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Molecular and phenotypic characterization of *Pichia fermentans* strains found among Boza yeasts

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ABSTRACT

Fifty-one yeasts were randomly isolated from four different preparations of Boza, a Turkish cereal-based beverage in order to throw light upon their biotechnological potential. Sequence analysis of 26S rDNA D1/D2 domain revealed that the isolated yeasts belonged to *Candida*, *Clavispora*, *Cryptococcus*, *Cystofilobasidium*, *Geotrichum*, *Coniochaeta*, *Pichia*, *Rhodotorula*, *Saccharomyces*, *Torulaspora* and *Trichosporon* genera. Interestingly, *Pichia fermentans* resulted the dominant species with 21 isolates; a high polymorphism among these yeasts resulted by RAPD-PCR analysis which allowed to distinguish 15 strains that were characterized for their biochemical and technological features. In addition, all *P. fermentans* strains did not assimilate D-maltose and D-sucrose and expressed a very low invertase activity, suggesting their possible coexistence with maltose- and sucrose-fermenting lactic acid bacteria in Boza microbial ecosystem. Three yeasts produced a high amount of the rose-like fragrance 2-phenylethanol that might be involved in flavor development of this traditional beverage. Moreover, these yeasts also displayed an antagonistic activity against *Trichosporon cutaneum*, a pathogen occurring in Boza. These selected yeasts are at disposal of Boza manufacturers who could apply them, in combination with LAB, for producing a high quality beverage in accordance with the Turkish quality standards.

1. Introduction

Boza is a cereal-based fermented beverage, originated in Mesopotamia 8000–9000 years ago. It is a light yellow, thick, sour drink produced in different parts of the world with light changes in the basic recipe and described in the literature under similar names. Closer variations of this drink are consumed in Balkans, Egypt and Kenya, even though these beverages are very acidic and alcoholic (Blandino, Al-Aseeri, Pandiella, Cantero, & Webb, 2003; Botes, Todorov, von Mollendorff, Botha, & Dicks, 2007; Sanni, 1993).

Different cereals such as wheat, rye, maize, millet can be used for Boza production. This beverage is usually stored at 4 °C and its shelf-life is short (one or two weeks); then, the product spoils and becomes undrinkable.

This slightly acid and highly viscous beverage is characterized by spontaneous fermentation, which is started by a daily back-slopping rising from a previous production involving heterofermentative lactic acid bacteria (LAB) belonging to *Lactobacillus*, *Lactococcus*, *Pediococcus* and *Leuconostoc* genera and yeasts belonging to *Candida*, *Geotricum*, *Torulaspora*, *Issatchenkia* and *Pichia* genera (Arici & Daglioglu, 2002; Botes et al., 2007; Gotcheva, Pandiella, Angelov, Roshkova, & Webb, 2000; Gotcheva, Pandiella, Angelov, Roshkova, & Webb, 2001; Hancioğlu & Karapinar, 1997; Zorba, Hancioglu, Genç, Karapinar, & Ova, 2003). Over the course of Boza processing and storage, the growth of LAB leads to an increase in free short-chain fatty acid concentration, mainly represented by acetic acid (Morea, 2008) that helps the product get its characteristic taste and reduces the growth of harmful bacteria as *Escherichia coli*, *Pseudomonas aeruginosa* and *Enterococcus faecalis* (Todorov & Dicks, 2006) and can include potential probiotic lactobacilli (Todorov et al., 2008). However, interactions among microorganisms are not controlled during the process, leading to variations in quality and stability of the product (Botes et al., 2007; Genç, Zorba, & Ova, 2002; Gotcheva et al., 2000; Hancioğlu & Karapinar, 1997).

Recently, yeasts, belonging to *Candida*, *Geotricum, Torulaspora, Issatchenkia* and *Pichia* genera, have been found in Boza (Botes et al., 2007) in spite of the fermentation of this beverage is occasionally started by bread sourdough or selected *Saccharomyces cerevisiae* strains altering the flavor and aspect of this traditional beverage (Todorov et al., 2009; Zorba et al., 2003). The occurrence of wild yeasts in Boza was mainly discussed on the basis of the potential issues regarding the transmission of food mycoses (Botes et al., 2007).

Despite the fact that lactic acid bacteria from Boza have been extensively studied, only few reports on the presence and the role of yeasts have been produced. Thus, in the present work we isolated and identified the mycobiota of four different preparations of Boza, spontaneously fermented, and characterized the dominant yeasts for their biotechnological properties. These selected yeasts are at

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disposal of Boza manufacturers who could apply them, in combination with LAB, for producing a high quality beverage in accordance with the Turkish quality standards.

2. Materials and methods

2.1. Method for Boza production

All Boza samples assayed were produced in different months of the year (from February 2008 to May 2009) by DENIZ PAZARLAMA, a traditional Boza factory located in Yildirim/Bursa/Turkey in accordance with the flow sheet shown in Fig. 1. The composition of four different kinds of Boza samples (named 1, 2, 3 and 4) analyzed in this work is shown in Table 1. Three replicates of each bottled Boza sample (3 L per bottle) were delivered under controlled temperature (about 4 °C) to the Institute of Sciences of Food Production (ISPA) in Bari and analyzed after 1 week of cold storage.

2.2. Physical and chemical analyses

The pH of Boza samples was measured with the Φ 340 pH/Temp Meter system (Beckman Coulter, Fullerton, CA, USA) equipped with a liquid food drill at 20 °C.

Total titratable acidity (TTA) was calculated following the AOAC protocol 947.05/2000 (AOAC, 2000).

