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Effect of dry-sausage starter culture and endogenous yeasts on *Aspergillus westerdijkiae* and *Penicillium nordicum* growth and OTA production

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ABSTRACT

Processed meat products frequently suffer from fungal and mycotoxin contamination, mostly ochratoxin A (OTA). *Penicillium nordicum* is considered responsible for this contamination, but *Aspergillus westerdijkiae* has recently been associated with high levels of OTA in meat products.

Several biocontrol agents have been tested against *P. nordicum* growth and OTA production in meat products, but *A. westerdijkiae* has not been considered. The aim of this work was to evaluate *in vitro* the effect of a commercial starter culture used in sausage fermentation and of sausage-native yeasts on OTA production by *A. westerdijkiae*, as compared with the highly studied *P. nordicum*, in meat-based culture media.

Four representative yeasts isolated from dry-cured sausage and a commercial starter culture were co-inoculated with both fungi in different meat-based media, under varying conditions. Fungal growth was determined by measuring colony diameter, and OTA production was quantified by HPLC-FLD.

A. westerdijkiae was significantly stimulated to produce OTA under all tested conditions, and, in ham, OTA production by *P. nordicum* was stimulated by co-culture with the starter culture.

In conclusion, endogenous or added microorganisms enrolled in fermentation or in biocontrol in meat products seem to exert varying responses on different ochratoxigenic fungi, thus leading to unforeseen safety problems.

1. Introduction

Processed meat products such as dry-cured ham, fermented sausage and others are foods of major importance in several European countries, both nutritionally and economically. Portuguese traditional fermented cured sausages (Salpicão, Linguiça, Chouriça) are prepared from raw pork meat with salt, and ingredients such as red/white wine, garlic and chili pepper. Some curing salts like nitrite and polyphosphates can also be added. The process includes a first stage of seasoning, stuffing of the mixture in natural pork casings, and a final stage of curing (with or without smoking), from one to four weeks, depending on the product and the process. (Cadavez et al., 2016). The fermentation of these traditional products occurs naturally, by the action of characteristic endogenous microbiota comprising essentially lactic acid bacteria (LAB), coagulase-negative staphylococci (CNS) and yeasts. Yeasts show important beneficial effects throughout curing, as they contribute with particular aroma and flavour resulting from their proteolytic and lipolytic activities (reviewed by Flores, Corral, Cano-García, Salvador, & Belloch, 2015). Although data on the yeast dynamic and biodiversity in sausages are limited, several studies revealed the presence of some genera such as *Debaryomyces*, *Candida, Yarrowia, Rhodotorula, Pichia* and *Trichosporon* (Andrade, Rodriguez, Casado, Bermudez, & Cordoba, 2010; Mendonça, Gouvêa, Hungaro, Sodré, & Querol-Simon, 2013; Nielsen, Jacobsen, Jespersen, Koch, & Arneborg, 2008; Romano, Capece, & Jespersen, 2006; Samelis & Sofos, 2003). Despite the fact that yeast population in dry-fermented meat products depends on meat flora and manufacture process, *Debaryomyces hansenii* has been the most frequently isolated yeast species along different manufacture processes, and nowadays selected strains are available as commercial starters.

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Dry-fermented sausages are considered safe meat products due to reduced water activity and pH achieved during processing and storage, which together inhibit the development of pathogenic microflora. However, due to their characteristics, they are highly exposed to mycotoxin producing fungi. Ochratoxin A (OTA) is the most significant mycotoxin found in processed meat products. *Penicillium nordicum* is considered to be responsible for OTA contamination of these products, as it is strongly adapted to salt and protein-rich matrices and is moderately psychrotrophic. However, another OTA-producing fungus, *Aspergillus westerdijkiae* (formerly known as *A. ochraceus*), associated with carbon-rich matrices such as cereals and coffee beans, has also been associated with meat products (Escher, Koehler, & Ayres, 1973; Iacumin, Milesi, Pirani, Comi, & Chiesa, 2011; Scaramuzza, Diaferia, & Berni, 2015) and its ability to produce high amounts of OTA in such products has been proved (Iacumin, Manzano, Andyanto, & Comi, 2017, as *A. ochraceus*; Vipotnik, Rodríguez, & Rodrigues, 2017).

Because of its relevance, OTA should be reduced to the lowest possible levels in foods, and for that reason it is mandatory to develop efficient strategies to avoid it from entering the food chain. Numerous chemical, physical and biological agents can be used for food detoxification purposes (EFSA, 2009). But one of the major problems with OTA, as with other mycotoxins, remains on its high stability (Boudra, Le Bars, & Le Bars, 1995) thus making it highly challenging to eliminate it from food products. For this reason, one of the most promising strategies under study is to prevent its accumulation by creating the best conditions to inhibit fungal growth and consequent OTA production.

Many studies have been done for the screening of yeasts, bacteria and moulds with antagonistic effect on OTA accumulation in food products, either by inhibition of fungal growth or by inhibition of OTA production. Several LAB and yeasts have been tested as biocontrol agents against *P. nordicum* growth and OTA production in meat products, mostly ham and Italian-style salami, with promising results (Andrade, Thorsen, Rodríguez, Córdoba, & Jespersen, 2014; Iacumin et al., 2017; Rodriguez et al., 2015; Simoncini, Virgili, Spadola, & Battilani, 2014; Virgili et al., 2012), but only one has considered the effect of these microorganisms on ochratoxigenic fungi other than *P. nordicum* (Iacumin et al., 2017).

