Relationships Between Flavoring Capabilities, Bacterial Composition, and Geographical Origin of Natural Whey Cultures Used for Traditional Water-Buffalo Mozzarella Cheese Manufacture

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ABSTRACT

Natural whey cultures (NWC) ($n = 29$) used for traditional water-buffalo Mozzarella cheese manufacture and arising from different geographical areas of production were characterized and grouped on the basis of their capability to develop neutral volatile compounds and according to their microbial diversity as revealed by molecular analysis. The flavoring properties of NWC were studied in dairy microcosms resembling the specific technological procedure used in the traditional water-buffalo Mozzarella cheese-making. Neutral volatile compounds were identified by high-resolution gas chromatography (HRGC)-mass spectrometry analysis while information on the microbial diversity occurring in the NWC was retrieved by PCR-denaturing gradient gel electrophoresis (DGGE) analysis of 16S rDNA after direct DNA extraction. Neoformation volatile substances ($n = 27$) were found; 23 were identified and some of them recognized as odor-conferring molecules. Eight different bands, referable to eight microbial species, were obtained by PCR-DGGE analysis of the NWC. Statistical analyses were applied to PCR-DGGE and HRGC data. Interestingly, the flavoring capabilities and the microbial diversity of the NWC proved to be closely linked and both related to the geographical origin of the NWC. These results suggested a possible use of the molecular characterization of the dairy products to support the traceability criteria of typical dairy products like water-buffalo Mozzarella cheese.

(Key words: natural whey culture, water-buffalo Mozzarella cheese, flavoring capability, microbial diversity).

Abbreviation key: CA = cluster analysis, HRGC-MS = high-resolution gas chromatography-mass spectrometry, MDS = multidimensional scaling, NWC = natural whey culture, PCA = principal component analysis,

PCR-DGGE = PCR-denaturing gradient gel electrophoresis.

INTRODUCTION

Typical fermented food products have received much attention in the last decades in many countries. The specific characteristics of these products mainly arise from the specific raw materials employed, the area of production, the environmental conditions, the traditional tools, and the manufacture. However, tools for the typing of these products are still seldom applied, and further study of the relationships among the characteristics leading to typical tastes, textures, and flavors is needed.

Water-buffalo Mozzarella cheese is a typical "pasta filata" cheese from Southern Italy, having high moisture (55 to 62%) and high fat in DM (>45%) and characterized by a soft body and a juicy appearance and by a pleasant, fresh, sour, and slightly nutty flavor. The manufacture of this cheese has been described in detail in previous works (Addeo and Coppola, 1983; Coppola et al., 1988; Coppola et al., 1990). Briefly, the cheese is made from whole raw water-buffalo milk by adding a natural whey culture (NWC; from the manufacture of the previous day) as a starter. After a curd-ripening phase (4.0 to 4.5 h at 35 to 37°C), the optimal pH (4.9 to 5.1) is reached, and the drained curd is stretched in hot water (90 to 95°C). Water-buffalo Mozzarella cheese from Campania ("Mozzarella di Bufala Campana") received European certification Product of Designated Origin (DOP, EEC Regulation n. 1107 12th June 1996) certifications, and its production zone includes seven provinces: Caserta and Salerno, where most of the cheese is usually produced, and part of the provinces of Benevento, Naples, Frosinone, Latina, and Rome. In characterizing the microflora of NWC, Coppola et al. (1988) found hundred millions of microorganisms belonging to a variety of groups. This biodiversity was recognized not only as responsible for curd ripening but also for conferring the typical flavor of Mozzarella cheese (Coppola et al., 1990).

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Flavoring properties of many strains of lactic acid bacteria isolated from NWC utilized within the traditional manufacture of water-buffalo Mozzarella cheese were studied by Mauriello et al. (2001) in pure cultures in whey. The complexity of the aroma composition arising from the activities of mixed cultures such as NWC has never been investigated.

In this work, 29 NWC were characterized on the basis of their capability to develop neutral volatile compounds and grouped according to their microbial diversity as revealed by molecular analysis.

The flavoring properties of NWC were studied in dairy microcosms resembling the specific technological procedure used in the traditional water-buffalo Mozzarella cheese-making. The main goal of this research was investigating relationships between geographical origin and microbiological as well as sensorial features of the dairy products. Furthermore, the correlation between microbial diversity of NWC and aromatic profiles of the products was defined in order to find out whether and how the species composition affects the technological strength of the natural starter.

MATERIALS AND METHODS

NWC Samples

The 29 NWC used in this study were collected in 29 different cheese factories located in Campania region: 17 were from the province of Salerno (NWC1 to NWC17) and 12 from the province of Caserta (NWC18 to NWC29). All the factories providing samples were part of an organized consortium for the preservation of the certified typical product "water-buffalo Mozzarella cheese from Campania." The NWC used as starter is so prepared: the whey drained from the curd during the manufacture of Mozzarella cheese on the previous day is stored overnight at room temperature and used the following day as starter in the next manufacture. After the collection, the NWC samples were cooled at 4°C and, having been transferred to the laboratory, were processed immediately.

