

## Diversity of yeasts and moulds in dairy products from Umbria, central Italy

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### Abstract

In this research communication we report on the diversity of yeast and mould species in 69 samples of milk and different dairy products from three plants located in Umbria, central Italy. Isolates were characterised both macroscopically and microscopically and then identified by PCR and genome sequencing of the ITS region and the D1–D2 domain of the large-subunit rRNA gene for filamentous fungi and yeasts, respectively. Out of the 69 samples analysed, 51 (73.9%) tested positive for the presence of yeasts, whereas moulds were detected in 25 (36.2%) samples. A total of 9 yeast species belonging to 8 different genera and 13 mould species belonging to 6 different genera were isolated. The most common genera isolated were *Debaryomyces* and *Kluyveromyces* among the yeasts and *Penicillium* and *Galactomyces* among the moulds. Microbiota play a key role in the formation of flavour, aroma, texture and appearance of dairy products. This complex microbial ecosystem includes both cultured and external bacteria, yeasts and moulds. Some of them have an important role in the production of cheeses, whereas others are responsible for dairy product spoilage, resulting in significant food waste and economic losses. Some species can produce mycotoxins, representing a potential hazard for the consumer's safety. This study provides interesting information on the diversity of fungi species in dairy products from central Italy that can be of major importance to identify these products and to develop adequate strategies for fungal spoilage control and consumer safety.

**Keywords:** Dairy products; diversity; moulds; yeasts

Because of their physical and chemical characteristics (pH,  $a_w$ , redox potential), dairy products provide a favourable habitat for the growth and survival of many microorganisms including fungi (Garnier et al., 2017). Determining the fungal microbiota is useful to improve production processes, dairy product quality and consumer safety. The variety and number of fungi in dairy products together with the microbial quality of the milk used, the heat treatment, the production and the ripening conditions, all vary widely. Some yeasts and moulds play a major role in the production of certain cheeses and in developing their flavour and aroma (Mounier et al., 2008). The most familiar examples are Camembert, Roquefort, Stilton and Gorgonzola, also known as blue cheese, made from two species of *Penicillium*, *P. camemberti* in Camembert cheese and *P. roqueforti* in other blue cheeses. In contrast, yeasts and moulds can also cause dairy product spoilage (Banjara et al., 2015).

According to the literature, the main genera involved in dairy product spoilage are *Galactomyces* and *Yarrowia* among yeasts and *Penicillium*, *Mucor* and *Cladosporium* among moulds, respectively (Pitt and Hocking, 2009). Contaminations can originate from the dairy-plant environment, including not only surfaces, air and the operators' equipment, but also from raw milk and other ingredients (Kure et al., 2004). Fungal spoilage of cheeses can be invisible, when it is due to fungal metabolism, resulting in the production of gas and catabolites, which leads to undesirable odours and flavours and texture alteration. On the contrary, when spoilage is linked to the growth of fungi (colony or thallus), it is clearly visible, e.g. so-called 'cat hair' (*Mucor* spp.) or 'toad skin' (*Galactomyces geotrichum*) (Ledenbach and Marshall, 2010). Furthermore, some fungi species are opportunistic pathogens and can represent a risk for some consumer categories (Jacques and Casaregola, 2008), and several mould species (*Aspergillus* spp. and *Penicillium* spp.) can produce mycotoxins (Huis in't Veld, 1996).

In this study, we aimed to analyse yeast and mould species in milk and dairy products from three plants located in Umbria, central Italy. Samples included raw milk, curds, fresh cheeses, medium and long-aged cheeses from bovine and ovine origin. Isolates were characterised based on macro- and microscopic aspect of colonies and subsequently identified by PCR and genome sequencing.

### **Materials and methods**

A total of 69 samples were taken from 3 dairy-plants located in Umbria, central Italy, over a period of 4 months (from March to June 2018). The following product categories were analysed: bovine and ovine raw milk (12 samples), curd (11 samples), cream cheese (10 samples), fresh cheese (12 samples), aged cheese at the medium and high end of the spectrum (24 samples). Samples were collected in sterile containers and immediately transported to the laboratory in a refrigerated container for subsequent analysis. The analysis procedure followed the ISO regulation number 21527-1 for samples with a water activity of over 0.95 and the ISO regulation number 21527-2 for samples with a water activity below or equal to 0.95. The actual procedure is described in the online

Supplementary File. Isolates were preliminarily characterised at the genus level, using phenotypic methods, including macro- and microscopic observations. The isolates morphology was studied macroscopically by observing the colony features (colour, shape, size and hyphae), and microscopically by a compound microscope with a digital camera using a lactophenol cotton blue (LCB) stained slide mounted with a small portion of the mycelium (conidia, conidiophores and arrangement of spores for moulds, cells shape for yeasts). Isolates were identified by PCR and genome sequencing of the ITS region and the D1–D2 domain of the large-subunit rRNA gene for filamentous fungi and yeasts, respectively. The primers, amplification condition and sequencing method used are described in the Supplementary File.

