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Selection and evaluation of *Debaryomyces hansenii* isolates as potential bioprotective agents against toxigenic penicillia in dry-fermented sausages

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ABSTRACT

Biocontrol using autochthonous *Debaryomyces hansenii* isolates is a potentially suitable strategy for inhibiting toxigenic moulds in dry-cured meat products. The antifungal activity of 280 *D. hansenii* isolated from dry-cured meat products as well as the mode of action of the most active isolates against toxigenic penicillia were evaluated in this work. A 13.9% of the *D. hansenii* isolates showed inhibitory activity in a radial inhibition assay. The effects on penicillia growth of both the cell-free culture filtrate and volatile compounds from active yeast isolates were analysed. Penicillia growth inhibition by *D. hansenii* was probably based on additive or synergistic effects of several inhibiting factors such as competition for nutrient and space, and production of soluble or volatile compounds. When four *D. hansenii* isolates were tested on dry-fermented sausage, two of them produced a significantly growth reduction of the ochratoxigenic *Penicillium verrucosum*, keeping its counts under the level considered as hazardous for the mycotoxin presence. Therefore, the use of these two *D. hansenii* isolates during the processing of dry-fermented meat product could be a promising tool to control toxigenic moulds in the meat industry.

1. Introduction

During the processing of dry-cured meat products, ecological conditions favour the development of an uncontrolled mould population, mainly composed by penicillia (Alapont et al., 2014; López-Dıaz et al., 2001; Núñez et al., 1996a). Several species of *Penicillium* are potentially hazardous for consumers, since they are able to produce mycotoxins such as ochratoxin A, patulin or cyclopiazonic acid on these products (Alapont et al., 2014; Iacumin et al., 2009; López-Dıaz et al., 2001; Núñez et al., 1996a, 2007; Rodríguez et al., 2012). These fungal metabolites have shown toxigenic, nephrotoxic, hepatotoxic, immunosuppressive, mutagenic and carcinogenic effects. To control the mycotoxin occurrence through dry-cured meat processing, the prevention of toxigenic mould growth is a key issue. Mould growth can be efficiently controlled in several foods with chemical preservatives or modified atmosphere packaging. However, these treatments are not appropriate for dry-cured meat products, since mould activity is essential for their sensorial characteristics (Martín et al., 2006). Moreover, chemical fungicides can leave residues and nowadays consumers demand residue-free chemical foods.

Biocontrol of toxigenic moulds by antagonistic microorganisms could be an alternative to those chemical and physical methods. In this sense, yeasts have been widely proposed for controlling diseases of fruits and vegetables due to mould growth (Droby et al., 1989; Zhao et al., 2008). Recently the use of autochthonous yeasts against toxigenic moulds has been also explored in dry-cured meat products (Andrade et al., 2014; Simoncini et al., 2014; Virgili et al., 2012). *Debaryomyces hansenii*, the predominant yeast species during the processing of dry-cured meat products (Andrade et al., 2009; Mendonça et al., 2013; Núñez et al., 1996b), is effective in reducing pathogenic moulds in foods such as dairy products (Liu and Tsao, 2009), fruits (Hernández-Montiel et al., 2010) or dry-cured meat products (Andrade et al., 2014; Simoncini et al., 2014; Virgili et al., 2012). *D. hansenii* also contributes to the adequate flavour development of dry-cured meat products (Andrade et al., 2010; Cano-García et al., 2014; Martín et al., 2006). In addition, *D. hansenii* has been included in the list of qualified presumption of

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safety (QPS) of the European Food Safety Authority (BIOHAZ, 2012). Therefore, autochthonous *D. hansenii* with antifungal activity could be useful for developing starter cultures to improve safety and sensorial quality in dry-cured meat products (Andrade et al., 2010, 2014).

An adequate knowledge about the mode of action of antagonistic yeasts is useful both to improve their performance against toxigenic moulds and to establish screening criteria for more effective strains (Liu et al., 2010; Sharma et al., 2009; Taczman-Brückner et al., 2005). Competition for nutrients and space has been suggested as the major mechanism of action of yeasts isolated from foods, including fruits (Droby et al., 1989), vegetables (Zhao et al., 2008) and dry-cured ham (Andrade et al., 2014). Several yeasts showed antifungal activity linked to volatile compounds (Fialho et al., 2009; Masoud et al., 2005; Taczman-Brückner et al., 2005) or killer proteins (Coelho et al., 2009; Hernández et al., 2008; Santos and Marquina, 2004). In addition, yeasts may decrease mycotoxin content by adsorption to cell wall molecules such as glyco mannoproteins (Caridi, 2007) or by blocking the biosynthetic pathway of mycotoxins (Gil-Serna et al., 2011).

The main objective of this work was to select *D. hansenii* isolates from dry-cured meat products with antifungal activity against toxigenic penicillia commonly found in those products. The mechanisms of action of antagonist *D. hansenii* were also evaluated. Finally, the antifungal activity of selected yeasts was checked under simulated ripening conditions for dry-fermented meat products.

2. Materials and methods

2.1. Yeast and mould strains

A total of 280 *D. hansenii* isolates collected from dry-cured meat products in different ripening stages and factories (Andrade et al., 2010) were used. Yeasts were isolated by repeated cultivation on malt extract agar (MEA, Scharlab, Barcelona, Spain) at 25 °C and maintained until use at −80 °C in malt extract broth (MEB, Scharlab) containing 20% v/v glycerol.