NWC Employment in Experimental Mozzarella Cheese Manufacture

Each sample of NWC was employed as starter in trials resembling the traditional water-buffalo Mozzarella cheese production. The same raw water-buffalo milk was used for all the manufactures. Added to 200 ml of milk were 5% (vol/vol) NWC and 0.01% liquid rennet (Clerici, Cadorago, Italy) to reach the coagulation in 20 min. The ripening was carried out for 6 h at 37°C, and then the curd was separated from the whey by centrifugation at $9,000 \times g$ for 20 min at 4°C. The whey was collected

and used for neutral volatile compounds extraction as described below. The whey arising from the coagulation of uninoculated milk was analyzed as control.

Extraction of Neutral Volatile Compounds

The volatile constituents of whey after curd ripening were isolated as previously reported (Mauriello et al., 2001). Briefly, whey samples were centrifuged for 20 min at $9,000 \times g$, and 100 ml of supernatant was maintained in ice bath and neutralized with 0.2 M NaOH up to pH 9.00 to salify the FFA. After adding of 0.1032 mg/kg of methyl decanoate and 0.1044 mg/kg of isopropyl decanoate as internal standards, the whey was extracted three times with freshly distilled dichloromethane (50 ml) with vigorous agitation by magnetic stirrer (Mauriello et al., 2001). The whey/CH₂Cl₂ emulsion formed during stirring was separated from the aqueous layer and frozen at -30°C (Moio and Etievant, 1995). The flask was then allowed to reach room temperature, and the CH₂Cl₂ solution, progressively separated from the remaining whey, was dried over anhydrous MgSO₄ and reduced to 0.5 ml under a stream of nitrogen.

High-Resolution Gas Chromatography (HRGC)

HRGC analysis was carried out with a GC-17A gas chromatograph (Shimadzu Italy, Milano, Italy) computer-controlled by CLASS-VP 4.3 software (Shimadzu Italy) and equipped with a split-splitless injector, a flame-ionization detector, and an HP-5 fused silica capillary column (30 m $\times$ 0.32 mm i.d.; film thickness, 1 μ m). The operating conditions were as follows: the temperature was programmed to rise from 40 to 210°C at a rate of 3°C/min. The injector and detector temperatures were set at 250 and 255°C, respectively. The hydrogen carrier velocity was 37 cm/s. Quantitative measurements of each component were obtained from the peak areas of the components relative to the internal standards, assuming that the extraction efficiency and the GC response were identical for all compounds separated.

HRGC-Mass Spectrometry (HRGC-MS)

The electron impact mass spectra were obtained with an HP 5970 quadrupole mass spectrometer coupled with an HP 5890 gas chromatograph connected directly to the ion source of the mass-spectrometer. The capillary column and operating HRGC conditions were the same as those described above. The mass spectra were determined at 70 eV while the ion source and the interface were held at 150 and 280°C, respectively (Moio et al., 2000). Acquisition and processing of mass spectra were carried out by using a computer, and the compounds

were identified with the aid of mass spectral databases (Nover de Brauw et al., 1987).

DNA Extraction from NWC and Curds

Each sample of natural starter was also subjected to DNA extraction as previously described (Ercolini et al., 2001b). The protocol described by the Wizard DNA purification kit (Promega, Madison, WI) was applied as follows: 1 ml of NWC sample was centrifuged at $10,000 \times g$ for 5 min at 4°C, and the resulting pellet was resuspended in 100 μ l of TE buffer (100 mM TRIS, 10 mM EDTA); then 160 μ l of 0.5 M EDTA/Nuclei Lysis Solution in 1/4.16 ratio, 5 μ l of RNase (10 mg/ml; Sigma, St. Louis, MO) and 20 μ l of pronase E (20 mg/ml, Sigma) were added, and the mixture was incubated for 60 min at 37°C. After incubation, 285 μ l of ammonium acetate 5 M was added to the sample that was then centrifuged at $10,000 \times g$ for 5 min at 4°C. The supernatant was precipitated with 570 μ l of isopropanol and centrifuged at $14,000 \times g$ for 5 min. Finally, the pellet was dried and resuspended in 50 μ l of DNA rehydration solution by incubation at 55°C for 45 min. The same method was applied for the DNA extraction from milk, as control, and from curds; the curds were fivefold diluted-suspended in TE buffer and the protocol was applied to 1 ml of suspension.

