TESIS DOCTORAL

EFECTO DE AGENTES PARA EL BIOCONTROL DE MOHOS OCRATOXIGÉNICOS EN EMBUTIDOS CURADO-MADURADOS

MARÍA MICAELA ÁLVAREZ RUBIO

PROGRAMA DE DOCTORADO EN CIENCIA DE LOS ALIMENTOS

2021

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Conformidad de los Directores "La conformidad del director/es de la tesis consta en el original en papel de esta Tesis Doctoral"

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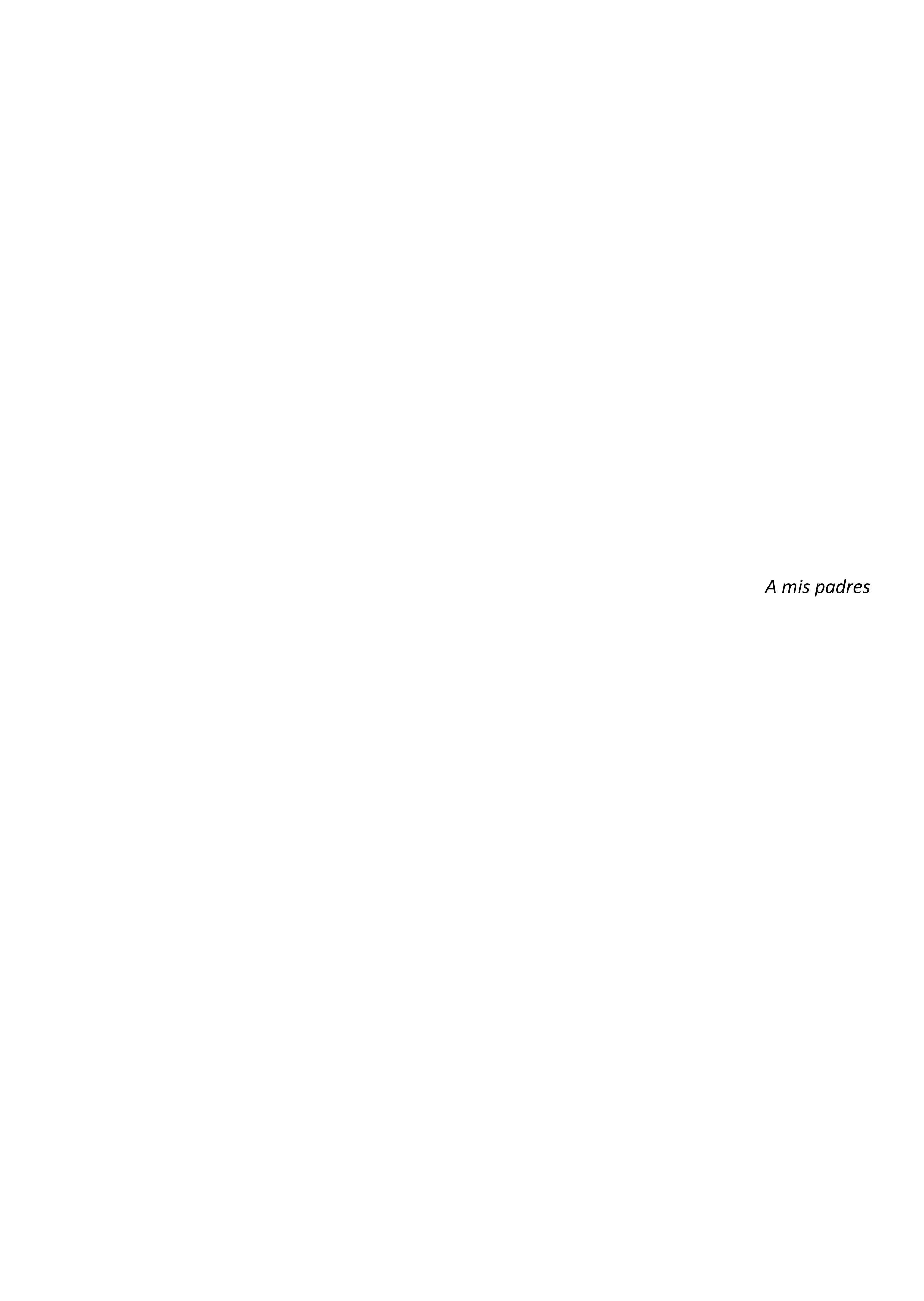
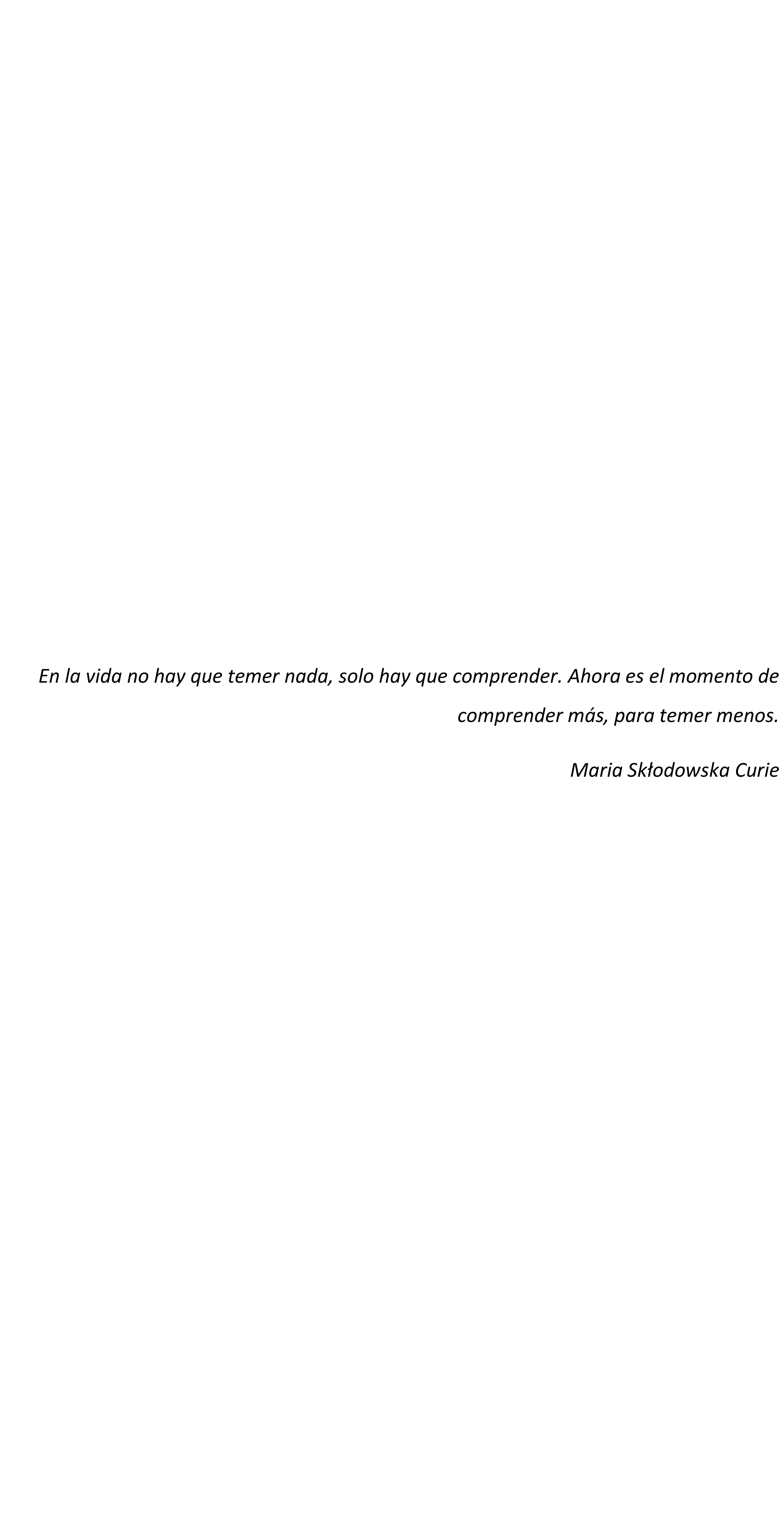


A mis padres



En la vida no hay que temer nada, solo hay que comprender. Ahora es el momento de comprender más, para temer menos.

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RESUMEN/ABSTRACT

Resumen

Para el control de mohos productores de ocratoxina A (OTA) en embutidos curado-madurados se han desarrollado estrategias naturales basadas en el uso de agentes de biocontrol (BCAs). El objetivo de esta Tesis Doctoral fue diseñar un método frente a mohos ocratoxigénicos (*Penicillium nordicum* y *Aspergillus westerdijkiae*) en embutidos empleando *Enterococcus faecium*, *Debaryomyces hansenii* y *Penicillium chrysogenum*. Además, se evaluaron BCAs de origen vegetal, como el orégano, tomillo, romero (incluyendo su aceite esencial (REO)) y un extracto de bellota. Tras evaluar su efecto sobre la producción de OTA, se investigaron los modos de acción implicados, así como los efectos sobre la población autóctona y los parámetros de calidad de los embutidos curado-madurados. El tomillo y el REO fueron descartados como BCAs al no reducir la OTA. El resto mostraron diferencias en cuanto a sus modos de acción. *P. chrysogenum* parece competir por nutrientes y espacio. *D. hansenii* redujo proteínas relacionadas con la biosíntesis de OTA y la integridad de la pared celular (CWI), mientras que la pared celular parece ser la diana del orégano. Las hojas de romero redujeron el ergosterol y las tripas maceradas en él afectaron a la CWI y a la síntesis de ergosterol y OTA en *P. nordicum*. La adición conjunta de hojas de romero como ingrediente o en el macerado y *D. hansenii* es una estrategia prometedora debido a la potenciación de la disminución de proteínas de la CWI y la ruta biosintética de OTA sin afectar negativamente a la calidad del producto final.

Abstract

To control ochratoxin A (OTA)-producing moulds in dry-cured fermented sausages, different natural strategies based on biocontrol agents (BCAs) have been developed. The main objective of this Doctoral Thesis was to design a method against ochratoxigenic moulds (*Penicillium nordicum* and *Aspergillus westerdijkiae*) in dry-cured fermented sausages using *Enterococcus faecium, Debaryomyces hansenii* and *Penicillium chrysogenum*. Oregano, thyme, rosemary (including rosemary essential oil (REO)) and an acorn extract were also tested. After OTA analysis, their modes of action and their effects on the native population and quality parameters of dry-cured fermented sausages were investigated. Thyme and REO did not show the desirable effect on the OTA production. Different modes of action were revealed depending on the BCAs. *P. chrysogenum* seems to compete by nutrients and space. *D. hansenii* decreased proteins involved in OTA biosynthesis and the cell wall integrity pathway (CWI), while the cell wall seems to be the oregano's target sites. Rosemary leaves reduced ergosterol content, while the casings macerated with them affected the CWI, ergosterol biosynthesis and OTA biosynthesis in *P. nordicum*. The addition of rosemary leaves as ingredient or by macerating the casings of dry-cured fermented sausages, together with *D. hansenii*, is a promising biocontrol strategy since their combination enhanced the reduction of proteins related to CWI and OTA biosynthesis without negatively altering the quality parameters.

I. INTRODUCCIÓN

I.1. Importancia de los derivados cárnicos curado-madurados

En el año 2020, España alcanzó el récord de producción de carne de porcino, llegando a 5 millones de toneladas, lo que supone un aumento de la producción del 8,2 % respecto al año 2019 y un 15 % desde el 2015. Este nivel sitúa a España como el cuarto mayor productor mundial de carne de porcino por detrás de China, Estados Unidos y Alemania (MAPA, 2020). Esta situación se ha traducido en un aumento de la producción de derivados cárnicos curado-madurados y de las exportaciones de carne y derivados del sector porcino situándose en el tercer exportador mundial en volumen en 2019 (MAPA, 2020).

Por definición, los derivados cárnicos curado-madurados son los productos sometidos a un proceso de salazón y de curado-maduración, suficiente para conferirles las características organolépticas propias y de estabilidad a temperatura ambiente. Pueden someterse opcionalmente a ahumado y se dividen en piezas y carnes troceadas o picadas (Ministerio de la Presidencia, 2014).

En el año 2020 el consumo per cápita en España de jamón y paleta curada fue de 1,70 kg, de jamón y paleta ibérica de 0,48 kg, aumentando en un 23 % su consumo respecto al año anterior, y 2,5 kg de embutidos (MAPA, 2020). Dividiendo los embutidos por producto, se consumieron 1,08 kg de chorizo, de 0,44 kg salchichón/salami y 0,72 kg de fuet/longanizas. Además, en el año 2020 se exportaron 66493 toneladas de embutidos curados y 61943 toneladas de jamón curado (ANICE, 2021). La tradición y el valor gastronómico de estos productos, elaborados en muchos casos de manera tradicional, hacen que sean apreciados tanto dentro como fuera de España (ANICE, 2021). Por lo tanto, debido a que forman parte de la dieta tradicional y están ampliamente distribuidos por toda la geografía, es necesario que sean seguros y no supongan ningún riesgo al consumidor.

I.2. Embutidos curado-madurados

En Europa, la fabricación de embutidos se remonta a la Grecia Clásica ya que era la manera de preservar la carne tras el sacrificio de los animales, mediante el secado y la fermentación. Los romanos sazonaban y condimentaban la carne picada que embutían en tripas que después secaban. Sin embargo, esta técnica la reprodujeron de los celtas que salaban y secaban las patas traseras de los jabalíes y cerdos (Leroy & De Vuyst, 2015). Estos productos considerados estables sirvieron de provisiones para viajeros y soldados facilitando expediciones y campañas militares. A lo largo de los siglos, sus ingredientes principales siguen siendo los mismos: carne picada, grasa, sal y especias. Sin embargo, dependiendo de la región se pueden encontrar claras

diferencias que hacen que formen parte de su entidad gastronómica y cultural. En Europa, éstos pueden diferenciarse en embutidos mediterráneos como el salami en Italia, la «*rosette*» en Francia o el chorizo en España y en embutidos del norte de Europa como el «*saucisson d'Ardennes*» belga o el salami húngaro (Leroy & De Vuyst, 2015).

Según la legislación española, los embutidos curado-madurados son los derivados cárnicos elaborados mediante carnes troceadas o picadas y grasa no identificables anatómicamente que, con carácter general y no limitativo, se han sometido a un proceso de picado más o menos intenso, mezclados con especias, ingredientes, condimentos y aditivos, embutidos en tripas naturales o envolturas artificiales, y sometidos a un proceso de salazón seguido de curado-maduración, acompañado o no de fermentación, suficiente para conferirles las características organolépticas propias y su estabilidad a temperatura ambiente (Ministerio de la Presidencia, 2014). Las carnes podrán ser todas del mismo tipo o ser una mezcla de carnes de distinta procedencia, naturaleza, parte anatómica y especie animal (Ministerio de la Presidencia, 2014), aunque la mayoría se fabrican con carne de cerdo.

I.2.1. Procesado de los embutidos curado-madurados

Las etapas del procesado de los embutidos curado-madurados se recogen en el diagrama de flujo descrito en la Figura 1.

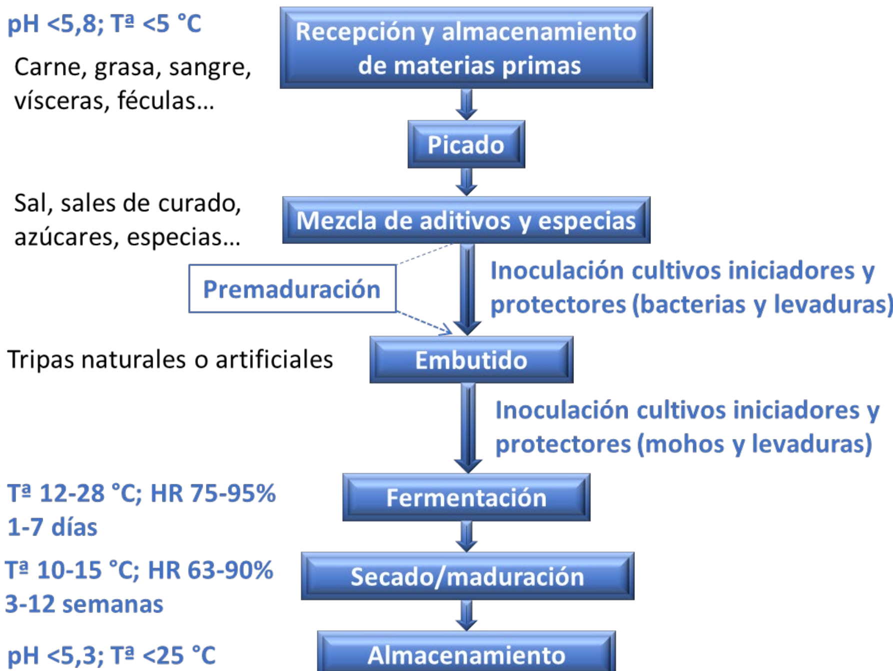

Figura 1. Diagrama de flujo del procesado de los embutidos curado-madurados. Elaboración propia a partir de Jiménez & Carballo (1989) y Ordóñez & de la Hoz (2001).

A continuación, se detallan las diferentes etapas del procesado de los embutidos curado-madurados:

a) Selección de materias primas

La carne empleada en la elaboración de embutidos es generalmente de cerdo, pero se pueden emplear otras especies, además de elaborarse con mezclas, como en el caso del salami italiano en el que se utiliza carne de vacuno y de porcino. Es recomendable utilizar carne cuyo pH sea menor de 5,8 ya que si es superior puede originar el desarrollo de microorganismos alterantes o patógenos, además de alteraciones en la textura como la falta de ligazón o una blandura excesiva (Jimenez & Carballo, 1989; Ordóñez & de la Hoz, 2001). Sin embargo, si se añaden cultivos iniciadores se puede emplear carne fresca con un valor de pH ligeramente superior, de hasta 6,2.

La grasa se añade procedente de tocino o panceta, fresca, refrigerada o congelada. Normalmente se opta por grasa refrigerada o congelada para aumentar la consistencia ya que así se evita la formación de un producto viscoso durante el picado que dificultaría la cohesión y la entrada de las sales de curado. Además, disminuye el enranciamiento y con ello la posible aparición de características indeseables (Hernández et al., 2003). En algunos embutidos se añade también sangre, vísceras como pulmones, corazón o estómago o hidratos de carbono como arroz o patata (Jimenez & Carballo, 1989).

La proporción de los ingredientes es diversa y dependerá de las características propias de cada embutido. Por ejemplo, la chistorra presenta un 50 % de grasa mientras que el chorizo solo un 20 %.

b) Picado

El picado de la materia prima puede realizarse en picadoras o trituradoras tipo *cutter*. El grado de picado puede ser grueso (chorizo), mediano (salami) o fino (sobrasada). La materia prima se añade refrigerada o congelada para evitar el calentamiento de la masa que produce pérdidas excesivas de agua y defectos posteriores durante el secado-maduración.

c) Mezclado de aditivos y especias y amasado

Tras el picado, se mezclan y amasan el resto de los ingredientes, como los aditivos y las especias, en máquinas mezcladoras-amasadoras hasta conseguir una masa uniforme. La proporción total habitual de estos aditivos oscila entre el 2,2 y el 3 % (Ordóñez & de la Hoz, 2001). La sal se añade para dar sabor y además inhibe el desarrollo de microorganismos indeseables contribuyendo al

descenso de la actividad de agua (a_w), condiciones que favorecen el crecimiento de las bacterias lácticas (Ordóñez & de la Hoz, 2001). Dentro de los aditivos más comunes se encuentran las sales de curado (nitratos y nitritos) y el ascorbato sódico (Fernández-López et al., 2008; Martín-Sánchez et al., 2011). Aunque la adición de nitratos no es necesaria, puede emplearse en procesos de maduración largos actuando como reservorio de nitritos. Las sales de curado se han empleado tradicionalmente como agentes antimicrobianos frente a bacterias patógenas como *Clostridium botulinum, Listeria monocytogenes* o *Salmonella* Typhimurium (Christieans et al., 2018; Ordóñez et al., 1999), aunque también contribuyen al desarrollo del color característico por formación de nitrosomioglobina. Las especias se añaden para conferir al producto un flavor, aroma y color característicos. Éstas varían dependiendo del producto, pudiendo añadirse semillas (mostaza), hojas (orégano, romero, etc.), bulbos (ajo o cebolla) o frutos (pimentón) en su forma natural o molidas (Toldrá, 2002). No suele añadirse al producto más del 2 % siendo la pimienta en grano, el pimentón y la nuez moscada las más frecuentes (Ordóñez & de la Hoz, 2001; Toldrá, 2002). Algunas especias presentan efecto estimulante de las bacterias lácticas debido a su contenido en manganeso y actividad antimicrobiana frente a patógenos y alterantes (Ordóñez & de la Hoz, 2001; Tajkarimi et al., 2010). También se puede añadir azúcar como sustrato para los microorganismos durante la fermentación.

Tras los aditivos y especias se pueden añadir los cultivos iniciadores que necesitan ser reconstituidos si se encuentran liofilizados. Tradicionalmente, estos cultivos están formados principalmente por bacterias ácido-lácticas productoras de ácidos y cocos Gram positivo catalasa positivo reductoras de nitratos a nitritos (Hernández et al., 2003). Las industrias que comercializan estos productos también han desarrollado cultivos con doble actividad, iniciadora y protectora frente a bacterias patógenas como *L. monocytogenes*, *Salmonella* o *Escherichia coli* (Raimondi et al., 2014; Työppönen et al., 2002).

A continuación, en algunas industrias se realiza una fase de premaduración de la masa en la que se almacena durante uno o dos días a 4 °C (fabricación difásica) con lo que se consigue una mayor consistencia al corte y estabilidad del color del embutido (Jimenez & Carballo, 1989).

d) Embutido

Las tripas empleadas en la etapa de embutido determinan tanto el tamaño como la forma y las características físico-químicas del producto debido a su calibre, resistencia o permeabilidad. Las tripas pueden ser naturales procedentes del intestino delgado o grueso o artificiales derivadas de celulosa, colágeno o plástico (Jimenez & Carballo, 1989).

Tras el embutido pueden inocularse mohos y levaduras en su superficie como cultivos iniciadores, que regulan la pérdida de humedad, haciendo que sea uniforme, contribuyen a la estabilización del color e inhiben el enranciamiento (Vignolo et al., 2010). Además, debido a su actividad proteolítica y lipolítica producen sustancias sápidas y aromáticas que contribuyen al olor y flavor característico del embutido (Vignolo et al., 2010). También pueden emplearse como cultivos protectores impidiendo el crecimiento de mohos no deseables por competición o mediante la producción de compuestos antifúngicos (Bernáldez et al., 2013; Núñez et al., 2015).

e) Fermentación

Los embutidos son llevados a secaderos o cámaras con temperatura, humedad relativa (HR) y velocidad de aire controladas, donde se realiza la fermentación y una etapa de ahumado en los productos que lo requieran. Generalmente la temperatura se mantiene entre 20-28 °C para facilitar el crecimiento de los microorganismos en los primeros días. Sin embargo, pueden fijarse valores por debajo de 15 °C para crear una fermentación «lenta», en la cual los embutidos adquieren una coloración más intensa (Hernández et al., 2003). Los valores de HR se encuentran inicialmente en torno al 95 % (24-48 h) para impedir el secado excesivo del producto, que disminuiría el agua disponible para los microorganismos y favorecería la formación de una costra externa (Ordóñez & de la Hoz, 2001). Después la HR puede disminuir hasta el 75 %. El proceso de fermentación puede ampliarse hasta una semana dependiendo del tipo de embutido (Vignolo et al., 2010). Al final de esta etapa, en algunos embutidos se realiza un ahumado, que consigue modificar el aroma y el sabor del producto y evita el desarrollo de mohos y levaduras no deseables (Ordóñez & de la Hoz, 2001).

f) Secado-maduración

La etapa de secado-maduración puede realizarse en las mismas cámaras donde se ha producido la fermentación modificando la temperatura y la HR. Estos parámetros dependen del tipo de embutido que se fabrique, encontrándose generalmente valores entre 10-17 °C y 65-85 % de HR (Jiménez & Carballo, 1989; Ordóñez & de la Hoz, 2001). Esta etapa puede realizarse a temperaturas más bajas entre 5-10 °C en el caso de maduraciones lentas consiguiéndose un color y sabor más intenso (Jimenez & Carballo, 1989). La velocidad del aire debe ser constante, de aproximadamente 1 m/s, y uniforme en toda la cámara. Este período discurre por un mínimo de 20 días pudiendo ampliarse hasta 4 meses dependiendo de la variedad de embutido (Benito et al., 2007; Ordóñez & de la Hoz, 2001).

Durante la maduración se degradan proteínas y lípidos y la a_w disminuye por debajo de 0,90 inhibiendo el crecimiento de microorganismos no deseables. Al final de la maduración el valor

de pH puede aumentar ligeramente debido a que los mohos utilizan el ácido láctico y producen amoníaco y se acumulan productos derivados de las proteínas (Ordóñez & de la Hoz, 2001; Toldrá et al., 2015).

g) Almacenamiento

Los embutidos son considerados productos microbiológicamente estables por lo que tras su procesado no necesitan almacenarse a temperaturas de refrigeración (Jimenez & Carballo, 1989). A esta estabilidad contribuyen la disminución del pH y a_w, la sal, las sales nitrificantes y especias (Tajkarimi et al., 2010; Ventanas et al., 2004; Vignolo et al., 2010). El pH final del producto puede encontrarse entre 4,8 y 5,3, y su descenso durante la maduración es debido a los procesos de fermentación producidos por los microorganismos presentes espontáneamente o añadidos como cultivos iniciadores (Leroy & De Vuyst, 2015). En embutidos con un periodo de maduración largo el pH puede ser superior a 5,5 debido al crecimiento fúngico descrito anteriormente, por lo que estos productos requieren valores de a_w menores para asegurar su preservación (Toldrá et al., 2015). Sin embargo, es necesario evitar las temperaturas mayores a 25 °C (Toldrá et al., 2015) ya que pueden conducir a una proliferación microbiana indeseada, desecaciones excesivas y endurecimiento (Jiménez & Carballo, 1989; Pardo et al., 1988).

I.2.2. Población microbiana de los embutidos curado-madurados

La población microbiana de los embutidos curado-madurados al principio de la fermentación se encuentra a niveles de entre 10^5 y 10^6 ufc/g (Ordóñez & de la Hoz, 2001), incluyendo una variedad de especies de bacterias, levaduras y mohos.

Entre las bacterias ácido lácticas (BAL) se pueden encontrar especies de lactobacilos, pediococos y enterococos predominando *Lactobacillus sakei*, *Lactobacillus curvatus*, *Lactobacillus plantarum, Enterococcus faecium* y *Pediococcus pentosaceus* (Benito et al., 2007; Correia Santos et al., 2017; Landeta et al., 2013; Ruiz-Moyano et al., 2008). La concentración inicial de este grupo es variable y depende del embutido presentando valores a partir de 10^4 ufc/g (Benito et al., 2007; Fonseca et al., 2013a), aunque cuando se añaden cultivos iniciadores pueden alcanzarse recuentos de 10^7 ufc/g (Casquete et al., 2012). Las BAL constituyen la población mayoritaria durante toda la fermentación alcanzando al final de ésta 10^7-10^8 ufc/g (Benito et al., 2007; Drosinos et al., 2005).

Los cocos Gram-positivos catalasa positivos (CGC+) pertenecen principalmente a los géneros *Staphylococcus, Micrococcus* y *Kocuria*. Entre las especies frecuentemente aisladas de los embutidos se encuentran *Staphylococcus xylosus, Staphylococcus saprophyticus,*

Staphylococcus equorum y *Kocuria varians* (Lorenzo et al., 2007; Ordóñez & de la Hoz, 2001). Los CGC+ son usados en ocasiones como cultivos iniciadores, ya que contribuyen a controlar la rancidez, al desarrollo del aroma debido al catabolismo de los aminoácidos y ácidos grasos y al desarrollo y estabilización del color debido a sus actividades catalasa y nitrato reductasa (Martín et al., 2007; Vignolo et al., 2010). Además, algunos CGC+ se han propuesto como cultivos protectores al limitar el crecimiento de *L. monocytogenes* y otras bacterias Gram-positivas (Martín et al., 2007). Los recuentos de estos microorganismos al final de la maduración de los embutidos se sitúan entre 10^4-10^6 ufc/g (Casquete et al., 2012; Martín et al., 2007).

Por otra parte, entre los grupos bacterianos que constituyen la población inicial de estos derivados cárnicos curado-madurados se incluyen bacterias alterantes y patógenas. Entre las responsables de alteraciones predomina las pertenecientes al género *Pseudomonas* y las enterobacterias (Casquete et al., 2012; Drosinos et al., 2005; Ordóñez et al., 1999). Sin embargo, en condiciones normales, estas bacterias van reduciéndose progresivamente debido a la bajada del pH y la a_w, los aditivos añadidos y la competición con el resto de población microbiana incluyendo los cultivos iniciadores (Casquete et al., 2012; Cenci-Goga et al., 2012; Drosinos et al., 2005).

Las principales bacterias patógenas que pueden encontrarse en embutidos son *L. monocytogenes*, *Salmonella spp*., *E. coli*, *Staphylococcus aureus*, y *Clostridium spp.* siendo las dos primeras las más frecuentes (Casquete et al., 2012; Christieans et al., 2018). Aunque el riesgo de microorganismos patógenos en embutidos se considera bajo debido a sus características físico-químicas y la adición de nitritos y cultivos protectores, su presencia puede deberse a un mal procesado, una alta contaminación de las materias primas y a una contaminación cruzada (Mataragas et al., 2015). En el año 2020, *L. monocytogenes* fue la causante de siete alertas alimentarias en chorizo en España y Dinamarca, en salchichón de España, en salami en Italia, Rumania y Francia y en *Andouille de Guémené* en Francia (RASFF, 2021). *Salmonella* puede sobrevivir a a_w reducidas y pH ácido habiéndose comunicado entre los años 2016 y 2020 ocho alertas alimentarias en embutidos comercializados en Europa (Serra-Castelló et al., 2021).

Las levaduras pueden encontrarse tanto en el interior de los embutidos como colonizando su superficie. *Debaryomyces hansenii* ha sido descrita como la especie predominante en los embutidos elaborados en distintos países (Aquilanti et al., 2007; Baruzzi et al., 2006; Encinas et al., 2000; Flores et al., 2015; Ramos et al., 2017). En el interior del producto se han descrito recuentos finales de levaduras entre 10^2-10^4 ufc/g, dependiendo de la industria de procedencia,

mientras que en la superficie pueden ser superiores a 10^5 ufc/g (Encinas et al., 2000; García-Béjar et al., 2020; Mendonça et al., 2013).

Las levaduras facilitan el proceso de secado protegiendo a los embutidos de los cambios en la HR e influyen en el desarrollo del flavor debido a sus capacidades proteolítica y lipolítica (Flores et al., 2015). Así, algunas cepas de *D. hansenii* producen compuestos volátiles derivados del catabolismo de los aminoácidos como el 3-metil-1-butanol y el 3-metil-1-butanal (Andrade et al., 2010; Corral et al., 2015) asociados al aroma a curado (Ruiz et al., 1999). Otras cepas muestran una alta actividad proteolítica (Patrignani et al., 2007) y a otras se les atribuye una capacidad antioxidante al disminuir la oxidación lipídica (Cano-García et al., 2014; Corral et al., 2015). Por otra parte, se han utilizado extractos de *D. hansenii* libres de células para mejorar la calidad de embutidos curado-madurados (Bolumar et al., 2006). Debido a estas características, algunas cepas de *D. hansenii* seleccionadas se comercializan como cultivos iniciadores (Domínguez et al., 2016; Meftah et al., 2018).

Los mohos colonizan la superficie de los embutidos, perteneciendo la mayoría a los géneros *Penicillium* y *Aspergillus* (Iacumin et al., 2009; López-Díaz et al., 2001; Pleadin et al., 2017), siendo las especies *Penicillium nalgiovense*, *Penicillium chrysogenum*, *Penicillium nordicum* y *Penicillium solitum* las más frecuentes en estos productos (Castellari et al., 2010; Iacumin et al., 2009; Pleadin et al., 2017; Sonjak et al., 2011; Susca et al., 2017). En algunos embutidos el desarrollo de mohos es deseable ya que se les atribuyen diferentes efectos beneficiosos relacionados con la textura y el flavor:

- Evitan un excesivo secado y favorecen una deshidratación homogénea (Toldrá et al., 2015).
- Intervienen en la estabilización del color al limitar la incidencia de luz sobre el embutido, consumir oxígeno y presentar una actividad catalasa (Leroy & De Vuyst, 2015).
- Reducen la oxidación lipídica previniendo la rancidez, como consecuencia de su actividad catalasa (Toldrá et al., 2015).
- Contribuyen a potenciar el flavor debido a las actividades lipolítica y proteolítica. Se han descrito una amplia producción de compuestos volátiles en el producto que dependen de la población fúngica y que interfieren en las características sensoriales del mismo creando aromas específicos (Bruna et al., 2003; Sunesen & Stahnke, 2003). El 2-metil-1-propanal y el 2- y 3-metil-1-butanal son algunos de los aldehídos ramificados generados por el catabolismo de aminoácidos de los mohos y asociados al aroma a curado en embutidos curado-madurados (Bruna et al., 2003).

A pesar del impacto positivo de la población de mohos, algunos ocasionan efectos indeseables. Algunas especies minoritarias, como las pertenecientes al género *Mucor*, producen un secado heterogéneo al principio de la fermentación y pueden producir un fuerte olor a amoniaco debido a su alta actividad proteolítica (Toldrá et al., 2015). Otras especies del género *Cladosporium* pueden producir manchas negras en la superficie de los embutidos (Lozano-Ojalvo et al., 2015) y originar alergias e infecciones fúngicas en el consumidor (Schmidt-Heydt et al., 2013). Sin embargo, el principal problema relacionado con la población de mohos es la capacidad de algunas especies de producir toxinas en el alimento.

I.3. Micotoxinas en embutidos curado-madurados

Las micotoxinas son metabolitos secundarios tóxicos producidos naturalmente por mohos filamentosos. Suponen un peligro para la salud de los animales y los humanos al contaminar la cadena alimentaria desde los cultivos vegetales hasta los alimentos de origen animal (Ostry et al., 2017). Algunas micotoxicosis son conocidas desde hace siglos, como el ergotismo o Fuego de San Antonio descrito en la Edad Media por el consumo de pan de centeno contaminado por alcaloides de *Claviceps purpurea,* produciendo gangrena y sensación de quemazón en las extremidades (Bryła et al., 2019). Hasta la actualidad, se han descrito al menos 400 tipos diferentes de micotoxinas (Cimbalo et al., 2020), centrándose la mayoría de los estudios en las que han mostrado mayor toxicidad en mamíferos como son las aflatoxinas (AFs), la ocratoxina A (OTA), la patulina (PAT) y el deoxinivalenol (DON) (Cimbalo et al., 2020; Ostry et al., 2017).

Las AFs se consideran de mayor interés debido a su alta toxicidad y su presencia en muchos tipos de alimentos y piensos (Kumar et al., 2017). Hay más de veinte AFs conocidas siendo las principales la B1, B2, G1 y G2 (Kumar et al., 2017). Son producidas fundamentalmente por *Aspergillus flavus* y *Aspergillus parasiticus* y están clasificadas como cancerígenas para los humanos en el Grupo 1A por la Agencia Internacional de Investigaciones sobre el Cáncer (IARC, 2012). También presentan hepatotoxicidad, teratogenicidad e inmunotoxicidad (Kumar et al., 2017). Contaminan principalmente productos de origen vegetal como el maíz, los frutos secos o las especias (El-Nezami & Gratz, 2011), pero también pueden encontrarse en los derivados cárnicos curado-madurados (Perrone & Rodriguez, 2019; Zadravec et al., 2020).

La OTA fue aislada por primera vez en 1965 de *Aspergillus ochraceus* en maíz (El Khoury & Atoui, 2010). Es producida por distintas especies de *Aspergillus* y *Penicillium* como *A. ochraceus*, *Aspergillus carbonarius*, *Aspergillus westerdijkiae, Aspergillus steynii, Aspergillus niger, P. nordicum* y *Penicillium verrucosum* (El Khoury & Atoui, 2010; Wang et al., 2016), aunque se han

aislado otras especies potencialmente productoras de OTA en alimentos como *Aspergillus affinis*, *Aspergillus cretensis* y *Aspergillus elegans* (Gil-Serna et al., 2020a). Los alimentos que más contribuyen a la exposición de OTA a través de la dieta son los cereales, debido a su alto consumo en todo el mundo, el queso y los productos cárnicos (EFSA CONTAM Panel, 2020).

La PAT es producida sobre todo por *Penicillium expansum* aunque se han descrito al menos 60 especies productoras (Vidal et al., 2019). Su exposición crónica produce neurotoxicidad, genotoxicidad, teratogenicidad e inmunotoxicidad (Lai et al., 2016; Vidal et al., 2019). Se encuentra principalmente en frutas y verduras destacando las manzanas y alimentos derivados (Vidal et al., 2019). Sin embargo, aunque con menor frecuencia, se ha detectado en otros alimentos como el queso (Pattono et al., 2013).

Las micotoxinas de *Fusarium*, zearalenona, fumonisinas y tricotecenos como el DON, producen distintos tipos de toxicidad. La zearalenona produce cardiotoxicidad, inmunotoxicidad y problemas reproductivos, las fumonisinas son neurotóxicas, hepatotóxicas y nefrotóxicas y el DON produce mayormente síndromes eméticos (Cimbalo et al., 2020). Las especies del género *Fusarium* contaminan frecuentemente granos de cereales, haciendo de los alimentos y piensos derivados la principal fuente de estas micotoxinas (Neuhof et al., 2008).

Las micotoxinas pueden llegar a los embutidos curado-madurados por dos vías. Por un lado, por la acumulación en el músculo del animal productor debido al consumo de pienso o forrajes contaminados con ellas (Adegbeye et al., 2020; Bertuzzi et al., 2013). Por otro lado, por su síntesis en los propios embutidos por el desarrollo de mohos toxigénicos en la superficie de estos productos durante la etapa de secado-maduración (Iacumin et al., 2009; Pleadin et al., 2017). Las micotoxinas derivadas de éstos últimos pueden penetrar al interior del producto y permanecer en él aunque se elimine la capa fúngica superficial (Ordóñez & de la Hoz, 2001; Peromingo et al., 2019a; Pleadin et al., 2017). Su capacidad de difusión depende del tipo de embutido, la tripa empleada y el tiempo de maduración, aumentando en periodos largos (Dall'Asta et al., 2010; Parussolo et al., 2019; Pleadin et al., 2017).

I.3.1. Ocratoxina A en embutidos curado-madurados

La OTA es la micotoxina más frecuente en los derivados cárnicos curado-madurados (Merla et al., 2018; Perrone & Rodríguez, 2019; Pleadin et al., 2017), siendo *P. nordicum* la especie ocratoxigénica aislada con mayor frecuencia en estos productos (Castellari et al., 2010; Iacumin et al., 2009; Sonjak et al., 2011). Sin embargo, existen otras especies como *A. westerdijkiae* que ha sido descrito recientemente como productor de OTA en los derivados cárnicos curado-madurados (Iacumin et al., 2020a; Rodrigues et al., 2019; Vipotnik et al., 2017).

La OTA es un pentacétido derivado de la familia de las dihidrocumarinas unido a una molécula de fenilalanina (Figura 2). Es un compuesto cristalino, sin olor, con una baja solubilidad en agua, pero soluble en compuestos polares como el cloroformo o el metanol. Debido a su estructura derivada de la isocumarinas, presenta fluorescencia de color verde bajo luz ultravioleta (UV) en condiciones ácidas y azul en condiciones alcalinas (Khoury & Atoui, 2010). Es una molécula muy estable a la acidez y altas temperaturas por lo que una vez que contamina un alimento es difícil eliminarla.

OH O=C H N H O OH O O H CH3 Cl

Figura 2. Estructura química de la ocratoxina A. Adaptado de Khoury & Atoui (2010).

