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Original Article

Analysis of the water-soluble compounds as a tool for discriminating traditional and industrial high moisture mozzarella made with citric acid

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Abstract High moisture mozzarella from cow milk is a pasta filata cheese that can be manufactured by different protocols. Fermentation by autochthonous starter is used for the traditional product, whereas direct acidification with citric acid is widely used at industrial level. Both types are stored immersed in a liquid for preserving freshness, but this packaging method is known to favour the changes of the quality characteristics. The present study aimed to assess the evolution of the soluble compounds and to evaluate their use as chemical indices for discriminating the two types of mozzarella. The contents of lactose, organic acids, water-soluble nitrogen and free amino acids differently changed over time. The whole patterns of these soluble compounds allowed discrimination of the two products during the whole storage period. The simple analyses of lactic acid and lactose could be a rapid tool to protect traditional mozzarella from industrial imitations obtained with citric acid.

Keywords Covering liquid, free amino acids, high moisture mozzarella, lactose, organic acids, sensory analysis.

Introduction

High moisture Mozzarella (HMM) is a soft 'pasta filata' cheese manufactured from water buffalo or cow milk; it is used as table cheese and is different from the low moisture type (semi-hard) that is used for topping pizza. The production technology is based on controlled acidification of the curd that determines casein demineralisation and makes it stretchable in hot water. For the product obtained from bovine milk, acidification can be done by fermentation of the curd by added starters (biological method) or by addition of citric or lactic acid to the milk (chemical method). This latter method is widely used at industrial level because is cheaper and can be managed more easily than fermentation; however, it gives rise to poor flavour (Faccia *et al.*, 2009; Natrella *et al.*, 2020a). Independently from the acidification method used, HMM is stored under refrigerated conditions packaged in plastic bags or trays containing a covering liquid, which can be pot water or a diluted brine (0.2–0.4% NaCl). The liquid has the role of preserving freshness, by avoiding dehydration of the surface and formation of the rind (Alinovi *et al.*, 2020). Unfortunately, such storage conditions determine fast deterioration of the product, since they favour the growth of spoilage microorganisms and the occurrence of mass exchange phenomenon between the product and the preserving liquid (Faccia *et al.*, 2019; Zappia *et al.*, 2020). The microbiological changes have been widely studied during the last years. Lucera *et al.* (2014), in a shelf-life study, reported that *Pseudomonas* spp. and *Enterobacteriaceae* rapidly grew in the product, approaching the levels of 7 log cfu g^{-1} and 5 log cfu g^{-1} after 7 days of refrigerated storage; no information was given about the acidification method used for preparing the samples. Ricciardi *et al.* (2015) analysed twenty samples of HMM purchased in local supermarkets the same day they were delivered from cheese making plants and analysed them immediately or after storage at 10 °C for 5 days. The samples had been produced by different acidification methods, and the authors found that the microbiological quality on the day of purchase varied greatly, and at the end of refrigerated storage, *Enterobacteriaceae* counts were highly variable, while counts of psychrotrophs and *Pseudomonas* always exceeded 10^6 cfu g^{-1}. Counts were significantly higher in samples produced by direct acidification. The role of *Pseudomonas* in

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mozzarella deterioration with particular reference to its proteolytic activity and ability of producing the 'blue discoloration' defect in this cheese was recently review by del Olmo *et al.* (2018). Differently from microbiological aspects, the chemical variations have been poorly investigated: a couple of studies have investigated the soluble metabolites in commercial samples, but have not considered their evolution during storage (Pisano *et al.*, 2016; Tirloni *et al.*, 2019); other studies have taken into consideration the changes of the volatile organic compounds over time (Ricciardi *et al.*, 2015; Natrella *et al.*, 2020b).

The chemical changes taking place during storage strongly influence the sensory characteristics of the cheese: the market operators perfectly know that the flavour tends to worsen already after a few days storage, making it difficult to distinguish between different types of mozzarella. Worsening of the flavour connected to the storage in the governing liquid has been reported in a previous paper (Faccia *et al*, 2019), but it has been poorly investigated. Discrimination of the traditional type from the industrial one is becoming an important challenge, for several reasons. First, it has been recently acknowledged at EU level as a Protected Designation of Origin cheese with the name 'Mozzarella of Gioia del Colle'; second, it is more appreciated by some consumers than the industrial type for some nutritional aspects; finally, its manufacturing cost is higher, and its replacement with the industrial type represents a form of unfair economic competition.