PCR-Denaturing Gradient Gel Electrophoresis (DGGE) Analysis

Primers spanning the 200-bp V3 region of the 16S ribosomal DNA of *Escherichia coli* were used in PCR amplification as previously described (Ercolini et al., 2001a). A GC-clamp was added to the forward primer, according to Muyzer et al. (1993). Amplification was performed in a programmable heating incubator (MJ Research Inc., Watertown, MA). Each mixture (final volume, 25 μ l) contained 20 ng of template DNA, each primer at a concentration of 0.2 μ M, each deoxynucleoside triphosphate at a concentration of 0.25 mM, 2.5 mM $MgCl_2$, 2.5 μ l of 10 $\times$ PCR buffer and 2.5 U of *Taq* polymerase (Gibco BRL, Gaithersburg, MD). Template DNA was denatured for 5 min at 94°C. A "touchdown" PCR was performed (Muyzer et al., 1993) to increase the specificity of amplification and to reduce the formation of spurious byproducts. PCR products were analyzed by DGGE using a Bio-Rad Dcode apparatus. Parallel electrophoresis experiments were performed at 60°C by using gels containing a 30 to 50% urea-formamide denaturing gradient (100% corresponded to 7 M urea and 40% (wt/vol) formamide) increasing in the direction of electrophoresis. Bands were automatically detected by using the software Phoretix 1 advanced version 3.01 (Phoretix International Limited, Newcastle upon Tyne, England).

Statistical Analysis

Statistical treatment of data obtained from both chromatographic and electrophoretic analyses was performed using the Systat software for Macintosh ver. 5.2.1. Principal component analysis (PCA) was performed using principal components procedure of factor function on data matrix, with two PC and without rotational factor. Cluster analysis (CA) and multidimensional scaling (MDS) were carried out by examining the data by the simple matching coefficient with the Corr procedure. Relationships were established using the average linkage method by the cluster procedure and Kruskal scaling by the MDS procedure.

RESULTS

HRGC-MS Analysis

The results of GC analysis showed that all NWC were able to produce several volatile compounds during curd ripening. As reported in Table 1, 18 of the 27 components isolated were identified. Moreover, retention time and aroma description (if known) for each component are listed in Table 1. The chromatograms of four representative NWC (two from the province of Salerno and two from the province of Caserta) and of the control are depicted in Figure 1. The quantity of each component was calculated with respect to the internal standards, assuming that the extraction efficiency and the GC response were identical for all compounds separated. Tables 2 and 3 show the concentration of neutral volatile compounds (mg/kg) isolated for each NWC analyzed. Neo-formation substances ($n = 23$) were recognized out of 27 compounds isolated and three of them (acetoin, peak 1; 2,3-butanediol, peak 7; dimethylsulphone, peak 9) were already present in the chromatogram of the control, but at lower concentration. These three substances together with 2-hydroxy-2-methyl propanal (peak 6) and isopropanol (peak 8) were detected in the extracts by all NWC, with exception of NWC14, whose chromatogram did not show the peaks 6, 7, and 8. On the other hand, several substances were produced by only one or two NWC. Furthermore, all the NWC from Caserta province, with exclusion of NWC25, and most of NWC from Salerno province, were able to produce at least six neoformation compounds. Only five NWC (NWC 7, 13, 14, 16, and 25) produced fewer than five neoformation compounds. The gas-chromatograms showing higher level of complexity were obtained from NWC6 (from Salerno) and NWC29 (from Caserta), which were able to produce 14 and 12 new compounds, respectively.

PCR-DGGE Analysis

Information on the microbial composition of the NWC was achieved by direct extraction of DNA from NWC,

Table 1. Volatile compounds produced by natural whey cultures during curd ripening.

Peak number	Retention time	Compound	Odor ¹
1	4.670	3-Hydroxy-2-butanone	Buttery
2	5.073	Pyridin	Sour, putrid, fishy
3	5.157	2-Methyl-1-butanol	Fused oil, whiskey
4	5.293	n.i. ²	
5	5.580	3-Methyl-2-buten-1-ol	Fruit, grass
6	6.713	2-Hydroxy-2-methyl propanale	
7	6.803	2,3-Butanediol	
8	7.297	Isopropanol	Slightly buttery
9	13.310	Dimethylsulfone	Sulfurous, burnt, hot milk
10	14.323	n.i.	
11	14.760	Benzaldehyde	Bitter almonds
12	15.173	n.i.	
13	16.737	Decane	
14	18.387	Limonene	Fruit, lemon
15	21.341	n.i.	
16	22.340	Undecane	
17	22.753	Nonanal	Flowers, citrus, orange, rose
18	23.903	2-Phenylethanol	Flowers, honey, rose
19	24.527	n.i.	
20	29.450	n.i.	
21	32.307	3-Ethyl-4-methyl-2,5-pyrrolidindione	
22	33.043	n.i.	
23	35.887	n.i.	
24	42.447	n.i.	
25	42.640	δ -Decalactone	Coconut, creamy, peach
26	46.340	Dodecanoic acid	Oil
27	54.310	Tetradecanoic acid	Wax, oil

¹Odor described in: Anon. 1998. Flavour and Fragrances, Aldrich, International Edition; Anon.