Esta micotoxina se excreta del organismo muy lentamente por lo que tiene capacidad de bioacumulación. Es nefrotóxica, inmunotóxica, neurotóxica, genotóxica y teratógena (EFSA CONTAM Panel, 2020). La genotoxicidad es secundaria a su efecto oxidativo sobre el ADN, aunque hay indicios de que intervienen otros factores no conocidos en la mutagenicidad en animales (EFSA CONTAM Panel, 2020). Ha sido clasificada por la IARC como posiblemente carcinógena para los humanos, incluyéndose en el grupo 2B (IARC, 1993). Sin embargo, en los últimos años, nuevos estudios sugieren que podría reclasificarse en el grupo 2A como probablemente carcinógena para los humanos ya que se ha constatado un aumento de la incidencia de tumores y aductos de ADN en roedores y cerdos (EFSA CONTAM Panel, 2020).

Debido a la toxicidad de la OTA la Unión Europea ha establecido un valor de ingesta semanal tolerable de 120 ng/kg de peso corporal y el contenido máximo en algunos alimentos de origen vegetal (Comisión Europea, 2006). Sin embargo, actualmente no existe legislación vigente en la Unión Europea que regule el contenido de esta micotoxina en los derivados cárnicos curado-madurados, aunque sí en algunos países miembros, como Italia, donde se ha establecido un contenido máximo de 1 µg/kg para carne de cerdo y productos derivados (Ministero della Sanità, 1999), valor que suele utilizarse como referencia en diferentes estudios.

El riesgo relativo de la presencia de OTA en derivados cárnicos ha sido estimado en un 75 % en España (Sánchez-Montero et al., 2019), lo que supone una exposición a esta micotoxina de los consumidores un 75 % mayor que los no consumidores. En EE. UU. se ha estimado que la carne de cerdo es responsable de entre el 7,91 % y el 27,86 %de la ingesta de OTA, dependiendo de la franja de edad (Mitchell et al., 2017). En Francia se considera a las carnes *delicatessen* como la tercera mayor fuente de OTA en alimentos por detrás del pan y la pasta (Sirot et al., 2013). En la República Checa los productos cárnicos se encuentran como una de las principales fuentes de exposición a la OTA (Ostry et al., 2015). Por el contrario, un estudio realizado en Bélgica ha concluido que la presencia de OTA en productos cárnicos no supone un riesgo para la salud de los consumidores (Meerpoel et al., 2021).

Sin embargo, en la última opinión científica de la Autoridad Europea de Seguridad Alimentaria (EFSA) sobre el riesgo de la OTA en alimentos se refleja la incertidumbre sobre la presencia de esta micotoxina en derivados cárnicos curado-madurados debido a los pocos datos disponibles (EFSA CONTAM Panel, 2020), destacando la necesidad de llevar a cabo más estudios. Concretamente, solo en Croacia e Italia (Tabla 1) se han llevado a cabo estudios sobre la incidencia de OTA en embutidos en países europeos, a pesar de que se han aislado especies potencialmente productoras de OTA en la superficie de estos productos en otros países como España y Eslovenia (Chaves-López et al., 2012; Sonjak et al., 2011). No obstante, la EFSA considera que, en función de los datos disponibles, los derivados cárnicos curados-madurados se encuentran entre los alimentos que más contribuyen a la exposición crónica a la OTA a través de la dieta (EFSA CONTAM Panel, 2020) por lo que es esperable que a medio plazo se establezca un contenido máximo permitido en este tipo de alimentos.

Tabla 1. Incidencia y niveles de ocratoxina A en embutidos curado-madurados.

Origen	Muestras positivas/totales	Rango (µg/kg)	Referencia
Croacia	58/90 (64 %)	1,23-7,83	Markov et al., 2013
	14/208 (7 %)	0,95-5,1	Pleadin et al., 2015
	29/133 (22 %)	1,36-9,95	Vulić et al., 2016
	7/93 (8 %)	2,74-4,14	Zadravec et al., 2020
Italia	22/172 (13 %)	0,07-5,66	Altafini et al., 2019
	5/50 (10 %)	0,06-103,7	Armorini et al., 2015
	-/6	0,42-0,76	Bertuzzi et al., 2013
	72/160 (45 %)	3-18	Iacumin et al., 2009
	108/450 (24 %)	0,11-625,7	Iacumin et al., 2011
	16/83 (19 %)	0,25-98,5	Roncada et al., 2020
	6/9 (66 %)	-	Andersen, 1995

I.4. Control de mohos ocratoxigénicos en embutidos curado-madurados

Dado que la OTA representa un peligro sanitario importante en estos alimentos, y es producida por los mohos que colonizan los productos cárnicos, pudiendo encontrarse tanto en la superficie como en el interior del mismo (Peromingo et al., 2019a), es preciso controlar su procesado para garantizar que no se desarrollen los mohos productores o al menos para minimizar su síntesis, favoreciendo al mismo tiempo la actividad de los microorganismos con efectos deseables. Las medidas higiénicas para prevenir la contaminación durante la elaboración se ven desbordadas en los secaderos y bodegas, donde no es factible eliminar totalmente la contaminación ambiental. Por lo tanto, pueden aplicarse diferentes métodos físicos, químicos o biológicos disponibles, con diferente efectividad.

Algunos métodos físicos como la radiación UV o los ultrasonidos podrían eliminar los mohos en las superficies de alimentos sin dejar residuos en los mismos (Asensio et al., 2014; Luo et al., 2018). Sin embargo, estas técnicas no son adecuadas para los embutidos curado-madurados ya que al ser poco selectivas también afectarían a la población microbiana deseable y, por consiguiente, a las características típicas del producto (Asensio et al., 2014).

La industria cárnica emplea comúnmente preparados químicos para limitar el desarrollo de la población fúngica. La legislación europea permite el uso de ácido sórbico, sorbato potásico, benzoatos o natamicina en la superficie de los embutidos curado-madurados (Comisión Europea, 2011). Sin embargo, estos compuestos afectan a toda la población fúngica, incluyendo a los mohos beneficiosos, al igual que los tratamientos físicos. El ácido sórbico limita la capacidad de los mohos para adaptarse a factores ambientales adversos, inhibiendo el desarrollo de mohos toxigénicos, pero no es totalmente eficaz, salvo a valores de pH ácidos o con altas concentraciones de sal (Razavi-Rohani & Griffiths, 1999; Sergeeva et al., 2009). Además, algunas especies de *Penicillium* como *Penicillium roqueforti*, *Penicillium puberulum*, *Penicillium cyclopium*, *Penicillium brevicompactum* o de *Aspergillus* como *A. niger* crecen en presencia de altas concentraciones de ácido propiónico o de sorbato potásico (Bullerman, 1985; Mann & Beuchat, 2008) e incluso algunos *Penicillium* descarboxilan el sorbato para producir 1,3-pentadieno originando un olor a petróleo (Mann & Beuchat, 2008). Por ello, se ha propuesto la aplicación combinada de distintos compuestos antifúngicos (Mann y Beuchat, 2008). Por otra parte, la aplicación incorrecta del producto a dosis subinhibitorias puede favorecer la aparición de resistencias e incluso estimular la producción de OTA (Alcano et al., 2016; Arroyo et al., 2005; Fodil et al., 2018). Además, estudios recientes han mostrado la toxicidad de algunos de estos fungicidas y el desarrollo de alergias (Abd-Elhakim et al., 2020; Jacob et al., 2016; Piper & Piper, 2017). Por último, existe una demanda creciente por parte de los consumidores de alimentos libres de aditivos, especialmente de conservantes, y de utilización de sustancias más naturales.

Como alternativa, se están desarrollado diversas estrategias aplicables durante el secado-maduración de los embutidos curado-madurados sin la utilización de compuestos sintéticos. Entre ellas se encuentran las basadas en microorganismos o sustancias derivadas de plantas. Tradicionalmente se han definido como agentes de biocontrol (BCAs) a los microorganismos y compuestos derivados de éstos, aunque actualmente, este término agrupa también a agentes naturales como los aceites esenciales u otros extractos de origen vegetal (Jing et al., 2018; Kong et al., 2016; Oliveira et al., 2018; Silva et al., 2019).

La eficacia de cada BCAs para el control de mohos toxigénicos deriva de su modo de acción. El conocimiento de estos mecanismos proporciona una importante información que contribuye a optimizar el método de aplicación de los BCAs durante el procesado de los alimentos y permite realizar combinaciones más eficaces debido al posible efecto sinérgico entre ellos (Delgado, Olivera et al., 2021; Kong et al., 2016).

I.4.1. Agentes de origen microbiano

Entre las estrategias para limitar el desarrollo de mohos toxigénicos se encuentra la aproximación ecológica aprovechando el potencial inhibidor de la microbiota de los embutidos curado-madurados. Esta aproximación asegura una adecuada implantación sin alterar significativamente la población microbiana natural y, en consecuencia, las características sensoriales del producto. Por esta razón, es necesario evaluar la capacidad de implantación en el producto de los potenciales BCAs y su impacto en las características físico-químicas y organolépticas del embutido. Además, debe descartarse que posean potencial para producir toxinas, antibióticos u otras sustancias no deseables. La EFSA publica un listado actualizado semestralmente de microorganismos con presunción cualificada de seguridad (QPS), en la que se recogen aquellos que son considerados seguros para su adición a alimentos y piensos (EFSA BIOHAZ Panel, 2019).

Entre los microorganismos que han sido considerados como potenciales BCAs con actividad antifúngica en derivados cárnicos curado-madurados se encuentran las BAL, los CGC+, las levaduras y los mohos no toxigénicos.

Distintas especies de BAL, han mostrado su capacidad para reducir el crecimiento de mohos toxigénicos (Coloretti et al., 2007; Dalié et al., 2010) y la producción de micotoxinas por *Fusarium spp.* y *Aspergillus spp.* (Chang & Kim, 2007; Franco et al., 2011; Juodeikiene et al., 2018; Luchese et al., 1992). Aunque no existen muchos estudios sobre el efecto antifúngico de las BAL en mohos ochratoxigénicos, *L. plantarum* reduce la producción de OTA por *A. niger* en medio de cultivo y en pan (Dallagnol et al., 2018; Gerez et al., 2014), *L. plantarum*, *Lactobacillus graminis* y *P. pentosaceus* disminuyen la producción de OTA por *A. carbonarius* en medio de cultivo (Belkacem-Hanfi et al., 2014), y *Lactobacillus brevis* inhibe el crecimiento de *A. carbonarius* y la producción de OTA en la superficie de uvas (Li et al., 2021). Además, algunas de estas cepas poseen efectos probióticos (Ruiz-Moyano et al., 2009).

Los modos de acción de estos microorganismos frente a mohos ocratoxigénicos han sido poco estudiados pero su actividad frente a otros mohos de interés en alimentos (Tabla 2) se ha asociado a la competición por nutrientes y espacio (Bianchini, 2015; Chen, Ju et al., 2021; Chen, Hong et al., 2021; Fernandez et al., 2017), a la capacidad de detoxificación de algunas BAL como *L. sakei*, *L. plantarum* y *E. faecium* (Bianchini, 2015; Fuchs et al., 2008; Juodeikiene et al., 2018), a la alteración de la pared celular (Belkacem-Hanfi et al., 2014; Sangmanee & Hongpattarakere, 2014) y a la inhibición de los genes implicados en la ruta biosintética de las micotoxinas (Zhao et al., 2019).

Tabla 2. Principales modos de acción de agentes de biocontrol (BCAs) de origen microbiano sobre el crecimiento y la producción de micotoxinas en alimentos.

Inhibición del crecimiento					
Modo de acción	**Compuesto responsable**	**BCAs**	**Moho diana**	**Sustrato**	**Referencia**
Alteración metabolismo fuentes de carbono	-	*Penicillium chrysogenum*	*Penicillium nordicum*	Agar jamón	Delgado et al., 2019
	-	*Lactobacillus plantarum*	*Aspergillus flavus*	Agar Mann, Rogosa y Sharpe	Zhao et al., 2019
Alteración en la integridad de la pared celular, permeabilización de la membrana	Compuestos extracelulares	*L. plantarum*	*A. flavus, Aspergillus parasiticus*	Agar Patata Dextrosa	Sangmanee & Hongpattarakere, 2014
	-	*D. hansenii*	*P. nordicum*	Agar jamón	Delgado et al., 2019
	Sanxiapeptina	*Penicillium oxalicum*	*P. digitatum*	Naranja	Yang et al., 2022
	Proteína PgAFP	*P. chrysogenum*	*Aspergillus tubingensis*	Caldo Patata Dextrosa	Delgado et al., 2016
Alteración de la germinación	Compuestos extracelulares	*Lactobacillus plantarum*	*Aspergillus flavus, Aspergillus parasiticus*	Agar Patata Dextrosa	Sangmanee & Hongpattarakere, 2014
	Compuestos extracelulares	*L. kefiri, Pediococcus acidilactici*	*P. expansum*	Zumo de manzana	Chen, Ju et al., 2021
	Compuestos volátiles (3,3-dimetil-1,2-epoxibutano)	*Staphylocccus saprophyticus*	*A. flavus*	Maíz y cacahuetes	Gong et al., 2019
	Compuestos extracelulares	*D. hansenii*	*P. nordicum*	Caldo extracto de carne	Andrade et al., 2014
	Compuestos extracelulares	*D. hansenii*	*Penicillium italicum Wehmer*	Lima	Hernández-Montiel et al., 2010
No descrito	Compuestos extracelulares	*L. plantarum, L. graminis, Pediococcus pentosaceus*	*Aspergillus carbonarius*	Caldo Lauril triptosa	Belkacem-Hanfi et al., 2014
	Compuestos extracelulares	*Lactobacillus rhamnosus* + *Bifidobacterium animalis* subsp. *lactis*	*P. chrysogenum*	Queso Cottage	Fernández et al., 2017

Tabla 2. Continuación.

		Inhibición del crecimiento			
Modo de acción	**Compuesto responsable**	**BCAs**	**Moho diana**	**Sustrato**	**Referencia**
	Compuestos extracelulares	*Lactobacillus spp.*	*Penicillium digitatum*	Agar Patata Dextrosa	Chen, Hong et al., 2021
	-	*Staphylococcus xylosus*	*Penicillium nordicum, Aspergillus flavus, Aspergillus parasiticus, Penicillium griseofulvum*	Agar extracto de carne, agar jamón	Cebrián et al., 2020
	-	*Rhodosporidium paludigenum*	*Alternaria alternata*	Tomate cherry	Wang et al., 2008
	Proteínas antifúngicas	*Penicillium expansum, Penicillium digitatum*	*A. alternata, Aspergillus, Byssochlamus, Fusarium, Penicillium*	Caldo Patata Dextrosa	Martínez-Culebras et al., 2021
	-	*Penicillium nalgiovense*	*Penicillium verrucosum*	Embutidos	Bernáldez et al., 2013
		Reducción en la concentración de micotoxinas			
Modos de acción	**Compuesto**	**BCAs**	**Moho diana**	**Sustrato**	**Referencia**
Detoxificación	Compuestos de la pared celular	*Lactobacillus* spp.	*Fusarium graminearum*	-	Franco et al., 2011
	-	*Lactobacillus sakei, Pediococcus spp.*	*Fusarium* spp.	Trigo	Juodeikiene et al., 2018
	-	*Lactobacillus* spp.	-	Caldo Mann, Rogosa y Sharpe	Fuchs et al., 2008
	Compuestos de la pared celular	*Sacharomyces cerevisiae*	-	Vino	Meca et al., 2010
	Compuestos de la pared celular	*D. hansenii*	*Aspergillus westerdijkiae*	Caldo extracto de levadura	Gil-Serna et al., 2011
Represión de genes asociados con la biosíntesis de micotoxinas	-	*Lactobacillus plantarum*	*A. flavus*	Agar Mann, Rogosa y Sharpe	Zhao et al., 2019
	-	*D. hansenii*	*P. verrucosum; P. nordicum; A. westerdijkiae*	Agar carne; Jamón; Agar Czapek-Dox con extracto de levadura	Cebrián et al., 2019; Gil-Serna et al., 2011; Peromingo et al., 2018

Los **CGC+** como *S. xylosus* y *S. saprophyticus* empleados con BCAs han mostrado la capacidad de reducir la producción de micotoxinas por distintas especies fúngicas. *S. xylosus* disminuyó la producción de OTA por *P. nordicum*, de aflatoxinas por *A. flavus* y *A. parasiticus*, y de ácido ciclopiazónico por *Penicillium griseofulvum* en sustratos cárnicos (Cebrián et al., 2020) y *S. saprophyticus* la producción de aflatoxinas por *A. flavus* en maíz y cacahuetes (Gong et al., 2019).

La actividad de *S. saprophyticus* se ha atribuido a la producción de compuestos volátiles capaces de reducir el micelio y la germinación (Gong et al., 2019). Además, los CGC+ podrían competir por nutrientes y sustrato en los embutidos curado-madurados al añadirse en muchos casos como cultivos iniciadores alcanzando recuentos altos (Casquete et al., 2012).

Las levaduras autóctonas de los embutidos han sido evaluadas frente a mohos toxigénicos, siendo *D. hansenii* la más estudiada. Distintas cepas de esta levadura, aisladas de derivados cárnicos curado-madurados, han mostrado capacidad de reducir la OTA producida por *P. nordicum*, *P. verrucosum*, *A. ochraceus* y *A. westerdijkiae* (Iacumin et al., 2017; Iacumin et al., 2020a; Meftah et al., 2018; Simoncini et al., 2014). *D. hansenii* provoca una reducción en la producción de OTA en jamón y embutidos superior al 80 % (Peromingo et al., 2018) siendo mayor a valores de a_w de 0,94 (Andrade et al., 2014), sugiriéndose que su inoculación debería realizarse en las primeras etapas del procesado. La inoculación de *D. hansenii* o *Saccharomycopsis fibuligera* en la superficie del derivado cárnico *speck* evita el desarrollo de *P. nordicum* y *A. ochraceus* y, por lo tanto, la contaminación con OTA (Iacumin et al., 2017).

La capacidad de las levaduras para reducir la presencia de OTA y el desarrollo de mohos no deseados (Tabla 2) se ha relacionado con la competición por nutrientes y espacio (Andrade et al., 2014; Hernández-Montiel et al., 2010; Yifei Wang et al., 2008), la producción de compuestos antifúngicos solubles y volátiles y proteínas *killer* que pueden afectar al desarrollo estructural del moho y a la germinación (Andrade et al., 2014; Çorbacı & Uçar, 2018; Núñez et al., 2015), la represión de la expresión de genes relacionados con la biosíntesis de la micotoxina (Gil-Serna et al., 2011; Peromingo et al., 2018) y la adsorción de la OTA a la pared celular de las levaduras (Gil-Serna et al., 2011; Meca et al., 2010).

También se ha estudiado la utilización como BCAs de mohos no toxigénicos procedentes de cultivos iniciadores comerciales o cepas aisladas de derivados cárnicos curado-madurados. Así, un cultivo comercial de *P. nalgiovense* redujo el crecimiento de mohos toxigénicos en la superficie de embutidos curado-madurados y la producción de OTA por *P. verrucosum* (Bernáldez et al., 2013). *P. chrysogenum* CECT 20922, productor de la proteína antifúngica PgAFP

es capaz de reducir la OTA producida por *P. nordicum* en sistemas modelos cárnicos (Cebrián et al., 2019; Delgado et al., 2019).

Los mecanismos de acción implicados en la actividad antifúngica de los mohos se han asociado principalmente a la competición por nutrientes y espacio (Bernáldez et al., 2013; Delgado et al., 2019), a la producción de proteínas antifúngicas que provocan alteraciones en la pared celular y permeabilización de la membrana (Delgado et al., 2019; Fodil et al., 2018; Martínez-Culebras et al., 2021), y a la represión de la expresión de genes que codifican enzimas implicadas en la ruta de síntesis de las micotoxinas (Cebrián et al., 2019; Tabla 2). Las proteínas antifúngicas pueden purificarse y añadirse como BCA sin necesidad de inocular el moho productor. Por ejemplo, la sanxiapeptina producida por *Penicillium oxalicum* es capaz de inhibir el crecimiento de *P. digitatum* en naranjas afectando a la estructura y extensión del micelio (Yang et al., 2022) y la proteína antifúngica PgAFP producida por *P. chrysogenum* inhibe la producción de OTA en *A. carbonarius* (Delgado et al., 2018).

I.4.2. Agentes de origen vegetal

El empleo de plantas como BCAs ha ganado interés en los últimos años debido a que la mayoría son consideradas seguras (GRAS) por la Administración de Alimentos y Medicamentos de EE. UU. (Food and Drug Administration, 2019).

Dentro de los agentes de origen vegetal, los aceites esenciales (AE) han sido ampliamente evaluados frente a mohos productores de toxinas, principalmente aflatoxinas (Prakash et al., 2015a). Los AE pueden obtenerse de las hojas, flores, brotes, raíces o cortezas empleando distintas técnicas siendo las más tradicionales la destilación con vapor, la maceración o la hidrodestilación (Loi et al., 2020; Oliveira et al., 2018; Sadeh et al., 2019). Los estudios frente a mohos productores de OTA son escasos y hasta el momento se desconoce la actividad antiocratoxigénica de los AE aplicados en embutidos curado-madurados. Los AE de hinojo, cardamomo, romero, menta o melisa son efectivos para reducir la producción de OTA por *A. carbonarius* en medio de cultivo elaborados a partir de uva (El Khoury et al., 2016, 2017). Por otra parte, el AE de orégano puede reducir la producción de OTA por *P. verrucosum,* pero estimularla por *A. westerdijkiae* en medios de cultivo (Schlösser & Prange, 2019). Sin embargo, los AE de orégano y de menta parecen ser efectivos inhibiendo la producción de OTA en *A. ochraceus* en medio de cultivo tras 21 días de incubación (Basílico & Basílico, 1999). Otros AE como el de salvia, lavanda o laurel son capaces de inhibir completamente el crecimiento y la producción de OTA por *A. carbonarius* (Dammak et al., 2019). Los aceites esenciales de canela, cúrcuma, albahaca, jengibre y palmarosa fueron capaces de reducir la producción de esta

micotoxina por *A. ochraceus* y *P. verrucosum* en maíz empleando concentraciones iguales o superiores a 1500 µg/g (Kalagatur et al., 2020).

El efecto antagonista de estos AEs se vincula a los terpenos o fenoles presentes mayoritariamente en su composición como el carvacrol, timol o eugenol. Además, la composición de los AEs es muy variable ya que depende de la variedad y edad de la planta, de la localización, la época de cosechado, las condiciones ambientales y el método de extracción (Da Cruz Cabral et al., 2013; Hu et al., 2017). Aunque se ha demostrado la actividad antiocratoxigénica de los terpenos mayoritarios aislados (Jeršek et al., 2014; Schlösser & Prange, 2019), su purificación eleva el coste por lo que sería más fácil y económico aplicar directamente el AE en el producto. Estos compuestos actúan modificando algunas funciones celulares (Tabla 3), afectando a la morfología mediante pérdidas de la integridad de la pared celular y la inhibición de la síntesis de ergosterol, alterando la permeabilidad de las membranas (a Cruz Cabral et al., 2013; Da Silva Bomfim et al., 2020; Hu et al., 2017; Tian et al., 2018), o inhibiendo la síntesis de micotoxinas (El Khoury et al., 2016; Hu et al., 2017). Además, se ha observado que algunos AE como los de romero, albahaca y alcaravea también afectan a la conidiogénesis (Da Silva Bomfim et al., 2020; Kocić-Tanackov et al., 2020). Otros AE como el de canela, limón, eucalipto y palmarosa han mostrado su capacidad de detoxificación frente a micotoxinas de *Fusarium* (Perczak et al., 2016; Xing et al., 2014).

Tabla 3. Principales modos de acción de agentes de biocontrol (BCAs) de origen vegetal sobre el crecimiento y la producción de micotoxinas en alimentos.

		Inhibición del crecimiento		
Modo de acción	**BCAs**	**Moho diana**	**Sustrato**	**Referencia**
Alteración metabolismo energético	Aceite esencial (AE) de cúrcuma	*Aspergillus flavus*	Maíz	Hu et al., 2017
Alteración en la integridad de la pared celular, permeabilización de la membrana	AE de romero	*A. flavus, Fusarium verticillioides*	Agar extracto de levadura, medio de cultivo	Da Silva Bomfim et al., 2020; 2015
	AEs de albahaca y de alcaravea	*Penicillium* spp.*, Mucor racemosus*	Agar Patata Dextrosa	Kocić-Tanackov et al., 2020
Alteración de la germinación	AE de cúrcuma	*A. flavus*	Maíz	Hu et al., 2017
	AE de romero	*A. flavus*	Agar extracto de levadura	Da Silva Bomfim et al., 2020
	AE de alcaravea	*Penicillium* spp.	Agar Patata Dextrosa	Kocić-Tanackov et al., 2020
Inhibición de la síntesis de ergosterol	Cinamaldehído, eugenol	*Aspergillus ochraceus*	Agar extracto de malta	Hua et al., 2014
	AE de cúrcuma	*A. flavus*	Maíz	Hu et al., 2017
	AE de romero	*A. flavus, Fusarium verticillioides*	Agar extracto de levadura, medio de cultivo	Da Silva Bomfim et al., 2020; Da Silva Bomfim et al., 2015
	Cimeno, timol, carvacrol	*A. flavus*	Agar Patata Dextrosa	Tian et al., 2018
		Reducción en la concentración de micotoxinas		
Modos de acción	**BCAs /Compuesto**	**Moho diana**	**Sustrato**	**Referencia**
Detoxificación	AE de canela, limón, eucalipto y palmarosa	-	-	Perczak et al., 2016
	AE de canela	-	-	Xing et al., 2014
Represión de genes asociados con la biosíntesis de micotoxinas	AE de hinojo, romero, cardamomo, camomila, anís, apio	*Aspergillus carbonarius*	Agar uva	El Khoury et al., 2016
	AE de cúrcuma	*A. flavus*	Caldo extracto de levadura	Hu et al., 2017
	Cimeno, carvacrol, γ-terpineno	*A. flavus*	Agar Patata Dextrosa	Tian et al., 2018; Moon et al., 2018
	Eugenol	*Aspergillus parasiticus*	Agar sabouraud dextrosa	Jahanshiri et al., 2015
	Cinamaldehído	*Alternaria alternata*	Caldo patata dextrosa	Liu et al., 2020
Reducción de especies reactivas de oxígeno	Cinamaldehído	*A. alternata*	Caldo patata dextrosa	Liu et al., 2020

Los extractos de plantas obtenidos mediante su extracción con agua y metanol o acetona han sido menos estudiados que los AEs, pero algunos son capaces de reducir el crecimiento de mohos toxigénicos o la producción de micotoxinas. Extractos de menta, comino o fenogreco añadidos a un medio de cultivo con uvas, redujeron significativamente la presencia de OTA en *A. carbonarius* (El Khoury et al., 2017). Estos extractos acuosos están formados mayoritariamente por fenoles como el eugenol, ya que se disuelven bien en solventes polares. El mecanismo de acción es similar al de los AEs (Tabla 3), ya que comparten componentes en distinta proporción. Compuestos como el cimeno, el carvacrol, el eugenol, o el cinamaldehído son capaces de inhibir la expresión de genes relacionados con la biosíntesis de micotoxinas. El cimeno y el carvacrol inhiben la expresión de los genes *aflD*, *aflK*, *aflQ* y *aflR* en *A. flavus* (Tian et al., 2018). El eugenol afecta a los genes *pksA* actualmente *aflC*, *omtA*, *nor-1*, *ver-1* y *aflR*, relacionados con la ruta biosintética de las aflatoxinas en *A. parasiticus* (Jahanshiri et al., 2015). El cinamaldehído reduce la expresión de genes relacionados con la producción de toxinas de *A. alternata* y reduce la producción de especies reactivas de oxígeno que se han asociado a su vez con la producción de micotoxinas (Liu et al., 2020).

Por último, es necesario considerar que el impacto sensorial de los AEs y otros extractos vegetales en los embutidos curado-madurados puede ser un factor limitante para su utilización. Normalmente, éstos son más activos en altas concentraciones pero pueden ser rechazados por el consumidor por alterar las características sensoriales (Oliveira et al., 2018). Por lo tanto, sería interesante aplicar agentes de origen vegetal que se usen normalmente en la elaboración de estos productos para evitar la aparición de flavores anómalos. Algunos de los vegetales empleados comúnmente como ingredientes son especias como el orégano, el romero o el tomillo (Ordóñez et al., 1999). Además, dado que extractos de corteza de bellotas de distintas variedades de roble han mostrado inhibición del crecimiento fúngico en mohos toxigénicos como *A. flavus*, *A. ochraceus* o *A. niger* (Elansary et al., 2019) en una región como Extremadura, también sería interesante probar el efecto antifúngico de las cáscaras de bellota que pueden considerarse un subproducto de la producción del cerdo ibérico, y que no han sido evaluadas frente a mohos productores de OTA.

I.5. Técnicas moleculares para la evaluación de los modos de acción de los agentes de biocontrol

Las técnicas ómicas son herramientas avanzadas y de alto rendimiento que facilitan la obtención de datos sobre un determinado tema empleando equipos modernos y métodos

computacionales avanzados (García-Cela et al., 2018). Estas técnicas incluyen a la genómica, transcriptómica, proteómica y metabolómica. Su estudio ha permitido avanzar en el conocimiento de los procesos fisiológicos de mohos toxigénicos a nivel molecular y su relación con la producción de micotoxinas (García-Cela et al., 2018). La figura 3 muestra la relación del dogma central de la biología molecular con la producción de micotoxinas.

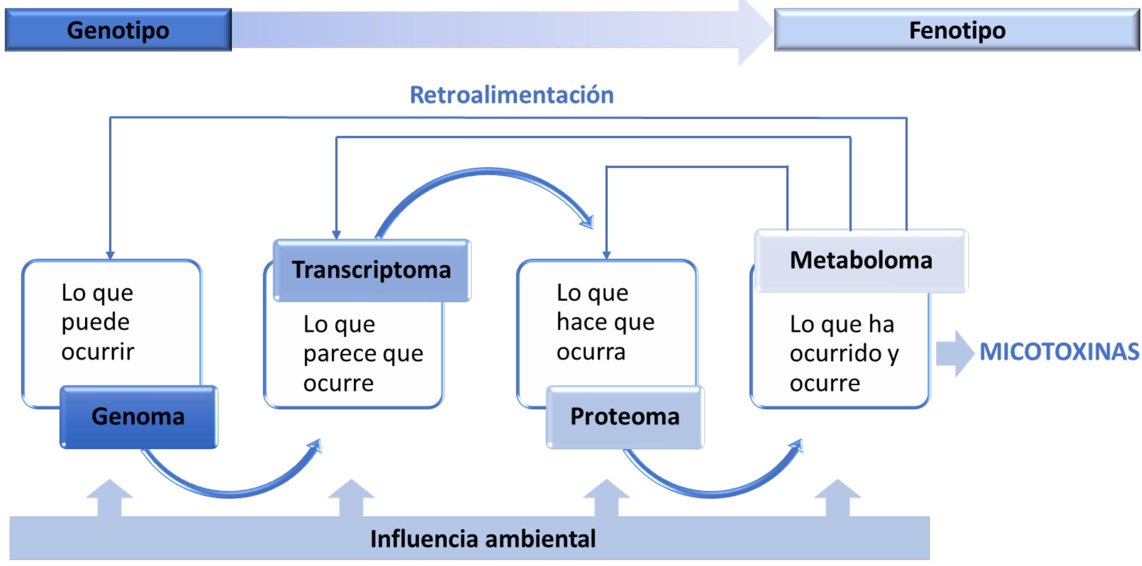

Figura 3. Relación entre el dogma central de la biología molecular y la producción de micotoxinas. Adaptado de García-Cela et al. (2018).

I.5.1. Expresión génica

El transcriptoma es el conjunto de transcritos o ARN presente en una célula, tejido u organismo bajo condiciones específicas. Ya que una secuencia de ARN es un reflejo de la secuencia de ADN, es posible determinar la actividad de los genes en un momento determinado mediante el estudio de la expresión génica. La influencia de parámetros ambientales y de la presencia de los BCAs sobre la expresión de genes relacionados con la síntesis de micotoxinas ha sido ampliamente estudiada (Tablas 2 y 3). Valores de temperatura y a_w cercanas a las óptimas de crecimiento estimulan la expresión de genes asociados a la producción de micotoxinas (Lozano-Ojalvo et al., 2013; Schmidt-Heydt et al., 2008). BCAs como *D. hansenii*, el AE de cúrcuma o el γ-terpineno derivado del AE de jengibre son capaces de inhibir la expresión de genes pertenecientes a la ruta biosintética de AFs (*aflR*, *aflS*) en algunas especies de A*spergillus* (Hu et al., 2017; Moon et al., 2018; Peromingo et al., 2019b).

Debido a que la producción de OTA en *P. nordicum* está regulada y relacionada con distintos genes (Figura 4), puede conocerse el efecto de los BCAs en la biosíntesis de OTA a nivel del transcriptoma.

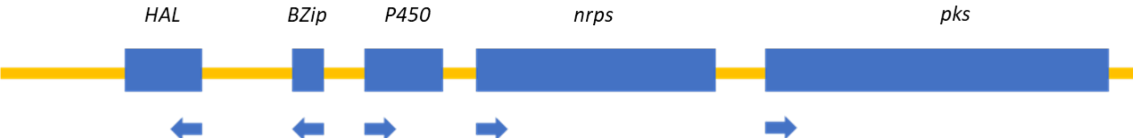

Figura 4. Posible clúster de la ruta biosintética de ocratoxina A en *Penicillium nordicum*. Adaptado de Gil-Serna et al. (2018).

Los genes de la ruta biosintética de OTA más estudiados en *P. nordicum* son el *otapks* y el *otanrps*. El gen *otapks* codifica una enzima policétido sintasa (PKS), perteneciente al grupo de PKSs fúngicas, esenciales en la producción de metabolitos secundarios. Estas enzimas incluyen múltiples subdominios como el β-cetoacil reductasa (KR), dehidratasa (DH) y enoil reductasa (ER) que actúan como una cadena encargada de las reacciones necesarias para producir la enzima (Gallo et al., 2013). El gen *otanrps* codifica la enzima péptido sintetasa, indispensable para la unión entre la ocratoxina β, la base de la dihidrohisocumarina, y la fenilalanina (Gallo et al., 2017), (Figura 5).

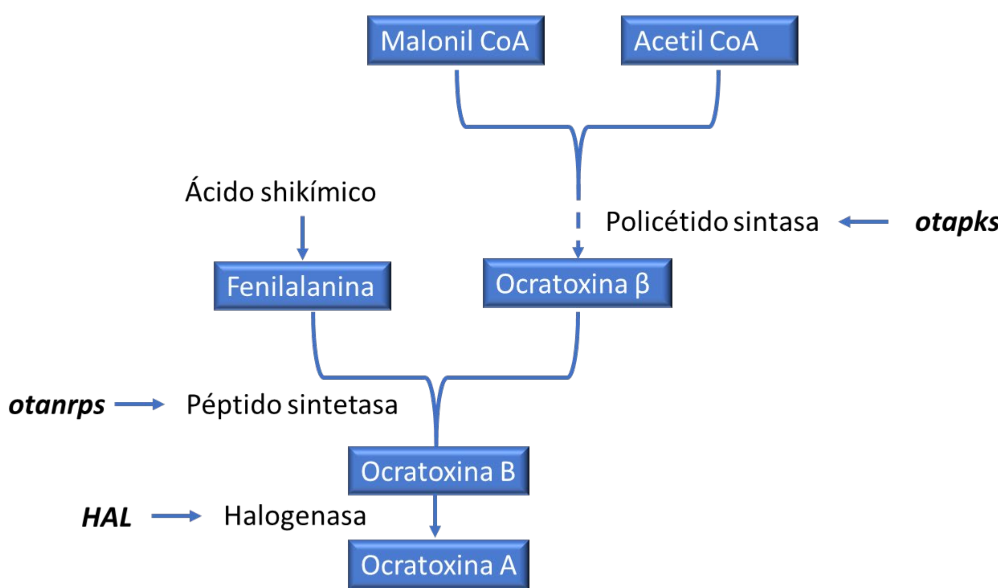

Figura 5. Representación de la ruta biosintética de ocratoxina A. Adaptado de Gallo et al. (2017).

Estos genes pertenecientes a la ruta biosintética de OTA han sido correlacionados con la producción de la micotoxina en distintas especies de *Penicillium* y *Aspergillus* (Bernáldez et al., 2018; Castellá et al., 2015; Delgado et al., 2018; Gallo et al., 2014; Peromingo et al., 2018; Rodríguez et al., 2011). Se ha documentado que algunos BCAs provocan la reducción en la

expresión de estos genes. *D. hansenii* inhibe la expresión relativa del gen *otanrps* y la producción de OTA en *P. verrucosum* en medios de cultivo (Peromingo et al., 2018), y la presencia de *D. hansenii* y *P. chrysogenum* reducen tanto la expresión del gen *otapks* como la concentración de OTA en jamón inoculado con *P. nordicum* (Cebrián et al., 2019).

Además, distintos tipos de estrés pueden estimular la producción de micotoxinas (Gil-Serna et al., 2020b; Jurado et al., 2008). En este sentido, el estrés producido por antifúngicos se ha relacionado con el aumento de OTA y citrinina en *P. nordicum* y *P. verrucosum* respectivamente (Schmidt-Heydt et al., 2013). Por el contrario, la proteína antifúngica PgAFP disminuye la síntesis de OTA por la represión del gen *Rho1*, implicada en la respuesta al estrés producido por alteraciones en la pared celular en *A. carbonarius* (Fodil et al., 2018). La activación de la ruta del glicerol de alta osmolaridad (HOG) activada en situaciones de estrés osmótico se ha relacionado con la producción de OTA por *P. verrucosum* (Stoll et al., 2013). Por lo tanto, genes como *Hog1* y *Rho1* (Delgado et al., 2019; Rodríguez et al., 2016) pueden ser empleados para estudiar el estrés causado por los BCAs en *P. nordicum*.

La reacción en cadena de la polimerasa en tiempo real de transcripción inversa (RT-qPCR) permite conocer la variación en la expresión de genes concretos sin necesidad de conocer el genoma completo. Esta técnica ha sido ampliamente utilizada para evaluar la expresión génica de los genes anteriormente citados en mohos ocratoxigénicos (Cebrián et al., 2019; Delgado et al., 2018; Ferrara et al., 2016; Gil-Serna et al., 2018; Peromingo et al., 2018).

La cuantificación de la expresión génica puede realizarse mediante el método de cuantificación absoluta o el de cuantificación relativa. La absoluta indica el número total de copias de un gen relacionando la señal de PCR con una curva estándar previamente realizada. En cambio, la cuantificación relativa relaciona la señal de PCR de un gen diana de interés en una muestra bajo un tratamiento específico (presencia de BCAs, cambios de temperatura, pH, etc.) con otra similar no sometida a dicho tratamiento o calibrador (Livak & Schmittgen, 2001). Este método necesita una normalización previa empleando genes endógenos que permanezcan constantes bajo los distintos tratamientos. Dentro de la cuantificación relativa existen dos metodologías para realizar el cálculo: el método $2^{-\Delta\Delta Ct}$ y el método $2^{-\Delta C't}$. En el primero, las eficiencias de los genes diana y endógeno deben ser aproximadamente iguales mientras que el segundo se aplica cuando no lo son (Livak & Schmittgen, 2001).

I.5.2. Proteómica

La proteómica tiene como objetivo el estudio del proteoma que es el conjunto de proteínas presentes en un organismo en unas condiciones específicas. Esta técnica ha mostrado que la

relación entre la secuencia de ADN y la estructura de las proteínas es menos predecible de lo que se pensaba (Merrill & Mazza, 2006). La transcriptómica permite conocer la expresión génica, pero un mismo gen puede dar lugar a diferentes formas proteicas ya que las proteínas pueden sufrir modificaciones post-transduccionales durante su intervención en procesos biológicos (Merrill & Mazza, 2006). Por ello, la proteómica puede ayudar a entender los cambios producidos por los BCAs a través del aumento o la disminución relativa de proteínas y sus efectos en el estado fisiológico de mohos toxigénicos.