In a previous paper, GC-MS analysis of volatile organic compounds was proposed as a possible tool for discriminating the two types of mozzarella (Natrella *et al.*, 2020b). Unfortunately, this technique cannot be used for routine analysis, due to difficult standardisation, high variability of the analytical results, high cost of the equipment and need of highly skilled personnel (Balasubramanian & Panigrahi, 2011). The analysis of the water-soluble fraction could represent a suitable alternative, since the most important soluble compounds can be quantified by well-established analytical techniques that are less prone to variability (Mullin & Emmons, 1997; Zeppa *et al.*, 2001; BUffa *et al.*, 2004; Zaky *et al.*, 2017). In this perspective, the changes of their concentrations during storage need to be investigated. The present research aimed to assess the evolution of NaCl, lactose, organic acids, water-soluble nitrogen fraction and free amino acids during storage of high moisture mozzarella packaged in water. The study was applied to industrial (direct acidification type with citric acid) and traditional (P.D.O. Mozzarella of Gioia del Colle) products, and the possibility of using these soluble compounds as indices for their discrimination was assessed.

Materials and methods

Cheese manufacturing and sample preparation

Traditional (MGC) and industrial (DAM) high moisture mozzarella were prepared at a local dairy from pasteurised cow milk (two replicates per type were made in two different days). The manufacturing processes of the two cheeses were very similar, except for the acidification phase, as reported in a previous paper (Natrella *et al.*, 2020a). In short, the main manufacturing steps for DAM were as follows: direct acidification of the milk with citric acid; coagulation at 36 °C with calf rennet (153 I.M.C.U., 92% chymosin, 8% pepsin, Sacco srl, Cadorago, Italy) at the ratio of 0.25 mL L^{-1} milk, followed by curd cutting; settling of curd grains until curd compaction (around 15 min), followed by whey drainage; curd mincing, addition of salt (28 g kg^{-1} curd) and mechanical stretching in hot water. The main steps for MGC were those included in the official PDO production protocol: addition of natural whey starter (50 mL L^{-1} milk); coagulation at 36 °C with calf rennet (153 I.M.C.U., 92% chymosin, 8% pepsin, Sacco srl, Cadorago, Italy) at the ratio of 0.25 mL L^{-1} milk, followed by curd cutting; settling of curd grains for curd compaction followed by fermentation for about 3 h; whey drainage, curd mincing, addition of salt (28 g kg^{-1} curd) and mechanical stretching in hot water. After moulding, all cheeses (round-shaped, 150 g weight) were cooled down in chilled water, transferred in plastic trays containing pot water as covering liquid (one piece per pack, volume of water to the weight of the cheese around 1.3), thermo-sealed with a plastic laminated film and transported to the University laboratory where they were stored at 4 ± 2 °C for 2 weeks. At each sampling time (1, 4, 8 and 14 days), two cheeses from each trial were taken as analytical duplicates.

Chemical analyses

The cheese samples were subjected to the following chemical analyses (in triplicate): total nitrogen by Kjeldahl method (ISO/IDF, 2014); fat by the Van Gulik method (ISO/IDF, 2008); pH by a pHmeter equipped with a penetration probe (HANNA Instruments, Woonsocket, RI, USA); dry matter by oven drying (ISO, 2004); and NaCl by Chloride Analyser (Sherwood Scientific Ltd., Cambridge, UK). NaCl and pH were also quantified in the governing liquid. For the analysis of the soluble compounds, a water extract was prepared as follows: around 10 g minced cheese were exactly weighed and homogenised with 80 mL distilled water at 35 °C for 15 min; the suspension was then cooled down to 4 °C and centrifuged at 3000 ***g*** × 10 min; the pellet was recovered for

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electrophoretic analysis, as described later on. After elimination of fat by a spatula, the supernatant was transferred into a 100 mL flask, made up to volume and subjected to analysis of lactose, organic acids, soluble nitrogen (WSN) and free amino acids (FAA). Lactose was determined by high-performance liquid chromatography (HPLC) with refractive index detection (Agilent, Palo Alto, CA, US) as reported by Trani *et al*. (2017); organic acids were analysed by HPLC with Diode Array detection (Waters, Milford, MA, USA) as reported by Buffa *et al*. (2004). All solvents were HPLC grade purchased from Merck Chemicals (Darmstadt, Germany); Milli-Q water (Millipore Corp., Bedford, MA, USA) was used for preparing the mobile phase and samples. Pure standards of lactose and individual organic acids were supplied by Sigma (Sigma Chemical Company, St Louis, MO, USA). WSN was quantified by the Kjeldahl method; FAA were analysed at days 1, 8 and 14 by gas chromatography (MFC800 gas chromatograph, Fisons, Milan, Italy) using EZ:faast™ amino acid analysis kit (KG0-7165, Phenomenex Inc., Torrance, CA, USA) as reported by Subramanian *et al*. (2011). The pellet obtained during preparation of the water extract was used for assessing the level of casein degradation (primary proteolysis) by urea polyacrylamide gel electrophoresis (Urea-PAGE), as reported by Andrews (1983) and Faccia *et al*. (2014). The electrophoretic gels were stained with Brilliant Blue Coomassie G250 overnight, destained with double distilled water and scanned by using an Image scanner II (Amersham Biosciences, Buckinghamshire, UK). The main casein fractions were identified by comparison with the data from the scientific literature (Veloso *et al*., 2002; Faccia *et al*., 2014).

