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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

P. verrucosum Pv21, the fibre was inserted in the parafilm seal through a septum for 30 min at 20 °C. Results of the volatile analysis are given in Arbitrary Area Units/10^6.

Finally, the antifungal activity of the unique volatile compounds identified from the headspace of all *D. hansenii* cultures in the presence of the mould (2-methyl-1-propanol, 3-methyl-1-butanol and 2-methyl-1-butanol) was evaluated according to the above described mouth-to-mouth method. However, in this experiment 15 μl of each volatile compound were placed in the lower position in an empty Petri dish. The effect of a mix of the three selected volatile compounds on *P. verrucosum* Pv21 growth was also studied. For controls, 15 μl of sterile deionized water were used. The experiment was done in triplicate.

2.6. Antifungal activity of D. hansenii on dry-fermented sausages

The effect of *D. hansenii* isolates on the growth of toxigenic penicillia was tested using slices of non-sterile commercial dry-fermented sausages "salchichón" simulating its industrial ripening conditions. Before purchase, the "salchichón" had been stuffed in natural beef casing and fermented for 3 days (25 °C, 90% relative humidity). The sausage showed a water activity (a_w) value of 0.95 before inoculation when measured with an a_w metre Novasina Lab Master from Novasina AG (Lachen, Switzerland). Slices of "salchichón" with a surface of *c.a.* 20 cm^2 were then aseptically prepared in a laminar flow cabinet (Bio Flow II, Telstar, Tarrasa, Spain). To simulate the evolution of a_w during sausages processing, the slices were separately placed in pre-sterilised receptacles with humidity kept constant at 84% after vapour–liquid equilibrium by a saturated potassium chloride solution. As inocula, four isolates of *D. hansenii* that gave the best overall results in the previous experiments and the ochratoxigenic *P. verrucosum* Pv21 were selected.

A volume of 100 μL of *D. hansenii* cell and *P. verrucosum* spore suspensions was spread to reach a final concentration of 10^6 cells/cm^2 and 10^4 spores/cm^2, respectively, on each slice. A positive control batch was inoculated with only *P. verrucosum*. Each treatment was done in three replicates. After inoculation, the samples were incubated at 20 °C for up to 10 days. Sampling was performed at 0, 7 and 10 days. The ochratoxigenic mould load was determined by real-time quantitative PCR (qPCR) using primers targeting the *otanps*PN gene, as described by Rodríguez et al. (2011). The mould counts as log cfu/cm^2 were determined after replacing the obtained Ct values in the standard curve previously calculated from dry-cured meat products artificially inoculated with ochratoxigenic mould strains (Rodríguez et al., 2011). Counts of *D. hansenii* were obtained by plating samples on Potato Dextrose Agar after incubating at 25 °C for 3 days. In addition, the a_w of the slices was measured at the end of the incubation period as above described.

2.7. Statistical analysis

Statistical analyses were performed with the IBM SPSS Statistics for Windows version 19.0 (IBM Corporation, New York, USA). One way analysis of variance (ANOVA) was carried out to determine significant differences within and amongst groups. Dunnet's test was applied to compare mean values of treated samples with control batch.

3. Results and discussion

3.1. Selection of active isolates of D. hansenii

A total of 280 *D. hansenii* isolates from dry-cured meat products at different stages of ripening were tested for their antifungal activity. The screening performed by radial inhibition assay (RI) revealed that 39 *D. hansenii* isolates (13.9%) were active against at least one of the tested toxigenic penicillia (Table 1). Most of antagonistic isolates (24) showed activity against both *P. expansum* PxIC2 and *P. verrucosum* Pv21, and only 3 (57G, 112G and 226G) were able to inhibit the three moulds. This antifungal ability of *D. hansenii* could provide an ecological advantage in dry-cured meat products since they consists of substrates where a substantial mould growth occurs (Alapont et al., 2014; López-Dıaz et al., 2001; Núñez et al., 1996a). *P. verrucosum* Pv21 was the most sensitive mould, since 34 *D. hansenii* isolates originated inhibition halo, which measured more than 15 mm of diameter for 15 yeasts.

