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# Metabolomics and microbiological profile of Italian mozzarella cheese produced with buffalo and cow milk

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ABSTRACT

Italian buffalo mozzarella (BM) cheese metabolite profile and microbial communities were characterised and compared to cow mozzarella (CM). Polar metabolite profiles were studied by gas-chromatography mass-spectrometry (GC–MS) and results elaborated by multivariate analysis (MVA). BM produced using natural whey starter cultures (NWS) exhibited a higher microbial diversity with less psychrotrophic bacteria. BM samples were higher in threonine, serine, valine, and lower in orotic acid and urea. CM produced with commercial starters (CMS) had the highest count of *Streptococcus thermophilus* and higher levels of galactose and phenylalanine. CM obtained by direct acidification (CMA) had lower microbial counts and higher levels of urea and sugars. Orotic acid was the only metabolite linked to milk animal origin. Results indicated that this metabolite pool well reflects the different production protocols and microbial complexity of these dairy products. This approach can help to protect the designation of origin of Italian buffalo mozzarella.



## 1. Introduction

Buffalo mozzarella (BM) is a fresh 'pasta filata' cheese, produced from Italian Mediterranean buffalo (*Bubalus bubalis*) milk, which in 1996 gained a Protected Designation of Origin (PDO) recognition under European Union disciplinary, the latter describing the origin and the conditions of transformation of buffalo milk obtained from autochthonous animals bred in local farms (Commission Regulation EC # 1107/96). BM is traditionally made from raw buffalo milk by adding natural whey cultures (NWS) as starters obtained from previous day manufacturing. After reaching pH values 4.9–5.1, the curd is first placed in boiling water and manually stretched and shaped, and then in cold water to provide a first hardening, followed by immersion in a sodium chloride solution. The NWS have the main function to ensure a rapid acidification of the curd, therefore promoting the transformation of dicalcium paracasein into monocalcium paracasein during stretching in hot water (Chapman & Sharpe, 1981). The composition of these natural starters is complex, variable and may be affected by either the environmental conditions or cheese-making technology, as well as the dairy's location within the production district (Bonizzi, Feligini, Aleandri, & Enne, 2006; Coppola, Blaiotta, Ercolini, & Moschetti, 2001; Coppola, Parente, Dumontet, & La Pecerella, 1988). Lactic acid bacteria (LAB) and yeasts are the major components of NWS (Aponte, Pepe, & Blaiotta, 2010; Ercolini, De Filippis, La Storia, & Iacono, 2012; Ercolini, Moschetti, Blaiotta, & Coppola, 2001; Suzzi et al., 2000) and are regarded as a microbial community with great importance for driving the fermentation and for determining the rheological and sensory characteristics of this traditional mozzarella (Coppola, Villani, Coppola, & Parente, 1990). On the other hand, in the production of the cheaper and more widespread cow mozzarella (CM), commercial starter cultures, composed mainly of *Streptococcus thermophilus*, are used; direct acidification with citric acid is also allowed. Direct acidification has many advantages that include shorter processing time, longer shelf life, pH control, lower costs, and more standardised products (Cruz, Ocampo, & Abella, 2013).

There have been many attempts to misappropriate and unlawfully produce BM from cow milk copying and counterfeiting the Italian PDO mark of quality. In 2010, the Italian agricultural authorities found that some BM from an area south of Rome (Italy) had fallen below standards after traces of cow's milk were found; moreover, anomalous blue colouration of Mozzarella cheese contaminated with a pigment-producing strain of *Pseudomonas fluorescens* has been reported (Cenci-Goga et al., 2014).

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Many investigations have been carried out to characterise BM. They dealt with establishing methods to detect the addition of cow's milk to buffalo milk in mozzarella preparation (Addeo et al., 1995), and with characterising its microbiological profile (Coppola et al., 1988; Romano, Ricciardi, Salzano, & Suzzi, 2001; Ercolini et al., 2012). Other manuscripts focused on the study of geographical origin of mozzarella; Mauriello, Moio, Genovese, and Ercolini (2003) found that the flavouring capabilities and the microbial diversity of the NWS, used for traditional BM cheese manufacture, are closely related to the geographical origin. In another study, based on a molecular approach, Bonizzi et al. (2006) found a relationship between the genetic diversity of the BM cheese microbiota and different areas of the PDO production district. Mazzei and Piccolo (2012) demonstrated that $^{1}$H HRMAS NMR spectroscopy can rapidly characterise the metabolic profile of mozzarella cheese and, through the application of multivariate statistical data analysis (MVA), the authors were able to distinguish the different production sites of BM. By means of analytical and spectroscopic determinations, Brescia, Monfreda, Buccolieri, and Carrino (2005) accomplished the same goal.

