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Candida milleri species reveals intraspecific genetic and metabolic polymorphisms

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Candida milleri, together with *Candida humilis*, is the most representative yeast species found in type I sourdough ecosystems. In this work, comparison of the ITS region and the D1/D2 domain of 26S rDNA gene partial sequences, karyotyping, mtDNA-RFLP analysis, Intron Splice Site dispersion (ISS-PCR) and $(GTG)_5$ microsatellite analyses, assimilation test of different carbohydrates, and metabolome assessment by FT-IR analysis, were investigated in seventeen strains isolated from four different companies as well as in type strains CBS6897^T and CBS5658^T. Most isolates were ascribed to *C. milleri*, even if a strong relatedness was confirmed with *C. humilis* as well, particularly for three strains. Genetic characterization showed a high degree of intraspecific polymorphism since 12 different genotypes were discriminated. The number of chromosomes varied from 9 to 13 and their size ranged from less than 0.3 to over 2 Mbp. Phenotypic traits let to recognize 9 different profiles of carbon sources assimilation. FT-IR spectra from yeast cells cultivated in different media and collected at different growth phases revealed a diversity of behaviour among strains in accordance with the results of PCR-based fingerprinting. A clear evidence of the polymorphic status of *C. milleri* species is provided thus representing an important feature for the development of technological applications in bakery industries.

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1. Introduction

Probably used since thousands of years and spread all over the world for the production of baked cereal-based products, sourdough is an interesting man-made ecosystem to be studied for the relationships that are established between prokaryotic and eukaryotic organisms (Vogel et al., 1999; Hammes et al., 2005; De Vuyst et al., 2013). Depending on raw materials, environmental factors and the technological practices, different species of lactic acid bacteria and yeasts can dominate the fermentative process, forming mutualistic associations or competing for carbon and nitrogen sources (Gobbetti, 1998; Gobbetti et al., 2005; De Vuyst and Neysens, 2005; De Vuyst et al., 2009; Vogelmann et al., 2009; Vogelmann and Hertel, 2011; Venturi et al., 2012). The development of this microbiota mainly causes the leavening and acidification of the dough, whereas the sensorial and nutritional properties of the resulting products are improved due to specific enzymatic activities and metabolites (Arendt et al., 2007; Hansen and Schieberle, 2005; Gänzle, 2009; Chavan and Chavan, 2011; Nuobariene et al., 2012). More than six hundred are the references that may be found on scientific databases (Web Of Knowledge^{SM}, Scopus SciVerse$^{®}$) by crossing keywords “sourdough” and “lactic acid bacteria”, while less than one quarter of this number are the documents retrieved when the same research is made with the word “yeast”. Actually, most of these articles describe studies on the detection, identification and sometimes genetic typing of yeast populations by culture dependent and independent techniques in different types of sourdough. *Candida humilis* (Gullo et al., 2003; Meroth et al., 2003; Foschino et al., 2004; Garofalo et al., 2008; Iacumin et al., 2009; Zhang et al., 2011; Lattanzi et al., 2013) and *Candida milleri* (Gänzle et al., 1998; Mantynen et al., 1999; Pulvirenti et al., 2004; Vernocchi et al., 2004; Venturi et al., 2012) are the dominant species detectable in type I sourdoughs (Vogel et al., 1996; De Vuyst and Neysens, 2005), where a stable microbial consortium with *Lactobacillus sanfranciscensis* is formed. This kind of sourdough is traditionally fermented at <30 °C and

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maintained by daily back-slopping. Also *Saccharomyces cerevisiae* is very often detected in type I sourdough (Corsetti et al., 2001; Succi et al., 2003; Pulvirenti et al., 2004; Iacumin et al., 2009); while the current opinion is that it originates from a cross contamination from bakery equipment, its presence is still debated since recent studies suggested a specific role of different strains selected by the environmental and technological conditions (Valmorri et al., 2010; Vrancken et al., 2010; Minervini et al., 2012; Venturi et al., 2012; Lattanzi et al., 2013). Other frequently reported yeasts are *Candida glabrata* (Succi et al., 2003; Vrancken et al., 2010; Moroni et al., 2011), *Kazachstania exigua* [formerly *Saccharomyces exiguus*, anamorph *C.* (*Torulopsis*) *holmii*] (Pulvirenti et al., 2004; De Vuyst and Neysens, 2005; Zhang et al., 2011; Minervini et al., 2012) and *Pichia kudriavzevii* (formerly *Issatchenkia orientalis*, anamorph *Candida krusei*) (Meroth et al., 2003; Succi et al., 2003; Pulvirenti et al., 2004; Vogelmann et al., 2009; Valmorri et al., 2010). Results of recent investigations on the yeast populations in sourdoughs by PCR-DGGE analysis (Gatto and Torriani, 2004; Garofalo et al., 2008; Iacumin et al., 2009; Valmorri et al., 2010; Zhang et al., 2011; Minervini et al., 2012) may not have correctly discriminated between *C. humilis* and *C. milleri* because, if the 26S rDNA has been used as target of amplification (Sandhu et al., 1995; Kurtzman and Robnett, 1998; Cocolin et al., 2000), it could not be sufficiently diverse to recognize the two species. In fact, molecular investigations have shown that the type strains, *C. humilis* CBS5658^T and *C. milleri* CBS6897^T, differ only by 1 substitution in the D1/D2 domains of 26S rDNA, whereas up to 10 and 11 substitutions have been detected in the ITS1-5.8S-ITS2 rDNA region (ITS region) and in the elongation factor 1α gene sequences, respectively (Kurtzman and Robnett, 2003). Indeed these two yeasts, once considered conspecific or possible synonym (Vaughan-Martini, 1995; Kurtzman and Robnett, 1998), are nowadays positioned into the *Kazachstania* clade according to Vaughan-Martini et al. (2011) and are designated as separated species by Lachance et al. (2011). On the basis of different RFLP patterns in the ITS region obtained by using the endonuclease *Hae*III, Pulvirenti et al. (2001) and Gullo et al. (2003) proposed a protocol able to distinguish between the two taxa. Furthermore, the two species differ in the assimilation of some carbon compounds; in particular, the type strain of *C. humilis* does not metabolize sucrose and raffinose (Van der Walt and Nel, 1968; Yarrow, 1978) whereas that of *C. milleri* does.