All the 39 active *D. hansenii* isolates were selected to stablish their inhibitory ability against a wider range of toxigenic penicillia belonging to species commonly isolated from dry-cured meat products. This screening was performed by coculturing such active *D. hansenii* isolates in solid media (CSM) with 8 toxigenic penicillia strains. No significant differences ($P > 0.05$) were found between the tested pH values (4.5 and 6.0) and only those obtained at pH 6.0 are shown in Table 2. In the CSM assay, most of the 39 yeasts produced a growth reduction higher than 60% of *P. nordicum*, *P. expansum*, *P. verrucosum* and *P. commune* strains. Only seven isolates of *D. hansenii* produced a significant ($P < 0.001$) decrease of all penicillia growth. Strong differences were found in the sensitivity of the penicillia in both RI and CSM assays. *P. verrucosum* Pv21 and *P. expansum* PxIC2 showed a great sensitivity in both assays, whereas *P. nordicum* CBS 323.92 and *P. camemberti* Pcm30 were the less sensitive in the RI and CSM assays, respectively. The highest sensitivity of Pv21 and PxIC2 strains makes them good candidates for a subsequent screening of the *D. hansenii* antifungal activity. Otherwise, *P. camemberti* Pcm30 strain seems to be the most resistant one to *D. hansenii* activity, which can be of great value when looking for broad-spectrum activities.

Even though only three *D. hansenii* isolates showed a limited antagonistic activity against *P. nordicum* CBS 323.92 on the RI assay, all the 39 selected isolates were able to strongly reduce the growth of this mould on the CSM assay. Similar differences against *P. expansum* PxIC2 and *P. verrucosum* Pv21 were also observed for few *D. hansenii* isolates. These facts could be explained by the different modes of action of yeasts. Several mechanisms for the antagonistic activity of yeasts have been suggested, including competition for nutrients and space (Hernández-Montiel et al., 2010), antagonism by production of extracellular hydrolytic enzymes, killer toxins (Masih and Paul, 2002; Walker et al., 1995) or volatile compounds (Fialho et al., 2009; Masoud et al., 2005; Taczman-Brückner et al., 2005). In the present work, the inhibition observed in the RI assay could be related with the production

Table 1
Number of *Debaryomyces hansenii* isolates with inhibitory activity against toxigenic penicillia tested by a radial inhibition assay on YMA. A total of 280 *D. hansenii* isolates were investigated.

Mould strains	Inhibition rate[a]			
	+	++	+++	Total
Penicillium expansum PxIC2	27	1	2	30 (10.7%)[b]
Penicillium nordicum CBS 323.92	3	0	0	3 (1.07%)
Penicillium verrucosum Pv21	13	6	15	34 (12.1%)
Number of active yeast isolates[c]				39 (13.9%)

[a] Inhibition rate was indicated by the diameter of the inhibition zone. +: diameter of inhibition halo<10 mm; ++: diameter of inhibition halo between 10 and 15 mm; +++: diameter of inhibition halo >15 mm.
[b] The values between brackets are the percentages of *D. hansenii* isolates showing inhibition of penicillia growth with respect to the 280 tested isolates.
[c] Number of *D. hansenii* isolates that showed inhibition against at least one of the tested toxigenic penicillia.

Table 2
Number of *Debaryomyces hansenii* isolates with inhibitory activity against toxigenic penicillia tested in cocultures in Malt Extract Agar with the addition of 6% NaCl. A total of 39 *D. hansenii* isolates were investigated.