## Results

The presence of filamentous fungi (moulds) was detected in 25 out of 69 samples (36.2%) for a total of 32 isolates belonging to 6 genera (5 from *Ascomycota* phylum and 1 from *Mucoromycotina* subphylum) and 13 species. Among *Ascomycota*, *Penicillium* was the most frequently isolated genus (18 isolates, 26.1%; 6 *P. oxalicum*, 5 *P. camemberti*, 3 *P. fuscoglaucum*, 1 *P. aurantiocandidum*, 1 *P. brevicompactum*, 1 *P. commune*, 1 *P. rugulosum*) followed by *Galactomyces* (7 isolates, 10.2%; all isolates were identified as *G. candidum*) and *Cladosporium* (3 isolates, 4.4%; 2 *C. cladosporoides* and 1 *C. asperulatum*). Only 1 isolate (1.5%) was identified as *Didymella pinodella* and another 1 (1.5%) as *Fusarium oxysporum*. *Penicillium* specimen were isolated mainly from hard cheese (14 out of 18) while *Galactomyces candidum* was isolated only from milk samples. Among *Mucoromycotina*, the only species isolated was *Mucor racemosus* (2 isolates, 2.9%); both specimen were isolated from hard cheese (Table 1).

**Table 1.** Diversity of moulds isolated from dairy products

Species	Raw milk <sup>a</sup>	Curd	Cream cheese	Fresh cheese	Hard cheese	Total
<i>Cladosporium asperulatum</i>	–	–	–	–	1	1 (1.5%)
<i>Cladosporium cladosporoides</i>	1	–	–	–	1	2 (2.9%)
<i>Didymella pinodella</i>	–	–	–	1	–	1 (1.5%)
<i>Fusarium oxysporum</i>	1	–	–	–	–	1 (1.5%)
<i>Galactomyces candidum</i>	7	–	–	–	–	7 (10.2%)
<i>Mucor racemosus</i>	–	–	–	–	2	2 (2.9%)
<i>Penicillium aurantiocandidum</i>	1	–	–	–	–	1 (1.5%)
<i>Penicillium brevicompactum</i>	–	–	–	–	1	1 (1.5%)
<i>Penicillium camemberti</i>	1	–	–	–	4	5 (7.3%)
<i>Penicillium commune</i>	–	–	–	–	1	1 (1.5%)
<i>Penicillium fuscoglaucum</i>	–	–	–	–	3	3 (4.4%)
<i>Penicillium oxalicum</i>	–	1	–	1	4	6 (8.7%)
<i>Penicillium rugulosum</i>	–	–	–	–	1	1 (1.5%)

<sup>a</sup>Raw milk samples were both bovine and ovine.

Yeasts were detected in 51 out of 69 samples (73.9%). Sixty isolates belonging to 8 genera (5 from *Ascomycota* phylum and 3 from *Basidiomycota* phylum) and 9 species. Among *Ascomycota*, *Debaryomyces hansenii* was the dominant species (22 isolates, 31.9%) followed

by *Kluyveromyces marxianus* (19 isolates, 27.5%) and *Kazachstania unispora* (11 isolates, 15.9%). These three species were common in every category of dairy products analysed during this study. Only 1 isolate (1.5%) was identified as *Candida inconspicua* and 1 other (1.5%) as *Wickerhamiella pararugosa*. Among *Basidiomycota*, the dominant genus was *Rhodotorula* (4 isolates, 2 *R. glutinis* and 2 *R. mucilaginosa*, 5.8%). The species *Filobasidium magnum* and *Sporidiobolus metaroseus* had 1 isolate each (1.5%) (Table 2).

