International Journal of Food Microbiology 250 (2017) 19–26

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Identification and characterization of lactic acid bacteria and yeasts of PDO Tuscan bread sourdough by culture dependent and independent methods

Michela Palla, Caterina Cristani, Manuela Giovannetti, Monica Agnolucci *

Department of Agriculture, Food and Environment, University of Pisa, Via del Borghetto 80, 56124 Pisa, Italy

ARTICLE INFO

Article history:
Received 30 November 2016
Received in revised form 21 March 2017
Accepted 22 March 2017
Available online 23 March 2017

Keywords:
ARDRA
ITS-RFLP
PCR-DGGE
Lactobacillus sanfranciscensis
Candida milleri
Saccharomyces cerevisiae

ABSTRACT

Sourdough fermentation has been increasingly used worldwide, in accordance with the demand of consumers for tasty, natural and healthy food. The high diversity of lactic acid bacteria (LAB) and yeast species, detected in sourdoughs all over the world, may affect nutritional, organoleptic and technological traits of leavened baked goods. A wide regional variety of traditional sourdough breads, over 200 types, has been recorded in Italy, including special types selected as worthy of either Protected Geographical Indication (PGI) or Protected Designation of Origin (PDO), whose sourdough microbiota has been functionally and molecularly characterized. As, due to the very recent designation, the microbiota of Tuscan bread sourdough has not been investigated so far, the aim of the present work was to isolate and characterize the species composition of LAB and yeasts of PDO Tuscan bread sourdough by culture-independent and dependent methods. A total of 130 yeasts from WLN medium and 193 LAB from both mMRS and SDB media were isolated and maintained to constitute the germplasm bank of PDO Tuscan bread. Ninety six LAB from mMRS medium and 68 yeasts from WLN medium were randomly selected and molecularly identified by ARDRA (Amplified Ribosomal DNA Restriction Analysis) and PCR-RFLP analysis of the ITS region, respectively, and sequencing. The yeast identity was confirmed by 26S D1/D2 sequencing. All bacterial isolates showed 99% identity with *Lactobacillus sanfranciscensis*, 65 yeast isolates were identified as *Candida milleri*, and 3 as *Saccharomyces cerevisiae*. Molecular characterization of PDO Tuscan bread sourdough by PCR-DGGE confirmed such data. The distinctive tripartite species association, detected as the microbiota characterizing the sourdough used to produce PDO Tuscan bread, encompassed a large number of *L. sanfranciscensis* and *C. milleri* strains, along with a few of *S. cerevisiae*. The relative composition and specific physiological characteristics of such microbiota could potentially affect the nutritional features of PDO Tuscan bread, as suggested by the qualitative functional characterization of the isolates. Investigations on the differential functional traits of such LAB and yeast isolates could lead to the selection of the most effective single strains and of the best performing strain combinations to be used as starters for the production of baked goods.

1. Introduction

Sourdough fermentation represents one of the oldest biotechnologies used by humans to ferment cereals for bread production. In recent times, it has been increasingly used worldwide, in accordance with the demand of consumers for tasty, natural and healthy foods (Gobbetti and Gänzle, 2013). Indeed, sourdough fermentation improves bread sensory, structural and nutritional properties, and prolongs its shelf-life (Arendt et al., 2007; Katina et al., 2005; Minervini et al., 2014). In addition, microbial metabolism during sourdough fermentation positively affects several bread functional features, producing bioactive compounds, such as peptides, beta-glucans and other exopolisaccharides (Gobbetti et al., 2014).

A large number of lactic acid bacteria (LAB) and yeast species, establishing positive interactions and often stable associations, has been isolated from sourdoughs all over the world, *i.e.* about 80 bacterial and 20 yeast species (De Vuyst and Neysens, 2005; Gänzle and Ripari, 2016). Notwithstanding, only a few species characterize single batches of sourdough, where no >6 different species have been usually identified. The typical most commonly detected bacterial species are *Lactobacillus brevis, Lactobacillus pontis, Lactobacillus reuteri, Lactobacillus plantarum* and *Lactobacillus sanfranciscensis*, the latter representing the dominant member of the microbiota, as it has been isolated in >75% of sourdoughs globally (Gänzle and Ripari, 2016). The most common sourdough yeast is *Saccharomyces cerevisiae*, although, as reviewed by De Vuyst et al. (2016) other species may occur in spontaneously developed stable sourdoughs (in decreasing order of abundance): *Candida humilis*/*Candida milleri*, *Wickerhamomyces anomalus*, *Torulaspora delbrueckii*,

