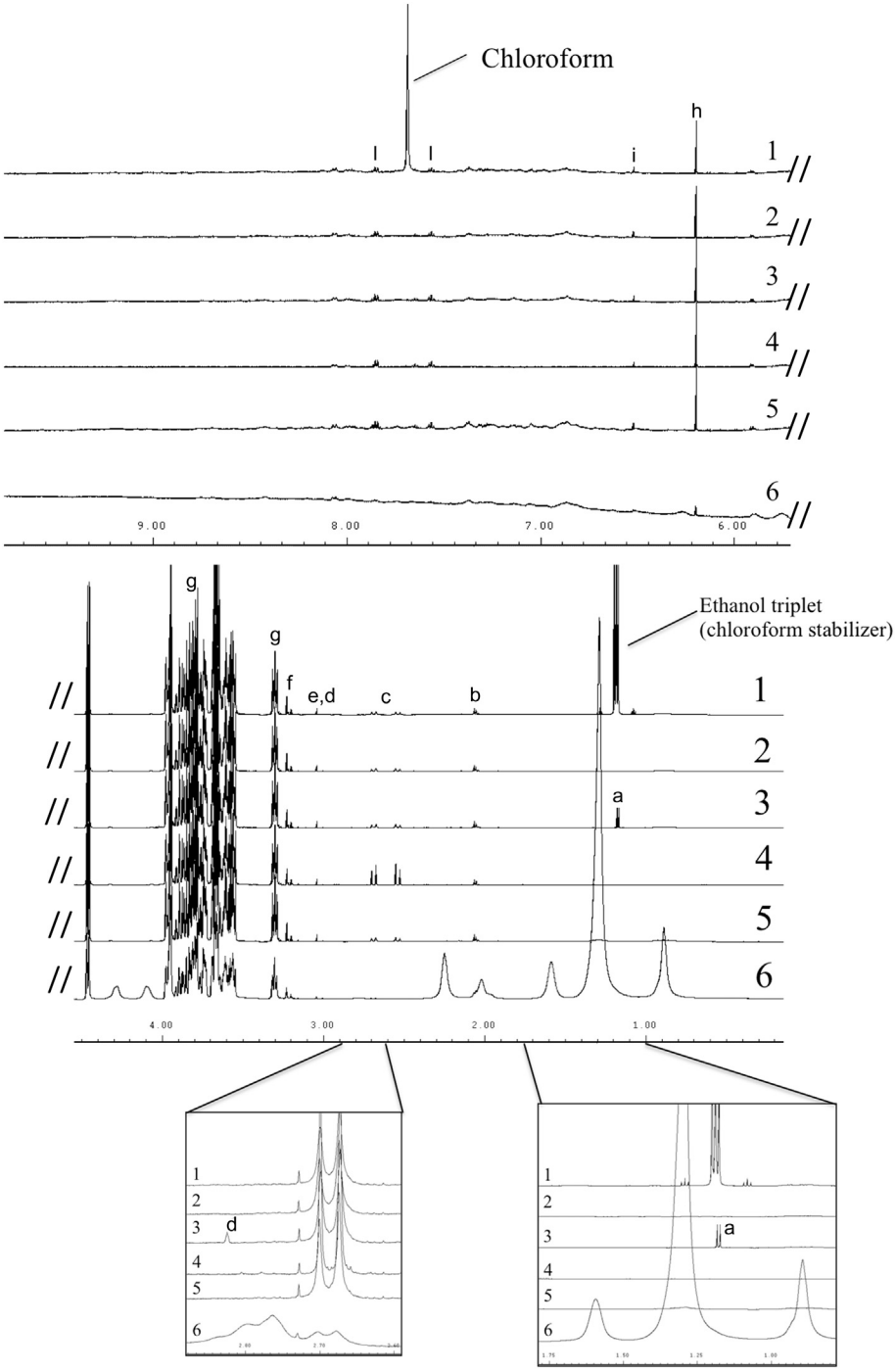


**Fig. 1.** Comparison among $^{1}$H NMR spectra obtained from the same milk sample using different extraction methods. Chloroform (1), deuterated chloroform (2), dichloromethane (3), filtration with 10 kDa cut-off (4), ultracentrifugation (5), raw milk (6). Some metabolites are highlighted: β-hydroxybutyrate (a), *N*-acetyl group (b), citrate (c), creatine (d), creatinine (e), choline (f), lactose (g), orotate (h), fumarate (i), hippurate (l).

to market different dairy products: the cows were actually the same, living in the same environment and receiving the same kind of feedings. Correctly considering these two group of samples as coming from a single farm, the cross-validated accuracy for the recognition (of nine farms) became very high (97%, see Supplementary Table 3), demonstrating that each farm is characterized by a specific metabolic fingerprint of its milk. The same situation (Fig. 3B) and similar average accuracy (97%) was found also for the 2014 collection. To test the effect of a different kind of normalization on the recognition accuracy, PQN (Dieterle et al., 2006) was also used, showing comparable results (97.5% and 96.8% of accuracy for the 2013 and 2014 collections, respectively). Total area normalization was then used in all the subsequent analyses.

The comparison between bulk raw milk samples coming from organic and non-organic farms, resulted in a very high cross-validated accuracy for the discrimination (about 99%, Supplementary Fig. 9).

