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NMR-based metabolomics of water-buffalo milk after conventional or biological feeding

Pierluigi Mazzei[*] and Alessandro Piccolo[*]

Abstract

Background: Biological farming in dairy production is often advocated as one of the most virtuous solutions to the environmental problems of conventional farming while improving the sustainability of production and cattle welfare. However, it is still under debate whether the conversion from conventional to biological farming has an influence on milk composition. In addition, the possible frauds related to biological dairy products call for analytical tools enabling the authentication of products quality and consumers protection. The aim of this work was to determine the composition of milk produced by water-buffaloes and to identify the specific metabolic profiles discriminating a biological from a conventional feeding diet.

Methods: Liquid-state ^{1}H, ^{13}C, and ^{31}P nuclear magnetic resonance (NMR) spectroscopies were used to study milk samples which were supplied during a 2-year-long experimentation by a single dairy farm and sampled from conventionally and biologically fed buffaloes (CFM and BFM, respectively). For each milk sample, we obtained NMR spectra of both raw milk and milk cream fractions comprising neutral lipids and phospholipids.

Results: The elaboration of multinuclear spectroscopic NMR results by the principal component analysis (PCA) enabled the identification of diagnostic differences in the milk composition between CFM and BFM samples. In particular, BFM were characterized by larger content of unsaturated lipids and phosphatidylcholine. Our findings confirmed that the conversion from a conventional to biological feeding regime influenced the buffalo milk composition, with possible implications for sensorial and nutritional properties of dairy products. Finally, the analytical methodology of NMR spectroscopy shown here may be considered as a useful tool to assess the quality and the authenticity of biological milk.

Keywords: Water-buffalo, Milk, Metabolomics, Multinuclear NMR spectroscopy, Principal component analysis, Biological diet

Background

Bovine milk is an essential constituent of the human diet, particularly in the Western world, being it nutrient-rich and well-balanced with respect to the nutritional values of carbohydrates, fats, and proteins [1, 2]. Milk consists in a complex mixture of metabolites including water, lactose, lipids, vitamins, minerals, and proteins, including caseins and immunoglobulins [3]. Milk metabolites derive from various cell types and originate downstream of different metabolic processes occurring in the bovine [4]. Milk is also processed into a variety of dairy products such as cream, butter, yogurt, ice cream, and cheese [5]. The sensorial and nutritional properties of dairy products depend strictly on milk composition, that, in turn, may be influenced by factors such as bovine lactation stage and age, farming methods, milk processing, and bovine diet [6–10]. The latter is particularly critical, since it is strongly involved in the metabolism of the mammalian organism and has a direct qualitative and quantitative influence on the lactation process [1, 11, 12].

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In respect to the conventional farming, the biological approach is increasing in European dairies due to a general reduced impact on the environment and an improvement of bovine welfare [13], but also to income increase of producers for the growing demand of organic milk and dairy products [14]. However, it is still under debate whether the variation from conventional to biological bovine diet influences the molecular composition of milk, and, consequently, the corresponding dairy products [10, 14, 15]. Advanced analytical techniques are thus required to identify metabolites in the complex milk matrix which can be directly attributable to a biological diet. Nuclear magnetic resonance (NMR) spectroscopy is one of the most powerful techniques to evaluate the total molecular composition of milk, since it enables the concomitant detection of lipids, organic acids, nucleotides, amino acids, and carbohydrates [16–18]. Despite its relatively low sensibility, NMR offers a number of unique advantages, including the application of different, complementary, and multidimensional experiments to reach a molecular profiling and perform both semi- and quantitative analyses [19]. In fact, it has been proven that NMR-based metabolomics enables the appraisal of both the authenticity and geographical origin of milk [14, 20–22] and assesses important milk aspects, such as quality-related technological parameters [16, 18, 23, 24], fats composition [25, 26], and nutritional quality [27–29].

While NMR applications were mainly devoted to evaluate bovine rather than buffalo milk (*Bubalus bubalis*) [25, 30], the latter is the essential component for the production of "mozzarella di bufala campana" cheese, that represents an economically valuable PDO (protected denomination of origin) dairy product of the Italian Campania region [31]. The aim of this work was then to apply multinuclear NMR-based metabolomics to differentiate buffalo milk after feeding animals with either biological or conventional fodder. In particular, liquid-state ^{1}H, ^{13}C and ^{31}P nuclear magnetic resonance (NMR) spectroscopies were employed on milk samples supplied by a single dairy farm breeding buffaloes fed by conventionally and biologically fodder (CFM and BFM, respectively) during a 2-year-long experimentation. For each milk sample, NMR spectra were obtained for both raw milk and milk cream fractions (neutral lipids and phospholipids).

Methods
Milk samples
Buffalo milk samples were supplied by the dairy "Allevamento Biologico Bufalino Di Mauro Nicola" that is located in Caserta (Italy). During a 2-year-long experimentation, milk samples were collected from the same 24 buffalo animals. The selection of these specific bovines was based on the facts that they calved in the same

period and were characterized by similar age and average productive levels. Half of these animals was fed with a conventional diet, whereas the other half was exclusively fed with a fodder mix from a controlled biological production, consisting in alfalfa hay, straw hay, and a biologic mix prepared with maize meal (40%), barley meal (17%), proteic peas (35%), and flax seeds (8%). Both types of feeding diet were modulated to be isoenergetic and isoproteic. In 2010 and 2011, milk samples from both the conventional (CFM) and biological (BFM) buffalo populations were collected once a month during the lactation period spanning from March to October. During sampling sessions, a milk volume between 150 and 200 mL was collected from each of the 24 selected bovines (12 CFM + 12 BFM), unless the selected animal was unable to produce milk due to specific reasons (i.e., relatively delayed birth, precocious end of lactation stage). In all cases, milk was machine-milked during mornings, immediately stored at 4 °C, and delivered to the NMR laboratory. Subsequently, each sample was divided into two aliquots corresponding to 5 and 95 mL, one of which was stored at − 20 °C until the NMR analysis, while the other one was immediately freeze-dried.