* Corresponding author.
E-mail address: monica.agnolucci@unipi.it (M. Agnolucci).

http://dx.doi.org/10.1016/j.ijfoodmicro.2017.03.015
0168-1605/

Kazachstania exigua, *Pichia kudriavzevii and Candida glabrata*. Some of these species, such as *K. exigua, C. humilis* and *C. milleri*, are maltose-negative yeasts reported to form stable mutualistic associations with L. *sanfranciscensis*, which is able to hydrolyze maltose (De Vuyst et al., 2014).

Several studies investigating the microbiota of sourdough from different countries, *e.g.* Belgium (Scheirlinck et al., 2007), France (Ferchichi et al., 2008; Vera et al., 2012), Turkey (Dertli et al., 2016), China (Liu et al., 2016; Zhang et al., 2011), showed that the diversity of microbial communities depends on process technologies, types of flour and other ingredients traditionally associated with local culture and origin. Such diversity is at the basis of differential metabolic products, affecting nutritional, organoleptic and technological traits of leavened baked goods. In particular, traditional or type I sourdough is characterized by a spontaneous fermentative process, based on continuous backslopping, carried out by LAB and yeasts originating from the flour, other dough ingredients and the environment. Type I sourdough is utilized to produce various leavened baked products, such as San Francisco bread, French bread, rye bread, Altamura bread (Corsetti, 2013).

A wide regional variety of traditional sourdough breads, over 200 types, has been recorded in Italy (INSOR, 2000), including special types selected as worthy of either Protected Geographical Indication (PGI) (*Coppia Ferrarese*, *Pane Casareccio di Genzano*, *Cornetto di Matera*) or Protected Designation of Origin (PDO) (*Pagnotta del Dittaino* and *Pane di Altamura*). The sourdoughs of such traditional regional breads have been functionally and molecularly characterized, *i.e. Cornetto di Matera* (Zotta et al., 2008), *Pane di Altamura* (Ricciardi et al., 2005), along with breads from Abruzzo (Valmorri et al., 2006, 2010), Marche (Osimani et al., 2009), Molise (Reale et al., 2005), Sicily (Pulvirenti et al., 2001) and Sardinia (Catzeddu et al., 2006).

Recently, *Pane Toscano* has obtained the PDO status from the European Community (Commission implementing regulation (EU) 2016/303 of 1 March 2016, Official Journal of the European Union L 58 of 04 March 2016). PDO Tuscan bread has to be manufactured by a typical method generally adopted in Tuscan bakeries. It requires the exclusive use of sourdough starters, water, the absence of added salt, and type '0' soft-wheat flour from wheat varieties grown in Tuscany, according to the production guideline described in the EU Regulation for PDO Tuscan bread (Official Journal of the European Union C 235 of 14 August 2013). Due to the very recent designation, the microbiota of Tuscan bread sourdough has not been investigated so far. The aim of the present work was to isolate and characterize the species composition of lactic acid bacteria and yeasts of PDO Tuscan bread sourdough. To this aim, we utilized *i*) a culture-independent approach, Polymerase Chain Reaction (PCR) Denaturating Gradient Gel Electrophoresis (DGGE), a molecular technique able to avoid underestimates deriving from the constraints of culture conditions and from the presence of microorganisms in Viable But Non-Culturable (VBNC) state; *ii*) a culture-dependent approach to isolate and molecularly identify LAB and yeast species; *iii*) a preliminary qualitative screening to characterize LAB and yeasts with functionally important traits.

2. Materials and methods

2.1. Sourdough sampling

The sourdough analyzed in this study was collected from the Consortium of Promotion and Protection of Tuscan Sourdough Bread - *Consorzio Pane Toscano a Lievitazione Naturale* (CPT).

