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Volatile organic compounds throughout the manufacturing process of *Mozzarella di Gioia del Colle* PDO cheese

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Abstract: The evolution of volatile organic compounds during the production process of *Mozzarella di Gioia del Colle* (traditional type) was investigated in comparison with citric mozzarella (industrial type). The total volatile concentration in the traditional curd was ten times higher than milk, versus only twice as much in the industrial type. In both technologies, the concentrations decreased from curd to mozzarella at the same rate, due to losses during the stretching phase. The higher microbial activity in the traditional product was responsible for a much more complex profile, characterised by 2- and 3-methylbutanal, acetoin, ethanol, 3-methyl-1-butanol, acetic acid and ethyl-acetate. In contrast, the industrial mozzarella had a very simple profile, with the most important compounds being directly derived from the milk, the oxidation reactions, or the activity of the adventitious microflora such as acetone, hexanal, nonanal, and hexanoic acid. According to the discriminant analysis, *Mozzarella di Gioia del Colle* had a very different profile than the milk used, whereas the industrial mozzarella was more similar to the milk.

Keywords: *Mozzarella of Gioia del Colle*; industrial mozzarella; volatile compounds; discriminant analysis

Mozzarella is a fresh cheese that originated in Southern Italy several centuries ago. It is a "pasta filata" cheese, obtained by stretching the acidified curd with hot water, giving it a shape, then cooling it in a pot of water. In 1993, high-moisture buffalo mozzarella became an EU "Protected Designation of Origin" (PDO) cheese. Nevertheless, the most widely produced mozzarella is made from cow's milk, due to the wide availability of the raw matter that allows manufacturing all over the world, also on an industrial level (Faccia et al. 2019). Recently, the traditional bovine type is also going to be acknowledged with the EU PDO label "*Mozzarella di Gioia del Colle*", the name of the town worldwide known for its production [EU Commission (2019/C 356/09), Publication of an application for registration of a name pursuant to Article 50(2)(a) of Regulation (EU) No. 1151/2012 of the European Parliament and of the Council on quality schemes for agricultural products and foodstuffs]. According to the official PDO protocol, the curd acidification can be made exclusively by using an autochthonous natural whey starter (NWS). It is an acidified whey obtained by a "backslopping" procedure (Demarigny et al. 2011), involving the incubation of fresh cheesemaking whey for about 12 h at 30–35 °C, until the pH reaches a value of 3.4–3.8. The backslopping procedure gives complex and specific flavour characteristics to the cheese (Parente 2006; Guidone et al. 2016). The microbiological profile of the NWS used in mozzarella has been deepened by several researchers (Ercolini et al. 2012; Pisano et al. 2016). Unfortunately, the volatile organic

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compounds (VOC), which are highly connected to the flavour of the product, have been poorly studied (Moio et al. 1993; Natrella et al. 2020). In particular, no information is available on the kinetics of their formation throughout the manufacturing process. The aim of the present research was to monitor the evolution of the VOC from the milk to the cheese during the production process of *Mozzarella of Gioia del Colle*, in comparison with the industrial type made by direct acidification.

MATERIAL AND METHODS

Samples. The study was conducted on milk, curd and mozzarella samples (22 for each type, 66 samples in total) taken from several dairies located in the geographical area of PDO *Mozzarella di Gioia del Colle* (province of Bari, Apulia, Southern Italy). One half of them were manufactured by the NWS method as contemplated by the PDO official protocol (the addition of about a 5% starter to the milk 15 min before coagulation), the other half was made by the industrial technology (direct acidification with citric acid). The manufacturing protocols are reported in Figure 1. The curd and mozzarella samples were coded as DA-C and DA-M (direct acidification) and as NWS-C and NWS-M (NWS method) respectively. All the samples were cooled to 6 °C in chilled water, packaged in plastic trays containing cold diluted brine and immediately transported under refrigeration to the laboratory for the analyses. The milk was analysed for its protein (Kjeldahl-method; ISO 17997-1|IDF 29:2004), fat (Gerber method, ISO 488|IDF 105:2008), and pH (pH-meter HANNA Instrument Inc, United Kingdom); all

