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Updating on the fungal composition in Sardinian sheep's milk by culture-independent methods

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This work applies culture-independent methods for the characterization of fungal populations (yeasts and moulds) naturally occurring in Sardinian ewe's milk sampled in the Italian areas with the largest dairy production (Sardinia and Lazio regions). Sequences of the D1/D2 variable domains at the 5′ end of the 26S rRNA gene were obtained by amplification of DNA directly isolated from milk, and this allowed identification of a total of 6 genera and 15 species of fungi. Among the 6 identified genera *Geotrichum* spp., *Candida* spp., *Phaeosphaeriopsis* spp., *Pestalotiopsis* spp. and *Cladosporium* spp. belong to the phylum of Ascomycota, while *Cryptococcus* spp. is part of the phylum of Basidiomycota. In particular, two genera (*Pestalotiopsis* and *Phaeosphaeriopsis*) and two species (*Plectosphaerella cucumerina* and *Pryceomyces carsonii*) have never been reported in dairy ecosystems before. Results provide evidence that several moulds and yeasts, previously described only in ovine cheeses, are transferred directly from raw milk. The knowledge of fungal consortia inhabiting sheep raw milk is a particularly relevant issue because several species are directly involved in cheese making and ripening, determining the typical aroma. On the other hand, spoilage yeasts and moulds are involved in anomalous fermentation of cheese and may be responsible for considerable economic losses and serious risks for consumers' health.

Keywords: Fungi, yeasts, moulds, milk, 26S rRNA.

In recent years, mycobiota have been increasingly acknowledged as important agents in the maturation of cheese. Indeed, the composition of the fungal communities significantly influences sensorial and organoleptic characteristics thanks to their peculiar metabolic properties. During the initial stages of ripening, yeasts metabolise lactic acid and increase the pH, thus enabling growth and metabolic activities of less acid tolerant bacteria (Mounier et al. 2008). Moreover, the lysis of yeast cells liberates vitamins and amino acids and provides flavour precursors (free amino acids and fatty acids) that may contribute significantly to the cheese flavour. During the same stage, a fungal antilisterial activity has been supposed (Goerges et al. 2006) as well as the inhibition of spore-forming bacteria by competitive or symbiotic interactions (Fatichenti et al. 1983).

Yeasts and moulds naturally occur in high numbers in sheep dairy products; nevertheless information on native communities inhabiting raw milk is still limited. Given that the majority of sheep's milk products in Italy is transformed into cheese using traditional procedures, we assume that the production site is important in providing them with definite and typical characteristics. The frequent use of raw milk in most cheeses represents a further, strong contribution to the myco- and microflora diversity of these products: the indigenous microbial communities inhabiting raw milk strongly influence the characteristics of the final product, exalting its peculiarities mainly related to the geographical origin (Delbès et al. 2007; Giannino et al. 2009). During the last decade, several fungi have been identified in sheep cheeses (Pereira-Dias et al. 2000; Cosentino et al. 2001; Fadda et al. 2004; Capece & Romano, 2009) but updated information on indigenous communities occurrence in milk is limited.

In addition to the described natural beneficial properties, several fungi widely distributed in the environment, in equipment of dairies or inhabiting raw milk can act as spoilage organisms of dairy products, with various spoilage effects. For example various species are potential aetiologic agents of mastitis, an inflammatory reaction of the udder that has undesirable consequences for cheese making and, consequently, for the quality of the dairy products (Crawshaw et al. 2005). In particular, they release abundant extracellular

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enzymes such as lipases and proteinases that modify the technological properties of milk, and therefore its suitability for dairy processing into cheese. Furthermore, some yeasts may be responsible for production of gas, alcohol and undesirable aromatic compounds leading to flavour and texture defects of cheese. Finally, other species are harmful to human health (Oliver et al. 2005).

In Italy, more than 400 000 tonnes of sheep's milk are produced annually and this milk is used for the production of numerous PDO cheeses (Pirisi et al. 2011). It is manifest that in order to avoid technological dairy problems, economic losses and risks to human health, a sound and reliable knowledge of the fungal communities inhabiting milk is highly desirable.

This work is a contribution to a more complete description of the mycoflora inhabiting raw sheep's milk sampled in the Italian Sardinia and Lazio regions. Yeasts and moulds were identified by direct genomic DNA amplification of the variable D1/D2 domain of the 26S rRNA gene bypassing conventional microbiological methods, that require selective enrichments and sub-culturing and could fail to detect some fungi, thus weakening the exhaustive characterisation of the community.