### 3.4. Metabolomic fingerprinting of seasonal variations

In order to evaluate whether the pattern recognition was preserved independently of the season, all the data collected for milk from the nine farms (the two different collections) were merged together. The

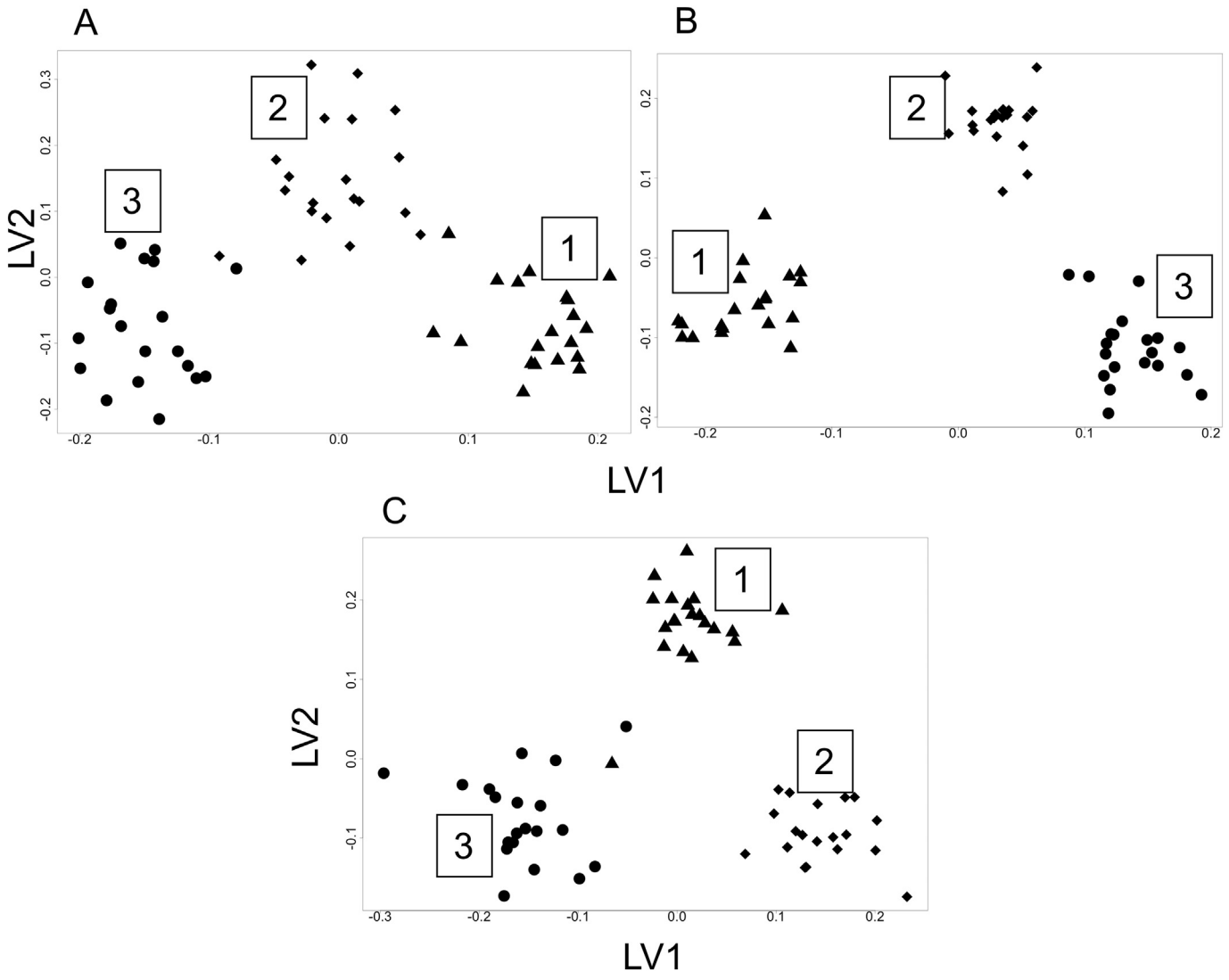


**Fig. 2.** PLS-CA plot of large scale distribution milk samples. Brand 1, triangles; brand 2, rhombus; brand 3, circles. Collection 2013, A; collection 2014, B; collection 2015, C.

cross-validated accuracy obtained for farms identification was 96% (Fig. 4A). Merging together the data for large-scale distribution milk (three different collections) the accuracy for brand identification was still 95% (Fig. 4C). These findings demonstrate that the characteristic metabolic fingerprint of milk samples exists independently of the season.