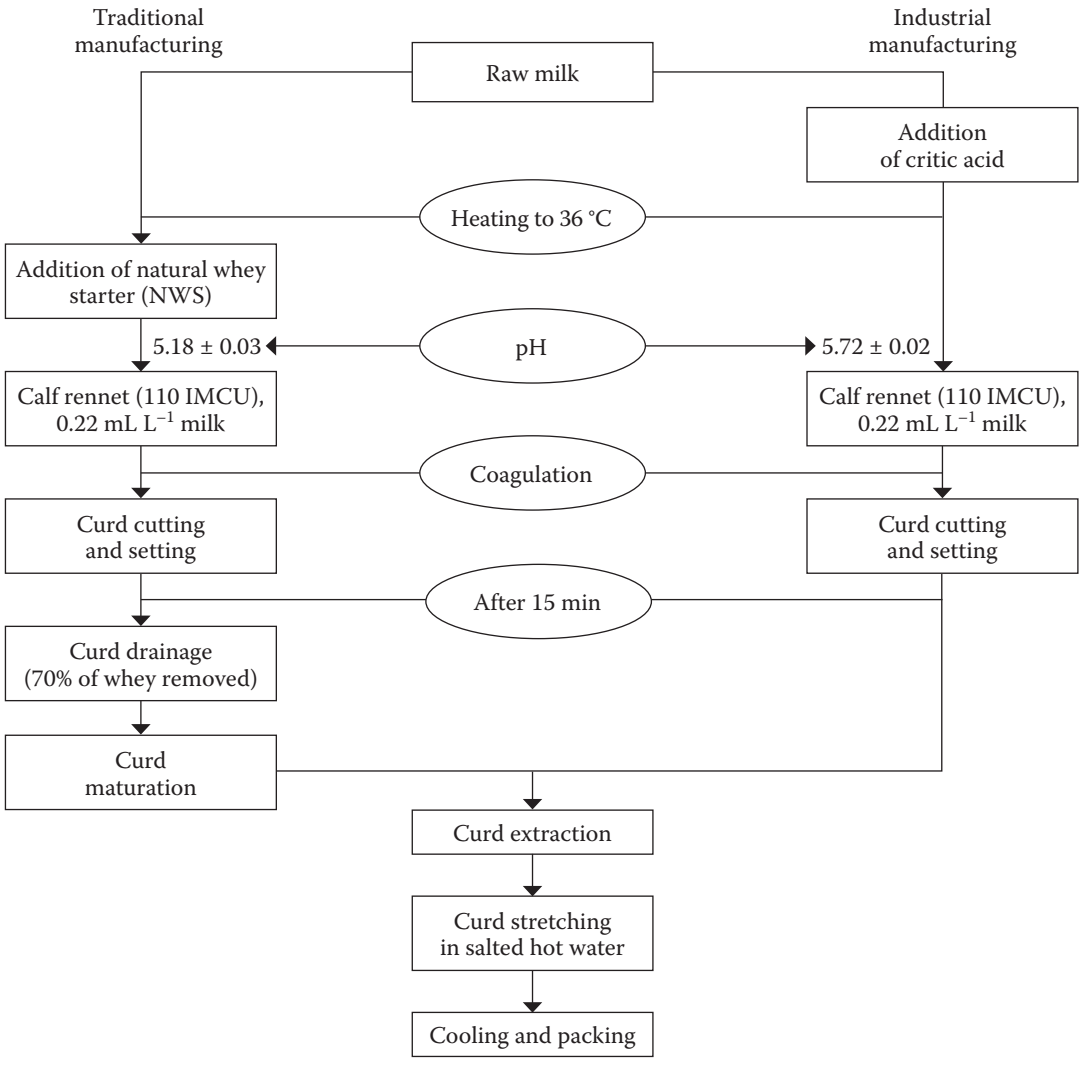

Figure 1. Technological scheme of manufacturing mozzarella

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the samples were subjected to an analysis of the volatile organic compounds.