Among different approaches, metabolomics is an effective way to characterise and to verify the authenticity of food products, being based on identification and quantification of characteristic metabolites through the application of MVA to data originating from different analytical techniques (Cevallos-Cevallos, Reyes-De-Corcuera, Etxeberria, Danyluk, & Rodrick, 2009; Marincola et al., 2012; Piras et al., 2013; Scano, Murgia, Pirisi, & Caboni, 2014). Metabolomics is able to identify a molecular fingerprint that accurately represents the food product, in all its aspects, and can discriminate from different or fraudulent varieties (Lindon, Nicholson, & Holmes, 2007). Metabolites found in food matrices can originate from the starting material, the manufacturing procedures, ageing and detrimental processes or are efficiently produced and/or consumed by the food microorganisms. Metabolites contribute to the taste, flavour and rheological properties of final food products. Moreover, among food-borne bacteria, LAB play an important role in fermented foods, where they improve the sensory profile, enhance shelf-life and microbial safety and contribute to the nutritional value and functional properties of these foods.

In this work, we applied a metabolomics approach, based on GC–MS, to characterise low-molecular-weight polar metabolites of Italian mozzarella cheese produced with buffalo and cow milk. The composition of predominant cultivable microbiota was also analysed, seeking potential correlations with the polar metabolite profiles. We also exploited the classification potential of this overall approach to discriminate these two typical Italian dairy products and, in the last instance, protect the authenticity of buffalo mozzarella PDO.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Methanol, chloroform, hexane, pyridine, methoxamine hydrochloride, potassium chloride, *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA), and all analytical standards were purchased from Sigma–Aldrich (Milano, Italy). Twice-distilled water was obtained from a Milli-Q purification system (Millipore, Milan, Italy).

### 2.2. Samples

Twenty commercial samples of BM and 18 of CM were purchased from a local retailer, all within the expiration date. Each mozzarella sample was sold in a sealed package containing the preserving liquid. Samples were classified according to their typologies, as claimed on the label: (i) $n = 20$ of BM of Campania PDO produced with whole raw milk and with the addition of NWS (BM 1–20); (ii) $n = 14$ of mozzarella cheese produced with pasteurised cow's milk and commercial starter cultures (CMS 1–14); (iii) $n = 4$ of mozzarella cheese produced with pasteurised cow's milk and citric acid (CMA 1–4).

### 2.3. Extraction and derivatization

Finely chopped mozzarella sample (1 g) was placed into a Falcon tube and 2.5 mL of methanol and 1.2 mL of chloroform were added. Samples were sonicated for 15 min to obtain the rupture of matrix micelles and then vortexed for 15 min. After 1 h, 3.8 mL of chloroform and 0.9 mL of aqueous potassium chloride (14.8 g/L) were added. The suspension was centrifuged at 32,000*g* for 10 min. Five-hundred microlitres of the aqueous layer were transferred into a glass vial and dried by a gentle nitrogen stream and derivatised with 50 μL of pyridine containing methoxamine hydrochloride at 10 mg/mL. After 17 h, 100 μL of MSTFA were added and after 1 h samples were resuspended with 800 μL of hexane.

### 2.4. GC–MS analysis

Derivatized samples (1 μL) were injected splitless into a 6850 gas chromatograph coupled with a 5973 Network mass spectrometer (Agilent Technologies, Santa Clara, CA). The injector temperature was 200 °C. The gas flow rate through the column was 1 mL/min. The fused silica capillary column was a 0.25 μm DB5-MS, 30 m × 0.25 mm ID (J&W Scientific, Folsom, CA, USA). The initial temperature program was as follows: 10 min of isothermal heating at 50 °C then increased to 300 °C at 10 °C/min and held at 300 °C for 10 min. Ions were generated at 70 eV with electron ionisation and were recorded at 1.6 scan/s over the mass range $m/z$ 50–550. GC/MS data analysis was conducted by integrating each resolved chromatogram peak. Identification of metabolites was performed using the standard NIST08 mass spectra library, a library developed at the Max Planck Institute of Golm, and, when available, by comparison with authentic standards (Scano et al., 2014).

### 2.5. Multivariate statistical data analysis and visualisation tools

For each sample, GC–MS peak intensity of metabolites was normalised to a total sum of 100. Lactate peak, due to its disproportionate intensity, was excluded from the normalisation. An ***X***-matrix composed of the analysed mozzarella cheese samples (38), and the chromatographic peak areas (51 variables), was constructed. A further 1-column ***Y***-matrix, containing sample classes, was prepared for discriminant analysis; three classes were defined depending on mozzarella cheese typologies (buffalo and cow) and the addition to cow's milk of commercial starter cultures or citric acid. Each variable in the ***X***-matrix was mean centred and unit variance scaled over all samples; when it presented skew distribution it was log-transformed and the improvement of the symmetry evaluated using the skewness test statistics as implemented in SIMCA-P+ program (Version 13.0, Umetrics, Sweden). Principal component analysis (PCA), partial least squares-discriminant analysis (PLS-DA) and its orthogonal variant (OPLS-DA) were performed with SIMCA-P software. The quality of the PLS-DA models and the optimum number of principal components were evaluated based on the cumulative parameters $R^2Y$ (classification power) and $Q^2Y$ (prediction power calculated in cross-validation), as implemented in SIMCA-P+ program. Useful parameters obtained from the PLS-DA models were the variable influence on projection