To compare the two collections of farm milk and the three collections of large-scale distribution milk, the statistical analysis described above for discrimination was repeated distinguishing samples from the same farm (or brand) originated in different collections as they were different farms (*pseudofarms*) or different brands (*pseudobrand*). In this case we obtained a total of 18 *pseudofarms* and 9 *pseudobrands*. The results of this analysis demonstrate that farm milk samples collected in the two seasons are indeed different, and the overall cross-validated accuracy is 96% (Fig. 4B). Considering the plot of the PLS-CA for pseudofarms, it is possible to observe that samples of 2014 are "shifted" to the bottom of the plot and mostly separated along the second latent variable. Instead, the first latent variable (LV1) of the same PLS-CA model tends to separate the samples according to farms. The loadings of this PLS-CA model are reported in Supplementary Fig. 10 to show which parts of the spectra contribute to the discrimination of the two different year of collection (LV2, Supplementary Fig. 10B), and which parts of the spectra mostly contribute to the farms characterization (LV1, Supplementary Fig. 10A). The two sets of discriminating variables are not completely orthogonal, meaning that at least some features are affected by both seasonality and provenance, thus making more difficult to recognise farms without considering also the season of collection. Consequently, using the samples of the first collection (Autumn 2013) as a training set to blindly predict the farms of the samples of the second collection (Spring 2014), the accuracy drops to 78.5%. Analogously, the accuracy is 87.3% for the reverse analysis (i.e. predicting the farms of the 2013 samples using the 2014 samples as training set). For large-scale distribution milk, we collected samples in three periods to have a clearer scenario of the seasonal variability. The first collection (Autumn 2013) and the second collection (Spring 2014) are well discriminated and the cross-validated accuracy is 90%. The third collection (Autumn 2014) is still different from both the collections mentioned before and this means that although the period of the year for the third collection is the same as the first, the composition of milk is different (Fig. 4D). In practice, although the recognition accuracy inside each collection is very high, seasonal variations need to be considered if the aim is to build a metabolomic method for origin assessment and traceability.

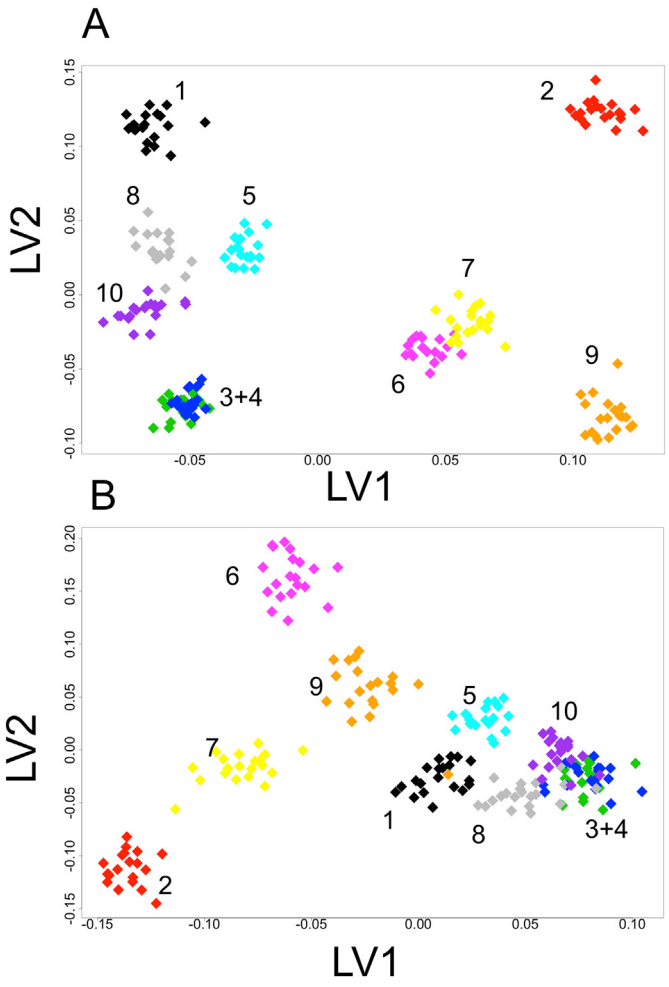


**Fig. 3.** PLS-CA plot of Mugello farms, each colour represents a distinct farm. Collection 2013, A; collection 2014, B.

### 3.5. Blind prediction of the origin of unknown samples

To evaluate the power of the metabolomic approach in the traceability of milk, we were provided with additional eight raw milk samples from the Mugello farms, collected in March 2015. We used these samples as a blind test set for assessing the predictive ability of the samples collected in 2013 and/or 2014 used as a training set. Three and four test samples were correctly assigned using, respectively, the 2013 and 2014 collections for training. Using both collection together in the training set, six out of eight test samples were unambiguously assigned to the correct farm. This latter result is quite promising, considering both the complexity of the task (identifying the correct group among ten possible choices) and the different period of the year in which these new samples were collected (late Winter) with respect to the samples included in the training set.

### 3.6. Analysis of cow feeding data

For each day of collection, raw milk samples coming from the Mugello's farms were accompanied by data about cow feeding, reported as kilograms of foods per bovine. These data are divided in eight different nutritional groups described in Materials and Methods section. By means of PCA analysis of the cow feedings data, we selected the farms presenting common nutritional profiles. Both for the 2013 and for the 2014 collection, we found three different nutritional groups (Supplementary Figs. 11A and 11B). For the 2013 collection, group 1 is composed by two farms that use silage and hays as cow feedings; group 2 is composed by four farms mostly using silage as cow feeding; group 3 is composed by four farms, and in this case hays and cereal flour constituted the major components of the feeding ration. In the 2014 collection, the feeding composition of the three groups did not change from those of 2013 and the only difference regarded group 1 that is composed by two more farms previously belonging to the group 3 of 2013 (farm nr 2 and 7, Fig. 5B). The features of the three groups with their composition in terms of feedings are reported in Supplementary Table 4 and bar-plots of the composition of the three groups in terms of foods are reported in Supplementary Figs. 12 and 13.