Sensory analysis

A sensory evaluation was performed to check the presence of possible connections between the profiles of the soluble compounds and the sensory characteristics of the two types of mozzarella. The analyses were performed by a panel composed of twelve trained assessors belonging to the Italian Association of Cheese Tasters (ONAF), selected following international standard (ISO, 8586-1:1993). All of them had attended a basic course for qualitative evaluation of cheese aroma, taste and texture (20 h), a professional course for quantitative evaluation of cheese attributes (25 h), and had at least a 3-year experience in mozzarella grading. The panellists evaluated the cheeses by quantitative descriptive analysis (QDA) as reported in a previous paper (Trani *et al*., 2016). The samples were presented randomly in white disposable dished marked with a three-digit code. The descriptors were quantified on a 6-point scale and were selected based on the weight percentage (frequency of citations × perceived intensity). Only descriptors with a weight percentage greater than 30% were considered (Table S2).

Statistical analysis

The analytical data were subjected to analysis of variance (ANOVA) using XLSTAT software package for Excel (Addinsoft, New York, USA). Modes were calculated for the data from sensory analysis. Multivariate statistic (Principal Component Analysis, PCA) was applied to two different data set, one including the soluble compounds (for amino acids only the total concentration was considered) and the other including the sensory attributes.

Results and discussion

Cheese manufacturing and sample preparation

Direct acidification with citric acid was chosen for making industrial mozzarella, since it is by far the most used technology in the territory involved in the production of PDO Mozzarella of Gioia del Colle. The amounts of citric acid used for milk acidification in the two DAM trials were 1.11 g (pH of milk 5.70) and 1.18 g L^{-1} (pH of milk 5.66). The amounts were established throughout the 'stretching trial', as reported in a previous paper (Faccia *et al*., 2009), and were slightly different, since the milk used in the two replicates was not the same and it is known that the level of acidification depends on several compositional variables. The study did not focus on microbiological aspects; however, the basic microbiological differences between direct acidified and traditional mozzarella are widely known. In the former, microbiota is mainly composed of adventitious microorganisms; in the latter, the primary compounds of microbiota are lactic acid bacteria (LAB) added with the starter; in addition, the overall microbial populations are much more abundant in all types of mozzarella made by lactic fermentation (Coppola *et al*., 2001; De Angelis *et al*., 2008; Guidone *et al*., 2016). The LAB species present in the autochthonous starters used in traditional mozzarella manufacturing can widely vary (de Candia *et al*., 2007), according to a huge number of variables, including the quality of raw milk and the incubation temperature used in their production, the hygienic conditions of processing, and time and temperature of cheese storage.

Gross composition and WSN quantitation

Analytical data are reported in Table S1. Moisture, fat and protein were not significantly different between the two types of mozzarella throughout the whole storage

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time, although the batch-to-batch variations in the industrial product caused higher standard deviation than the traditional one. Differently from macroconstituents, pH and WSN allowed discrimination of the samples in some phases of storage. In particular, pH discriminated industrial and traditional mozzarella until day 4 (at this time, the values were 6.02 ± 0.04 for DAM and 5.86 ± 0.03 for MGC), and then, the differences became not statistically significant. During storage, the pH value increased, and that of the governing liquid decreased, until day 8 in MGC; differently, pH continuously decreased in DAM, both in the cheese and governing liquid. These trends indicated the occurrence of mass exchange phenomena for reaching the equilibrium, in conjunction with acidification; the mass exchange was particularly evident in MGC, since the initial values in cheese and liquid were very different. The equilibrium was approached at the end of the storage period. WSN was more effective than pH in discriminating the samples, since the concentration was significantly higher in MGC at days 1, 4 and 14, with a final level of 0.29 ± 0.00 versus 0.22 ± 0.05 g $100\ g^{-1}$ cheese. This result suggested a more intense proteolysis in this type of cheese, in connection with the presence of the autochthonous starter microflora, as reported by De Angelis *et al.* (2008). In MGC cheese, the evolution of the WSN concentration followed a sort of U-pattern, with initial decrease and final increase, as a result of the balance between the amounts transferred into the governing liquid and those newly formed by proteolysis. Differently, the variations in DAM over time were not significant, suggesting that new WSN formation progressively compensated the loss into the liquid. Such a sort of 'multifactorial evolution' and the high standard deviation observed in DAM samples suggest the need of further investigation before proposing WSN as a discrimination index, also because the quantities formed are very low. A further contribution to understand the proteolytic events was supplied by the urea-PAGE analysis (Figure 1). In general, a more intense αS-1 and β-casein degradation was found in the traditional product, but the patterns of the two trials evidenced some differences, suggesting that primary proteolysis proceeded according to different kinetics. In fact, in trial A casein degradation was faster in MGC until day 8, but the bands of both αS1- and β-casein were thinner at the end of storage in DAM: something happened that accelerated proteolysis in this sample after day 8. Differently, in trial B proteolysis proceeded faster in MGC throughout the entire period of storage. Further information derived from the observation of the band corresponding to the primary product of αs1-casein hydrolysis (αS1-I-casein 24-199 fragment), which is released by the activity of the residual rennet entrapped into the curd (Mc Sweeney, 2004). The formation of this polypeptide was faster in DAM, and it was much more evident in trial B. It has been reported that its formation in mozzarella starts during coagulation and continues in the curd until stretching, when the rennet enzymes are more or less denatured (Faccia *et al.*, 2014). Of course, the rate of formation is closely connected to the extent of rennet retention during cheesemaking: according to Bansal *et al.* (2007), the retention of chymosin is favoured when pH of milk at rennet addition is below pH 6.1 and the pH at whey drainage is below pH 5.7. Such values match with those applied in direct-acidified mozzarella and can explain the differences observed between the cheeses. The reason why the αS1-I-casein band was more intense in trial B remains unclear and is hard to make an hypothesis: it could depend on different characteristics of the raw matter used, or by activity of adventitious microflora of the cheese. In fact, it is known that HMM obtained by direct acidification is a more favourable substrate for the growth of adventitious proteolytic microorganisms, such as *Pseudomonas* and yeasts, than the cheese obtained by biological acidification (Baruzzi *et al.*, 2012; Ricciardi *et al.*, 2015; Guidone *et al.*, 2016). However, the connection between primary proteolysis and WSN formation is not that straightforward, since the latter mostly derives from secondary proteolysis whose main agents are LAB (Mc Sweeney, 2004).