²n.i. = Not identified.

amplification of V3 region of the 16S rDNA and DGGE analysis. Figure 2 shows the eight typologies of pattern obtained out of the 29 NWC, the pattern of the milk as control, and of the curd derived from milk without adding NWC. The variability of NWC in matter of both number and electrophoretic mobility of bands was low. Eight bands were detected, differentially distributed in the 29 patterns (the software Phoretic 1 advanced was used for band detection). The number of bands for each profile ranged from 3 (NWC 2, 4, 7, 8, 9, 10, 13, 14, 16, 17, 19, and 27) to 7 (NWC1).

DNA extraction and DGGE analysis were applied to the curds obtained by using all the NWC and to the curd obtained from uninoculated milk. The DGGE profile of each curd was always identical to that shown by the corresponding NWC employed (data not shown). The profiles of the water-buffalo milk (Figure 2, lane M) and of the corresponding curd (Figure 2, lane C) were completely different from those of the NWC, showing bands migrating different distances in the gel.

Statistical Analysis

The GC analysis data were treated by PCA, CA, and MDS to evaluate distances among NWC. PCA was performed considering quantitative data of 11 isolated com-

pounds, excluding the substances that were not produced by at least five NWC as well as dodecanoic and tetradecanoic acids. The score plot of neutral volatile compounds produced by NWC is shown in Figure 3. All NWC were separated on the plane, although nine NWC (seven from Salerno and two from Caserta) were grouped in the left part of the plane as highlighted in Figure 3. The total variance accounted for was 53%, with a good discrimination among NWC from Salerno and Caserta provinces. In fact, 13 NWC from Salerno out of 17 were located on the left part of the plane, as well as 12 NWC from Caserta out of 9 analyzed were located on the right part of the plane.

Qualitative CA and MDS were performed considering only the presence or absence of each isolated compound; in this data processing all peaks were considered. For clustering and scaling purposes, concentrations of peak ≥ 0.01 mg/kg were coded as 1; concentrations of peak < 0.01 mg/kg were coded as 0. In Figure 4 the bidimensional-scaling plot of NWC is shown and, on the same plot, clusters arising from hierarchical CA are highlighted. MDS analysis confirmed definite differences in NWC from Salerno and Caserta provinces. As result of CA, three main clusters were obtained: clusters A and C (94 and 93% similarity level, respectively) including about all NWC from Salerno and cluster B (83% similarity

Figure 1. Whey extract chromatograms of representative natural whey cultures (NWC) from Salerno and Caserta provinces. IS1: methyl decanoate as first internal standard; IS2: isopropyl decanoate as second internal standard. A: control; B: NWC3; C: NWC10; D: NWC24; E: NWC29.

Table 2. Concentration (mg/kg) of neutral volatile compounds produced by natural whey cultures (NWC) from Salerno province during curd ripening.¹

Peak number	NWC								
	1	2	3	4	5	6	7	8	9
1	57.546 ± 7.10	9.055 ± 0.304	48.123 ± 1.058	14.898 ± 2.282	17.700 ± 1.949	50.617 ± 3.491	5.357 ± 1.35	11.074 ± 1.66	8.929 ± 0.33
2	0.061 ± 0.007	—	—	—	—	0.051 ± 0.003	—	—	—
3	1.056 ± 0.130	0.020 ± 0.001	0.695 ± 0.015	0.067 ± 0.010	0.214 ± 0.024	0.696 ± 0.048	—	0.025 ± 0.004	0.042 ± 0.00
4	0.122 ± 0.015	0.029 ± 0.001	0.135 ± 0.003	—	—	0.111 ± 0.008	—	—	—
5	—	—	—	—	—	0.032 ± 0.002	—	—	—
6	0.256 ± 0.032	0.099 ± 0.003	0.531 ± 0.012	0.314 ± 0.048	0.309 ± 0.034	0.334 ± 0.023	0.455 ± 0.115	0.073 ± 0.011	0.387 ± 0.01
7	0.328 ± 0.040	0.371 ± 0.012	0.531 ± 0.012	0.314 ± 0.048	0.309 ± 0.034	0.334 ± 0.023	0.455 ± 0.115	0.073 ± 0.011	0.387 ± 0.01
8	0.292 ± 0.036	0.230 ± 0.008	0.275 ± 0.006	0.155 ± 0.024	0.180 ± 0.020	0.165 ± 0.011	0.182 ± 0.046	0.339 ± 0.051	0.219 ± 0.00
9	0.308 ± 0.038	0.285 ± 0.010	0.265 ± 0.006	0.298 ± 0.046	0.165 ± 0.018	0.229 ± 0.016	0.153 ± 0.039	0.220 ± 0.033	0.262 ± 0.01
10	—	—	0.068 ± 0.001	—	—	0.041 ± 0.003	—	—	—
11	—	0.020 ± 0.001	—	0.041 ± 0.006	—	—	—	—	—
12	—	—	—	—	—	—	—	—	—
13	—	—	—	0.067 ± 0.010	—	—	—	—	—
14	—	—	—	—	0.031 ± 0.003	0.033 ± 0.002	—	—	—
15	—	—	—	—	—	—	—	—	—
16	—	—	—	0.077 ± 0.012	0.065 ± 0.007	0.066 ± 0.005	—	0.031 ± 0.005	—
17	—	0.021 ± 0.001	—	—	—	—	—	—	—
18	0.442 ± 0.054	0.027 ± 0.001	0.261 ± 0.006	—	0.094 ± 0.010	0.182 ± 0.013	—	—	—
19	—	—	0.329 ± 0.007	—	—	—	0.112 ± 0.028	—	—
20	—	—	0.117 ± 0.003	—	—	—	—	—	—
21	0.069 ± 0.008	0.061 ± 0.002	—	0.048 ± 0.007	0.031 ± 0.003	0.038 ± 0.003	—	0.042 ± 0.006	0.049 ± 0.00
22	—	0.023 ± 0.001	—	—	—	0.032 ± 0.002	—	0.026 ± 0.004	—
23	—	—	—	—	—	—	—	—	—
24	—	—	—	—	—	—	—	—	—
25	—	0.021 ± 0.001	—	—	—	—	—	0.030 ± 0.005	—
26	0.099 ± 0.012	0.063 ± 0.002	—	0.047 ± 0.007	0.093 ± 0.010	0.038 ± 0.003	—	0.034 ± 0.005	0.044 ± 0.00
27	0.193 ± 0.024	0.112 ± 0.004	—	0.078 ± 0.012	0.031 ± 0.003	0.066 ± 0.005	—	0.072 ± 0.011	0.086 ± 0.00