VOC analysis. The VOC were extracted by solid-phase microextraction (SPME) and analysed by Gas Chromatography Mass Spectrometry (GC-MS). For the milk, mozzarella and curd, a 1 ± 0.05 g sample was weighed into a 20 mL vial, with the addition of an internal standard (81.3 ng of 3-pentanone) and closed by a rubber septum and an aluminium cap. The samples were then loaded into a Triplus RSH autosampler (ThermoFisher Scientific, Italy) for extraction at 37 °C for 15 min, using a divinylbenzene/carboxen/polydimethylsiloxane 50/30 mm SPME fibre assembly (Supelco, USA). The fibre was desorbed at 220 °C for 2 min in the injection port of the gas chromatograph, operating in the splitless mode. The GC-MS analyses were performed using a Trace1300 gas chromatograph equipped with an ISQ Series 3.2 SP1 mass spectrometer (ThermoFisher Scientific, Rodano, Italy). The compounds were separated on a VF-WAX MS thermo capillary column (60 m, 0.25 mm, 0.25 mm), under the following conditions: injection port temperature, 220 °C; oven temperatures, 40 °C for 0.1 min then 4 °C min^{-1} to 140 °C, 10 °C min^{-1} to 220 °C and a final isothermal for 7.5 min. The mass detector was set at the following conditions: detector voltage, 1700 V; source temperature, 250 °C; ionisation energy, 70 eV; scan range 33–200 amu. The tentative identification of the peaks was undertaken by means of software Xcalibur 2.0 (Thermo Fisher Scientific, USA), by matching their spectra with the reference mass spectra of the NIST (National Institute of Standards and Technology) library. A semi-quantitation was performed by the internal standard method, and the amounts were expressed as mg L^{-1} for the milk and mg kg^{-1} for the curd and mozzarella.

Statistical analysis. All the samples were analysed in triplicate, the Discriminant Analysis (DA), means and Analysis of Variance (ANOVA) were calculated with XLSTAT-Sensory software (Addinsoft, France).

RESULTS AND DISCUSSION

No significant differences were observed in the chemical composition of the two milk groups: the average protein content was the same (3.69%), the average fat content was similar (3.99% DA and 3.88% NSW), as was the pH value (6.8 for DA and 6.7 for NWS).

For both technologies, the total VOC concentration increased from the milk to the curd, but strongly decreased in the mozzarella, as a consequence of the partial volatilisation and dissolution into the water during the stretching phase (Table 1). The processing technology had a strong influence in the first phase since the concentration increased about 15-fold from the milk to the curd in the NWS versus only twice in the DA methods. It was due to the different fermentation intensity since a starter had only been added in the NWS. The decrease in the second phase was not linked to the technology, since, in both cases, the VOC concentration fell by about a quarter. To the best of our knowledge, these are the first data on this subject: Sacchi et al. (2020) performed a similar study on buffalo milk and mozzarella, reporting that the total VOC amounts in the two matrices were similar, but the curd was not analysed.

The VOC were grouped in 8 chemical classes. The NWS curd was characterised by high amounts of alcohols (1 631.35 mg kg^{-1}) and esters (892.50 mg kg^{-1}), whereas alcohols (296.39 mg kg^{-1}), ketones (156.70 mg kg^{-1}) and aldehydes (131.59 mg kg^{-1}) were the most represented chemical classes in the corresponding moz-

Table 1. VOC chemical groups in raw milk (RM), curd and cheese in the *Mozzarella of Gioia del Colle* (NWS-C and NWS-M) and the industrial mozzarella (DA-C and DA-M)