Esta técnica puede ser empleada para comprender rutas metabólicas complejas. En algunos casos, las proteínas de estas rutas transmiten señales desde la superficie de las células hasta el núcleo estimulando o reprimiendo la expresión génica (Merrill & Mazza, 2006). Ante estímulos estresantes en la pared celular se activan proteínas como Rho1 y Hog que inician la señal necesaria para activar los factores de transcripción encargados de regular la expresión de genes relacionados con la biosíntesis de la pared celular o con el aumento de glicerol intracelular respectivamente (Rodríguez et al., 2016; Yun et al., 2014).

La proteómica comparativa es el análisis comparado del proteoma de un organismo en diferentes condiciones, como puede ser el originado tras un tratamiento frente a un control no tratado. Esta técnica se ha empleado para elucidar la respuesta de mohos toxigénicos frente a BCAs, antifúngicos comerciales o agentes estresantes (Delgado et al., 2017; Li et al., 2020; Stoll et al., 2014). Un estudio ha revelado la disminución en condiciones ácidas de diversas proteínas de *Penicillium citrinum* relacionadas con la regulación de la síntesis de OTA (Zhao et al., 2017). El estudio proteómico del citral como BCA frente a *A. ochraceus* ha mostrado un mecanismo de acción complejo, relacionado con la captación de nutrientes, la biosíntesis de ergosterol, la biogénesis ribosomal, el estrés oxidativo y el metabolismo energético y de los aminoácidos (Wang et al., 2021). El compuesto 2-fenil-1-etanol producido por *Candida intermedia* induce el aumento de proteínas relacionadas con la respuesta al estrés en *A. carbonarius* (Tilocca et al., 2019). *D. hansenii* y *P. chrysogenum* disminuyen la producción de OTA por *P. nordicum* en un sustrato cárnico debido a la afectación de proteínas implicadas en la ruta de integridad de la pared celular (CWI) y además *P. chrysogenum* compite por nutrientes afectando a la ruta de represión catabólica de fuentes de carbono (Delgado et al., 2019).

Para llevar a cabo el estudio de las proteínas se puede emplear la electroforesis en gel de poliacrilamida (PAGE) o la cromatografía líquida de alta resolución acoplada a un detector de masas (HPLC). Las proteínas separadas mediante PAGE pueden ser visualizadas al teñirse con colorantes como el azul coomassie. La espectrometría de masas (MS) permite conocer el peso

molecular y la estructura química de péptidos, proteínas, carbohidratos, oligonucleótidos, productos naturales y metabolitos (Büyükköroğlu et al., 2018), por lo que también se emplea comúnmente para el análisis de micotoxinas (Peromingo et al., 2019a). Sus ventajas incluyen el empleo de una pequeña cantidad de muestra y una alta sensibilidad sin necesidad de realizar un marcaje con isótopos en las proteínas o péptidos deseados. Además, permite comparar los patrones obtenidos con el genoma e identificar rápidamente un grupo de proteínas sin necesidad de caracterizarlas exhaustivamente una a una (Merrill & Mazza, 2006).

Debido a que esta técnica se basa en la separación de las moléculas en función del ratio masa-carga (m/z) se emplean diversas fuentes de ionización para fragmentarlas. Las más comunes son la desorción/ionización láser asistida por matriz (MALDI) y la ionización mediante electropulverización (ESI). Para la separación de las moléculas ionizadas existen distintos analizadores de masas como el de tiempo de vuelo (TOF), la trampa iónica (IT-MS), los cuadrupolos o el Orbitrap. El analizador de masas Orbitrap consta de dos electrodos exteriores y uno central que le permite actuar a la vez como analizador y detector. Los iones que entran en el Orbitrap oscilan a diferente frecuencia provocando su separación con unos valores de resolución muy altos, superiores a 140 000 FWHM, necesarios para separar picos con una diferencia de masa pequeña (0,01 u). Tras la detección de los iones, el espectro de masas se compara a través de bases de datos online para completar su identificación. Tanto la identificación como la cuantificación puede llevarse a cabo mediante herramientas bioinformáticas como el software MaxQuant que analiza grandes conjuntos de datos (Cox & Mann, 2008). Para organizar los resultados y realizar el análisis estadístico pueden utilizarse otros softwares como Perseus (Delgado et al., 2019).

I.5.3. Análisis de metabolitos

Los mohos y levaduras tienen una gran capacidad para adaptarse a diferentes nichos ecológicos y competir con otros microorganismos. Para aumentar su supervivencia sintetizan una gran variedad de compuestos. Muchos de ellos son metabolitos secundarios empleados como señales químicas para comunicarse, defender su hábitat o inhibir el crecimiento de otros microorganismos (Brakhage, 2012). Los metabolitos secundarios producidos por mohos, y especialmente las micotoxinas ha sido previamente estudiados en derivados cárnicos curado-madurados (Peromingo, et al., 2019; Perrone et al., 2015) utilizando diferentes metodologías.

Hay numerosos métodos analíticos para detectar micotoxinas en alimentos incluyendo las técnicas inmunoenzimáticas (ELISA) y cromatográficas entre las cuales se encuentran la cromatografía en capa fina (TLC), la cromatografía de gases (GS-MS) y la HPLC (Pizzolato

Montanha et al., 2018). Aunque las técnicas inmunológicas son rápidas y permiten analizar simultáneamente múltiples muestras (Markov et al., 2013; Pleadin et al., 2017), los métodos basados en HPLC presentan mejores valores de recuperación y precisión (Pleadin et al., 2013).

En estos últimos, aunque se pueden emplear detectores como el diodo array (DAD) o el de fluorescencia (FLD) (Meftah et al., 2018; Soleas et al., 2001), los analizadores cuadrupolos son actualmente de los más usados en la detección de micotoxinas (Eshelli et al., 2018), por su capacidad de resolución y su alta sensibilidad. Su funcionamiento se basa en crear tensiones entre la corriente continua y alterna manteniendo un equilibrio constante. Los iones con una determinada m/z presentan una trayectoria estable y consiguen llegar al detector. Estos analizadores pueden trabajar en modo *single ion monitoring* (SIM) dejando pasar solo un ion o *scan* donde se selecciona un determinado intervalo de m/z. El empleo de tres cuadrupolos ha permitido la monitorización mediante *selective reaction monitoring* (SRM) en la cual se emplea la fragmentación del ion precursor. Al conjunto de los dos valores de m/z específicos del ion precursor y el ion fragmentado se conoce como "transición", siendo altamente selectiva para un analito determinado (Lange et al., 2008). Esta técnica ha sido utilizada para detectar OTA en derivados cárnicos curado-madurados (Delgado et al., 2019; Peromingo et al., 2018). Además, se pueden realizar múltiples transiciones (MRM) para aumentar la selectividad del método (Merla et al., 2018; Roncada et al., 2020).

I.6. Efecto de los BCAs en los embutidos curado-madurados

Una vez seleccionados los BCAs por su capacidad antiocratoxigénica se debe comprobar si se implantan correctamente en el producto y si afectan negativamente a las características físico-químicas y organolépticas del mismo. En los embutidos curado-madurados, la población microbiana naturalmente presente durante la fermentación y el secado-maduración contribuye tanto a la estabilidad del producto (apartado I.2.2.) como a las propiedades sensoriales que los caracterizan (Vignolo et al., 2010). Por ello, es necesario estudiar si los BCAs interfieren en el crecimiento de la población autóctona además de su efecto sobre la calidad sensorial ya que las sustancias responsables del flavor y el aroma están producidas por la oxidación lipídica, la proteólisis o la adición de especias (Flores et al., 2004). Para comprobar estos efectos se pueden realizar ensayos de cultivo dual, análisis físico-químicos, texturales y evaluaciones sensoriales.

Los ensayos de cultivo dual han sido ampliamente probados para definir las interacciones entre microorganismos y estudiar la capacidad antimicrobiana de los BCAs a distancia (Belkacem-Hanfi et al., 2014; Kogkaki et al., 2015; Magan & Lacey, 1984). El método consiste en la inoculación de

dos microorganismos separados entre sí a una distancia conocida. Dependiendo del tipo de interacción se puede observar la inhibición de uno sobre otro en contacto o a distancia, una inhibición mutua o que convivan sin afectarse. A cada tipo de interacción se le puede asignar un valor para calcular el índice de dominancia (I_D). Estas interacciones se ven afectadas por la a_w, la temperatura de incubación y el sustrato por lo que deben estudiarse bajo condiciones típicas del procesado del producto. La inoculación de *D. hansenii* o de CGC+ puede provocar cambios en la población autóctona de las BAL en embutidos (Cano-García et al., 2014; Domínguez et al., 2016) lo cual podría conllevar alteraciones en la fermentación y la actividad proteolítica alterando el flavor característico del producto (Benito et al., 2007).

La evolución del pH, la a_w y la humedad del producto es crucial para obtener productos estables microbiológicamente. Además, entre los valores de pH de 5,1 y 5,5 se encuentra el punto isoeléctrico de las proteínas cárnicas, en el cual se desnaturalizan y se agregan generando su textura característica (Hernández et al., 2003). Por lo tanto, la medida de estos tres parámetros es realizada frecuentemente para controlar el procesado de los embutidos curado-madurados y puede ser empleada para comprobar la influencia de los BCAs sobre ellos (Cano-García et al., 2014; Corral et al., 2017; Fonseca et al., 2013b). La inoculación de *D. hansenii* en embutidos se ha empleado tradicionalmente por su capacidad de mejorar la fermentación retardando la rancidez y aumentando el pH obteniendo embutidos más aromáticos (Corral et al., 2017). Otro parámetro decisivo en la decisión de compra del consumidor es el color, que puede ser medido mediante el espacio CIE L*a*b* (Corral et al., 2017; Pérez-Alvarez et al., 1999). El color está influido por las reacciones microbianas, químicas y enzimáticas afectadas por el pH, los agentes del curado, la temperatura y la humedad del producto. La inoculación con BAL y *S. xylosus* en embutidos interfieren en el pH, la humedad y las enzimas microbianas y por lo tanto en la formación del color (Hu et al., 2019). Por ejemplo, *S. xylosus* aumenta el color a* (rojo) mediante el aumento de la formación de nitrosilmioglobina (Hu et al., 2019).

Las características texturales del producto es un aspecto importante para la aceptación del consumidor (Herrero et al., 2007), y en los productos cárnicos dependen del contenido de grasa y sal y el valor de pH que puede variar dependiendo de la población microbiana (Casquete et al., 2011; Corral et al., 2017; Gimeno et al., 2000). El método instrumental más utilizado para evaluarlas es el análisis del perfil de textura (TPA) en el cual se imita el proceso de masticación (Bourne, 1978). Este método aporta información sobre la dureza, la adhesividad, la cohesividad, la elasticidad o la masticabilidad. El TPA se ha empleado como índice para determinar la calidad del producto final o seleccionar los mejores ingredientes (Herrero et al., 2007). La disminución de la dureza en los embutidos curado-madurados puede ser de gran interés en productos de

larga maduración (Casquete et al., 2011). Este parámetro está influenciado por la desnaturalización y adhesión proteica y la pérdida de agua que aumentan con el tiempo de maduración (Chaves-López et al., 2012). La adición de la proteasa Pronasa E individualmente o junto a un extracto de *Penicillium aurantiogriseum* durante la elaboración de salchichón mejora la textura del producto final disminuyendo la dureza, la elasticidad, la gomosididad y la resistencia a la masticación (Bruna et al., 2000). Por otro lado, la inoculación de cultivos iniciadores reducen la dureza y masticabilidad de distintos embutidos (Casquete et al., 2011; Corral et al., 2017). Además, la incorporación de antifúngicos como el sorbato potásico o el AE de orégano disminuyen la dureza de estos productos (Chaves-López et al., 2012).

Los compuestos volátiles juegan un papel fundamental en el aroma y flavor de los embutidos curado-madurados, y pueden ser modificados por la utilización de cultivos iniciadores o BCAs como *D. hansenii* (Flores et al., 2004; Fonseca et al., 2013b; Montanari et al., 2018). Los compuestos volátiles suelen ser extraídos mediante la técnica de microextracción en fase sólida (SPME). Su fundamento se basa en la afinidad de los analitos por la fase sólida compuesta por una fibra de diferentes materiales. Para la extracción de volátiles a partir de productos cárnicos se usan comúnmente fibras dobles o tripes de carboxeno/polidimetilsiloxano (CAR/PDMS) o de divinilbenceno/carboxeno/polidimitilsiloxano (DVB/CAR/PDMS) (Andrade et al., 2010; Iacumin et al., 2020b; Latorre-Moratalla et al., 2011). La desorción de los compuestos suele producirse en un cromatógrafo de gases mediante la acción del gas portador (generalmente helio) a una temperatura concreta. Las BAL producen principalmente ácidos y compuestos como el etanol o el acetaldehído (Flores et al., 2015). Dentro de las levaduras, se ha observado que la producción de volátiles es diversa y depende de la cepa estudiada. Por ejemplo, distintas cepas de *D. hansenii* aumentan o disminuyen la concentración de aldehídos, cetonas o ácidos (Cano-García et al., 2014; Flores et al., 2004). Los mohos producen frecuentemente compuestos alifáticos de ocho carbonos como el 1-octen-3-ol. Este junto a otros compuestos como el 3-octanona contribuyen al aroma a rancio (Sunesen & Stahnke, 2003).

El análisis sensorial evalúa los atributos organolépticos de un alimento mediante los sentidos, es indispensable para conocer la aceptación de un producto por el consumidor y se utiliza ampliamente en los estudios de mercado. Existen distintas pruebas dependiendo del objetivo que se quiera evaluar (Chambers & McGuire, 2003). Para conocer si hay diferencias entre muestras se emplean test discriminatorios o diferenciales que muestran el porcentaje de consumidores que distingue los productos. Si el objetivo es conocer la preferencia se emplean test de aceptación o hedónicos en los que se realizan preguntas sencillas sobre el flavor general o específico, color, textura, etc. Para realizar estos tests los panelistas pueden colocar cada

muestra dentro de una escala de aceptación, ordenar varias muestras que se les presentan según la preferencia o utilizar escalas de intensidad de olor o de sabor (Hein et al., 2008; Solomando et al., 2020). En un análisis sensorial realizado en salami inoculado con *S. xylosus*, los panelistas asociaron la intensidad, el olor a fermentado y el olor a salami con la presencia de esta bacteria (Stahnke, 1994). Además, los tests de preferencia contribuyen a añadir la concentración idónea de un microorganismo sin perjudicar las características sensoriales del producto. Por ejemplo, los embutidos inoculados con *D. hansenii* a un concentración de 6,69 log ufc/g fueron preferidos con respecto al control sin inocular mientras que una concentración de 7,17 log ufc/g produjo un efecto negativo en el aroma final (Flores et al., 2004).

Teniendo en cuenta lo anterior, es necesario comprobar el efecto de la inoculación de BCAs durante el procesado industrial en los parámetros de calidad de los embutidos curado-madurados. Además, la mayoría de los estudios sobre la capacidad antifúngica de los BCAs han sido realizados en laboratorio a partir de sistemas modelo cárnicos y bajo condiciones controladas por lo que es imprescindible estudiar su capacidad frente a mohos toxigénicos bajo las condiciones ambientales habituales de las industrias elaboradoras. El grupo de investigación de Higiene y Seguridad Alimentaria (HISEALI) de la Universidad de Extremadura (UEX) dispone de algunos BCAs previamente seleccionados como *D. hansenii* FHSCC 253H y *P. chrysogenum* CECT 20922, capaces de reducir la OTA bajo condiciones controladas (Andrade et al., 2014; Delgado et al., 2019). También es necesario estudiar el potencial como BCA de algunas plantas añadidas frecuentemente como ingredientes a los embutidos como el orégano, el romero o el tomillo.

II. OBJETIVOS

II.1. Objetivos

El presente trabajo está integrado dentro de la línea de investigación sobre el empleo de BCAs frente mohos productores de OTA en derivados cárnicos curado-madurados desarrollada por el Grupo de Investigación HISEALI dentro del Instituto Universitario de Investigación de la Carne y Productos Cárnicos de la UEX.

El objetivo general ha sido diseñar un método de biocontrol de mohos ocratoxigénicos en embutidos curado-madurados empleando los microorganismos *D. hansenii*, *E. faecium* y *P. chrysogenum* (productor de la proteína antifúngica PgAFP) y vegetales como el romero, el orégano, el tomillo y las bellotas, así como estudiar sus modos de acción y sus efectos sobre el producto final. Para llevarlo a cabo se han realizado los siguientes objetivos parciales:

1. Evaluar el potencial de *E. faecium* como BCA.
2. Evaluar la influencia del romero, el orégano, el tomillo y el extracto de cáscara de bellota sobre el crecimiento de *P. nordicum* y producción de OTA.
3. Estudiar el efecto de los BCAs seleccionados en mohos productores de OTA a nivel molecular.
4. Evaluar las interferencias entre los BCAs seleccionados y la población microbiana habitual de los embutidos curado-madurados.
5. Conocer el impacto de la utilización de los BCAs sobre las características físico-químicas y sensoriales de los embutidos curado-madurados.
6. Estudiar el efecto de la adición de los BCAs durante la elaboración de los embutidos curado-madurados sobre la producción de OTA y el proteoma de *P. nordicum*.

II.2. Diseño experimental

Para la obtención de los objetivos de la Tesis Doctoral se siguió el diseño experimental indicado en la Figura 6.

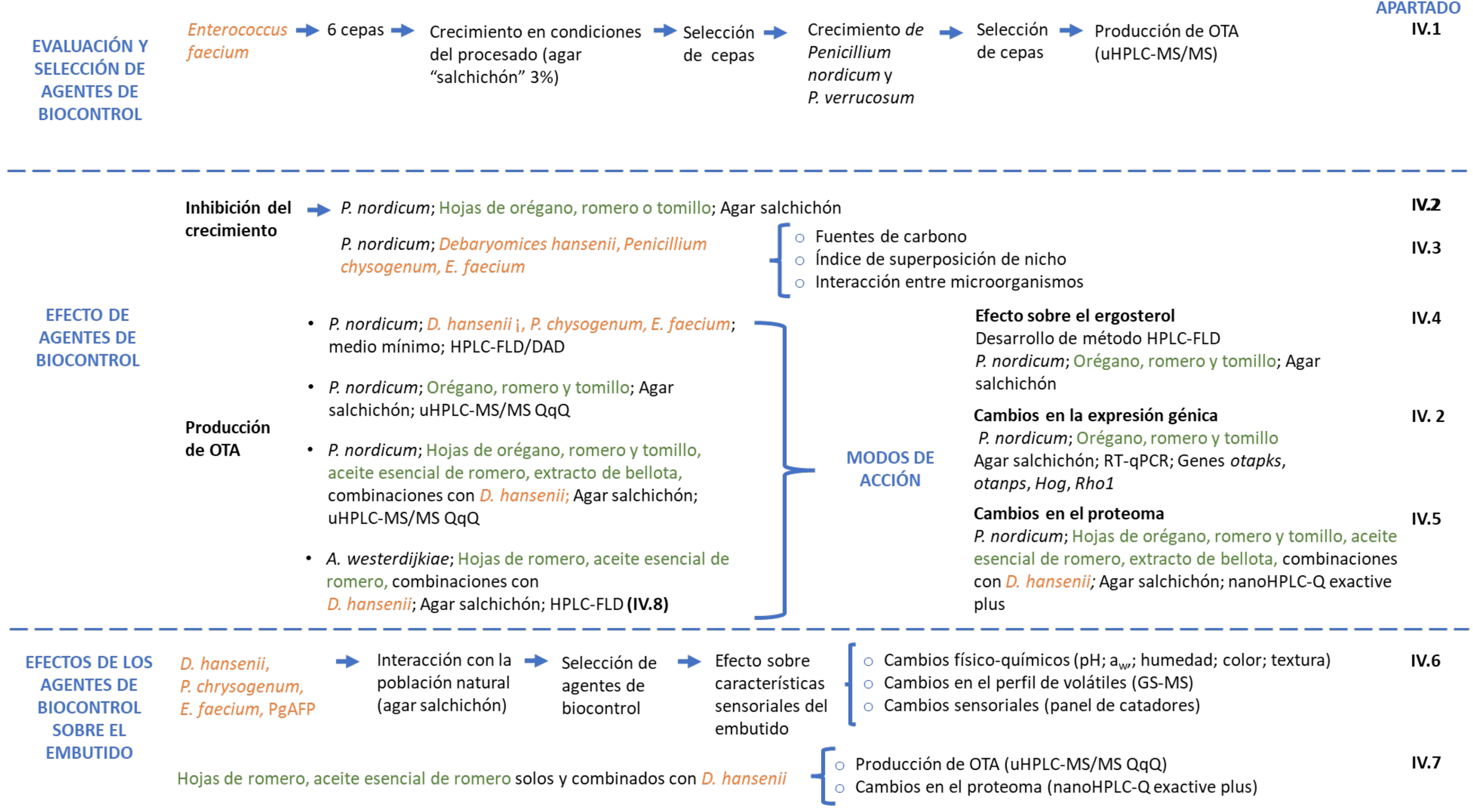

Figura 6. Diseño experimental de la Tesis Doctoral dividida en 8 apartados.

III. MATERIALES Y MÉTODOS

III.1. Materiales

III.1.1. Reactivos químicos

En este trabajo se emplearon productos químicos de calidad reactivo suministrados por las firmas comerciales Scharlab S.L. (Barcelona, España), Panreac Química S.L.U. (Barcelona, España), Thermo Fisher Scientific (Waltham, Massachusetts, EE. UU.) y Biolife (Milán, Italia).

La resazurina, la OTA y el ergosterol fueron suministrados por Sigma-Aldrich (San Luis, EE. UU.).

Los azúcares y aminoácidos empleados fueron de las marcas Sigma-Aldrich, Acros Organics de Thermo Fisher Scientific y LabKen (Barcelona, España).

Para la extracción de ARN se empleó el kit Spectrum™ Plant Total RNA de Sigma-Aldrich.Los reactivos para la RT-qPCR SYBR green, ROX y el kit PrimeScript™ RT Reagent fueron proporcionados por Takara Bio Inc (Kusatsu, Japón). El kit RNeasy® Plant Mini empleado para la extracción de ARN fue de Quiagen (Hilden, Alemania). La enzima DNase I, RNase free fue suministrada por Fermentas (Vilnius, Lituania) y los cebadores utilizados por Biomol S.L. (Sevilla, España).

Para el análisis de proteómica los reactivos empleados fueron suministrados por la firma Sigma-Aldrich.

III.1.2. Medios de cultivo

Los medios de cultivo comerciales empleados en esta Tesis Doctoral pertenecieron a las casas comerciales Scharlab S.L. y Panreac Química S.L.U.

A continuación, se describen los diferentes medios de cultivo empleados a lo largo del desarrollo experimental de la presente Tesis Doctoral:

a) Medios de cultivo generales:

Para el crecimiento de bacterias se emplearon el caldo Man, Rogosa y Sharpe (MRS) y el caldo Brain Heart Infusion (BHI). Para el cultivo de las levaduras se empleó caldo Extracto de Levadura y Sacarosa (YES) y para los mohos Agar Patata Dextrosa y Agar Extracto de Levadura y Sacarosa (agar YES). La composición de los medios de cultivo se detalla en la tabla 4.

Tabla 4. Composición de los medios de cultivo generales empleados en esta Tesis Doctoral.

	Medios de cultivo*					
Ingredientes	**MRS**	**BHI**	**YES**	**PDB**	**PDA**	**Agar YES**
Agua destilada	1000 mL	1000 mL	1000 mL	1000 mL	1000 mL	1000 mL
Agar bacteriológico	-	-	-	-	20 g	20 g
MRS	52,25 g	-	-	-	-	-
BHI		37 g	-	-	-	-
Extracto de levadura			20 g	-	-	20 g
Sacarosa			125 g	-	-	125 g
Patata dextrosa				24 g	24 g	-

*MRS: Caldo Man, Rogosa y Sharpe; BHI: Caldo Infusión Cerebro Corazón; YES: Caldo Extracto de Levadura y Sacarosa; PDB: Caldo Patata Dextrosa; PDA: Agar Patata Dextrosa; Agar YES: Agar Extracto de Levadura y Sacarosa.

b) Medios de cultivo cárnicos:

La composición de los medios de cultivo realizados a base de embutidos curado-madurados se detalla en la tabla 5.

Tabla 5. Composición de los medios cárnicos empleados en esta Tesis Doctoral empleando salchichón liofilizado.

Ingredientes	**Agar salchichón 3 %**	**Agar salchichón 25 %**	**Agar salchichón + especias (orégano, romero o tomillo)**
Salchichón liofilizado	30 g	250 g	250 g
Agar bacteriológico	20 g	20 g	20 g
Agua destilada	1000 mL	1000 mL	1000 mL
Especia	-	-	0,4 g

c) Medios mínimos:

Para la preparación de los medios mínimos se añadieron 20 g de agar bacteriológico, 0,32 g de NaH_2PO_4, 1,09 g de Na_2HPO_4, NaCl (50 ó 90) y 1000 mL de agua destilada. Además, a cada medio mínimo se añadieron distintas fuentes de carbono en una proporción de 20 g/L (Tabla 6).

Tabla 6. Fuentes de carbono añadidas a los medios mínimos PnEf, PnDh y PnPc.*

Fuente de carbono	PnEf	PnDh	PnPc
Fructosa	X		X
Glucosa		X	X
Lactosa		X	X
Sacarosa		X	X
Ribosa		X	X
Glicina		X	X
Alanina		X	X
Arginina	X	X	X
Asparagina	X	X	X
Cisteína			X
Fenilalanina		X	X
Glutamina	X	X	X
Histidina	X		X
Isoleucina	X	X	X
Leucina		X	
Lisina	X	X	X
Metionina	X	X	
Prolina		X	X

*PnEf: medio mínimo para *Penicillium nordicum* FHSCC IB4 y *Enterococcus faecium* SE920; PnDh: medio mínimo para *P. nordicum* FHSCC ib4 y *Debaryomyces hansenii* FHSCC 253H; PnPC: medio mínimo para *P. nordicum* FHSCC IB4 y *Penicillium chrysogenum* CECT 20922.

Todos los medios de cultivo fueron esterilizados a 121 °C durante 20 min. Las fuentes de carbono para los medios mínimos fueron esterilizadas mediante filtración y añadidas al medio tras la esterilización térmica del resto de componentes. Los medios sólidos se atemperaron hasta 45-50 °C antes de ser distribuidos en placas de Petri de 9 ó 5,5 cm de diámetro. Los medios se

almacenaron a 4 °C antes de su uso excepto los medios mínimos que fueron elaborados en el mismo día de su uso.

III.1.3. Tampones

Para la recogida de esporas se empleó el tampón fosfato-salino (PBS) compuesto por 0,32 g de NaH_2PO_4, 1,09 g de Na_2HPO_4, 9 g de NaCl y 1000 mL de agua destilada.

Para la extracción de la proteína antifúngica PgAFP mediante cromatografía líquida de proteínas a alta velocidad (FPLC) se emplearon dos tampones: acetato de sodio 20 mM (pH 4,5) y acetato de sodio 20 mM (pH 4,5) con NaCL 1M.

Para la extracción de OTA se elaboraron los siguientes tampones: agua/ácido acético 0,1% (v/v) y acetonitrilo/ácido acético 0,1% (v/v).

Para la detección y cuantificación de OTA mediante HPLC se emplearon distintas fases móviles dependiendo del equipo empleado. En el caso del HPLC con detectores de fluorescencia y diodo array (HPLC-FLD/DAD) agua/acetonitrilo/ácido acético 41/57/2 (v/v/v) y para el HPLC con detector de fluorescencia (HPLC-FLD) agua/acetonitrilo/ácido acético (29.5:70:0.5). Para el cromatógrafo líquido de ultra alta resolución con detector de masas (uHPLC-MS) se empleó ácido fórmico 0,1% (v/v) con 10 mM de formato amónico (eluyente A) y acetonitrilo (eluyente B) y para el uHPLC con triple cuadrupolo (uHPLC-MS/MS QqQ) agua/ácido fórmico 0,1% (v/v) (eluyente A) y acetonitrilo/ácido fórmico 0,1% (v/v) (eluyente B).

Para la detección y cuantificación de ergosterol mediante HPLC-FLD/DAD se optimizó el uso de 3 fases móviles: MP1 compuesto por metanol/ácido acético 0,05% (v/v) 90/10, MP2 formado por metanol/ácido acético 0,1% (v/v) 90/10 (v/v) y MP3 compuesto por metanol/ácido acético 0,05% (v/v) 95/5 (v/v).

En la extracción de proteínas para su posterior análisis mediante proteómica comparativa se empleó un tampón de lisis compuesto por Tris-HCL 100 mM, NaCL 50 mM, EDTA 20 mM, glicerol 10% (v/v), PMSF 1 mM y 1µ/mL de pepstatina A. Su pH fue ajustado a 7,5.

III.1.4. Microorganismos

Los microorganismos empleados procedían de distintas colecciones de cultivos: la colección de cultivos del Grupo de Investigación HISEALI de la UEX (FSHCCC, Cáceres, España), la colección de cultivos del Grupo de Investigación de Calidad y Microbiología de Alimentos de la UEX (CAMIALI, Badajoz, España), la Colección Española de Cultivos Tipo (CECT, Valencia, España), la *Centraalbureau voor Schimmelcultures* (CBS, Utrecht, Holanda), la del *Federal Research Centre*

for Nutrition and Food (BFE, Karlsruhe, Alemania) y la colección de micología de la *Universidade do Minho* (MUM, Braga, Portugal); (Tabla 7).

Tabla 7. Microorganismos empleados en el desarrollo de esta Tesis Doctoral.

Especie	**Denominación***
Enterococcus faecium	CAMIALI QE233, CAMIALI SE260, CAMIALI SE920, CAMIALI SE45, CAMIALI HE238, CAMIALI HE 107
Staphylococcus vitulinus	FHSCC MSA19
Debaryomyces hansenii	FHSCC 253H y FHSCC 46P
Penicillium verrucosum	FHSCC Pv45,
Penicillium nordicum	FHSCC IB4, FHSC Pn15, BFE 856, CBS 110.769 y CBS 323.92
Penicillium chrysogenum (renombrado *Penicillium alli-sativi*)	CECT 20922
Penicillium nalgiovense	FHSCC Pj261
Aspergillus westerdijkiae	MUM 16.142

*CAMIALI: Grupo de Investigación de Calidad y Microbiología de Alimentos de la Universidad de Extreamdura; FHSCC: Grupo de Investigación de Higiene y Seguridad Alimentaria de la Universidad de Extremadura; CBS: *Centraalbureau voor Schimmelcultures*; CECT: Colección Española de Cultivos Tipo; BFE: *Federal Research Centre for Nutrition and Food*; MUM: colección de micología de la Universidade do Minho.

III.1.5. Plantas y especias

Las plantas empleadas fueron el romero (*Rosmarinus officinalis*), el orégano (*Origanum vulgare*) y el tomillo (*Thymus zygis*). También se utilizaron bellotas de encina (*Quercus ilex*). Todas ellas fueron recolectadas en Extremadura.

III.1.6. Otro material

Las tripas de colágeno para la realización de los modelos cárnicos fueron suministradas por Viscofan (Cáseda, España).

El antifúngico comercial (AP) empleado fue suministrado por Taberner S.L. (Valencia, España). Éste incluía en su composición sorbato potásico (8,3%) y natamicina (2%), a los cuales se añadieron 60 g/L de NaCl, siguiendo las instrucciones del fabricante.

III.1.7. Equipos

Las pesadas rutinarias para elaborar los medios de cultivo, tampones, etc., se realizaron en dos balanzas. Una de ellas fue de la marca COBOS (Barcelona, España) modelo CB COMPLET con una precisión de 10 mg. Para las pesadas de mayor precisión se utilizó una balanza SARTORIUS (Goettingen, Alemania) modelo LA310S con una precisión de 0,1 mg.

El agua destilada y el agua ultrapura fueron obtenidas mediante un equipo de purificación de agua modelo Milli-Q® Integral water system (Merck, Darmstadt, Alemania).

El salchichón liofilizado en los medios de cultivo cárnicos se obtuvo con un liofilizador VirTis Advantage de SP Scientific (Suffolk, Reino Unido).

El salchichón liofilizado, el romero y las bellotas fueron trituradas en una picadora JATA PC123N (Tudela, España).

Para mezclar los ingredientes de los medios de cultivo se utilizaron agitadores magnéticos con calor SELECTA (Barcelona, España).

La esterilización de los medios de cultivo y otros materiales se llevó a cabo en un autoclave SELECTA modelo Presoclave Plus II.

Cuando fue necesario trabajar en condiciones de esterilidad y para inocular los distintos microorganismos se utilizaron dos campanas de flujo laminar TELSTAR modelos AV-30/70 y BV-100 (Barcelona, España).

La incubación de los medios de cultivo y otras matrices inoculadas se realizó en estufas de las marcas VELP Scientifica FOC 225E, SARTORIUS modelo CERTOMAT® IS y SELECTA® modelo HOTCOLD GL y dos incubadoras con agitación orbital Certomat™ IS de Sartorius (Goettingen, Alemania) y Optic Ivymen 2000 de COMECTA (Barcelona, España).

La observación microscópica se llevó a cabo mediante los microscopios NIKON 125-2 (Tokio, Japón), LaborLux 12 (Leitz, Stuttgart, Alemania) y un microscopio de flourescencia Eclipse E200 con cámara digital DS-Fi2 (NIKON). El recuento de esporas fúngicas se realizó mediante una cámara de recuento Thoma marca BRAND™ (Wertheim, Alemania) o de Neubauer marca Labor Optik (Lancing, Reino Unido).

Se emplearon distintas centrífugas marca EPPENDORF (modelos Centrifuge 5430, 5430R y 5417R) y LAN Technics modelo D2012 Plus (Esparza de Galar, España).

Para homogeneizar muestras se utilizó un agita-tubos VELP SCIENTIFICA (Usmate, Italia) modelo Vortex mixer y un Stomacher modelo 400 circulator de Seward (Worthing, Reino Unido).

Las muestras que requerían incubación a temperatura precisa o un tratamiento por calor se procesaron en un bloque térmico modelo TEMBLOC de SELECTA.

Las pipetas empleadas fueron de las marcas Eppendorf (Hamburgo, Alemania) y Kartell (Noviglio, Italia).

Las placas multipocillo empleadas fueron de la marca Thermo Fisher Scientific (Waltham, Massachusetts, EE. UU.). Tanto la absorbancia como la fluorescencia fueron medidas en un lector de placas Thermo Fisher Scientific modelo Varioskan Lux.

Las soluciones de azúcares, aminoácidos, resazurina, micotoxinas y ergosterol fueron esterilizadas con filtros de 0,22 µm de la marca Cosela (Sevilla, España). Los viales, tapones e insertos fueron suministrados por la misma empresa.

La concentración de ADN y de proteínas fueron medidas con un biofotómetro NanoDrop 2000C de la marca Thermo Fisher Scientific.

Las reacciones de PCR convencional se llevaron a cabo en un termociclador modelo Mastercycler epgradient de Eppendorf. Las reacciones de qPCR se realizaron en el equipo Applied Biosystems 7500 Fast de Thermo Fisher Scientific.

Para la extracción de la proteína PgAFP se filtraron los medios de cultivo mediante una bomba de succión LABOPORT (Trenton, EE. UU.) y se empleó un equipo de filtración con placa porosa de vidrio y un matraz kitasato de FisherBrand (Thermo Fisher Scientific) con filtros de membrana de acetato de celulosa con distintos tamaños de poro 8, 1,2, 0,45, y 0,22 µm de Sartorius (Goettingen, Alemania).

La purificación de la proteína PgAFP se realizó en un cromatógrafo FPLC modelo AKTA^{TM}FPLC (Amersham Pharmacia Biotech, Inc., Amersham, Reino Unido). y Se emplearon columnas de sefarosa de intercambio catiónico HiTrap^{TM}SP y de filtración en gel HiLoad 26/60 Superdex75^{TM} de Amersham Biosciences (Uppsala, Suecia).

Para la preparación del extracto de bellota se empleó un rotavapor modelo VV2000 de Heidolph (Kelheim, Alemania) y el AE fue extraído mediante hidrodestilación en un Clevenger de marca Scharlab con una malla calefactora de SELECTA.

La concentración de ergosterol fue medida mediante HPLC-FLD/DAD Agilent 1260 Infinity (Agilent Technologies, Santa Clara, California, EE. UU.) con una columna C18 (5 µm de tamaño de partícula) de Phenomenex (Torrance, EE. UU.).

Para la detección, identificación y cuantificación de OTA se utilizaron además del HPLC-FLD /DAD citado anteriormente un HPLC-FLD Smartline pump 1000 (Knauer, Germany) con detector FP-2020 (Jasco, USA). La columna usada fue una C18 PLRP-S 300 Å (250 x 4.6 mm, 8µm, Polymer Labroratories, Church Stretton, Reino Unido). Se emplearon también otros dos HPLC con detector de espectrometría de masas. Uno de ellos modelo Scientific Dionex UltiMate 3000 de Thermo Fisher Scientific acoplado a un espectómetro de masas con analizador de trampa de iones de Bruker Daltonics Inc., (Billerica, Massachusetts, EE. UU.), equipado con una columna C18 de fase reversa de 100 mm x 2,1 mm y 2 µm de tamaño de partícula la marca Agilent Technologies. El otro equipo dotado de un detector triple cuadrupolo 6470 (uHPLC MS/MS QqQ) modelo Agilent 1290 Infinity II uHPLC de Agilent Technologies y una columna Zorbax C18 de 100 mm x 2,1 mm y 1,8 µm de tamaño de partícula de la marca Agilent Technologies.

Para la evaporación de las muestras de proteómica se empleó un evaporador Speed Vac Concentrator de Thermo Fhiser Scientific. Las electroforesis de los geles de poliacrilamida se realizaron en los equipos Mini-PROTEAN® Tetra Cell y PowerPac™ HC Power Supply de BIO-RAD (Hercules, EE. UU.). Los geles se tiñeron en movimiento empleando un agitador Heidolph Unimax 2010 (Schwabach, Alemania).

El análisis de las muestras de proteómica se realizó mediante un espectrómetro de masas híbrido cuadrupolo Orbitrap™ Q Exactive™ Plus (Thermo Scientific) acoplado a un sistema UltiMate™ 3000 RSLCnano (Thermo Scientific).

El pH se determinó con un medidor de Hanna Instruments S.L. modelo FC232D (Eibar, España). La a_w fue determinada con un medidor modelo LabMaster de Novasina AG (Lachen, Suiza). Para evaluar los parámetros de textura se empleó un texturómetro TA.XT plus Texture Analyser de Stable Micro Systems Ltd. (Godalming, Reino Unido). El color fue medido con un colorímetro Minolta CR-300 dotado con un iluminador D65 de Konica Minolta, Inc. (Nieuwegein, Holanda).

El análisis de compuestos volátiles se realizó con un cromatógrafo de gases 6890 GC de Agilent Technologies acoplado a un detector de masas 5975C (Agilent Technologies). Para extraer los compuestos volátiles se empleó una fibra DVB/CAR/PMDS 50/30 µm (Merck, Darmstadt, Alemania). La columna empleada fue una HP-5 (5% fenil-95% dimitilpolisiloxano) (Hewlett Packard, California, EE. UU.).

III.1.8. Programas informáticos y páginas web

Los resultados del FPLC se analizaron con el programa UNICORN versión 4.12.

La señal de la OTA detectada mediante espectrometría de masas fue procesada con el programa MassHunter Software de Agilent Technologies o Hystar versión 3.2 de Bruker Daltonics.

Los compuestos volátiles fueron identificados mediante la base de datos NIST/EPA/NIH.

El análisis de los resultados de proteómica comparativa fue realizado mediante el software MaxQuant (versión 1.6.15.0.; https://www.maxquant.org/). Para el enriquecimiento del análisis se empleó el software ClueGo (versión 2.5.6.). Para determinar la función de las proteínas detectadas se empleó la base de datos UniProt (https://www.uniprot.org/).

Para el análisis estadístico se emplearon los programas IBM SPSS Statistics versión 20 (Armonk, Nueva York, EE. UU.) y Perseus versión 1.6.0.7.