Table 2 (continued). Concentration (mg/kg) of neutral volatile compounds produced by natural whey cultures (NWC) from Salerno province during curd ripening.¹

Peak number	NWC							
	10	11	12	13	14	15	16	17
1	11.804 ± 0.57	89.048 ± 11.23	19.698 ± 1.93	7.657 ± 0.66	0.425 ± 0.18	5.348 ± 0.11	6.754 ± 0.48	20.072 ± 3.140
2	—	0.101 ± 0.010	—	—	—	—	—	—
3	—	0.377 ± 0.040	0.365 ± 0.03	—	—	—	—	0.183 ± 0.029
4	—	0.230 ± 0.020	—	—	—	—	—	0.042 ± 0.007
5	—	—	—	—	—	—	—	—
6	0.481 ± 0.02	0.636 ± 0.080	0.389 ± 0.03	0.402 ± 0.035	—	0.275 ± 0.006	0.182 ± 0.013	0.549 ± 0.086
7	0.481 ± 0.02	0.636 ± 0.080	0.389 ± 0.03	0.402 ± 0.035	—	0.275 ± 0.006	0.182 ± 0.013	0.549 ± 0.086
8	0.234 ± 0.01	0.358 ± 0.040	0.205 ± 0.02	0.199 ± 0.017	—	0.135 ± 0.003	0.057 ± 0.004	0.268 ± 0.042
9	0.233 ± 0.01	0.222 ± 0.020	0.173 ± 0.01	0.145 ± 0.013	0.055 ± 0.024	0.274 ± 0.006	0.174 ± 0.012	0.327 ± 0.051
10	—	—	—	—	—	—	—	—
11	—	—	—	—	—	—	—	—
12	—	—	—	—	—	0.044 ± 0.001	—	—
13	—	—	0.054 ± 0.00	—	—	—	—	—
14	—	—	—	—	—	0.040 ± 0.001	—	—
15	—	—	—	—	—	—	—	—
16	—	—	0.065 ± 0.00	—	—	0.070 ± 0.001	—	—
17	—	—	—	—	—	—	—	—
18	—	0.049 ± 0.000	0.077 ± 0.00	—	—	—	—	—
19	—	—	—	—	—	—	—	—
20	—	—	—	—	—	—	—	—
21	0.047 ± 0.00	0.042 ± 0.000	—	—	0.046 ± 0.020	—	—	0.072 ± 0.011
22	—	—	—	—	0.067 ± 0.029	—	—	—
23	—	—	—	—	—	—	—	—
24	—	—	—	—	0.049 ± 0.022	—	—	—
25	—	—	—	—	—	—	—	—
26	0.047 ± 0.00	—	—	—	—	0.094 ± 0.002	—	0.058 ± 0.009
27	0.073 ± 0.00	0.047 ± 0.000	0.055 ± 0.00	0.058 ± 0.005	0.095 ± 0.042	0.086 ± 0.002	0.063 ± 0.005	0.057 ± 0.009

¹Values are means of three experiments ± SD; — = absence of peak.

