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Short communication: Identification and technological characterization of yeast strains isolated from samples of water buffalo Mozzarella cheese

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ABSTRACT

Sixty yeast cultures were isolated from samples of water buffalo Mozzarella, a popular "pasta filata" cheese, originating on 16 farms located in the provinces of Salerno, Caserta, and Frosinone (Italy). Strains were identified by means of 5.8S internal transcribed spacer rDNA PCR-RFLP combined with 26S rRNA gene partial sequencing and characterized for their ability to exert biochemical properties of technological interest. The recorded dominance of fermenting yeasts such as the lactose-fermenting *Kluyveromyces marxianus* (38.3% of the total isolates) and the galactose-fermenting *Saccharomyces cerevisiae* (21.6% of the total isolates) suggests that these yeasts contribute to the organoleptic definition of the water buffalo Mozzarella. The speciographic analysis revealed the presence of 7 other species rarely or never reported in a dairy environment belonging to the genera *Pichia* and *Candida*, whose role in Mozzarella cheese organoleptic properties need to be further investigated.
Key words: yeast community, *Kluyveromyces marxianus*, water buffalo Mozzarella cheese

Water buffalo Mozzarella cheese (**WBMC**) is a popular "pasta filata" cheese from southern Italy, manufactured in a traditional fashion from whole raw water buffalo milk using natural whey cultures as fermentation starters. This cheese is characterized by high moisture (55 to 62%) and high fat in dry matter (>45%), by a soft body and a juicy appearance, and by a pleasant, fresh, sour, and slightly nutty flavor. The manufacture has been described in detail in previous works (Coppola et al., 1988, 1990). Briefly, the cheese is made from whole, raw water buffalo milk by adding a natural whey culture (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 from Campania ("Mozzarella di Bufala Campana") has been endowed with the European Product of Designated Origin (PDO) certification. The production district of this cheese is defined in its law specifications and encompasses 7 provinces across 2 regions of southern Italy (Campania and Lazio).

Previous works demonstrated that the use of unselected starters during cheese making of Mozzarella allows the development of a complex and largely undefined microbial population, whose composition may be related to the manufacturing context and to the dairy's location within the production district (Coppola et al., 2001).

The dominant microflora of cheeses including WBMC comprises lactic acid bacteria (**LAB**) that are responsible for acid production and the ripening process. An important input to cheese maturation is that of the secondary microbiota, mainly comprising enterococci, micrococci, nonstarter LAB, and yeasts (Beresford et al., 2001). In recent years, yeasts have been increasingly considered as important agents in the maturation process of some cheeses (Eliskases-Lechner and Ginzinger, 1995; Mounier et al., 2005), but their contribution to cheese ripening remains unclear (Jakobsen and Narvhus, 1996; Addis et al., 2001). Recently, several studies performed on WBMC found that yeasts represent a significant part of the natural microflora, with total numbers ranging from 10^4 to 10^6 cfu/g (Coppola et al., 1988; Romano et al., 2001). In light of these observations, the aim of this study was the identification and technological characterization of yeasts isolated from samples of WBMC from 16 farms located in the provinces of Salerno (9 samples), Caserta (6 samples), and Frosinone (1 sample) in southern Italy (Table 1). Decimal dilutions of samples were performed in sterile solution of 0.9% (wt/vol) NaCl and inoculated in triplicate on WL nutrient agar (Oxoid, Basingstoke, UK). After incubation at 28°C for 72 h, yeast colonies from counting plates were sorted based on morphology and streaked on dichloran rose-bengal chloramphenicol agar

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Table 1. Yeast species and relative distribution in the 16 sampled farms (A to Q)

Province/farm	Yeast, log cfu/g	Strains, n	Strain[1]									
			SC	*KM*	*CB/A*	*CP*	*CS*	*CL*	*PC*	*PB*	*PN*	*PP*
Caserta												
A	4.4	4	1	3								
B	2.8	4	4									
C	1.9	2	2									
D	2.3	2				1			1			
E	2.8	1							1			
F	1.2	2	1							1		
Salerno												
G	3.9	6	1	5								
H	3.8	5		1	1			2			1	
I	2.4	3					1				2	
L	3.3	5	4			1						
M	4.1	5		5								
N	4.3	4		3								1
O	4.2	4		3							1	
P	3.9	5		2				2		1		
R	3.8	3		1			2					
Frosinone												
Q	4.2	5								5		
Strains, n		60	13	23	1	2	3	4	2	7	4	1
Prevalence, %			(21.60)	(38.30)	(1.70)	(3.30)	(5.00)	(6.70)	(3.30)	(11.70)	(6.70)	(1.70)

[1]*SC* = *Saccharomyces cerevisiae*; *KM* = *Kluyveromyces marxianus*; *CB/A* = *Candida butiry/aaseri*; *CP* = *Candida pararugosa*; *CS* = *Candida sorbophila*; *CL* = *Clavispora lusitaniae*; *PC* = *Pichia cactophila*; *PB* = *Pichia barkeri*; *PN* = *Pichia norvegensis*; *PP* = *Pichia pastoris*.

base (Oxoid) with chloramphenicol selective supplement.

