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Diversity of spoilage fungi associated with various French dairy products

Lucille Garnier [a,b], Florence Valence [b], Audrey Pawtowski [a], Lizaveta Auhustsinava-Galerne [a], Nicolas Frotté [a], Riccardo Baroncelli [a], Franck Deniel [a], Emmanuel Coton [a], Jérôme Mounier [a,*]

[a] Université de Brest, EA 3882, Laboratoire Universitaire de Biodiversité et Ecologie Microbienne, ESIAB, Technopôle Brest-Iroise, 29280 Plouzané, France
[b] STLO, Agrocampus Ouest, INRA, 35000 Rennes, France

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ABSTRACT

Yeasts and molds are responsible for dairy product spoilage, resulting in significant food waste and economic losses. Yet, few studies have investigated the diversity of spoilage fungi encountered in dairy products. In the present study, 175 isolates corresponding to 105 from various spoiled dairy products and 70 originating from dairy production environments, were identified using sequencing of the ITS region, the partial β-tubulin, calmodulin and/or EFα genes, and the D1–D2 domain of the 26S rRNA gene for filamentous fungi and yeasts, respectively. Among the 41 species found in spoiled products, *Penicillium commune* and *Penicillium bialowiezense* were the most common filamentous fungi, representing around 10% each of total isolates while *Meyerozyma guilliermondii* and *Trichosporon asahii* were the most common yeasts (4.8% each of total isolates). Several species (*e.g. Penicillium antarcticum*, *Penicillium salamii* and *Cladosporium phyllophilum*) were identified for the first time in dairy products or their environment. In addition, numerous species were identified in both spoiled products and their corresponding dairy production environment suggesting that the latter acts as a primary source of contamination. Secondly, the resistance to chemical preservatives (sodium benzoate, calcium propionate, potassium sorbate and natamycin) of 10 fungal isolates representative of the observed biodiversity was also evaluated. Independently of the fungal species, natamycin had the lowest minimum inhibitory concentration (expressed in gram of preservative/l), followed by potassium sorbate, sodium benzoate and calcium propionate. In the tested conditions, *Cladosporium halotolerans* and *Didymella pinodella* were the most sensitive fungi while *Yarrowia lipolytica* and *Candida parapsilosis* were the most resistant towards the tested preservatives. This study provides interesting information on the occurrence of fungal contaminants in dairy products and environments that may help developing adequate strategies for fungal spoilage control.

1. Introduction

Food spoilage is a major problem for the food industry and consumers because it renders products unacceptable for consumption and consequently leads to significant food waste and economic losses. Such spoilage is frequently due to microbial activities. Indeed, because of their physico-chemical characteristics (pH, a_w, redox potential), food products, especially fresh products provide a favorable niche for the survival and growth during storage of certain undesirable microorganisms including bacteria and fungi.

In the context of dairy product spoilage, the presence and effect of spoilage or pathogenic bacteria is well documented (Brooks et al., 2012; Canganella et al., 1998; Huis in't Veld, 1996; Ledenbach and Marshall, 2010). In contrast, despite the fact that yeasts and molds also cause important dairy food waste, only few studies (Banjara et al., 2015; Kure, 2001; Kure and Skaar, 2000) have been conducted on the diversity of spoilage fungi. Moreover, much more emphasis has been put on yeast than on mold diversity. According to the literature, the main genera involved in dairy product spoilage are *Candida*, *Galactomyces* and *Yarrowia* and, *Penicillium*, *Mucor* and *Cladosporium* for yeasts and molds, respectively (Deák, 2008; Pitt and Hocking, 2009). These contaminants mainly originate from the dairy environment including the air, the surfaces, the equipment and the personnel as well as raw materials and ingredients (Kure et al., 2004; Vacheyrou et al., 2011).