Mould strains	Inhibition rate[a]				
	+	++	+++	++++	Total
Penicillium expansum PxIC2	1	7	29	0	37 (94.9%)[b]
P. expansum CECT 2279	0	0	16	23	39 (100%)
P. expansum CECT 2278	0	1	14	24	39 (100%)
Penicillium nordicum CBS 323.92	0	0	8	31	39 (100%)
P. nordicum PnICR4	0	10	26	3	39 (100%)
Penicillium verrucosum Pv21	1	9	26	3	39 (100%)
P. commune Pc13	0	1	17	21	39 (100%)
P. camemberti Pcm30	0	1	7	0	8 (20.5%)

[a] Inhibition rate was indicated by the reduction of diameter of mould colonies when cocultured with D. hansenii compared to control mould without yeast. +: reduction of 10–40% ($P < 0.001$); ++: reduction of 40–60% ($P < 0.001$); +++: reduction of 60–80% ($P < 0.001$); ++++: reduction >80% ($P < 0.001$).
[b] The values between brackets are the percentages of *D. hansenii* isolates showing inhibition of penicillia growth with respect to the 39 tested isolates.

Table 3
Number of *Debaryomyces hansenii* isolates with active cell-free filtrates or volatile compounds against toxigenic *Penicillium verrucosum* Pv21. A total of 21 *D. hansenii* isolates were investigated.

	Inhibition rate[c]			
	+	++	+++	Total
Cell-free culture filtrates[a]	12	0	0	12 (57.1%)[d]
Volatile compounds[b]	3	12	6	21 (100%)

[a] Inhibition rate was indicated by the reduction of mould growth measured as optical density in liquid culture in the presence of *D. hansenii* cell-free filtrate compared to control *P. verrucosum* Pv1 cultured without the filtrate.
[b] Inhibition rate was indicated by the reduction of diameter of *P. verrucosum* Pv1 colonies in mouth-to-mouth plates with *D. hansenii* compared to control mould in mouth-to-mouth plates without *D. hansenii* inoculum.
[c] +: reduction of 10–40% ($P < 0.001$); ++: reduction of 40–60% ($P < 0.001$); +++: reduction of 60–80% ($P < 0.001$); ++++: reduction >80% ($P < 0.001$).
[d] The values between brackets are the percentages of *D. hansenii* isolates showing inhibition of penicillia growth with respect to the 21 tested isolates.

of extracellular compounds, while the antagonism in the CSM assay could be also due to competition for space and nutrients. Competition is considered the most common way involved in biocontrol of filamentous moulds by yeasts (Spadaro and Gullino, 2004). Moreover, it seems that the antifungal ability is a strain-related attribute and does not exclusively depend on species, as it has been reported for yeasts isolated from different foods (Bleve et al., 2006; Suzzi et al., 1995).

3.2. Antifungal effect of D. hansenii cell-free culture filtrate and volatile compounds

To elucidate the mechanisms of action involved in the antagonistic activity based on the production of extracellular compounds, the effect of both cell-free culture filtrate (CF) and volatile compounds obtained from 21 *D. hansenii* isolates was investigated. They were selected among the most active isolates from different geographical origins and ripening stages, and all of them had showed activity against at least two toxigenic penicillia in the RI assay. *P. verrucosum* Pv21 was chosen as reference mould because it was one of the most sensitive moulds and had been isolated from dry-cured meat products.

The microspectroscopic tests showed a reduction less than 40% of the mould growth ($P < 0.001$) in the presence of the CF from only 12 *D. hansenii* isolates (Table 3). The yeast isolates showing inhibition might produce extracellular compounds with potential antifungal activity. In this sense, *D. hansenii* is able to synthesize killer proteins effective for sensitive yeasts (Hernández et al., 2008; Marquina et al., 2001). Moreover, other yeast species produce killer proteins with antifungal activity, such as *Zygosaccharomyces bailii* that synthesizes zygocin effective against *Fusarium oxysporum* (Weiler and Schmitt, 2003) and *Candida guilliermondii* and *Pichia membranifaciens* that produce proteins with antagonistic effect against *P. expansum* (Coelho et al., 2009). Although the inhibition observed by the CF can not explain completely the inhibition obtained in the CSM and RI assays, further studies must be conducted to identify and characterize antifungal compounds produced by these isolates of *D. hansenii*.