Materials and methods

Sampling

Thirty six bulk raw milk samples were collected monthly (March to May) in the Spring 2012 from 12 farms, 4 of which were located in Southern Sardinia, 4 in the North of Sardinia and 4 in the Lazio region (Centre of Italy). Once collected, samples were immediately frozen and stored at − 20 °C until analysis.

DNA extraction

Aliquots of bulk raw milk (40 ml-each) were thawed at 40 °C for 1 h and centrifuged at 1000 ***g*** for 10 min at 4 °C. The pellet was separated from the supernatant and recovered with 1 ml lysis buffer (10 mmol/l TRIS–HCl [pH 7·5], 1 mmol/l EDTA, 51 mmol/l NaCl, 2 mg/ml SDS). Each suspension was incubated with 120 U lyticase (Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) on a linear shaker at 37 °C for 2 h, and then 50 µl of a 1 mg/ml proteinase K solution (Fermentas Life Sciences, Burlington, Ontario, Canada) was added on a linear shaker, at 42 °C for 6 h. DNA was isolated by phenol/chloroform extraction, followed by ethanol precipitation. In detail, the lysate was extracted twice with an equal volume of phenol, then once with an equal volume of phenol and chloroform, and finally once with one volume of chloroform, always performing a centrifugation step at 11 000 ***g*** for 15 min at 4 °C after each extraction. DNA was precipitated by centrifugation (14 000 ***g*** for 30 min at 4 °C) after adding 0·1 volumes of sodium acetate (pH 5·2) and 2·5 volumes of ethanol cooled at − 20 °C. The pellet was washed gently with 300 µl 70% ethanol and centrifuged at 9000 ***g*** for 2 min. DNA pellet was finally suspended in 100 µl sterile and acid nucleic-free water. DNA concentration and purity were measured using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA).

Amplification and sequencing

Amplifications were assembled in a total volume of 30 µl that contained: 10 mM Tris-HCl pH 8·3, 50 mM KCl, 0·2 mM dNTPs, 2·5 mM $MgCl_2$, 1 µM of each primer, 3·5 U hot start. Taq polymerase (Euroclone, Milan, IT), and about 100 ng template genomic DNA. Primers NL1 (GCATATCAATA AGCGGAGGAAA) and NL4 (GGTCCGTGTTTCAAGA CGG) targeting the variable D1/D2 domains located at the 5′ end of the 26S rRNA gene (Kurtzman & Robnett, 1997) were added at a final concentration of 0·2 µM. All reactions were performed on a Bioer LifePro thermal cycler (Bioer Technology Co, Ltd, Tokyo, Japan); amplification parameters comprised an initial denaturation step (95 °C 10 min) followed by 30 cycles at 94 °C 30 s, 60 °C 30 s, 72 °C 1 min, and by a final elongation (72 °C 10 min). Amplicons were visualised on 2% TBE agarose gel stained with ethidium bromide, followed by ultraviolet exposure. The bands were excised, purified using a Nucleospin® Extract II (Macherey Nagel, Düren, Germany) and sequenced in both directions on a 310 ABI PRISM automated DNA sequencer (Life Technologies, Foster City, CA) using NL1 and NL4.

Sequence analysis

After checking the electropherograms using the Bioedit program (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html) (Hall, 1999), sequences were subjected to BLAST searches (www.ncbi.nlm.nih.gov/BLAST) and, when necessary, aligned by means of the ClustalW2 algorithm (http://www.ebi.ac.uk/Tools/msa/clustalw2/). In some cases, from a single PCR of milk samples we obtained two sequences corresponding to different microorganisms.

Results and discussion

Culture-independent methods were applied for the characterisation of fungal species inhabiting sheep bulk raw milk samples from the Italian geographic areas with the largest dairy production. Sequences of the D1/D2 variable domains located at the 5′ end of the 26S rRNA gene, obtained by amplification of DNA directly isolated from milk, allowed identification of a total of 6 genera and 15 species of fungi (Table 1). Only the sequences showing the highest similarity in terms of closest relative species and per cent of identity (97 to 100%) were aligned with reference sequences by ClustalW to confirm BLAST results. Among the 6 identified genera *Geotrichum* spp., *Candida* spp., *Phaeosphaeriopsis* spp., *Pestalotiopsis* spp. and *Cladosporium* spp. belong to

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Table 1. Fungal communities in raw sheep's milk sampled at North of Sardinia (A), Southern Sardinia (B) and Lazio (C). Identification was based on D1/D2 region sequence of the 26S rRNA gene. Multiple sequences have been sometimes obtained from single PCR of bulk milk samples ($n = 36$)

