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Biocontrol of aflatoxigenic *Aspergillus parasiticus* by native *Debaryomyces hansenii* in dry-cured meat products

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ABSTRACT

Dry-cured meat products, such as dry-cured ham or dry-fermented sausages, are characterized by their particular ripening process, where a mould population grows on their surface. Some of these moulds are hazardous to the consumers because of their ability to produce mycotoxins including aflatoxins (AFs). The use of native yeasts could be considered a potential strategy for controlling the presence of AFs in dry-cured meat products. The aim of this work was to evaluate the antagonistic activity of two native *Debaryomyces hansenii* strains on the relative growth rate and the AFs production in *Aspergillus parasiticus*. Both *D. hansenii* strains significantly reduced the growth rates of *A. parasiticus* when grown in a meat-model system at different water activity (a_w) conditions. The presence of *D. hansenii* strains caused a stimulation of AFs production by *A. parasiticus* at 0.99 a_w. However, at 0.92 a_w the yeasts significantly reduced the AFs concentration in the meat-model system. The relative expression levels of the *afl*R and *afl*S genes involved in the AFs biosynthetic pathway were also repressed at 0.92 a_w in the presence of both *D. hansenii* strains. These satisfactory results were confirmed in dry-cured ham and dry-fermented sausage slices inoculated with *A. parasiticus*, since both *D. hansenii* strains significantly reduced AFs amounts in these matrices. Therefore, both tested *D. hansenii* strains could be proposed as biocontrol agents within a HACCP framework to minimize the hazard associated with the presence of AFs in dry-cured meat products.

1. Introduction

Some typical dry-cured meat products, such as dry-cured ham or dry-fermented sausages, are appreciated worldwide and represent important items for the EU export market (Resano et al., 2011). During the ripening process of these products, a huge mould population grows on their surface. Some of the most usually found moulds are potentially producers of mycotoxins (Rodríguez et al., 2012b). Although ochratoxin A (OTA) is the most important mycotoxin encountered in dry-cured meat products (Ferrara et al., 2016; Markov et al., 2013; Rodríguez et al., 2012a), recent studies have highlighted the presence of concerning amounts of aflatoxins (AFs) in these foods (Markov et al., 2013; Pleadin et al., 2015; Rodríguez et al., 2012b). Moreover, the main AFs-producing moulds *Aspergillus flavus* and *Aspergillus parasiticus* can be isolated from dry-cured meat products (Aziz et al., 1991; Cvetnić and Pepeljnjak, 1995; Rojas et al., 1991). AFs have immunosuppressive and hepatotoxic properties and have been classified as carcinogenic to humans (group 1 A) by the International Agency for Research on Cancer (IARC, 2012). Consequently, it is necessary to prevent the presence of both aflatoxigenic moulds and AFs in dry-cured meat products to protect the health of consumers.

Biocontrol by antagonistic microorganisms have been proposed for controlling toxigenic moulds in foodstuffs, including fruits, wheat, dairy and meat products (Asensio et al., 2014). Some bacteria and non-toxigenic moulds have been studied as potential biocontrol agents against aflatoxigenic moulds in foods. Various lactic-acid bacteria, such as *Lactobacillus plantarum*, *Lactobacillus fermentum* and *Bifidobacterium bifidum* are able to reduce AFs production and *A. flavus* or *A. parasiticus* growth (Ahlberg et al., 2017; Ghazvini et al., 2016). *Bacillus* strains from several origins have successfully controlled the growth of *Aspergillus* spp. and production of mycotoxins (Kong et al., 2014). In the same way, *Penicillium chrysogenum* and non-aflatoxigenic *A. flavus* reduced AFs contamination in dry-cured ham (Bernáldez et al., 2014) and peanuts (Alaniz Zanon et al., 2016), respectively. Some yeasts, such as *Kluyveromyces lactis* and *Saccharomyces cerevisiae,* have been able to reduce the bioavailability of AFs in contaminated foods by adsorbing the toxins to the cell wall (Hamad et al., 2017). However, to the best of our knowledge, there are no studies focused on the effect of yeasts as

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potential biocontrol agents against aflatoxigenic moulds.

In this sense, throughout the ripening process of dry-cured meat products, a yeast population spontaneously grows on their surface, reaching different count levels depending on the processing stage and type of product (Núñez et al., 1996a; Simoncini et al., 2007). Some of the native yeasts have been reported to be positive contributors to the sensory quality of these products (Andrade et al., 2009; Martín et al., 2003, 2006). Therefore, biocontrol of aflatoxigenic moulds by native strains could be considered a possible strategy to improve food safety, without affecting the typical sensory properties of dry-cured meat products. *Debaryomyces hansenii*, the most commonly occurring yeast species in dry-cured meat products (Andrade et al., 2009; Aquilanti et al., 2007; Núñez et al., 1996a), has been considered a good candidate as biocontrol agent against OTA-producing moulds in dry-cured meat products (Andrade et al., 2014; Iacumin et al., 2017; Núñez et al., 2015; Peromingo et al., 2018; Simoncini et al., 2014). However, its activity against aflatoxigenic moulds in these foods has not been evaluated yet. The knowledge about the mechanisms involved in the potential antifungal activity by yeasts is required to maximize their effect on AFs production (Sharma et al., 2009; Taczman-Brückner et al., 2005). The main mechanism of action in antifungal yeasts seems to be competition by nutrients and space (Andrade et al., 2014; Droby et al., 1989; Simoncini et al., 2014; Virgili et al., 2012). Production of volatile compounds with antifungal activity (Masoud et al., 2005; Núñez et al., 2015; Taczman-Brückner et al., 2005) or killer proteins (Coelho et al., 2009; Hernández et al., 2008) have been also reported as potential modes of action. In addition, yeasts may decrease mycotoxin content by adsorption to cell wall molecules, such as glyco-mannoproteins (Fiori et al., 2014; Hamad et al., 2017). Moreover, the effect of antifungal yeasts on the reduction of mycotoxin biosynthesis at transcriptional level has been studied. A repression of the expression of genes involved in OTA biosynthesis has been reported in *Aspergillus* and *Penicillium* species as consequence of the presence of yeasts, such as *S. cerevisiae* on Yeast Peptone Dextrose medium (Cubaiu et al., 2012) or *D. hansenii* on Czapek-Dox Yeast modified agar (Gil-Serna et al., 2011), and on meat model systems (Peromingo et al., 2018). Regarding aflatoxigenic moulds, *Bacillus megaterium* has shown to be able to inhibit both the mould growth and AFs biosynthesis by means of altering gene transcription, especially in the regulatory *afl*S (Kong et al., 2014), *afl*D and *afl*R genes (Al-Saad et al., 2016). Similarly, *L. plantarum* and *Lactobacillus delbrueckii* subsp. *lactis* significantly decreased the growth, the production of AFs and the level of expression of the *afl*R gene when they were co-cultivated with *A. parasiticus* (Ghanbari et al., 2018). Therefore, studies related to the effect of yeasts on the gene expression involved in AFs biosynthetic pathway would be of utmost interest. Further investigations focused on such modes of action should be necessary to enhance the effect of protective cultures of *D. hansenii* on dry-cured meat products.

The efficacy of potential biocontrol agents is influenced by the nutritional sources and the inherent environmental factors of the dry-cured meat products process, especially water activity (a_w) and temperature (Al-Saad et al., 2016; Andrade et al., 2014). In fact, the tolerance of *D. hansenii* species to high salt concentrations (Breuer and Harms, 2006; Gori et al., 2005) makes its application as biopreservative in dry-cured meat products very adequate. Consequently, it would be interesting to identify the environmental conditions in which the biocontrol agents have the highest effectiveness against the common toxigenic moulds in these products.

The objective of this study was to evaluate the potential activity of two *D. hansenii* yeast strains isolated from dry-cured meat products against an aflatoxigenic *A. parasiticus* strain. Their effects on the growth rate and AFs production by *A. parasiticus* on meat-based media at different a_w conditions were determined. The ability of *D. hansenii* strains to reduce the presence of AFs by detoxifying (degrading and adsorbing) or by altering gene expression in *A. parasiticus* was also studied. Finally, the effect of *D. hansenii* strains against *A. parasiticus* was tested in dry-cured ham and dry-fermented sausage to confirm their use as biocontrol agents.

2. Material and methods

2.1. Microorganisms, culture media and inoculum preparation

Two strains of yeast *D. hansenii* and one of *A. parasiticus* were used in this study. Both yeast strains (FHSCC 125G and FHSCC 253H) were isolated from dry-cured meat products and belong to the Culture Collection of Food Hygiene and Safety at the University of Extremadura (Spain). These *D. hansenii* strains were selected due to their activity against ochratoxigenic moulds (Andrade et al., 2014; Núñez et al., 2015). The strain *A. parasiticus* CECT 2682 producer of aflatoxin B_1 (AFB_1) and aflatoxin G_1 (AFG_1) was obtained from the Spanish Type Culture Collection (CECT, Spain).

A meat-based medium containing lyophilized fresh pork meat (Peromingo et al., 2016) was used to evaluate the effect of both *D. hansenii* strains at different a_w on the growth rate of *A. parasiticus*, as well as on AFs production and expression of genes involved in the biosynthetic pathway of AFs. The a_w of the medium (0.99) was modified to 0.97 a_w by adding 50 g/L of NaCl (Fisher Scientific, UK), and to 0.92 a_w by adding 50 g/L of NaCl and 150 g/L of glycerol (Fisher Scientific). The 0.99 a_w is the value of the unmodified meat-based medium. The remaining values were chosen since they are usually found during the ripening process of dry-cured meat products. Concretely, 0.97 a_w is observed in the first stages, while 0.92 a_w is reached in the intermediate stages, when moulds achieve their highest growth level in these foods (Núñez et al., 1996b). The a_w was checked using the a_w meter "Lab Master" (Novasina AG, Switzerland). The culture media were autoclaved for 20 min at 121 °C.

For inocula preparation, the yeast strains were inoculated on Yeast broth (YES; 20 g/L of yeast extract and 125 g/L of sucrose, Scharlab S.L., Spain) and incubated at 25 °C for 72 h under stirring (150 rpm). The mould strain was inoculated on Malt Extract Agar [MEA; 20 g/L of malt extract (Scharlab S.L.), 1 g/L of bactopeptone (Scharlab S.L.), 20 g/L of D (+) glucose monohydrate (Scharlab S.L.), bacto agar 20 g/L, Scharlab S.L.)] and incubated at 25 °C for 7 days. The spores were then collected using 5 mL of phosphate-buffered saline [PBS; 0.32 g/L of NaH_2PO_4 (Scharlab S.L.) and 1.09 g/L of Na_2HPO_4 (Scharlab S.L.), and rubbing the surface with a glass rod in order to remove conidia. Yeast cells and mould spores were quantified by using a Thoma counting chamber Blaubrand® (Brand, Germany). Suspensions were adjusted to concentrations used as inoculum in each assay.

2.2. Effect of D. hansenii strains on A. parasiticus growth and AFs production

2.2.1. Growth assessment

The relative growth rate of *A. parasiticus* CECT 2682 in the presence of *D. hansenii* 125G and 253H strains was evaluated in meat-based media. Firstly, 1 mL of a suspension containing 10^6 yeast cells/mL was mixed with 20 mL of melted (45 °C) meat-based agar in Petri dishes. After agar solidification, the plates were centrally inoculated with 10 μL of a suspension of *A. parasiticus* containing 10^5 spores/mL. Plates inoculated only with *A. parasiticus* were also prepared to be used as controls. All the batches were incubated at 25 °C for 14 days. The experiments were performed in triplicates.

