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Unpasteurised commercial boza as a source of microbial diversity

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ABSTRACT

Boza is a cereal-based fermented beverage widely consumed in many countries of the Balkans. The aim of this study was to investigate the microbiota of three Bulgarian boza samples through a combination of culture-dependent and -independent methods with the long-term objective of formulating a multi-strain starter culture specifically destined for the manufacture of new cereal-based drinks. The isolation campaign for lactic acid bacteria (LAB) allowed the identification of *Lactobacillus parabuchneri*, *Lactobacillus fermentum*, *Lactobacillus coryniformis*, *Lactobacillus buchneri*, *Pediococcus parvulus* and members of the *Lactobacillus casei* group. Concerning yeasts, the following isolates were identified: *Pichia fermentans*, *Pichia norvegensis*, *Pichia guilliermondii* (synonym *Meyerozyma guilliermondii*) and *Torulaspora* spp. A high intra-species diversity was revealed by Randomly Amplified Polymorphic DNA (RAPD) analysis. In parallel, microbial DNA was directly extracted from the three boza samples, and portions of the rrn operons were analysed through Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE). The molecular fingerprinting partially confirmed the results of culturing. Among LAB, the species *Weissella confusa*, *Weissella oryzae*, *Leuconostoc citreum*, *Lactococcus lactis*, *Pediococcus parvulus* and *Pediococcus ethanolidurans* were detected together with members of the *Lb. casei* group. Among the yeasts, the species *P. fermentans*, *M. guilliermondii*, *Galactomyces geotrichum* and *Geotrichum fragrans* were found. The overall results confirmed boza as having a rich and heterogeneous biodiversity both in terms of species and genetically diverse strains, thus encouraging its exploitation for the isolation and future technological characterisation of cultures to be selected for the manufacture of innovative cereal-based drinks.

1. Introduction

A great variety of beverages worldwide are produced from cereals, including beer, sake, chicha, mahewu and boza (Blandino et al., 2003). The latter is a viscous low-alcoholic drink manufactured both in the Balkan Peninsula, namely, Bulgaria, Albania, the Republic of Macedonia, Southern Romania and Turkey and in Northern Africa (Altay et al., 2013; Arici and Dağlioğlu, 2002; Blandino et al., 2003; Gotcheva et al., 2001; Hayta et al., 2001; Kabak and Dobson, 2011; LeBlanc and Todorov, 2011). Because of its palatable sweet taste and slightly acid savour, boza is greatly appreciated and consumed by individuals of different ages (Altay et al., 2013; Blandino et al., 2003; Gotcheva et al., 2001; Kabak and Dobson, 2011).

Boza is traditionally produced from the fermentation of various cereals, which are used individually or in combination, such as barley, oats, millet, maize, wheat or rice (Kabak and Dobson, 2011). In more detail, the grains are usually washed, reduced to the size of semolina and cooked in a steam boiler with water (Kabak and Dobson, 2011). For the industrial production of boza, the grains are cooked in an autoclave for about 2 h at 4–5 atmospheres (Blandino et al., 2003); the cooked mixture is then left to cool, strained and added with cold water and sugar or saccharine (15–20%, w/w) (Kabak and Dobson, 2011; LeBlanc and Todorov, 2011). The mixture obtained is subsequently fermented at 30 °C for 24 h by adding boza obtained from a prior production, sourdough, or yogurt as inoculum (at 2–3%, v/v). After fermentation, boza is cooled to +4 °C, packed in plastic bottles and kept under refrigerated conditions (Caputo et al., 2012; Kabak and Dobson, 2011; LeBlanc and Todorov, 2011).

Fermentation confers sensory quality, digestibility and nutritional properties to the end product; in fact, boza is considered a healthy and nutritious beverage because of its fat, protein, carbohydrate, fibre, vitamin, amino acid and lactic acid content. The latter compound is widely recognised as having positive effects on the intestinal microflora and digestion trophism (Arici and Dağlioğlu, 2002). Moreover, during the production of boza, the decrease in pH to values around 3.0–4.0 produces optimal conditions for the enzymatic degradation of phytates, helps the product to acquire its typical flavour and prevents the growth of pathogenic and/or spoilage bacteria (Kabak and Dobson, 2011). This latter aspect could be amplified by the effect of antimicrobial substances, such as bacteriocines, synthesised by lactic acid bacteria (LAB), as extensively documented (LeBlanc and Todorov, 2011; Todorov et al., 2008, 2009; Todorov, 2010; Todorov and Dicks, 2006).

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Recently, Kancabaş and Karakaya (2012) have demonstrated that boza can also be a source of ACE-inhibitory peptides, thus contrasting hypertension.

From the available literature, the microbiota responsible for the fermentation of boza seems to be particularly heterogeneous, including either homo- or hetero-fermentative LAB and yeasts (Altay et al., 2013; Gotcheva et al., 2000). However, to the authors' knowledge, only a few papers have been published on the microbial composition of boza (Botes et al., 2007; Caputo et al., 2012; Gotcheva et al., 2000, 2001; Hancioğlu and Karapinar, 1997), and most of them report the identification of LAB and yeasts through traditional phenotype-based methods (Gotcheva et al., 2000, 2001; Hancioğlu and Karapinar, 1997). The latter are often unreliable and lack the resolving power to analyse the microbial composition of mixed microbial populations (Ben Amor et al., 2007), especially composed of LAB, which are notoriously characterised by analogous nutritional requirements and growth under similar environmental conditions (Aquilanti et al., 2007).

Over the last decade, a panoply of molecular approaches based on the analysis of DNA sequences have been applied for species and strain identification of food-borne microorganisms. Among these, the Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) technique has shown great potential for outlining the microbial diversity of fermented foods including cereal-based products (Cocolin et al., 2013).

Based on the above premise, the present study was aimed at disclosing the microbial diversity of unpasteurised Bulgarian boza through a combination of culture-dependent and -independent methods, with the long-term objective of investigating the technological traits of LAB and yeasts isolated from boza in order to formulate a multi-strain starter culture specifically destined for the manufacture of new cereal-based drinks. To describe the microbiota of the three commercial beverages, a representative pool of LAB and yeasts was isolated on suitable solid media, and further identified and typed by sequencing of portions of the rrn operons and Randomly Amplified Polymorphic DNA (RAPD) analysis, respectively. In parallel, the total microbial DNA was extracted directly from the three boza and portions of the rRNA coding genes were analysed through PCR-DGGE. No details regarding the technological traits of the isolated cultures are provided in this study.

2. Materials and methods

2.1. Reference strains and culture conditions

Four bacterial reference strains (*Lactobacillus paracasei* NRRL 4560^T, *Lactobacillus plantarum* DSMZ 2601, *Pediococcus pentosaceus* DSMZ 20336^T and *Lactobacillus parabuchneri* DSMZ 5708) and four yeast reference strains (*Wickerhamomyces anomalus* DBVPG 6613, *Starmerella bombicola* DBVPG 3827, *Candida humilis* CBS 6897^T and *Saccharomyces cerevisiae* CBS 1171^T) were used as controls in the PCR-DGGE analyses. These cultures were purchased from (i) the "National Center for Agricultural Utilization Research" (NRRL Collection, Preoria, USA, http://nrrl.ncaur.usda.gov/); (ii) the "Deutsche Sammlung von Mikrorganismen und Zellkulturen" (DSMZ Collection, Braunschweig, Germany, http://www.dsmz.de/); (iii) the "Department of Agricultural, Food and Environmental Science, University of Perugia" (Industrial Yeast Collection DBVPG Collection, Perugia, Italy, http://www.dbvpg.unipg.it/index.php/en/); and (iv) the "Centraalbureau voor Schimmelcultures" (CBS Collection, Utrecht, the Netherlands, http://www.cbs.knaw.nl/index.php/collection).