III.2. Métodos analíticos

III.2.1. Preparación de los inóculos

Para la preparación de los inóculos bacterianos se emplearon distintos medios de cultivo. Los enterococos fueron incubados en caldo MRS o caldo BHI mientras que *S. vitulinus* fue inoculado en caldo BHI. Todos ellos se incubaron durante 48 h a 30 °C en agitación (150 rpm). La concentración de células bacterianas establecida para cada ensayo (Tabla 8) fue ajustada mediante turbidimetría a una densidad óptica de 600 nm.

Los mohos fueron inoculados en 3 puntos en PDA o agar YES durante 7 o 10 días a 25 °C dependiendo del ensayo realizado. Las esporas fueron recogidas raspando la superficie de cada placa con ayuda de un asa de Drigalsky y posteriormente diluidas en tampón PBS. Las esporas fueron visualizadas y contadas en el microscopio empleando una cámara de Thoma o de Neubauer, siendo ajustada su concentración a la establecida para cada ensayo (Tabla 8).

Las levaduras fueron inoculadas en caldo YES e incubadas durante 48 horas en agitación. El número de células fue ajustado, siguiendo la metodología descrita para los mohos, según la concentración determinada para cada ensayo (Tabla 8).

Tabla 8. Cepas microbianas y concentraciones empleadas en los distintos ensayos realizados en el desarrollo de la Tesis Doctoral.

Especie	Concentración	Apartado
Penicillium nordicum CBS* 110.769 y *Penicillium verrucosum* FHSCC Pv45	10^6 esporas/mL	Apartado IV.1
Enterococcus faecium CAMIALI Q233, SE920, SE260, HE238, HE107, SE45	10^4 ufc/mL	
P. nordicum CBS 323.92	10^6 esporas/mL	Apartado IV.2
P. nordicum FHSCC IB4 y *Penicillium chrysogenum* CECT 20922	10^5 esporas/mL	Apartado IV.3
E. faecium CAMIALI SE920	10^6 ufc/mL	
Debaryomyces hansenii FHSCC 253H	10^6 células/mL	
P. nordicum CBS 323.92 y BFE 856, *Aspergillus flavus* CBS 573.65 y *Penicillium griseofulvum* IBT 14319	10^6 esporas/mL	Apartado IV.4
P. nordicum FHSCC Pn15 y BFE 856.	10^6 esporas/mL	Apartado IV.5
D. hansenii FHSCC 253H	10^6 células/mL	
P. chrysogenum CECT 20922 y FHSCC Pg222 y *Penicillium nalgiovense* FHSCC Pj261	10^6 esporas/mL	Apartado IV.6
E. faecium CAMIALI SE920 y *Staphylococcus vitulinus* FHSCC MSA19	10^6 ufc/mL	
D. hansenii FHSCC 253H y FHSCC 46P	10^6 células/mL	
P. nordicum FHSCC Pn15	10^6 esporas/mL	Apartado IV.7
D. hansenii FHSCC 253H	10^6 células/mL o células/g	
Aspergillus westerdijkiae MUM 16.142	10^6 esporas/mL	Apartado IV.8
D. hansenii FHSCC 253H	10^6 células/mL	

*CBS: *Centraalbureau voor Schimmelcultures*; FHSCC: Grupo de Investigación de Higiene y Seguridad Alimentaria de la Universidad de Extremadura; CAMIALI: Grupo de Investigación de Calidad y Microbiología de Alimentos de la Universidad de Extreamdura; CECT: Colección Española de Cultivos Tipo; BFE: *Federal Research Centre for Nutrition and Food*; MUM: colección de micología de la *Universidade do Minho*.

III.2.2. Elaboración de medios de cultivo a partir de salchichón liofilizado

Para elaborar los medios de cultivo a partir de salchichón liofilizado se empleó masa fresca de este producto, la cual fue dispuesta sobre rejillas de plástico hasta alcanzar 0,5 cm de grosor. Tras cubrirlas con una película de plástico transparente, se congelaron a -80 °C durante 24 h antes de ser introducidas en el liofilizador. Una vez obtenido la masa liofilizada, ésta se homogenizó hasta conseguir una textura de polvo que se envasó al vacío y se almacenó a - 20 °C.

Para la elaboración del agar salchichón suplementado con especias se emplearon las hojas de orégano, romero y tomillo. Para ello se deshojaron todas las plantas y se trituraron las hojas de romero para hacerlas más pequeñas. El romero y el tomillo fueron empleados frescos, mientras que el orégano se secó previamente a temperatura ambiente.

La composición de los medios de cultivo a partir de salchichón liofilizado se detalla en el apartado III.1.2.

III.2.3. Elaboración de embutidos en planta piloto

Para elaborar los embutidos empleados en esta Tesis Doctoral se realizaron 18 lotes distintos (Tabla 9).

Las hojas de romero fueron picadas y añadidas en la masa antes de embutir los lotes R, R+Dh_i y R+Dh_s. Las tripas fueron maceradas durante 24 h en hojas de romero picadas antes del proceso de embutido de los lotes CMR, CMR+Dh_i y CMR+Dh_s.. El AE de romero se añadió a los lotes REO, REO+Dh_i y REO+Dh_s en la superficie tras embutir. En los lotes R+REO, R+REO+Dh_i y R+REO+Dh_s se adicionó tanto romero en el interior de la masa como el AE de romero en la superficie. Finalmente, como control positivo, se sumergieron en AP los lotes AP, AP+Dh_i y AP+Dh_s siguiendo el procedimiento habitual en una industria. Además, se fabricaron los mismos lotes con la levadura *D. hansenii* FHSCC 253H añadida a la masa antes de embutir o inoculada en la superficie tras el embutido mediante inmersión en una suspensión de esta misma levadura (Tabla 9). Finalmente, *P. nordicum* FHSCC Pn15 fue inoculado en la superficie de todos los lotes mediante inmersión.

Tabla 9. Lotes de salchichones fabricados en planta piloto incluyendo distintos agentes de biocontrol.

	Tratamientos*						
Lote	***Penicillium nordicum***	**R**	**CMR**	**REO**	**AP**	**Dh_i**	**Dh_s**
C	X						
R	X	X					
CMR	X		X				
REO	X			X			
R+REO	X	X		X			
AP	X				X		
Dh_i	X					X	
R+Dh_i	X	X				X	
CMR+Dh_i	X		X			X	
REO+Dh_i	X			X		X	
R+REO+Dh_i	X	X		X		X	
AP+Dh_i	X				X	X	
Dh_s	X						X
R+Dh_s	X	X					X
CMR+Dh_s	X		X				X
REO+Dh_s	X			X			X
R+REO+Dh_s	X	X		X			X
AP+Dh_s	X				X		X

**Penicillium nordicum*: *P. nordicum* FHSCC Pn15 inoculado en la superficie mediante inmersión en una solución de 10^6 esporas/mL; R: hojas de romero añadidas a la masa cárnica (2 g/kg); CMR: Tripas maceradas con hojas de romero; REO: aceite esencial de romero aplicado en la superficie mediante spray después del embutido; AP: preparación antifúngica (sorbato potásico + natamicina) aplicada en superficie mediante inmersión después del embutido; Dh_i: *Debaryomyces hansenii* FHSCC 253H (Dh) inoculada a 10^6 células/g en la masa cárnica antes del embutido; Dh_s: Dh inoculada en la superficie mediante inmersión en una suspensión de 10^6 células/mL tras el embutido.

Los embutidos se maduraron en cámaras durante 3 días a 4 °C y una humedad relativa (HR) del 85 %, seguidos por 1 día a 13 °C y 84 % HR y 17 días a 12 °C y 84 % HR.

III.2.4. Producción y purificación de la proteína PgAFP

Para la extracción de la proteína PgAFP, se inocularon 200 µL de una suspensión de 10^7 esporas/mL de *P. chrysogenum* CECT 20922 en 500 mL de PDB pH 4,5. Tras 15 días a 25 °C se eliminó el micelio y el sobrenadante se filtró al vacío pasando el extracto a través de un soporte

de vidrio poroso cubierto por una membrana. Se emplearon tres membranas con distinto tamaño de poro (8; 1,2 y 0,45 µm) y un matraz kitasato.

El filtrado resultante se sometió a FPLC según el método descrito por Acosta et al. (2009). Primero se fraccionó mediante una columna de intercambio catiónico y posteriormente la fracción que contenía la proteína PgAFP se purificó tras pasar por una columna de filtración en gel. Finalmente, la proteína se esterilizó mediante filtros de 0,22 µm. La concentración fue medida siguiendo el método descrito por Lowry et al. (1951) y se almacenó a -20 °C hasta su uso.

III.2.5. Evaluación del crecimiento de las cepas de *E. faecium*

Para evaluar la capacidad de 6 cepas de *E. faecium* (QE233, SE920, SE260, HE238, HE107 y SE45) (Tablas 7 y 8) de crecer en condiciones similares a las del procesado de los embutidos, éstas se inocularon en el medio salchichón al 3 % durante 5 días a 3 temperaturas (15, 20 y 25 °C). El crecimiento fue evaluado mediante observación visual.

III.2.6. Efecto de *E. faecium* sobre el crecimiento de mohos toxigénicos

Las cepas de *E. faecium* que presentaron un mejor desarrollo en las condiciones explicadas en el apartado III.2.5. fueron seleccionadas para estudiar su efecto sobre el crecimiento de *P. nordicum* y *P. verrucosum* productores de OTA. Para ello, se inocularon 50 µL de cada cepa de *E. faecium* en la superficie del agar salchichón al 3 % (DFS;Tabla 8). Tras dejar secar, las placas fueron inoculadas con 2 µL de cada del moho en el centro y se incubaron durante 7 días a 15, 20 y 25 °C.

Tras este tiempo se midió el diámetro de cada colonia fúngica en dos direcciones perpendiculares. Mediante la relación entre el diámetro de la colonia y el tiempo de incubación (días) se obtuvo un modelo lineal que permitió el cálculo de la velocidad de crecimiento (µ) y la fase de latencia (λ) de los mohos. La velocidad de crecimiento expresada en mm/día es la pendiente de la recta de regresión y la fase de latencia se calcula mediante la siguiente fórmula a partir de la ecuación de la recta (y=ax+b):

$$\lambda = \frac{\mu L \ inoculados - b}{a}$$

Con estos parámetros pudo establecerse el porcentaje de reducción en el crecimiento de cada moho respecto a cultivos de ese moho en ausencia de enterococos.

III.2.7. Análisis de OTA

a) Extracción de OTA

Para la extracción de OTA se tomó 1 g de muestra (agar, micelio o tripa + micelio) que se depositó en tubos tipo Falcon. La extracción se llevó a cabo siguiendo el método previamente optimizado en el Grupo de Investigación HISEALI (Delgado et al., 2018). Concretamente, se añadieron 2 mL de agua con ácido acético (0,1% v/v) y se agitó durante 30 s. Posteriormente, se añadieron 2 mL de acetonitrilo con ácido acético (0,1% v/v) y se volvió a agitar durante 1 min. Tras ello se añadieron 0,4 g de NaCl y 1,6 g de $MgSO_4$, agitándose la mezcla manualmente durante 15 s. Las muestras se centrifugaron durante 5 min a 5000 rpm. El sobrenadante se trasfirió a viales opacos de 2 mL. Antes de realizar el análisis mediante HPLC, todos los sobrenadantes se filtraron a través de membranas de 0,22 mm de tamaño de poro.

b) Detección y cuantificación de OTA

Para la detección y cuantificación de OTA se emplearon 4 métodos distintos dependiendo del equipo cromatográfico empleado.

Para el uHPLC MS/MS las fases móviles empleadas se recogen en el apartado III.1.3. El análisis se llevó a cabo mediante un gradiente desde 2 a 98 % de fase B. El volumen de inyección fueron 10 µL a un flujo constante de 0,25 ml/min. Para la detección se empleó el ion precursor 404 m/z y para la cuantificación el ion 358 m/z. El tiempo de carrera fueron 15 min y se detectó la OTA en el minuto 6,5. Los límites de detección y cuantificación fueron 1,3 ng/g y 3,9 ng/g respectivamente.

Para el uHPLC MS/MS QqQ se empleó el siguiente gradiente: 0-3,5 min 20 % eluyente B; 3,5-10 min incremento lineal desde 20 a 95 % del eluyente B; fase de meseta hasta 100 % de eluyente B hasta el 12 min; 12-15 min reequilibrio hasta 20 % eluyente B. Los eluyentes están descritos en el apartado III.1.3. El volumen de inyección fueron 7 µL y el tiempo de carrera de 15 min. La OTA se detectó en el minuto 6,5. El nitrógeno fue empleado para el proceso de desolvatación. Para el proceso de ionización el gas de secado se encontraba a 300 °C y un flujo de 12 L/min. El gas nebulizador fue inyectado una presión de 30 psi y el gas de envoltura a una temperatura de 350 °C y un flujo de 10 L/min. El voltaje del capilar fue 3000 V y el voltaje de fragmentación 100 V. Las condiciones óptimas desde el punto de vista de la sensibilidad y selectividad del Monitoreo de Reacción múltiple (MRM) fueron las transiciones desde el ion 404 al 358 m/z a 10 V de energía de colisión y desde 404 a 239 m/z a 25 V de energía de colisión.

En el HPLC-FLD/DAD se estableció el el flujo en modo isocrático a 1 mL/min con un volumen de inyección de 20 µL. El tiempo de carrera fue de 15 min detectando la OTA en el min 7,5. La detección por fluorescencia fue realizada a una longitud de onda de 333 nm de excitación y 460 nm de emisión.

Para el HPLC-FLD Smartline pump 1000 se estableció el flujo en modo isocrático a una velocidad de 0,8 mL/min. El volumen de inyección fue 20 µL. La OTA fue detectada a 330 y 463 nm de emisión y excitación respectivamente. El tiempo total de carrera fue de 15 min.

El límite de detección (LOD) de OTA se calculó siguiendo la siguiente fórmula siendo S la desviación estándar de la ordenada del blanco analítico y b la pendiente de la recta de calibrado.

$$LOD = \frac{3\mathrm{S}}{b}$$

De forma similar al cálculo del LOD, la Unión Internacional de Química Pura y Aplicada (IUPAC) propone el cálculo del límite de cuantifiación (LOQ) empleando la siguiente fórmula:

$$LOQ = \frac{10\mathrm{S}}{b}$$

El efecto sinérgico de la combinación de dos BCAs sobre la producción de OTA se calculó empleando la fórmula descrita por Moreno et al. (2003):

$$I_e = X + Y - (XY/100)$$

donde I_e es el porcentaje esperado de inhibición de OTA de la combinación de dos BCAs, X e Y son los porcentajes de inhibición para cada BCA por separado y XY el porcentaje de inhibición de la combinación de ambos BCAs. El cociente entre el porcentaje de inhibición observado (I_o) *y el* I_e indica sinergia si el valor es superior a 1,5.

III.2.8. Métodos para evaluar los modos de acción de los BCAs

III.2.8.1. Estudio de la competición por nutrientes y espacio

Para evaluar las fuentes de carbono que utilizaban *P. nordicum* FHSCC IB4, *E. faecium* CAMIALI SE920, *D. hansenii* FHSCC 253H y *P. chrysogenum* CECT 20922 se emplearon placas multipocillos (Figura 7). Se utilizó como base un medio mínimo elaborado con PBS suplementado con 50 ó 90 g/L de NaCl para alcanzar valores de actividad de agua (a_w) de 0,97 y 0,94, respectivamente (PBS50 y PBS90). Cuando se inocularon los mohos filamentosos y la levadura, a estos medios se añadió 0,02 g/L del colorante resazurina (R-PBS50 y R-PBS90), el cual es un indicador de

reacciones de oxidación-reducción que cambia de color azul a rosa cuando un microorganismo utiliza una fuente de carbono.

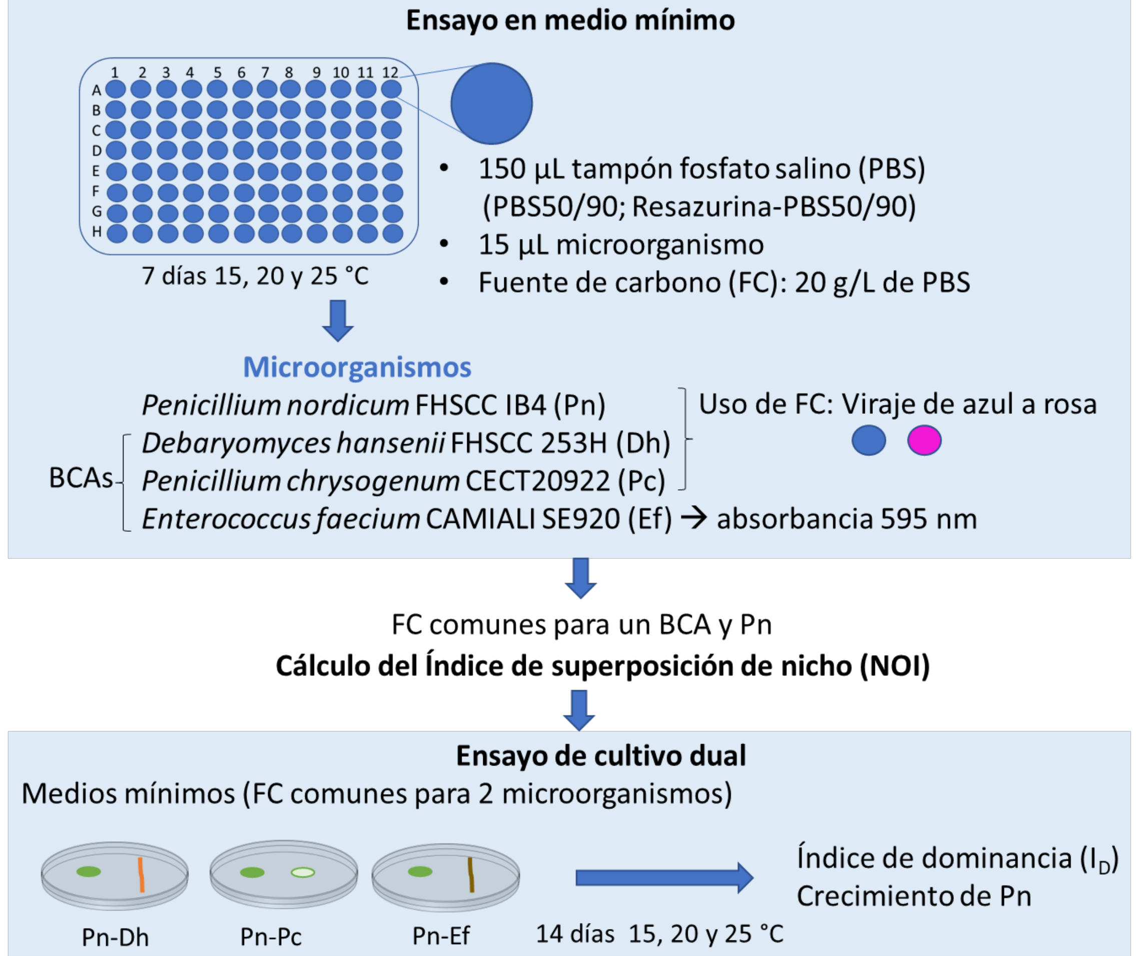

Figura 7. Esquema del método empleado en el estudio del modo de acción basado en la competición por nutrientes y sustrato.

La inoculación de los microorganismos se realizó mediante la adición de 15 µL de cada inóculo resuspendido en PBS50 o PBS90. Se emplearon placas sin inocular como control negativo. Las placas se dejaron incubar durante 7 días a 15, 20 y 25 °C. El crecimiento de mohos y levaduras fue evaluado visualmente por cambio de color de la resazurina, mientras que para *E. faecium* se midió la densidad óptica cada 24 h a una longitud de onda de 595 nm.

A partir de los datos obtenido se procedió a realizar el cálculo del índice de superposición de nicho (NOI) para cada microorganismo a cada valor de a_w y temperatura, siguiendo la siguiente fórmula:

$$NOI = \frac{Número\ de\ fuentes\ de\ carbono\ en\ común\ entre\ dos\ microorganismos}{Número\ de\ fuentes\ de\ carbono\ utilizadas\ por\ uno\ de\ los\ microorganismos}$$

Un valor mayor de 0,9 indica el mismo nicho nutricional mientras que valores por debajo de 0,9 indica diferentes nichos nutricionales. La relación entre los distintos valores de NOI para cada pareja de microorganismos expresados como coordenadas indica si hay dominancia nutricional de uno sobre otro, coexistencia o nichos separados.

Posteriormente, para evaluar la interacción de dos microorganismos se realizaron co-cultivos en medios mínimos que contenían las fuentes de carbono en común empleadas por los microorganismos que se enfrentaban (Tabla 6, apartado III.1.2.). Los microorganismos fueron inoculados a 20 mm de distancia uno del otro adaptando la metodología descrita por Mohale et al. (2013) y Verheecke et al. (2014) (Figura 7). Los mohos fueron depositados en un punto mientras que *E. faecium* y *D. hansenii* se inocularon en forma de estría. Las placas fueron incubadas durante 14 días a 15, 20 y 25 °C. Al final de la incubación se observaron las interacciones macroscópicamente. A cada tipo de interacción se le asignó un valor (Tabla 10) que se empleó para el cálculo del índice de dominancia (I_D) a través de la suma de los valores obtenidos para cada microorganismo (Magan & Lacey, 1984). Además, se midió el diámetro de las colonias de *P. nordicum* durante el tiempo de incubación para calcular su crecimiento en presencia de los BCAs.

Tabla 10. Valores asignados según el tipo de interacción entre microorganismos en el ensayo de cultivo dual.

Descripción	Valor
Mezcla de ambas especies*	1
Inhibición mutua en contacto o con un especio entre colonias < 2 mm	2
Inhibición mutua a distancia (> 2 mm)	3
Inhibición de un organismo en contacto. La inhibidora continúa creciendo	4
Inhibición de un organismo a distancia (> 2 mm). La inhibidora continúa creciendo	5

*Las especies crecen entremezclándose sin inhibición.

III.2.8.2. Evaluación de la síntesis de ergosterol

El análisis del contenido de ergosterol de mohos toxigénicos en presencia de los BCAs permite identificar una de las posibles dianas de acción de éstos. Los métodos previamente descritos presentan tiempos de análisis superiores a 30 min (Neuhof et al., 2008; Pastinen et al., 2017; Porep et al., 2015) por lo que es necesario optimizar un método para evitar la degradación del ergosterol y aumentar la productividad mediante el incremento del número de muestras por día y la reducción de reactivos. Además, ninguno de estos métodos emplea una matriz cárnica por lo que el método de extracción también debe ser optimizado.

Por esta razón en la presente Tesis Doctoral se procedió a optimizar un método de cuantificación de ergosterol en mohos toxigénicos en matrices cárnicas. Para ello, inicialmente se preparó una solución estándar de ergosterol en acetona a una concentración de 1 mg/mL. Las posteriores soluciones de trabajo se prepararon en viales opacos a partir de la estándar mediante su dilución en metanol y se filtraron con membranas de 0,22 µm de tamaño de poro. Las soluciones se prepararon cada día para evitar la degradación del ergosterol.

Para la detección y cuantificación del ergosterol se emplearon el HPLC-FLD/DAD y la columna descritos anteriormente en el apartado III.1.7. La temperatura de la columna fue establecida a 25 °C y el flujo en 1 mL/min. El volumen de inyección fue de 20 µL. El ergosterol fue detectado a una longitud de onda de 282 nm e identificado con el espectro de absorción del estándar. Las curvas de calibración se construyeron diluyendo la solución madre desde 0,1 a 100 µg/mL.

Los gradientes y la composición de las fases móviles empleadas fueron optimizados a partir de las soluciones estándar. Se evaluaron 10 gradientes diferentes con la fase móvil MP1 descrita en el apartado III.1.3 como eluyente A y metanol como eluyente B (Tabla 11).

Tabla 11. Gradientes empleados en la optimización del método de detección y cuantificación de ergosterol en mohos toxigénicos.

Gradiente	Características
1	0-10 min 10 % B*, 10-15 min crecimiento lineal desde 10 hasta 100 % B, 15-20 min 100 % B, 20-40 min disminución lineal desde 100 hasta 10 % B
2	0-10 min 10 % B, 10-18 min crecimiento lineal desde 10 hasta 100 % B, 18-23 min 100 % B, 23-40 min disminución lineal desde 100 hasta 10 % B
3	0-10 min 10 % B, 10-18 min crecimiento lineal desde 10 hasta 10 0% B, 18-25 min 100 % B, 25-40 min disminución lineal desde 100 hasta 10 % B
4	0-5 min 10 % B, 5-15 min crecimiento lineal desde 10 hasta 100 % B, 15-20 min 100 % B, 20-35 min disminución lineal desde 100 hasta 10 % B
5	0-7 min 10 % B, 7-17 min crecimiento lineal desde 10 hasta 100 % B, 17-22 min 100 % B, 22-37 min disminución lineal desde 100 hasta 10 % B
6	0-7 min 10 % B, 7-12 min crecimiento lineal desde 10 hasta 100 % B, 12-17 min 100 % B, 17-32 min disminución lineal desde 100 hasta 10 % B
7	0-5 min 10 % B, 5-10 min crecimiento lineal desde 10 hasta 100 % B, 10-15 min 100 % B, 15-30 min disminución lineal desde 100 hasta 10 % B
8	0-5 min 10 % B, 5-8 min crecimiento lineal desde 10 hasta 100 % B, 8-12 min 100 % B, 12-27 min disminución lineal desde 100 hasta 10 % B
9	0-5 min 10 % B, 5-10 min crecimiento lineal desde 10 hasta 100 % B, 10-15 min 100 % B, 15-25 min disminución lineal desde 100 hasta 10 % B
10	0-5 min 10 % B, 5-8 min crecimiento lineal desde 10 hasta 100 % B, 8-12 min 100 % B, 12-22 min disminución lineal desde 100 hasta 10 % B

*B: metanol como eluyente B.

Tras seleccionar el gradiente más adecuado se evaluaron las fases móviles MP2 y MP3 descritas en el apartado III.1.3. La selección del gradiente y la fase móvil más adecuada se basó en la obtención de un pico cromatográfico estrecho y simétrico en el menor tiempo de retención y el menor tiempo de carrera.

Para evaluar la efectividad del método para estudiar los modos de acción de los agentes antifúngicos, se incubó un patrón de ergosterol (10 µg/mL) en presencia de tres concentraciones de un antifúngico comercial (10, 50 y 90 %) durante distintos tiempos de incubación (0, 8 y 24 h). Se emplearon muestras sin compuesto antifúngico como control positivo.

Posteriormente se procedió a evaluar 6 métodos de extracción de ergosterol basados en dos previamente descritos por Neuhof et al., (2008) y Pastinen et al. (2017). Se analizaron distintas soluciones, concentraciones y tiempos de incubación (Tabla 12). En todos se prepararon soluciones de ergosterol para que al final de la extracción hubiera una concentración de 10 µg/mL suponiendo una recuperación del 100 %. El proceso de extracción fue llevado a cabo en condiciones de oscuridad.

Tabla 12. Características de los métodos de extracción de ergosterol fúngico evaluados en este estudio.

Métodos	**Características de cada método**	**Referencias**
Método 1	2 mL NaOH 10 % (p/v) en metanol + vórtex 30 s + 1 h at 60 °C + 2 mL agua destilada + 5 mL hexano + evaporar hexano + resuspender en 1 mL MP3*	Neuhof et al., 2008
Método 2	2 mL NaOH 18 % (p/v) en agua destilada + 2 mL 1-butanol + vórtex 30 s + 1 h at 90 °C + 2 mL tolueno + centrifugar 5,000 rpm for 5 min + evaporar la fase orgánica+ resuspender en 1 mL MP3	Pastinen et al., 2017
Método 3	2 mL NaOH 18 % (p/v) en agua destilada + 2 mL 1-butanol + vórtex 30 s + 1 h at 90 °C + 2 mL cloroformo + centrifugar 5,000 rpm for 5 min + evaporar fase orgánica + resuspender en 1 mL MP3	Este estudio
Método 4	2 mL NaOH 18 % (p/v) en agua destilada + 2 mL 1-butanol + vórtex 30 s + 30 min at 90 °C + 2 mL cloroformo + centrifugar 5,000 rpm for 5 min + evaporar fase orgánica + resuspender en 1 mL MP3	Este estudio
Método 5	2 mL NaOH 18 % (p/v) en agua destilada + 2 mL 1-butanol + vórtex 30 s + 1 h at 60 °C + 2 mL cloroformo + centrifugar 5,000 rpm for 5 min + evaporar fase orgánica + resuspender en 1 mL MP3	Este estudio
Método 6	2 mL NaOH 18 % (p/v) en agua destilada + 2 mL 1-butanol + vórtex 30 s + 1 h at 90 °C + 3 mL cloroformo + centrifugar 5,000 rpm for 5 min + evaporar fase orgánica + resuspender en 1 mL MP3	Este estudio

[1]MP3: metanol/ácido acético 0.05% (v/v) 95/5 (v/v).

La precisión del método fue evaluada mediante el cálculo del porcentaje de recuperación (RA) siendo seleccionado el método con los mejores resultados (≈ 100 %). Para ello se añadieron concentraciones conocidas a las soluciones antes de la extracción y fueron comparadas con el área de un patrón a la misma concentración (10 µg/mL). Todas las muestras se realizaron por triplicado. El porcentaje de recuperación fue calculado con la siguiente ecuación:

$$RA\ (\%) = \frac{media\ del\ área\ de\ la\ muestra}{media\ del\ área\ del\ estándar}$$

La validación del método se llevó a cabo mediante la determinación del LOD y el LOQ, la linealidad, precisión, repetibilidad, reproducibilidad intra-laboratorial y exactitud. El LOD y el LOQ del método optimizado se calcularon mediante las fórmulas descritas en el apartado III.2.7. La linealidad fue determinada a través del coeficiente de determinación (R^2) en un rango de 0,1 a 100 µg/mL y la precisión a través de la desviación estándar relativa también llamada coeficiente de variación (CV) que se calcula como el cociente entre la desviación estándar y el valor de la media y multiplicado por 100. Ambos parámetros fueron analizados por triplicado.

Para evaluar los modos de acción de agentes antifúngicos sobre el ergosterol se emplearon 4 cepas toxigénicas: *P. nordicum* CBS 323.92 y BFE 856, *A. flavus* CBS 573.65, *P. griseofulvum* IBT 14319. Éstas fueron incubadas en medio agar salchichón 25% en presencia de 25 µL de AP en la superficie (Tabla 8). Además, el efecto sobre el ergosterol de las especias orégano, romero y tomillo fue estudiado en *P. nordicum* CBS 323.92. Para ello, el medio agar salchichón 25% fue suplementado con 2 g/kg de la especia correspondiente (Tabla 2). Estas especias se probaron individualmente y en combinación con AP. Tras incubar 14 días a 12 °C se recogió 1 g de micelio y se introdujo en nitrógeno líquido para detener su actividad metabólica. Se almacenó a -80 °C hasta la extracción de ergosterol siguiendo el método optimizado anteriormente. Antes de la extracción el micelio se homogeneizó hasta conseguir un polvo mediante el uso de un mortero con nitrógeno líquido.

III.2.8.3. Cambios en la de expresión de genes implicados en la biosíntesis de OTA

La inhibición de la biosíntesis de micotoxinas puede ser uno de los modos de acción de los BCAs. Por lo tanto, se estudió la expresión génica relativa de genes involucrados en la biosíntesis de OTA por *P. nordicum* en presencia de orégano, romero y tomillo.

a) Extracción de ARN

Para llevar a cabo el estudio de la expresión génica relativa, las muestras de micelio fueron congeladas mediante nitrógeno líquido y machacadas en un mortero hasta conseguir un polvo.

Para la extracción de ARN se empleó el kit Spectrum™ Plant Total RNA kit. Se añadieron 500 µL de tampón de lisis junto a 10 µL de β-mercaptoetanol y se siguieron las instrucciones del fabricante. A continuación, las muestras se trataron con el kit DNase I, RNase-free para eliminar posibles contaminaciones de ADN genómico. La concentración y pureza del ARN se determinaron mediante la medida de 1,5 µL de muestra en un espectrofotómetro NanoDrop. La pureza se consideró adecuada cuando la ratio A260/A280 fue superior a 2. Las muestras de ARN se almacenaron a -80 °C hasta la síntesis de ADN complementario (ADNc).

b) Síntesis de ADNc

La síntesis de ADNc a partir del ARN se llevó a cabo utilizando la metodología descrita en el kit PrimeScript™ RT Reagent. De cada muestra se tomó una alícuota de 5 µL y se le añadió 2 µL de tampón PrimeScript 5X, 0,5 µL de OligoDT 50 µM, 0,5 µL de cebadores Random 6 mers 100 µM y la enzima trascriptasa inversa PrimerScript RT Enzyme Mix I. Se añadió agua ultrapura estéril hasta un volumen final de reacción de 10 µL.

Las condiciones de amplificación de la reacción de transcripción inversa consistieron en un ciclo de 15 min a 37 °C, un ciclo de 5 s a 85 °C y una fase final de enfriamiento a 4 °C. El ADNc se almacenó a -20 °C hasta su uso.

c) RT-qPCR

Para el estudio de la expresión génica relativa se seleccionó como gen endógeno (*housekeeping*) el gen estructural de la β-tubulina. La pareja de cebadores había sido previamente diseñada por Bernáldez et al. (2017). Los genes diana estudiados fueron *otapks* y *otanps,* implicados en la ruta biosintética de OTA y los genes *Hog1* y *Rho1* relacionados con distintos tipos de estrés. Los cebadores para dichos genes han sido previamente optimizados por otros autores siguiendo la metodología SYBR Green (Bernáldez et al., 2017; a Cruz Cabral et al., 2018; Rodríguez et al., 2011, 2016); (Tabla 13). Para ello, se empleó el termociclador Applied Biosystems 7500 Fast Real-Time PCR. Los volúmenes empleados en cada reacción se recogen en la tabla 14.

Tabla 13. Genes y cebadores empleados en las reacciones en cadena de la polimerasa en tiempo real de transcripción inversa (RT-qPCR).

Gen	Cebadores	Secuencia 5'-3'	Tamaño del producto (pb)	Referencia
β-tubulina	β-tubF1	GCCAGGGTGACAAGTACGT	72	Bernáldez et al., 2017
	β-tubR1	TACCGGGCTCCAAATCGA		
otapksPN	otapksF3	CGCCGCTGCGGTTACT	80	Bernáldez et al., 2017
	otapksR3	GGTAACAATCAACGCTCCCTCTT		
otanpsPN	F-npstr	GCCGCCCTCTGTCATTCCAAG	113	Bernáldez et al., 2017
	R-npstr	GCCATCTCCAAACTCAAGCGTG		
Hog1	HogF2	GGTAGACATCTGGAGCGCGG	143	Rodríguez et al., 2016
	HogR2	TCACATCATCGGGAGGAGTA		
Rho1	Rho1-F1	CTTTCCCCGAGGTCTACGTC	119	Da Cruz Cabral et al., 2018
	Rho1-R2	TCGTAATCCTCCTGACCAGC		

Tabla 14. Volúmenes (μL) de los reactivos empleados en las reacciones en cadena de la polimerasa en tiempo real de transcripción inversa (RT-qPCR).

	Genes				
Reactivos	*β-tubulina*	*otapks*	*nrps*	*Hog*	*Rho1*
SYBR®	6,25	6,25	6,25	6,25	6,25
Rox	0,125	0,125	0,125	0,125	0,125
Cebadores	0,375 (300 nM)	0,375 (300 nM)	0,375 (300 nM)	0,187 (150 nM)	0,25 (200 nM)
ADNc	2,5	2,5	2,5	2,5	2,5
H_2O milliQ	3,25	3,25	3,25	3,43	3,37

Las condiciones de reacción para cada pareja de cebadores fueron 1 ciclo a 50 °C durante 2 min, 1 ciclo de 95 °C durante 10 min y 40 ciclos a 95 °C 15 s y 60 °C 1 min a excepción de los cebadores Rho1-F1/R1 en los cuales la temperatura de hibridación fue 55 °C. Tras el último ciclo se le añadió una curva de disociación (*Melting curve*) para comprobar la especificidad de los productos amplificados tras su calentamiento a 95 °C 15 s, 60 °C 1 min y 95 °C 15 s. Se analizaron muestras a las que no se les adicionó ADN (blanco) como control negativo. Todas las reacciones fueron realizadas por triplicado.

Los valores de ciclo umbral (*cycle threshold*, C_T) indican el ciclo en el que se acumula suficiente producto como para generar el primer incremento de fluorescencia destacable. Éstos son inversamente proporcionales a la cantidad de ADNc de la que se parte.

d) Análisis de la expresión génica relativa

Tras comprobar que en los controles negativos no se produjo la amplificación de ningún producto de PCR y las temperaturas Tm de cada muestra eran las correctas, se procedió a analizar la expresión génica según el método $2_T^{-\Delta\Delta C}$ (Livak y Schimittgen, 2001) para la cuantificación relativa de los niveles de expresión de los genes diana respecto al control endógeno. Éste emplea como control endógeno los valores de C_T de las muestras del gen estructural *β-tubulina* para normalizar la cuantificación del ARNm. Los valores de C_T de las muestras de *P. nordicum* que crecieron sin la adición de ningún agente de biocontrol fueron empleados como calibrador para obtener los valores relativos de las demás muestras.

III.2.8.4. Proteómica comparativa

El análisis de proteómica comparativa permite conocer la influencia de los BCAs en el metabolismo del moho toxigénico a nivel del proteoma y, por lo tanto, elucidar los modos de acción de los BCAs de origen microbiano y vegetal. En la presente Tesis Doctoral esta técnica se empleó para el estudio del proteoma de *P. nordicum* en medio de cultivo en presencia de AE de romero y la combinación de romero con *D. hansenii* y en embutidos curado-madurados cuando se añadió romero, *D. hansenii* y sus combinaciones.

a) Extracción de proteínas

Se rasparon alrededor de 200 mg de micelio de la superficie de las muestras (apartados IV.5, IV.7) se congelaron empleando nitrógeno líquido y se almacenaron a -80 °C. El protocolo de extracción de proteínas se basó en el descrito por Delgado et al. (2019) con algunas modificaciones. Concretamente, se añadieron 400 mL de tampón de lisis a cada muestra, se sonicaron y los lisados fueron centrifugados para eliminar las células. Estos lisados se sometieron

a una electroforesis en gel de acrilamida (SDS-PAGE) y se digirieron siguiendo el método descrito por Shevchenko et al. (2006) con algunas modificaciones. Después las bandas fueron sometidas a un proceso de reducción con ditiotreitol y una alquilación con iodoacetamida. Tras ello, se digirieron con tripsina y proteasaMAX durante 1 h a 50 °C.

b) Detección y cuantificación de proteínas

Se analizaron 5 µg de la muestra digerida empleando el equipo Q-Executive Plus acoplado a Dionex Ultimate 3000 RSLCnano. El gradiente empleado fue desde el 8 al 30 % de B (A: 0,1% ácido fórmico (A), B: acetonitrilo, 0,1% AF) durante 4 h en una columna Acclaim PepMap RSLC C18, de 2 µm, 100 Å, 75 µm i.d. × 50 cm a una temperatura de 45 °C.

Los resultados fueron recogidos con el método Top15 descrito por Dolan et al. (2014). La cuantificación de proteínas y el análisis de los datos se realizó con el software MaxQuant. Las proteínas fueron identificadas mediante la búsqueda en una base de datos de *P. nordicum*. Las tasas máximas de falso descubrimiento de péptidos/proteínas (FDR) se establecieron en 1 % en base a la comparación con la base de datos inversa. Para generar intensidades espectrales normalizadas e inferir la abundancia relativa de proteínas se utilizó el algoritmo *label free quantitative* (LFQ). Se eliminaron las proteínas que coincidían con contaminantes o la base de datos inversa, y las proteínas solo se retuvieron en el análisis final si se detectaban en al menos tres réplicas con el mismo tratamiento.

También se realizó un análisis cualitativo para detectar proteínas que se encontraron en al menos dos o tres réplicas de un tratamiento en particular, pero no detectables con la muestra a comparar.

