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Characterization of yeasts from Portuguese brined olives, with a focus on their potentially probiotic behavior

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ABSTRACT

The functional properties of the dominant adventitious yeast strains in Portuguese cultivars of brined olives were evaluated. Identification followed traditional taxonomic methods, complemented with molecular biology approaches. The yeast population ranged in 3–5 $\log_{10}$ (cfu/mL), and included chiefly *Pichia membranaefaciens*, *Pichia fermentans*, *Saccharomyces cerevisiae* and *Candida oleophila*. A few strains exhibited desirable technological features, viz. absence of pectinolytic and lipolytic activities, positive catalase response, high osmotolerance, ability to uptake oleuropein and lactic acid, and capacity to produce B-complex vitamins. Furthermore, antimicrobial activity against selected food-borne bacterial pathogens was observed, as well as release of mycocin. *P. membranaefaciens* and *C. oleophila* appeared as the most promising candidates for eventual inclusion in tailor-made probiotic starter/adjunct cultures.

1. Introduction

Olive fruits are a traditional Mediterranean product, characterized by relatively low levels of sugars (20–50 g/kg) but high fat contents (200–350 g/kg); they normally exhibit a bitter taste caused by oleuropein, so edibility normally requires prior debittering. There are several ways to process table olives – either on the farm or industrial levels (Gómez, García, & Navarro, 2006). Directly brined olives are obtained in Portugal via a traditional process based on fermentation by their adventitious microflora, which encompass a great many different microorganisms; however, lactic acid bacteria (LAB) become dominant, together with yeasts, towards the end of fermentation (Nychas, Panagou, Waldron, & Tassou, 2002).

Yeasts are ubiquitous eukaryotic microorganisms, which play an active role in several fermented foods and beverages. They have also been implicated in synthesis of such interesting secondary products as antioxidants and vitamins (Fleet, 2006). Viable numbers of yeasts in olives typically range in 4–6 $\log_{10}$ (cfu/mL) (Marquina et al., 1992; Nisiotou, Chorianopoulos, Nychas, & Panagou, 2009) – and they play desirable roles in table olive fermentation, via e.g. production of volatile compounds (for flavour development), synthesis of antioxidant compounds (for long-term preservation) and biodegradation of polyphenols (for LAB growth enhancement) (Arroyo-López, Querol, Bautista-Gallego, & Garrido-Fernández, 2008). Yeasts are thus particularly important in directly brined olives; since their population is often larger than that of LAB during most part of the processing period (Peito et al., 2006), well-adapted yeast strains with desirable metabolic features may in principle be used, as starter or adjunct cultures, to improve the final quality of those olives (Psani & Kotzekidou, 2006) – especially aiming at large scale manufacture.

Therefore, assessment of the metabolic features of adventitious yeasts is in order, to back up strain selection afterwards. Such features include pectinolytic, lipolytic and catalase activities, oleuropein and lactic acid assimilation, osmotolerance and B-complex vitamin production; they might as well serve as objective functions for optimum technological performance (Hernandéz, Martín, Aranda, Pérez-Nevado, & Córdoba, 2007; Psani & Kotzekidou, 2006; Ruiz-Barba & Jiménez-Díáz, 1995). Furthermore, their antimicrobial features – viz. ability to secrete mycocins that are lethal to undesirable yeasts coupled with other activities against food-borne pathogens, would complement their added-value in biocontrol strategies, besides leading to design of novel probiotic products (Hernandéz et al., 2007; Psani & Kotzekidou, 2006).

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The aim of this research effort was thus to study the technological features of the dominant wild yeasts in the brines of "Galega" and "Cordovil" table olives – with the final goal of assessing them as starter cultures with a probiotic potential.

2. Materials and methods

2.1. Isolation and identification of yeasts

Microbiological analyses were performed on aliquots of spontaneously fermented olive brines from three distinct batches, collected at different fermentation times (see Fig. 1). For yeast counts, samples were plated on GYPA (Glucose Yeast extract Peptone Agar), supplemented with chloramphenicol (0.1 g/L), and incubated at 25 °C for 5 d; a total of 273 yeast strains were accordingly isolated. Yeasts were first identified according to morphological and physiological characteristics (Barnett, Payne, & Yarrow, 1990, chap. 9; Kreger-van Rij, 1984, chap. IV). Afterwards, identification was confirmed by PCR-RFLP, as described by Esteve-Zarzoso, Belloch, Uruburu, and Querol (1999); the DNA was extracted as previously described by Baleiras-Couto, Reizinho, and Duarte (2005).

Representative strains of each PCR-RFLP profile were identified to the species level, by sequencing the D1/D2 region of the 26S rRNA gene (Kurtzman & Robnett, 1998). The sequences obtained were compared by BLASTn with homolog 26S rRNA gene partial sequences from the DNA databank.

2.2. Technological performance of yeasts

Tests to ascertain tolerance to low pH values (2.5), resistance to 3 g/L bile salts and assimilation of 1 g/L oleuropein were performed according to Psani and Kotzekidou (2006). Hydrolysis of tributyrin (to assay for esterase activity) and lipolysis of olive oil (to assay for lipase activity) were monitored using the double layer method designed by Fryer and Reiter (1967). Osmotolerance to 50 and 100 g/L NaCl, assimilation of lactic acid and extracellular pectinolytic activity were assayed for using the assimilation tests of carbon compounds described by Kreger-van Rij (1984). Catalase activity was determined by adding 30 volumes of H_2O_2 to the cultured colonies; release of gas was indicative of a positive result.