The bacteria were grown on MRS agar (Oxoid, Basingstoke, UK) incubated at 30 °C for 48 h under anaerobiosis, while yeasts were grown on YPD agar (Sigma-Aldrich, St. Louis, USA) incubated at 25 °C for 72 h.

2.2. Sampling

Three industrially produced unpasteurised bottled boza (coded bz1, bz2, and bz3) from different manufacturers were purchased from local retail points located in Sofia (bz1), Koynare (bz2) and Mezdra (bz3) (Bulgaria) during storage at +4 °C. The sampling was performed extensively prior to the expiry date reported on the labels of the three boza, with a declared shelf life of 10 (bz1) and 6 (bz2 and bz3) days, respectively. After purchasing, the three samples were kept under refrigerated conditions using a portable refrigerator plugged to the car power supply and analysed within 36 h. All three boza samples were manufactured using water, durum wheat flour and saccharose; bz3 also contained ascorbic acid (E-300) and the following sweeteners: acesulfame-K (E-950), aspartame (E-951), sodium cyclamate (E-952) and saccharin (E-954).

2.3. pH measurements

The pH potentiometric measurements on boza samples were carried out with a model 300 pH meter equipped with an HI2031 solid electrode (Hanna Instruments, Padova, Italy). For each sample, three independent measurements were performed.

2.4. Microbial counting and isolation of LAB and yeasts

Aliquots (10 mL) of each boza sample were serially diluted in 90 mL of sterile saline peptone water (0.9% NaCl, 0.1% peptone, pH 7.0), and 100 μL of each dilution was streaked in duplicate on the suitable selective solid media listed below.

LAB were counted as previously described by Aquilanti et al. (2013); briefly, (i) lactococci and thermophilic cocci on M17 agar (Oxoid); (ii) lactobacilli on MRS agar (Oxoid) under anaerobiosis; and both media were added with cycloheximide (Osimani et al., 2009) to inhibit the growth of eumycetes.

Yeasts were counted on Wallerstein Laboratory Nutrient (WLN) agar (Oxoid) added with chloramphenicol (Osimani et al., 2009). The results of the viable counts were expressed as means of the log of colony forming units (cfu) per mL of sample ± standard deviation.

For each boza sample, colonies were selected on the basis of colony morphology and sub-cultured to purity on the same solid substrates. In all cases, a representative number of colonies were picked up, corresponding to about 10% of the colonies counted on MRS, M17 and WLN plates seeded with the highest sample dilution. For each isolate, the cell morphology was examined using a light microscope under oil-immersion (100×). Bacterial isolates were tested for Gram and catalase reactions. Gram-positive and catalase-negative bacteria were then stored at −80 °C in a mixture of glycerol and MRS (1:1); yeasts were stored in glycerol and YPD (1:1).

2.5. Molecular identification of LAB and yeast isolates

Cryopreserved LAB and yeast isolates were first cultured on suitable media. For LAB some colonies were suspended in 300 μL of sterile water; the suspension underwent DNA extraction using the method proposed by Hynes et al. (1992) with some modifications. Briefly, after centrifugation of the LAB suspensions, the cell pellets were resuspended in 1 mL of STE buffer [10 mM Tris–HCl pH 8.0, 100 mM NaCl, 1 mM EDTA pH 8.0, 20% sucrose (w/v)] containing 25 mg mL^{-1} of lysozyme. After incubation at 37 °C for 3 h, samples were centrifuged at 14,000*g* for 3 min, and the pellets suspended in 1 mL of lysis buffer consisting of 50 mM KCl, 10 mM Tris–HCl pH 8.0, 0.45% Tween 20 (w/v) 0.45% Triton X (w/v) supplemented with 100 μg mL^{-1} proteinase K. After incubation at 60 °C for 3 h, samples were heated at 95 °C for 10 min.

For yeasts, the DNA was extracted as described by Makimura et al. (1999).

The DNA quantity and purity were assessed by optical readings at 260, 280 and 234 nm, respectively, using a UV-Vis Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

The LAB isolates were subjected to molecular identification by sequencing of the 16S rRNA gene, using universal primers for eubacteria