	RM (mg L^{-1})	NWS-C (mg kg^{-1})	DA-C (mg kg^{-1})	NWS-M (mg kg^{-1})	DA-M (mg kg^{-1})
Acids	26.10^{c}	273.15^{a}	219.55^{ab}	93.92^{bc}	9.87^{c}
Alcohols	29.34^{b}	1631.35^{a}	25.34^{b}	296.39^{b}	6.33^{b}
Aldehydes	10.60^{b}	175.33^{a}	55.37^{ab}	131.59^{ab}	4.46^{b}
Esters	10.80^{b}	892.50^{a}	10.40^{b}	42.49^{b}	2.49^{b}
Ketones	117.28^{b}	349.70^{a}	73.66^{b}	156.70^{b}	71.21^{b}
Sulfur compounds	6.06^{a}	0.72^{c}	4.95^{ab}	2.28^{bc}	2.19^{bc}
Total	200.17	3322.75	389.27	723.37	96.55

Values in the same row bearing different superscripted are different at $P < 0.05$

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zarella. The industrial product had a different pattern: acids (219.55 mg kg^{-1}) and ketones (73.66 mg kg^{-1}) were the most represented classes in the DA curd, whereas the profile was quantitatively very poor in the mozzarella, and only ketones reached an appreciable level (71.21 mg kg^{-1}).

Sixty-three single compounds were identified in the entire set of samples: 11 aldehydes, 9 ketones, 21 alcohols, 13 acids, 2 sulfur compounds and 7 esters (Table 2). The NWS-C had the highest number of molecules (54) followed by the NWS-M (45), raw milk (38), DA-C (33) and DA-M (18). As regards to the car-

Table 2. VOC found in raw milk (RM), curd and cheese in the *Mozzarella of Gioia del Colle* (NWS-C and NWS-M) and the industrial mozzarella (DA-C, DA-M)

	P	RM (mg L^{-1})	NWS-C (mg kg^{-1})	DA-C (mg kg^{-1})	NWS-M (mg kg^{-1})	DA-M (mg kg^{-1})
Acids						
Acetic acid	**	2.70^{c}	176.42^{ab}	199.77^{a}	46.84^{bc}	1.20^{c}
Propanoic acid	*	ND	0.04^{b}	ND	3.54^{a}	ND
Propanoic acid, 2-methyl	**	ND	4.14^{a}	ND	0.05^{b}	ND
Propanoic acid, 2,2-dimethyl		0.07	ND	ND	ND	ND
Butanoic acid	**	9.55^{b}	30.16^{a}	3.70^{b}	8.47^{b}	2.80^{b}
Butanoic acid, 2-methyl	*	ND	1.08	ND	ND	ND
Butanoic acid, 3-methyl	*	0.03^{b}	4.52^{a}	ND	1.02^{ab}	ND
Pentanoic acid	*	0.04^{b}	0.08^{b}	ND	0.12^{a}	ND
Hexanoic acid	**	9.21^{bc}	43.89^{a}	8.87^{bc}	25.05^{b}	4.22^{c}
Heptanoic acid	**	0.12^{b}	0.81^{a}	0.48^{ab}	0.65^{a}	ND
Octanoic acid	**	3.32^{b}	11.73^{a}	4.56^{b}	7.08^{ab}	ND
Nonanoic acid	**	ND	0.17^{b}	0.77^{a}	0.44^{ab}	ND
n-Decanoic acid	**	0.96^{ab}	0.06^{c}	1.40^{ab}	0.65^{bc}	1.65^{a}
Alcohols						
Ethanol	**	20.52^{b}	704.08^{a}	3.27^{b}	109.43^{b}	2.76^{b}
Isopropanol		0.35	ND	ND	0.33	ND
1-propanol,2-methyl	**	1.51^{b}	47.35^{a}	ND	6.90^{b}	ND
1-butanol, 3-methyl	**	2.11^{b}	867.84^{a}	ND	170.84^{b}	ND
1-pentanol	*	0.63^{b}	4.75^{a}	0.69^{b}	3.64^{a}	ND
1-hexanol	*	0.28^{b}	0.67^{a}	0.12^{b}	0.71^{a}	0.10^{b}
1-hexanol,2-ethyl	**	1.88^{a}	ND	0.27^{b}	0.95^{ab}	1.18^{ab}
2-heptanol	*	ND	0.10	ND	0.11	ND
1-octanol	**	0.14^{b}	ND	3.36^{a}	ND	ND
2-nonanol	*	ND	0.06	ND	0.04	ND
2-(octyloxy)-ethanol	**	0.31^{b}	ND	15.41^{a}	ND	1.63^{b}
2-propanol, 1-(2-methoxypropoxy)	*	0.47	0.48	ND	0.49	ND
2-propyl-1-pentanol	**	0.37^{b}	1.60^{a}	ND	0.94^{ab}	ND
2-buten-1-ol,2-methyl	*	ND	0.12	ND	0.04	ND
2-buten-1-ol,3-methyl	*	0.02^{b}	0.19^{a}	ND	0.31^{a}	ND
3-buten-1-ol, 3-methyl	**	0.12^{b}	1.06^{a}	ND	0.83^{a}	ND
1-octen-3-ol		0.03	ND	ND	ND	ND
1,6-octadien-3-ol, 3,7-dimethyl	*	ND	0.35	ND	0.45	ND
2-furanmethanol		ND	ND	0.12	ND	ND
Benzyl alcohol		0.38	0.21	0.50	ND	0.66
Phenylethyl alcohol	**	0.21^{c}	2.48^{a}	1.60^{ab}	0.36^{bc}	ND