In order to evaluate the influence of cow feedings on the metabolic fingerprint of milk, we considered the discriminant plot obtained through PLS-CA and we checked the distribution of the three nutritional groups into this plot. It appears that the metabolic fingerprint of milk reflects cow feeding both in the 2013 and the 2014 collections. The collection of 2013, as shown in Fig. 5A, is characterized by three distinct groups corresponding to the kind of nutrients given: in particular is clearly visible the separation from farms that use silage (groups 1 and 2) and farms not using silage (groups 3). The same finding is true for the 2014 collection (Fig. 5B), where quite good separation of the three groups is maintained and the separation of farms that use silage (groups 1 and 2) from farms not using silage is mainly visible along the second latent variable (LV2). Farms 2 and 7 are more shifted towards group 3 along the LV1. These are the two farms belonged to group 3 in the 2013 collection, moving to silage feed, are now belonging to group 1 (collection 2014, Fig. 5B). Finally, the PLS-CA model built to distinguish farms using silage from farms not using silage revealed 98.8% predictive accuracy in discriminating the two kinds of feed (Supplementary Figs. 14 and 15).

### 3.7. Effects of silages on the metabolic profile of milk

A total of 19 metabolites were assigned in milk spectra. The variations in metabolites content have been analysed in order to point out differences between farms using or not using silages. Both collections show some metabolites that are statistically different between the two categories: for example the farms that did not use silages in the collection of 2013 present a weak but significant increase (positive $Log_2$ FC values in Fig. 6A) of 2-oxoglutarate, choline, methionine, hippurate, acetone, alanine and glutamate, and statistically significant lower levels of acetate, citrate, *N*-acetyl carbohydrates, creatinine, creatine, lactate and very low levels of an unknown metabolite (< 2.5-fold) if compared with the farms that use silages (negative $Log_2$ FC values in Fig. 6A). The unknown metabolite presents the same trend also in the 2014 collection: in this case, samples from the two farms that did not use silages present a 3-fold reduction of this metabolite. This unknown peak could belong to a trimethylamine group. However, this signal cannot be unambiguously assigned to any corresponding molecule. A good candidate could be lecithin, considering the intense signal resonating at 1.28 ppm (δH), 30 ppm (δC) that could be attributed to the methylene groups of the acyl chains (Supplementary Fig. 6). Less marked but statistically relevant common variations are also present for other metabolites in both collection (Fig. 6A and B). Six metabolites present a statistically relevant common trend: three metabolites are lower (choline, methionine, hippurate) and three are higher (the unknown, creatinine and lactate) in milk samples coming from farms were silages are used.

## 4. Discussion

The peculiar features of several dairy products are related to the quality and the origin of the milk used (Lamanna et al., 2011). In fact, milk of bovine origin is both consumed fresh and processed into a variety of dairy products (cheese, fermented milk products) and the nutritional quality and the processing capabilities are closely associated to composition and origin. In this sense, metabolomics is an ideal