The isolated yeasts were characterized by the 5.8S internal transcribed spacer (**ITS**) rDNA PCR-RFLP method developed by Esteve-Zarzoso et al. (1999) by using the restriction endonucleases *Cfo*I, *Hinf*I, and *Hae*III (Promega, Madison, WI). Extraction of DNA, PCR, and RFLP analysis were performed as previously reported (Aponte et al., 2010). Representative strains of each PCR-RFLP profile were identified at the species level by sequencing of the D1/D2 region of the 26S rRNA gene (Kurtzman and Robnett, 1997) according to a procedure previously described (Aponte et al., 2010).

Lipolytic activity was evaluated according to the procedure described by Fadda et al. (2004); for proteolytic activity, 2 protocols were used (Fadda et al., 2004; Gardini et al., 2006). The assimilation and fermentation of glucose, lactose, and galactose was assessed in yeast nitrogen base broth (Biolife Italiana, Milan, Italy) supplemented with 2% of each carbohydrate in test tubes containing Durham tubes. The assimilation of lactic acid and citric acid was evaluated according to Disegna et al. (1997).

Yeasts counts on WL agar ranged from 1 to 4 log cfu/g (Table 1), lower than those reported by other authors in WBMC (Romano et al., 2001). Sixty strains, isolated from WL agar plates seeded with the highest sample dilutions, were grouped according to the PCR-RFLP method developed by Esteve-Zarzoso et al. (1999); results are shown in Table 2. Ten PCR products, ranging from 380 to 800 bp, were obtained (Table 2). Digestion using 3 restriction endonucleases (*Cfo*I, *Hinf*I, and *Hae*III) presumptively confirmed the existence of 10 species, because strains characterized by the same ITS profile showed identical restriction patterns with all enzymes (Table 2). By sequencing the D1/D2 region of the 26S rRNA gene, 1 strain was assigned to the species *Pichia pastoris*, 4 to *Clavispora lusitaniae*, 2 to *Candida pararugosa*, 3 to *Candida sorbophila*, 2 to *Pichia cactophila*, 7 to *Pichia barkeri*, 4 to *Pichia norvegensis*, 1 to *Candida butiry/aaseri*, 23 to *Kluyveromyces marxianus*, and 13 to *Saccharomyces cerevisiae*, confirming previous presumptive identifications obtained by restriction analysis (Table 2). In agreement with evidence obtained by Romano et al. (2001) on WBMC, *K. marxianus* and *S. cerevisiae* represented the dominant species, with about 38.3% and 21.6% of the total isolates, respectively. The different species were not uniformly distributed in the samples (Table 1). Twenty strains of *K. marxianus* (out of 23) were recovered in samples originating in the province of Salerno. In the sample taken in Frosinone, the sole species retrieved was *P. barkeri* (Table 1). Compared to data reported by Romano et al. (2001), higher biodiversity was recorded in the present study; this inconsistency can be related to the number of factories sampled and to the wider geographical area surveyed in the present work.

With emphasis on the safety assessment of dairy hemiascomycetous yeasts, the occurrence of yeast spe-

Table 2. Result of 5.8S internal transcribed spacer (ITS) rDNA PCR-RFLP analysis by using 3 restriction endonucleases

ITS	*Cfo*I	*Hin*f	*Hae*III	Species	Strains[1]
380	A	I	a	*Pichia pastoris*	42 (1)
400	B	II	b	*Clavispora lusitaniae*	22, 25, 51, 52 (4)
440	C	III	c	*Candida pararugosa*	12, 34 (2)
450	D	IV	d	*Candida sorbophila*	28, 62, 63 (3)
480	E	V	e	*Pichia cactophila*	11, 13 (2)
490	F	VI	f	*Pichia barkeri*	14, 53, 56, 57, 58, 59, 60 (7)
500	G	VII	g	*Pichia norvegensis*	26, 27, 30, 49 (4)
640	H	VIII	h	*Candida butiry/aaseri*	23 (1)
700	I	IX	i	*Kluyveromyces marxianus*	1, 2, 3, 17, 18, 19, 20, 21, 24, 36, 37, 38, 39, 40, 41, 43, 44, 46, 48, 50, 54, 55, 61 (23)
880	L	X	l	*Saccharomyces cerevisiae*	4, 5, 6, 7, 8, 9, 10, 15, 16, 31, 32, 33, 35 (13)

[1]Underlined strains were identified at species level by sequencing the D1/D2 region of the 26S rRNA gene; number of isolates in parentheses.

