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Selection of lactic acid bacteria isolated from Tunisian cereals and exploitation of the use as starters for sourdough fermentation

Asma Mamhoud [a], Luana Nionelli [b], Taroub Bouzaine [a], Moktar Hamdi [a], Marco Gobbetti [b], Carlo Giuseppe Rizzello [b,*]

[a] *Laboratoire d'Écologie et de Technologie Microbienne, Institut National des Sciences, Appliquées et de Technologie (INSAT), University of Carthage, 1080 Tunis, Tunisia*
[b] *Department of Soil, Plant and Food Science, University of Bari Aldo Moro, 70126 Bari, Italy*

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A B S T R A C T

Wheat bread is the most popular staple food consumed in Tunisia and, despite the niche production of some typical breads (*e.g. Tabouna*, *Mlawi*, *Mtabga*), the major part is currently produced with baker's yeast at industrial or, mainly, at artisanal level, while the use of sourdough fermentation is rarely reported. Considering the growing national demand for cereal baked goods, it can be hypothesized that sourdough fermentation through the use of selected lactic acid bacteria as starters could improve the overall quality and the diversification of local products. Different cereal grains were collected from the regions of Ariana, Bizerta, Beja Nabeul, and Seliana, and the autochthonous lactic acid bacteria were isolated, identified, characterized and selected on the basis of the kinetics of acidification, the proteolytic activity, and the quotient of fermentation. *Lactobacillus curvatus* MA2, *Pediococcus pentosaceus* OA2, and *Pediococcus acidilactici* O1A1 were used together as mixed starter to obtain a selected sourdough. According to the backslopping procedure, a type I sourdough was made from a Tunisian flour (spontaneous sourdough). Compared to the use of the spontaneous sourdough, the one obtained with selected and mixed starters by a unique fermentation step, favored the increase of the concentrations of organic acids, phenols, and total free amino acids, the most suitable quotient of fermentation, and the most intense phytase and antioxidant activities, that increased *ca.* 20% compared to the control. Moreover, the selected starters improved the *in vitro* protein digestibility (*ca.* 82% when selected sourdough was used), textural and sensory features of the breads, as determined by textural profile analysis and panel test, respectively.

This study aimed at exploiting the potential of selected autochthonous lactic acid bacteria and extending the use of a sourdough (type II), thanks to the set-up of a two-step fermentation protocol designed for application at the industrial level, and the confirmed nutritional, textural, and sensory advantages of the final product.

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1. Introduction

Tunisia, the northernmost Country in Africa, is a big consumer, producer, and importer of cereal grains (Lyddon, 2014). According to the International Grains Council (IGC) Tunisia produced and imported 1.2 and 1.7 Mt of wheat in 2013 and 2014, respectively. Import also concerns large quantities of maize and barley (Lyddon, 2014). Nevertheless, cereal production in the country is growing and, in 2014, an increase of 49% compared to the previous season was registered (Lyddon, 2014; EI, 2014). Tunisia has one of the highest rates of wheat consumption in North Africa, estimated at 258 kg per capita per year, with a total consumption of *ca.* 2.8 Mt per year (Lyddon, 2014). Tunisia's government promotes the increased cereal production by a specific policy started in 2008 (Lyddon, 2014).

Wheat bread is the most popular staple food consumed in Tunisia (M'hir et al., 2007) and, despite the niche production of some typical breads (*e.g. Tabouna*, *Mlawi*, *Mtabga*), the major part is currently produced with baker's yeast at industrial or, mainly, at artisanal level (EI, 2014; M'hir et al., 2007). The use of sourdough fermentation for baked goods production is rarely reported (M'hir et al., 2007). Overall, baked goods production in Tunisia is expected to further increase, following the trend of the last years, also thanks to the improved trend in the national economic environment (EI, 2014).

Sourdough is a leavening agent traditionally obtained through a backslopping procedure, without the addition of starter microorganisms, whose use in bread making has a long history (Hammes and Ganzle, 1998). Novel biotechnological options, including the obtainment of the sourdough through a unique fermentation step instead of the

Abbreviations: BC, Baker's yeast bread; BHT, butylated hydroxytoluene; BSLS, breads including SLS; BSS, breads including SS; DPPH, 1,1-diphenyl-2picrylhydrazyl; DY, dough yield; FAAs, free amino acids; IVPD, *in vitro* protein digestibility; ME, methanol extract; *p*-NPP, *p*-nitrophenyl phosphate; QF, quotient of fermentation; SLS, selected sourdough; SS, spontaneous sourdough; TPA, texture profile analysis; TTA, titratable acidity; WSE, water/salt-soluble extract.

* Corresponding author.
E-mail address: carlogiuseppe.rizzello@uniba.it (C.G. Rizzello).

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backslopping procedure (type II sourdough) and the use of lactic acid bacteria starter cultures have been adopted aiming at shortening and simplifying the fermentation process at artisanal/industrial level (Corsetti, 2013).

The advent of baker's yeast in modern baking has not decreased the importance of sourdough fermentation, thanks to the many advantages on the sensory, structural, nutritional, and shelf-life properties that it can confer to leavened baked goods (Gobbetti et al., 2014). The use of sourdough, thanks to the effects of lactic acid bacteria metabolism, improves dough workability, bread structure and organoleptic and nutritional properties of the raw ingredients; moreover, it increases the content of biogenic compounds, decreases the level of anti-nutritional factors and the value of the glycaemic response, and increases the uptake of minerals (Gobbetti et al., 2014). Nevertheless, fermentation processes depend on specific determinants, which have to be strictly controlled to get standardized and agreeable products (Hammes and Ganzle, 1998). Among these determinants, the characteristics of the microrganisms involved, the environment, the type of flour, the fermentation parameters and the modality of sourdough propagation are of great importance. It was largely demonstrated that, to exploit the potential of local flours, the selection of adequate starter cultures is necessary (Coda et al., 2014). Nevertheless, the use of industrial starter cultures for cereal fermentation is limited, and when used, starters often lack biochemical properties to differentiate the products and to exploit the potential of the various flour matrices (Coda et al., 2014). Mainly based on the above considerations, the manufacture of bakery products with local flours and tailor made starter cultures deserves great interest to get high quality products (Coda et al., 2014).

Nowadays, the selection of an autochthonous starter suitable for industrial and artisanal bread making together with the use of a proper biotechnological protocol of fermentation would represent a useful tool to better address the production choices of the Tunisian baked goods, improving their qualitative standards (M'hir et al., 2009). Despite the above mentioned great scientific and economic interest, there are no previous studies focused on the characterization of lactic acid bacteria isolated from local cereals or flours, and their potential use in the bakery industry.

This study aimed at selecting lactic acid bacteria isolated from different Tunisian cereal grains to be used as starters for a two-step sourdough fermentation process. A comparison between spontaneous and started (autochthonous lactic acid bacteria) sourdough breads was also done based on the main technological, nutritional, functional, and sensory features.

2. Materials and methods

2.1. Cereal grains and isolation of lactic acid bacteria (LAB)

Different cereal grains (durum wheat, *Triticum durum*; soft wheat, *Triticum aestivum*; triticale, *Triticosecale* Wittm; barley, *Hordeum vulgare*; corn, *Zea mays*; and sorghum, *Sorghum bicolor*) were collected from Tunisia (geographical regions of Ariana, Bizerta, Beja Nabeul, and Seliana) and used for the isolation of LAB. The harvesting was made during the season 2013–2014.

LAB were isolated from grains according to the protocol proposed by Hartnett et al. (2002). Ten grams of each grain sample were put into 50 ml of modified-MRS (mMRS) broth (maltose, 1% *w*/*v*; lactose, 1% *w*/*v*, and fresh yeast extract 10% *v*/*v*; final pH 5.6) and incubated at 30 °C for 4 days. For the isolation of LAB, the supernatants were diluted and plated in mMRS (Oxoid, Basingstoke, Hampshire, UK) agar medium, (Corsetti and Settani, 2007). To avoid yeast growth, cycloheximide (0.1 g/l) was added to the media. Incubation was carried out at 30 °C for 48 h under anaerobic conditions. Colonies characterized by different morphologies were randomly picked from the agar plates at the highest dilution. Gram-positive, catalase-negative, non-motile rod and cocci isolates were cultivated in the broth medium at 30 °C for 24 h, and restreaked into the same agar medium. All isolates considered for further analyses were able to acidify the culture medium.