Water activity (a_w) of Boza was determined with the DECAGON AquaLab Serie 3TE system (Aqualab, Pullman, WA, USA), following the manufacturer's instructions. The mean values were obtained from three replicates.

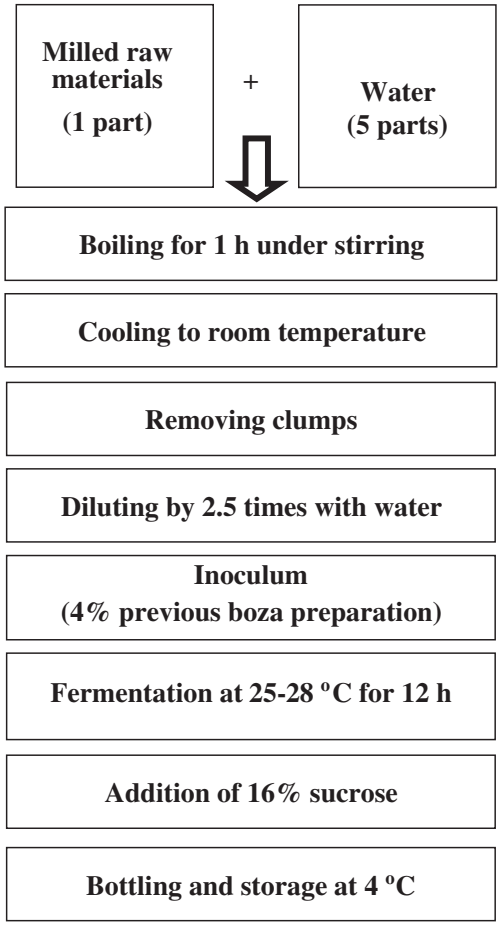

Fig. 1. Boza production process flow chart.

Table 1
Composition of four different kinds of Boza.

Ingredients (%, w/w)						
Boza sample	Water	Maize	Wheat	Bulgur [a]	Rice	Potato
1	83.30	9.20	7.50	_	_	_
2	83.67	7.00	3.50	_	5.83	_
3	83.30	4.20	_	7.50	5.00	_
4	83.34	5.83	3.33	_	_	7.50

[a] Bulgur is a durum wheat germinated, steamed and subsequently dried and milled.

2.3. Microbiological analyses and selection of yeast colonies

All media and ingredients, if not differently specified, were purchased from Oxoid (Basingstoke, Hampshire, UK).

A 10-g portion of each beverage sample was aseptically homogenized in 90 mL of autoclaved saline peptone water (0.85% NaCl, 0.1% peptone, pH 7) for 2 min in a stomacher apparatus (400 Circulator, PBI International, Milan, Italy). Appropriate 10-fold dilutions of the homogenized sample were carried out in saline peptone water and plated in triplicate on different media: Plate Count Agar (PCA) for total bacterial count; M17 with 0.5% lactose and MRS agar for presumptive Gram positive cocci and bacilli, respectively; Potato Dextrose Agar (PDA) supplemented with chloramphenicol (100 mg L^{-1}; Sigma-Aldrich S.r.l., Milan, Italy) for enumerating yeasts and molds. PCA plates were kept at 30 °C for 48 h under aerobic condition, whereas M17 and MRS plates were incubated at the same temperature under anaerobic condition (AnaerogenTM system, Oxoid S.p.A., Garbagnate, Milano, Italy). PDA plates were kept at 25 °C for 5 days under aerobic conditions.

Microbial counts were log-transformed and expressed as mean values (± standard deviation) of at least three independent experiments.

For each beverage sample, 11–17 yeast colonies, representing from 10 to 20% of the colonies counted onto PDA plates seeded with the highest sample dilutions, were randomly picked up, inoculated in YPD broth (2% peptone, 1% yeast extract and 2% glucose), and incubated at 25 °C, 170 rpm for 24 h. After centrifugation (6,000×*g* for 5 min), the isolates were suspended in YPD, containing 20% glycerol, and stored at −80 °C for further characterization.

2.4. Genomic DNA extraction and molecular analyses

All enzymes and reagents for molecular analyses were from Sigma-Aldrich S.r.l. (Milan, Italy), if not differently specified.

Yeast DNA was isolated using the protocol of Hofman and Winston (1987). Quantity and quality of DNA were determined with the Nanodrop ND-1000 (Nanodrop Technologies, Inc., Wilmington, DE, USA) system, following the manufacturer's instructions. DNA samples were kept at −20 °C until use for PCR tests.

Yeast isolates were identified amplifying and sequencing the 26S rDNA D1/D2 domain, as reported by Yarrow (1998); sequences are available through GenBank Accession numbers from JN417604.1 to JN417654.1.

The DNA sequences were obtained using an ABI PRISM™ Big Dye Terminator Cycle Sequencing Kit version 3.1 (PE Applied Biosystems, Inc., Foster City, CA, USA) with both the forward and reverse primers being used. The reaction products were analyzed with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The software package of the Applied Biosystems (Sequencing Analysis release 3.3 and MT Navigator PPC release 1.0.2) was used for the analysis and comparison of DNA sequences. Taxonomic strain identification was performed comparing the rDNA sequences of yeast isolates with sequences present in the Basic BLAST Search (http://www.ncbi.nih.gov/BLAST), as described by Altschul et al. (1997).

Strain typing of isolates belonging to *Pichia fermentans* was carried out applying the "two-step RAPD PCR" protocol, as described by Baruzzi, Morea, Matarante, and Cocconcelli (2000) using M13: