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Research Article

The microbiota of high-moisture mozzarella cheese produced with different acidification methods

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ABSTRACT

The microbiota of high-moisture Mozzarella cheese made from cow's milk and produced with different acidification methods was evaluated at the end of refrigerated storage by pyrosequencing of the 16S rRNA gene. The cheeses were clearly separated on the basis of the acidification methods. Cheeses produced with the addition of starters were dominated by *Streptococcus thermophilus*, but a variety of lactic acid bacteria and spoilage microorganisms appeared at low levels (0.01–1%). Cheeses produced by direct addition of citric acid were dominated by a diverse microbiota, including both lactic acid bacteria and psychrotrophic γ-proteobacteria. For five brands the acidification system was not declared on the label: the microbiota was dominated by thermophilic lactic acid bacteria (*S. thermophilus*, *Lactobacillus delbrueckii*, *Lactobacillus helveticus*) but a variety of other subdominant lactic acid bacteria, psychrotrophs and *Enterobacteriaceae* were present, with a diversity comparable or higher to cheeses produced by direct acid addition. This led to the conclusion that undefined starters were used for acidification. Both ordination methods and network analysis were used for the representation of beta-diversity: matrix cluster analysis, principal coordinate analysis and OTU networks uncovered different aspects of the microbial community structure. For three cheese brands both biological replicates (cheeses from different lots) and technical replicates (replicate cheeses from the same lot) were analyzed. Repeatability was acceptable for OTUs appearing at frequencies >1%, but was low otherwise. A linear mixed model showed that the starter system was responsible for most differences related to dairies, while difference due to psychrotrophic contaminants was more related to lot-to-lot variability.

1. Introduction

High-moisture Mozzarella cheese is a soft, unripened pasta filata cheese manufactured from cow's milk using a variety of acidification methods, including direct acidification by addition of citric acid, or natural acidification by addition of thermophilic defined or undefined strain starters, including natural whey or milk cultures (De Angelis and Gobbetti, 2011). In the direct acid addition process citric acid (or more rarely lactic acid) is added to pasteurized milk before rennet addition, and the curd is ready for stretching soon after coagulation (Faccia et al., 2009). Addition of acids must be declared by the producer. A variety of starter cultures are used for cultured high-moisture Mozzarella cheese. Complex undefined whey cultures or milk cultures are required by the standards of identity of Protected Designation of Origin water-buffalo Mozzarella cheese (De Filippis et al., 2014a; Ercolini et al., 2004; Ercolini et al., 2012) or Traditional Specialty Guaranteed cow's milk Mozzarella cheese (De Angelis and Gobbetti, 2011) and are used for traditional mozzarella cheese production (Coppola et al., 2006; de Candia et al., 2007; Parente et al., 1997). These cultures are dominated by *Streptococcus thermophilus*, but other thermophilic (*Lactobacillus delbrueckii*, *Lactobacillus helveticus*) and mesophilic (*Lactococcus lactis*, *Leuconostoc*, other lactobacilli) lactic acid bacteria (LAB) and *Enterococcus* are often present as subdominant organisms (Coppola et al., 2006; de Candia et al., 2007; De Filippis et al., 2014a; Ercolini et al., 2004; Ercolini et al., 2012; Parente et al., 1997). Defined starter cultures for cultured Mozzarella cheese usually include *S. thermophilus* alone, or in combination with *L. delbrueckii* subsp. *bulgaricus* or *L. helveticus* (De Angelis and Gobbetti, 2011), although more complex starters have been proposed (De Angelis et al., 2008), and the use of defined starters must be declared on the product label.

After production, high-moisture mozzarella cheese is packaged in liquid (water, whey, stretching water, brine (Faccia et al., 2013) and stored under refrigerated conditions. Because of its high moisture content (50–60%) and relatively high pH (>5.5), Mozzarella cheese

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has a short shelf life, which usually does not exceed 5 days at refrigeration temperature. Addition of coatings or of preservatives has been proposed to increase the shelf-life of Mozzarella (Del Nobile et al., 2009; Lucera et al., 2014; Sinigaglia et al., 2008).

Spoilage is often caused by proteolytic psychrotrophic microorganisms (Baruzzi et al., 2012); or by discoloration (Andreani et al., 2014; Nogarol et al., 2013), and members of the genus *Pseudomonas* have been found to dominate the spoilage association (Baruzzi et al., 2012).