Table 3. Concentration (mg/kg) of neutral volatile compounds produced by natural whey cultures (NWC) from Caserta province during curd ripening.¹

Peak number	NWC					
	18	19	20	21	22	23
1	38.379 ± 3.539	19.859 ± 1.774	100.672 ± 25.001	79.716 ± 5.715	22.095 ± 4.000	9.126 ± 0.466
2	0.041 ± 0.004	—	0.092 ± 0.023	0.078 ± 0.006	—	—
3	0.321 ± 0.030	0.258 ± 0.023	1.587 ± 0.394	0.234 ± 0.017	0.520 ± 0.094	0.061 ± 0.003
4	0.071 ± 0.007	0.039 ± 0.003	0.422 ± 0.105	0.114 ± 0.008	0.139 ± 0.025	—
5	—	—	—	—	—	—
6	0.577 ± 0.053	0.459 ± 0.041	0.080 ± 0.020	0.057 ± 0.004	0.060 ± 0.011	0.044 ± 0.002
7	0.577 ± 0.053	0.459 ± 0.041	0.615 ± 0.153	0.376 ± 0.027	0.186 ± 0.034	0.454 ± 0.023
8	0.271 ± 0.025	0.299 ± 0.027	0.268 ± 0.067	0.417 ± 0.030	0.392 ± 0.071	0.354 ± 0.018
9	0.240 ± 0.022	0.251 ± 0.022	0.250 ± 0.062	0.313 ± 0.022	0.213 ± 0.039	0.281 ± 0.014
10	—	—	0.160 ± 0.040	0.052 ± 0.004	—	—
11	—	—	—	—	—	—
12	—	—	—	—	—	—
13	—	—	—	—	—	0.027 ± 0.001
14	—	—	—	—	—	—
15	—	—	—	—	—	—
16	—	—	—	—	—	0.050 ± 0.003
17	—	—	—	—	—	—
18	0.058 ± 0.005	0.033 ± 0.003	0.152 ± 0.038	0.062 ± 0.004	0.096 ± 0.017	0.029 ± 0.002
19	—	—	—	—	—	—
20	—	—	—	—	—	—
21	0.042 ± 0.004	0.043 ± 0.004	0.045 ± 0.011	0.073 ± 0.005	0.052 ± 0.009	0.053 ± 0.003
22	—	0.032 ± 0.003	—	—	—	0.031 ± 0.002
23	—	—	—	—	—	—
24	—	—	—	—	—	—
25	—	—	—	—	—	—
26	—	—	—	—	—	0.026 ± 0.001
27	0.041 ± 0.004	0.063 ± 0.006	—	0.046 ± 0.003	0.065 ± 0.012	0.074 ± 0.004

Peak number	NWC					
	24	25	26	27	28	29
1	58.574 ± 7.979	7.868 ± 0.666	23.504 ± 4.082	15.537 ± 3.371	33.243 ± 3.847	44.117 ± 2.564
2	0.062 ± 0.008	—	—	—	0.040 ± 0.005	0.048 ± 0.003
3	0.614 ± 0.084	0.066 ± 0.006	0.579 ± 0.100	0.788 ± 0.171	0.188 ± 0.022	0.447 ± 0.026
4	0.120 ± 0.016	—	0.073 ± 0.013	0.217 ± 0.047	—	0.094 ± 0.005
5	—	—	—	—	—	—
6	0.268 ± 0.036	0.083 ± 0.007	0.763 ± 0.133	0.084 ± 0.018	0.521 ± 0.060	0.019 ± 0.001
7	0.475 ± 0.065	0.419 ± 0.035	0.763 ± 0.133	0.173 ± 0.037	0.521 ± 0.060	0.616 ± 0.036
8	0.244 ± 0.033	0.207 ± 0.131	0.308 ± 0.096	0.156 ± 0.036	0.385 ± 0.222	0.285 ± 0.019
9	0.221 ± 0.030	0.250 ± 0.021	0.248 ± 0.043	0.237 ± 0.051	0.236 ± 0.027	0.195 ± 0.011
10	—	—	—	0.334 ± 0.072	—	—
11	—	—	—	—	—	—
12	—	—	—	—	—	—
13	—	—	—	—	—	—
14	—	—	—	—	—	—
15	0.053 ± 0.007	—	—	—	—	—
16	—	—	—	—	—	0.019 ± 0.001
17	0.141 ± 0.019	—	—	—	—	—
18	—	—	0.097 ± 0.017	0.141 ± 0.031	0.040 ± 0.005	0.107 ± 0.006
19	—	—	—	—	—	—
20	—	—	—	—	—	—
21	—	—	—	0.044 ± 0.010	0.041 ± 0.005	0.032 ± 0.002
22	—	—	—	0.034 ± 0.007	—	0.034 ± 0.002
23	—	—	—	0.032 ± 0.007	—	—
24	—	—	—	—	—	0.018 ± 0.001
25	—	—	—	—	—	—
26	—	—	—	—	—	0.018 ± 0.001
27	0.071 ± 0.010	0.070 ± 0.006	0.058 ± 0.010	0.056 ± 0.012	0.049 ± 0.006	0.045 ± 0.003

¹Values are means of three experiments ± SD; — = absence of peak.