Samples preparation for NMR analysis
The molecular composition of raw milk (RM) was assessed for refrigerated samples, which were prepared by diluting 0.01 mL of milk with 0.99 mL of deuterated (99.8% D_2O/H_2O, Armar Chemicals) phosphate buffer (90 mM, pH 6.5). 0.05 mg of 3-(tri-methylsilyl)propionic-2,2,3,3-d4 acid (TMSPA, EurisoTop, France) was also added to the mixture as an internal standard.

The freeze-dried milk fraction was redissolved in water and centrifuged at 10,000 rpm at 4 °C for 90 min to precipitate the caseins, and concomitantly isolate the milk cream emerging on the supernatant surface. The apolar lipids (AL) were extracted from 5 g of milk cream by shaking twice in 200 mL of acetone (HPLC grade, Merck). The extracted material was then roto-evaporated to dryness and suspended in a 2:1 chloroform/acidic water solution (v/v), and the organic phase separated and again roto-evaporated until dried. Prior ^{13}C NMR analysis, AL extracts (150 μL) were diluted with 700 μL of deuterated chloroform (99.8% D, H_2O < 0.01%, Eurisotop) and added with 23.33 μL mL^{-1} of tetramethylsilane (TMS, 99.9%, Eurisotop) as an internal standard.

The phospholipid (PL) fraction was isolated from the AL extract by suspending this solid residue into 60 mL of a 2:1 chloroform/methanol solution (v/v) (HPLC grade, Merck) and then filtering the supernatant (PVDF filters, 0.45 μm polyvinylidene fluoride, Sigma-Aldrich) to which 60 mL of a Na$_4$-EDTA (0.01 M) and NaCl (0.1 M) aqueous solution was added to remove metals

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possibly affecting NMR resolution [32]. The PL-containing organic phase was separated, dried with anhydrous Na_2SO_4, filtered (PVDF filters, 0.45 μm), and roto-evaporated at 37 °C [26, 33]. Prior to ^{31}P NMR analysis, PL were redissolved in 1 mL of a 1:3.3 mixture of triethylamine (> 99%, Aldrich Chemie) and deuterated dimethylformamide (99.5% $D/D_2O + H_2O < 0.05\%$, Eurisotop) (v/v) [26], containing 76.9 mg mL^{-1} of guanidine chloride (Sigma-Aldrich). 0.75 mg mL^{-1} of triphenylphosphate (TPP, > 99%, Sigma-Aldrich) was also added as an internal standard. Each sample was centrifuged at 12,000 rpm and 6 °C for 15 m to remove possible suspensions before loading the NMR tube.

NMR spectroscopy

All NMR experiments were conducted at 25 ± 1 °C on a 400 MHz Avance magnet (Bruker Biospin, Rheinstetten, Germany), equipped with a 5 mm Bruker Broadband Inverse (BBI) probe working at the ^{13}C, ^{31}P, and ^{1}H frequencies of 100, 161, and 400 MHz, respectively.

^{1}H spectra of RM samples (84 replicates per treatment and per year) were acquired through conventional, T_2-edited, and diffusion-edited NMR experiments. The setup for the conventional pulse sequence consisted in a 2 s-long on-resonance presaturation of water signal (54–65 dB for power level attenuation), 32k data point, and 248 scans. T_2-edited spectra were achieved through the spin-echo-based CPMG (Carr–Purcell–Meiboom–Gill) pulse sequence. In particular, the total spin–spin relaxation delay $(2n\tau)$, to make the T_2-filter effective, was 1320 ms and was composed by single echo times (τ) of 3 ms. The T_2-edited acquisitions consisted in a spectral width of 12 ppm (5 kHz), 32 K time domain points, and 248 scans. Diffusion-edited spectra were achieved using a stimulated echo pulse sequence based on bipolar gradients and combined with a watergate 3–9–19 pulse train to suppress the water signal. Spectra were acquired by setting 2000 μs-long sine-shaped gradients (δ) at 32.030 G cm^{-1} and selecting a diffusion delay of 0.2 s (Δ) between encoding and decoding gradients. The acquisition implied a recycle delay of 2 s, 16k time domain points, a spectral width of 12 ppm (5 kHz), 96 scans, and 4 dummy scans. The conventional and T_2-edited spectra were Fourier transformed multiplying by a 0.1 Hz exponential function and without zero-filling, whereas diffusion-edited spectra were processed multiplying by a 0.6 Hz exponential function and applying a twofold zero-filling.

AL samples (35 replicates per treatment and per year) were examined through proton-decoupled ^{13}C spectra. Acquisitions were attained through the inverse-gated pulse sequence and consisted in 32k time domain points, a spectral width of 299.34 ppm (30,120.482 Hz), 5 s of recycle delay, 248 scans, and a carbon 45° pulse in the range 8–10 μs. ^{13}C spectra were processed by multiplying by a 2 Hz exponential function and applying a twofold zero-filling.

^{31}P NMR spectroscopy was used to evaluate PL fractions (35 replicates per treatment and per year). A decoupling scheme, based on the inverse-gated sequence, was applied to remove long-range proton-phosphorous couplings and improve spectral resolution. The setup consisted in 7 s of recycle delay, a phosphorous 45° pulse ranging within 7 and 8.5 μs, a spectral width of 250.9 ppm (40,650 Hz), 32k time domain points, and 6500 scans. ^{31}P NMR spectra were Fourier transformed by multiplying by a 3 Hz exponential function and applying a twofold zero-filling.