High-throughput sequencing (HTS) of rRNA genes is being increasingly used in the study of microbial communities in cheese (De Filippis et al., 2014a; Delcenserie et al., 2014; De Pasquale et al., 2014a, 2014b; Dolci et al., 2014; Ercolini et al., 2012; Riquelme et al., 2015; Schornsteiner et al., 2014). Its unprecedented depth of analysis compared to other molecular methods (Ercolini, 2013) is extremely appealing and its use is significantly improving the understanding of the role of microorganisms in cheese. The objective of this work was to analyze the composition of the microbiota of commercial samples of high-moisture cow's milk Mozzarella cheese after refrigerated storage by using high throughput sequencing methods, in order to evaluate the diversity of starter and spoilage organisms in cheeses produced with different acidification methods. Moreover, analyses on replicate samples for the same lot and on different lots for three different products were used to evaluate the effect of dairy and lot on the occurrence of starter and spoilage organisms.

2. Materials and methods

2.1. Sampling

Samples (20 samples, named from A to N, Table 1) of high-moisture cow's milk Mozzarella cheese belonging to 14 different commercial brands were purchased in local supermarkets over one month shortly after delivery (<1 day) from cheese plants and were stored for 5 days at 10 °C before analysis. The shelf-life duration indicated on the packages varied between 5 and 20 days, and 5 days was the most frequent consume by date. Of the 14 brands, five were produced by industrial cheese plants and nine by artisanal cheese plants. For three brands (one industrial, A; two artisanal, C and F) cheeses were purchased in three different days during the sampling period. The commercial brands were chosen to be representative of large industrial cheese making plants (G, H) available on the Italian market, medium-sized industrial plants with a regional distribution (A, B, D, L) and small artisanal plants. The cheeses were produced using different acidification systems: five brands declared the use of citric acid, four the use of starter cultures, while for the remaining five brands no indication was provided on the label. However, interviews with the cheese-makers confirmed that for three (C, D, F) out of five brands for which no indication was provided undefined starters were used.

2.2. DNA extraction

Two individual cheeses (125 g) obtained from two packages were used for each sample. For the three brands for which three replicate samplings were carried out, cheeses from two packages were used separately for DNA extraction, while for all the other cheeses the two cheeses were pooled before extraction. Cheese samples (10–20 g) were aseptically homogenized (1:3) in 2% sterile sodium citrate using a Stomacher 400 Lab Blender (International PBI, Milan, Italy), 40 °C. The suspension was centrifuged (13,000 × *g*, 3 min) and the pellet was washed in the same solution to remove precipitated casein. MoBio Power food Bacterial DNA Extraction kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) was used as described in the manufacturer's instructions and a FastPrep®-24 Instrument (MP BIOMEDICALS) was used for the lysis step (speed 4, 3 × 20 sec). The purified DNA was stored at −80 °C until used.

2.3. Pyrosequencing

The microbial diversity was studied by pyrosequencing of the amplified V1–V3 region of the 16S rRNA gene by using primers Gray28F 5′-

Table 1
Number of sequences analyzed, observed diversity and estimated sample coverage for 16S rRNA amplicons obtained by pyrosequencing of microbial DNA extracted from high moisture Mozzarella cheese stored for 5 days at 10 °C. The average and standard deviation from ten replicate subsamples are indicated. The first letter of each sample indicates the cheese brand. The category of the cheese-making plant (artisanal or industrial), the use of citric acid (E330) and of defined starter, as declared in the label, are indicated.