Inhibition tests were carried out against 8 toxigenic mould strains chosen among the most frequent toxigenic *Penicillium* spp. found in dry-cured meat products. They were obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain), the Centraalbureau voor Schimmelcultures (CBS, Utrecht, The Netherlands), or the microbial collection of Food Hygiene and Safety of the University of Extremadura (Cáceres, Spain): *Penicillium camemberti* Pcm30, *Penicillium commune* Pc13, *Penicillium expansum* CECT 2278, *P. expansum* CECT 2279, *P. expansum* PxIC2, *Penicillium nordicum* CBS 323.92, *P. nordicum* PnICR4 and *Penicillium verrucosum* Pv21.

2.2. Radial inhibition assay of toxigenic moulds

The ability of *D. hansenii* isolates to inhibit toxigenic moulds was carried out by agar plate inhibition assay similarly to described by Santos et al. (2000), using Yeast Morphology Agar (YMA: 1% (w/v) glucose, 0.3% (w/v) yeast extract, 0.3% (w/v) malt extract, 0.5% (w/v) proteose peptone and 1.8% (w/v) agar), pH 4.5, containing 6% NaCl that enhances killer activity (Marquina et al., 2001). One hundred microliters of a *c.a.* 10^6 spores/ml suspension of each mould strain were spread onto the YMA surface. After drying, 5 μl of a *c.a.* 10^6 cells/ml suspension of each *D. hansenii* isolate were deposited, and then incubated for 7 days at 20 °C. The inhibition rate was determined by measuring the diameter of the inhibition zone outside the yeast colony. The experiment was performed in triplicate using *P. expansum* PxIC2, *P. nordicum* CBS 323.92 and *P. verrucosum* Pv21 as reference moulds. *D. hansenii* isolates showing radial inhibition were selected for further studies.

2.3. Effect of selected D. hansenii isolates on mould growth in solid media

This assay was carried out using 39 active *D. hansenii* isolates against 8 toxigenic penicillia. The test was performed on MEA containing 6% NaCl, adjusted to pH values of 4.5 and 6. One hundred microliters of a *c.a.* 10^6 cells/ml suspension of *D. hansenii* isolates were spread on the MEA plates and, after drying, 10 μl of a *c.a.* 10^6 spores/ml suspension of moulds were spotted. After the incubation for 14 days at 20 °C, the mould growth was estimated measuring the diameter of each colony. The inhibitory activity (IA) was expressed as the percentage of average diameter of mould colonies when cocultured with *D. hansenii* compared to control mould without yeast as follows: $IA\,(\%) = [(C-T)/C] \times 100$, where C was the average diameter of mould colonies in the absence of *D. hansenii* and T was the average diameter of mould colonies in the cocultured plates. The assay was conducted by triplicate.

2.4. Antifungal effect of cell-free culture filtrate of D. hansenii

Each 21 selected active *D. hansenii* was inoculated on Yeast Morphology Broth (YMB: 1% (w/v) glucose, 0.3% (w/v) yeast extract, 0.3% (w/v) malt extract and 0.5% (w/v) proteose peptone). After 120 h of incubation at 20 °C, cultures were centrifuged at 2630 × *g* for 15 min. The resultant cell-free supernatant was filtered through a 0.22 μm-pore-size membrane (GE Healthcare Europe GmbH, Freiburg, Germany).

A quantitative assay for mould growth inhibition was carried out by the microspectroscopic method reported by Acosta et al. (2009). The inhibition test was performed in 96-well microtiter plates. One hundred microliters of the cell-free filtrate together with 100 μl of double-strength MEB containing *c.a.* 10^6 spores/ml of *P. verrucosum* Pv21 were inoculated per well. The assay was run in triplicate. Growth was monitored by measuring the optical density variation at 595 nm after 72 h of incubation at 20 °C. An extract was considered active when the average absorbance in the wells was significantly lower than that in the control wells containing only *P. verrucosum* Pv21.

2.5. Antifungal effect of volatile compounds produced by D. hansenii

The effect of volatile compounds generated by 21 selected *D. hansenii* isolates was assessed using two agar plates facing each other according to the "mouth-to-mouth" method (Taczman-Brückner et al., 2005). The upper plate containing MEA was inoculated on three spots with 10 μl of a *c.a.* 10^6 spores/ml suspension of *P. verrucosum* Pv21. On the lower plate, 100 μl of a *c.a.* 10^7 cells/ml suspension of *D. hansenii* were spread onto YMA. After drying, the two plates were faced each other, sealed with parafilm and incubated at 20 °C during 14 days. Mouth-to-mouth plates without *D. hansenii* inoculum on the lower plate were used as controls. Trials were done in triplicate. Inhibition was determined by measuring the mould colony diameter. The inhibitory activity was expressed as previously described in Section 2.3.

Thereafter, the volatiles produced by four of active *D. hansenii* were extracted by Solid Phase Micro-Extraction technique and analysed by GC-MS according to Ruiz et al. (1998), using a Hewlett-Packard 5890-II gas chromatograph coupled with a Hewlett-Packard 5971A mass selective detector (Hewlett-Packard, Palo Alto, CA, USA). To extract the volatile compounds from the headspace of *D. hansenii* cultures in either presence or absence of