Structural identification of compounds detected in RM and AL samples was reached on the basis of existing literature [4, 16, 17, 21, 25] and two-dimensional (2D) NMR experiments, such as homonuclear ^{1}H–^{1}H correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), J-RES, heteronuclear ^{1}H–^{13}C heteronuclear single-quantum correlation (HSQC), and heteronuclear multiple-bond correlation (HMBC). All 2D NMR experiments were acquired with a spectral width of 16 (6410.3 Hz) and 300 (30,186.8 Hz) ppm for ^{1}H and ^{13}C nuclei, respectively, and a time domain of 2048 points (F2) and 256 experiments (F1). Homonuclear 2D spectra were based on 16 dummy scans and 64 total transients. In addition, a mixing time of 80 ms and a trim pulse length of 2500 ms were set for the TOCSY experiment. HSQC and HMBC heteronuclear experiments were acquired with 16 dummy scans, 80 total transients, and 0.5 μs of trim pulse length. The experiments were optimized by considering 145 and 6.5 Hz as the optimal ^{1}H–^{13}C short- and long-range J couplings, respectively.

For all 1D and 2D NMR spectra, the axes of ^{1}H, ^{13}C, and ^{31}P nuclei were referred to the resonance frequencies of respective internal standards (δ ^{13}C and $^{1}H = 0$ ppm for both TMS and TMSPA standards; δ $^{31}P = -17.7$ ppm for TPP standard). All spectra were processed using both Bruker Topspin software (version 2.1, Bruker Biospin, Rheinstetten, Germany) and MNOVA software (version 9.0, Mestrelab Research, Santiago de Compostela, Spain).

Multivariate analysis

All detected signals in ^{1}H, ^{13}C and ^{31}P spectra were integrated and divided by the area of the respective internal standard (TMSPA, TMS and TPP, respectively). The standardized data were Pareto-scaled prior to perform principal component analysis (PCA). One-way ANOVA test was used to examine and filter the loading-vectors (variables) mostly represented in the applied PCA axes. In all cases, only the loading-vectors of variables which

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contributed significantly (*p* values < 0.05 at a significance α of 0.05) to differentiate the conventional from biological treatments were considered and superimposed in related score-plot. Statistical elaboration was conducted by the XLStat software (version 2012, Addinsoft, Paris, France).

Results and discussion

Buffalo raw milk (RM)

NMR spectra of RM samples provided rapid qualitative and quantitative information on the most abundant milk metabolites. These samples were prepared by diluting a small volume of fresh milk in an aqueous solution buffered at pH 6.5, to minimize the effect of signal broadening due to suspended components (i.e., proteins and liposoluble compounds). Our preliminary experiments have shown that the best compromise to reach maximum NMR detectability and sample stability consisted in a dilution of 1:99 (v/v, milk/aqueous solution). A 90:10 (v/v, milk/deuterated water) dilution was previously proposed to evaluate raw milk samples through liquid-state NMR [17]. However, while the latter dilution was meant to favor the NMR detection of relatively insensitive and low naturally abundant ^{13}C nuclei [17], a greater amount of milk was not desirable in our case, because both an increased time-dependent phases separation and abundance of suspended fraction represent interferences for any reliable metabolomic analysis.

The residual water peak at 4.704 ppm was suppressed during proton NMR acquisitions of RM, thus enabling emergence of overlapped peaks and detection of low-intense signals, otherwise incompatible with the dynamic range of the digitizer. A relevant number of overcrowded signals were detected in the 7–0.5 ppm range of conventional proton spectra of RM samples (Fig. 1a) and included intense signals in the alkyl region (2.4–08 ppm), typically ascribable to lipidic components.

Filtered spectra (also referred to as edited spectra) based on specific NMR pulse sequences enabled selective suppression of signals modulated by intrinsic metabolites properties, such as molecular tumbling rate, diffusivity, hydrodynamic radius, and aggregation extent [31, 34]. T_2-edited and diffusion-edited spectra are most used in NMR-based metabolomics studies, since they are very robust, reliable, and advantageous [35, 36]. In particular, a T_2-filtered NMR acquisition eliminates signals corresponding to slow-moving molecules, including lipid compounds, which are characterized by a relatively shorter spin–spin relaxation. This is due to the inverse correlation existing between the compound dimension and its mobility, so that the larger is the molecular size of a compound, the shorter is the NMR spin–spin relaxation time (T_2) [37]. Conversely, a diffusion-based pulse sequence selectively suppresses signals of metabolites with small hydrodynamic radius and faster diffusivity, thus enhancing visibility and detectability of larger molecules.

Both diffusion-edited (Fig. 1b) and T_2-edited (Fig. 1c) NMR experiments were hence acquired to simplify and enhance readability of proton spectra of RM samples. However, as compared to the conventional spectrum (Fig. 1a), the NMR filters may lead to both a partial reduction of signal intensity and a distortion in J-coupling resolution. Nevertheless, T_2-edited spectra (Fig. 1c) allowed a clearer observation of singlets and multiplets of relatively fast-moving molecules, such as carbohydrates, organic acids, and nitrogen-containing compounds. The most intense signals in T_2-edited spectra were attributed to lactose, choline, creatine, and several organic acids, including fumarate, citrate, and isobutyrate. Remarkably, most signals within the 5.3–3.33 ppm range rose from the magnetically non-equivalent hydroxy-alkyl protons of glucopyranosyl and galactopyranosyl moieties of lactose disaccharide. Moreover, the most deshielded signals at 5.25, 4.7, and 4.48 ppm are assignable to protons bound to lactose anomeric carbons.