cies in dairy environments has been reviewed recently (Jacques and Casaregola, 2008). With the exception of *K. marxianus*, *S. cerevisiae*, and *Cl. lusitaniae* (an emerging pathogen), none of the species retrieved in our samples was reported by Jacques and Casaregola (2008) as a usual component of the dairy microflora. *Pichia barkeri* has been frequently recovered in yeast communities of the cactus *Pilosocereus arrabidae* (Morais et al., 1994), but never in a dairy environment. In the present study, this species was found in 100% of the samples from Frosinone province. Equally, *P. cactophila* has only been associated with necrotic stems of cacti (Moraes et al., 2005). *Pichia norvegensis* has been isolated from humans and is considered to be an opportunistic human pathogen able to cause septicemia (Maxwell et al., 2003). Nevertheless, *P. norvegensis* has been recovered in other food ecosystems such as boza, a low-alcohol fermented beverage (Botes et al., 2007), and in other cheeses (Rohm et al., 1992; Westall and Filtenborg, 1998). Similarly, there is scarce information about the normal habitat of *C. pararugosa,* a species phylogenetically related to *Candida rugosa*, which has recently been recovered from commercial red wines (Jensen et al., 2009). *Kluyveromyces lactis*, *Debaromyces hansenii*, and *Yarrowia lipolytica*, yeast species commonly found in dairy products (Jacques and Casaregola, 2008), were not detected in the present study. Because of their intrinsic characteristics, *D. hansenii* and *Y. lipolytica* usually dominate the later stages of maturation in ripened cheeses (Gardini et al., 2006); even Romano et al. (2001) did not retrieve these species in WBMC. Nevertheless, the latter authors (Romano et al., 2001) recovered both *K. marxianus* and *K. lactis*; these species are closely related and share the same biochemical activities.

One of the objectives of this study was to investigate the ability of the isolated yeasts to exert properties of industrial interest and to contribute to the desirable

Table 3. Biochemical characteristics of technological interest for yeasts isolated from natural whey culture samples

	Strain[1]										
Characteristic	*SC*	*KM*	*CB/A*	*CP*	*CS*	*CL*	*PC*	*PB*	*PN*	*PP*	Positive strains, n
Lipolytic activity	2	21	1	2	2	4	2	6	4	1	45
Proteolytic activity											
Fadda et al. (2004)	7	16	1	1	2	2	0	3	1	1	34
Gardini et al. (2006)	0	3	0	0	2	0	0	0	0	0	5
Assimilation of:											
Glucose	13	23	1	2	3	4	2	7	4	1	60
Lactose	13	23	1	2	3	4	2	7	4	1	60
Galactose	13	23	1	2	3	4	2	7	4	1	60
Fermentation of:											
Glucose	13	23	1	0	0	4	0	6	2	0	49
Lactose	0	23	0	0	0	0	0	0	0	0	23
Galactose	12	23	0	0	0	4	0	0	0	0	39
Assimilation of:											
Lactate	11	23	1	2	3	3	2	7	3	1	56
Citrate	3	13	1	0	0	2	0	1	3	1	24
Strains, n	13	23	1	2	3	4	2	7	4	1	60

[1]*SC = Saccharomyces cerevisiae*; *KM = Kluyveromyces marxianus*; *CB/A = Candida butiry/aaseri*; *CP = Candida pararugosa*; *CS = Candida sorbophila*; *CL = Clavispora lusitaniae*; *PC = Pichia cactophila*; *PB = Pichia barkeri*; *PN = Pichia norvegensis*; *PP = Pichia pastoris.*

organoleptic characteristics of the fermented product. All species showed the ability to assimilate glucose, lactose, and galactose, whereas the ability to ferment these carbohydrates was limited to a lower number of isolates: 49, 39, and 23 strains out of 60 were able to ferment glucose, galactose, and lactose, respectively (Table 3). The ability to ferment lactose, in agreement with current knowledge (Suleau et al., 2006; Fonseca et al., 2008), is limited to isolates of the species *K. marxianus* and *K. lactis*. The assimilation of lactate was widely held among species, whereas the assimilation of citrate was restricted to a few strains in the case of *S. cerevisiae* (3 of 13). Lipolytic activity was widespread in *K. marxianus* strains (22 out of 23), but infrequent in *S. cerevisiae* (2 of 13). Results regarding proteolytic activity appeared to be greatly affected by the detection method: only 5 of the total isolates were able to hydrolyze proteins according to the procedure described by Gardini et al. (2006), whereas the load of proteolytic strains increased up to 34 on plate count agar plus skim milk (Fadda et al., 2004). Only some strains of *K. marxianus* and *C. sorbophila* exhibited proteolytic activity with the 2 procedures used (Fadda et al., 2004; Gardini et al., 2006; Table 3). None of the unusual species recovered in the WBMC environment displayed specific activities expected from cheese yeasts (Table 3); therefore, their presence may be considered contamination. The dominance of fermenting yeast such as the lactose-fermenting *K. marxianus* and the galactose-fermenting *S. cerevisiae* could significantly contribute to the organoleptic profile of the final product by producing CO_2 as well as precursors of flavoring compounds, such as ethanol, acetaldehyde, and ethyl acetate (Dahl et al., 2000).

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