III.2.9. Efectos de los agentes de biocontrol en los parámetros físico-químicos y sensoriales del embutido

El proceso seguido para la evaluación de los efectos de los BCAs sobre la población autóctona y los parámetros físico-químicos y sensoriales del embutido se muestra en la Figura 8.

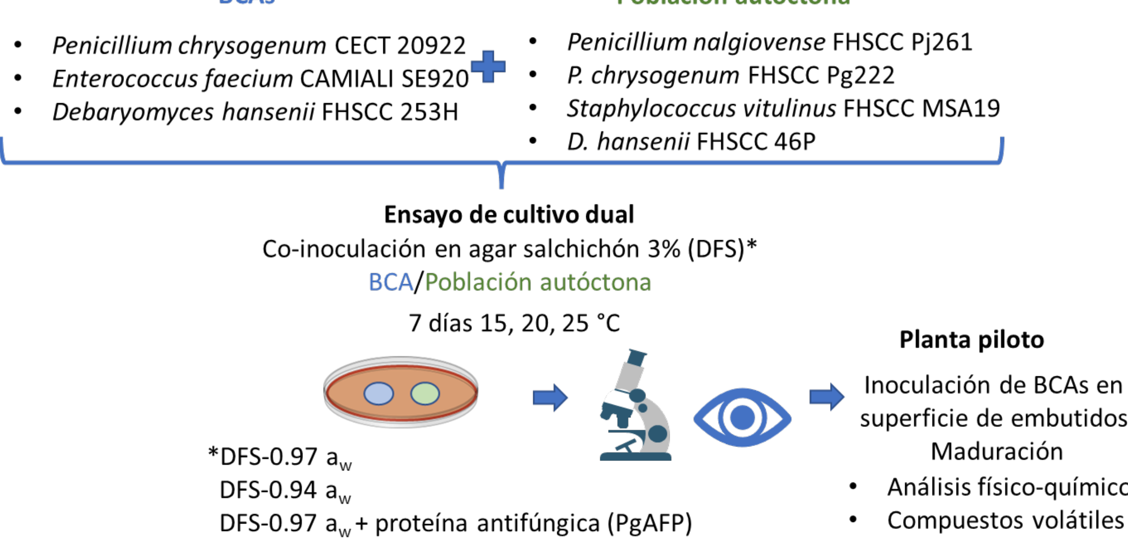

Figura 8. Esquema del procedimiento seguido para la evaluación de los agentes de biocontrol (BCAs) sobre la población autóctona y los parámetros físico-químicos y sensoriales de los embutidos curado-madurados.

III.2.9.1. Ensayo de cultivo dual

Los agentes de biocontrol *E. faecium* SE920, *D. hansenii* FHS253H y *P. chrysogenum* CECT 20922 fueron co-inoculados con microorganismos presentes de manera natural en embutidos (Tabla 8, apartado III.2.1.). Para ello, se realizó un ensayo de cultivo dual de uno de los BCA con un microorganismo perteneciente a la población autóctona de los embutidos siguiendo la metodología descrita en la Figura 8. El medio de cultivo empleado fue DFS al cual se le añadió 50 ó 90 g/L de NaCl para alcanzar valores de a_w de 0,97 (DFS-0,97) y 0,94 (DFS-0,94) respectivamente. La proteína antifúngica PgAFP fue añadida a una concentración de 20 µg/mL al medio ya autoclavado (DFS-0,97P y DFS-0,94P).

Las placas fueron incubadas durante 7 días a 15, 20 y 25 °C. Las interacciones entre microorganismos fueron observadas macroscópica y microscópicamente a través de la observación al microscopio de muestras de micelio.

III.2.9.2. Ensayo en planta piloto

Los embutidos sin madurar fueron comprados en una industria local e inoculados en su superficie mediante inmersión en soluciones que contenían distintos microorganismos. Se realizaron 5 lotes distintos: un lote control sin inocular (C), un lote con *E. faecium* SE920 (E), un lote con *D. hansenii* FHSCC 253H (D), otro con *P. chrysogenum* CECT 20922 (P) y un lote que incluía la mezcla de todos (M). Se realizaron 5 réplicas de cada lote.

Los embutidos se maduraron en cámaras de maduración durante 3 días a 5 °C y 85 % HR, seguidos por 1 día a 13 °C y 84 % HR y 17 días a 12 °C y 84 % HR.

a) Análisis físico-químico

El pH, la a_w y la humedad fueron medidos al final del periodo de maduración. La humedad fue medida con el método de referencia 935.29 de la AOAC (2000) mediante el secado a 105 °C de muestras de salchichón picadas.

El análisis del perfil de textura fue llevado a cabo tras cortar rodajas de 1 cm de grosor usando un texturómetro TA.XT plus Texture Analyser. Las muestras fueron axialmente comprimidas hasta un 50 % a una velocidad de 2 mm/s en una secuencia de dos ciclos usando un émbolo plano de 50 mm de diámetro (P/50). Los parámetros de textura dureza, adhesividad, elasticidad, cohesividad y masticabilidad descritos por Bourne (1978) fueron obtenidos a partir de las curvas de deformación.

El color fue evaluado en rodajas de 1 cm de grosor empleando un colorímetro para determinar el espacio CIE $L^*a^*b^*$ relacionado con la luminosidad (L^*), color rojo (a^*) y color amarillo (b^*). El área de medición fue de 2,5 cm y se realizó con un iluminador D65 con un ángulo de 0°.

Todas las muestras fueron analizadas por triplicado.

b) Análisis de compuestos volátiles

Para la extracción de compuestos volátiles se empleó la técnica SPME tras calentar las muestras a 37 °C durante 30 min. El análisis se realizó en un GC-MS con una columna HP-5 (5 %). La temperatura del horno fue de 40 °C durante 5 min y se incrementó hasta 280 °C a una velocidad de 7 °C/min. El tiempo de desorción fue de 30 min a 250 °C. La temperatura de la línea de transferencia fue establecida en 280 °C. El gas portador fue helio a un flujo de 1,2 mL/min. La detección de masas fue realizada mediante un escaneo completo desde 50 a 350 unidades de masa atómica (amu). La identificación de los compuestos fue realizada mediante la deconvolución de los espectros comparados con los espectros de masas de patrones y la base de datos NIST/EPA/NIH.

c) Evaluación sensorial

La evaluación sensorial se realizó con 22 voluntarios de la Facultad de Veterinaria de la UEX. Ésta consistió en una prueba hedónica y una escala de intensidad de olor. Las muestras fueron presentadas a los panelistas en tubos tapados para evitar influencias visuales. A cada muestra se le asignó un código numérico de tres cifras. Para la prueba hedónica se pidió a los panelistas

que ordenasen las muestras desde el olor menos preferido al más preferido. Para la escala de intensidad de olor, los panelistas debían ordenar las muestras desde la menos intensa a la más intensa. Se permitió volver a oler las muestras para confirmar el ranking. A cada posición en la escala se le asignó un valor, siendo la muestra con más puntuación la más preferida o con mayor intensidad de olor. Tras ello, se presentó a los panelistas una rodaja de cada lote y se les preguntó si los comprarían o no para detectar si la apariencia podría influir en su intención de compra.

III.2.10. Tratamiento estadístico

Para los conjuntos de datos de crecimiento, producción de OTA, expresión génica y concentración de ergosterol inicialmente se realizaron las pruebas de normalidad *Saphiro-Wilk* y *Kolmogorov-Smirnov* dependiendo del número de muestras en cada análisis. Tras no seguir una distribución normal se realizaron las pruebas paramétricas *Kruskal-Wallis* y *U de Mann-Whitney*. Se usó el coeficiente de correlación de *Spearman* para calcular, por un lado, las correlaciones entre la producción de OTA y la expresión génica y, por otro, la relación entre los parámetros que definen el color. Los resultados de la evaluación sensorial se analizaron mediante el test de *Friedman*. Para el análisis de los datos de proteómica comparativa se empleó la prueba *T de Student*. Para el análisis de enriquecimiento funcional, se empleó el software ClueGo que permite la visualización en forma de gráficos de proteínas relacionadas funcionalmente agrupándolas en "grupos" y subdividiéndolas en "términos". Para definir las interrelaciones término-término y los grupos creados a partir de genes compartidos entre los términos se estableció la puntuación Kappa en 0,4. Se establecieron tres términos GO (función molecular, componente celular, procesos biológicos) y al menos el 4 % de los genes identificados con una ruta deben mostrar valores significativos para ser seleccionados. La significación estadística se estableció en $p \leq 0,05$.

IV. RESULTADOS

IV.1. Evaluación y selección de *E. faecium* como agente de biocontrol frente a *Penicillium nordicum*

Enterococcus faecium*: a promising protective culture to control growth of ochratoxigenic moulds and mycotoxin production in dry-fermented sausages*

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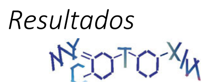

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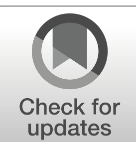

Enterococcus faecium: a promising protective culture to control growth of ochratoxigenic moulds and mycotoxin production in dry-fermented sausages

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Abstract
Moulds positively contribute to the development of typical characteristic flavour and aroma of dry-fermented sausages. However, some mould species, such as *Penicillium nordicum* and *Penicillium verrucosum*, may contaminate this product with ochratoxin A (OTA). For this reason, the control of toxigenic moulds is needed. Strategies based on the use of antifungal microorganisms present in the native microbial population in the dry-fermented sausage processing could be a promising strategy. The aim of this work was to study the effect of *Enterococcus faecium* strains on *P. nordicum* and *P. verrucosum* growth and OTA production in a dry-fermented sausage-based medium at conditions of temperature and water activity similar to those occurring during the ripening of these meat products. Six strains were screened to evaluate their growth capacity and antifungal activity against *P. nordicum* and *P. verrucosum* at three fixed temperatures related to the sausage ripening. The two *E. faecium* strains that decreased growth of both species were chosen to further evaluate their effect on growth of *P. verrucosum* and *P. nordicum* and their mycotoxin production under conditions simulating the dry-fermented sausage ripening. The presence of *E. faecium* SE920 significantly reduced OTA production of *P. nordicum* although it did not affect *P. verrucosum*. *E. faecium* SE920, isolated from dry-fermented sausages, could be a good candidate to reduce OTA production by *P. nordicum* in dry-fermented sausages.

Keywords Lactic acid bacteria · Ochratoxin A · *E. faecium* · Antifungal effect · Dry-fermented sausages

Introduction

Moulds can be found as natural contaminants in dry-cured meat products and most of them can contribute positively to the development of typical characteristic flavour and aroma (Sunesen and Stahnke 2003; Martín et al. 2006). Conversely, some moulds may produce mycotoxins that represent a hazard to consumers (EFSA 2006; Payne 2016). In fact, some toxigenic mould species such as *Penicillium nordicum* and *Penicillium verrucosum* producers of ochratoxin A (OTA) have been commonly found in dry-cured meat products including dry-fermented sausages or dry-cured ham (Battilani et al. 2007; Iacumin et al. 2009; Rodríguez et al. 2012). OTA has nephrotoxic, genotoxic, teratogenic and hepatotoxic effects (EFSA 2006; Lioi et al. 2004; Petzinger and Ziegler 2000) and has been classified as possibly carcinogenic to humans within Group 2B by the International Agency for Research on Cancer (IARC 1993). For this reason, many countries and international organisations have regulated OTA content in several food commodities (European Commission 2006), although only some producer countries such as Italy have established a guideline value in meat and meat products for this mycotoxin of 1 μg/kg (Ministero della Sanità 1999).

Biopreservation has been described as a promising approach to improve food safety without changing the sensory quality of products (Goerges et al. 2006). The dominant microbial population during the dry-fermented sausage processing is composed by moulds, yeasts and lactic acid bacteria (LAB). The latest are frequently added to dry-fermented sausages at levels around 5–6 log CFU/g as starter cultures and they can reach counts higher than 7–8 log CFU/g at the end of the sausage processing (Benito et al. 2007; Fraqueza 2015; Rantsiou and Cocolin 2006). Despite more studies have been performed about the effect of protective moulds and yeasts on

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the growth of ochratoxigenic moulds and their mycotoxin production in dry-cured meat-system models (Bernáldez et al. 2013; Peromingo et al. 2018), recently the use of LAB as biopreservative to minimise OTA amounts in food is gaining interest (Belkacem-Hanfi et al. 2014; Dallagnol et al. 2018; Gerez et al. 2014; Lappa et al. 2018; Meftah et al. 2018; Perczak et al. 2018). In addition, a large number of LAB, including those used as protective cultures, has been considered as safe for human consumption as they possess the GRAS (Generally Recognised As Safe) and/or QPS (Qualified Presumption of Safety) status from the US FDA and the EFSA, respectively (Di Gioia et al. 2016).

On another side, LAB commonly used as biocontrol agents against toxigenic moulds mainly belong to *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Pediococcus* genera (Dalié et al. 2010; Belkacem-Hanfi et al. 2014; Dallagnol et al. 2018; Delgado et al. 2018b; Saladino et al. 2016). Few studies of the antifungal potential of *Enterococcus* species have been conducted yet (Belguesmia et al. 2013; Taghi-zadeh and Nejati 2017), despite the fact that *Enterococcus* species are members of the native microbiota in traditional dry-fermented sausages, and some species, such as *Enterococcus faecium*, have been used as a starter culture and demonstrated its antimicrobial and probiotic activity (Barbosa et al. 2010; Belgacem et al. 2010; Correia Santos et al. 2017; Franz et al. 2011; Landeta et al. 2013; Rubio et al. 2013; Ruiz-Moyano et al. 2009; Zommiti et al. 2018). Therefore, the autochthonous *E. faecium* that remain throughout the processing of dry-fermented sausages could be investigated as biopreservative agent to reduce OTA in these meat products.

Therefore, the aim of this study was to evaluate the potential activity of *E. faecium* strains on growth of *P. nordicum* and *P. verrucosum* and their OTA production in a dry-fermented sausage-based-medium simulating conditions of temperatures and water activities (a_w) used in the ripening of dry-fermented sausages.

Materials and methods

Bacterial and fungal strains

One strain of *P. nordicum*, one strain of *P. verrucosum* producers of OTA and six strains of *E. faecium* were used in this study. *P. nordicum* CBS 110.769 was obtained from the Centraalbureau voor Schimmelcultures fungal collection (Utrecht, The Netherlands) and *P. verrucosum* FHS Pv45 belongs to the culture collection of Food Hygiene and Safety Research Group of the University of Extremadura (Cáceres, Spain). The ability to produce OTA of both mould strains on malt extract agar (MEA) and yeast extract-sucrose agar (YES) at 25 °C for 12 days has been confirmed in this study.

The strains of *E. faecium* were obtained from the culture collection of Food Quality and Microbiology Research Group of the University of Extremadura (Badajoz, Spain) (Ruiz-Moyano et al. 2008).

Moulds and enterococci strains were maintained for long-term storage as stocks in Potato Dextrose (PDB, Scharlab, Spain) and enterococci in Man Rogosa Sharpe (MRS; Scharlab) broth, respectively, supplemented with 20% sterile glycerol (Fisher Scientific, UK) at − 80 °C.

Inocula preparation

Each mould was grown on Potato Dextrose Agar (PDA, Scharlab) for 7 days at 25 °C. The spores were collected using 3 ml of phosphate-buffered saline (PBS) buffer and rubbing the surface with a glass rod. The spores were counted using a Thoma counting chamber Blaubrand® (Brand, Germany), and their concentration was adjusted to 10^6 spores/ml by diluting with PBS.

The enterococci strains were inoculated on MRS broth and incubated for 48 h at 30 °C. The concentration of the inocula was turbidimetrically adjusted to 10^4 CFU/ml.

Fresh inocula were prepared before starting each experiment. All the mould and enterococci strains used in this study were routinely grown in PDA and MRS, respectively.

Experimental settings

Evaluation of the growth of E. faecium *strains in a dry-fermented sausage-based medium*

To check whether the enterococci strains were able to grow at conditions similar to those found in the ripening process of dry-fermented sausages, all the strains were tested at 3 different temperatures in a dry-fermented sausage-based medium. To prepare this medium, commercial industrial dry-fermented sausage ("salchichón") was first lyophilized in a freeze dryer for 2 days (VirTis Advantage 2.0 ES, SP. Industries VirTis, USA). Next, the meat-based medium was made using 30 g/l of the lyophilized dry-fermented sausage and 20 g/l of technical agar (CONDA, Spain). The medium was modified with 8.5% glycerol to reach 0.95 a_w. This a_w value is frequently found in meat during the first ripening stages coinciding when moulds start to grow (Lizaso et al. 1999). The a_w was measured using the a_w meter Lab Master (Novasina AG, Switzerland). After autoclaving at 121 °C and 103 kPa for 16 min, the culture medium was cold down up to 45–50 °C. Next, it was vigorously shaken and poured into 5-cm diameter Petri plates.

In the first screening, the six strains of *E. faecium* were inoculated in stripes in the dry-fermented sausage-based medium and incubated for 5 days at three temperatures commonly used during the dry-fermented sausage ripening (25, 20 and 15

°C). The growth was visually evaluated. This experiment was carried out in triplicate.

Examination of the antifungal activity of E. faecium *strains*

The four *E. faecium* strains showing the best adaptation to grow on a dry-fermented sausage-based medium at common temperatures used during the ripening of dry-fermented sausages were selected to study their antagonist effect against growth of *P. nordicum* and *P. verrucosum*. Fifty microlitres of each fresh *E. faecium* inoculum were separately spread plated on the medium surface. After allowing the inoculum to dry out, 2 μL of the inoculum of *P. verrucosum* or *P. nordicum* were inoculated onto the centre of each plate. The plates were incubated at 25, 20 and 15 °C for 1 week. This experiment was carried out in triplicate. At the end of the incubation period, the measurement of the diameter of the mould colonies in two directions at right angle to each other to know the growth of the moulds was performed. The antagonistic activity of the *E. faecium* strains was checked by comparing the diameter in the presence of enterococci to the absence of them (control batches) and calculating the percentage of growth reduction.

Assessment of the effect of two E. faecium *strains on growth and OTA production by* P. verrucosum *and* P. nordicum *under conditions simulating dry-fermented sausage ripening*

The two *E. faecium* strains showing the antifungal activity against both ochratoxigenic moulds, at three temperatures evaluated on the sausage-based medium, were chosen to examine their effect against growth of *P. verrucosum* and *P. nordicum* and their mycotoxin production under conditions simulating the dry-fermented sausage ripening.

The inoculation of the plates with both *E. faecium* and mould strains was conducted as explained in the previous section. Next, the plates were incubated for 2 days at 22 °C, 1 day at 19 °C and 18 days at 15 °C to simulate the wide range of conditions occurring throughout the dry-fermented sausage ripening in an industrial seasoning process (about 21 days length). All experiments were done with three replicates.

Growth assessment

The diameter of the colonies was daily measured in two directions at right angle to each other to know the growth of the moulds. In order to determine the relative growth rates of strains, primary modelling was carried out on the temporal radial colony expansion data. Data was fitted using a linear model obtained by plotting the results against time. Only the linear parts were used. From this primary model, the growth rates (radius mm/day) were obtained as the slope of the curve (Garcia et al. 2009).

OTA quantification

For OTA analysis, 1 g from the mould colonies was taken at 7, 15 and 21 day of processing and placed in a 2-ml microcentrifuge tube. All replicates were stored at− 20 °C prior to their extraction for the quantification of OTA.

OTA from samples was extracted using the QuEChERS-based procedure proposed by Kamala et al. (2015), with some modifications (Delgado et al. 2018a). Briefly, samples were dissolved in acetonitrile MS-grade acidified with 1% (v/v) formic acid after phase partitioning with aqueous salted solution. A 1 mL aliquot of the organic layer was taken and filtered through a 0.2-mm nylon filter for subsequent analyses.

OTA was analysed by an UHPLC system, Thermo Scientific Dionex UltiMate 3000 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). The stationary phase was a C_{18} reversed-phase column (100 mm × 2.1 mm, 2 μm; Agilent Technologies, USA). The mobile phase consisted of 0.1% formic acid, 10 mM ammonium formate (solvent A) and acetonitrile (solvent B). Analysis was done in a gradient mode from 2 to 98%. The injection volume was 10 μl and the flow rate was 0.25 ml/min. Mass spectrometry (MS) detection of OTA was performed using the precursor ion m z^{-1} 404 and the quantification ion m z^{-1} 358. The run time was 15 min, being OTA detected at 6.5 min. To determine OTA amounts (ng/g) the signals were processed by the Hystar 3.2 software (Bruker Daltonics Inc.).

The calibration curves for OTA (1–20 and 20–100 ng/g) by UHPLC-MS revealed a linear relationship ($r^2 \geq 0.99$) between detector response and amounts of OTA standards. The limit of detection (LOD) and limit of quantification (LOQ) were calculated as described by (Long and Winefordner 1983). The LOD and LOQ values were 1.3 ng/g and 3.9 ng/g, respectively. Mean recovery (accuracy) was around 95%. Quantities reported were corrected by recovery.

Statistical analysis

Statistical analysis was performed using the SPSS v.20 software. Data of growth and OTA production were tested for normality using the Shapiro-Wilk test. All data sets failed the normality test. Therefore, non-parametric data analysis was performed using the Kruskal-Wallis rank sum test. After that, the Mann-Whitney *U* test was applied to compare the median values obtained. The statistical significance was set at $p \leq 0.05$.

Results

Growth of *E. faecium* strains on a dry-fermented sausage-based medium

All the six *E. faecium* strains were able to grow at 25 °C, but the strains H238 and H107 grew neither at 20 nor at 15 °C (Table 1). Therefore, the strains Q233, SE920, SE260 and SE45 were selected to carry out further tests to analyse their antifungal activity against ochratoxigenic moulds.

Antifungal effect of *E. faecium* strains in a dry-fermented sausage-based medium

The antagonistic activity of the four *E. faecium* strains was evaluated by comparing the diameter of ochratoxigenic mould colonies in the presence of enterococci in relation to their absence. The effect of *E. faecium* against ochratoxigenic moulds was very different depending on the strain tested (Table 2). The strains Q233 and SE260 only affected the growth of *P. verrucosum*, causing a reduction between 3.57% and 33.33%. However, the strains SE920 and SE45 minimised the growth of both moulds between 10.71% and 26.66% with respect to the control batches at different temperatures (15 and 25 °C).

Antifungal activity of *E. faecium* under conditions simulating dry-fermented sausage ripening

Effect on growth

The effect of *E. faecium* strains SE920 and SE45 on *P. nordicum* and *P. verrucosum* growth rates in a dry-fermented sausage-based medium under ecological conditions usually reached during processing of this product is shown in Fig. 1. *P. nordicum* (2.9 mm/day) grew faster than *P. verrucosum* (1 mm/day) in all cases ($p \leq 0.05$). The presence of *E. faecium* strains did not significantly affect the growth *of P. nordicum*, but they stimulated the growth rate of *P. verrucosum*, being this effect more pronounced with *E. faecium* SE45 (2.02 mm/day) than with *E. faecium* SE920 (1.73 mm/day).

Table 1 Growth of *Enterococcus faecium* strains on a dry-fermented sausage-based medium at 25, 20 and 15 °C after a 5-day incubation period

Strains	Temperature (°C)		
	25	20	15
QE233	+[a]	+	+
SE920	++	+	+
SE260	++	+	+
HE238	+	n.d.	n.d.
HE107	+	n.d.	n.d.
SE45	++	+	+

[a] : ++: abundant growth; +: normal growth; n.d.: growth no detected

Effect on mycotoxin production

Figure 2 shows the effect on OTA production by the two ochratoxigenic mould strains when co-inoculated with *E. faecium* SE920 and SE45 at 7, 15 and 21 days compared to the control batches. *P. verrucosum* produced higher amounts of OTA than *P. nordicum* at 7 and 15 days ($p \leq 0.05$). However, *P. nordicum* produced the highest OTA amounts at 21 days (25.12 ± 3.50 ng/g).

Despite OTA production by *P. nordicum* significantly increased throughout the incubation time ($p \leq 0.05$), a significant decrease was observed when it was co-inoculated with both *E. faecium* strains, especially with the SE920 strain at 21 days (4.51 ± 1.14 ng/g). However, in *P. verrucosum*, a significant decrease in OTA production was observed in the presence of *E. faecium* SE45 only at the first sampling day; whilst at the end of the incubation period, no influence on OTA concentration was observed.

Discussion

The use of LAB as protective culture against toxigenic moulds has been extensively described in the literature (Dalié et al. 2010). However, only a reduced number of studies have focused on the antifungal potential of *Enterococcus* sp. (Belguesmia et al. 2013; Taghi-zadeh and Nejati 2017). Besides, the interaction between ochratoxigenic moulds and *E. faecium* in a meat substrate has not been studied yet.

In this work, two different ochratoxigenic *Penicillium* species, *P. nordicum* and *P. verrucosum*, usually found in dry-cured meat products (Castellari et al. 2010; Iacumin et al. 2009), were chosen to test the antifungal activity of some enterococci as biopreservative candidate. This study has examined the effect of *E. faecium* strains selected for probiotic meat cultures (Ruiz-Moyano et al. 2008, 2009), on the growth and OTA production by these *Penicillium* strains.

To simulate the real environmental conditions occurring in a dry-fermented sausage industry, *E. faecium* strains were selected according to their capacity to grow on a culture medium made with dry-fermented sausages under temperature and a_w conditions commonly reached throughout the processing of these products. For this assay, three fixed temperatures (25, 20 and 15 °C) related to their manufacture were tested. All strains tested grew at 25 and 20 °C, but only 4 were able to grow at 15 °C (QE233, S260, SE45 and SE920). This result could be related to the origin of the strains. The selected 4

Table 2 Percentage of growth reduction of *Penicillium verrucosum* FHS Pv45 and *Penicillium nordicum* CBS 110.769 when co-inoculated with four *Enterococcus faecium* strains (QE233, SE260, SE920 and SE45) on a dry-fermented sausage-based medium at 25, 20 and 15 °C for 7 days

	Temperature (°C)	*Enterococcus faecium* strains			
		QE233	SE260	SE920	SE45
P. verrucosum	25	11.76 ± 0.51[a]	-[b]	-	
	20	33.33 ± 1.81	-	-	-
	15	-	3.57 ± 2.52	10.71 ± 5.21	14.28 ± 5.38
P. nordicum	25	-	-	15.55 ± 2.84	26.66 ± 4.26
	20	-	-	-	-
	15	-	-	-	-

[a] Percentage of growth reduction respect to the control

[b] No significant growth reduction of ochratoxigenic moulds

strains were isolated from Iberian dry-fermented sausages, so they are better adapted to this niche than the other 2 strains (Ruiz-Moyano et al. 2008).

The selected enterococci were co-inoculated with ochratoxigenic strains to test their effect on the fungal growth. After one week of incubation, *E. faecium* strains showed different behaviours. The strains SE920 and SE45 inhibited the growth of both fungal strains depending on the incubation temperature. However, the strains QE233 and SE260 only reduced the growth of *P. verrucosum*. The antifungal activity is then strain-dependent, as it has been previously proven in other studies conducted with LAB such as *L. plantarum* (Dal Bello et al. 2007; Guimarães et al. 2018; Russo et al. 2015; Taghi-zadeh and Nejati 2017).

Due to the effect on the growth of both moulds, the *E. faecium* strains SE920 and SE45 were selected for the next study based on the environmental conditions reached in an industrial process on a small scale. Given that the usual temperatures during the dry-fermented sausage ripening ranged from 22-12 °C (Bernáldez et al. 2013; Ferrara et al. 2016; Pleadin et al. 2016), a simulation of the above conditions was made, by incubating enterococci and moulds for 21 days length, decreasing the temperatures from 22 to 15 °C progressively. In this assay, *P. nordicum* grew much faster than *P. verrucosum*. This agrees with previous studies that have stated that *P. nordicum* is ecologically adapted to niches containing moderate NaCl quantities (Rodríguez et al. 2015; Schmidt-Heydt et al. 2012;). The optimum growth on a dry-fermented sausage-based medium for *P. nordicum* was reported at 0.94 a_w and 20 °C whilst for *P. verrucosum* was at 0.94 a_w and 25 °C (Rodríguez et al. 2015).

The growth of *P. nordicum* was not affected by the presence of enterococci, but that of *P. verrucosum* was significantly increased. This different effect has been also found by other

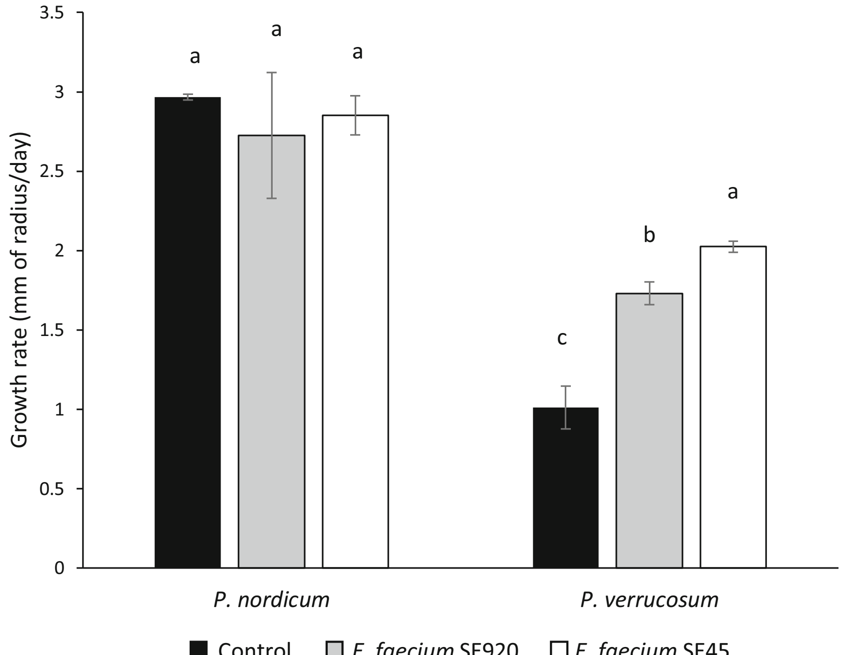

Fig. 1 Effect of the *Enterococcus faecium* SE920 and *Enterococcus faecium* SE45 on growth of *Penicillium nordicum* CBS 110.769 and *Penicillium verrucosum* FHS Pv45 on a dry-fermented sausage-based medium for 21 days of incubation. Bars indicate standard deviation of the means. Statistically significant differences represented by letters

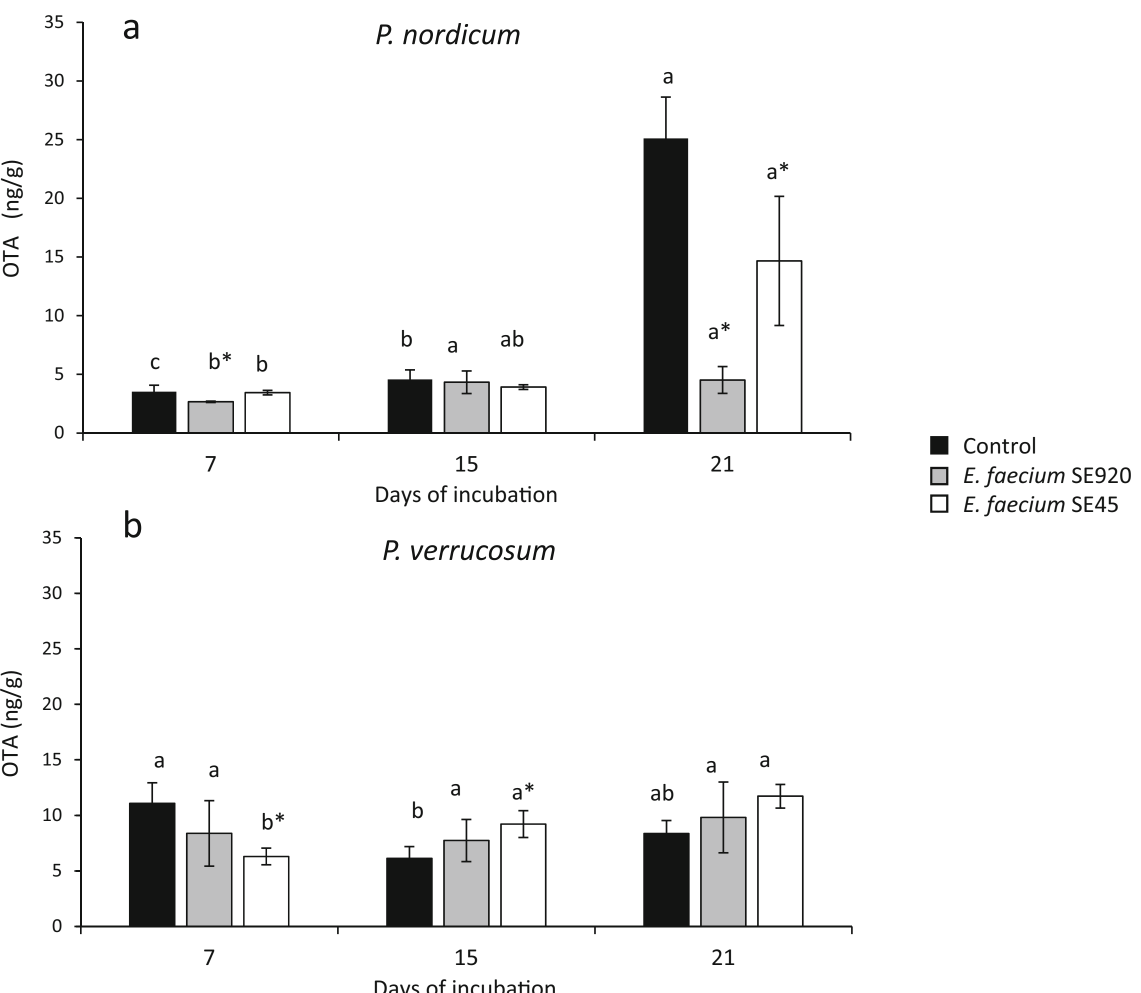

Fig. 2 Effects of **a** *Enterococcus faecium* SE920 and *Enterococcus faecium* SE45 on ochratoxin A (OTA) production by *Penicillium nordicum* CBS 110.769 and **b** *Penicillium verrucosum* FHS Pv45 on a dry-fermented sausage-based medium at 7, 15 and 21 days of the incubation. Bars indicate standard deviation of the means. Different letters mean significant differences at different incubation times. Significance differences between co-inoculated and control batches at each incubation time is represented by an asterisc (*)

authors, indicating that it could be due to the removal of acids and/or to the production of stimulatory metabolites by the bacteria (Gourama and Bullerman 1995; Moore-Landecker and Stotzky 1974; Wiseman and Marth 1981). Furthermore, likely the count level of enterococci used could be too low to inhibit the growth of moulds (Fernandez et al. 2017), so a higher inoculum concentration would probably be needed. On the other hand, given that the assay was designed to simulate the conditions of the dry-fermented sausage processing, the culture conditions were more appropriate for mould growth than for enterococci metabolism. The temperature (15–25 °C) were nearer to the optimal growth of *P. nordicum* and *P. verrucosum* (Rodríguez et al. 2015), whilst the optimum for *E. faecium* strains is generally around 37 °C (Yang et al. 2018). In addition, the incubation was conducted under aerobic conditions, which favour mould growth but not enterococci, which prefer microaerophilic conditions to grow.

In relation to OTA production, *P. verrucosum* FHS Pv45 produced more mycotoxin than *P. nordicum* CBS 110.769 during the first 15 days. These results agree with similar studies in dry-cured sausage-based media, where *P. verrucosum* produced more OTA amounts after 12 days at similar a_w and temperatures (Rodríguez et al. 2015). Nevertheless, at day 21 of incubation, the OTA amounts were higher in *P. nordicum*.

Although a growth reduction of *P. nordicum* and *P. verrucosum* was not observed, the presence of *E. faecium* SE920 resulted in a significant decrease in the OTA concentration in *P. nordicum* after 21 days of incubation. This is the most important aspect when a control strategy is searched for limiting the production of mycotoxins in foods.

As discussed above, the low initial inoculum concentration of enterococci could be related to the limited effect on OTA production by *P. verrucosum*. However, in previous studies, LAB was able to significantly reduce mycotoxin production

when they were inoculated at the same ratio (50:50) that mould spores (Al-Saad et al. 2016; Belkacem-Hanfi et al. 2014). Then, it could be suggested that the *E. faecium* strains SE920 and SE45 are able to reduce OTA production by *P. nordicum*, but they are not effective against *P. verrucosum*. Therefore, the production of mycotoxins does not correlate with the growth rate in the presence of enterococci. Recent studies have demonstrated that OTA production by *P. nordicum* and *P. verrucosum* is increased at suboptimal growing conditions (Sánchez-Montero et al. 2019), and the mould growth should not be considered as a good predictor of OTA contamination (Gil-Serna et al. 2014). Hence, the effect of *E. faecium* over the OTA concentration by strains of *P. nordicum* is due to mechanisms of action different than those limiting mould growth. In this sense, OTA detoxification mediated by LAB has been recorded for different species mainly from *Bifidobacterium* and *Lactobacillus* genera by enzymatic degradation or by the adsorption on the cell wall (Luz et al. 2018). *E. faecium* strains are able to bind both aflatoxin B_1 and patulin from aqueous solutions (Topcu et al. 2010). Nevertheless, no information about the detoxification of OTA by *E. faecium* in meat substrates is still available, and further investigations are required to assess the modes of action responsible for reduction of OTA production by *P. nordicum* of the selected *E. faecium* strains SE920 and SE45.

As it can be observed in Fig. 2, although the two enterococci strains had an antifungal effect at 21 days of incubation against *P. nordicum*, *E. faecium* SE920 controlled better the production of OTA than *E. faecium* SE45. Although *E. faecium* SE920 is not able to reduce OTA amounts to levels below those established in some producer countries (Ministero della Sanità 1999), it is capable of minimising the mycotoxin content up to 82.01% regarding the control batch. Then, although the effect of the latter strain was not enough to fully suppress the OTA production by *P. nordicum* in meat substrates, *E. faecium* SE920 could contribute to reduce accumulation of this mycotoxin together with other protective cultures, such as yeasts or non-toxigenic moulds isolated from dry-cured meat (Bernáldez et al. 2013; Delgado et al. 2018b; Peromingo et al. 2018). Given that the growth of enterococci normally occurs in the first phases of the processing of dry-cured meat products, according to our results, a delay in mycotoxin production can be expected for long enough to allow the development of other biocontrol agents that can help to effectively control the hazard associated with OTA.

In addition, *E. faecium* SE920 has been considered safe with regards to its low aminogenic potential, low antibiotic resistance pattern and absence of virulence determinants genes (Ruiz-Moyano et al. 2009). Then, this strain exhibits favourable characteristics to be used as a protective culture in dry-fermented sausages. Despite the influence of this strain over *P. nordicum* is promising, it would be necessary to study higher levels of initial bacterial inoculum or the co-inoculation with other biocontrol agents to achieve the complete control of ochratoxigenic moulds. In addition, further experiments would be necessary to study whether the reduction of OTA is due to detoxification (degradation or adsorption) or alteration of the expression of the genes involved in OTA biosynthesis. Moreover, the use of *E. faecium* SE920 should be carefully evaluated during the processing of this food to study its effects on the sensory characteristics of final products.