The intended aims of this work were to study, *in vitro*, the effect of a commercial starter culture composed by LAB, CNS and *D. hansenii* and of several Portuguese dry-sausage endogenous yeasts on *P. nordicum* and *A. westerdijkiae* growth and OTA production ability in a Portuguese-style dry-fermented sausage-based medium. Throughout the work, some unexpected results were obtained for the matrix under study, and further studies were developed to test the influence of the matrix on the effect of biocontrol agents. For this subsequent study, two types of fermented dry-cured sausages were included. While the main interest of the work was fermented dry-cured sausage, dry-cured ham was also included, as a control, since information on OTA production ability by *P. nordicum* and *A. westerdijkiae* were available (Vipotnik et al., 2017).

2. Materials and methods

2.1. Selection and identification of yeasts

A group of 100 yeasts previously isolated from two dry-fermented sausage plants and preserved at −20 °C in 30% glycerol were grown on Potato Dextrose Agar (PDA, Liofilchem-ITALY) for 3 days at 28 °C and preliminarily grouped according to their colony and cell morphology. From these, ten yeasts were selected from morphologically distinct groups and were biochemically identified by the rapid miniaturised system API 20 C AUX System (bioMérieux, France), following supplier instructions. The following yeast species were identified: *Candida albicans*, *Candida colliculosa, Candida krusei* (2 isolates), *Candida zeylanoides* (2 isolates), *Cryptococcus laurentii*, *Rhodotorula glutinis*, and *Rhodotorula mucilaginosa* (2 isolates). From these, four strains – phenotypically identified as *C. krusei*, *C. zeylanoides*, *R. glutinis* and *R. mucilaginosa* – were further selected based on relative frequency on Portuguese dry-fermented sausage and on other similar products (Andrade et al., 2010; Mendonça et al., 2013). The identification of the four selected yeasts was confirmed molecularly by amplification and sequencing of the D1/D2 region of 26S rRNA gene. Yeasts were grown on Potato Dextrose Agar (PDA, Liofilchem-ITALY) for 3 days at 28 °C, and genomic DNA extraction was performed as described by Rodrigues, Venancio, Kozakiewicz, and Lima (2009). PCR amplification was achieved with the primers NL1 (5′GCATATCAATAAGCGGAGGAAAAAG3′) and NL4 (5′GGTCCGTGTTTCAAGACGG3′), as described by Kurtzman and Robnett (1998). PCR reactions were carried out in a thermal cycler BioRad Mycycler, in a final volume of 50 μL, containing 10 μL of 5x Go-Flexi Taq $MgCl_2$-free reaction Buffer (Promega), 1.5 mM $MgCl_2$, 1.25 U of Go-Flexi Taq polymerase (Promega), 200 μM of each dNTP (Bioron), and 2 μL of genomic DNA. PCR programme was optimized from the procedure described by Kurtzman and Robnett (1998), and was carried out as follows: initial denaturation of DNA for 4 min at 95 °C; 34 cycles of denaturation at 95 °C for 50 s, annealing at 55 °C for 50 s, and extension at 72 °C for 1 min; and a final extension cycle at 72 °C for 7 min. PCR products were purified with the commercial kit GF-1 PCR CleanUp Kit (Vivantis), according to manufacturer's instructions. Sequence analysis was performed on an ABI 3730xl DNA Analyzer (Applied Biosystems), by outsourcing. PCR products were sequenced in both directions, and a consensus sequence was created from the assembly of the forward and backward sequences using the package Sequencher 4.9 (Gene Codes, Ann Arbor Michigan) (Rodrigues, Santos, Venancio, & Lima, 2011). The consensus sequences were manually adjusted by chromatogram comparison and then aligned with the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/) using the BLAST algorithm. Yeast phenotypic identification was confirmed as *C. krusei*, *C. zeylanoides*, *R. glutinis* and *R. mucilaginosa*.

2.2. Preparation of inocula

2.2.1. Yeasts and starter culture

The selected yeasts were sub-cultured from stock vials onto PDA and incubated at 28 °C for 3 days. For the preparation of the pre-inoculum, one colony from 3 day old cultures was suspended in Potato Dextrose Broth (PDB, Liofilchem-ITALY) and incubated at 28 °C for 24 h in a rotary shaker (120 rpm). Optical density of the suspension was determined by spectrophotometry at 600 nm wavelength (OD600). Cell concentration was estimated by using the correspondence OD600 = 1.0 equals 3×10^7 cells/mL, as described by Day, Schneider, and Schneider (2004). An inoculum of approximately 10^5 cells/mL was used in all assays.

The commercial starter culture Texel®ELCE Br (Danisco) – composed of *Pediococcus pentosaceus, Lactobacillus sakei, Staphylococcus carnosus, Staphylococcus xylosus* and *D. hansenii* – was also used for biocontrol tests. The freeze-dried starter culture (0.01% w/v) was inoculated in MRS (de Man, Rogosa, Sharpe) broth (Liofilchem-ITALY) and incubated at 37 °C for 24 h. An inoculum of 2 μL of cell suspension per mL of meat extract media was used in all assays.

2.2.2. Ochratoxigenic fungi

Two species of OTA-producing fungi previously isolated from