Diffusion-edited acquisitions isolated broad signals of slow-diffusing lipidic moieties (Fig. 1b), with short spin–spin relaxation and relatively large molecular weights. In particular, the signal at 5.37 ppm corresponds to protons bound to *sp*2-hybridized carbon atoms in unsaturated lipids, whereas the three multiplets resonating between 5.3 and 4.05 ppm correspond to methylene and methine protons of glycerol moieties (Fig. 1b). The signals at 2.08 and 2.82 are due to methylene protons geminally bound to a single unsaturated carbon (i.e., oleic acyl group) and two unsaturated carbons (i.e., linoleic and linolenic acyl groups), respectively. The 2.3 and 1.63 ppm signals may be assigned to protons bound to C2 and C3 carbons in acyl chains, whose NMR frequencies result from progressively longer distance from the deshielding carboxyl group (C1). Finally, the signals at 0.92 and 1.33 ppm derive from terminal CH_3 and superimposed methylene $-(CH_2)_n-$ protons in acyl chains, respectively (Fig. 1b).

T_2-edited and diffusion-edited NMR spectra of CFM (collected in 2010, during the first year of experimentation) and BFM (collected in 2011, during the second year of experimentation) samples were compared to evaluate the short-medium effect of the two diets on buffalo RM composition. From a qualitative standpoint, BFM samples did not show any new signal directly related to the specific diet or at least at a concentration compatible with the instrumental detection limit. To further evaluate NMR data and attempt to reveal even small diet-related semiquantitative differences, we applied the principal component analysis (PCA). This multivariate statistical procedure is an unsupervised pattern-recognition

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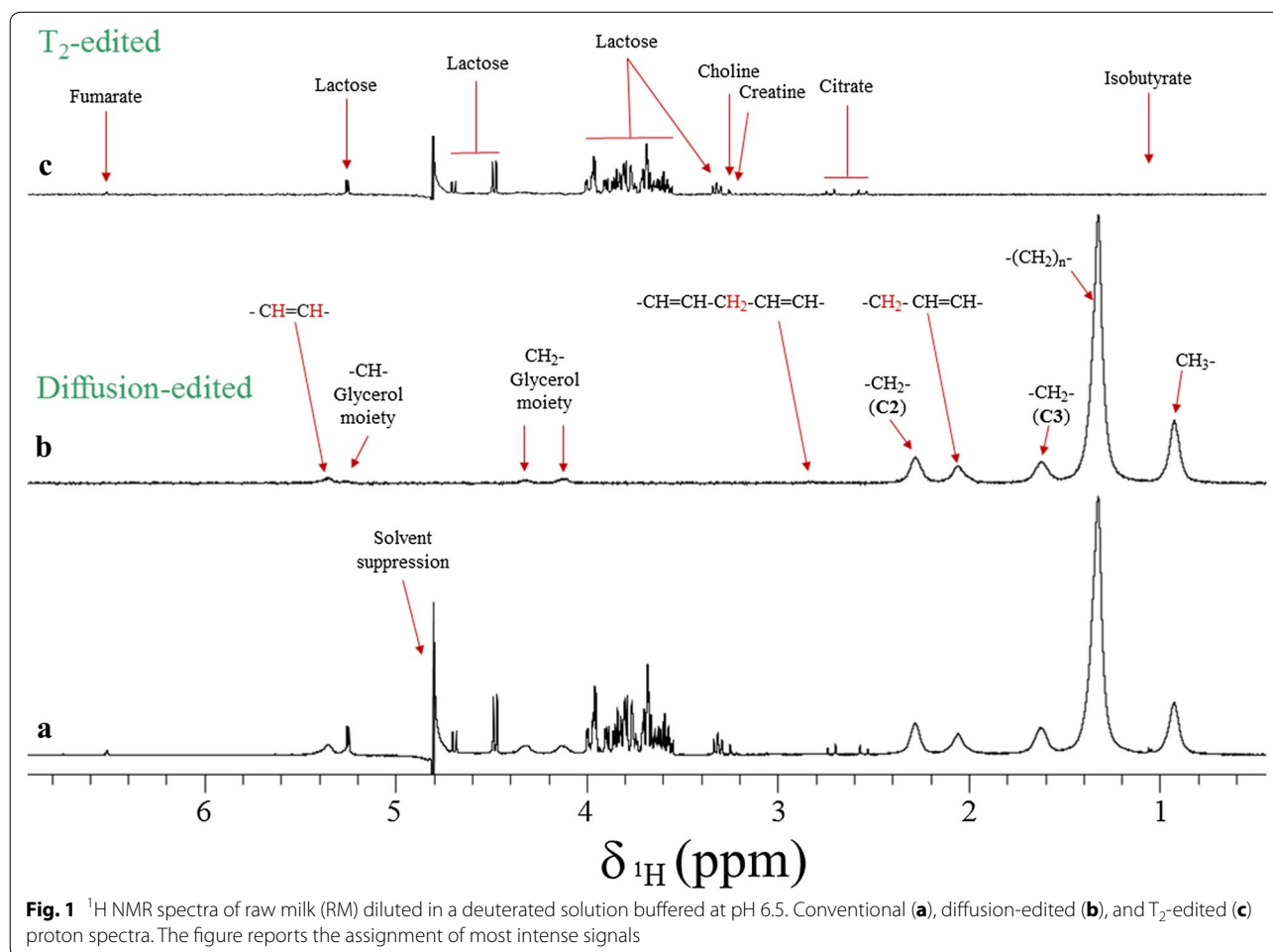
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technique that explores the intrinsic variations within different samples types [38, 39]. It offers the practical advantage to evaluate in a single output, referred to as score-plot, the response induced on the metabolome by numerous variables (described by the loading-plot).
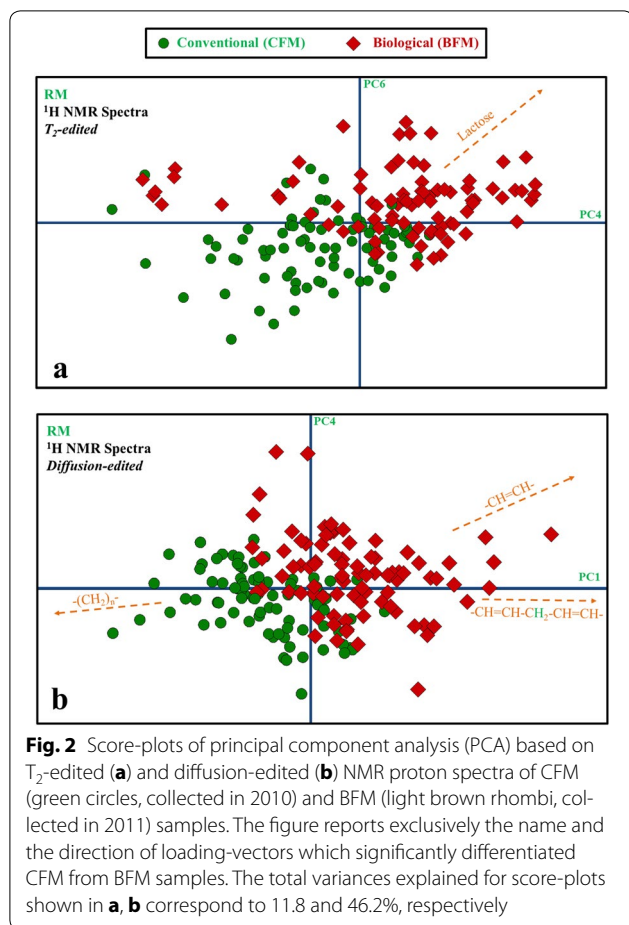
The PCA score-plots based on T_2-edited (Fig. 2a) and diffusion-edited (Fig. 2b) spectra revealed a difference in milk composition attributable to the buffaloes feeding regime. In fact, the relatively distant spread between CFM (2010) and BFM (2011) samples suggests that the biological diet systematically affected the metabolome of buffalo milk involving both small non-lipidic (Fig. 2a) and lipidic (Fig. 2b) components. It is noteworthy that replicates from the same feeding treatment were projected relatively close to each other, thereby revealing a good reproducibility of the proposed method.