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Table 2 to be continued

	P	RM (mg L^{-1})	NWS-C (mg kg^{-1})	DA-C (mg kg^{-1})	NWS-M (mg kg^{-1})	DA-M (mg kg^{-1})
Aldehydes						
Acetaldehyde	**	ND	44.73^{a}	0.46^{b}	ND	ND
Benzaldehyde	*	ND	1.45^{a}	0.05^{b}	0.85^{ab}	ND
Benzeneacetaldehyde		ND	ND	0.43	ND	ND
Butanal, 2-methyl	*	ND	13.88	ND	28.91	ND
Butanal, 3-methyl	**	3.02^{c}	87.22^{a}	18.77^{b}	87.46^{a}	ND
Hexanal	*	6.34^{ab}	0.18^{b}	12.73^{a}	0.94^{b}	2.70^{b}
Octanal	**	0.19^{b}	0.11^{b}	1.68^{a}	0.25^{b}	0.30^{b}
Nonanal	**	0.92^{b}	0.98^{b}	13.26^{a}	1.54^{b}	1.45^{b}
Decanal	**	0.13^{b}	0.10^{b}	2.80^{a}	0.10^{b}	ND
Propanal, 2-methyl	*	ND	26.67^{a}	5.16^{b}	11.54^{ab}	ND
Furfural		ND	ND	0.01	ND	ND
Esters						
Ethyl acetate	**	10.46^{b}	867.36^{a}	10.40^{b}	42.49^{b}	2.49^{b}
Ethyl propanoate	**	ND	3.27	ND	ND	ND
Ethyl butanoate	*	ND	1.87	ND	ND	ND
Ethyl hexanoate	**	ND	0.58	ND	ND	ND
n-Propyl acetate	*	ND	3.83	ND	ND	ND
Isobutyl acetate	*	ND	1.29	ND	ND	ND
Ketones						
Acetone	**	85.05^{a}	18.69^{c}	43.54^{bc}	31.41^{bc}	56.55^{ab}
Acetoin	**	2.19^{b}	305.57^{a}	ND	94.39^{b}	ND
2-butanone	*	29.40^{a}	4.31^{c}	27.57^{a}	11.91^{b}	14.22^{b}
2-heptanone	**	0.21^{b}	14.72^{a}	ND	15.60^{a}	ND
2-nonanone	**	0.12^{b}	1.30^{a}	ND	1.54^{a}	ND
2-hydroxy-3-pentanone	**	ND	2.42	ND	1.43	ND
2-butanedione		ND	ND	1.55	ND	ND
2,3-pentanedione	**	ND	2.55	ND	ND	ND
5-hepten-2-one, 6-methyl	**	0.30^{b}	0.12^{b}	1.01^{a}	0.43^{b}	0.44^{b}
Sulphur compounds						
Dimethyl sulfide	*	2.58^{a}	0.26^{d}	0.74^{c}	1.76^{b}	0.33^{d}
Dimethyl sulfone	**	3.47^{ab}	0.45^{c}	4.20^{a}	0.52^{c}	1.86^{bc}