When *P. verrucosum* Pv21 was exposed to the headspace of all the tested *D. hansenii* cultures in the mouth-to-mouth assay, its growth was significantly ($P < 0.001$) reduced (Table 3). Six of *D. hansenii* isolates reduced the mould growth in 60–80%. The production of antifungal volatile compounds has been described for other yeast such as *Candida maltosa*, *Hanseniaspora uvarum*, *Kluyveromyces lactis*, *Pichia anomala*, *Pichia kluyveri* and *Saccharomyces cerevisiae* isolated from different foods (Ando et al., 2012; Druvefors et al., 2002; Fialho et al., 2009; Masoud et al., 2005; Taczman-Brückner et al., 2005). Although some differences in the inhibition rate were observed, for most of *D. hansenii* the growth reduction of *P. verrucosum* Pv21 produced by volatiles was less pronounced than that found in the CSM assay. Only five isolates (125G, 14G, 123G, 226G and 357G) produced a reduction of mould growth at a similar level (60–80%) to that described in the previous CSM assay.

The volatile compounds generated by four of the active *D. hansenii* isolates (253H, 14G, 226G and 357G) in the presence of *P. verrucosum* were analysed by GC-MS. Although, a total of 51 volatile compounds were detected (results not shown), only three of them (2-methyl-1-propanol, 3-methyl-1-butanol and 2-methyl-1-butanol) were generated by all the tested *D. hansenii* isolates in the presence of *P. verrucosum* (Table 4). The tested branched alcohols, derived from amino acid metabolism, are commonly found on the volatile fraction of dry-cured meat products (Andrade et al., 2009, 2010; Martín et al., 2006). Some differences in the amount of such volatiles were observed depending on the *D. hansenii* isolate. The 226G isolate produced the high amount of all these three branched alcohols. Otherwise, only one of those alcohols, 3-methyl-1-butanol, was found in lower amount in the cultures of *P. verrucosum* in the absence of *D. hansenii*. The alcohol

Table 4
Volatile compounds (expressed as Arbitrary Area Units/10^6) detected in all the headspaces in the mouth-to-mouth assay when coculturing the four *Debaryomyces hansenii* isolates (253H, 14G, 226G and 357G) and *Penicillium verrucosum* Pv21.

Volatile compounds	Inoculated batches				
	Pv21	Pv21 + 253H	Pv21 + 14G	Pv21 + 226G	Pv21 + 357G
2-Methyl-1-propanol	n.d.[a]	12	14	25	16
2-Methyl-1-butanol	n.d.	33	51	76	55
3-Methyl-1-butanol	8	76	102	158	107

[a] n.d.: not detected.

Table 5
Growth inhibition of *Penicillium verrucosum* Pv21 exposed to individual or combined volatile compounds 2-methyl-1-propanol, 2-methyl-1-butanol and 3-methyl-1-butanol after incubation at 20 °C for 14 days. Concentration of volatiles in the headspace was expressed as Arbitrary Area Units/10^6.

Inhibition rate[b]	Batches[a]			
	A	B	C	D
	−	+	−	+
Volatile compounds				
2-Methyl-1-propanol	400	n.d.[c]	n.d.	230
2-Methyl-1-butanol	n.d.	1184	10	856
3-Methyl-1-butanol	10	3	811	881

[a] Volatile compounds tested in the mouth-to-mouth assays against *P. verrucosum* Pv21. A: 2-methyl-1-propanol (15 μl/dish); B: 2-methyl-1-butanol (15 μl/dish); C: 3-methyl-1-butanol (15 μl/dish); D: 2-methyl-1-propanol (15 μl/dish) + 2-methyl-1-butanol (15 μl/dish) + 3-methyl-1-butanol (15 μl/dish).
[b] Inhibitory activity was indicated by the reduction of diameter of mould colonies incubated in the presence of volatile compounds over control mould incubated in air. −: no reduction ($P > 0.05$); +: reduction of 10–40% ($P < 0.001$); ++: reduction of 40–60% ($P < 0.001$); +++:reduction of 60–80% ($P < 0.001$); ++++: reduction >80% ($P < 0.001$).
[c] n.d. not detected.

accumulation has been reported as a strategy of yeasts to inhibit competitive microorganisms (MacLean and Gudelj, 2006). The antimicrobial activity of alcohols is attributed to their absorption in cell membrane, increasing the permeability and accelerating the diffusion of essential ions and metabolites through the membrane (Ingram and Buttke, 1984). In addition, lipophilic alcohols, such as 2-methyl-1-butanol, have high affinity for the membrane, producing higher toxicity than other less lipophilic alcohols as the ethanol (Heipieper et al., 1994).