Soluble compounds

Figure 2 shows the evolution of the NaCl contents in cheese and governing liquid. The concentration at day 1 was slight higher in DAM than in MGC, as well as in the corresponding liquid. In the successive days, the values deeply changed since salt was highly involved in diffusive phenomena, leading the concentration in the two matrices to be similar at day 4. The equilibrium of NaCl was reached much more rapidly than pH, because this latter is a dynamic parameter influenced by both the microbial activities and mass exchange phenomena. At this time, the difference between traditional and industrial samples was still significant. After 8 days, the salt concentration in the MGC liquid exceeded that in the corresponding cheese and approached that in the DAM liquid, whereas the levels in the two products remained different. At day 14, the cheeses reached the same NaCl content, as well as the two governing liquids; these results indicated that mass exchange continued throughout the whole storage time. Despite of the significant differences observed until day 8, the NaCl content cannot be proposed as a discrimination index of traditional and industrial products, since it is not a specific technological variable. In fact, salt is added during the curd-stretching phase, at

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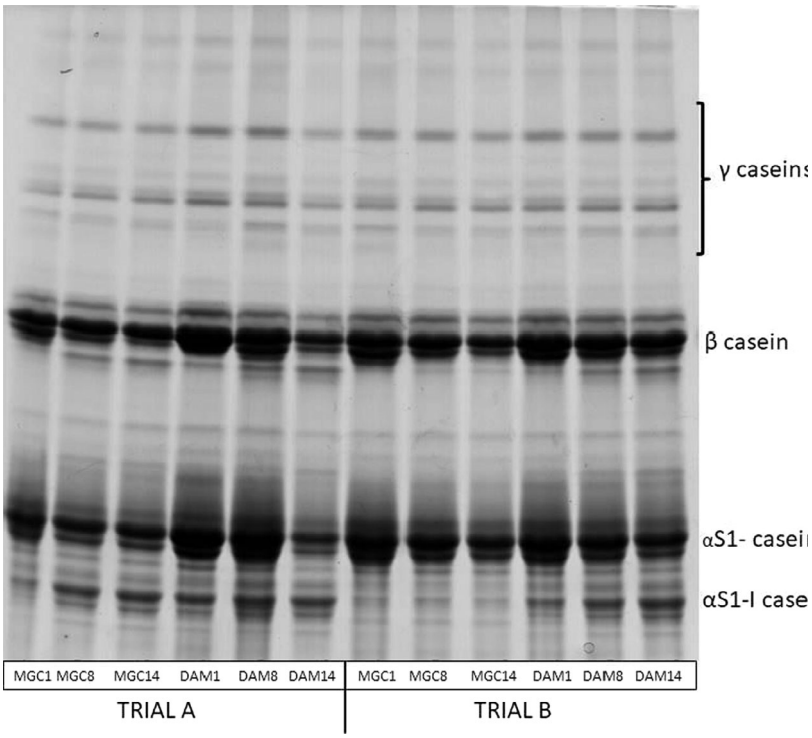

Figure 1 Urea-PAGE electrophoresis of traditional (MGC) and industrial (DAM) high moisture mozzarella during refrigerated storage (1, 8 and 14 days)