The PCA based on T_2-edited spectra (Fig. 2a) showed that most of BFM samples were positioned in the direction of the first quadrant, with a correlation to the lactose loading-vectors. This indicates that BFM samples exhibited a larger abundance than CFM samples of this disaccharide after a 1-year of biological feeding regime. While

the partial superimposition of CFM and BFM replicates (Fig. 2a) also suggests that the differences in lactose content between these two types of RM samples were not so large, the ANOVA test confirmed that, though small, the differences were statistically significant (p values < 0.05 at a significance α of 0.05). Besides being the most abundant saccharide in milk (> 98%, ≈ 30% of the caloric value of the whole milk), lactose plays the important biological role of osmotic regulator during lactation, thus generally undergoing only small content variations in milk. Nevertheless, our results indicate that the biological diet contributed to enhance lactose biosynthesis in buffaloes, even though to a small extent. A possible explanation may reside in (i) an enhanced glucose uptake from blood exerted by secretory cells of mammary gland, and (ii) an up-regulation of genes involved in lactose biosynthesis [40].

The PCA score-plot of diffusion-edited spectra (Fig. 2b) again suggested a role played by the administered diet on milk composition. In fact, BFM samples were differentiated from CFM along the first and the third quadrant. On the basis of the loading-plot, the placement of BFM
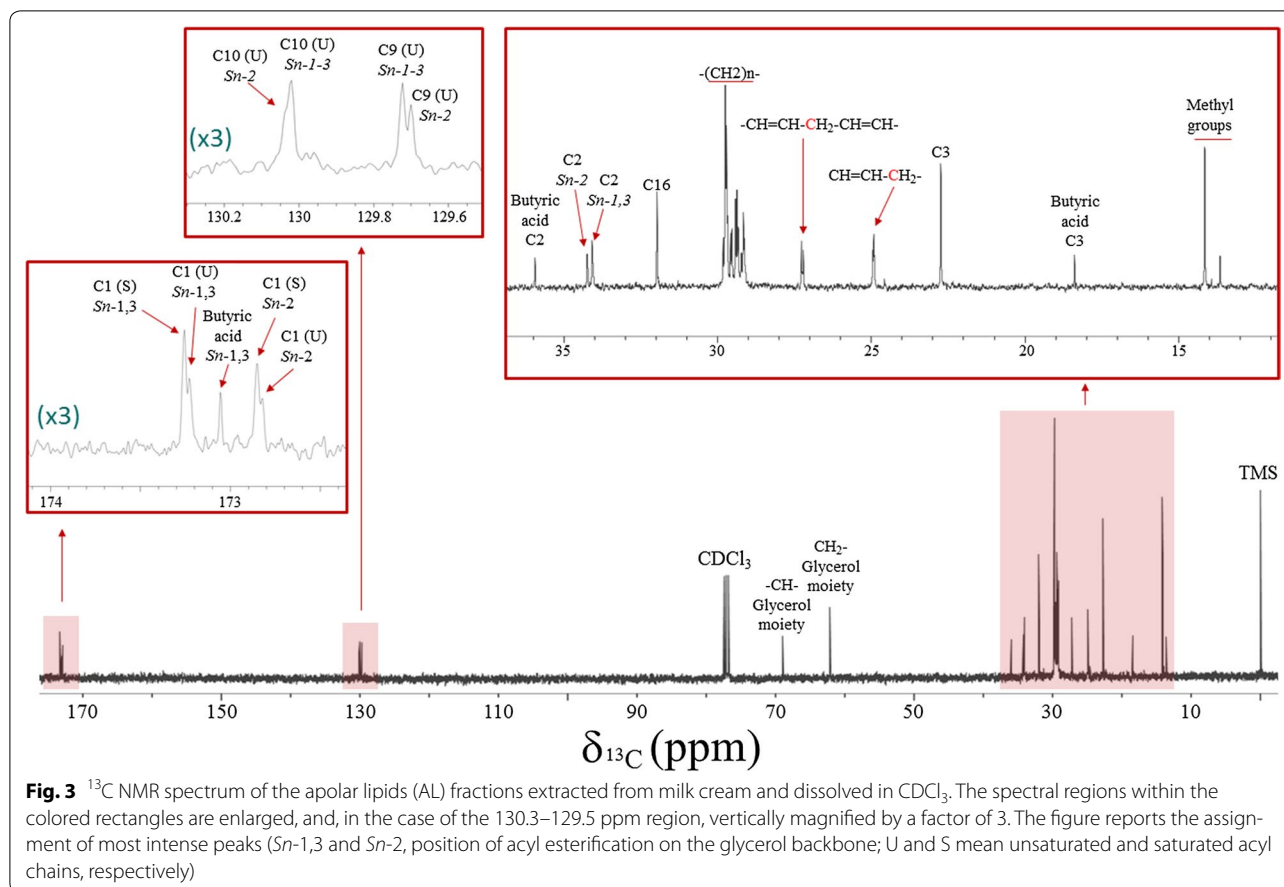