**Table 2.** Diversity of yeasts isolated from dairy products

Species	Raw milk <sup>a</sup>	Curd	Cream cheese	Fresh cheese	Hard cheese	Total
<i>Candida inconspicua</i>	–	–	1	–	–	1 (1.5%)
<i>Debaryomyces hansenii</i>	4	2	3	5	8	22 (31.9%)
<i>Filobasidium magnum</i>	1	–	–	–	–	1 (1.5%)
<i>Kazachstania unispora</i>	2	1	2	2	4	11 (15.9%)
<i>Kluyveromyces marxianus</i>	4	2	4	3	6	19 (27.5%)
<i>Rhodotorula glutinis</i>	–	–	–	2	–	2 (2.9%)
<i>Rhodotorula mucilaginosa</i>	–	–	–	1	1	2 (2.9%)
<i>Sporidiobolus metaroseus</i>	1	–	–	–	–	1 (1.5%)
<i>Wickerhamiella pararugosa</i>	–	–	–	–	1	1 (1.5%)

<sup>a</sup>Raw milk samples were both bovine and ovine.

## Discussion

These results highlighted a wide diversity of fungi in dairy products of central Italy, with a total of eleven different genera and twenty-two species. It is worth noticing the substantial difference between the fungal species isolated from raw milk samples and those isolated from dairy products obtained after pasteurisation. This is obviously linked to the heat treatment of the milk. Therefore, it appears safe to say that the majority of the species found in cheeses during this study came from the environment of the dairy plant as contaminants.

The *Penicillium* genus was found in the majority of product types analysed, representative of local production, and was very common in hard cheeses. *P. brevicompactum* has been reported not only as a major dairy product contaminant in southern Italy (Montagna et al., 2004) and Spain (Barrios et al., 1998), but also as the most common species isolated from the internal environment of three plants in Norway (Kure et al., 2004). The presence of *Penicillium* species belonging to the Brevicompacta section is not surprising, considering the fact that they grow well at refrigerated temperatures and are often considered as the most xerophilic penicillia (Pitt and Hocking, 2009). Species of Fasciculata section (e.g. *P. commune*) are well-known spoilage agents for dairy products (Pitt and Hocking, 2009; Ledenbach and Marshall, 2010). *P. camemberti* is used in the production of Camembert, Brie, Coulommiers and Cambozola cheeses, on which its colonies form a hard, white crust and are responsible for the typical taste. It is worth mentioning that the products analysed in this study are not produced with the addition of *P. camemberti*, so it can be considered as a contaminant. The prevalence of *P. oxalicum* reported in this study is similar to what is reported by Kandasamy et al. (2020) in the Korean dairy product and plant environment.

The species of the *Galactomyces* genus are spoilage fungi, often isolated from fresh dairy products (Banjara et al., 2015): they are well adapted to dairy matrices and are able to grow at refrigeration temperatures. In our study, all the *G. candidium* isolates came from raw milk samples.

*Cladosporium* and *Didymella* are two genera that have undergone major taxonomical changes in the last decade. Even though the presence of *Cladosporium* spp. was reported in various dairy products in the past, its identification was only based on phenotypic characteristics without species level being identified. Instead, in this study, a molecular approach allowed us to identify 2 *Cladosporium* species (*C. alsperulatum* and *C. cladosporoides*) from hard cheese and raw milk, respectively. Other authors noticed the *Sphaerospermum* complex from butter identifying it by molecular tools for the first time. The isolation of *Didymella pinodella* from fresh cheese in our study is worth mentioning because previously its presence was only reported in cream cheese (Garnier et al., 2017). Among *Didymella* spp., it is also worth mentioning that *D. glomerata* was shown to be responsible for the abnormal colouration of Mozzarella cheese and the necessity of a correct differential diagnosis with the alterations caused by *Pseudomonas* spp. (Cenci-Goga et al., 2014). The genus *Mucor* includes many species of spoilage fungi: their presence was also reported in Taleggio cheese (Dragoni et al., 1997). In our study, *M. racemosus* was isolated from two samples of hard cheese.

*Debaryomyces hansenii* is one of the most common yeast species isolated from dairy products: it plays a major role in cheese ripening and in limiting the growth of spoiling bacteria (Cholet et al., 2007). This species is an important component in the production of many cheeses, in particular the Italian 'Pecorino cheese' (Del Bove et al., 2009). In our study, *D. hansenii* was the most common species isolated, with a total of 22 specimen identified: 4 from raw milk, 2 from curd, 3 from cream cheese, 5 from fresh cheese and 8 from hard cheese. The other yeast species isolated in this study have previously been reported to spoil dairy products and are known to be well adapted to growing in dairy products (Garnier et al., 2017).