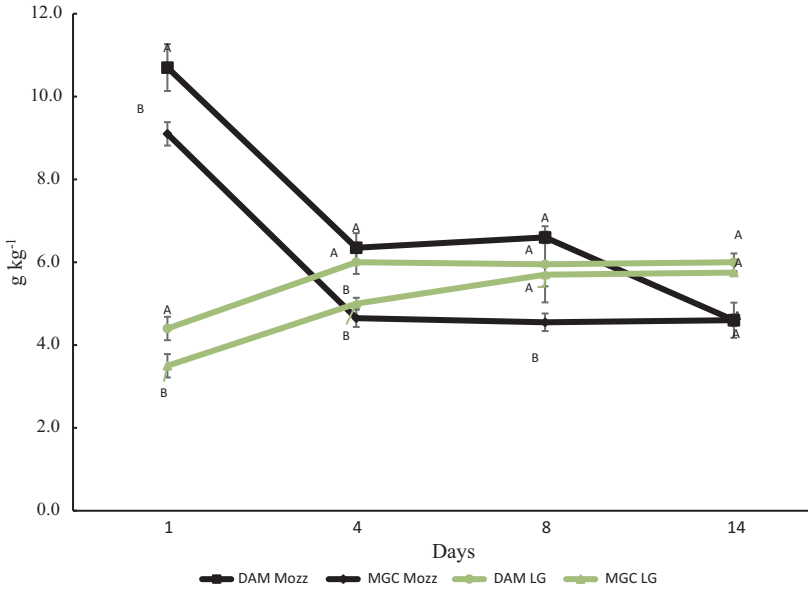

Figure 2 Changes of the NaCl content in traditional (MGC) and industrial (DAM) high moisture mozzarella and corresponding governing liquid during refrigerated storage. Letters indicate significant difference at $P < 0.05$

a dosage that depends on the level of savouriness required (Faccia *et al.*, 2012); therefore, it can widely vary from product to product.

As for lactose, the absence of the fermentation phase in DAM determined an almost double content with respect to MGC at the beginning of storage (11.6 ± 0.85 vs 5.8 ± 0.64 g kg^{-1}). This finding agrees with the results of Pisano *et al.* (2016), who reported a higher level of total sugars in 4 commercial samples of cow mozzarella obtained by direct acidification method with respect to 14 samples manufactured with selected starter. Unfortunately, they did not considered

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traditional mozzarella nor analysed the samples during storage. In the present study, the concentration of the disaccharides rapidly decreased with time in both DAM and MGC, disappearing at day 8 in the traditional product and reaching the minimum value of 3.9 ± 0.78 g kg^{-1} in the industrial one after 14 days. Both microbial degradation and transfer into the governing liquid should have contributed to the decrease, and the difference between the two products remained statistically significant throughout the entire storage time. The only exception was the DAM value at day 14, which was included within the range of the values observed in MGC in the interval 1–4 days. Table 1 shows profile of the organic acids. As expected, lactic was the most represented acid in MGC, whereas citric prevailed in DAM; acetic acid was a minor compound. The higher lactic acid content in the traditional product matched with the lower lactose content and was expected, since it is greatly produced by starter fermentation into the curd, which is absent in direct-acidified mozzarella. It decreased in both cheeses at day 4 probably due to mass exchange with the liquid, and then, it started to increase until the end of the storage, indicating that the amount formed by the microbial activity exceeded that lost into the liquid. On the other hand, citric acid continuously decreased, since it is metabolised very rapidly in dairy products by lactic acid bacteria, such as *Lactococcus lactis* subsp. *diacetylactis* and *Leuconostoc* spp. (Izco *et al.*, 2002) and other microorganisms that can be present such as yeasts (Ferreira & Viljoen, 2003) and *Enterobacteriaceae* (Chaves-López *et al.*, 2006). Overall, both acids were able to discriminate the two products at days 1 and 4, but only lactic acid continued to do it in the successive days. These findings partially matched with the results reported by Tirloni *et al.* (2019) in a study on thirty-three mozzarella samples of different brands, which reported that in most of the cases lactic acid was able to discriminate between products obtained by direct acidification or by selected starter fermentation, whereas citric acid was not. Unfortunately, the study did not considered traditional mozzarella and was only performed on samples obtained on the day of production. Finally, acetic acid was not able to discriminate the cheeses, except at day 14. As said before, it is a secondary compound that mostly derive from secondary fermentations (such as heterolactic), which became relevant only at the end of the storage time. It is likely that the natural whey starter also contained some heterolactic species, as reported in the literature (de Candia *et al.*, 2007). Considering the low levels observed, further study is needed to understand whether this compound could be useful for cheese discrimination.