Values in the same row bearing different superscripted are different at $^{*}P < 0.05$ or $^{**}P < 0.0001$; ND – not detected

bonyl compounds, the mostly observed differences between the curds were seen in the aldehydes. Acetaldehyde, 2- and 3-methyl-butanal were more abundant in the NWS-C, being connected to the microbial activity (De Palencia et al. 2004; Cadwallader et al. 2009). Acetaldehyde was not detected in the mozzarella, probably because it was lost during the stretching phase due to it being totally miscible with water. Differently, the DA-C and DA–M were characterised by the higher presence of linear aldehydes (hexanal, octanal, nonanal and decanal) that have an oxidative origin (Karatapanis et al. 2006). Acetone was the most important ketone in the milk, in which it is associated with the nutritional status of the cattle and silage feeding (Villeneuve et al. 2013). It was highly lost into the whey, and was less abundant in the NWS curd since any additional whey is expulsed during the fermentation (the DA does not require fermentation). The most im-

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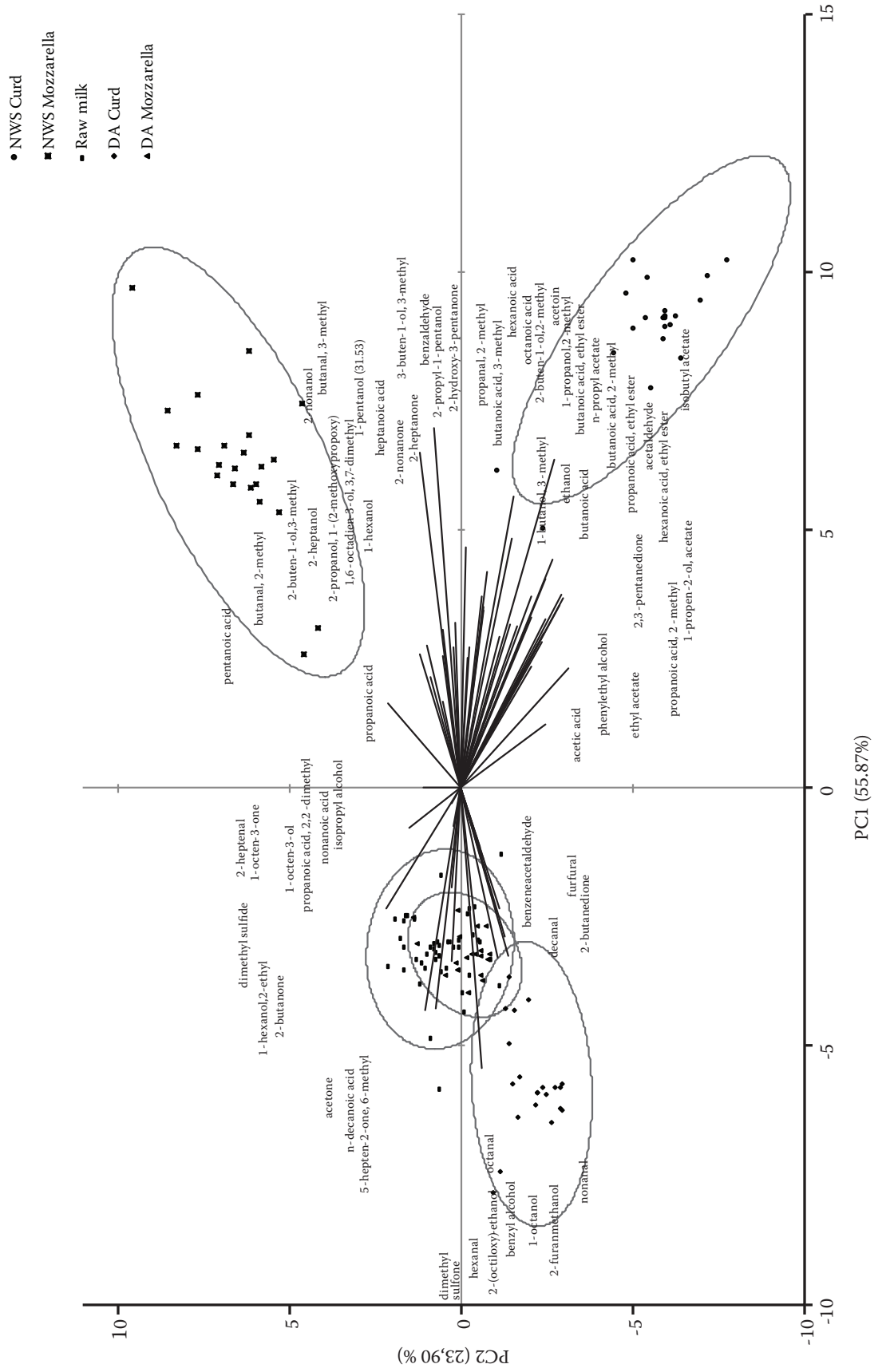