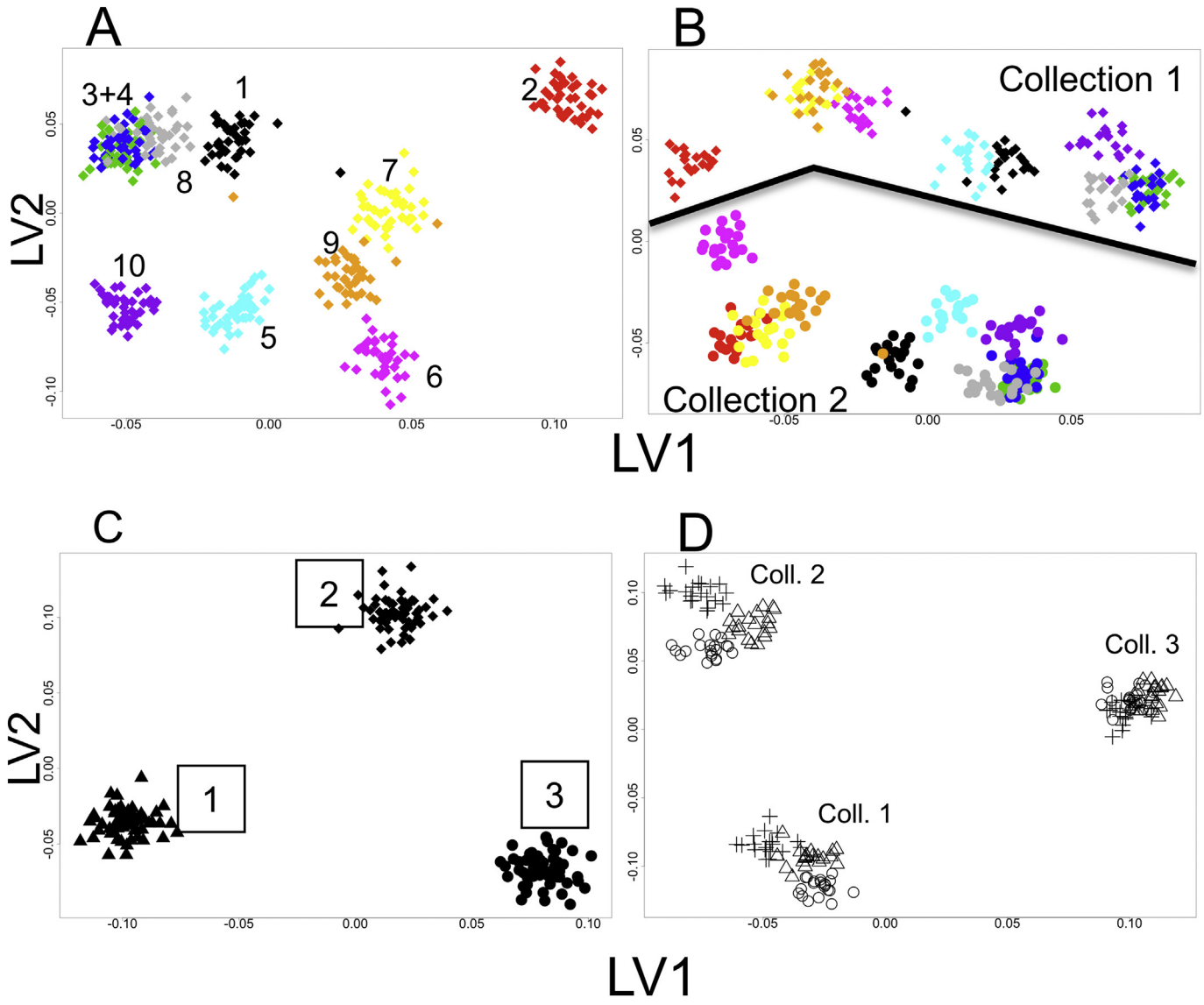


**Fig. 4.** PLS-CA plot of milk samples considering all the collections together. Discrimination among different farms, aggregating samples from the same farm originated in different collections, A; discrimination among different farms, distinguishing samples from the same farm originated in different collections as they were different farms (pseudofarms), B (diamonds: samples from Collection 1, circles: samples from Collection 2); discrimination among different brands, aggregating samples from the same brand originated in different collections, C; discrimination among different brands, distinguishing samples from the same brand originated in different collections as they were different brands (pseudobrands), D (the same symbol identify the same brand in the three collections). The separation line in panel B is manually drawn only for visualization purposes.

approach because it provides a good profiling of low-molecular-weight compounds in milk (Sundekilde, Poulsen, et al., 2013). Many applications related to the analysis of milk through NMR have been proposed, most of them focusing on linking milk metabolites content with nutritional aspects, discovery of biomarkers of cow's diseases(Enjalbert, Nicot, Bayourthe, & Moncoulon, 2001; M. S. Klein et al., 2010; Matthias S. Klein et al., 2012; Sundekilde, Poulsen, et al., 2013) and analysis of bioactive compounds(Garcia et al., 2012; Holmes, Snodgrass, & Iles, 2000).

In the present study, before considering metabolic fingerprints of different kinds of milk samples, a novel procedure for NMR-based metabolomics analysis of milk samples has been developed. Comparing different pre-analytical procedures for obtaining soluble metabolites from a heterogeneous fluid such as milk, the use of dichloromethane has given good results in terms of quality and reproducibility of the spectra. Spectra of milk samples obtained using ultracentrifugation and filtration with 10 kDa cut-off are good, especially in the case of filtration, in which no broader signals are present. Our experienceconfirm that sample preparation based on organic solvent such as chloroform or dichloromethane is a viable alternative. The quality of the spectra obtained is comparably good: the signals of metabolites are clearly visible, and no broader signals are present. Although chloroform and dichloromethane provide similar spectra, the preparation with dichloromethane appears preferable. First, dichloromethane is less toxic than chloroform, second the signals of β-hydroxybutyrate, and creatine are better visible after the extraction with $CH_2Cl_2$. The detection of these two compounds can provide important information: β-hydroxybutyrate is known to be a diagnostic biomarker for cow ketosis (Matthias S. Klein et al., 2012) and it also increases in relation to somatic cells count that is normally used as an indicator of mastitis infection (Sundekilde, Poulsen, et al., 2013). Creatine plays a key role in cellular energetics and its deficiency is associated with several neurological manifestations. Creatine is also found in milk and dairy products but in a relatively small amount. Estimating the amount of creatine can be useful to assess the nutritional value of milk.