Table 1 Organic acids (mg kg^{-1}) in traditional (MGC) and industrial (DAM) high moisture mozzarella (average values of the two cheesemaking trials) packaged in water during refrigerated storage

Day	Sample	Lactic acid	Citric acid	Acetic acid
1	DAM	9.4 (±5.4)^{e}	578.1 (±32)^{a}	2.0 (±0.7)^{c}
	MGC	1,559.2 (±178.3)^{ab}	416.5 (±23.3)^{b}	20.3 (±5.5)^{b}
4	DAM	3.3 (±0.7)^{e}	313.3 (±26.9)^{c}	nd
	MGC	1,166.3 (±150.7)^{c}	166.7 (±34.6)^{d}	12.1 (±3.4)^{b}
8	DAM	77.2 (±26.05)^{d}	114.0 (±68.1)^{de}	nd
	MGC	1,414.0 (±99.1)^{bc}	41.0 (±5.7)^{ef}	24.2 (±8.8)^{b}
14	DAM	122.0 (±20.2)^{d}	54.2 (±7.6)^{ef}	15.0 (±5.6)^{b}
	MGC	1,878.2 (±142.1)^{a}	nd	82.4 (±24)^{a}

For each acid, values bearing different superscripts are different at $P < 0.05$. nd, not detected (<0.05 mg kg^{-1})

Finally, the FAA were quantified in the water-soluble extract (Table 2). As expected, the total concentration increased with time, but the values in the two types of mozzarella were strongly different. Higher amounts were detected in MGC at all sampling time, which should be connected to higher peptidasic activity of the autochthonous microbiota (De Angelis *et al.*, 2008). A few compounds did not accumulated over time: proline continuously decreased, phenylalanine, leucine and lysine increased during the first week then evidenced a decrease or remained constant, glycine varied only slightly. The different behaviours of the single amino acids should be the result of several concurrent events: the specificity of the strains growing in the cheese to release them by peptidasic activities; the solubility in water of the single compounds in connection with their isoelectric point and with the evolution of pH in cheese; their microbial catabolism. Overall, the aminoacidic patterns of the two mozzarella types evidenced some differences. Glutamic acid, isoleucine, valine, threonine, serine, alanine and proline were the most represented compounds after 14 days of storage in the traditional product, and their sum accounted for about 54% of total. On the other hand, the most abundant amino acids in the direct-acidified product at day 14 were glutamic acid, isoleucine, valine, threonine, alanine, phenylalanine and asparagine, whose sum corresponded to about 74% of total. By calculating the ratio between WSN and FAA, it was found that FAA represented 19.95% and 12.31% of WSN in MGC and DAM, respectively, at day 1 and 34.23% and 18.39%, respectively, at day 14. Such differences seem sufficiently relevant for considering FAA as suitable indices of discrimination, but, as reported for WSN, it must be validated on a large number of samples; they should also include samples obtained from raw milk, in which the contribution of the milk indigenous microbiota could be a further source of variability of the proteolysis rate.

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Table 2 Free amino acids (mg kg^{-1}, average values of the two cheesemaking trials) in traditional (MGC) and industrial (DAM) high moisture mozzarella packaged in water during refrigerated storage

	1		8		14	
Compound	**MGC**	**DAM**	**MGC**	**DAM**	**MGC**	**DAM**
ala	53.9 ± 6.2^{b}	18.2 ± 1.9^{e}	53.6 ± 7.1^{b}	21.2 ± 5.0^{d}	69.1 ± 4.4^{a}	30.1 ± 2.9^{c}
gly	8.8 ± 0.5^{a}	nd	7.2 ± 0.0^{c}	3.6 ± 0.2^{e}	7.8 ± 0.2^{b}	4.4 ± 0.4^{d}
val	71.5 ± 4.4^{c}	39.3 ± 1.7^{f}	81.1 ± 3.9^{b}	43.5 ± 2.0^{e}	96.4 ± 8.6^{a}	50.5 ± 4.2^{d}
leu	33.1 ± 1.8^{a}	18.4 ± 1.0^{d}	25.6 ± 0.7^{b}	16.7 ± 0.9^{d}	23.5 ± 0.6^{c}	17.2 ± 1.2^{d}
ile	75.6 ± 6.3^{c}	27.7 ± 1.2^{f}	103.6 ± 8.9^{b}	32.2 ± 3.1^{e}	128.7 ± 10.7^{a}	60.9 ± 2.8^{d}
thr	70.8 ± 2.1^{c}	19.2 ± 1.5^{f}	77.7 ± 3.9^{b}	28.6 ± 0.9^{e}	109.7 ± 12.5^{a}	42.4 ± 4.5^{d}
ser	50.1 ± 2.2^{c}	5.4 ± 0.0^{f}	54.5 ± 2.2^{b}	6.4 ± 1.0^{e}	71.0 ± 4.1^{a}	11.4 ± 0.8^{d}
pro	111.4 ± 8.3^{a}	27.1 ± 1.1^{d}	86.9 ± 10.1^{b}	23.3 ± 2.0^{e}	66.0 ± 4.4^{c}	17.7 ± 0.9^{f}
asn	10.2 ± 0.8^{e}	7.6 ± 1.8^{f}	27.4 ± 3.0^{b}	15.4 ± 1.0^{d}	48.2 ± 2.4^{a}	20.3 ± 1.9^{c}
asp	28.6 ± 1.3^{b}	15.7 ± 0.5^{d}	27.6 ± 1.7^{b}	7.2 ± 0.3^{e}	55.9 ± 4.0^{a}	16.3 ± 0.9^{d}
met	8.7 ± 0.0^{d}	nd	9.5 ± 0.3^{c}	4.4 ± 0.1^{e}	52.7 ± 3.8^{a}	12.5 ± 0.9^{b}
glu	79.6 ± 2.9^{b}	46.3 ± 1.5^{e}	73.9 ± 0.8^{c}	43.5 ± 0.9^{f}	129.3 ± 4.2^{a}	69.4 ± 1.7^{d}
phe	14.1 ± 1.3^{d}	9.5 ± 0.3^{f}	75.0 ± 6.0^{a}	11.6 ± 0.8^{e}	62.9 ± 4.1^{b}	28.9 ± 2.0^{c}
gln	39.2 ± 2.3^{c}	11.6 ± 0.6^{d}	48.6 ± 2.1^{b}	5.9 ± 0.3^{e}	55.7 ± 2.9^{a}	11.8±.8.1^{d}
lys	22.6 ± 1.0^{b}	7.8 ± 0.4^{e}	26.1 ± 1.4^{a}	9.9 ± 0.9^{d}	15.7 ± 2.1^{c}	10.9 ± 1.5^{d}
TOTAL	678.2 ± 405^{c}	246.2 ± 12.1^{f}	778.3 ± 504^{b}	273.4^{e}	992.6 ± 574^{a}	404.7 ± 35.9^{d}