Fig. 1 ^{1}H NMR spectra of raw milk (RM) diluted in a deuterated solution buffered at pH 6.5. Conventional (**a**), diffusion-edited (**b**), and T_2-edited (**c**) proton spectra. The figure reports the assignment of most intense signals

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Fig. 2 Score-plots of principal component analysis (PCA) based on T_2-edited (**a**) and diffusion-edited (**b**) NMR proton spectra of CFM (green circles, collected in 2010) and BFM (light brown rhombi, collected in 2011) samples. The figure reports exclusively the name and the direction of loading-vectors which significantly differentiated CFM from BFM samples. The total variances explained for score-plots shown in **a**, **b** correspond to 11.8 and 46.2%, respectively

samples was due to a greater intensity of –CH=CH– and –CH=CH–CH$_2$–CH=CH– proton signals, as well as by a smaller intensity for saturated –(CH$_2$)$_n$– methylene groups. This indicates a larger abundance of mono- and polyunsaturated lipids in BFM samples. Remarkably, the behavior of these variables is consistent since the larger the abundance of unsaturated carbons, the lower the number of protons contributing to the –(CH$_2$)$_n$– methylenes signals in acyl chains. Moreover, such a dissimilarity in polyunsaturated milk components is in line with previous reports on the differentiation between biological and conventional cow milk based on the larger concentration of α linolenic acid [14, 41]. Finally, the ANOVA test confirmed again that, though to a small extent, all these variables showed a statistically significant difference (*p* values < 0.05 at a significance α of 0.05) between CFM to BFM. Interestingly, the larger content of polyunsaturated lipids also denotes an enhanced quality for biological milk, due to the beneficial properties for human health commonly recognized to these compounds.

The apolar lipids (AL) fraction in buffalo milk

Lipids are present in the milk matrix as globules emulsified in the aqueous phase. In buffalo milk, the total lipid content ranges between 8.3 and 15% [11] and about 98% is represented by triacylglycerides, whereas the rest is composed by phospholipids, diacylglycerols, monoacylglycerols, and free fatty acids [42]. In bovine animals, the long-chain fatty acids (mainly C16 and C18, saturated, monounsaturated and polyunsaturated fatty acids) in milk are of dietary origin, while short-chain fatty acids (C4–C10, mainly saturated) are biosynthesized in the mammary gland [43, 44].

The previous works showed the high potential of ^{13}C NMR spectroscopy in evaluating the fatty acids in bovine milk [25, 42, 43]. Therefore, ^{13}C NMR spectra of the AL fraction extracted from buffalo milk were acquired here and revealed more than 30 signals due to acyl chains and glycerol carbon atoms. In particular, signals common to most of triacylglycerides consisted in terminal methyls (14.5–13.3 ppm), C3 carbons (22.7 ppm), –(CH$_2$)$_n$– methylenes (30–28.8 ppm), and both methine and methylene of glycerol moiety (68 and 62 ppm, respectively) (Fig. 3). Other signals permitted to distinguish different acyl types and identify the position of acyl esterification on the glycerol backbone. The carboxyl (C1) signals resonating from 173.4 to 172.7 ppm identified the related acyl position (*Sn*-1,3 or *Sn*-2) in the glycerol backbone and differentiated between saturated (S) and unsaturated (U) acyl chains (Fig. 3). The two signals at 34.24 and 34.03 ppm indicated C2 carbons of acyl chains in *Sn*-1,3 and *Sn*-2 positions, respectively. The signals detected in the spectral region of *sp2* hybridized carbons (130.2–129.6 ppm) confirmed the presence of unsaturated acyls in either *Sn*-1,3 or *Sn*-2 positions (Fig. 3). The resonances at 24.9 and 27.2 ppm corresponded to carbons directly bound to either one or two *sp2* hybridized carbons, respectively. Finally, signals at 18.37, 35.9, and 173.1 ppm were assigned, respectively, to C3, C2, and C1 carbons of butyric acid, which is the most abundant short-chain acyl group in buffalo milk triacylglycerides [44].