Metabolic profiles of milk samples reflect with high accuracy the

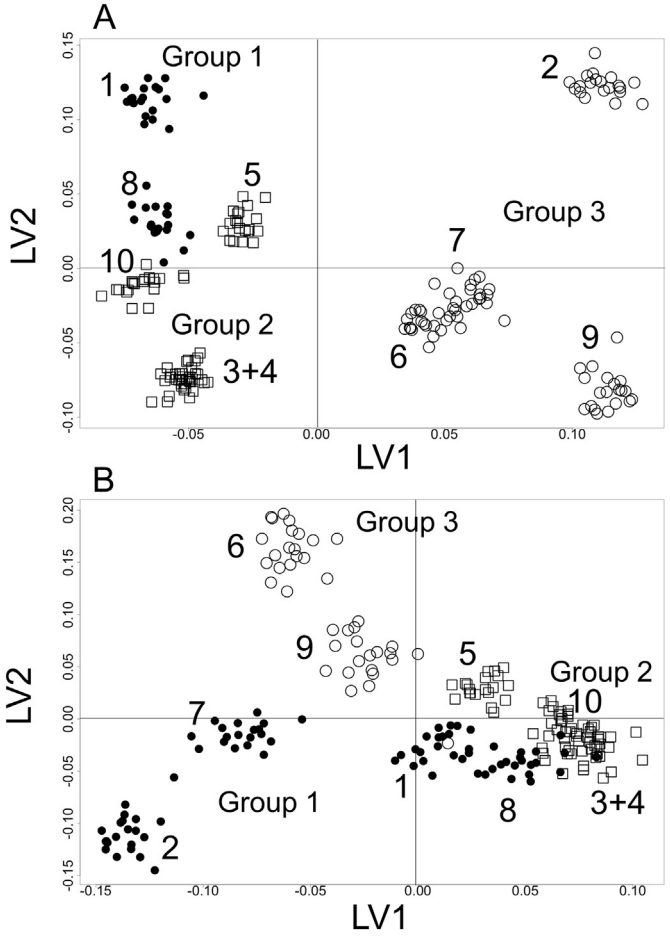


**Fig. 5.** PLS-CA plot for the discrimination among farms, highlighting the nutritional groups obtained by means of PCA on nutritional data. Black dots, group 1; white squares, group 2; white dots, group 3. Collection 2013, A; collection 2014, B.

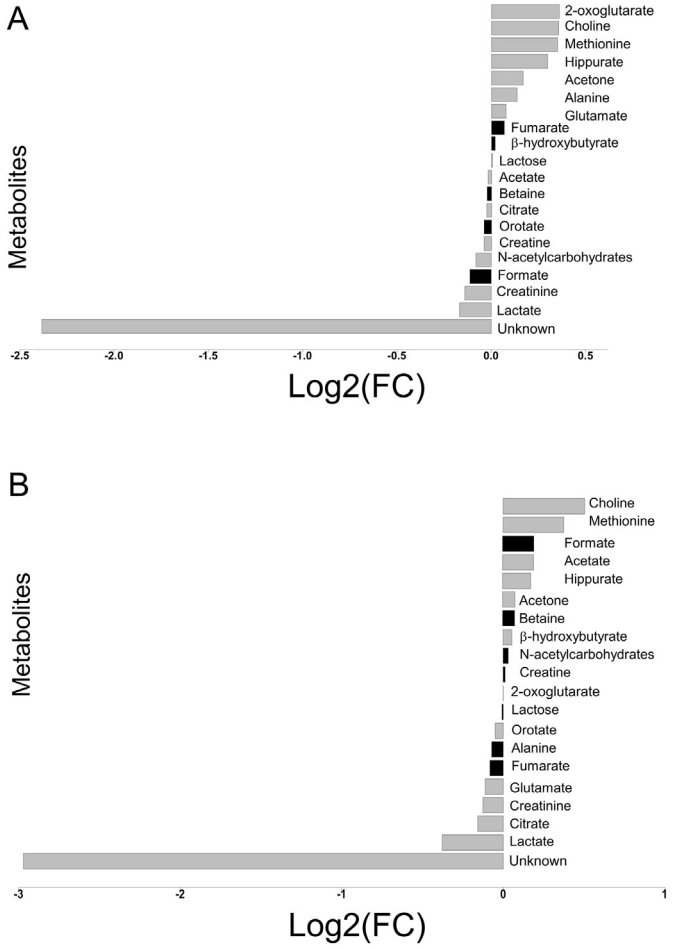


**Fig. 6.** $Log_2$(FC) analysis between farms using feeding without silages (Log2 FC positive values) vs. farms using feeding with silages (Log2 FC negative values). Grey bars are related to metabolites statistically different between the two groups ($p < 0.05$). Collection 2013, A; collection 2014, B.

farm of origin. Also considering two distinct milk collections (summer/autumn 2013 and spring 2014), the results did not change and the accuracy for the discrimination among farms is very high. Milk coming from organic and non-organic farm can also be identified, and this is likely due to the different cow feeding. Extending our approach to large-scale distribution milk it is possible to observe a clear separation between the three brands that we have considered, although in this case each brand of milk originates from a more extensive geographical area.

Milk samples collected in different periods are indeed different. Identifying the reasons of this seasonal variability is not an easy task: in different seasons several factors can change, e.g. living environment, nutrition, metabolism, etc. Our results show that considering each collection period separately, milk presents some features that allow us to correctly identify each farm (for the Mugello samples) or each brand (for the supermarket samples). For raw milk coming from Mugello farms, the distribution of the two collections in the PLS-CA plot is almost the same; however, the 2014 samples are shifted to the bottom part of the plot, demonstrating a seasonal metabolic shift. Due to this variability, the accuracy for predicting the farm of provenience in one collection, using the other as training, is suboptimal (78.5% and 87.3%). However, pooling together the two collections permits the correct prediction of 6 out of 8 “unknown” samples, demonstrating that repeated collections can average these variations, and confirming the possible role of metabolomics for the traceability of products. In any case, further analyses with a larger number of samples in different seasons are needed to fully validate this approach.