The activity of the purified 2-methyl-1-propanol, 3-methyl-1-butanol and 2-methyl-1-butanol against *P. verrucosum* Pv21 was then investigated. Among individual compounds, only 2-methyl-1-butanol slightly reduced the growth of *P. verrucosum* Pv21 after 14 days of exposure (Table 5). The mixture of the three alcohols showed a similar effect to 2-methyl-1-butanol alone. The 2-methyl-1-butanol has been identified as one of the compounds responsible for the antifungal activity of *Trichoderma* spp. and *Muscodor albus* (Braun et al., 2012; Wheatley et al., 1997). In spite of the fact that 3-methyl-1-butanol produced by *C. maltosa* inhibited spore germination of *Penicillium chrysogenum* and *Penicillium citrinum* (Ando et al., 2012), no effect of this compound on the growth of *P. verrucosum* Pv21 has been observed. Such discrepancy could be due to the different amount of 3-methyl-1-butanol used. Thus, while Ando et al. (2012) reported a minimum inhibitory dose of 20 μl/dish, in the present work 15 μl/dish were used. However, after the incubation period this amount generated peak areas higher than those previously showed by every tested *D. hansenii* isolates in the mouth-to-mouth assay. Moreover, 3-methyl-1-butanol was detected in the control cultures of *P. verrucosum* Pv21 and it should be not expected that this mould generates a self-inhibiting compound. Given that the inhibition produced by the commercial alcohols was lower than that observed in the mouth-to-mouth assay with yeasts, the antifungal effect could be linked to the full pattern of volatiles produced by *D. hansenii*, and not to any individual compounds.

Even though the reduction of penicillia growth has been recorded in all the assays carried out in the present work, the *D. hansenii* antagonism found in the CSM assay could not be fully reproduced in neither the CF assay nor the mouth-to-mouth assay. Consequently, the efficient mould inhibition might require the additive or synergistic effects of inhibiting factors described for yeasts such as competition for nutrients and space and antagonistic activity by soluble or volatile compounds.

3.3. Antifungal activity of *D. hansenii* isolates on dry-fermented sausages

The biocontrol efficacy of yeasts depends on their colonization and survival in the food and can be influenced by extrinsic factors such as temperature and relative humidity, or intrinsic factors such as pH, nutrients and water activity reached during the processing of dry-cured meat products. Consequently, the procedures of antagonism screening should simulate conditions under which the biocontrol agent will be used. To investigate the potential application in the meat industry, four of the most active *D. hansenii* isolates (253H, 226G 14G and 357G) were selected according to their overall *in vitro* antagonistic effect, geographical origin and processing stage of isolation. The antifungal effect was tested on dry-fermented sausages slices against the ochratoxigenic *P. verrucosum* Pv21 isolates from dry-cured meat product.

The a_w of sausages decreased from 0.95 before inoculation to around 0.91 after 10 days of incubation (20 °C, 84% RH). No significant differences ($P > 0.05$) in a_w values were detected among batches. Both the incubation conditions and final a_w in "salchichón" could be considered usual for this product (Lizaso et al., 1999). All four *D. hansenii* isolates were able to grow in the inoculated sausages, reaching levels around log 7 cfu/cm^2 (Table 6), showing significant differences ($P < 0.001$) with the control batch inoculated only with *P. verrucosum*. On the other hand, lower levels of ochratoxigenic moulds were detected in the batches inoculated with *D. hansenii* 253H and 226G isolates compared to the positive Pv21 batch ($P < 0.001$) at the end of incubation. However, *D. hansenii* 14G and 357G did not show antagonistic effect in this assay.