The PCA score-plot based on ^{13}C NMR spectra of the AL fraction showed an almost neat separation of BFM (2011) from CFM (2010) samples along the horizontal PC2 (15.57% variance explained) (Fig. 4). The related loading-plot revealed that the variables responsible for the differentiation consisted in C9(U) and C10(U) carbons in both glycerol positions, the –CH=CH–CH$_2$–CH=CH– carbons and the (CH$_2$)$_n$ methylene signals. Excluding the latter, all variables resulted associated with positive PC2 values. The fact that most of BFM
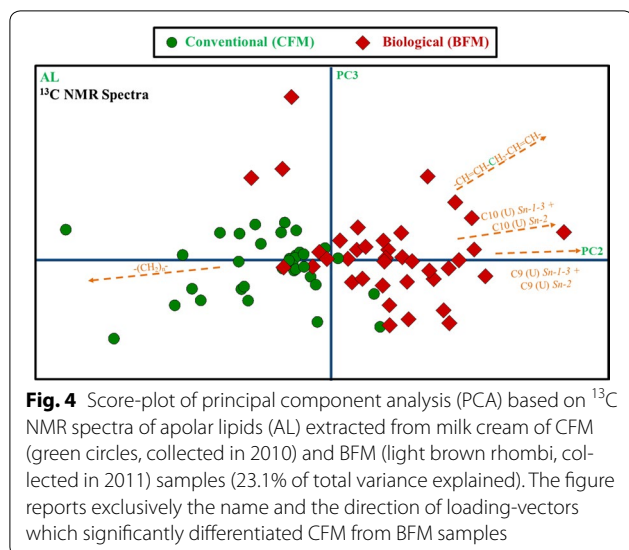
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Fig. 3 ^{13}C NMR spectrum of the apolar lipids (AL) fractions extracted from milk cream and dissolved in CDCl$_3$. The spectral regions within the colored rectangles are enlarged, and, in the case of the 130.3–129.5 ppm region, vertically magnified by a factor of 3. The figure reports the assignment of most intense peaks (*Sn*-1,3 and *Sn*-2, position of acyl esterification on the glycerol backbone; U and S mean unsaturated and saturated acyl chains, respectively)

observations were placed in the positive PC2 region suggests a larger content of mono- and polyunsaturated lipids in the milk generated by the biological diet. In agreement with diffusion-edited RM results (Fig. 2), the abundance of mono- and polyunsaturated lipids was accompanied by a lower intensity for (CH$_2$)$_n$ methylene signals. Our findings are in line with a previous work proving that a different diet in bovines may influence the amount and the composition of mono and polyunsaturated fatty acids in milk [45].

The phospholipids (PL) fraction in buffalo milk
Phosphorous (P) in milk is mainly present as inorganic orthophosphate, occurring as Ca-phosphate salt in casein micelles or in solution. The rest of P is in organic forms as phosphate monoesters, phosphodiesters and, in small amount, in nucleotides [33]. The phosphodiesters are principally represented by PL, whose important role is due to their bioactive functions and capacity to emulsify fat globules in the whole milk. More than 65% of PL in milk is composed by external bilayer of fat globule membranes that surround the lipid droplets secreted by mammary gland cells [46].

Glycerophospholipids and sphingolipids are the two main PL groups. The former consists of fatty acids esterified to glycerol backbone, a phosphate group, and a hydrophilic residue (e.g., choline, ethanolamine, serine, and inositol), while the latter differ from glycerophospholipids because presents a long-chain amino-alcohol sphingosine, rather than the glycerol backbone [46]. Sphingomyelin, that is composed of a phosphorylcholine head group linked to the ceramide, is the dominant Sphingolipid species in milk [47].

^{31}P NMR spectroscopy was applied to examine the principal phospholipids in buffalo milk (Fig. 5). Phosphatidylcholine (0.02 ppm), sphingomyelin (0.82 ppm), phosphatidylethanolamine (PE, 0.55 ppm), and phosphatidylinositol (1.05 ppm) are the most prevalent classes of bovine milk phospholipids, whereas phosphatidylserine (0.48 ppm), phosphatidylglycerol (1.2 ppm), monomethyl P.E., and P.E. plasmalogens (0.57 ppm) are present to a lesser extent [7] (Fig. 5). Signals at 0.22 and 0.18 ppm are attributed to adducts in which one or more molecules of triethylamine are linked, via hydrogen bonds, to the amine protons of the polar head of Plasmalogens and PE, respectively [26] (Fig. 5).
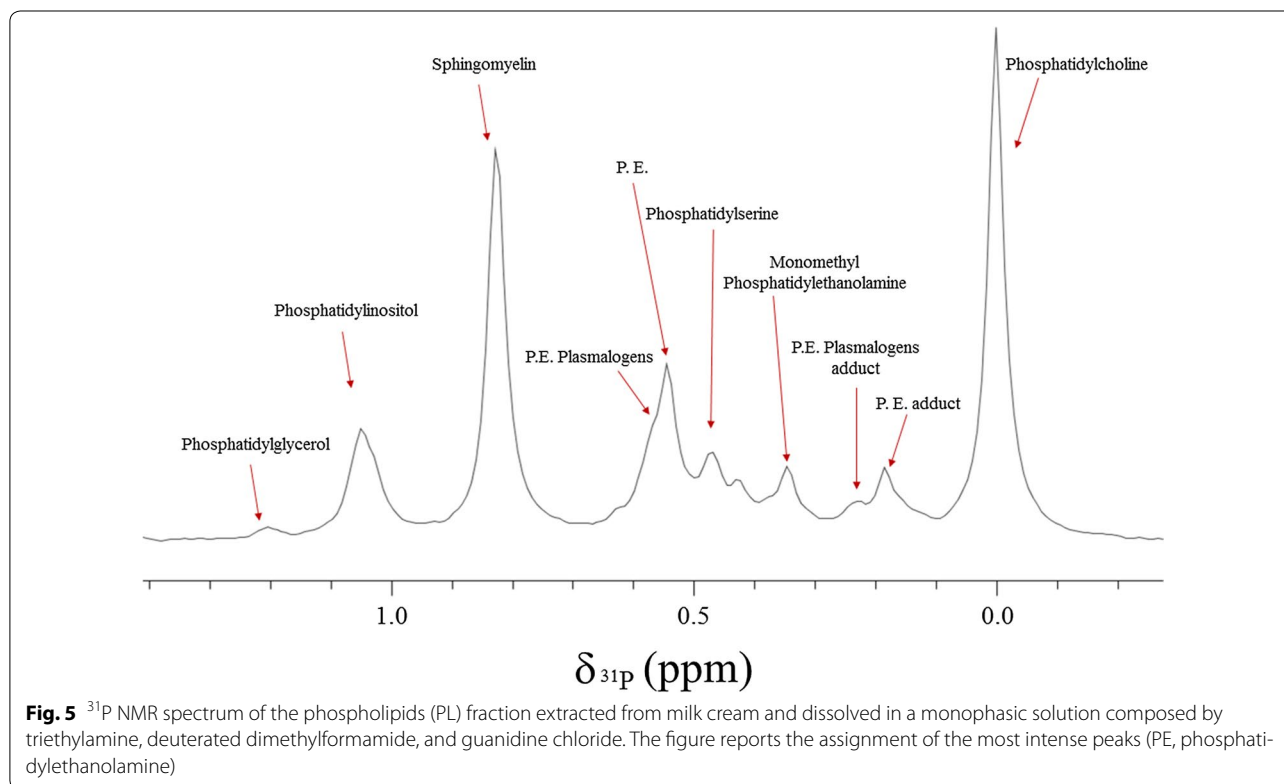
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Fig. 4 Score-plot of principal component analysis (PCA) based on ^{13}C NMR spectra of apolar lipids (AL) extracted from milk cream of CFM (green circles, collected in 2010) and BFM (light brown rhombi, collected in 2011) samples (23.1% of total variance explained). The figure reports exclusively the name and the direction of loading-vectors which significantly differentiated CFM from BFM samples