In conclusion, this study provides new and interesting information on the diversity of fungal species found in dairy products in Umbria, central Italy. Some of the yeast and mould species isolated during this study are part of the normal flora of milk and dairy products and have often been reported in similar studies in other parts of the world, while other species are able to cause spoilage of these products. This data is of great importance to develop new strategies to decrease the occurrence of fungal spoilage and/or control its growth in dairy products and thus reduce food waste and increase the quality and safety of these products.

## References

- Banjara, N, Suhr, MJ and Hallen-Adams, HE (2015) Diversity of yeast and mould species from a variety of cheese types. *Current Microbiology* 70, 1–9.
- Barrios, MJ, Medina, LM, Lopez, MC and Jordano, R (1998) Fungal biota isolated from Spanish cheeses. *Journal of Food Safety* 18, 151–157.
- Cenci-Goga, BT, Karama, M, Sechi, P, Iulietto, MF, Novelli, S and Mattei, S (2014) Evolution under different storage conditions of anomalous blue coloration of Mozzarella cheese intentionally contaminated with a pigment-producing strain of *Pseudomonas fluorescens*. *Journal of Dairy Science* 97, 6708–6718.
- Cholet, O, Henaut, A, Casaregola, S and Bonnarne, P (2007) Gene expression and biochemical analysis of cheese-ripening yeasts: focus on catabolism of L-methionine, lactate, and lactose. *Applied and Environmental Microbiology* 73, 2561–2570.
- Del Bove, M, Lattanzi, M, Rellini, P, Pelliccia, C, Fatichenti, F and Cardinali, G (2009) Comparison of molecular and metabolomic methods as characterization tools of *Debaryomyces hansenii* cheese isolates. *Food Microbiology* 26, 453–459.
- Dragoni, L, Papa, A and Vallone, L (1997) Mycological quality standards of Italian cheese Taleggio. Typical and unacceptable surface moulds – mycological paste standard [Lombardia]. *Archivio Veterinario Italiano* 48, 154–160.
- Garnier, L, Valence, F, Pawtowski, A, Auhustsinava-Galerie, L, Frotté, N, Baroncelli, R, Deniel, F, Coton, E and Mounier, J (2017) Diversity of spoilage fungi associated with various French dairy products. *International Journal of Food Microbiology* 241, 191–197.
- Huis in't Veld, JHJ (1996) Microbial and biochemical spoilage of foods: an overview. *International Journal of Food Microbiology* 33, 1–18.
- Jacques, N and Casaregola, S (2008) Safety assessment of dairy microorganisms: the hemiascomycetous yeasts. *International Journal of Food Microbiology* 126, 321–326.
- Kandasamy, S, Park, WS, Yoo, J, Yun, J, Kang, HB, Seol, KH, Oh, MH and Ham, JS (2020) Characterisation of fungal contamination sources for use in quality management of cheese production farms in Korea. *Asian-Australasian Journal of Animal Sciences* 33, 1002–1011.
- Kure, C, Skaar, I and Brendehaug, J (2004) Mould contamination in production of semi-hard cheese. *International Journal of Food Microbiology* 93, 41–49. CrossRefGoogle ScholarPubMedCheck for full text via OCLC
- Ledenbach, LH and Marshall, RT (2010) Microbiological spoilage of dairy products. In Sperber, WH and Doyle, MP (eds), *Compendium of the Microbiological Spoilage of Foods and Beverages*. Food Microbiology and Food Safety. New York: Springer, pp. 41–67.
- Montagna, MT, Santacroce, MP, Spilotros, G, Napoli, C, Minervini, F, Papa, A and Dragoni, I (2004) Investigation of fungal contamination in sheep and goat cheeses in southern Italy. *Mycopathologia* 158, 245–249.

Mounier, J, Monnet, C, Vallaey, T, Ardit, R, Sarthou, AS, Hélias, A and Irlinger, F (2008) Microbial interactions within a cheese microbial community. *Applied and Environmental Microbiology* 74, 172–181.

Pitt, JI and Hocking, AD (2009) *Fungi and Food Spoilage*, 3rd Edn, New York, USA: Springer Science & Business Media.