Values in the same row bearing different superscripts are different at $P < 0.05$. nd, not detected (<0.01 mg kg^{-1}).

Multivariate statistic and sensory evaluation

The values of the soluble compounds were included in a data set for multivariate statistical analysis (Figure 3). Overall, six factors were obtained from the analysis, and PCA1 and PCA2 explained 86.1% of variance, with clear separation of the DAM and MGC samples along PC1 (63.44% of variability). Along this principal component, DAM samples were positioned at the right side of the plot and MGC at left, except for one sample at day1. The most distant samples were DAM at day 1 and GMC at day 14: the former was highly discriminated by the contents of lactose, citric acid and soluble nitrogen, the latter by lactic acid, acetic acid and total AA. The plot clearly evidenced that these soluble compounds discriminated less effectively the cheeses at the intermediate sampling times; the 'most critical' samples were DAM at day 4, followed by those at day 8, which tended to be closer to MGC samples at days 1 and 4. At these phases of storage, it should be preferable considering the whole pattern of the soluble compounds for avoiding possible overlapping. PC2 accounted for 22.57% of total variability, allowing a certain separation of the samples according to the storage time. In fact, all samples at 8 and 14 days laid in the bottom part of the graph

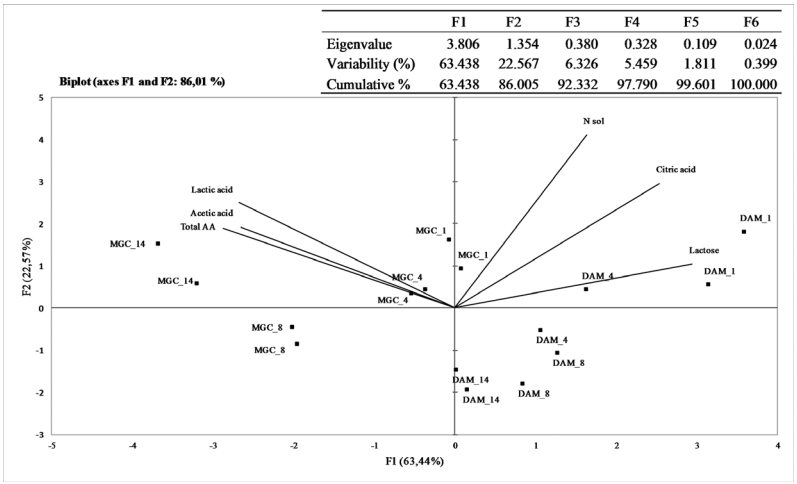

Figure 3 Principal component analysis of traditional (MGC) and industrial (DAM) high moisture mozzarella during refrigerated storage using the soluble compounds as variables

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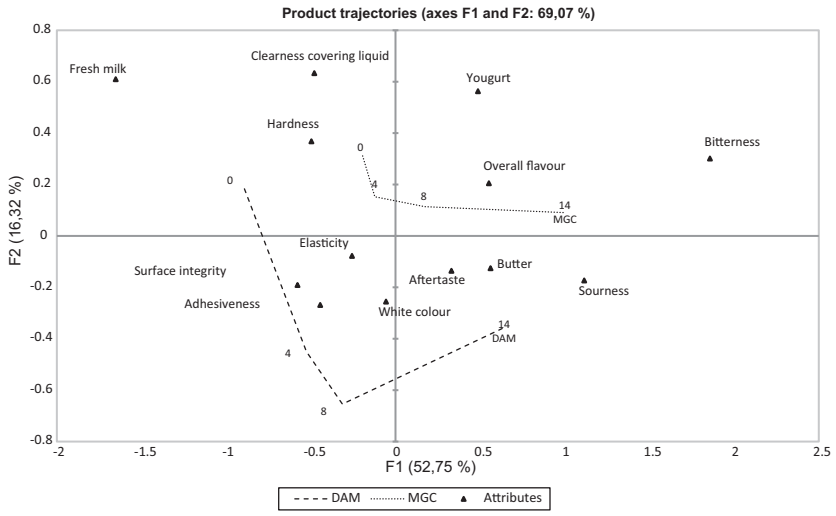