Analysis of nutritional profiles during the collection periods has been performed in order to evaluate the influence of cow nutrition on the metabolic profile of milk. Both for the 2013 and for the 2014 collections, we obtained three different groups: the composition of the three groups in terms of foods did not change drastically, except that two farms that in 2013 did not use silages started to use silages in 2014. The three nutritional groups are perfectly discriminated in the PLS-CA plot of the metabolic profiles, confirming the great influence of cow feeding in the composition of milk. In particular, the three groups are well-discriminated in both collections, even if in the 2014 PLS-CA plot two farms belonging to nutritional group 1 are shifted near group 3. We have noticed also that the most important difference between the three groups is the evident separation between farms using and not using silages. In fact, in both collections, groups 1 and 2 are composed by farms that use high quantity of silages, at variance with farms belonging to group 3 that mainly use hays. This finding is very important for the production and authentication of dairy products: for example, the production protocol of the Italian cheese “Parmigiano Reggiano” (Parmesan cheese), that is strictly regulated by the European Union, forbids the use of milk coming from bovines fed with silages (Council Regulation 510/2006) to avoid contamination of the product by clostridium bacteria that cause malformations in the wheels of cheese during maturation. Thus, analysis of metabolic profiles could be a fast and reliable tool to screen milk for the presence of silage in the animal feedings, and possibly to prevent fraud in the preparation of original cheeses. Although in the two collections it is possible to identify several common metabolites that are statistically different between farms

where silages are used or not, the most important difference is related to an unknown signal resonating at 3.11 ppm (δH), that is strongly reduced in farms where silages are not used, both in 2013 (2.5-fold reduction) and 2014 (3-fold reduction) collections.

## 5. Conclusion

The present work supports NMR-based metabolomics as a useful tool for the analysis of milk. Sample preparation is very important in order to obtain reliable results, and it should be fast and accurate in order to analyse several samples together and to obtain good quality and reproducible spectra. For this purpose, after testing protocols from the literature and comparing them with the one developed in our laboratory, we propose extraction using dichloromethane: it proved to be a fast and cheap method, and provides information about two important metabolites for the metabolic status of the cow (β-hydroxybutyrate) and nutritive value of the milk (creatine). Further, the metabolomic approach has proven to be a valid method in assessing the origin of authentic products, both considering a small geographic valley (Mugello) and a larger area (large-scale distribution). However, seasonal variations need to be considered using repeated collections, due to the changes over time of the composition of milk. This method is also able to provide information about the nutrition of the cows, opening scenarios for the monitoring of the production of dairy products (e.g. Parmesan cheese), although its potential in fraud detection needs to be further investigated. In this respect, the NMR analysis performed directly on cheese samples can be the natural continuation of the present work.

## Acknowledgments

We acknowledge the Cooperlatte s.c.a. associated farmers for providing us with the milk samples.

## Conflict of interest

The authors declare no conflicts of interest.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2018.06.066.

## References

Bittante, G., Penasa, M., & Cecchinato, A. (2012). Invited review: Genetics and modeling of milk coagulation properties. *Journal of Dairy Science, 95*(12), 6843–6870. https://doi.org/10.3168/jds.2012-5507.

Bonferroni, C. E. (1935). Il calcolo delle assicurazioni su gruppi di teste. *Studi in Onore del Professore Salvatore Ortu Carboni* (pp. 13–60). (Rome).

Brescia, M. A., Monfreda, M., Buccolieri, A., & Carrino, C. (2005). Characterisation of the geographical origin of buffalo milk and mozzarella cheese by means of analytical and spectroscopic determinations. *Food Chemistry, 89*(1), 139–147. https://doi.org/10.1016/j.foodchem.2004.02.016.

Brown-Woodman, P. D., Hayes, L. C., Huq, F., Herlihy, C., Picker, K., & Webster, W. S. (1998). In vitro assessment of the effect of halogenated hydrocarbons: Chloroform, dichloromethane, and dibromoethane on embryonic development of the rat. *Teratology, 57*(6), 321–333. https://doi.org/10.1002/(SICI)1096-9926(199806)57:6<321::AID-TERA5>3.0.CO;2-Z.

Cover, T., & Hart, P. (1967). Nearest neighbor pattern classification. *IEEE Transactions on Information Theory, 13*(1), 21–27. https://doi.org/10.1109/TIT.1967.1053964.

Dieterle, F., Ross, A., Schlotterbeck, G., & Senn, H. (2006). Probabilistic quotient normalization as robust method to account for dilution of complex biological mixtures. Application in 1H NMR metabonomics. *Analytical Chemistry, 78*(13), 4281–4290. https://doi.org/10.1021/ac051632c.

Enjalbert, F., Nicot, M. C., Bayourthe, C., & Moncoulon, R. (2001). Ketone bodies in milk and blood of dairy cows: Relationship between concentrations and utilization for detection of subclinical ketosis. *Journal of Dairy Science, 84*(3), 583–589. https://doi.org/10.3168/jds.S0022-0302(01)74511-0.

Ergon, R. (n.d.). PLS post-processing by similarity transformation (PLS + ST): A simple alternative to OPLS. Journal of Chemometrics, 19(1), 1–4. doi:https://doi.org/10.1002/cem.899.

Garcia, C., Lutz, N. W., Confort-Gouny, S., Cozzone, P. J., Armand, M., & Bernard, M. (2012). Phospholipid fingerprints of milk from different mammalians determined by 31P NMR: Towards specific interest in human health. *Food Chemistry, 135*(3), 1777–1783. https://doi.org/10.1016/j.foodchem.2012.05.111.