Again, the PCA score-plot revealed an almost neat separation between BFM (2011) and CFM (2010) samples (Fig. 6), whose difference, according to the loading-plot, was due to a diverse content of phosphatidylcholine and sphingomyelin. In particular, BFM showed the largest and the smallest amount of phosphatidylcholine and sphingomyelin, respectively (Fig. 6). This indicates that the diet fed to buffaloes during the 2-year-long experimentation not only influenced the composition of major metabolites in milk, but also their minor components, such as PL. Remarkably, the detection of the largest content of phosphatidylcholine in BFM is related to the quality and beneficial value of dairy products derived from biological feeding regimes, since the assumption of PL has potential implications for human health [48]. In fact, it has been shown that phosphatidylcholine mitigates inflammatory processes, such as rheumatoid arthritis [49], and ulcerative colitis [50], while reducing the risk of cardiovascular diseases [51].

Conclusions

This work revealed that liquid-state multinuclear NMR spectroscopy enables to determine the buffalo milk metabolome and concomitantly differentiate milk deriving from buffaloes fed with either a conventional or a biological diet. The T_2-edited proton spectra of RM indicated that BFM samples were characterized by a slightly larger content of lactose than CFM samples. Moreover, BFM milk showed consistently more abundant mono- and polyunsaturated lipids in both ^{1}H diffusion-edited and ^{13}C spectra. Moreover, the ^{31}P NMR spectra of PL extracts revealed that the biological diet further influenced milk biosynthesis by inducing a larger production

Fig. 5 ^{31}P NMR spectrum of the phospholipids (PL) fraction extracted from milk cream and dissolved in a monophasic solution composed by triethylamine, deuterated dimethylformamide, and guanidine chloride. The figure reports the assignment of the most intense peaks (PE, phosphatidylethanolamine)

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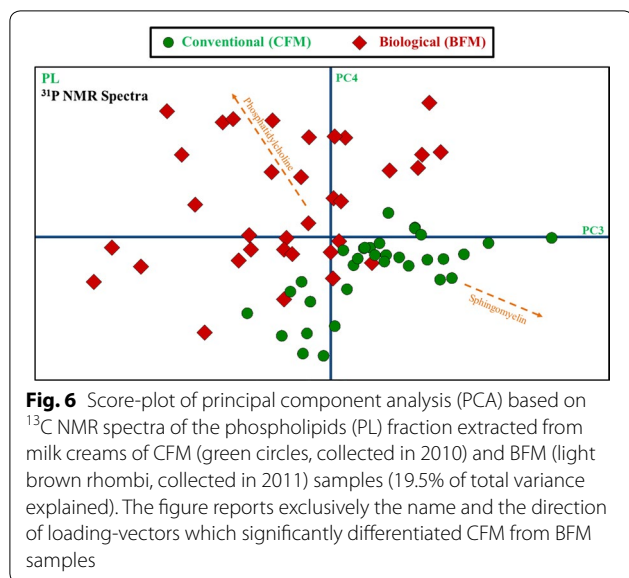
Fig. 6 Score-plot of principal component analysis (PCA) based on ^{13}C NMR spectra of the phospholipids (PL) fraction extracted from milk creams of CFM (green circles, collected in 2010) and BFM (light brown rhombi, collected in 2011) samples (19.5% of total variance explained). The figure reports exclusively the name and the direction of loading-vectors which significantly differentiated CFM from BFM samples

of phosphatidylcholine. It is noteworthy that these differences in milk composition were relatively small and resulted significant only after a 2-year-long application of the biological feeding diet. In fact, the comparison of the 2010 CFM samples with either the 2010 BFM or the 2011 CFM samples did not show any significant difference (data not shown).

Our results indicate that the conversion from a conventional to a biological feeding diet for water-buffalo animals exerts a significant influence on milk composition, but only after a short-medium period (> 1 year), especially if the feeding regimes were carefully modulated to be isoenergetic and isoproteic. Finally, our findings suggest the capacity of liquid-state multinuclear NMR spectroscopy to assess the quality of biological dairy products and its potential contribute to identify and contrast the possible frauds in the realm of biological farming.

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