E. faecium SE920, isolated from dry-fermented sausages, triggers a reduction of OTA concentration in *P. nordicum*. Then, this strain may be a feasible biocontrol agent to reduce OTA levels in dry-fermented sausages in the context of the preventive measures within the HACCP system in the meat industry.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

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IV.2. Selección de especias como agentes de biocontrol frente a *Penicillium nordicum*

In vitro *antifungal effects of spices on ochratoxin A production and related gene expression in* Penicillium nordicum *on a dry-cured fermented sausage medium*

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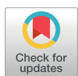

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Spices

ABSTRACT

Penicillium nordicum, an ochratoxin A (OTA) producer, widely contaminates the surface of dry-cured fermented sausages. Although the meat industry uses antifungal synthetic compounds during ripening, consumers' preferences currently stand up for natural preservatives. The aim of this study was to evaluate the antifungal effect of spices commonly added to dry-cured fermented sausages during their processing on *P. nordicum* for establishing their value as an alternative strategy to synthetic compounds. The mould strain was grown on a dry-cured fermented sausage-based medium containing oregano, rosemary or thyme. Moreover, an antifungal commercial preparation containing potassium sorbate and natamycin was tested alone and in combination with the spices. The growth rate, OTA accumulation and relative expression of genes involved in OTA biosynthesis (*otapks* and *otanps*) and some stress pathways (*Hog1* and *Rho1*) were evaluated. In the growth assessment, the oregano alone or in combination with the antifungal preparation significantly reduced the growth rate. Different patterns were observed at both sampling times for the OTA production and gene expression analyses, and even differences between treatments at the same incubation period were observed. Regarding OTA accumulation, significant reductions were found when adding oregano or rosemary in the presence and absence of the antifungal compounds. While a stimulation of the expression of most of the tested genes was obtained at the initial stages, a repression was generally found at the end of the incubation. This makes sense since the mycotoxin accumulation was usually higher at the initial stages than at the end of the incubation. Accordingly, such spices would allow satisfying the current consumers' demand for natural preservatives minimising the hazard associated with the OTA presence in cured meats at the same time.

1. Introduction

Dry-cured fermented sausages are manufactured and consumed worldwide, being highly appreciated by consumers, which demand high quality and safe products (Asefa et al., 2010; Ferrara et al., 2016). The surface of such products is colonised throughout their ripening by moulds, being some of them able to produce ochratoxin A (OTA), which is the mycotoxin most commonly found in dry-cured fermented sausages (Iacumin, Milesi, Pirani, Comi, & Chiesa, 2011, 2009; Markov et al., 2013). The accumulation of this mycotoxin in meat derived products has been generally related to the presence of *Penicillium nordicum* and *Penicillium verrucosum* on their surfaces during ripening (Iacumin et al., 2009; Rodríguez, Rodríguez, Martín, Delgado, & Córdoba, 2012). OTA has negative effects in tested animals, including nephrotoxicity, immunotoxicity or neurotoxicity, leading to a possible risk on human health (EFSA Panel on Contaminants in the Food Chain, 2006; Pfohl-Leszkowicz & Manderville, 2007). Moreover, OTA has been classified as a possible carcinogenic to human (Group 2B; IARC, 1993).

The development of strategies to control the OTA presence in dry-cured meat products has gained increasing interest in the last years. Nowadays, the meat industry usually uses antifungal synthetic compounds to avoid the growth of both toxigenic and spoilage moulds throughout the processing of dry-cured fermented sausages. Therefore, the utilisation of natamycin and sorbic acid or benzoic acid and their

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salts is now permitted in the European Union (European Commission, 2011). Nevertheless, the consumers' preferences are currently based on food free of synthetic compounds, which could be influenced by some studies that suggest the potential toxicity of some preservatives for human beings (Piper & Piper, 2017; Saatci et al., 2016) and their effect on the development of allergies (Jacob, Hill, Lucero, & Nedorost, 2016). Thus, last tendencies try to reduce the use of synthetic fungicides and increase that of natural preservatives, such as plant products. Furthermore, it has been reported that the continuous or inappropriate application of the synthetic compounds at sub-inhibitory concentrations to control the mould growth could favour their resistance to them and even the stimulation of the OTA production (Alcano et al., 2016; Arroyo, Aldred, & Magan, 2005). In addition, strategies relied on an adjustment of environmental manufacturing conditions, biopreservation and matrix composition have been described for minimising the mycotoxin hazard in dry-cured fermented sausages (Delgado, Peromingo, Rodríguez, & Rodríguez, 2019; Peromingo, Andrade, Delgado, Sánchez-Montero, & Núñez, 2019; Peromingo, Núñez, Rodríguez, Alía, & Andrade, 2018; Sánchez-Montero, Córdoba, Alía, Peromingo, & Núñez, 2019a; Sánchez-Montero, Córdoba, Peromingo, Álvarez, & Núñez, 2019b; Álvarez, Rodríguez, Peromingo, Núñez, & Rodríguez, 2019). Until now the effect of the substrate composition on reducing or preventing the OTA presence has been scarcely studied in cured meat products. Concretely, the influence of different concentrations of NaCl on the growth and OTA production by *P. nordicum* and *P. verrucosum* has been evaluated (Rodríguez, Capela, Medina, Córdoba, & Magan, 2015; Rodríguez, Medina, Córdoba, & Magan, 2014; Schmidt-Heydt, Graf, Stoll, & Geisen, 2012). Furthermore, Andrade, Peromingo, Rodríguez, and Rodríguez (2019) have recently reported that the use of KCl instead of NaCl or the addition of sucrose were able to reduce the OTA accumulation *in vitro*. Concerning dry-cured fermented sausages, a variable number of other additives are used for their manufacturing, such as spices, which have proved to contribute to their flavour (Kargozari et al., 2014) and prevent oxidation reactions (Oswell, Thippareddi, & Pegg, 2018; Shah, Bosco, & Mir, 2014). Additionally, spices have a long history of use over an extensive range of human exposures without known adverse effects. In fact, rosemary, oregano and thyme are generally recognized as safe (GRAS) for their intended use in foods by the Food and Drug Administration (2019). Regarding the antimicrobial activity of spices, although their role as preservative is well-known, the existing literature is only related to paprika and essential oils obtained from several spices but not to the plants. The addition of 2–3% of paprika favoured the growth of moulds but reduced their OTA production on a dry-cured meat substrate (Sánchez-Montero et al., 2019a). Several kinds of essential oils from different herbs and plants have also exhibited antagonistic activity against unwanted bacteria in meat and meat products, such as *Salmonella* spp. or *Listeria monocytogenes* (García-Díez et al., 2016; Pesavento et al., 2015; Vasilijević et al., 2019). Despite the fact that the antifungal properties of essential oils have been reported, literature focused on their effect against toxigenic moulds with interest in dry-cured meat product is not available yet. So far, only the impact of essential oils on OTA biosynthesis has been studied in Aspergilla, being attributed to the tested extracts different levels of efficacy to control the mycotoxin accumulation (Basílico & Basílico, 1999; Chaves-López et al., 2012; El Khoury et al., 2017; Lappa, Simini, Nychas, & Panagou, 2017b).

During last years many studies have analysed the relationship between the OTA biosynthesis and the expression of different genes governing such production when applying different treatments. Thus, the effect of ecophysiological conditions (Lappa, Kizis, & Panagou, 2017a; Rodríguez et al., 2014), protective cultures (Cebrián, Rodríguez, Peromingo, Bermúdez, & Núñez, 2019; Cubaiu, Abbas, Dobson, Budroni, & Migheli, 2012; Gil-Serna, Patiño, Cortés, González-Jaén, & Vázquez, 2011; Peromingo et al., 2018), essential oils (El Khoury et al., 2016; Lappa, Kizis, & Panagou, 2017a; Tannous et al., 2015) or commercial antifungal compounds (Fodil, Delgado, Varvaro, Yaseen, & Rodríguez, 2018) on the OTA production and the expression of genes potentially involved in the OTA biosynthesis has been evaluated. The expression of the key genes in Penicillia for the OTA biosynthesis *otapks* or *otanps* has been thus studied (Peromingo et al., 2018; Rodríguez et al., 2014). Moreover, stressful situations have been described as a possible inductor of secondary metabolite synthesis by filamentous fungi such as mycotoxins (Medina et al., 2014; Ponts, 2015; Schmidt-Heydt, Stoll, & Geisen, 2013). Therefore, the expression of genes related to the pathways used for moulds to overcome different kind of stress, such as *Hog1* and *Rho1*, has been associated with mycotoxin production (Delgado, da Cruz Cabral, Rodríguez, & Rodríguez, 2018; Fodil et al., 2018). The *Hog1* gene is the core gene of the high osmolarity glycerol (HOG) response pathway, which is responsible for the survival of moulds in osmotic and oxidative stresses (Brewster & Gustin, 2014; Miskei, Karányi, & Pócsi, 2009). The *Rho1* gene is considered the master regulator of the cell wall integrity pathway (Levin, 2005), which seems to be involved in the tolerance of moulds against antifungal compounds (Hayes, Anderson, Traven, van der Weerden, & Bleackley, 2014).

The aim of the study was to evaluate the effect of rosemary, oregano and thyme added as ingredients during the manufacturing of dry-cured fermented sausages on the *P. nordicum* growth and OTA accumulation on a dry-cured fermented sausage-based medium. The expression of two genes associated with the target mycotoxin production and two stress response pathways (osmotic and cell wall integrity routes) was also checked.

2. Materials and methods

2.1. Plant materials

The herbal samples consisted of three spices plants namely the leaves of rosemary (*Rosmarinus officinalis*), oregano (*Origanum vulgare*) and thyme (*Thymus zygis*). They were recollected in the region of Extremadura, located in the southwest of Spain. While oregano leaves were received dried at the laboratory, raw rosemary and thyme leaves were used. When the raw material was not used at the same day of harvest, it was kept at −20 °C until further use.

2.2. Culture media

Yeast Extract Sucrose agar (YES) was prepared by adding 20 g/L of yeast extract (Scharlab, S.L., Spain), 20 g/L of bacteriological agar (Scharlab, S.L.) and 125 g/L of sucrose (Scharlab, S. L.).

A dry-cured fermented sausage-based agar (FS) containing 250 g/L of lyophilised dry-cured fermented sausage "salchichón" and 20 g/L of bacteriological agar was used in this study to simulate the composition of dry-cured fermented sausages. The meat product was lyophilised in a freeze dryer (Labconco®, USA). The basic FS was supplemented with 2 g/kg of the three evaluated spices, rosemary (FS-R), oregano (FS-O) and thyme (FS-T), which is the amount commonly used during the dry-cured fermented sausage manufacturing (Vignolo, Fontana, & Fadda, 2010).

After autoclaving for 20 min at 121 °C, both YES and FS were cold down up to 45–50 °C and vigorously shaken prior to pouring into 9-cm and 5-cm diameter Petri plates, respectively.

To simulate the processing conditions of industrial dry-cured fermented sausages, a collagen casing (Viscofan, Spain) was placed on the surfaces of FS, FS-R, FS-O and FS-T. The casings had been previously treated by immersion in ethanol 100% (v/v) for 1 s and subsequently exposed to UV radiation for 24 h in a laminar flow cabinet (Telstar, Japan).

Additionally, a mix containing a commercial antifungal preparation (8.3% of potassium sorbate and 2% of natamycin) was supplemented with 60 g/L of NaCl (Fisher Scientific S.L., USA) according to the manufacturer's instructions (Taberner S.A., Spain). This antifungal preparation was used as positive control (FS-AP). Concretely, 25 μL of

the antifungal preparation were spread on the surface of the casings previously placed on the FS using a Drigalsky spatula. Besides, the antifungal preparation was spread on plates previously supplemented with rosemary (FS-R-AP), oregano (FS-O-AP) and thyme (FS-T-AP).

2.3. Mould strain, inoculum and inoculation

One strain of *P. nordicum* (CBS 323.92) belonging to Centraalbureau voor Schimmelcultures (The Netherlands) was used. The mould strain was 3-point inoculated on YES and incubated for 10 days at 25 °C to obtain a heavily sporulating culture. The spores were then collected by scraping the surface of the plate, diluting them in phosphate-buffered saline (PBS) buffer [0.32 g of NaH_2PO_4 (Scharlab, S. L.), 1.09 g of Na_2HPO_4 (Scharlab, S.L.), 9 g of NaCl, 1 L of distilled water]. The total amount of spores was quantified by using a Thoma counting chamber Blaubrand® (Brand, Germany) and visualised in a microscope (NIKON, Japan). The spore suspension was adjusted to 10^6 spores/mL and used as an inoculum.

Agar plates containing the different treatments (FS, FS-R, FS-O, FS-T, FS-AP, FS-R-AP, FS-O-AP, FS-T-AP) were then centrally inoculated with 2 µL of the mould inoculum and incubated for 14 days at 12 °C, which is a common temperature used throughout the processing of industrial dry-cured fermented sausages (Bernáldez et al., 2013; Lorenzo, González-Rodríguez, Sánchez, Amado, & Franco, 2013). Three replicates of each treatment were evaluated.

2.4. Growth assessment

The diameter of the mould colony was daily measured in two perpendicular directions to determine the lag phase prior to growth (λ, in days) and the maximum growth rate (μ_m, in mm of radius/day). The radii of the colony were plotted against each incubation time. Data plots showed, after a lag phase, a linear regression and μ_m was obtained as the slope of the line. The λ was calculated by equalling the regression line formula to the original inoculum size (diameter, mm).

2.5. OTA analysis

OTA production by *P. nordicum* CBS 323.92 was evaluated at days 7 and 14 of incubation. Additionally, two kinds of samples were obtained at each sampling time: 1) mould mycelium (including casing), and 2) ≈ 1 g agar within the area below the casing where the mould grew. All samples were weighed before storing at −20 °C until their extraction. The QuEChERS procedure previously described by Delgado et al. (2018) was used for OTA extraction. An Agilent 1290 Infinity II UHPLC coupled with 6470 triple quadrupole (QqQ) (Agilent Technologies, Inc., USA) was used. The UHPLC was equipped with a built-in auto-degasser, binary pump and column thermostat. The separation was achieved through the use of a Zorbax C_{18} column, 100 mm × 2.1 mm, 1.8 µm (Agilent Technologies, Inc.) working at 45 °C. The mobile phase was composed by two solutions: (A) milli-Q water acidified with formic acid 0.1% and (B) acetonitrile acidified with formic acid 0.1%. The separation was carried out at 0.4 mL/min using a binary gradient solvent: 0–3.5 min 20% eluent B, 3.5–10 min linear increase from 20 to 95% eluent B, a plateau phase after eluent B raised to 100% eluent B and kept until 12 min and a re-equilibration of the column for 3 min at 20% eluent B. OTA was eluted at 6.5 min. The injection volume was 7 µL and the run time was 15 min.

The LC-MS detector was equipped with Agilent Jet Stream electrospray ionisation (ESI) Source, which conducted the assays in positive ionisation mode. Nitrogen was used as nebulising, drying, sheath and collision gas. The operating ionisation source parameters were drying gas temperature at 300 °C and flow rate at 12 L/min, nebuliser at 30 psi, sheath gas temperature at 350 °C and flow rate of 10 L/min, capillary voltage at 3000 V and fragmentor voltage at 100 V. The optimum Multiple Reaction Monitoring (MRM) conditions were from the sensitivity and selectivity points of view the transitions from 404 to 358 at 10 V collision energy (CE) and from 404 to 239 at 25 V CE, respectively. Signals were processed by the MassHunter Software (Agilent Technologies Inc.).

The limits of detection (LOD) and quantitation (LOQ) defined as the concentrations of the analyte producing the signal-to-noise ratios of 3:1 and 10:1, respectively, were calculated using the following equations: LOD = 3SD and LOQ = 10SD, where SD is the standard deviation of the average (n = 3) of the concentration obtained for the calibration solution of the lowest concentration by the calibration curve. Concretely, the LOD and LOQ were 0.20 and 0.50 ng/mL, respectively.

2.6. Gene expression studies

2.6.1. Sampling and sample preparation

Sampling for gene expression studies was performed at days 5 and 12 of incubation (2 days before collecting samples for OTA determination). After each incubation time, mycelia containing the whole colony was removed under sterile conditions and weighed in sterile foils. Mycelia were immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

2.6.2. RNA extraction and cDNA synthesis

Frozen mycelia were ground to fine powder using a pre-frozen mortar and a pestle. The RNA extraction was carried out by using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, USA) following the manufacturer's instructions (Protocol A). The RNA concentration (µg/µL) and purity (A_{260}/A_{280} ratio) were spectrophotometrically determined using a 1.5 µL aliquot on a NanoDrop™ (Thermo Fisher Scientific, USA). Samples were then diluted to 0.1 µg/µL and treated with the DNAse I RNase-free kit (Thermo Fisher Scientific) according to the manufacturer's instructions in order to remove genomic DNA. Retrotranscription was performed using 5 µL of total RNA (≈500 ng) using the PrimeScript™ RT reagent Kit (Takara Bio Inc., Japan) as described by the manufacturer. The cDNA samples were stored at −20 °C until analysis.

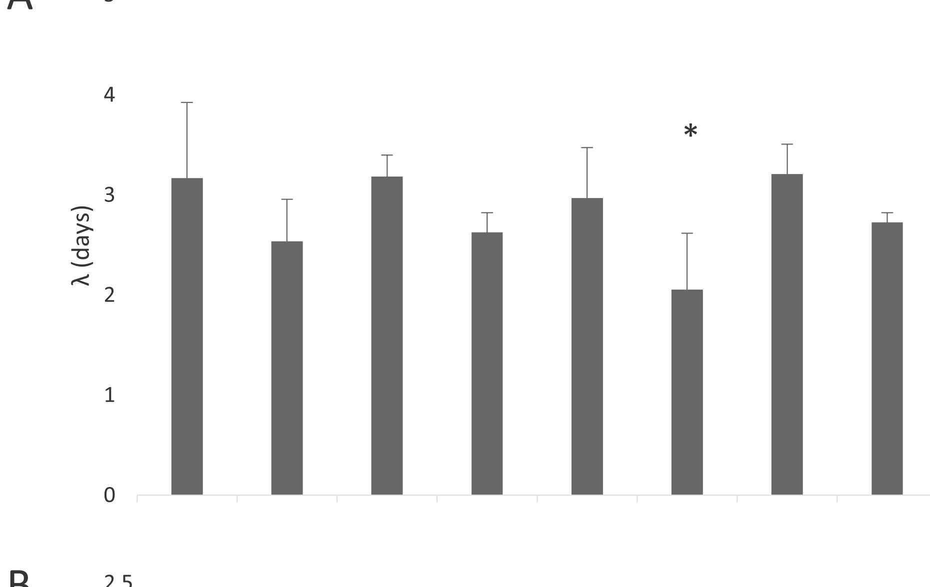
2.6.3. Real-time PCR reactions and relative gene expression determination

The relative expression of four genes was quantified using the SYBR Green technology on the Applied Biosystems 7500 Fast Real-Time PCR (Applied Biosystems, USA). Primer sets, mix reactions and qPCR thermal conditions were the same that those previously used for the amplification of the *otapks*, *otanps*, *Hog1* and *Rho1* genes (Bernáldez, Córdoba, Andrade, Alía, & Rodríguez, 2017; da Cruz Cabral, Delgado, Andrade, Rodríguez, & Rodríguez, 2018; Rodríguez, Rodríguez, Luque, Justesen, & Córdoba, 2011, Rodríguez, Medina, Córdoba, & Magan, 2016). The threshold cycle (C_T) value, corresponding to the PCR cycle number at which fluorescence was detected above threshold, was calculated by the 7500 Fast System SDS software (Applied Biosystems).

The relative quantification of gene expression was calculated according to the $2_T^{-\Delta\Delta C}$ method reported by Livak and Schmittgen (2001). As endogenous control, the *β-tubulin* gene was used to normalise the quantification of the mRNA target. The samples of *P. nordicum* in FS at each incubation time were used as calibrator.

2.7. Statistical analysis

Data analysis was carried out using the SPSS v. 20 software (IBM Corporation, USA). The results obtained from the growth assessment, OTA production and relative gene expression were tested for normality. Due to the fact that all data failed the normality test, the non-parametric analyses were performed using the Kruskal-Wallis and Mann-Whitney U tests. The relationships between the OTA production and the gene expression were analysed by calculating the Spearman's correlation coefficients. The statistical significance was stablished at $p \leq 0.05$.



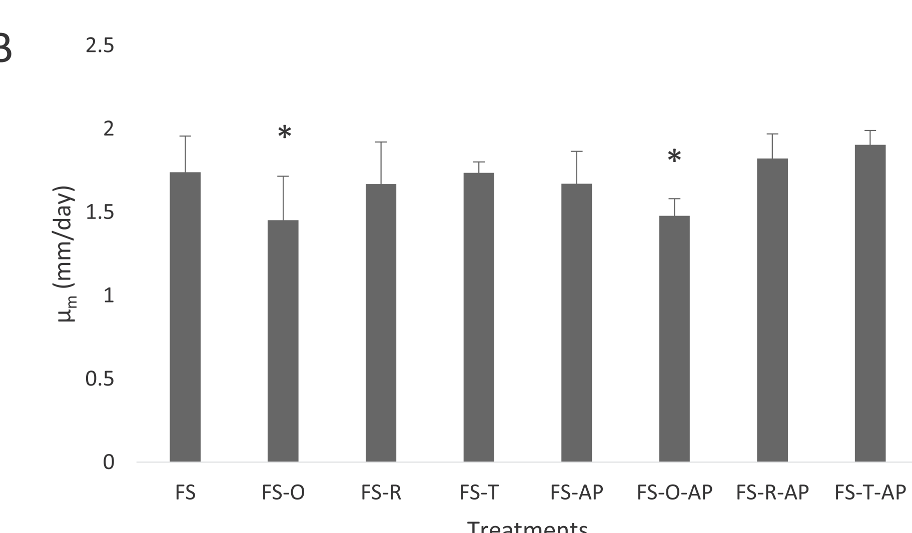


Figure 1. Lag phase prior to growth (λ, days) and maximum growth rate (μ_m, mm/day) of *Penicillium nordicum* CBS 323.92 grown during 14 days at 12 °C in a dry-cured fermented sausage-based agar (FS) with the addition of different spices (oregano, rosemary and thyme) and a commercial antifungal preparation.
Treatments: FS: non-treated control; FS-O: oregano; FS-R: rosemary; FS-T: thyme; FS-AP: antifungal preparation; FS-O-AP: oregano + antifungal preparation; FS-R-AP: rosemary + antifungal preparation; FS-T-AP: thyme + antifungal preparation. Statistical differences between the non-treated control and treatments are indicated by an asterisk ($p \leq 0.05$).

3. Results and discussion

3.1. Growth assessment

Regarding the growth assessment, the effect of the different treatments on the λ and μ_m of *P. nordicum* CBS 323.92 is shown in Fig. 1. Only the combination of oregano with the antifungal preparation (FS-O-AP) significantly shortened its λ compared to the non-treated control (FS; Fig. 1A) but any of them significantly lengthened this parameter. Concerning the μ_m, both the individual application of oregano (FS-O) and its combination with the antifungal preparation (FS-O-AP) provoked a significant slowdown. As shown in Table 1, there were significant inter-treatment differences when they were compared for both the λ and the μ_m. It is remarkable that the μ_m of the combination of oregano and the antifungal preparation (FS-O-AP) showed significant differences with the remaining treatments. Although the λ was shortened when combining oregano and the antifungal preparation, which means that the mould starts to grow earlier, this treatment reduced the μ_m. These results are of great importance from the food safety point of view since the time window to take preventive measures for avoiding mycotoxin production increases.

3.2. OTA analysis

Fig. 2 summarised the effect of the different treatments on the OTA accumulation in the modified and unmodified FS. The quantities of OTA found in the superficial portion of the non-treated control (FS) were 32.8 and 97.2 ng/g at days 7 and 14, respectively. At day 7 of incubation, none of the treatments significantly reduced the OTA presence with respect to the control (FS) in the casing. However, the addition of oregano (FS-O), the antifungal preparation (FS-AP) and the combination of thyme and the antifungal preparation (FS-T-AP) significantly stimulated the OTA production compared to the non-treated control. By contrast, at day 14 of incubation the treatments that included oregano or rosemary, with or without the addition of the antifungal preparation (FS-O, FS-R, FS-O-AP, FS-R-AP), reduced significantly the levels of OTA. These latter findings are in accordance with several studies where essential oils from different herbs or plants have shown effective inhibition of mycotoxin accumulation (Basílico & Basílico, 1999; El Khoury et al., 2016; Hu, Zhang, Kong, Zhao, & Yang, 2017; Razzaghi-Abyaneh et al., 2009; Sun, Wang, Lu, & Liu, 2015). El Khoury et al. (2017) evaluated the effect of essential oils from rosemary, oregano and thyme against ochratoxigenic *Aspergillus*

Table 1
Mann Whitney U statistical analysis performed with data about the lag phase prior to growth (λ) and maximum growth rate (μ_m) of *Penicillium nordicum* CBS 323.92 grown in a dry-cured fermented sausage-based agar (FS) with the addition of different spices (oregano, rosemary and thyme) and a commercial antifungal preparation.

	p-value of studied factors													
	λ							μ_m						
Treatments[a]	FS-O	FS-R	FS-T	FS-AP	FS-O-AP	FS-R-AP	FS-T-AP	FS-O	FS-R	FS-T	FS-AP	FS-O-AP	FS-R-AP	FS-T-AP
FS-O	–	0.05*	0.82	0.31	0.18	0.03*	0.82	–	0.11	0.10	0.11	1.00	0.05*	0.25
FS-R	0.05*	–	0.05*	0.69	0.02*	1.00	0.05*	0.11	–	0.45	0.89	0.04*	0.22	0.05*
FS-T	0.82	0.05*	–	0.48	0.10	0.34	0.51	0.10	0.45	–	0.90	0.02*	0.28	0.12
FS-AP	0.31	0.69	0.48	–	0.20*	0.36	0.81	0.11	0.89	0.90	–	0.05*	0.39	0.05*
FS-O-AP	0.18	0.02*	0.10	0.20	–	0.01*	0.02*	1.00	0.04*	0.02*	0.05*	–	0.01*	0.02*
FS-R-AP	0.03*	1.00	0.34	0.36	0.01*	–	0.03*	0.05*	0.22	0.28	0.39	0.01*	–	0.28
FS-T-AP	0.82	0.05*	0.51	0.81	0.02*	0.03*	–	0.02*	0.05*	0.12	0.05*	0.02*	0.28	–

*Indicates statistical differences ($p \leq 0.05$).
[a] FS-O: oregano; FS-R: rosemary; FS-T: thyme; FS-AP: antifungal preparation; FS-O-AP: oregano + antifungal preparation; FS-R-AP: rosemary + antifungal preparation; FS-T-AP: thyme + antifungal preparation.

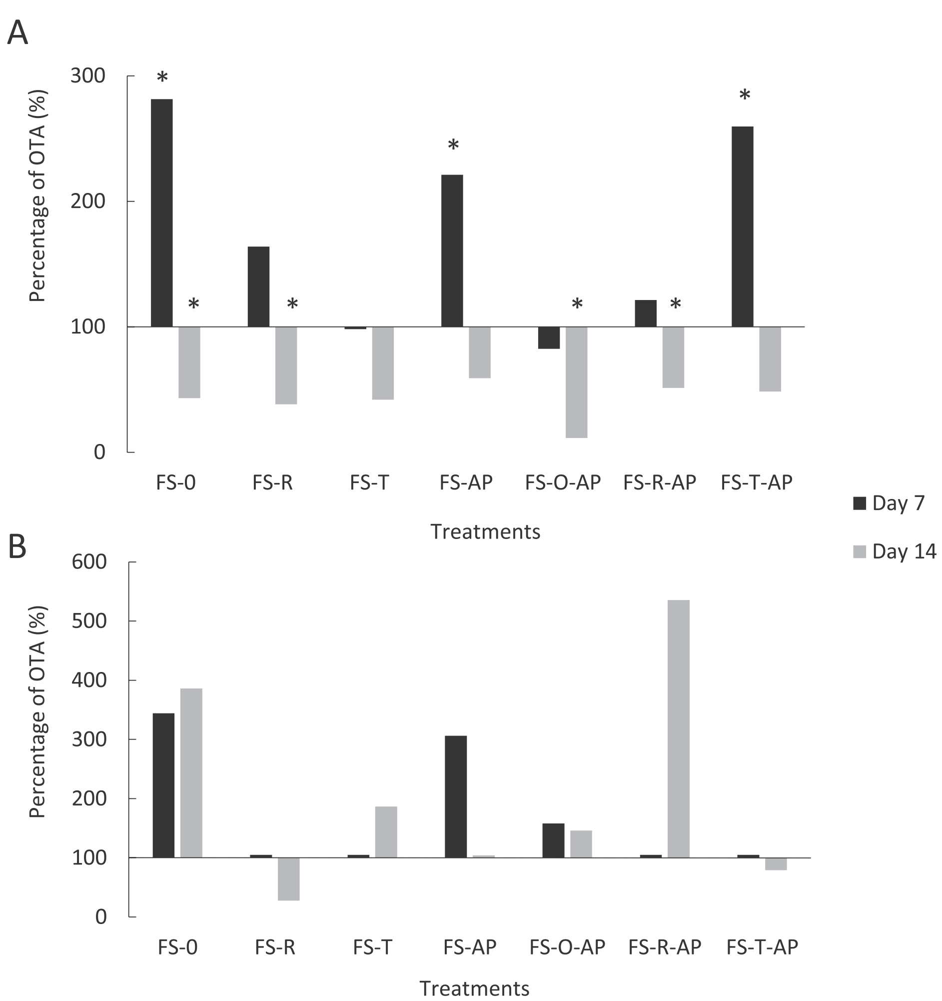


Fig. 2. Ochratoxin A (OTA) amounts produced by *Penicillium nordicum* CBS 323.92 grown during 14 days at 12 °C in a dry-cured fermented sausage-based agar (FS) with the addition of different spices (oregano, rosemary and thyme) and a commercial antifungal preparation. It is expressed as percentage of OTA compared to the control (mould grown in the absence of antifungal compounds, value = 100%) in the portion containing the mycelium and the casing (A) and in the portion containing the agar below the casing (B). Samples were taken at days 7 and 14 of incubation.
Treatments: FS-O: oregano; FS-R: rosemary; FS-T: thyme; FS-AP: antifungal preparation; FS-O-AP: oregano + antifungal preparation; FS-R-AP: rosemary + antifungal preparation; FS-T-AP: thyme + antifungal preparation.
Statistical differences between the non-treated control (100%) and treatments are indicated by an asterisk ($p \leq 0.05$). The statistical analysis was performed using the mean concentration ± standard deviation of each treatment.

carbonarius, being that from oregano the most effective to reduce the OTA presence since it did not allow its production. An appropriate effect of essential oils from oregano on inhibiting OTA production by *Aspergillus ochraceus* has been also reported (Basílico & Basílico, 1999). The increase of the OTA amount found when the antifungal preparation was individually applied could be attributed to a stimulation of the mycotoxin production by the use of sub-inhibitory levels of these compounds (Alcano et al., 2016; Arroyo et al., 2005; Lappa, Kizis, & Panagou, 2017a). Thus, it is of crucial importance that the meat industry tests and applies the appropriate concentrations of the antifungal compounds in order to reduce the possibility of OTA accumulation in their products.

Although OTA diffused from the surface into the inner core of the meat model system (deep portion) in most of the treatments, the concentration of this mycotoxin decreased as it diffused inwards (Fig. 2B). In fact, no OTA was detected at day 7 in the control (FS) and only 1.80 ng/g of OTA were found at day 14. The collagen casing could thus act as a barrier preventing the OTA diffusion to the agar, as it has been

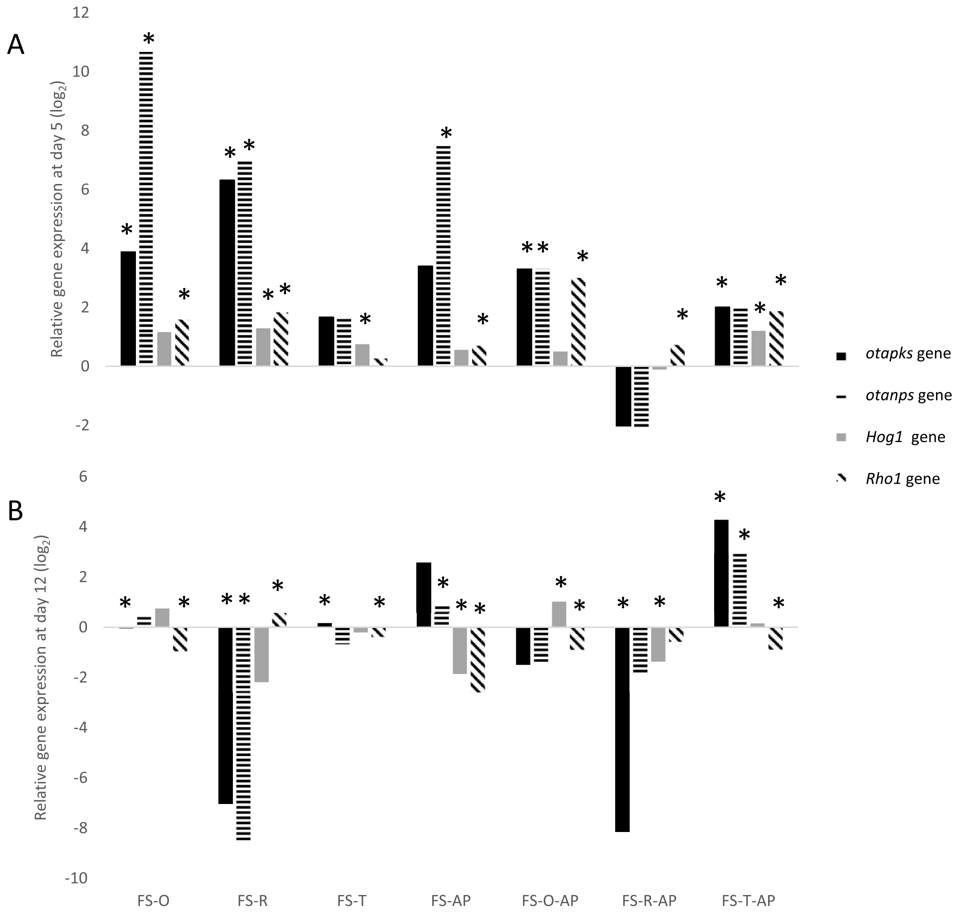


Fig. 3. Relative expression of the *otapks*, *otanps*, *Hog1* and *Rho1* genes in *Penicillium nordicum* CBS 323.92 grown during 14 days at 12 °C in a dry-cured fermented sausage-based agar (FS) with the addition of different spices (oregano, rosemary and thyme) and a commercial antifungal preparation. Samples were taken at days 5 (A) and 12 (B) of incubation.
Treatments: FS: non-treated control; FS-O: oregano; FS-R: rosemary; FS-T: thyme; FS-AP: antifungal preparation; FS-O-AP: oregano + antifungal preparation; FS-R-AP: rosemary + antifungal preparation; FS-T-AP: thyme + antifungal preparation.
Statistical differences between the non-treated control and treatments are indicated by an asterisk ($p \leq 0.05$).

described in dry-cured fermented sausages (Parussolo et al., 2019). It should be also highlighted that the statistical analysis revealed the absence of significant effect of the applied treatments on OTA accumulation in the deep portion.

3.3. Gene expression studies

The effect of the different treatments on the gene expression of *P. nordicum* CBS 323.92 was finally assessed to investigate the mode of action of the spices throughout the incubation time. Until now, no studies based on the level of gene transcription in the presence of spices have been performed.

The quantification of the relative gene expression performed in this work at days 5 and 12 of incubation is shown in Fig. 3. Different expression patterns were observed at both sampling times and, even, differences were observed between treatments at the same incubation period. In general, the results showed an overexpression of the tested genes at day 5. The two OTA-biosynthetic genes, *otapks* and *otanps*, displayed significant differences with the calibrator when oregano (FS-O) or rosemary (FS-R) was added. Moreover, the addition of the antifungal preparation (FS-AP) significantly enhanced the expression of the *otanps* gene. When oregano and thyme were combined with the antifungal preparation (FS-O-AP, FS-T-AP), a stimulation in the relative expression of both genes occurred, even though no statistical differences respect to the calibrator in the *otanps* gene were found for thyme (FS-T-AP). Nonetheless, both genes were inhibited due to the combination of rosemary and the antifungal preparation (FS-R-AP) despite the absence of significant differences.

In relation to the stress-related genes, the expression of the *Rho1* gene was significantly enhanced respect to the calibrator when compared to that of the *Hog1* gene in most treatments. It is also noticeable that at this first sampling time the *Rho1* gene showed the strongest correlation with the OTA accumulation in most of the treatments (Table 1 Supplementary Material). Such results make sense since the *Rho1* gene expression is activated by compounds affecting the maintenance of the cell wall (Hayes et al., 2014) to overcome the effect of compounds against its stability. This fact may indicate that the cell wall integrity pathway is overactivated in *P. nordicum* in the presence of the tested spices (Fig. 3), allowing to the mould to cope with this stress and keep growing normally (Fig. 1). In addition, the activation of this stress-response gene appears to trigger the OTA production as a response to the imposed stress (Schmidt-Heydt et al., 2013; Yun et al., 2014). Similarly, the expression of the *Rho1* gene has been linked to the production of alternariol monomethyl ether by *Alternaria tenuissima* (da Cruz Cabral, Delgado, Patriarca, & Rodríguez, 2019) and OTA by *A. carbonarius* (Fodil et al., 2018) in the presence of the antifungal protein PgAFP.

At day 12, the variability of the results was higher than at day 5, being found a significant repression of most of the genes. The reduction of the expression level of the *otapks* gene during the ripening of sausages inoculated with *P. nordicum* has been attributed to a degradation of mRNA transcripts (Ferrara et al., 2016). In the present work, it should be noted the significant decrease in the relative expression of both OTA-biosynthetic genes in samples that individually included rosemary (FS-R). On the contrary, the expression of the *otapks* gene was stimulated in the treatments including thyme (FS-T and FS-T-AP).

Regarding the stress-related genes, most treatments presented significant differences in the relative expression of the *Rho1* gene, being significantly inhibited in the treatments with oregano or thyme (FS-O, FS-T, FS-O-AP and FS-T-AP) and in the presence of the antifungal

preparation (FS-AP). Regarding the *Hog1* gene, while a significant reduced expression was detected for the antifungal preparation (FS-AP) and when it was combined with rosemary (FS-R-AP), a significant overexpression was obtained when it was added together with oregano (FS-O-AP). Owing to the fact that the strongest positive correlation at the end of the incubation period was obtained between the OTA production and the relative expression of the *otapks* gene (Table 2 Supplementary Material), it seems that after the adaptation of the mould to the stressful environment, the level of transcription of the biosynthetic gene is the main responsible for OTA accumulation. The correlation between the *otapks* gene and the phenotypic OTA production has been demonstrated for *P. nordicum* (Bernáldez, Rodríguez, Delgado, Sánchez-Montero, & Córdoba, 2018; Geisen, Schmidt-Heydt, & Karolewiez, 2006).

Although there are no previous studies exploring the influence of spices or the antifungal synthetic preparation on the transcriptional response of *P. nordicum*, the variability on the gene expression results when applying different treatments is in accordance with Lappa, Kizis, & Panagou, 2017a who highlighted the complex regulation of the OTA related gene expression in *A. carbonarius*. Similarly, El Khoury et al. (2016) showed different effects on the OTA reduction at transcriptomic level in *A. carbonarius*, since some essentials oils including that from rosemary downregulated regulatory genes (*laeA* and *veA*) and consequently reduced the expression of the *otapks* and *otanps* genes, but other essential oils directly downregulated the biosynthetic genes. Consequently, the production of mould secondary metabolites could be influenced by numerous signals that may provoke inhibitory or promotional effect on the regulatory genetic systems (El Khoury et al., 2016; Lappa, Kizis, & Panagou, 2017a).

The implication of other mechanisms to reduce OTA could be also deduced in this study since the mycotoxin accumulation was higher at the initial stages than at the end of the incubation period when adding oregano or rosemary. These findings are consistent with other studies indicating the potential effect of essential oils or extracts to detoxify mycotoxins *in vitro* (Perczak et al., 2016; Sun, Wang, Lu, & Liu, 2015; Xing et al., 2014).

4. Conclusions

The addition of oregano and rosemary as ingredients to dry-cured fermented sausages showed an antagonistic activity against *P. nordicum* based on the reduction of the OTA accumulation. These findings suggest the feasibility of using both spices to gain acceptability among consumers preserving the food safety in comparison to antifungal synthetic compounds. Although the reduction of the OTA amount correlated with a repression of the tested stress-related genes, further studies are required to elucidate the mode of action of oregano and rosemary since a clear relationship between the OTA presence and transcriptional response could not be established. This association should be deeper evaluated together with other mechanisms that may be implied in the OTA synthesis by *P. nordicum* in the presence of spices. Additionally, the synergism, compatibility and activity of a combination of both spices in the real meat matrix should be evaluated.