Both *D. hansenii* 253H and 226G isolates were able to keep the counts of *P. verrucosum* under 4.6 log cfu/g. Counts over this level have been previously considered as hazard for the ochratoxin A presence on hams (Rodríguez et al., 2012). *D. hansenii* have also demonstrated biopreservation potential in foods, including plant

Table 6
Quantification of ochratoxigenic moulds and yeasts (log cfu/cm^2) on dry-fermented sausage slices after 10 days of incubation. *Penicillium verrucosum* Pv21 was inoculated alone (Pv21) or together with the selected *Debaryomyces hansenii* isolates (253H, 14G, 226G and 357G).

Inoculation batch	Yeasts counts[a] (log cfu/cm^2)			Mould counts[b] (log cfu/cm^2)		
	Day 0	Day 7	Day 10	Day 0	Day 7	Day 10
Pv21	<4.0	<4.0	<4.0	4.5 ± 0.16	4.8 ± 0.31	5.7 ± 0.12
Pv21 + 253H	4.9 ± 0.01*	7.4 ± 0.04*	7.7 ± 0.25*	4.5 ± 0.13	4.4 ± 0.75	3.9 ± 0.51*
Pv21 + 14G	4.6 ± 0.05*	7.3 ± 0.25*	7.2 ± 0.23*	4.6 ± 0.06	3.7 ± 0.44	5.6 ± 0.16
Pv21 + 226G	4.8 ± 0.10*	7.6 ± 0.17*	7.2 ± 0.40*	4.5 ± 0.08	3.7 ± 0.37	3.8 ± 0.32*
Pv21 + 357G	4.9 ± 0.08*	7.1 ± 0.32*	6.9 ± 0.52*	4.6 ± 0.24	3.6 ± 0.68	5.9 ± 0.12

*Batches with statistically different ($P < 0.001$) counts compared to control batch (Pv21).
[a] Counts of yeasts obtained by counting plate in Potato Dextrose Agar.
[b] Counts of ochratoxigenic moulds obtained by qPCR as described by Rodríguez et al. (2011).

foods (Gil-Serna et al., 2011; Hernández-Montiel et al., 2010), dairy products (Liu and Tsao, 2009) and other dry-cured meat products (Andrade et al., 2014; Simoncini et al., 2014). Specifically, *D. hansenii* 253H used in the present work had previously showed ability for reducing the growth of *P. nordicum* and the presence of ochratoxin A on dry-cured ham (Andrade et al., 2014). In addition to the inhibition of the toxigenic mould growth, *D. hansenii* could decrease mycotoxin content by cell wall adsorption and blocking the expression of biosynthetic genes, as it has been described for OTA in mixed cultures of *D. hansenii* with *Aspergillus westerdijkiae* (Gil-Serna et al., 2011).

Therefore, the use of the *D. hansenii* 253H and 226G isolates to control toxigenic moulds for dry-fermented meat product seems to be a promising approach, since these isolates are evolutionarily adapted to this ecological niche and, additionally, *D. hansenii* is safe for use in foods (BIOHAZ, 2012). However, the mould concentration has been described as a key factor for *D. hansenii* effectiveness, being this biocontrol more effective at low mould counts (Liu and Tsao, 2009; Virgili et al., 2012). Thus, for a successful application of antagonistic *D. hansenii*, the population of toxigenic moulds should be kept as low as possible with good manufacturing practice through HACCP procedures (Asefa et al., 2009).

In conclusion, the results of this study indicate that there are some native *D. hansenii* isolates which are eligible for the biocontrol of toxigenic moulds in meat products. Their antifungal effect is probably due to a combination of competition for space and nutrients and production of yeast metabolites. *D. hansenii* 253H and 226G isolates showed an antifungal effect in dry-fermented sausages, being able to colonize and prevail in this product reaching high population levels. Thus, these two isolates could be considered for their potential use as protective culture against toxigenic moulds in dry-fermented meat products.

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