Fungal spoilage may be visible due mainly to the organism growth (colony or thallus), such as the “toad skin” or the “cat hair” defects caused by *Galactomyces geotrichum* and *Mucor* spp., respectively, or non-visible, *via* fungal metabolism resulting in production of off-odors and/or off-flavors, gas production or texture alteration (Ledenbach and Marshall, 2010). Furthermore, although spoilage yeasts have never been involved in food outbreaks, certain species are considered

* Corresponding author at: Laboratoire Universitaire de Biodiversité et Ecologie Microbienne, Parvis Blaise-Pascal, Technopôle Brest-Iroise, 29280 Plouzané, France.
E-mail addresses: lucille.garnier@univ-brest.fr (L. Garnier), florence.valence@inra.fr (F. Valence), audrey.pawtowski@univ-brest.fr (A. Pawtowski), liza.galerne2@orange.fr (L. Auhustsinava-Galerne), nicolas.frotte@univ-brest.fr (N. Frotté), riccardobaroncelli@gmail.com (R. Baroncelli), Franck.deniel@univ-brest.fr (F. Deniel), emmanuel.coton@univ-brest.fr (E. Coton), jerome.mounier@univ-brest.fr (J. Mounier).

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as opportunistic pathogens and may represent a risk for immuno-compromised people (Jacques and Casaregola, 2008). In addition, several mold spoilage species such as *Aspergillus* and *Penicillium* spp. are able to produce mycotoxins which may also be toxic for humans (Filtenborg et al., 1996; Huis in't Veld, 1996; Westall and Filtenborg, 1998a, 1998b). However, it is worth mentioning that no food poisoning cases related to the consumption of mycotoxin-contaminated dairy products have been documented so far (Hymery et al., 2014).

In order to avoid or delay fungal spoilage, and thus to extend product shelf life and safety, prevention methods combined with hurdle technologies are applied by dairy manufacturers. Prevention methods include the application of good manufacturing and hygiene practices, implementation of Hazard Analysis Critical Control Point (HACCP) system and the use of air filtration or decontamination equipment. The hurdle technologies can correspond to the use of heat treatment, modified atmosphere packaging, cold storage, addition of sodium chloride or sugar or fermentation with beneficial microorganisms, including bacteria and fungi (Bourdichon et al., 2012; Phillips, 1996; Sakkas et al., 2014). In certain dairy product types, the use of chemical preservatives (*e.g.* natamycin or organic acids such as acetic, lactic, propionic, sorbic, benzoic acids and their salts) is also permitted. For example, within the EU, propionate and sorbate, as well as natamycin can be used for the surface-treatment of hard, semi-hard and semi-soft cheese at *quantum satis* level and at a maximum level of 1 mg/dm^2 (Commission Regulation (EU) No 1129/2011, 2011). Moreover, benzoate can be added in non-heat-treated dairy-based desserts (at 300 mg/kg or mg/l) while sorbates can be added in a much larger range of dairy products (*i.e.* fermented milks, cheese and cheese-derived products) with maximum limits varying from 300 to 2000 mg/kg (Commission Regulation (EU) No 1129/2011, 2011). It is now well established that the intrinsic resistance of fungi to these preservatives is species-dependent (López-Malo et al., 2005; Rodrigues and Pais, 2000).

In the present study, we first investigated the biodiversity of spoilage fungi associated with a large range of dairy products (including butter, cream, fresh and semi-hard smear cheese, yoghurt drink and yoghurt) and in the air of dairy production environments. Then, we determined the resistance of 10 fungal isolates representative of the observed biodiversity to chemical preservatives commonly used in the dairy industry (*e.g.* calcium propionate, sodium benzoate, potassium sorbate and natamycin).

2. Materials and methods

2.1. Fungal isolates

In the present study, 175 fungal isolates, corresponding to 105 isolates from spoiled products and 70 isolates from air in dairy production environments were identified. Sixty-three isolates originating from contaminated products were directly provided by 4 dairy manufacturers over a period of 6 months and 32 fungi were isolated from spoiled products that were supplied by 3 dairy manufacturers over a period of 6 months. These products included cream cheese (7 samples), fresh cheese (6 samples), hard cheese (12 samples), yoghurt (4 samples) and yoghurt drink (3 samples). Sensorial properties of contaminated dairy products were not studied. In addition to these products, 70 isolates from the air of 3 industrial dairy plants were also identified after collection using an air sampler on yeast glucose chloramphenicol (YGC) agar.