Sites of grazing	Identification	Sequences (n)	GenBank ID	Similarity (%)
A, B, C	*Galactomyces geotrichum* (anamorph†: *Geotrichum candidum*)	9	JF701182	100
C	*Geotrichum* spp.	4	EU360776	100
C	*Galactomyces candidum*	1	JN_974273	100
A	*Candida* spp.	1	AM420304	99
B, C	*Kluyveromyces marxianus* (anamorph: *Candida kefyr*)	5	JN938927	100
A, C	*Yarrowia lipolytica* (anamorph: *Candida lipolytica*)	2	JQ672588	99
C	*Meyerozyma guilliermondii* (anamorph: *Candida guilliermondii*)	1	KC494715	98
B	*Debaryomyces hansenii* (anamorph: *Candida famata*)	1	KF214439	99
C	*Wickerhamomyces anomalus*‡ (anamorph: *Candida beverwijkiae*	1	KC494721	100
B	*Rhodotorula mucilaginosa*	1	GU373744	100
B	*Phaeosphaeriopsis* spp.	1	JX401986	100
C	*Pestalotiopsis* spp. (teleomorph: *Pestalosphaeria* spp.)	1	JF773635	97
A, B	*Plectosphaerella cucumerina* (anamorph: *Fusarium tabacinum*)	2	U17399	100
B	*Priceomyces carsonii*	5	JX456534	100
B	*Cladosporium* spp.	1	JQ732937	99
A, C	*Cladosporium herbarum* (teleomorph§: *Davidiella tassiana*)	4	KC311478	99
A	*Cladosporium cladosporioides*	1	KC585410	100
B	*Gibberella zeae* (anamorph: *Fusarium graminearum*)	1	XM_389605	100
C	*Cryptococcus* spp. (teleomorph: *Filobasidiella*)	1	KC442264	100
B	*Cryptococcus magnus*	2	JX129907	100
B	*Cryptococcus curvatus*	1	AJ555468	100
C	Soil fungus DM2-009	2	AB438625	100

†Anamorph: asexual form.
‡Equiprobable species: *W. subpelliculosa* (JX049439).
§Teleomorph: sexual form.

the phylum of Ascomycota, while *Cryptococcus* spp. is part of the phylum of Basidiomycota.

Cosmopolitan fungal populations in raw sheep's milk

Among the most abundant genera/species, *Galactomyces geotrichum* was retrieved in all the sites of grazing (Table 1). Indeed, *Gal. geothricum* is a species with a worldwide distribution and its anamorph, *Geotrichum candidum,* is usually associated with dairy products, together with the yeast *Debaryomyces hansenii*. *Geo. candidum* plays an important role during the ripening of cheese for its contribution to the flavour, that is the result of metabolic activities such as glycolysis, lipolysis and proteolysis. For this reason it is widely used as an adjunct culture during the ripening process (Boutrou & Guéguen, 2005). It has been found in Italian sheep's cheeses such as Pecorino, Feta and Ricotta and Fiore Sardo (Cosentino et al. 2001; Fadda et al. 2004). The results of the present paper evidence that this yeast may be transferred in cheese directly from the raw milk.

A group of fungi representing the sexual forms of *Candida* genus is represented by five species, already described in various cheeses and here reported for the first time in raw milk. The most frequent fungus belonging to this group is *Kluyveromyces marxianus* retrived in Southern Sardinia and in Lazio. Many strains of this dairy yeast were discovered in a large variety of habitats and with a notable metabolic diversity, showing that the potential biotechnological applications of this yeast can be numerous (Fonseca et al. 2008). Another sexual form of the *Candida* genus, *Yarrowia lipolytica,* has been found in milk samples from Northern Sardinia and Lazio. Previous studies show the presence of this yeast in relevant sheep's cheeses as Feta, Pecorino and Caciotta (Cosentino et al. 2001; Gardini et al. 2006) and its contribution to cheese ripening has been confirmed (Lucia et al. 2001). Another yeast, *Meyerozyma guilliermondi* previously named *Pichia guilliermondii*, has been isolated from the French Salers and from the Italian Taleggio cheeses (Callon et al. 2006; Giannino et al. 2011). The antifungal activity of this yeast in dairy products could represent a natural agent for the biological control of pollutants, as shown in a recent study (Coda et al. 2013).