Figure 2. Discriminant analysis of the samples related to the volatile organic compounds

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portant ketone in NWS curd and cheese was acetoin, whereas 2-butanone was in the DA: the former derives from the citrate fermentation, the latter from the feed or metabolism of the adventitious microflora (Marsili et al. 2003; Massouras et al. 2006). The acid and alcohol compounds discriminated (differentiated??) the samples both quantitatively and qualitatively. The acetic, propanoic, butanoic, an hexanoic acid and the ethanol, 1-butanol, 3-methyl and 1-propanol,2-methyl, almost always connected to the activity of the lactic acid bacteria and yeasts (Yang et al. 2020), were much more abundant in the NWS. Ethanol (at lower concentration), 2-(octyloxy)-ethanol and 1-hexanol,2-ethyl were the most represented in the industrial curd and mozzarella. Acetic acid was found at the highest concentration in the curds, without any significant difference between the groups. It was expected to be more abundant in the traditional samples, but it could be derived from the fermentation of the citric acid added for the milk acidification by indigenous microflora (Andic et al. 2010 not 6). Ethyl acetate was the only ester present in all the samples, and its concentration followed the trend of its precursors, ethanol and acetic acid. The highest level was found in the NWS-C, whereas the concentration in the DA-C was almost the same as the milk. Moreover, the curd stretching caused a strong reduction in mozzarella. Many other esters were formed in the traditional curd, but they disappeared in the mozzarella after stretching. The only detected sulfur compounds were dimethyl sulfide and dimethyl sulfone, its oxidative product. Milk had the highest content, and mozzarella the lowest.

Figure 2 shows the DA of all the samples by their volatile profile. Being that the profiles of the two milk groups were almost identical; the raw milk (RM) was considered as a single qualitative variable category. About 79.77% of the variability was explained by two principal components (PC). Considering PC1, the NWS-M and NWS-C were at the positive side of the plot, clearly separated from the other samples. The majority of the VOC are on this side of map, related to these samples. In contrast, the RM and DA-M were present on the negative side: they overlapped, and DA-C was close to them. Considering PC2, the NWS-M laid on the positive side whereas the curds were well-separated on the negative side. Straddling them, almost in the middle, were the RM and DA-M. These results better highlight the differences previously discussed: the curds were clearly separated from the cheeses due to the reduction of the VOC after the stretching phase, as well as that the NWS-C was clearly differentiated from the DA-C.

CONCLUSION

The study deepened the kinetics of the VOC formation in the PDO *Mozzarella of Gioia del Colle* and in the industrial mozzarella. In the PDO cheese, the poor volatile profile of the milk became very complex during processing, whereas the changes were much less relevant in the industrial product, due to scarce presence of the fermentative metabolites. The volatile profile of the PDO mozzarella should be described as "fermentative", whereas that of the industrial type as "oxidative". The results obtained could be useful in developing a method for protecting the traditional product from imitations.

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