Ghadiri, K., Rezaei, M., Tabatabaei, S. M., Shahsavari, M., & Shahsavari, S. (2016). *Use of LDA combined with PLS for classification of lung cancer gene expression data.* 7.

Holmes, H. C., Snodgrass, G. J., & Iles, R. A. (2000). Changes in the choline content of human breast milk in the first 3 weeks after birth. *European Journal of Pediatrics, 159*(3), 198–204.

Honma, T., & Suda, M. (1997). Changes in plasma lipoproteins as toxicity markers for carbon tetrachloride, chloroform, and dichloromethane. *Industrial Health, 35*(4), 519–531. https://doi.org/10.2486/indhealth.35.519.

Kalo, P., Kemppinen, A., & Kilpeläinen, I. (1996). Determination of positional distribution of butyryl groups in milkfat triacylglycerols, triacylglycerol mixtures, and isolated positional isomers of triacylglycerols by gas chromatography and 1H nuclear magnetic resonance spectroscopy. *Lipids, 31*(3), 331–336.

Klein, M. S., Almstetter, M. F., Schlamberger, G., Nürnberger, N., Dettmer, K., Oefner, P. J., ... Gronwald, W. (2010). Nuclear magnetic resonance and mass spectrometry-based milk metabolomics in dairy cows during early and late lactation. *Journal of Dairy Science, 93*(4), 1539–1550. https://doi.org/10.3168/jds.2009-2563.

Klein, M. S., Buttchereit, N., Miemczyk, S. P., Immervoll, A.-K., Louis, C., Wiedemann, S., ... Gronwald, W. (2012). NMR metabolomic analysis of dairy cows reveals milk glycerophosphocholine to phosphocholine ratio as prognostic biomarker for risk of ketosis. *Journal of Proteome Research, 11*(2), 1373–1381. https://doi.org/10.1021/pr201017n.

Lamanna, R., Braca, A., Di Paolo, E., & Imparato, G. (2011). Identification of milk mixtures by 1H NMR profiling. *Magnetic Resonance in Chemistry, 49*, S22–S26. https://doi.org/10.1002/mrc.2807.

Leslie, R. B., Irons, L., & Chapman, D. (1969). High resolution nuclear magnetic resonance studies of alpha S-1, beta- and kappa-caseins. *Biochimica et Biophysica Acta, 188*(2), 237–246.

Lu, J., Antunes Fernandes, E., Páez Cano, A. E., Vinitwatanakhun, J., Boeren, S., van Hooijdonk, T., ... Hettinga, K. A. (2013). Changes in milk proteome and metabolome associated with dry period length, energy balance, and lactation stage in Postparturient dairy cows. *Journal of Proteome Research, 12*(7), 3288–3296. https://doi.org/10.1021/pr4001306.

Monakhova, Y. B., Kuballa, T., Leitz, J., Andlauer, C., & Lachenmeier, D. W. (2011). NMR spectroscopy as a screening tool to validate nutrition labeling of milk, lactose-free milk, and milk substitutes based on soy and grains. *Dairy Science & Technology, 92*(2), 109–120. https://doi.org/10.1007/s13594-011-0050-5.

R Core Team (2014). R: A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria (n.d.). Retrieved from http://www.R-project.org.

Sacco, D., Brescia, M. A., Sgaramella, A., Casiello, G., Buccolieri, A., Ogrinc, N., & Sacco, A. (2009). Discrimination between southern Italy and foreign milk samples using spectroscopic and analytical data. *Food Chemistry, 114*(4), 1559–1563. https://doi.org/10.1016/j.foodchem.2008.11.056.

Sundekilde, U. K., Poulsen, N. A., Larsen, L. B., & Bertram, H. C. (2013). Nuclear magnetic resonance metabonomics reveals strong association between milk metabolites and somatic cell count in bovine milk. *Journal of Dairy Science, 96*(1), 290–299. https://doi.org/10.3168/jds.2012-5819.

Sundekilde, U. K., Larsen, L. B., & Bertram, H. C. (2013). NMR-based milk metabolomics. *Metabolites, 3*(2), 204–222. https://doi.org/10.3390/metabo3020204.

Sundekilde, U. K., Frederiksen, P. D., Clausen, M. R., Larsen, L. B., & Bertram, H. C. (2011). Relationship between the metabolite profile and technological properties of bovine milk from two dairy breeds elucidated by NMR-based metabolomics. *Journal of Agricultural and Food Chemistry, 59*(13), 7360–7367. https://doi.org/10.1021/jf202057x.

Tian, H., Zheng, N., Wang, W., Cheng, J., Li, S., Zhang, Y., & Wang, J. (2016). Integrated metabolomics study of the milk of heat-stressed lactating dairy cows. *Scientific Reports, 6*. (srep24208) https://doi.org/10.1038/srep24208.

Wishart, D. S. (2008). Metabolomics: Applications to food science and nutrition research. *Trends in Food Science & Technology, 19*(9), 482–493. https://doi.org/10.1016/j.tifs.2008.03.003.

Yu, H., & MacGregor, J. F. (2004). Post processing methods (PLS–CCA): Simple alternatives to preprocessing methods (OSC–PLS). *Chemometrics and Intelligent Laboratory Systems, 73*(2), 199–205. https://doi.org/10.1016/j.chemolab.2004.04.006.