Wickerhamomyces anomalus, also known as *Pichia anomala* or *Hansenula anomala*, is able to propagate in a wide range of environments for its capacity to grow on various carbon sources, at low pH, under high osmotic pressure and with little or no oxygen. This yeast, frequently associated with food processing, may contribute to aroma through the production of volatile compounds (Fleet, 2003). The last species belonging to the group of the sexual forms of *Candida* spp. is *Debaryomyces hansenii*, a popular yeast widely diffused in natural ecosystems and manufactured foodstuff. Only rarely has it been reported in cow's raw milk, whereas it is one of the species most frequently retrieved in

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many cheeses, where its proteolytic and lipolytic activities provide positive attributes during ripening (Lopandic et al. 2006).

Cladosporium herbarum was retrieved in North of Sardinia and Lazio. *Cladosporium* is a mould that grows on different plant substrates; the genus includes more than fifty species that are difficult to distinguish, and can cause allergies. In general, the presence of *Cladosporium* represents a potential risk for human health especially in the agricultural environment, and may result in respiratory and subcutaneous phaeohyphomycosis (Millner, 2009). The species *C. herbarum* was found in Northern Sardinia and Lazio region. It occurs in abundance on dead leaves of herbaceous and woody plants present in pastures. It has frequently been isolated from air, food, paints, fabrics and many other substrates. It seems to be involved in 'thread mould', a defect which occurs sporadically in maturing vacuum-packaged Cheddar cheese (Hocking & Faedo, 1992). The defect is caused by the growth of fungi in the folds and wrinkles of the plastic film in which the cheese is packaged. *C. cladosporioides*, retrieved only in a bulk milk from a flock grazing in the North of Sardinia and recently isolated from a brine solution used for salting cheese (Panelli et al. 2012), is a fungal plant pathogen that affects wheat and contaminates many types of seeds.

Gibberella zeae, discovered in bulk milk from a flock grazing in Southern Sardinia and also known as *Fusarium graminearum*, is a plant pathogen which causes fusarium head blight, a devastating disease of wheat and barley with a worldwide distribution (McMullen et al. 1997). This mould produces two mycotoxins named deoxynivalenol (DON) and zearalenone that have been linked to feed refusal and toxicoses in livestock and also present a danger to human safety when present in milk. Concerning the other species listed in Table 1, *Cryptococcus magnus* and *Cryptococcus curvatus* already retrieved in goat milk by Callon et al. (2007). *Cryptococcus* genus includes species widely distributed in nature, some of which deserve particular attention as aetiological agent of cryptococcosis in immunocompromised patients.

Newcomers in the dairy environment

This work proved for the first time the presence in raw milk of four fungi: *Pestalotiopsis spp.* In Lazio and *Phaeosphaeriopsis spp., P. cucumerina* and *P. carsonii* in Sardinia. The *Pestalotiopsis* genus is common in tropical and temperate ecosystems and its species (about 20) are plant pathogens, historically named according to the host from which they were first observed. Endophytic strains of *Pestalotiopsis*, together with some pathogenic strains, have been found to produce more than 130 secondary metabolites useful for medicinal, agricultural and industrial applications (Maharachchikumbura et al. 2011). Two strains isolated from the medicinal plant 'espinheira santa' have demonstrated the capacity to inhibit the growth of *Escherichia coli, Klebsiella pneumoniae, Micrococcus luteus* and *Staphylococcus aureus* (Gomes-Figueiredo et al. 2007). As well as in the sheep's milk (see this work), the genus *Phaeosphaeriopsis* has been recently isolated in the deep-sea from the South China Sea (Zhang et al. 2013), and from soybean in Brazil, with an a hypothesised role in the biological control of soybean pathogens (de Souza Leite et al. 2013).

P. cucumerina is well-known as a pathogen of several plant species causing fruit, root and collar rot. This fungus was isolated from egg masses of the plant parasitic nematode *Meloidogyne hapla* and investigated for its ability to control the potato cyst nematode (Atkins et al. 2003). Sporadic cases of fungal keratitis caused by its anamorph form, *Fusarium tabacinum,* have been observed (Kamada et al. 2012).

P. carsonii also known as *Debaryomyces carsonii* is one of the five species belonging to the genus *Priceomyces*. It was isolated in red wine after long fermentative maceration (GenBank: JX456534) and in dry-cured meat products (Andrade et al. 2006).

This group of fungi appears particularly interesting and as such deserves further studies in relation to human health and to the fact that the majority of ovine cheeses are produced from raw milk (Pirisi et al. 2011). A further molecular and biochemical characterisation would thus be highly desirable in order to elucidate their properties as well as the potential consequences on cheese making and benefits/risks for the consumer.

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