The diameter of the colonies was daily measured in two perpendicular directions. These data were utilized for determining the relative growth rate of the aflatoxigenic *A. parasiticus* strain. The mould colony radius was plotted against time, and linear regression was applied to obtain the growth rate (mm/day) as the slope of the line.

2.2.2. Extraction and quantification of AFs

Five to six agar plugs with a diameter of *c. a.* 4 mm and about 0.5 g

were removed from the *A. parasiticus* cultures every two days, placed in 2 mL microcentrifuge tube, weighed and stored at −20 °C. AFs were extracted using the methodology described by Peromingo et al. (2016) consisting of an overnight shaking of the agar plugs together with chloroform at room temperature followed by the evaporation to dryness of the obtained chloroform layer. The residue was then redissolved in 200 μL of HPLC-grade acetonitrile (Scharlab S.L.) and filtered through a 0.45 μm pore size nylon membrane (Jet Bio-Filtration Co., China).

AFs were analyzed by ultrahigh performance liquid chromatography (uHPLC-MS/MS) in a Thermo Scientific Dionex UltiMate 3000 Rapid Separation LC (RSLC) system with an autosampler thermostat (UltiMate® 3000 Rapid Separation Autosampler, Thermo Scientific, USA) coupled to an Ion Trap Mass Spectrometer System amaZon SL (Bruker Daltonics Inc., Germany). A reversed-phase column C_{18} (100 mm × 2.1 mm, 2 μm; Agilent Technologies, USA) was used. The mobile phase consisted of 0.1 % formic acid-10 mM ammonium formate (solvent A) and acetonitrile (solvent B). Analysis was performed in a gradient mode from 2 to 98 % ([0 min] 2 % B, [0–0.1 min] 40 % B, [0.1–4 min] 60 % B, [4–7 min] 80 % B, [7–8.5 min] 80 % B, [8.5–8.51 min] 98 % B, [8.51–12 min] 98 % B, [12–12.01 min] 2 % B and [12.01–15 min] 2 % B). The injection volume was 5 μL and flow rate was set at 0.2 mL/min. MS detection of AFB_1 and AFG_1 was performed using the precursor ions 313 and 329, and the quantitation ions 285 and 311, respectively. The run time was 15 min, being detected AFB_1 at 6.4 ± 0.5 min and AFG_1 at 6.3 ± 0.5 min. Data were processed by Hystar v. 3.2 software (Bruker Daltonics Inc.).

The calibration curves for AFB_1 and AFG_1 (1–500 ppb) by uHPLC-MS revealed a linear relationship ($r^2 \geq 0.99$) between detector response and amounts of the AFB_1 and AFG_1 standards. The minimum detectable value or limit of detection (LOD) was estimated from the calibration curve, according to the equation: $LOD = 3\,(s_B{}^2 + s_i{}^2 + (i/m)^2\, s_m)^{1/2}/m$ (Long and Winefordner, 1983) being "m" the slope of the calibration curve, "i" the intercept term and "s_B", "s_i" and "s_m" the standard errors of the blank, the intercept term and the slope of the calibration curve, respectively. Assuming a normal distribution of the estimated quantities, α (error of the first type) = β (error of the second type) = 0.05, the quantification limit (LOQ) was 3.04 LOD (Currie, 1999). The LOD obtained in this study were 4 ng/g (AFB_1) and 1.5 ng/g (AFG_1), and the LOQ were 12 ng/g (AFB_1) and 4.5 ng/g (AFG_1).

2.3. Detoxification of AFs

To evaluate the capacity of *D. hansenii* strains to degrade or adsorb AFs, 1 mL of YES spiked with 100 ng of AFB_1 and 100 ng of AFG_1 (Sigma-Aldrich Co., USA) was inoculated with 10^6 yeast cells/mL prepared as described in section 2.1. Positive controls containing only AFs were also included. After incubating at 25 °C for 48 h, the cultures were centrifuged at 3.500 rpm for 5 min. AFs were extracted, detected and quantified as described in section 2.2.2. The experiment was carried out in triplicate.

2.4. Gene expression studies

The effect of *D. hansenii* strains on the relative expression of the *afl*R and *afl*S genes involved in the biosynthetic pathway of AFs was evaluated in cultures of *A. parasiticus* grown on the meat-based media described in section 2.1 at the incubation conditions defined in section 2.2.1. For gene expression studies, the mycelia from *A. parasiticus* cultures were daily scraped under sterile conditions, placed in 2 mL microcentrifuge tube, quickly frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

2.4.1. RNA extraction and cDNA synthesis

RNA extraction from *A. parasiticus* was performed using the Spectrum Plant Total RNA Kit (Sigma-Aldrich Co.) as described by Peromingo et al. (2017). To remove possible trace amounts of contaminating DNA, DNase I, RNase-free (Fermentas, Life Sciences, Thermo Fisher Scientific, Germany) was used according to the manufacturer's protocol. RNA concentration and purity were spectrophotometrically determined (A_{260}/A_{280} ratio) using the NanoDrop 2000c (Thermo Scientific). RNA aliquots were stored at −80 °C.

2.4.2. Reverse transcriptase quantitative PCR

The transcription profiles of the *afl*R and *afl*S genes of the AFs biosynthesis cluster, were analyzed by reverse transcriptase quantitative PCR (RT-qPCR). Nucleotide sequences of primers used in the RT-qPCR assays are shown in Supplementary Table S1. Firstly, cDNA was synthesized using 150–500 ng of RNA according to the PrimeScript™ RT Reagent kit protocol instructions (Takara Bio Inc., Japan) and subsequently used for qPCR.

qPCR reactions were performed using the Applied Biosystems ViiA™ 7 Real-Time PCR System (Applied Biosystems, USA). The SYBR Green methodology was used. Reaction mixtures were prepared in triplicate in MicroAmp Optical 96-well reaction plates (Applied Biosystems) reaching a final volume of 12.5 μL per well. Plates were then sealed with optical adhesive covers (Applied Biosystems). The reaction mixture consisted of 6.25 μL of SYBR® *Premix Ex Taq*™ (Takara Bio Inc.), 0.05 μl of 50x ROX™ Reference Dye (Takara Bio Inc.), different concentrations of each primer (Supplementary Table S1) and 2.5 μL of cDNA. Non-template controls were also included for each primer pair. The thermal cycling conditions included an initial step of 10 min at 95 °C, 40 cycles at 95 °C for 15 s and 60 °C for 30 s. A dissociation curve was performed after amplification by a gradual rise in temperature from 72 °C to 95 °C, with the fluorescence signal measured to differentiate PCR products of interest rather than primer dimers or non-specific PCR products. Threshold cycle (Ct) determinations were automatically performed by the instrument using default parameters.

The relative quantification of the expression of the *afl*R and *afl*S genes was performed using the housekeeping gene *β-tubulin* as an endogenous control (Peromingo et al., 2017). The calibrator for each target gene was that corresponding to *A. parasiticus* grown in the absence of *D. hansenii* on each meat-based medium.

2.5. Antagonistic effect of D. hansenii against aflatoxigenic A. parasiticus in dry-cured meat products

The antagonistic activity of *D. hansenii* against the aflatoxigenic *A. parasiticus* CECT 2682 was also tested in dry-cured meat products under conditions modelling industrial ripening. For that, slices of approximately 25 cm^2 from cross sections of commercial dry-fermented sausage and dry-cured ham were aseptically obtained in a laminar flow cabinet (Bio Flow Telstar, Spain). Microbial contamination was reduced by dipping each slice in 70 % ethanol (v/v) (Scharlab S.L.) for a few seconds and left to dry under UV light for 30 min. To simulate the evolution of a_w during meat product processing, slices were separately placed in sterilized receptacles, where relative humidity was kept at 84 % by disposing at the bottom of the containers a saturated potassium chloride solution. Three batches were set: a control with only *A. parasiticus* and two for the combination of *A. parasiticus* with each *D. hansenii* strain. Each batch contained three replicates. Microbial suspensions were prepared as described in section 2.1 to reach a final concentration of 4×10^4 cfu/cm^2 of *D. hansenii* and 4×10^3 spores/cm^2 of *A. parasiticus* on the slice surfaces. For this, 100 μL of such suspension were spread onto the whole surface of each slice with a sterile glass rod. After incubation at 25 °C for 21 days, each slice was put into a filter bag BagPage (Interscience, France) together with 10 mL of 0.1 % peptone water (Panreac Quimica S.L.U., Spain) and homogenized using a Stomacher (Seward Stomacher®, UK). The obtained filtrate was used for counting moulds and yeasts on DG18 (Scharlab S.L.) after incubation at 25 °C for 48 h. Finally, 5 g of each sample was used for AFs extraction according to the method reported by Bernáldez et al. (2013). Briefly, after maceration of the samples with a mixture of

hexane and acetonitrile-water containing formic acid, the acetonitrile-water phase was filtered through anhydrous sodium sulphate and washed with hexane. The resulting acetonitrile layer was filtered and evaporated to dryness. The obtained extracts were redissolved in 200 µL of HPLC-grade acetonitrile and filtered through a 0.45 µm pore size nylon membrane for their analysis by uHPLC-MS as described in section 2.2.2. The experiment was repeated three times.

2.6. Statistical analysis

Statistical analyses of data were performed using IBM SPSS Statistics for Windows v. 22.0 (IBM Corporation, USA). Data sets of relative growth rates, mycotoxin production and expression of the *afl*R and *afl*S genes were tested for normality using the Shapiro-Wilk test. Since all of them failed the normality test, variable transformation was performed to improve normality or homogenize the variances but without any success. For that reason, non-parametric data analysis was performed using the Kruskal-Wallis rank sum test. The U Mann-Whitney test was then applied to compare the median values obtained. The statistical significance was set at $p \leq 0.05$. The effect of each factor (a_w and yeast presence) and their interaction on AFs production was evaluated by Kruskall-Wallis test.

3. Results

3.1. Effect of D. hansenii strains on A. parasiticus growth

The relative growth rates of *A. parasiticus* in the presence and absence of *D. hansenii* 125G and 253H in meat-based agar at 25 °C and at different a_w values (0.99, 0.97 and 0.92) are shown in Fig. 1, Supplementary Fig. S1 and Supplementary Table S2. In general, the growth rate of *A. parasiticus* increased significantly ($p \leq 0.05$) when the a_w decreased, reaching the fastest growth at the lowest a_w level. Both *D. hansenii* strains significantly reduced *A. parasiticus* growth rates ($p \leq 0.05$) at the three tested a_w. The antagonistic activity of *D. hansenii* 125G strain did not differ among the three different a_w values. However, the effect of *D. hansenii* 253H strain on the *A. parasiticus* growth rates was significantly affected by a_w ($p \leq 0.05$). In the presence of the later antagonistic yeast, the fastest growth rate of *A. parasiticus* was observed at 0.97 a_w.

3.2. Effect of D. hansenii strains on AFs production by A. parasiticus

Fig. 2 shows the temporal accumulation of AFB_1 and AFG_1 in the presence and absence of *D. hansenii* 125G or 253H strains in meat-based agar at 25 C and at different a_w (0.99, 0.97 and 0.92). In general, the AFG_1 production was higher than that of AFB_1 at every tested condition. The highest levels of both AFs were reached at 0.92 a_w, regardless of the presence or absence of the yeast strains. The effect of antagonistic yeasts depended on the environmental conditions. At 0.99 a_w, both yeasts strains led an overproduction of AFs by *A. parasiticus*, when comparing with the non-treated batch ($p \leq 0.05$). However, *D. hansenii* strains did not influence the AFs production by *A. parasiticus* at 0.97 a_w. Finally, at 0.92 a_w both *D. hansenii* strains displayed an inhibitory effect on AFs production, particularly after the day 9 of incubation ($p \leq 0.05$). Statistical analysis showed that a_w and its interaction with antagonistic yeasts significantly affected the AFs production by *A. parasiticus* during a 14-day incubation period (Table 1).

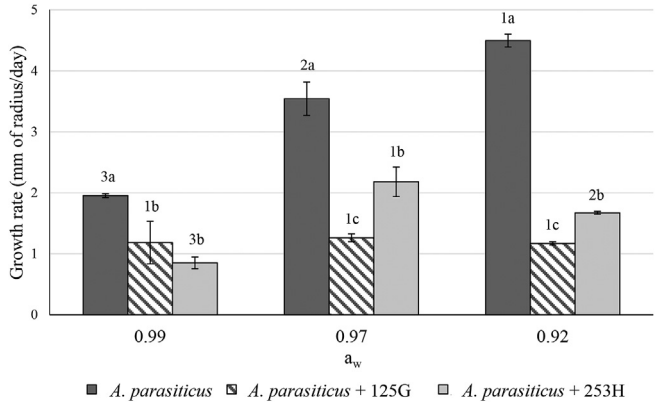

Fig. 1. Effect of *Debaryomyces hansenii* 125G and 253H strains on the growth rate of *Aspergillus parasiticus* in meat-based media with different a_w (0.99, 0.97 and 0.92) during 14 days of incubation at 25 °C. Significant differences between treatments within each a_w are indicated by different letters ($p \leq 0.05$). Significant differences between each a_w in the three treatments are indicated by different numbers ($p \leq 0.05$).

3.3. AFs degradation by D. hansenii

Neither of the two *D. hansenii* strains studied led to a significant decrease in the concentration of AFB_1 and AFG_1 ($p > 0.05$) in YES after 48 h of incubation at 25 °C (Supplementary Table S3). Concretely, the percentages of AFB_1 removal were 6.12 and 9.96 % and those of AFG_1 6.13 and 11.92 % in the presence of *D. hansenii* 125G and 253H, respectively.

3.4. Effect of D. hansenii strains on aflR and aflS gene expression

The relative expression levels of the *A. parasiticus* regulatory genes *afl*R and *afl*S in the presence and absence of *D. hansenii* 125G and 253H in meat-based agar at 25 °C and at different a_w (0.99, 0.97 and 0.92) after 6 and 12 days of incubation are shown in Fig. 3. Slight differences were found between the patterns of gene expression. Although at 0.99 a_w and 0.97 the highest relative expression average of both *afl*R and *afl*S genes was recorded when *A. parasiticus* was co-cultured with *D. hansenii* 253H no significant differences between treated samples with respect to *A. parasiticus* control were found ($p > 0.05$). However, at 0.92 a_w after 6 days of incubation, *D. hansenii* 253H significantly stimulated the relative expression of the *afl*R and *afl*S genes, while *D. hansenii* 125G significantly stimulated only the expression of the *afl*R gene. Conversely, at day 12 the expression of both genes was significantly inhibited at 0.92 a_w in the presence of antagonistic *D. hansenii* strains.

3.5. Antagonistic effect of D. hansenii on against aflatoxigenic A. parasiticus in dry-cured meat products

The ability of *D. hansenii* strains as protective cultures against *A. parasiticus* was evaluated on dry-fermented sausage and dry-cured ham slices. *A. parasiticus* properly grew in both cured meat matrices (Table 2). None yeast strain was able to reduce the *A. parasiticus* growth, neither in dry-fermented sausage nor in dry-cured ham. However, both *D. hansenii* strains significantly reduced the AFs amounts in dry-fermented sausage and dry-cured ham slices (Table 2). *D. hansenii* 253H produced the highest AFs reduction levels when comparing with the control batch.

4. Discussion

To the best of our knowledge, this is the first study to evaluate the efficacy of *D. hansenii* as biocontrol agent against AFs-producing moulds in dry-cured meat products at the environmental conditions usually found throughout their ripening. *A. parasiticus* is commonly isolated from the surface of these kind of meat products and might cause accumulation of AFs in them (Aziz et al., 1991; Cvetnić and Pepeljnjak, 1995; Rodríguez et al., 2012b; Rojas et al., 1991). On the other hand, *D. hansenii*, the most common yeast species in this kind of food (Andrade et al., 2009; Aquilanti et al., 2007; Núñez et al., 1996a), has been regarded as potential biocontrol agent against ochratoxigenic moulds

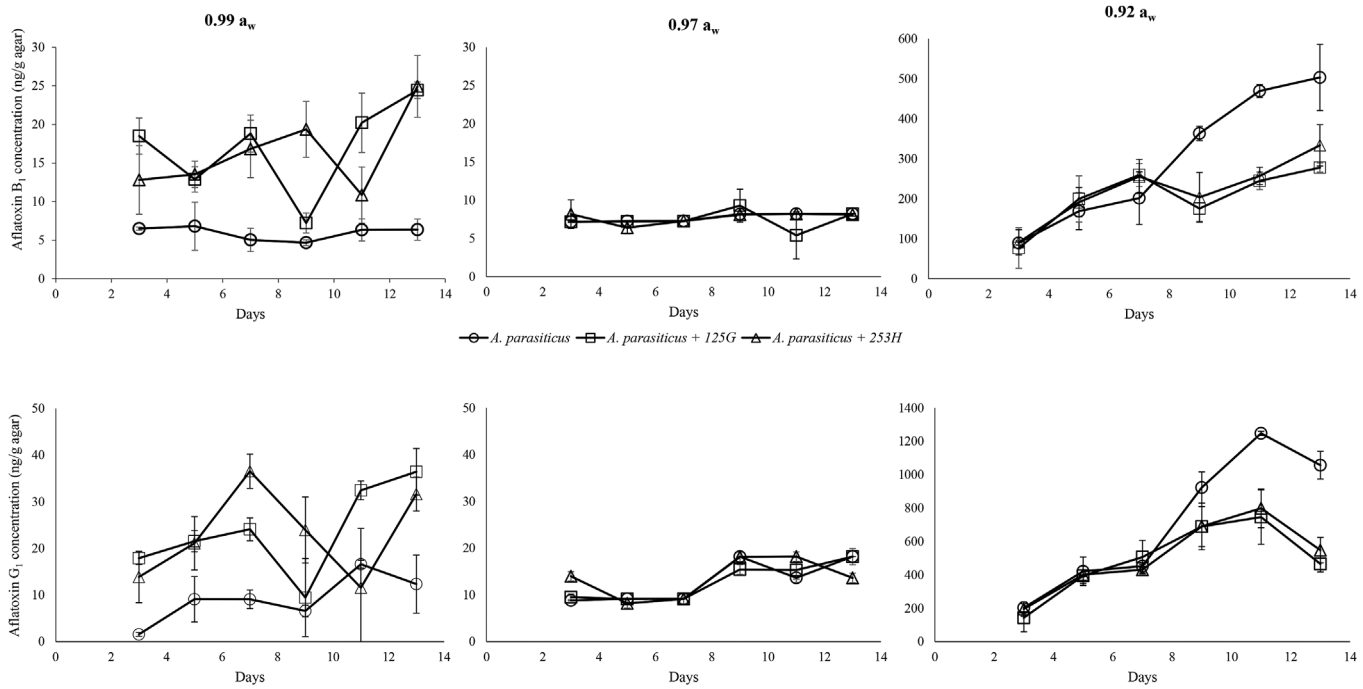

Fig. 2. Effect of *Debaryomyces hansenii* 125G and 253H strains on *Aspergillus parasiticus* aflatoxins (AFB_1 and AFG_1) production in meat-based media with different a_w (0.99, 0.97 and 0.92) during 14 days of incubation at 25 °C.

Table 1
Summary of statistical analyses of the effect of various factors on the aflatoxin B_1 (AFB_1) and aflatoxin G_1 (AFG_1) production by *Aspergillus parasiticus* grown in meat-based media.

Factor studied	*p*-value	
	AFB_1	AFG_1
a_w	0.000***	0.000***
Yeast	0.770	0.821
a_w - Yeast	0.022*	0.004**

[a]: Significant differences are indicated by an asterisk ($p \leq 0.05$), two asterisks ($p \leq 0.01$) and three asterisks ($p \leq 0.001$).

(Andrade et al., 2014; Núñez et al., 2015; Peromingo et al., 2018; Virgili et al., 2012). Since *D. hansenii* is included in the Qualified Presumption of Safety list (EFSA BIOHAZ Panel, 2017), it can be intentionally added to meat products without the requirement of further studies. Therefore, it would be interesting to study the ability of *D. hansenii* to control the growth and AFs production by *A. parasiticus* in the common environmental conditions throughout ripening of dry-cured meat product.

In this study, the tested *D. hansenii* strains significantly influenced ($p \leq 0.05$) the *in vitro* growth of the aflatoxigenic *A. parasiticus*. The growth rate of *A. parasiticus* decreased in the presence of yeasts at every a_w values, mainly at 0.92 (Fig. 1). These results agree with those obtained by Peromingo et al. (2018), which reported that the same *D. hansenii* strains showed the most effective inhibition of the growth of ochratoxigenic *Penicillium verrucosum* at 0.92 a_w. It has been previously proposed that the mechanism of action of these yeast strains against ochratoxigenic moulds in meat products consists of the combination of competition for nutrients and space and the production of extra-cellular inhibitory compounds, such as volatile compounds or killer proteins (Andrade et al., 2014; Núñez et al., 2015; Peromingo et al., 2018). For that reason, it is expected that these mechanisms of action could also be responsible for the activity against *A. parasiticus*.

Even though yeast strains reduced the growth of *A. parasiticus* in meat-based culture media at every tested condition, at 0.99 a_w both *D. hansenii* strains increased the production of AFs by *A. parasiticus* (Fig. 2). These results are in accordance with the stimulation of AFs production described in co-cultures of *A. flavus* and the yeast *Candida guilliermondii* in corn (Wicklow et al., 1980) and after the treatment of *A. parasiticus* with sub-inhibitory levels of some fungicides in different substrates (D'Mello et al., 1998). In the same way, sausage-native yeasts, such as *Rhodotorula mucilaginosa*, *Rhodotorula glutinis*, *Candida zeylanoides* and *Candida krusei*, and commercial starter cultures composed by *Pediococcus pentosaceus*, *Lactobacillus sakei*, *Staphylococcus carnosus*, *Staphylococcus xylosus* and *D. hansenii* significantly stimulated OTA production by *A. westerdijkiae* in meat substrates and by *Penicillium nordicum* in ham (Meftah et al., 2018). At 0.97 a_w the presence of both *D. hansenii* strains did not affect the level of AFs in comparison to those detected in their absence. By contrast, at 0.92 a_w, *D. hansenii* 125G and 253H strains significantly reduced the AFs accumulation in meat-based medium. The antagonistic effect of native yeasts on OTA accumulation has been also reported in meat substrates at 0.92 a_w (Simoncini et al., 2014), including the effect of *D. hansenii* 125G and 253H against *P. verrucosum* (Peromingo et al., 2018).

The modes of action of *D. hansenii* responsible for the increase or the decrease of the AFs concentration are not completely elucidated yet. On the one hand, it has been suggested that the mould adaptive response to sublethal stress due to growth-limiting treatments includes the upregulation of secondary metabolism increasing the mycotoxin production (Schmidt-Heydt et al., 2013). Thus, the incomplete growth inhibition by other microorganisms could be a stress factor to the mould, which potentially activates mycotoxin production (Meftah et al., 2018). Given that this stimulation of AFs production by yeast was not observed at other a_w values, it could be deduced that the environmental conditions play a key role in the response of *A. parasiticus* in presence of competitive *D. hansenii*. On the other hand, several modes of action of antagonistic yeasts in the decrease mycotoxin concentration have been suggested, such as mycotoxin adsorption or degradation, and blockage of the biosynthetic pathway (Asensio et al., 2014), that could explain the decrease of AFs levels at 0.92 a_w observed in the present work. Some yeasts can degrade mycotoxins, such as AFB_1 (Mann and Rehm, 1977), OTA (Fiori et al., 2014; Kapetanakou et al., 2012) or patulin

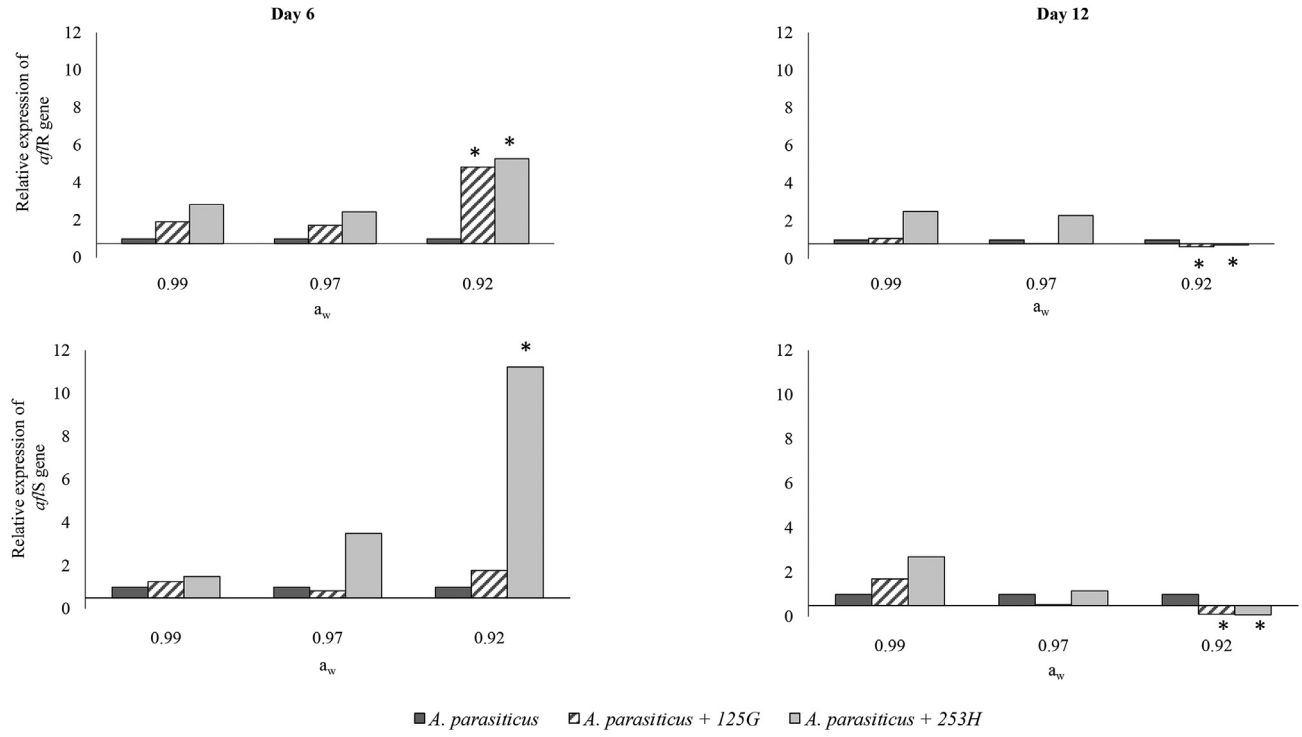

Fig. 3. Effect of *Debaryomyces hansenii* 125G and 253H strains on the relative expression of the *afl*R and *afl*S genes in *Aspergillus parasiticus* in meat-based media with different a_w (0.99, 0.97 and 0.92) after 6 and 12 days of incubation at 25 °C. Significant differences between mean values of the *afl*R and *afl*S gene expression in *Aspergillus parasiticus* in presence of each *Debaryomyces hansenii* strain at the same a_w and sampling time with respect to *Aspergillus parasiticus* control, are indicated by an asterisk ($p \leq 0.05$).

(Castoria et al., 2011), to less toxic compounds. Furthermore, some yeast strains, mainly from *S. cerevisiae* species, are able to decontaminate media and foods by AFs surface binding (Campagnollo et al., 2015; Hamad et al., 2017; Sahebghalam et al., 2013). However, no evidences of AFs degradation or binding by *D. hansenii* strains after 48 h were found in the present work (section 3.3). These results are similar to those previously obtained with the same *D. hansenii* strains with respect to adsorption or degradation of OTA (Peromingo et al., 2018).