2.2. Genotypic characterization by randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) analysis

Genomic DNA of LAB was extracted according to De Los Reyes-Gavilan et al. (1992). Three oligonucleotides, P4 (5′-CCGCAGCGTT-3′), P7 (5′-AGCAGCGTGG-3′) (Corsetti et al., 2003), and M13 (5′-GAGGGT GGCGGTTCT-3′) (Stendid et al., 1994), with arbitrarily chosen sequences, were used for bio-typing of LAB isolates. Reaction mixture and PCR conditions for primers P4 and P7 were those described by Corsetti et al. (2003), whereas those reported by Zapparoli et al. (1998) were used for primer M13. RAPD-PCR profiles were acquired through the Gel Doc 2000 Documentation System and compared using the Fingerprinting II Informatix™ Software (Bio-Rad Laboratories). Dice coefficients of similarity and UPGMA algorithm were used to estimate the similarity of the electrophoretic profiles.

2.3. Genotypic identification of lactic acid bacteria

To identify presumptive LAB, two primer pairs (Invitrogen Life Technologies, Milan, Italy), LacbF/LacbR and LpCoF/LpCoR, were used for amplifying the 16S rDNA (De Angelis et al., 2006). Electrophoresis was carried out on agarose gel at 1.5% (wt/vol) (Gellyphor, EuroClone) and amplicons were purified with GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare). Sequencing electrophoregram data were processed with Geneious (http://www.geneious.com). rDNA sequence alignments were carried out using the multiple sequence alignment method (Edgar, 2004) and identification queries were fulfilled by a BLAST search in GenBank (http://www.ncbi.nlm.nih.gov/genbank/).

2.4. Characterization and selection of lactic acid bacteria

Aiming at the selection of LAB strains to be used as starters for sourdough making, 28 strains were singly inoculated in dough made with a Tunisian soft wheat flour, and characterized. In particular, the flour, commonly used for making local white wheat bread, was purchased from an industrial mill (Les Grands Moulins de Tunis, Tunisia). Moisture, protein, starch, fat, dry gluten, ash, falling number and aflatoxin were determined according to AACC methods: 44-15.02, 46-14.03, 76-13.01, 30-10.01, 38-12.02, 56-81.03 and 45-15.01, respectively (AACC, 2003).

Flour strength (W), tenacity (P) and extensibility (L) were determined through the Chopin Alveograph (MA82, Villeneuve-la-Garenne, France).

LAB in wheat flour were enumerated using MRS agar medium, supplemented with cycloheximide (0.1 g/l). Plates were incubated under anaerobiosis (AnaeroGen and AnaeroJar, Oxoid), at 30 °C for 48 h. The cell density of yeasts was estimated on Malt extract Agar (Oxoid) medium, supplemented with chloramphenicol (0.1 g/l), at 30 °C for 72 h.

Aiming at the characterization and the selection of LAB, 28 autochthonous LAB strains were cultivated into mMRS broth at 30 °C for 24 h. Cells were harvested by centrifugation (10,000 $\times g$, 10 min, 4 °C), washed twice in 50 mM sterile potassium phosphate buffer (pH 7.0) and re-suspended in tap water. Sixty-two grams of the Tunisian flour and 37.5 ml of tap water, containing the cells of each lactic acid bacterium (cell density in the dough of *ca.* log 7.0 cfu/g), were used to prepare 100 g of dough. Dough yield (DY (dough weight / flour weight) $\times$ 100) was 160. Mixing was done manually for 5 min. Doughs were fermented at 30 °C for 16 h, according to the optimal growth temperature of the selected LAB and the fermentation time allowing the obtaining of the proper biochemical properties (Coda et al., 2009; Nionelli et al., 2014). The pH value of doughs was determined by a pH meter (Model 507, Crison, Milan, Italy) with a food penetration probe. Total titratable acidity (TTA) was determined after homogenization of 10 g of dough with

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