2.3. Production of B-complex vitamins by yeasts

The capacity of our yeast strains to produce B-complex vitamins was assessed according to Ruiz-Barba and Jiménez-Díáz (1995).

2.4. Production of mycocins by yeasts

The mycogenic activity was determined as described by Marquina et al. (1992). The strains showing inhibition zones with, at least, 10 mm of diameter were considered mycocin-producing strains.

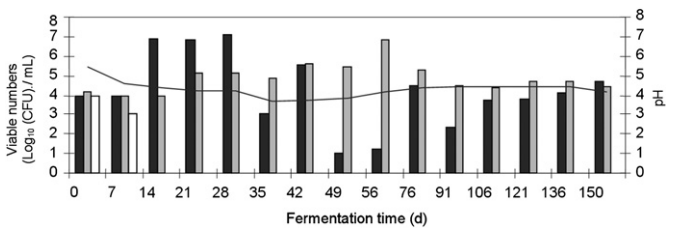

Fig. 1. Evolution of viable numbers of the adventitious microflora throughout brining of olives – (▫) Yeasts, (■) Lactic acid bacteria and (□) Gram-negative bacteria, and of (·) pH.

2.5. Antimicrobial activity of yeasts

A few common food-borne pathogens were selected, viz. *Escherichia coli* (ATCC 25922), *Listeria monocytogenes* (ATCC 7644), *Salmonella enteritidis* (ATCC 13076) and *Staphylococcus aureus* (ATCC 25923); they were maintained on Trypticase Soy Soft (TSS) agar (20 mL, with 8 g/L agar), and incubated at 37 °C for 24 h. The capacity of each yeast strain to inhibit the aforementioned bacterial pathogens was determined using TSS agar medium. One loopful (i.e. 2 mm in diameter) of yeast was streaked as a line (2–3 cm) onto the soft-agar surface; the plates were then incubated at 27 °C for 24 h, and screened for inhibition zones around the bacterial colonies.

2.6. Potential probiotic features of yeasts

The yeasts were screened for potential probiotic roles – via *in vitro* assays for their ability to grow at 37 °C, and resist low pH and presence of bile salts. The former was studied in YM broth. The effect of exposure to low pH was qualitatively evaluated in YM broth, acidified to pH 2.5, and incubated for up to 10 d. The effect of exposure to bile salts was examined using Yeast Nitrogen Base agar plates containing 3 g/L Oxgall, incubated at 27 or 37 °C for 3 d (Psani & Kotzekidou, 2006).

3. Results and discussion

3.1. Quantitative and qualitative profiles of yeasts

LAB and yeasts coexisted in the table olive brines studied; the yeast population ranged in 3–5 $\log_{10}$ (cfu/mL), whereas the LAB viable counts were 4–7 $\log_{10}$ (cfu/mL) (Fig. 1). Despite these differences, yeasts appeared chiefly during early brining – so a practical interest exists with regard to wild yeast ecology in olive brines. Substrate availability, low pH and high polyphenolic content may be considered as relevant factors upon microflora survival and dynamics (Oliveira et al., 2004); the content of NaCl (80 g/L) added at start-up constrains growth of LAB and less resistant yeasts species.

Identification of yeast isolates was carried out using morphological, biochemical and physiological features – and 16 species were successfully identified from a total of 273 strains, viz. *Candida boidinii*, *Citeromyces matriensis*, *Pichia membranaefaciens, Pichia fermentans, Candida oleophila*, *Candida citrea*, *Candida sake*, *Candida silvae*, *Candida valida*, *Candida norvegica, Metschnikovia pulcherrima*, *Rhodosporidium capitatum*, *Torulaspora delbrueckii*, *Trichosporum pullulans*, *Saccharomyces cerevisiae* and *Kloeckera apiculata*. The species most frequently found were *P. membranaefaciens*, *P. fermentans*, *S. cerevisiae* and *C. oleophila*; viable counts showed that *P. membranaefaciens* reached usually the highest levels. These species had been consistently found in olive brines elsewhere (Hernandéz et al., 2007; Nisiotou et al., 2009; Oliveira et al., 2004; Peito et al., 2006).

All subsequent studies were logically conducted with the predominant species: e.g. the tests for killer protein and vitamin synthesis (see Tables 2 and 3, respectively) encompassed only those species that were more frequently found by the end of fermentation (i.e. *P. membranaefaciens*, *S. cerevisiae*, *C. oleophila* and *P. fermentans*) because only those may bring about a competitive advantage over other species based on ecological pressure. Hence, only those were identified by molecular methods. The isolates yielded different ITS-PCR product sizes, ranging from 450 to 880 bp in length; the PCR products from strains of the same species had identical, or somewhat similar molecular sizes. After digestion with *Hinf I* and *Cfo I* enzymes, 7 different ITS-RFLP profiles could be