2.2. Isolation of spoilage fungi from contaminated products

Contaminated pieces were removed with a sterile scalpel and deposited at the center of M2Lev (20 g/l malt extract (ME), 3 g/l yeast extract (YE), 15 g/l agar, 5 mg/l penicillin, 5 mg/l streptomycin) and M5S5 (50 g/l ME, 50 g/l sodium chloride, 15 g/l agar, 5 mg/l penicillin, 5 mg/l streptomycin) media prior to incubation for 2 to 7 days at 25 °C. For food materials with no visible fungal growth on their surfaces, isolation was also performed after successive dilution in physiological water supplemented with 0.005% Tween 80 and surface-plating on M2Lev medium incubated for 2 to 7 days at 25 °C. Isolates were then purified on the same agar medium and stored at −80 °C in 20% glycerol until further use.

2.3. Identification of fungal isolates

Isolates were preliminarily characterized at the genus level using phenotypic methods including macro- and microscopic observations. For species identification, barcode markers were sequenced. DNA was extracted from scraped colonies or mycelial plugs using the FastDNA SPIN Kit (MP Biomedicals, Illkirch, France) according to the manufacturer's instructions. DNA was then diluted to 50–100 ng/μl and kept at −20 °C until further analysis.

Depending on the studied fungal isolate, 1 to 3 DNA regions were amplified and sequenced. For filamentous fungi, the internal transcribed spacer (ITS) region (all isolates except presumptive *Penicillium* spp.), the partial β-tubulin gene (*Penicillium* spp.), the partial elongation factor α (EFα) (*Cladosporium* spp.) were PCR-amplified using primers ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) (White et al., 1990), Bt2a (5′-GGTAACCAAATCGGTGCTGCTTTC-3′) and Bt2b (5′-ACCCTCAGTGTAGTGACCCTTGGC-3′) (Glass and Donaldson, 1995), EF1-728F (5′-CATCGAGAAGTTCGAGAAGG-3′) and EF1-986R (5′-TACTTGAAGGAACCCTTACC-3′) (Carbone and Kohn, 1999), respectively. In addition, the partial calmodulin gene was amplified using primers cmd5 (5′-CCGAGTACAAGGAGGCCTTC-3′) and cmd6 (5′-CCGATAGAGGTCATAACGTGG-3′) (Hong et al., 2005) for three *Penicillium* isolates related to *Penicillium antarcticum* in order to confirm their species identity. For yeasts, primers NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) (Kurtzman and Robnett, 1997) were used to amplify the D1-D2 domain of the 26S rRNA gene. PCR amplifications were carried out in a FlexCycler thermocycler (Analityk Jena, Germany) and PCR products were checked by gel electrophoresis.

Sequencing was performed at the Biogenouest sequencing platform in the "Station Biologique de Roscoff" (http://www.sb-roscoff.fr/SG/) using the same primer pairs as those used for PCR amplifications. Sequences were assembled into contig using the DNA Baser software (Heracle software, Germany), and compared with the GenBank database using the "Basic Local Alignment Search Tool" (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST). Phylogenetic analyses (multiple alignment using ClustalW and phylogenetic reconstruction using the neighbor-joining method) were performed with sequences retrieved from the NCBI database using the MEGA6 software (Tamura et al., 2013). Sequences have been deposited in GenBank under accession numbers KX928780 to KX928952.

2.4. 2.3. Resistance to chemical preservatives

Resistance to calcium propionate, potassium sorbate, sodium benzoate and natamycin was evaluated for 10 isolates representative of the determined fungal species, namely, *Cladosporium halotolerans* UBOCC-A-116001, *G. geotrichum* UBOCC-A-216001, *Mucor racemosus* UBOCC-A-116002, *Penicillium commune* UBOCC-A-116003, *Didymella pinodella* UBOCC-A-116004, *Candida parapsilosis* UBOCC-A-216002, *Meyerozyma guilliermondii* UBOCC-A-216003, *Rhodotorula mucilaginosa* UBOCC-A-216004, *Trichosporon asahii* UBOCC-A-216005 and *Yarrowia lipolytica* UBOCC-A-216006. All chosen isolates were deposited at the Université de Bretagne Occidentale Culture Collection (UBOCC, Plouzané, France) and were collected from contaminated dairy products. Resistance to calcium propionate, potassium sorbate and sodium benzoate was evaluated *in vitro* at pH 5 for concentrations ranging from 0 to 3 g/l (tested concentrations: 0, 0.04, 0.08, 0.1, 0.15, 0.3, 0.5, 1, 1.5, 2, 2.5 and 3 g/l).