Figure 2. Representative PCR-denaturing gradient gel electrophoresis profiles of natural whey cultures (NWC) from Salerno and Caserta provinces. Lanes: 1, NWC28; 2, NWC22; 3, NWC23; 4, NWC18; 5, NWC3; 6, NWC2; 7, NWC6; 8, NWC1; M, raw water-buffalo milk as control; C, curd obtained from raw water-buffalo milk without NWC addition.

level) comprising eight NWC from Caserta. Moreover, six single NWC clusters were distinguished.

A qualitative CA was performed for the DGGE profiles as well; according to the presence or absence of the bands within the patterns, the dendrogram depicted in Figure

Figure 3. Principal Component Analysis of neutral volatile compounds produced in raw water-buffalo milk by 29 natural whey cultures from Salerno (●) and Caserta (■) provinces.

Figure 4. Multidimensional Scaling and Cluster Analysis of 29 natural whey cultures from Salerno (●) and Caserta (■) provinces on the basis of their production of neutral volatile compounds in raw water-buffalo milk. Cluster A: 94% similarity level; Cluster B: 93% similarity level; Cluster C: 83% similarity level.

5 was obtained. At 70% similarity level, three main clusters were observed: 1) the cluster A including nine NWC all from the province of Caserta; 2) the cluster B comprising all the NWC from the province of Salerno together with NWC 19, 25, and 27 from Caserta; and 3) the cluster C only including NWC 1 and 6, both from Salerno.

DISCUSSION

This study dealt with the characterization of NWC for traditional water-buffalo Mozzarella cheese manufacture on the basis of their flavoring capabilities, bacterial composition, and geographical origin. A total of 27 components were isolated in the neutral extracts of NWC, and 18 of them were identified on the basis of electron impact spectra (five alcohols, three aldehydes, two ketones, two n-alkanes, two fatty acids, one sulfur compound, one nitrogen compound, one lactone, one terpene). The number of compounds isolated in this work is low if compared with other similar studies (Moio et al., 1993a, 1993b), most likely due to the different extraction methods employed. On the other hand, few volatile compounds were also found in other studies on dairy samples (Grosch et al., 1994; Preininger et al., 1996; Ben Lawlor et al., 2001).

Among 27 components isolated, we did not find esters, but this class of substances is generally extracted from dairy samples at very low concentration, indicating the

Figure 5. Simplified dendrogram showing the degree of similarity (%) of PCR-denaturing gradient gel electrophoresis profiles of the natural whey culture (NWC) analyzed in this study. NWC 1 to 17 are from the province of Salerno and NWC 18 to 29 are from the province of Caserta. Clusters A, B, and C are at 70% of similarity level. ¹The PCR-DGGE profiles of these NWC are depicted as representative in Figure 2.

importance of the extraction method. Alcohols were the main neutral volatile constituents of most of the extracts. A similar result was found in both water-buffalo milk and water-buffalo Mozzarella cheese by Moio et al. (1993a, 1993b), who reported an abundance of alcohols at least twofold higher than the other components. The 3-methyl-1-butanol (peak 3) and 2-phenylethanol (peak 18) were produced by most NWC and are considered odor-active volatile compounds (Moio et al., 1993b; Kotsieridis and Baumes, 2000). Moio et al. (1993b) reported 3-methyl-1-butanol as the most interesting among the alcohols found, having a pleasant aroma of fresh cheese. Furthermore, 2-phenylethanol and 3-methyl-1-butanol were reported as produced by some lactococcal and *Strep-*

tococcus thermophilus strains (Mauriello et al., 2001) and yeasts (Antonelli et al., 1999). On the other hand, heavy presence of yeasts, lactococci, and thermophilic streptococci in the microbial composition of NWC was reported by Coppola et al. (1988) and Ercolini et al. (2001b).

Surprisingly, in spite of the neutralization of the extracts, peaks identified as dodecanoic and tetradecanoic acids were shown by GC-chromatograms of 12 and 26 NWC extracts, respectively. This result could be due to an abundant production of these two acids and to an incomplete salification. This hypothesis has been confirmed by the results of free fatty acids analysis of some NWC extracts (data not shown). For this reason these

acids were excluded from the statistical analysis. As shown in GC chromatogram of the control, we found acetoin (most abundant substance), 2-3-butandiol, and dimethylsulfone to be most likely produced by the natural microflora of raw milk. Moio et al. (1993a), in the neutral extract of raw water-buffalo milk, found acetoin at a concentration only a little higher than other substances and did not find 2-3-butandiol and dimethylsulfone at all. Furthermore, the same authors (Moio et al., 1993b) found the acetoin as the most abundant substance and dimethylsulfone as the unique sulfur compound in the neutral extract of water-buffalo Mozzarella cheese. However, in contrast with our results, they did not find 2-3-butandiol. Finally, the NWC chromatograms, compared with the control, showed that the microflora of NWC was able to increase acetoin, 2-3-butandiol, and dimethylsulfone concentrations during curd ripening. Particularly, the acetoin concentration ranged from 0.425 mg/kg (NWC14) to 100.670 mg/kg (NWC20). Although it is well known that acetoin, together with diacetyl, plays an important role in the aroma of dairy products, Moio et al. (1993c) found only a trace impact of acetoin on water-buffalo Mozzarella cheese aroma.