CRediT authorship contribution statement

Micaela Álvarez: Investigation, Formal analysis, Visualization, Writing - original draft, Writing - review & editing. **Alicia Rodríguez:** Conceptualization, Methodology, Supervision, Writing - review & editing. **Félix Núñez:** Writing - review & editing, Funding acquisition. **Antonio Silva:** Investigation, Writing - review & editing. **María J. Andrade:** Conceptualization, Methodology, Supervision, Writing - original draft, Writing - review & editing, Funding acquisition, Project administration.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2020.107222.

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IV.3. Competición por nutrientes y espacio como modo de acción de agentes de biocontrol frente a *Penicillium nordicum*

Competitiveness of three biocontrol candidates against ochratoxigenic Penicillium nordicum *under dry-cured meat environmental and nutritional conditions*

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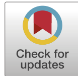

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A B S T R A C T

The environmental conditions during the ripening of dry-cured meats and their nutritional composition promote the colonisation of their surface by *Penicillium* spp., including *P. nordicum* producer of ochratoxin A (OTA). The objective of this work was to study the competitiveness of three potential biocontrol candidates (*Debaryomyces hansenii* FHSCC 253H, *Enterococcus faecium* SE920 and *Penicillium chrysogenum* CECT, 20922) against the ochratoxigenic *P. nordicum* FHSCC4 under environmental and nutritional conditions simulating the ripening of dry-cured meat products. For this, the nutritional utilisation pattern, niche overlap index (NOI), interactions by dual-culture assays and OTA production were determined. The number of carbon sources (CSs) metabolised depended on the microorganism and the interacting water activity (a_w) x temperature conditions. The number of CSs utilised by both filamentous fungi was quite similar and higher than those utilised by *D. hansenii* and *E. faecium*. The yeast isolate metabolised a number of CSs much larger than the bacterium. The NOI values showed that, in general, *P. nordicum* nutritionally dominated *E. faecium* and *D. hansenii* regardless of the environmental conditions evaluated. The relationship between the toxigenic and non-toxigenic fungal isolates depended on the a_w x temperature combinations, although in none of the conditions a dominance of *P. nordicum* was observed. According to the interaction assays, both *D. hansenii* and *P. chrysogenum* decreased the growth of *P. nordicum*. The effect of *D. hansenii* could be attributed to the production of some extra-cellular compounds, while the action of *P. chrysogenum* is likely related to nutritional competition. In addition, both *P. chrysogenum* and *D. hansenii* reduced the OTA levels produced by *P. nordicum*. The effect of the yeast was more pronounced decreasing the concentration of OTA at quantities lower than the limit established by the Italian legislation. Therefore, *P. chrysogenum* and *D. hansenii* can be suggested as biocontrol candidates in the manufacture of dry-cured meat products.
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1. Introduction

Both the particular environmental conditions of the ripening process of dry-cured meat products and their nutritional composition promote their surface colonisation by a profuse mycobiota, mainly composed by species belonging to *Penicillium* genus (López-Dıaz et al., 2001; Núñez et al., 1996a). These filamentous fungi play a key role in the development of their distinctive aroma and flavour (Martín et al., 2006), but some of them are responsible for the synthesis of toxic secondary metabolites including ochratoxin A (OTA) (Battilani et al., 2007; Iacumin et al., 2009; Rodríguez et al., 2012b). *Penicillium nordicum* is the most commonly ochratoxigenic fungus found on dry-cured meats (Battilani et al., 2007; Rodríguez et al., 2012a, 2012b). The ability of this species to grow and produce OTA in cured meats may be derived from its adaptability to intermediate water activity (a_w) values (0.86–0.94), temperature (11–30 °C) and salt content (5–9 %) which are

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characteristic of the processing of these foods (Battilani et al., 2007; Delgado et al., 2018; Rodríguez et al., 2014, 2015b; Sánchez-Montero et al., 2019). Besides, *P. nordicum* is specially adapted to NaCl-rich niches due to its capability to cope with osmotic stress conditions (Delgado et al., 2018; Rodríguez et al., 2016). OTA has shown side effects to human health including nephrotoxic, hepatotoxic, and immunotoxic effects (Petzinger and Ziegler, 2000). In addition, it has been classed as Group 2B by the International Agency for Research on Cancer due to its possible carcinogenicity to humans (IARC, 1993). The toxicity of this mycotoxin led to the European Commission to set up maximum permitted levels of OTA for certain foodstuffs (European Commission, 2006) but not for dry-cured meats yet. Only Italy, one of the major producers of dry-cured hams and sausages, has established a maximum guide value for this mycotoxin at 1 μg/kg in fresh pork and derived meat products (Ministero della Sanità, 1999).

Only a few reports about the prevalence of OTA in dry-cured meat products are available. However, the contamination of traditional ripened meat products from Mediterranean countries by this toxin is quite frequent ranging from 5.7 to 76 % of the samples exceeding the OTA level set up by the Italian regulation (Iacumin et al., 2009, 2017; Pleadin et al., 2015; Rodríguez et al., 2012a). For this reason, the development of efficient strategies to keep the OTA levels as low as possible in dry-cured meats is of utmost importance in order to protect consumer health.

The use of the native microbiota isolated from cured meats, including lactic acid bacteria (LAB), yeasts and non-toxigenic filamentous fungi as biocontrol agents is a promising strategy to minimise the presence of OTA in these foods. LAB have been widely applied as bioprotective agents in various types of foods, showing an inhibitory capacity against *P. nordicum* growth and OTA accumulation in Yeast Extract Sucrose (YES) agar (Guimarães et al., 2018). *Enterococcus faecium* SE920, isolated from Spanish dry-fermented sausages, has shown probiotic activity in this kind of foods (Ruiz-Moyano et al., 2009), and a promising antifungal activity (Álvarez et al., 2019). Among yeasts, *Debaryomyces hansenii* is the most commonly found throughout the whole ripening process of dry-cured meat products (Andrade et al., 2009; Mendonça et al., 2013; Núñez et al., 1996b). It has been also reported that several *D. hansenii* strains were able to reduce between 21 and 100 % of OTA amounts in dry-cured meat products depending on the environmental conditions (Andrade et al., 2014; Iacumin et al., 2017; Peromingo et al., 2018; Simoncini et al., 2014; Virgili et al., 2012). Focusing on non-toxigenic filamentous fungi, *Penicillium chrysogenum* is one of the most frequently isolated species from dry-cured meat products (Núñez et al., 1996a). *P. chrysogenum* CECT 20922 efficiently limited the growth of ochratoxigenic fungi and OTA accumulation in Iberian dry-cured ham throughout its processing (Rodríguez et al., 2015a). Furthermore, this non-toxigenic isolate produces the antifungal protein PgAFP (Acosta et al., 2009; Rodríguez-Martín et al., 2010), which possesses a fungistatic effect against mycotoxin-producing fungi, including *P. nordicum* (Delgado et al., 2015). Thus, this fungus can be also useful to control OTA in dry-cured meat products.

The elucidation of the mode of action of potential biocontrol agents would be helpful to optimise the correct timing and handling for their application during the processing of meat products. Competition by nutrients and space has been considered one of the most common mechanisms responsible for the antagonistic activity of biocontrol agents (Andrade et al., 2014; Asensio et al., 2014; Núñez et al., 2015; Virgili et al., 2012). The interactions and competitiveness between microorganisms are mainly affected by interacting environmental factors such as a_w and temperature (Lee and Magan, 1999; Marín et al., 1998), and the utilisation of food nutrients (Arroyo et al., 2008). Some studies have evaluated the level of coexistence or dominance based on different indexes and nutrient utilisation profiles (Arroyo et al., 2008; Lee and Magan, 1999; Marín et al., 1998; Wilson and Lindow, 1994). However, none has checked the ability of biocontrol candidates to vie with usual ochratoxigenic species in the environmental and nutritional conditions associated with the ripening of dry-cured meats.

The main objective of the present work was to study the competitiveness of three potential biocontrol microorganisms against an ochratoxigenic *P. nordicum* isolate and their ability to minimise OTA in an environment simulating ripening of cured meats. For this, their nutritional utilisation pattern, their niche overlap index (NOI) and interactions by dual-culture assays were determined.

2. Materials and methods

2.1. Microorganisms

P. nordicum FHSCC IB4 and *D. hansenii* FHSCC 253H belonged to the culture collection of Food Hygiene and Safety Research Group of the University of Extremadura (Cáceres, Spain). *E. faecium* SE920 was supplied by the culture collection of Food Quality and Microbiology Research Group of the University of Extremadura (Badajoz, Spain). *P. chrysogenum* CECT 20922 (formerly named RP42C) was obtained from the Spanish Type Culture Collection (Valencia, Spain). All the microorganisms used in this study had been isolated from dry-cured meat products and previously identified by DNA sequencing (Andrade et al., 2009; Rodríguez-Martín et al., 2010; Ruiz-Moyano et al., 2009; Sánchez-Montero et al., 2019).

2.2. Preparation of inocula

The inocula of *P. chrysogenum* and *P. nordicum* were prepared after growing on Potato Dextrose Agar (PDA, Scharlab S.L., Spain) at 25 °C for 7 d. Conidia were harvested by scrapping the surface with a glass rod after adding 3 mL of phosphate-buffered saline (PBS) on the surface of inoculated plates. PBS was supplemented with 50 or 90 g/L of NaCl to reach 0.97 (PBS50) and 0.94 (PBS90) a_w, respectively. Conidia suspensions were then quantified using a Thoma counting chamber Blaubrand® (Brand, Germany), and the concentrations were adjusted to 10^5 spores/mL and used as inocula.

D. hansenii was inoculated on YES Broth (containing 20 g/L yeasts extract, and 125 g/L sucrose) at 25 °C for 72 h under stirring (150 rpm). After the incubation period, the culture was centrifuged, the supernatant removed, and the pellet resuspended in PBS50 and PBS90. Next, the cells were quantified using the Thoma counting chamber and their concentration adjusted to 10^6 cfu/mL. *E. faecium* was cultured on Brain Heart Infusion (BHI, Scharlab S.L.) broth and incubated for 48 h at 30 °C under stirring (150 rpm). The bacterium culture was then centrifuged, and the pellet resuspended in PBS50 and PBS90. Finally, the cell suspension was turbidimetrically adjusted to 10^6 cfu/mL. For this, a calibration curve relating the plate counts with the absorbance at 595 nm of suspensions of the bacterium was firstly built using a BioPhotometer model 6131 (Eppendorf AG, Germany).

2.3. Carbon sources pattern and niche overlap index studies

2.3.1. Microtiter plate preparation

For carbon source (CS) utilisation patterns and obtaining the NOI values for the biocontrol agents and the ochratoxigenic fungus, sterile 96-well microtiter plates were used. For *E. faecium*, minimal PBS medium supplemented with 50 or 90 g/L of NaCl was prepared (PBS50 and PBS90, respectively). In the case of filamentous fungi and yeast, a resazurin sodium salt solution (RSS; Sigma–Aldrich,

USA) was used as minimal medium. This solution was prepared by supplementing 0.02 g/L of RSS to PBS modified with NaCl, either PBS50 (R-PBS50) or PBS90 (R-PBS90). Resazurin is a non-fluorescent blue dye, which is converted by reduction into the pink-coloured highly fluorescent resorufin, and is used to reveal the viability of fungal cells (Monteiro et al., 2012). Glycerol was not used to modify the a_w as it is also a CS. NaCl was used to modify the a_w of the medium (50 or 90 g/L to reach 0.97 and 0.94 a_w, respectively) since this solute is commonly used in the manufacture of dry-cured meats. A total of 20 CSs (Sigma–Aldrich), which are among the main chemical constituents of cured meats (Table 1), were separately added into the minimal media at a final concentration of 20 g/L (carbon equivalent to 2 % (w/v) glucose). All the CSs were sterilised by filtration through a 0.22 μm-pore size membrane (Thermo Fisher Scientific, Spain) before addition.

2.3.2. Inoculation of the microtiter plates

One hundred and fifty μL of the minimal medium (PBS50 or PBS90 for the bacterium and R-PBS50 or R-PBS90 for the fungi) containing one CS were poured into the wells. Next, the wells were inoculated with 15 μL of each of the corresponding microorganism inoculum resuspended in PBS50 or PBS90 as required. Microtiter plates without inoculum were prepared and incubated as non-growth controls. The plates were covered with lids and incubated at 15, 20 and 25 °C for 7 d. These temperatures are reached during the ripening of traditional dry-cured meat products (Núñez et al., 1996b). The assay was run in triplicate wells, using separate plates for each isolate.

The growth of *E. faecium* was monitored by measuring the optical density (OD) at 595 nm every 24 h. The use of a CS was defined as a significant increase of OD during the incubation time compared to the initial OD value. The utilisation of the CSs by filamentous fungi and yeast was visually checked. A blue colour means no CS metabolisation by the microorganism, while a fluorescent pink colour indicates the metabolic use of the corresponding CS.

2.3.3. Calculation of niche overlap index

The NOI was calculated for each potential biocontrol agent against *P. nordicum* and vice versa, using the total number of different CSs used by each isolate for the different sets of interacting $a_w \times$ temperature conditions as follows (Wilson and Lindow, 1994):

$$NOI = \frac{\text{Number of CSs in common between two microorganisms}}{\text{Number of CSs utilised by the tested microorganism}}$$

The NOI values obtained are between 0 and 1. NOI values > 0.90 indicate coexistence in the same nutritional niche and values < 0.90 indicate the occupation of different niches.

2.4. Dual-culture assay to test antagonism of biocontrol candidates against P. nordicum

The dual-culture assay was adapted from Magan and Lacey (1984) to observe the interaction of two microorganisms inoculated on the same culture plate (co-cultivation). The dual-culture assays of *P. nordicum* and the potential antagonists were performed on three different culture media depending on the CS utilised by the ochratoxigenic fungi and the candidate (Table 1). The three culture media, PnEf (interaction between *P. nordicum* and *E. faecium*), PnDh (interaction between *P. nordicum* and *D. hansenii*), and PnPc (interaction between *P. nordicum* and *P. chrysogenum*), were prepared by mixing 20 g of agar (Scharlab S.L.) and sterilised PBS50 or PBS90 with 1000 mL distilled water. Culture media were autoclaved at 121 °C for 15 min and a mix of CSs used by both *P. nordicum* and the biocontrol agent at a concentration of 20 g/L was added to each medium before its cooling. The CS mixes were sterilised by filtration through a 0.22 μm-pore size membrane. The sterile media were then left to cool before being poured into 5.5 cm diameter Petri plates. The a_w of each medium was determined using a Novasina Lab Master water activity meter (Novasina AG, Switzerland).

P. nordicum was point-inoculated approximately 10 mm from the plate wall and approximately 20 mm from the antagonist on the three culture media adapting the methodology proposed by Mohale et al. (2013) and Verheecke et al. (2014). Point inoculation was preferred for the filamentous fungal candidate (*P. chrysogenum*) and streak inoculation was chosen for bacterial and yeast candidates (*E. faecium* and *D. hansenii*). Plates inoculated with only *P. nordicum* on the three culture media without

Table 1
The major dry-cured meat carbon sources (CSs) and the specific CSs added to prepare the culture media PnEf, PnDh and PnPc to evaluate the interaction of *Penicillium nordicum* with each of the three biocontrol candidates: *Enteroccus faecium*, *Debaryomyces hansenii* and *Penicillium chrysogenum*, respectively.

Carbon sources[a]	*E. faecium* (PnEf)	*D. hansenii* (PnDh)	*P. chrysogenum* (PnPc)
Fructose	x[b]		x
Glucose		x	x
Lactose		x	x
Sucrose		x	x
Ribose		x	x
Glycine		x	x
Alanine		x	x
Arginine	x	x	x
Asparagine	x	x	x
Cysteine			x
Phenylalanine		x	x
Glutamine	x	x	x
Histidine	x		x
Isoleucine	x	x	x
Leucine		x	
Lysine	x	x	x
Methionine	x	x	
Proline		x	x
L-tryptophan			
Valine			

[a] CSs used to study the carbon sources pattern.
[b] “x” indicates the carbon sources used to prepare each medium for the interaction assay.

inoculation of any potential antagonist were used as negative controls. The experiment was conducted with three replicates and incubated for 14 d at 15, 20 and 25 °C.

At the end of the incubation period, the interactions between *P. nordicum* and the biocontrol candidates were macroscopically observed. The types of interaction were numerically scored according to the description and classification provided by Magan and Lacey (1984). The numerical scores obtained for *P. nordicum* in combination with the other microorganisms were used to determine the Index of dominance (I_D) (Magan and Lacey, 1984).

The diameter of the *P. nordicum* colonies in the presence and absence of the biocontrol candidates was measured 10 out of 14 d of incubation in two perpendicular directions.

2.5. OTA extraction and quantification

After 14 d of incubation agar plugs of about 1 g including mycelium from *P. nordicum* colonies in the nearest area to biocontrol agents' colonies were collected from each plate used for the interaction study (see section 2.4) following the methodology described by Mohale et al. (2013) and Verheecke et al. (2014). Samples were transferred to 10 mL tubes and extracted using the QuEChERS-based procedure described by Delgado et al. (2018). Briefly, the extraction method consisted of extracting the mycotoxin with water and acetonitrile (Scharlab S.L.) acidified with acetic acid 0.1 % (v/v; Fisher Scientific, USA) and phase partitioning with NaCl (Scharlab S.L.) and anhydrous $MgSO_4$ (Scharlab S.L.). Next, the mixture was hand-shaken and centrifuged for 5 min at 4 °C at 5300 rpm (Diglicen 21R, Spain). A 0.75 mL aliquot from the supernatants was then evaporated to dryness and redissolved in 0.75 mL HPLC-grade acetonitrile (Scharlab S.L.), filtered through a 0.22 μm-pore size nylon membrane (Jet Bio-Filtration Co., Ltd., China) and stored at −20 °C until analysis.

OTA was analysed by high-performance liquid chromatography with fluorescence detection (HPLC-FLD) in an Agilent 1260 Infinity equipment (Agilent Technologies, USA) coupled to a fluorescence detector (Agilent Technologies, UK) using a Phenomenex C_{18} column of 250 × 4.6 mm, 5 μm particle size (Phenomenex, USA) as stationary phase according to the method described by Rodríguez et al. (2014). The mobile phase was water:acetonitrile:acetic acid (41:57:2 v/v/v) with a flow rate of 1.0 mL/min in an isocratic mode, and an injection volume of 20 μL. The run time was 15 min being the retention time for OTA 7.5 ± 0.2 min. FLD detection was performed using 333 nm and 460 nm as excitation and emission wavelengths, respectively. The full FLD spectra data were acquired and processed using the Agilent Technologies software (Agilent Technologies, USA). OTA was quantified using a calibration curve built with solutions of standard OTA (Sigma−Aldrich) from 1 to 100 ng/mL with a linear relationship ($R^2 = 0.998$). Limits of detection and quantification (LOD and LOQ) were determined as previously described (Long and Winefordner, 1983), and were 1.06 μg/kg and 3.24 μg/kg, respectively.

2.6. Statistical analysis

Statistical analysis was carried out using the SPSS v22.0 software. Normality of data was analysed by means of the Shapiro−Wilk test and all data failed the normality test. Therefore, the non-parametric Kruskal Wallis and Mann Whitney U tests were applied. The statistical significance was set at $p \leq 0.05$.

3. Results

3.1. CS utilisation patterns

Fig. 1 shows the total number of CSs utilised by *P. nordicum* and the three biocontrol candidates at all the tested environmental conditions. In general, a larger number of CSs were metabolised under warmer and wetter conditions by all the microorganisms. Besides, the number of CSs metabolised by both filamentous fungi was quite similar and higher than that utilised by *D. hansenii* and *E. faecium*. Meanwhile, the yeast isolate metabolised a number of CSs much larger than the bacterium. At all a_w and temperature ≥20 °C both filamentous fungal isolates were able to utilise at least 16 CSs relevant for dry-cured meats. For the filamentous fungi, at 0.97 a_w the number of CSs used decreased slightly when the temperature was lowered, whilst at 0.94 a_w, the opposite effect was generally observed. *D. hansenii* metabolised at least 13 CSs regardless of the temperature and a_w evaluated, although the niche sizes narrowed at the lowest temperatures and reduced a_w. *E. faecium* used the lower number of CSs independently of the evaluated environmental conditions. This bacterium was able to utilise 8 CSs at 25 °C and 0.97 a_w, while it used no more than 3 CSs at lower temperatures. At 0.94 a_w *E. faecium* only grew at 25 °C, using 9 CSs.

3.2. Niche overlap index

Water activity and temperature had an impact on the NOI values of the isolates evaluated (Fig. 2). Generally, regardless of the environmental conditions assayed, *P. nordicum* nutritionally dominated *E. faecium* (Fig. 2). Regarding *D. hansenii*, *P. nordicum* dominated the niche under most of the tested a_w x temperature conditions, except at 0.97 a_w x 20 °C (coexistence) and at 0.94 a_w x 25 °C (separate niches). In the case of *P. chrysogenum,* the nutritional competitiveness varied depending on the environmental conditions. Both toxigenic and non-toxigenic filamentous fungi co-existed in the same niches at most of the evaluated conditions. However, at 0.97 a_w x 20 °C *P. chrysogenum* nutritionally dominated *P. nordicum* and at 0.97 a_w x 15 °C, and 0.94 a_w x 25 °C they occupied separate niches.

3.3. Interaction between P. nordicum *and biocontrol agents*

The macroscopic interactions (Fig. 3) between the colonies were numerically classified and the I_D obtained at different a_w and temperature levels. Table 2 shows the type of interactions and the total I_D between *P. nordicum* and the different antagonists. Interactions between each pair toxigenic:antagonist isolates were similar regardless of a_w and temperature conditions. At both a_w assayed, *P. nordicum* was dominated at distance by *D. hansenii* (0/15, 0/15) while both toxigenic and non-toxigenic filamentous fungi showed mutual antagonism (6/6, 7/7). In the case of *E. faecium*, its growth was very slow in the tested conditions and its interactions with *P. nordicum* could not be examined after 14 d of incubation.

Fig. 4 compares the diameter of *P. nordicum* in the presence and absence of *D. hansenii* and *P. chrysogenum* under various sets of a_w x temperature conditions. Generally, the growth of *P. nordicum* was significantly slower in the presence of both *D. hansenii* and *P. chrysogenum* ($p \leq 0.05$). At the lowest a_w assayed, the effect of the antagonists was less pronounced on the *P. nordicum* growth at the three temperatures tested.

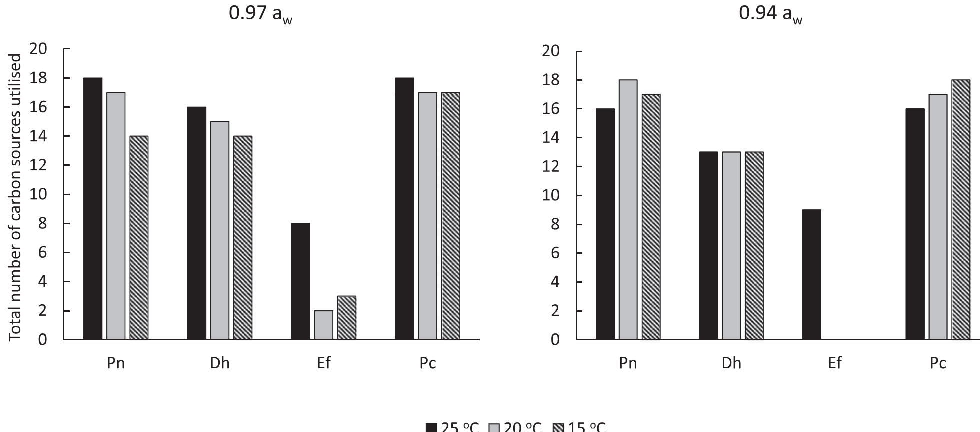
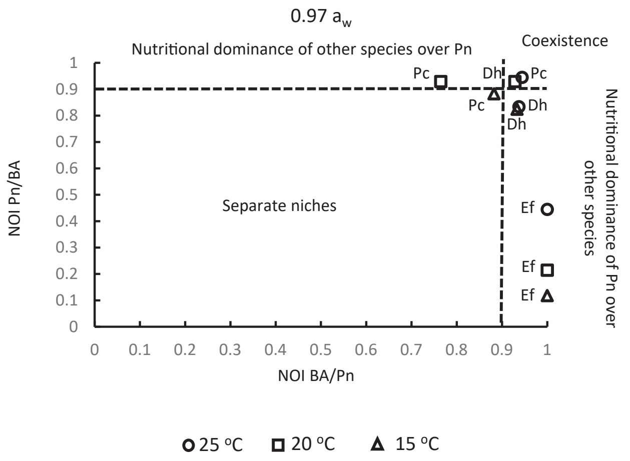


Fig. 1. Total number of carbon sources used by *Penicillium nordicum* (Pn), *Debaryomyces hansenii* (Dh), *Enterococcus faecium* (Ef), and *Penicillium chrysogenum* (Pc) in each combination of temperatures and water activity (a_w) tested.



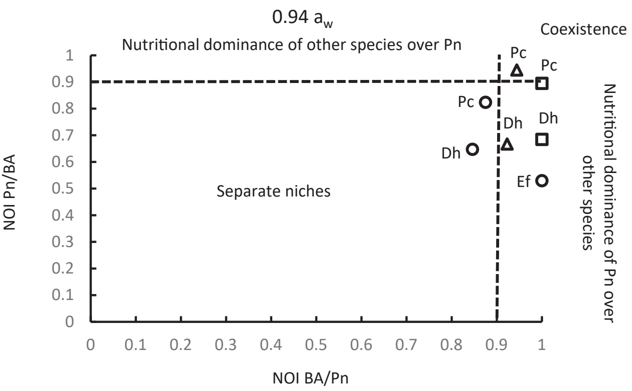


Fig. 2. Effect of temperature and water activity (a_w) on niche overlap indices (NOIs) of *Penicillium nordicum* (Pn) in relation to potential biocontrol agents (BA), for determining nutritional dominance or coexistence. Dh, *Debaryomyces hansenii*; Ef, *Enterococcus faecium*; Pc, *Penicillium chrysogenum*.

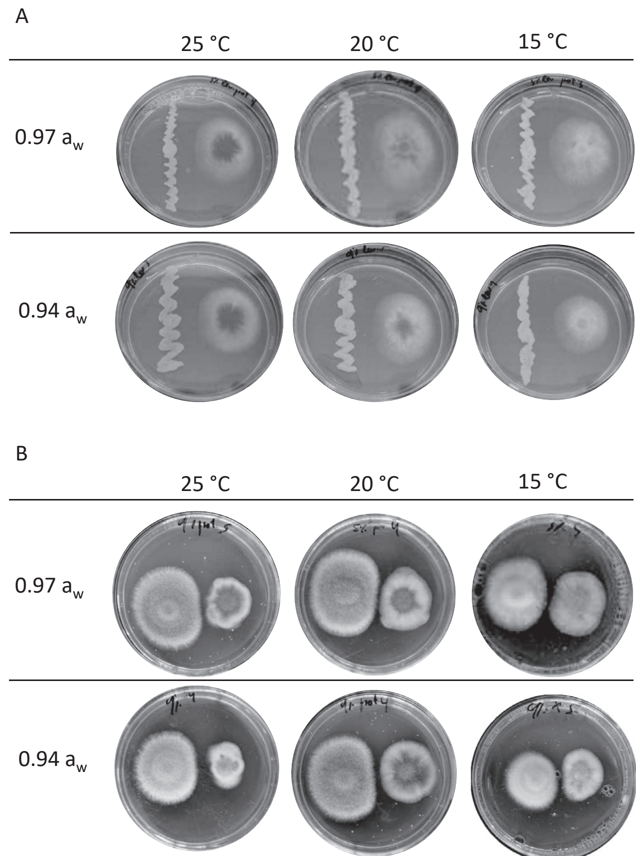


Fig. 3. Macroscopic interactions between *Penicillium nordicum* and *D. hansenii* (panel A), and *Penicillium chrysogenum* (panel B) after 14 d of incubation at different combinations of temperature (25, 20, and 15 °C) and water activity (a_w) (0.97, and 0.94). The toxigenic species is placed on the right side of the Petri dish while the biocontrol agent is on the left side.

3.4. Effect of biocontrol candidates on OTA production

P. nordicum did not produce OTA at detectable levels (<1.06 μg/kg) regardless of the temperature or a_w in the PnEf medium. *P. nordicum* synthesised OTA in the medium PnPc under all tested environmental conditions, while in the PnDh medium the mycotoxin was detected at the three temperatures assayed at a_w 0.94, but only at 15 °C at the highest a_w (Fig. 5). In both media (PnPc and PnDh), the highest OTA amounts were found at 15 °C x 0.97 a_w. Overall, the presence of *P. chrysogenum* or *D. hansenii* significantly decreased the OTA quantities produced by *P. nordicum*, except at 15 °C x 0.94 a_w in the medium PnPc.

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Table 2
Interaction scores and Index of Dominance (I_D) between *Penicillium nordicum* and the biocontrol agents on culture media at 0.97 and 0.94 water activity (a_w) and 15, 20 and 25 °C after 14 d of incubation. The first score is for *P. nordicum* and the second for the biocontrol candidate.

	0.97 a_w			I_D	0.94 a_w			I_D
	25 °C	20 °C	15 °C		25 °C	20 °C	15 °C	
Enterococcus faecium	n.d.	n.d.	n.d.	-	n.d.	n.d.	n.d.	-
Debaryomyces hansenii	0/5	0/5	0/5	0/15	0/5	0/5	0/5	0/15
Penicillium chrysogenum	2/2	2/2	2/2	6/6	3/3	2/2	2/2	7/7

Interaction scores: 1/1: intermingling; 2/2: mutual antagonism on contact or space between colonies <2 mm; 3/3: mutual antagonism at a distance (>2 mm); 4/0: dominance by the latter over the former species; 5/0: dominance at a distance by the latter species.
n.d.: not determined.

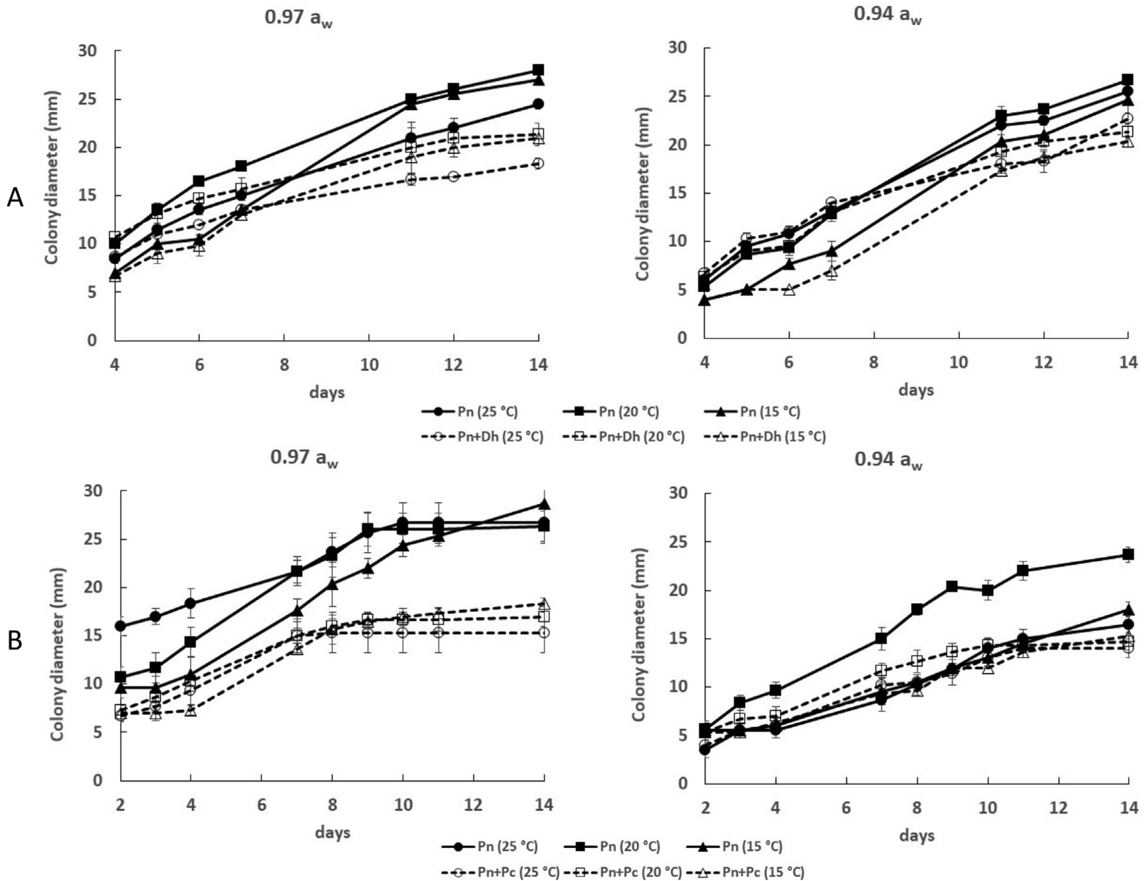

Fig. 4. Effect of *Debaryomyces hansenii* (A) and *Penicillium chrysogenum* (B) on the growth of *Penicillium nordicum* in each combination of temperature and water activity (a_w) tested. Pn, *P. nordicum*; Dh, *D. hansenii*; Pc, *P. chrysogenum*.

4. Discussion

This is the first study to examine the effect of the environmental conditions usually reached during the ripening of dry-cured meat products on the growth, nutritional utilisation patterns, NOI values and interactions between the toxigenic *P. nordicum* and three biocontrol agents: *D. hansenii* FHSCC 253H, *P. chrysogenum* CECT 20922 and *E. faecium* SE920. In addition, the influence of the biocontrol agents on OTA production by *P. nordicum* was tested.

The three biocontrol candidates evaluated had previously demonstrated their antagonist activity against different toxigenic fungi in dry-cured meat model systems (Álvarez et al., 2019; Andrade et al., 2014; Delgado et al., 2019a; Peromingo et al., 2018, 2019). However, there are needs to in-depth investigate their main mode of action in order to optimise their application in meat products. These biocontrol agents were also chosen as they belong to the most frequently found microorganisms' species in dry-cured meat product (Núñez et al., 1996a, 1996b).

The number and the type of CSs that a biocontrol candidate is able to catabolise compared to that of pathogenic species give information about its potential degree of dominance and their potential efficacy to be used with such purpose. Comparisons of the relative utilisation of CSs by *P. nordicum* and the biocontrol candidates showed differences in the CSs total number and the types used under the different a_w x temperature conditions examined. Both *P. chrysogenum* and *P. nordicum* utilised higher number of CSs than the other two microorganisms regardless of the environmental conditions. In addition, it was observed that at 0.97 a_w the assimilation of CSs tended to decrease as temperature dropped. These results are in accordance with those obtained by Mohale et al. (2013) and Samsudin et al. (2016). However, at 0.94 a_w, in both the toxigenic and non-toxigenic filamentous fungi the

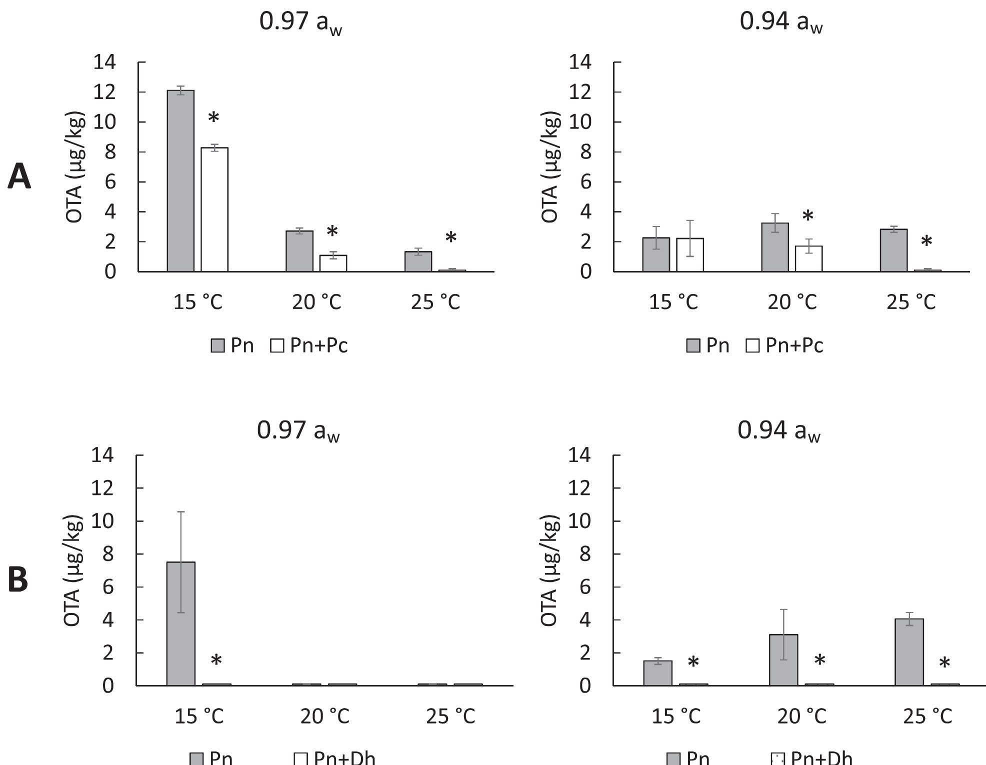

Fig. 5. Effect of *Penicillium chrysogenum* (A) and *Debaryomyces hansenii* (B) on the production of ochratoxin A (OTA) by *Penicillium nordicum* in each combination of temperature and water activity (a_w) tested. Pn, *P. nordicum*; Dh, *D. hansenii*; Pc, *P. chrysogenum*. Statistically significant differences of the OTA production of *P. nordicum* in the presence of each of the biocontrol agents with respect to the control are indicated by an asterisk ($p \leq 0.05$).

number of CSs increased or stayed the same as the temperature fell.

Co-existence of microorganisms in a same niche is regulated by nutritional-resource partitioning. The patterns of carbon utilisation were used to determine the NOI values of potential biocontrol microorganisms relative to the ochratoxigenic species and, therefore, their ecological similarity (Arroyo et al., 2008; Magan and Aldred, 2007). In this research, the NOIs are significant indexes to comprehend whether biocontrol and toxigenic microorganisms co-exist or occupy different niches. In general, *P. nordicum* nutritionally dominated *E. faecium* and *D. hansenii* (Fig. 2). Regarding the interactions between *P. chrysogenum* and *P. nordicum*, the dominance varied depending on the a_w x temperature combination checked. This environmental influence on nutritional dominance has been also described in previous studies where similar conditions of temperature and a_w were used (Mohale et al., 2013; Samsudin et al., 2016). Mohale et al. (2013) studied the interaction between toxigenic and atoxigenic strains of *Aspergillus flavus*, while Samsudin et al. (2016) evaluated the impact of the biocontrol fungus *Clonostachys rosea* on toxigenic *Fusarium verticillioides*.

Taking together the findings obtained by the determination of CSs utilisation profiles and NOIs, there is no doubt that the toxigenic *P. nordicum* predominantly controlled the niche evaluated in this study, which simulates the nutritional composition of dry-cured meats. The high presence of amino acids in dry-cured meats has been already described as a consequence of proteolysis reactions during the ripening process (Córdoba et al., 1994). In addition, the a_w of the media was modified by the addition of the ionic solute NaCl, given that this salt is habitually added to dry-cured meat products. The findings initially observed seem to not favour the utilisation of either *D. hansenii* or *E. faecium* as antagonist microorganisms against *P. nordicum*. *P. chrysogenum* is capable of utilising as much CSs as *P. nordicum* and is able to coexist or even dominate the toxigenic isolate. Then, this isolate of *P. chrysogenum* may be a good biocontrol agent to avoid the growth of ochratoxigenic fungi in dry-cured meats.