2.2. Microbiological analysis and isolation of LAB and yeasts

Three samples of about 10 g of PDO Tuscan bread sourdough were homogenized in a sterile stomacher bag containing 90 mL of saline-peptone water (9 g/L NaCl, 1 g/L bacteriological peptone, Oxoid, Milan, Italy) for 2 min at 260 rpm, using a Stomacher (Stomacher 400, Laboratory Blender). Further, a tenfold serial dilution (10^{-1} to 10^{-6}) was carried out in the same solution and aliquots (100 μL) were added in triplicate into a Petri dish containing the agar media listed below. LAB were counted on Sourdough Bacteria (SDB) (Kline and Sugihara, 1971) medium and on mMRS agar (De Man et al., 1960) modified by adding 20 g/L maltose and 50 mL/L fresh yeast extract and adjusted to pH 5.6. To inhibit yeast growth, media were supplemented with 100 mg/L cycloheximide. Inoculated plates were incubated for 7 days at 28 °C under anaerobic conditions (AnaeroGen, Oxoid). Yeasts were counted on Wallerstein Laboratory Nutrient (WLN) agar (Oxoid, Basingstoke, UK) and on Yeast Extract Peptone Dextrose (YEPD) agar. Both media were added with 100 mg/L chloramphenicol and incubated at 28 °C for 48 h.

LAB were randomly selected picking up at least 20 colonies from each plate of both media and purified by streaking four times onto the same medium used for isolation. About 15 yeast colonies were randomly selected from each plate of WLN medium on the basis of phenotypic colony characteristics and then purified as described above.

Each strain was named with the acronym of the Collection of the Department of Agriculture, Food and Environment of the University of Pisa (IMA, International Microbial Archives), followed by a progressive number plus "Y" or "LAB" for yeasts or bacteria, respectively. Purified strains were stored at −80 °C in the appropriate broth medium, supplemented with 20% (w/v) glycerol.

2.3. Molecular identification of LAB and yeast isolates

DNA of isolates and reference strains, listed in Table 1, was extracted from microbial liquid cultures grown at 28 °C using "MasterPure™ Yeast DNA Purification Kit" (Epicentre®) according to the manufacturer's protocols. LAB strains were identified by Amplified Ribosomal DNA Restriction Analysis (ARDRA) and yeast strains by ITS region amplification and its Restriction Fragment Length Polymorphism (RFLP) analysis. Amplification reactions were carried out in a final volume of 50 μL, containing 5 μL of 10× Ex Taq Buffer (Takara Biotechnology), 0.2 mM of each dNTP (Takara Biotechnology), 0.5 μM of each primer (Eurofins), 1.25 U of Takara Ex Taq polymerase (Takara Biotechnology) and 10–20 ng of DNA. The 16S rRNA gene was amplified using 27f (5′-GAG AGT TTG ATC CTG GCT CAG-3′) and 1495r (5′-CTA CGG CTA CCT TGT TAC GA-3′) primers (Lane, 1991; Weisburg et al., 1991) and ITS region was amplified using ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) primers (White et al., 1990). PCR amplifications were carried out with an iCycler-iQ Multicolor Real-Time PCR Detection System (Bio-Rad) using the following conditions: 94 °C initial denaturation for 1 min; 35 amplification cycles of 30 s at 94 °C, 30 s at

Table 1
Lactic acid bacteria and yeast reference strains used in this study.

Strains[a]	Source of isolation
Lactobacillus panis DSMZ 6035^T	Sourdough
Lactobacillus sanfranciscensis DSMZ 20451^T	San Francisco sourdough
Lactobacillus fermentum DSMZ 20052^T	Fermented beets
Lactobacillus brevis DSMZ 20054^T	Faeces
Lactobacillus plantarum IMA B23	Boza
Lactobacillus curvatus IMA LB51	Sourdough
Saccharomyces cerevisiae ATCC 32167	Unknown
Dekkera bruxellensis IMA 1 L	San Giovese Tuscan wine
Candida milleri DBVPG 6753^T	San Francisco sourdough
Candida humilis DBVPG 7219^T	Bantu beer
Candida humilis DBVPG 6754	Sourdough, Finland
Kazachstania exigua DBVPG 6956	Wheat sourdough, Italy

^{T}Type Strain.
[a] DSMZ = Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; IMA = International Microbial Archives, Department of Agriculture, Food and Environment, University of Pisa, Pisa, Italy; ATCC = American Type culture Collection, Manassas, Virginia, USA; DBVPG = International Collection of Department of Agricultural, Food and Environmental Science, University of Perugia, Perugia, Italy.