The GC chromatograms of NWC 2 and 8 showed the peak of δ -decalactone (peak 25) at 0.021 and 0.030 mg/kg, respectively. The presence of this compound could be explained by lactonization of hydroxy acids, a chemical cycling caused by high temperature (Boldingh and Taylor, 1962). Therefore, we supposed that the NWC 2 and 8 were from dairy factories producing water-buffalo Mozzarella cheese by using heat-treated milk. In the NWC 2 and 4 extracts, 0.020 and 0.041 mg/kg of benzaldehyde (peak 11) were detected, respectively. This almond-like-odor aromatic compound is important for its impact, mainly due to its low odor threshold (Poll and Lewis, 1987). Similarly, nonanal (peak 17) was detected in the chromatograms from the NWC 2 and 24 extracts at 0.021 and 0.141 mg/kg, respectively. This substance was already found as strongly affecting the aroma of water-buffalo Mozzarella cheese, although detected at low concentration (Moio et al., 1993c). In fact, Poll and Lewis (1987) found an odor threshold of nonanal as 0.001 mg/kg.

The microbial composition of the NWC analyzed was investigated by a molecular PCR-DGGE approach. The scope of this analysis was only retrieving information about the diversity of the NWC in matter of species composition; for this reason, the identification of the bands was not performed. This molecular tool, combined to the statistical analysis, has been already shown as useful for the differentiation of dairy products in our previous works (Coppola et al., 2001; Ercolini et al., 2002). The low number of bands detected, if directly referred to the number of species occurring in the NWC,

is not in agreement with the wide spectrum of species revealed in previous works by a traditional cultivation-dependent approach (Coppola et al., 1988, 1990). On the other hand, the bias of culture-independent PCR-DGGE approach for describing the NWC microflora was already reported in a previous study (Ercolini et al., 2001b). However, the detected microbial diversity has allowed a satisfactory differentiation among the NWC analyzed. According to DGGE analysis, the fermentation of the milk, with the consequent metabolization of the nutrients and the production of aroma compounds, has been shown to be driven by the microflora of the NWC since the profiles obtained by the analysis of the curds were in every case identical to the profile of the NWC; furthermore, the profile of the milk did not match any of the NWC profiles, suggesting that none of the microbial species occurring in the milk was involved in the processing of Mozzarella cheese.

According to a statistical analysis, the distribution of the NWC on the basis of their flavoring capabilities clearly is related to the geographical origin based on both quantitative and qualitative data. However, quantitative analysis, performed taking into account the concentration of each volatile compound produced, was able to show a higher closeness among the NWC from Salerno (see Figure 3). Moreover, the NWC that were far from all the others and consequently located toward the edges of the plot were not the same in quantitative and qualitative interpretation of data. This result suggests that the analysis of quantitative data does affect the interpretation of the results; otherwise no difference in the distance among the NWC would be detected by using both approaches. The high similarity of the NWC from Salerno in the PCA plot can be explained by the fact that both the water-buffalo farms supplying the milk and the cheese factories are located in a geographically restricted area. On the contrary, farms and factories from Caserta are distributed in a larger geographical area. As a matter of fact, the higher similarity of the NWC from the province of Salerno might also arise from the usual custom of NWC exchanges among cheese factories of the same zone.

The CA of DGGE profiles showed a very clear separation of the NWC from the two different provinces. Moreover, all the NWC in each cluster were closely related, suggesting a low diversity occurring in the NWC. As a result of the statistical analysis applied to the GC data, the NWC 1, 3, 6, and 11 appeared separated from the other NWC from Salerno. In fact, in MDS as well as in PCA plot, they are located in the right part, among the NWC from Caserta. Interestingly, NWC 1 and 6 are isolated in the dendrogram of the DGGE profiles as well. NWC 23 and 25, both from Caserta, resulted in being separated from the other NWC from the same geographi-

cal area in PCA as well as MDS plot. Again, interestingly, NWC25 is related to NWC from Salerno even as result of the DGGE analysis.

The coherence found in the correlation between geographical origin and chromatographic as well as electrophoretic data does suggest a relationship between the microbial diversity of the NWC and their flavoring capabilities.

Although the NWC proved to be microbiologically related, the bands detected are related to at least eight different species. Therefore, the variability in volatile compounds appreciated in the GC profiles is probably due to a variability at strain level as well as to an accessory microflora undetectable by PCR-DGGE analysis. The difference between the two provinces could be attributed to the different ecological conditions (water-buffalo farm typology, feed, environment of the cheese factory, equipment, plant, etc.), probably selecting wild strains capable of specific metabolic pathways.

The characterization of the aromatic features of cheeses obtained in a restricted geographical area, owing to the reliability and the correlation to the zone of production, could be suggested for supporting the traceability criteria of typical products like water-buffalo Mozzarella cheese.

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