The regulatory *afl*R and *afl*S genes are necessary for the AFs biosynthesis, and both have been used as targets to detect and quantify AFs-producing moulds (Medina et al., 2015; Peromingo et al., 2017; Schmidt-Heydt et al., 2010). It has been reported that *B. megaterium* inhibits both the growth and AFs biosynthesis in *A. flavus* through altering its gene transcription, especially because of a high reduction of *afl*S gene expression (Kong et al., 2014). Nevertheless, the effect of antagonistic yeasts on the expression of the genes involved in the AFs biosynthesis has not been studied yet. In order to evaluate whether there was some relationship between the temporal relative expression of the *afl*R and *afl*S genes over time and the presence of *D. hansenii* 125G and 253H strains, samples were taken at the middle and at the end of the incubation time. The yeast strains only provoked significant changes in the expression of the two regulatory genes at 0.92 a_w (Fig. 3). However, at the highest a_w levels, mainly at 0.99 a_w, the highest relative expression average of both *afl*R and *afl*S genes was observed in the presence of yeasts, matching with the increase of AFs production in this batches (Fig. 2). At 0.92 a_w, the highest levels of gene expression in the presence of yeasts were found at day 6, whereas at the end of the incubation period a repression of both genes was observed in the co-inoculation batches. Similarly, Peromingo et al. (2018) found that *D. hansenii* 125G and 253H inhibited both the OTA production and the expression of the *otanps*PN gene in *P. verrucosum* at 0.92 a_w. Therefore, the obtained results could suggest that the reduction of AFs

Table 2
Effect of *Debaryomyces hansenii* 125G and 253H strains on the *Aspergillus parasiticus* CECT 2682 growth and aflatoxin B_1 (AFB_1) and aflatoxin G_1 (AFG_1) accumulation in dry-cured meat products.

Meat product	Treatment	Mould counts (log cfu/cm^2)	AFB_1 content (ng/g)	Reduction of AFB_1 (%)[b]	AFG_1 content (ng/g)	Reduction of AFG_1 (%)[b]
Dry-fermented sausages	Control[a]	6.55 ± 0.12	151.43 ± 63.64	–	356.51 ± 91.57	–
	125G	6.55 ± 0.34	69.88 ± 45.10*	53.85	168.85 ± 80.46**	52.63
	253H	6.67 ± 0.34	55.81 ± 29.13**	63.14	145.94 ± 59.75**	59.06
Dry-cured ham	Control[a]	6.59 ± 0.05	< LOD[c]	–	6.83 ± 5.53	–
	125G	6.39 ± 0.35	< LOD	–	< LOD	100
	253H	6.25 ± 0.12	< LOD	–	< LOD	100

The results are presented as mean of triplicate ± standard deviation.
*: Significant differences with respect to control ($p \leq 0.01$).
**: Significant differences with respect to control ($p \leq 0.001$).
[a] Control batch inoculated only with *A. parasiticus*.
[b] Reduction of aflatoxin content (%) compared to control.
[c] LOD: Limit of detection.

production triggered by *D. hansenii* strains is mediated at transcriptional level.

After the *in vitro* promising results, the effect of the yeast strains on the growth of *A. parasiticus* and AFs production in dry-cured meat products under the typical ripening conditions was studied. The results suggest that the composition of meat product has a great impact on the biosynthesis of AFs by *A. parasiticus*, since their concentration was greatly higher in dry-fermented sausage than in dry-cured ham (Table 2). The amount of AFs detected in dry-fermented sausage were much higher than those described in the literature for naturally contaminated products (Markov et al., 2013; Pleadin et al., 2015; Rodríguez et al., 2012b), but the levels in dry-cured ham were closer to the range previously reported for samples taken from industries or markets (Markov et al., 2013; Pleadin et al., 2015). Apart from that, the presence of both *D. hansenii* strains provoked a dramatic reduction of AFs contamination in both meat matrices, mainly in dry-cured ham where AFs decreased at non-detectable level (Table 2) despite no effect on the *A. parasiticus* counts. A similar lack of relationship between the mould growth and OTA production has been observed when evaluating the antagonistic activity of *D. hansenii* species in meat substrates (Iacumin et al., 2017), including the *D. hansenii* 125G and 253H strains in dry-cured ham and dry-fermented sausage (Peromingo et al., 2018). Consequently, according to the results obtained in meat-based media in this work, the decrease of AFs concentration in dry-cured meat products could be attributable to the effect of *D. hansenii* 125G and 253H on *A. parasiticus* at transcriptional level. This hypothesis had been previously formulated for the antagonistic effect of native yeasts against ochratoxigenic moulds (Peromingo et al., 2018), but not yet for aflatoxigenic moulds in cured meat products.

The effect of the tested strains was sufficient for suppressing the AFs production by *A. parasiticus* in ham, but not completely in dry-cured sausage. Thus, their application should be considered a preventive measure together with good manufacturing practices to control the hazard related to the AFs presence. Moreover, the ability of *D. hansenii* 125G and 253H strains to reduce the accumulation of OTA has been previously demonstrated in both dry-cured ham and dry-fermented sausage (Peromingo et al., 2018).

The results obtained in this study suggest that the environmental conditions, specially a_w, play an important role in the antifungal capacity of yeasts. The inhibition of AFs production by *D. hansenii* was effective when a_w reached values of about 0.92, which occurs at drying stages in dry-cured ham (Rodríguez et al., 1994). Additionally, the stimulation of AFs production at the highest a_w suggests that the ecological parameters are crucial factors to be controlled to avoid the potential stress that could lead to mycotoxin production. Consequently, it is of critical importance to establish the most appropriate timing for the addition of the antagonistic yeasts throughout the ripening of dry-cured meat products and thus minimize the hazard associated with AFs. Concretely, to prevent the stimulation of AFs production detected at 0.99 a_w (Fig. 2), the yeast strains should be inoculated during the post-salting stage, when the a_w values are below 0.97 in the product (Arnau et al., 1995).

In conclusion, *D. hansenii* 125G and 253H strains could be proposed as biocontrol agents to reduce the growth of toxigenic moulds and the production of mycotoxins in dry-cured meat products. Their antifungal effect is probably due to a combination of competition for space and nutrients, production of antifungal compounds and alteration of the expression of genes involved in the biosynthetic pathway of mycotoxins. In addition, the knowledge of the optimal ecological conditions in which the antagonistic yeasts are most effective at controlling AFs accumulation would have a key role in the decisions made during the ripening of dry-cured meats within the HACCP framework to minimize the hazard associated with the presence of AFs in these products.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.01.024.

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