Sample[1]	Sequences	Ind./art.	E330	Starter	Observed species	Shannon	Simpson	Chao1	Goods coverage
E	4354	A	1	0	106.9 ± 0.6	4.331 ± 0.008	0.893 ± 0.001	116.3 ± 1.4	0.9962 ± 0.0001
J	5533	A	1	0	111.0 ± 1.0	4.412 ± 0.008	0.886 ± 0.001	119.9 ± 1.3	0.9967 ± 0.0001
K	5670	A	1	0	112.4 ± 1.5	3.862 ± 0.011	0.856 ± 0.001	128.4 ± 2.2	0.9952 ± 0.0002
C1A	4020	A	0	0	104.4 ± 0.6	3.444 ± 0.016	0.771 ± 0.002	118.0 ± 1.7	0.9950 ± 0.0002
C1B	4404	A	0	0	99.3 ± 0.8	3.140 ± 0.009	0.734 ± 0.001	113.8 ± 2.7	0.9956 ± 0.0003
C2A	4219	A	0	0	78.8 ± 1.1	2.472 ± 0.017	0.641 ± 0.003	92.2 ± 1.1	0.9952 ± 0.0001
C2B	3220	A	0	0	72.9 ± 1.2	2.628 ± 0.013	0.675 ± 0.002	88.2 ± 3.9	0.9940 ± 0.0004
C3A	5150	A	0	0	64.7 ± 0.8	1.990 ± 0.008	0.552 ± 0.002	74.6 ± 1.6	0.9971 ± 0.0002
C3B	5035	A	0	0	71.9 ± 0.7	2.035 ± 0.021	0.552 ± 0.004	84.7 ± 1.8	0.9964 ± 0.0002
F1A	3486	A	0	0	101.6 ± 1.8	3.337 ± 0.020	0.755 ± 0.003	118.4 ± 2.7	0.9929 ± 0.0003
F1B	2762	A	0	0	99.2 ± 1.1	3.664 ± 0.015	0.809 ± 0.001	115.3 ± 3.0	0.9912 ± 0.0006
F2A	3897	A	0	0	145.5 ± 1.6	4.466 ± 0.019	0.862 ± 0.002	161.7 ± 2.3	0.9931 ± 0.0003
F2B	4194	A	0	0	144.9 ± 1.0	4.675 ± 0.014	0.907 ± 0.001	160.2 ± 2.4	0.9941 ± 0.0002
F3A	4705	A	0	0	121.8 ± 0.9	3.551 ± 0.019	0.756 ± 0.002	137.5 ± 2.3	0.9949 ± 0.0003
F3B	2502	A	0	0	74.0 ± 0.8	2.749 ± 0.015	0.634 ± 0.003	88.7 ± 3.2	0.9925 ± 0.0006
I	5527	A	0	0	126.0 ± 0.4	3.867 ± 0.010	0.822 ± 0.001	141.1 ± 1.9	0.9957 ± 0.0002
N	5197	A	0	0	117.4 ± 1.0	3.775 ± 0.021	0.793 ± 0.003	131.4 ± 2.3	0.9953 ± 0.0003
B	3768	I	1	0	92.6 ± 0.9	4.284 ± 0.010	0.912 ± 0.001	102.8 ± 2.1	0.9959 ± 0.0003
G	4767	I	1	0	40.5 ± 0.3	1.361 ± 0.007	0.379 ± 0.002	52.8 ± 3.8	0.9973 ± 0.0002
D	4881	I	0	0	96.1 ± 1.2	2.994 ± 0.016	0.715 ± 0.002	106.2 ± 2.1	0.9968 ± 0.0003
A1A	3808	I	0	1	73.0 ± 1.0	1.527 ± 0.018	0.314 ± 0.003	82.5 ± 1.8	0.9961 ± 0.0003
A1B	3982	I	0	1	79.7 ± 0.9	1.557 ± 0.018	0.321 ± 0.004	99.6 ± 2.2	0.9940 ± 0.0002
A2A	3982	I	0	1	32.8 ± 0.8	0.767 ± 0.012	0.179 ± 0.003	40.2 ± 1.7	0.9970 ± 0.0002
A2B	8580	I	0	1	66.6 ± 1.3	1.223 ± 0.012	0.304 ± 0.003	82.6 ± 3.4	0.9959 ± 0.0003
A3A	5169	I	0	1	90.9 ± 0.9	1.977 ± 0.008	0.420 ± 0.002	106.7 ± 3.0	0.9961 ± 0.0002
A3B	4672	I	0	1	98.5 ± 0.8	1.992 ± 0.014	0.411 ± 0.003	110.4 ± 3.1	0.9958 ± 0.0003
H	6567	I	0	1	20.0 ± 0.6	0.403 ± 0.008	0.094 ± 0.002	27.9 ± 2.0	0.9985 ± 0.0001
L	5675	I	0	1	66.9 ± 0.7	1.796 ± 0.011	0.425 ± 0.002	73.2 ± 1.4	0.9977 ± 0.0002

1 XyZ is used for coding. X, indicates the cheese-making plant. For plants C, F and A three samples were purchased on different days (y) and for each sample DNA extraction was performed in duplicate (Z) on two cheeses obtained from two packages of the same lot. Therefore A1A indicates cheese produced from plant A, first sampling day, first replicate extraction.