Although during sourdough fermentation the contribution is mainly linked to leavening, the yeasts have an important role for the production of several metabolites that contribute to the development of the bread flavour and texture (Hansen and Schieberle, 2005; Katina et al., 2006; Salim-ur-Rehman et al., 2006; Vernocchi et al., 2008; Poutanen et al., 2009; Chavan and Chavan, 2011). In particular, as it happens for other fermented foods or beverages, more than one strain of the same species can have an influence on the sensorial and nutritional characteristics of the final product (Katina et al., 2005; Zannini et al., 2012). Thus, the possibility of discriminating at strain level is the basis for a successful cultures selection and starter development. The present work aims to investigate the genetic and phenotypic biodiversity among strains potentially belonging to *C. milleri* species, collected from sourdoughs of traditional Italian bread-making in four different companies during a longstanding sampling.

2. Materials and methods

2.1. Yeast strains and media

Seventeen yeasts isolated from sourdoughs for different Italian bakery products of different origin were used in this study (Table 1). For fourteen strains, isolation was carried out from dough samples homogenized 1:1 (w/v) with peptone-water (10 g/L peptone, pH = 6.2) and serially diluted. One hundred microlitres of appropriate dilutions were spread on YGC agar (Merck, Germany); after 4 days of incubation at 25 °C in aerobic condition, yeast colonies were isolated and cultivated in YPD medium (10 g/L Yeast Extract, 20 g/L Peptone, 20 g/L Glucose, pH 5.6) for 2 days at 25 °C. Yeast cultures were maintained at −80 °C in YPD medium plus 20% (v/v) glycerol. Three *C. milleri* strains, kindly provided by prof. Andrea Pulvirenti (Università degli Studi di Modena e Reggio Emilia), were also added to the yeast collection (Table 1). Finally, the strains *Kazachstania barnetti* Y27223^T, from the ARS (Agricultural Research Service) Culture Collection (NRRL), as well as *C. humilis* CBS5658^T and *C. milleri* CBS6897^T from the CBS (Centraal Bureau voor Schimmelcultures) collection were used as reference strains.

The experiments for the FT-IR analysis were performed on cells cultivated in Yeast Nitrogen Base (YNB) medium added with glucose (20 g/L) and in *Medium Farinae*. *Medium Farinae* was prepared in order to mimic the wheat flour environment. Briefly, commercial wheat flour was dissolved 1:2 in distilled water and the suspension was stirred for 1 h at 150 rpm and 25 °C to enable the release of soluble components from flour into water. The suspension was then centrifuged at 15,000 *g* for 20 min at 25 °C. Finally, the supernatant was autoclaved at 112 °C for 30 min and centrifuged in sterile tubes at 15,000 *g* for 40 min; the supernatant collected was the *Medium Farinae*.

2.2. Species identification

Species identification of the isolates was carried out by genetic and phenotypic analyses. Amplification and partial sequencing of ITS1-5.8S-ITS2 rDNA region (ITS region) and D1/D2 domain of the 26S rDNA were performed using amplification conditions as well as primers ITSY1/ITSY4 and NL1/NL4 as reported by Esteve-Zarzoso et al. (1999) and Kurtzman and Robnett (1998), respectively. DNAs were extracted according to the protocol described by Querol et al. (1992) using 500 μg/mL of Zymolyase 100T (US Biological, Massachusetts, USA). PCR reactions were carried out by a Mastercycler Gradient S (Eppendorf, Hamburg, Germany) in a final volume of 25 μL containing 80–100 ng of genomic DNA, 0.1 μM of each primers, 200 μM dNTPs, 1× reaction buffer $MgCl_2$ free, 2.5 mM $MgCl_2$ and 1U *Taq* polymerase (Fermentas, Vilnius, Lithuania). Amplification fragments were resolved in 0.8% (w/v) agarose gels containing 0.4 μg/mL of ethidium bromide at 100 V for 1 h using 1× TAE (0.4 M Tris-acetate, 0.01 M Na_2EDTA, pH 8.0) as running buffer. Bands were visualized under UV exposition (GelDOC, Bio-Rad). The amplified products of the ITS region were subjected to endonuclease restriction analysis using *Hae*III according to Pulvirenti et al. (2001). PCR products were also submitted for sequencing to an outdoor provider (Primm, Milan, Italy). The obtained sequences were identified through BLAST algorithm (by comparison with the sequences listed in databases (www.ncbi.nlm.nih.gov/)).

For the phenotypic analysis, the assimilation of different carbohydrates and the resistance to cycloheximide of the yeast isolates were tested using the API/ID32C® kit (bioMérieux SA, Marcy-L'Etoile, France) according to the manufacturer's instructions. Positive growth results were attributed by the turbidity increasing in comparison to the negative control after 24 and 48 h of incubation at 25 °C.

2.3. Genome characterization

For karyotyping, agarose inserts containing chromosome-size DNA were prepared as follow: fresh cells (1.5 OD_{600nm}) were mixed to 1.5% low melting agarose, 0.125 M EDTA pH 7.5 added with 2 mg L^{-1} Zymolyase 100T. After solidification, plugs were

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