Figure 4 Principal component analysis of traditional (MGC) and industrial (DAM) high moisture mozzarella during refrigerated storage using the sensory characteristics as variables

(negative side), except for MGC samples at 14 days that were in the positive side in connection with their content of lactic acid, acetic acid and total AA content.

The results of the sensory analyses during the storage period have been reported in Table S2. The sensory profiles were characterised by thirteen validated attributes, three of which regarded appearance, three texture and seven flavour. As expected, the scores of the most part of the attributes strongly changed over time, and the changes were mostly undesired since gave rise to off-flavours (such as excessive sour and butter odour, and bitterness), and texture deterioration (such as loss of clearness of the governing liquid and disruption of the surface). The data were used for obtaining the PCA plot shown in Figure 4. It contains the 'product trajectories' depicting how the two types of mozzarella evolved over time. The line related to MGC lied in the upper quadrant of the graph and in a rather restricted area, whereas DAM occupied a wider area, mostly positioned in the lower quadrant. These results indicate that the most characterising attributes of the traditional product were the yogurt odour and overall flavour, which matched well with the higher concentration of lactic acid and free amino acids with respect to the industrial type. A second information deriving from the data is that the sensory profile in MGC underwent to less marked changes over time. It is worth mentioning that the two trajectory lines of the two products never overlapped, indicating that the panellists were always able to discriminate the samples.

Conclusions

The present study allowed quantifying the changes over time of the main soluble compounds in high moisture mozzarella stored in water. The research has to be considered as a sort of model study demonstrating that the analysis of the water-soluble fraction could potentially allow discrimination of traditional and industrial products made with citric acid throughout a 14 days storage time. It was found that some compounds could better act as discriminating indices, in particular organic acids and lactose. In fact, even under the limited conditions of this experimentation, NaCl and WSN (and pH, too, which is linked to many water-soluble compounds) did not appear as sufficiently reliable. The concentration of free amino acids was another highly discriminating parameter, but since they derive (like WSN) from proteolysis that is a multifactorial event, further investigation is needed, on a large number of samples. Finally, although the chemical changes caused a marked evolution of the sensory characteristics, the panellists were always able to distinguish the two products. Nevertheless, all that matters under the practical point of view is the perception of consumers, and it should be very interesting to widen the sensory study by a consumer science approach.

Conflicts of interest

The authors hereby declare there is no conflict of interests.

Author Contributions

Michele Faccia: Conceptualization (lead); writing–original draft (lead). **Giuseppe Natrella:** Investigation (lead); software (lead). **Giuseppe Gambacorta:** Data curation (lead); software (supporting).

Ethical guidelines

Ethics approval was not required for this research.

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Peer Review

The peer review history for this article is available at https://publons.com/publon/10.1111/ijfs.15091.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

This paper dealt with the behavior of water in high moisture mozzarella stored under refrigeration or frozen. Even though the topic was not the same than that included in our research, it has been cited since it is one of the few studies available in the literature that has stressed the importance of deepening the relationships between the governing liquid and the product during storage.

This work deepened the role of adventitious microflora in the hydrolysis of the protein fraction in high moisture mozzarella. We have chosen to cite it since provides very useful information on the role of some bacteria species in the fast decay of the characteristics of this cheese

This article has been cited since it focused with high moisture mozzarella manufactured with different acidification methods, including the traditional one. It is important because it describes the connections between the proteolytic activity of LAB added with the natural whey starter and the formation of water soluble nitrogen.

This paper has been cited because dealt with primary proteolysis in direct acidified high moisture mozzarella. It is useful in the discussion of proteolysis (required by the referees) since it helps to understand the role played by residual rennet in casein degradation.

This work has been cited because presented the results of an investigation aiming at characterizing the bacteria populations in high moisture mozzarella. In particular, it contains very useful data for understanding the connections between the method of acidification and microbiota present in the cheese.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Gross composition (g 100 g^{-1} cheese, except for pH; average values of the two cheesemaking trials) of traditional (MGC) and industrial (DAM) high moisture mozzarella packaged in water during refrigerated storage. GL, governing liquid; WSN, water soluble nitrogen. Values in the same row bearing different superscripts are different at $P < 0.05$.

Table S2 Scores (modal values) of Quantitative Sensory Analysis of DAM and MCG mozzarella samples taken at different time throughout refrigerated storage. Edonic 6 point scale from 0 (not perceived) to 5 (intensely perceived).

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