The different use of the CSs by the bacterium, yeast, and fungal isolates makes sense since bacteria are the predominant microorganisms at the beginning of the dry-cured meat processing before their ripening falling substantially their counts during the ripening, and then levelled off (Rodríguez et al., 1994). In the case of filamentous fungi and yeasts, their counts increase slowly during the first stages of the processing before soaring substantially during maturation (Núñez et al., 1996a, 1996b) where the number of small molecule metabolites rises. For this reason, as the evaluated nutrients are those that can be found in dry-cured meats, the number of CSs metabolised by *E. faecium* is much lower than those used by other microorganisms. Besides, the dominance of the ochratoxigenic *P. nordicum* is consistent with the results obtained by Rodríguez et al. (2014, 2015b) and Delgado et al. (2018) who demonstrated that this species is able to successfully colonise NaCl-rich cured meat niches because of its ability to overcome osmotic stress (Delgado et al., 2018; Rodríguez et al., 2016).

The interactions between *P. nordicum* and the three potential biocontrol agents were also evaluated at a colony level by the numerical scoring system proposed by Magan and Lacey (1984). For this, three different culture media made up with the CSs metabolised by both, the tested biocontrol agent and the ochratoxigenic fungus were prepared. Due to the differences in composition, the *Penicillium nodicum* colonies showed visible morphological variations between the distinct culture media (Fig. 3). Anyway, the results showed that *D. hansenii* exerted dominance over *P. nordicum* at a distance, suggesting that its mode of action would consist in the production of some extra-cellular compounds, such as volatile

compounds or killer proteins. Although the production of these antifungal compounds has been described in yeasts (Núñez et al., 2015), the antagonist mechanisms of *D. hansenii* FHSCC 253H against ochratoxigenic fungi had been mainly attributed to the competition for nutrients and space (Andrade et al., 2014). However, Magan and Aldred (2007) stated that the a_w x temperature combinations influence the type of interactions that might occur and the total I_D, which both are in a state of flux and change with interacting environmental conditions.

P. chrysogenum CECT 20922 showed a mutual antagonism on contact with the ochratoxigenic *P. nordicum*. Therefore, under the environmental and nutritional parameters simulating the maturation process and composition of dry-cured meats, the antagonist mechanism of *P. chrysogenum* against *P. nordicum* is mainly based on competition by nutrients and space as previously suggested (Bernáldez et al., 2014; Rodríguez et al., 2015a). It has been reported that the presence of *P. chrysogenum* triggers significant changes in proteins associated with carbohydrate metabolic processes in *P. nordicum*, including the increase of the glucose-repressible protein Grg1, decrease of the hexokinase HxkA and the absence of the catabolite repressor protein CreC, that further supporting the nutritional competition (Delgado et al., 2019a). It has been also previously shown that the tested *P. chrysogenum* isolate possesses other modes of action that basically involve the production of the antifungal protein PgAFP which has a great impact on the growth of various toxigenic fungal species commonly found on dry-cured meat products (Delgado et al., 2015). The comparison of the colony diameters of *P. nordicum* during the incubation time in the absence and presence of the antagonists corroborates the important effect of *D. hansenii* and *P. chrysogenum* on minimising the growth of the toxigenic isolate. These results agree with those published by Peromingo et al. (2018).

The most important effect of the biopreservative microorganisms during ripening of dry-cured meats should be the minimisation, and ideally, the prevention of OTA contamination. *P. nordicum* did not synthesise OTA at detectable levels (<1.06 μg/kg) either alone or in the presence of *E. faecium* in the PnEf medium. This may be due to the fact that only 8 CSs were used to prepare this medium avoiding the normal growth of *P. nordicum*. However, *P. nordicum* was able to produce OTA in the other two media (PnDh and PnPc), at the most a_w x temperature conditions evaluated, except in the PnDh medium at 0.97 a_w x either 20 or 25 °C. This was not expected since normally filamentous fungi grow and produce mycotoxins at wetter and warmer conditions (Magan, 2007). In addition, the PnDh medium does not contain fructose which is a known repressor of OTA production (Medina et al., 2008). Further studies are needed to deeply investigate the mechanisms and interactions of each of the CSs used on OTA production by *P. nordicum*.

The maximum production of OTA by *P. nordicum* was detected at 0.97 a_w x 15 °C in both PnDh and PnPc media. From findings, it can be deduced that the *P. chrysogenum* isolate decreased the production of this mycotoxin at all the conditions evaluated. Nevertheless, the reduction of the mycotoxin amounts provoked by *D. hansenii* was much more pronounced reaching values higher than 90 % in all cases. This efficacy is similar to those described for *D. hansenii* in dry-cured meat products against both OTA-producing *P. nordicum* and *Penicillium verrucosum* (Andrade et al., 2014; Iacumin et al., 2017; Peromingo et al., 2018; Simoncini et al., 2014; Virgili et al., 2012).

The influence of *P. chrysogenum* on decreasing mycotoxin synthesis, including OTA in meat substrates has been reported (Bernáldez et al., 2014; Delgado et al., 2019a, 2019b; Rodríguez et al., 2015a). The observed effect of *P. chrysogenum* on the decrease of OTA production by *P. nordicum* is likely due to the above described nutritional competition, which is in turn related to a reduction in the secondary metabolism (Delgado et al., 2019b).

The utilisation of *D. hansenii* FHSCC 253H as biocontrol agent restricted the presence of OTA at quantities lower than 1 μg/kg, the guide value established by the Italian legislation for pork and by-products (Ministero della Sanitá, 1999). The effectiveness of *D. hansenii* FHSCC 253H on controlling the OTA synthesis by *P. nordicum* by repressing the expression of genes involved in the OTA biosynthesis has been previously reported (Peromingo et al., 2018).

The obtained results support the strategies of using native *P. chrysogenum* and *D. hansenii* as protective cultures to control ochratoxigenic *P. nordicum* in dry-cured meat products, since they are able to minimise both the growth and OTA production in the environmental and nutritional conditions related to the ripening of these foods. In addition, owing to the fact that *D. hansenii* is included in the European Qualified Presumption of Safety (QPS) list as a safe microorganism (EFSA BIOHAZ Panel, 2019), it can be added to foods without carrying out further studies. Besides, *P. chrysogenum* CECT 20922 does not produce any of 22 major foodborne mycotoxins, including citrinin and roquefortin C (Acosta et al., 2009). Therefore, the use of these isolates as a protective culture in dry-cured meat products would not suppose a food safety issue in the meat industry.

5. Conclusions

The application of the three biocontrol candidates displayed different antagonistic activities against the evaluated ochratoxigenic *P. nordicum* isolate. *E. faecium* was not able to compete against *P. nordicum* under the environmental and nutritional conditions related to the ripening of dry-cured meats. The ability of either *D. hansenii* or *P. chrysogenum* to utilise similar CSs, interact with and co-exist in some environmental conditions with *P. nordicum* may provide an ecological advantage to the two candidates in controlling the growth of *P. nordicum* and reduce the OTA production. The mode of action of *P. chrysogenum* CECT 20922 is based on competition for nutrients and space, without ruling out the production of the antifungal protein PgAFP. *D. hansenii* FHSCC 253H seems to produce some extra-cellular compounds although this antagonistic mechanism may be modified by prevailing environmental conditions. Therefore, both *P. chrysogenum* and *D. hansenii* could be proposed as biocontrol candidates in the manufacture of dry-cured meat products, and particularly *D. hansenii* FHSCC 253H due to its ability to control OTA at negligible levels.

Declaration of Competing Interest

The authors declare no conflict of interest.

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IV.4. Desarrollo de una metodología para cuantificar cambios en el ergosterol fúngico debido a la presencia de agentes antifúngicos

Development of a methodology for estimating the ergosterol in meat product-borne toxigenic moulds to evaluate antifungal agents

Article

Development of a Methodology for Estimating the Ergosterol in Meat Product-Borne Toxigenic Moulds to Evaluate Antifungal Agents

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Abstract: Antifungal agents are commonly used in the meat industry to prevent the growth of unwanted moulds, such as toxigenic ones, on dry-cured meat products. For enhancing the application of antifungals, their mode of action must be evaluated. Their effect on the mould ergosterol content is one of the most studied ones, since it is the target site of some commercialised antifungals or of those that are in development. The aim of this study was to develop a methodology for determining how the antifungal agents used in the meat industry work. A method for analysing ergosterol was firstly developed using high-performance liquid chromatography with fluorescence detection coupled to a diode array detector (HPLC-FLD/DAD). The chromatographically optimised conditions (gradient and mobile phases) allowed us to reduce the time per analysis with respect to previously published methods up to 22 min. Withing the six checked extraction methods, method 5, showing the best mean recovery values (99.51%), the shortest retention time (15.8 min), and the lowest standard deviation values (9.92) and working temperature (60 °C), was selected. The limit of detection and limit of quantification were 0.03 and 0.1 μg/mL, respectively. All the validation parameters corroborated the method's suitability. Finally, its feasibility for evaluating the effect of a commercial antifungal preparation (AP) and different herbs that are frequently added to meat products on the ergosterol content of several toxigenic moulds was studied. Differences at the strain level were obtained in the presence of AP. Moreover, the addition of herbs significantly reduced the ergosterol content in *Penicillium nordicum* up to 83.91%. The developed methodology is thus suitable for screening the antifungals' role in altering mould ergosterol biosynthesis before their application in real meat products.

Keywords: antifungals; meat products; toxigenic moulds; ergosterol; HPLC-FLD/DAD

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1. Introduction

Toxigenic moulds are frequently found on the surface of a wide range of dry-cured meat products [1,2]. The concern associated with this kind of moulds is linked to their capability to produce mycotoxins like aflatoxins, ochratoxin A (OTA), or cyclopiazonic acid (CPA) [1,3,4]. Examples of their toxicity are the immunosuppression and liver cancer caused by aflatoxins [5], the nephrotoxicity and the carcinogenicity provoked by OTA [6], and the immunotoxicity on human cells due to CPA [7].

The presence of these unwanted moulds has necessitated the development of several methods to avoid mycotoxin production. The most recently described strategies in dry-

cured meat products are related to biocontrol agents employing essential oils [8] or microorganisms, such as yeasts, lactic acid bacteria, and non-toxigenic moulds [9–11]. Nutritional and environmental factors greatly affect the growth of the protective cultures, which is a critical drawback for their application. Extensive studies of implementation and viability of such cultures in each particular case are thus necessary [11,12]. Due to such difficulties finding a universal biocontrol agent, the meat industry commonly employs legally authorised antifungals, such as benzoic acid, potassium sorbate (PS), and natamycin [13]. The recently developed alternatives to such synthetic preservatives are based on applying natural compounds of plant origin, such as essential oils or spices, in dry-cured fermented sausages [14,15].

To enhance an antifungal treatment, its mode of action has to be studied before its application. Despite the fact that this aspect has not been fully clarified, several reports have proposed ergosterol as the target site of their activity. Ergosterol is a lipid of the cellular membrane of moulds, which is essential for the growth of their mycelium [16]. Natamycin, one of the most applied antifungals in the meat industry, has been described as blocking mould growth by binding specifically to ergosterol [17]. Similarly, essential oils from oregano and cinnamon have been reported to reduce the ergosterol content of *Fusarium* spp. in maize samples [18]. *Eremanthus erythropappus* essential oil has been also described as a reducer of ergosterol content in *Aspergillus* spp. [19]. Moreover, compounds from essential oils, such as thymol and citral, have shown their effect on ergosterol content in *Fusarium graminEareum* and *Alternaria alternata*, respectively [20,21]. Nevertheless, there is no data about the targets of antifungals of plant origin when utilised against toxigenic moulds in dry-cured meat products.

Ergosterol is not water soluble, but appropriately dissolves in acetone and ethanol [22]. Accordingly, different kinds of organic solvents like butanol and hexane have been used for ergosterol extraction [23,24]. The analytical methods for its detection have been usually based on high-performance liquid chromatography (HPLC), with methanol as eluent [23–25], or gas chromatography (GC) with mass spectrometry detection [26]. Concretely, HPLC coupled with a UV detector is the most used method for analysing ergosterol [27,28], since it is easy to handle and has lower maintenance costs and technical requirements than other detectors or chromatographic techniques [29,30]. Whatever the method chosen, it is necessary to consider that the loss of ergosterol due to decomposition and light sensitivity can occur because of the time required for the different steps of the procedure [31]. Therefore, a reduction of 43% of its content after 24 h has been found under light action [25]. Due to the fact that the use of GC for ergosterol quantification implies previous sample derivatisation [26], such a technique could thus negatively affect the results and collaterally increase the time and the cost per analysis. For all the above-mentioned reasons, it is required that a faster and more convenient method be developed for checking the effect of antifungals on mould ergosterol content.

The aim of this study was to develop a methodology for evaluating the impact of antifungal agents on the ergosterol content of meat product-borne toxigenic moulds. Thus, a method for analysing ergosterol using HPLC with fluorescence detection coupled to a diode array detector (HPLC-FLD/DAD), as well as an extraction procedure based on previously published literature, were firstly optimised. After validating both protocols, their viability to evaluate the effect of a commercial antifungal preparation (AP) and different herbs frequently added to cured meat products on the ergosterol content of several toxigenic moulds was evaluated.

2. Materials and Methods

2.1. Standard and Reagents

All the chemical and chromatographic reagents were HPLC or analytical grade. Acetone, NaOH, 1-butanol, and chloroform were purchased from Scharlab, S.L. (Barcelona, Spain). Hexane, methanol, and acetic acid were obtained from Fisher Scientific S.L.

(Hampton, United States). Toluene was purchased from Panreac Química S.A. (Barcelona, Spain), and ergosterol ≥95% HPLC grade from Sigma-Aldrich (San Luis, United States).

The AP containing natamycin and PS was supplemented with 60 g/L of NaCl (Fisher Scientific S.L.), following the manufacturer's instructions (Manufacturas Taberner, S.A., Valencia, Spain).

2.2. Preparation of Standard Solutions

A stock solution (1 mg/mL) was prepared by dissolving standard ergosterol in acetone following the manufacturer's recommendations. Working solutions were daily prepared in limited light conditions using amber vials by diluting the standard solution in methanol, since it was the main constituent of the mobile phase (Cosela S.L., Sevilla, Spain). They were filtered through a 0.22 μm pore size nylon membrane (Cosela S.L.).

2.3. HPLC Method

Ergosterol was analysed by a HPLC-FLD/DAD model 1260 Infinity (Agilent Technologies, Santa Clara, United States). The column was a Phenomenex Luna C_{18}, (250 × 4.6 mm, 5 μm particle size; Phenomenex, Macclesfield, United Kingdom). The column temperature was established at 25 °C. The flow rate was set at 1 mL/min, and the injection volume was 20 μL. Ergosterol was detected at λ = 282 nm by the DAD and identified by the absorption spectrum of the standard. Calibration curves were built by diluting the stock solution from 0.1 to 100.0 μg/mL.

To optimise the analytical method for detecting and quantifying ergosterol, the gradients and composition of the mobile phase were assessed using the standard solutions. Firstly, 10 different gradients of mobile phase 1 (MP1), consisting of methanol/acetic acid 0.05% (*v*/*v*) 90/10 (*v*/*v*) (eluent A [24]) and methanol (eluent B) were evaluated (Table S1, Supplementary Materials).

After selecting the most appropriate gradient, three mobile phases were checked: MP1; mobile phase 2 (MP2), consisting of methanol/acetic acid 0.1% (*v*/*v*) 90/10 (*v*/*v*); and mobile phase 3 (MP3), composed by methanol/acetic acid 0.05% (*v*/*v*) 95/5 (*v*/*v*). The selection of the most adequate gradient and mobile phase relied on obtaining a chromatographic peak with a narrow width and symmetric shape, showing the earliest retention time and the shortest run time.

To evaluate the ability of the HPLC-FLD/DAD method to study the effect of antifungal agents, the ergosterol standard (10 μg/mL) was incubated in the presence of three AP concentrations (10%, 50%, and 90%) at different incubation times (0, 8, and 24 h). These concentrations represented different potential quantities of the AP found in meat products: 10% being a low level of the AP, 50% as a medium level, and 90% as a high level. Samples without AP (0%) were used as positive controls.

2.4. Extraction of Ergosterol

A total of six methods for extracting mould ergosterol were evaluated. They consisted of two previously reported ones [23,24] and modifications of such methods (Table 1).

Table 1. Main procedure steps and results of the methods for extraction of mould ergosterol evaluated in this study.

Methods	Main Steps Involved in the Procedure	Recovery (%)	Standard Deviation (%)[2]	Retention Time (min)	References
Method 1	2 mL NaOH 10% (*w/v*) in methanol + vortex 30 s + 1 h at 60 °C + 2 mL distilled water + 5 mL hexane + evaporation of the hexane extract + resuspension in 1 mL MP[1]	9.11*	±6.97	16.7	[24]
Method 2	2 mL NaOH 18% (*w/v*) in distilled water + 2 mL 1-butanol + vortex 30 s + 1 h at 90 °C + 2 mL toluene + centrifugation (5000 rpm for 5 min) + evaporation of the organic phase + resuspension in 1 mL MP	47.45*	±73.69	16.4	[23]
Method 3	2 mL NaOH 18% (*w/v*) in distilled water + 2 mL 1-butanol + vortex 30 s + 1 h at 90 °C + 2 mL chloroform + centrifugation (5000 rpm for 5 min) + evaporation of the organic phase + resuspension in 1 mL MP	103.10	±26.97	15.8	This study
Method 4	2 mL NaOH 18% (*w/v*) in distilled water + 2 mL 1-butanol + vortex 30 s + 30 min at 90 °C + 2 mL chloroform + centrifugation (5000 rpm for 5 min) + evaporation of the organic phase + resuspension in 1 mL MP	82.12	±80.37	15.9	This study
Method 5	2 mL NaOH 18% (*w/v*) in distilled water + 2 mL 1-butanol + vortex 30 s + 1 h at 60 °C + 2 mL chloroform + centrifugation (5000 rpm for 5 min) + evaporation of the organic phase + resuspension in 1 mL MP	99.51	±9.92	15.8	This study
Method 6	2 mL NaOH 18% (*w/v*) in distilled water + 2 mL 1-butanol + vortex 30 s + 1 h at 90 °C + 3 mL chloroform + centrifugation (5000 rpm for 5 min) + evaporation of the organic phase + resuspension in 1 mL MP	81.09*	±21.63	15.9	This study

[1] MP: mobile phase, composed of methanol/acetic acid 0.05% (*v/v*) 95/5 (*v/v*). [2] The experiment was performed in triplicate. * Significant differences regarding 100% recovery ($p \leq 0.05$). Ergosterol solutions were prepared from the standard to achieve a final concentration of 10 μg/mL. Different extraction solutions, reagent concentrations, and temperature and incubation times were checked. The extraction was always performed under limited light conditions.

The accuracy of each method was evaluated by the percentages of recovery (absolute recoveries) and the method with the best results (≈100% of recovery) was selected. The neat solvent was spiked by adding 10 μg/mL of ergosterol and then extracted following the above-mentioned methods. The results were compared with the area of a standard at the same concentration (10 μg/mL). The percentage of recovery (*RA*) was calculated according to the following equation:

$$RA\ (\%) = \frac{\text{average area (samples)}}{\text{average area (standards)}} \times 100 \quad (1)$$

All samples were tested in triplicate.

2.5. Validation Assays

The HPLC method was validated by determining the limit of detection (LOD) and limit of quantification (LOQ), linearity, precision, repeatability, within-lab reproducibility, and accuracy [7].

The LOD and LOQ were calculated as the minimum ergosterol concentration level at which the signal exceeded the noise level by a factor of 3 (signal-to-noise) and 10, respectively.

Linearity was determined by applying the coefficient of determination (R^2) in the range 0.1–100.0 μg/mL, and the precision by the analysis of three extractions was determined from the same samples under specified conditions. Both parameters were tested in triplicate.

The intra-day repeatability was analysed by three calibration curves in the range 0.1–100.0 μg/mL, conducted by the same operator in duplicate. The inter-day repeatability was checked by three calibration curves performed on three different working days. The within-lab reproducibility was conducted by two different operators, who built three independent calibration curves.

The accuracy was determined by *RA*, as explained in Section 2.4.

2.6. Mould Assessment

To check the suitability of the developed methodology for extracting and quantifying ergosterol when determining the modes of action of antifungal agents, four toxigenic mould strains were incubated in the presence of AP: the ochratoxigenic *Penicillium nordicum* CBS 323.92, belonging to Centraalbureau voor Schimmelcultures (The Netherlands), and *P. nordicum* BFE 856 from the Federal Research Centre for Nutrition and Food (Germany); the aflatoxigenic *Aspergillus flavus* CBS 573.65 from Centraalbureau voor Schimmelcultures; and the CPA producer *Penicillium griseofulvum* IBT 14319, supplied by the Type Culture Collection of the Department of Biotechnology from the Technical University of Denmark. In addition, *P. nordicum* CBS 323.92, one of the main OTA producers in meat products, was also selected to analyse the ergosterol content after applying rosemary, oregano, and thyme, which are herbs recently described as having antifungal activity in dry-cured fermented sausages [15] (Figure 1).

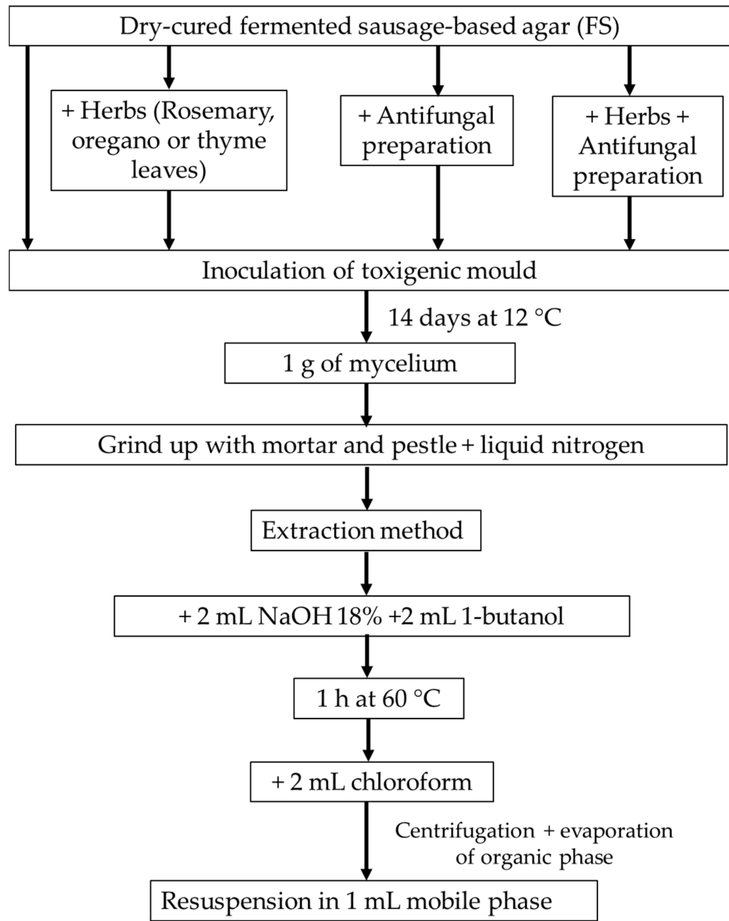


Figure 1. Flow chart showing the assay to check the suitability of the developed methodology for extracting and quantifying ergosterol when determining the effect of antifungal agents against meat product-borne toxigenic moulds. Mobile phase was composed of methanol/acetic acid 0.05% (*v/v*) 95/5 (*v/v*).

The mould inocula were prepared by growing them on potato dextrose agar (PDA; Scharlab, S.L.) at 25 °C for 7 days. Spores were harvested by scraping the plate surface with a glass rod after adding 3 mL of phosphate-buffered saline (0.32 g of NaH_2PO_4 (Scharlab, S.L.), 1.09 g of Na_2HPO_4 (Scharlab, S.L.), 9 g of NaCl (Scharlab, S.L.), and 1 L of distilled water). Spore suspensions were quantified using a Thoma Blaubrand counting chamber (Brand, Germany). A concentration of 10^6 spores/mL of each mould strain was individually inoculated on a dry-cured fermented sausage-based agar (FS; [15]). A volume of 25 µL of AP was distributed on the surface of the FS before the mould inoculation. To test the herbs, the FS was supplemented with 2 g/kg of rosemary (FS-R), oregano (FS-O), and thyme (FS-T), as described by Álvarez et al. [15]. Fresh rosemary and thyme leaves were used, while oregano leaves were added dried. The herbs were harvested in the region of Extremadura, located in the southwest of Spain. The effect of the herbs on the mould ergosterol content was evaluated alone and in combination with AP. As negative and positive controls, *P. nordicum* CBS 323.92 grown without herbs and in the presence of AP was used, respectively. The assay was performed in triplicate. After incubating for 14 days at 12 °C, 1 g of the mycelia was collected by scraping the surface with a scalpel and frozen with liquid nitrogen to stop the metabolic activity of the moulds before storing them at –80 °C. For the ergosterol extraction, the samples were firstly homogenised using a mortar and pestle with liquid nitrogen, and the obtained mycelium powder was subjected to the selected extraction method (Figure 1).

2.7. Statistical Analysis

Data analysis was carried out using the SPSS v. 20 software (IBM Corporation, Armond, United States). The non-parametric Kruskal–Wallis and Mann–Whitney U tests were applied, since the data failed the Shapiro–Wilk and Levene tests. The statistical significance was established at $p \leq 0.05$. The standard deviations were determined using Microsoft Excel 365 (Microsoft, Albuquerque, United States).

3. Results and Discussion

3.1. Improvement of the Chromatographic Conditions

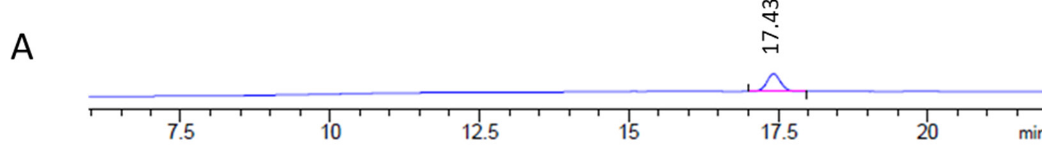
A reversed-phase column (C_{18}) was selected for the HPLC-FLD/DAD detection method, due to its good peak resolution [23,27]. Firstly, the selection of the most appropriate mobile phase was performed (Table S1, Supplementary Materials). Gradient 10 was the most suitable, since its retention time was the earliest (17.43 min) and its run time the shortest (22 min). Gradient 8 gave similar results, but it was dismissed because of its longer run time (27 min). The chosen gradient managed to reduce the analysis time to 45% compared to the previously developed methods for ergosterol detection, which employed between 30 and 40 min per analysis [23,24,32]. Such a method would thus allow minimising the potential degradation of ergosterol because of light exposure, with a subsequent decrease in false-negative results.

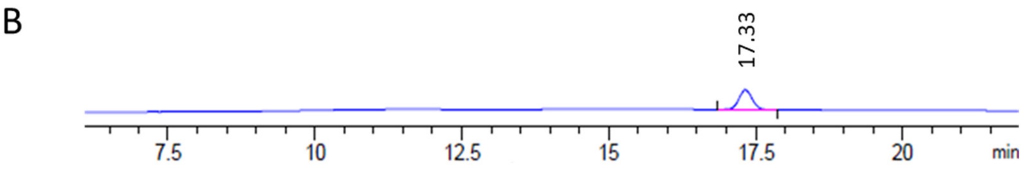
Regarding the mobile-phase composition, MP3, containing less acetic acid than MP1 and MP2, considerably reduced the retention time without losing precision, as its coefficient of variation value (7.59%) was less than 15% (Table 2). This is considered adequate by different official agencies [33,34]. MP3 also showed the best results for the peak width and asymmetry values (Table 2). A symmetrical, sharp peak with a narrow width (asymmetry value close to 1) has higher precision and is better separated than a more curved peak [35,36]. Such differences between mobile phases are shown in the chromatograms included in Figure 2. The gradient and mobile phase improvement was thus associated with shorter analysis time, enabling increased laboratory productivity because of the increase of the amount of analysis per day and the reduction of reagent costs.

Table 2. Results from the evaluation of three mobile phases (MP1–3) for detecting ergosterol (10 μg/mL) with Gradient 10 (0–5 min 10% B[1], 5–8 min linear increase from 10% to 100% B, 8–12 min 100% B, and 12–22 min linear decrease from 100% to 10% B).

Mobile Phases [2]	Retention Time (min)	Coefficient of Variations (%) [3]	Width of the Peaks (min)	Asymmetry
MP1	17.43	12.75	0.24	0.90
MP2	17.33	8.79	0.23	0.91
MP3	15.44	7.59	0.18	0.97

[1] Eluent A was composed of the corresponding MP and Eluent B by methanol. [2] MP1: methanol/acetic acid 0.05% (*v*/*v*) 90/10 (*v*/*v*); MP2: methanol/acetic acid 0.1% (*v*/*v*) 90/10 (*v*/*v*); MP3: methanol/acetic acid 0.05% (*v*/*v*) 95/5 (*v*/*v*). [3] The experiment was performed in triplicate.





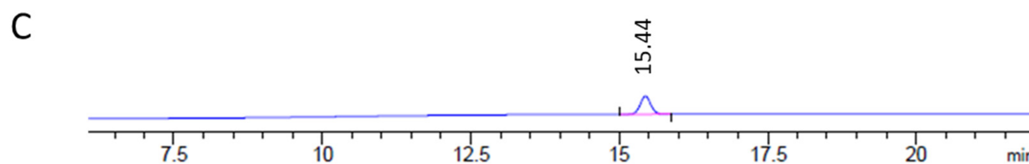


Figure 2. High-performance liquid chromatography with fluorescence detection coupled to a diode array detector (HPLC-FLD/DAD) chromatograms using an ergosterol standard (10 μg/mL) and the three different mobile phases tested in the present work. (A) Mobile phase 1, composed of methanol/acetic acid 0.05% (*v*/*v*) 90/10 (*v*/*v*); retention time: 17.43 min. (B) Mobile phase 2, composed of methanol/acetic acid 0.1% (*v*/*v*) 90/10 (*v*/*v*); retention time: 17.33 min. (C) Mobile phase 3, composed of methanol/acetic acid 0.05% (*v*/*v*) 95/5 (*v*/*v*); retention time: 15.44 min. Eluent A was composed of the corresponding mobile phase, and Eluent B of methanol. The gradient consisted of 0–5 min 10% B, 5–8 min linear increase from 10% to 100% B, 8–12 min 100% B, 12–22 min linear decrease from 100% to 10% B, with methanol being used as eluent B.

When the ability of the HPLC-FLD/DAD method to study the mode of action of antifungal agents was evaluated in the presence of different amounts of AP, a lack of detection of ergosterol was observed in the presence of the highest amount (Table 3). These findings corroborate those previously reported for both AP's components (PS and natamycin) when evaluating their effect on mould ergosterol biosynthesis. Therefore, PS has been associated with changes in the lipid composition, decreasing the sterol content [37] with the resultant reduction in ergosterol, the principal sterol in moulds [26]. In contrast, natamycin specifically binds to ergosterol, blocking mycelial growth [17]. In the present work, interference between AP and ergosterol depended on the applied concentration and not on the exposition time, which is of crucial interest for the meat industry, since the use of sub-inhibitory doses of antifungals has been related to undesirable mould growth, the emergence of mould resistance, and mycotoxin production [38–40].

Table 3. The concentration of ergosterol (μg/mL) when co-inoculated (10 μg/mL) with different amounts of antifungal preparation (AP) at different sampling times.

AP (%, *v*/*v*)	**Concentration of Ergosterol**		
	0 h	**8 h**	**24 h**
0	8.74 ± 1.28 [1]	8.44 ± 1.60	9.11 ± 0.72
10	9.28 ± 0.71	8.85 ± 0.56	9.03 ± 0.69

50	0.50 ± 0.43 *	n.d *	n.d *
90	n.d [2]*	n.d *	n.d *

[1] The experiment was performed in triplicate. [2] n.d: not detected (<limit of detection) * Significant differences regarding the absence of AP at the same sampling time ($p \leq 0.05$).

3.2. Extraction Method

When searching for reductions in the analysis time, the extraction method is considered to be as important as the detection one. The results obtained when evaluating the six different methods for extracting ergosterol are shown in Table 1. When testing the two previously reported methods (Methods 1 and 2), the lowest recoveries values were obtained. In addition, Method 2 presented a high standard deviation, which could be due to the fact that this method was originally designed to be used in yeast [23] instead of filamentous fungi that have a more complex cell wall. On the contrary, the best mean recovery values, together with the shortest retention times, were shown by Methods 3 and 5. The saponification step, with a higher concentration of NaOH in Methods 2 to 6, seems to be crucial for ergosterol extraction by increasing cell wall permeability and facilitating the entry of alcohols [23]. This feature, as well as the use of methanol instead of 1-butanol for extraction, could be the reason for the low recovery value found in Method 1. This is in accordance with Pastinen et al. [23], who described that alcohols with four carbons, such as 1-butanol, are better solvents for ergosterol extraction than alcohols with fewer carbons like methanol, which was that employed in Method 1.

Furthermore, when comparing Method 2 and those developed from it (Methods 3–6), it appears that the addition of chloroform instead of toluene triggers the improvement of the recoveries. This could be due to the good solubility of ergosterol in chloroform [41], which might also help to increase the permeability of the cell wall. Additionally, the permeability could increase by prolonging the incubation time, as better results were obtained when samples were incubated for 1 h (Method 3) than for 30 min (Method 4).

Within the procedures with the best outcomes, Methods 3 and 5 were the most appropriate. Method 5 was finally selected because of its lower standard deviation values and temperature than Method 3, since the detection of ergosterol can be negatively affected by high temperatures [42]. Consequently, Method 5 has been able to improve the results obtained from the previously reported methods [23,24].

3.3. Validation Parameters

The values for LOD and LOQ were 0.03 and 0.1 µg/mL, respectively, lower than those previously obtained by other authors using HPLC-FLD [24,30,42].

The linearity defined by calibration lines relating to ergosterol concentration and peak area gave an optimum value (Figure 3). The R^2 value is considered an indicator of linearity, and values close to the unit are synonymous to a straight line [29,43], while some studies have demonstrated curved relationships and suggest a visual inspection of calibration plots to confirm the linearity [44,45]. In our study, the linear trend of the calibration line is clearly visualised in Figure 3, supporting the good linearity of the method.

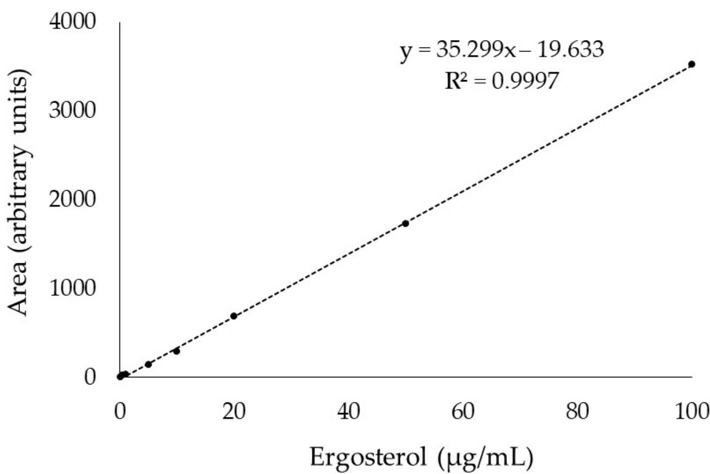


Figure 3. Standard calibration curve built in the range 0.1–100.0 μg/mL of ergosterol using the optimised HPLC-FLD/DAD method.

The calibration curves for intra-day and inter-day repeatability were similar, and demonstrated no significant differences between them, like the ones used for within-lab reproducibility ($p \leq 0.05$) (data not shown). These features support the use of the method independently of the operator.

3.4. Ergosterol Content of Toxigenic Moulds in the Presence of Antifungal Compounds

The extraction and HPLC-FLD/DAD methods were finally used to evaluate the effect of the AP and the selected herbs on the ergosterol content of toxigenic moulds (Tables 4 and 5). It should be pointed out that the assay was carried out using a meat-based model and not a real meat matrix, since the presence of the native microbial population of the latter having ergosterol would not give feasible results about the effect of the antifungals on the content of such compound. Nonetheless, a full understanding of the targets of antifungal strategies is desirable, since it would allow the establishment of the best conditions for their addition, minimising mycotoxin exposure, and consequently, improving food safety. Accordingly, FS has been selected to perform this assay, due to the fact that this culture medium efficiently simulates the composition of dry-cured, fermented sausages [15].

Table 4. Effect of an antifungal preparation (AP) on the ergosterol content (μg/g mycelium) of four toxigenic mould strains.

	Mould Strain			
	***Penicillium nordicum* CBS 323.92**	***P. nordicum* BFE 856**	***Penicillium griseofulvum* IBT 14319**	***Aspergillus flavus* CBS 573.65**
Control	731.55 ± 183.54[1]	365.57 ± 45.96	1121.61 ± 486.34	248.09 ± 174.49
Mould + AP	513.59 ± 92.84*	187.15 ± 92.14*	185.89 ± 158.75*	297.22 ± 207.47

[1] The experiment was performed in triplicate. * Significant differences regarding the control ($p \leq 0.05$).

Table 5. Effect of rosemary, oregano, and thyme on the ergosterol content (μg/g of mycelium) of *Penicillium nordicum* CBS 323.92.

Treatment[1]	Ergosterol Content
FS	731.55 ± 183.54[2]
FS-R	184.34 ± 44.50*
FS-O	177.40 ± 17.50*
FS-T	117.70 ± 69.30*
FS-R+AP	132.16 ± 44.50*
FS-O+AP	204.73 ± 50.85*
FS-T+AP	237.57 ± 11.99*

[1] FS: dry-cured, fermented, sausage-based agar (control); FS-R: FS with rosemary; FS-O: FS with oregano; FS-T: FS with thyme; FS-R+AP: FS-R with antifungal preparation; FS-O+AP: FS-O with antifungal preparation; FS-T+AP: FS-T with antifungal preparation. [2] The experiment was performed in triplicate. * Significant differences regarding the control ($p \leq 0.05$).

On the other hand, the novelty of the method applied with moulds has to be highlighted. The method consists of blocking the moulds' metabolic activity after sampling using liquid nitrogen (Figure 1), thereby reducing ergosterol instability following the mould death.

It has been described that ergosterol production depends on the mould species and colony age, as well as on the substrate [46]. Accordingly, our results show that *Penicillium* species were able to produce higher ergosterol amounts than *A. flavus* under control conditions. To our knowledge, there are no studies about mould ergosterol levels in a meat-based substrate, but previous studies have demonstrated a higher production of ergosterol in Penicillia than in Aspergilla in PDA and rice [46,47]. Furthermore, the influence of the AP on the ergosterol content in the tested toxigenic Penicillia moulds was corroborated, because it was significantly reduced in those samples incubated in its presence. The reducing effect of the components of AP on ergosterol content has been previously reported in Penicillia. Thus, PS has been described as significantly reducing the sterol content in *Penicillium roqueforti*, affecting the lipid bilayer [37], while natamycin has shown a high binding to ergosterol during germination in *Penicillium discolor* [48]. Additionally, ergosterol has shown to be the target of antifungal drugs used to treat fungal infections, such as azoles, which inhibit ergosterol biosynthesis in *Aspergillus fumigatus* [49].

On the contrary, the ergosterol content of *A. flavus* CBS 573.65 was not affected in the presence of the AP in this study. This lack of effect of natamycin on mould ergosterol has been previously described in *Aspergillus ochraceus*, and has been associated with the existence of resistances due to its continuous use; it even could enhance resistance to other polyene antifungals by cross-tolerance [39].

Regarding the effect of the addition of rosemary, oregano, and thyme leaves against the ochratoxigenic *P. nordicum* CBS 323.92, all of them significantly reduced the ergosterol content regardless of whether they were in the presence or absence of AP (Table 5). These findings are in accordance with previous studies testing different essential oils against toxigenic moulds. Thus, it has been reported that the essential oil extracted from rosemary decreased *in vitro* ergosterol content in toxigenic *A. flavus* and *Fusarium verticillioides*, although the minimum inhibitory concentration differs depending on the species [50,51]. Regarding oregano and thyme essential oils, some of their main components, such as carvacrol or thymol, have been reported to diminish the ergosterol content in *A. flavus* [52]. Despite the fact that there are not many studies focused on Penicillia, some essential oils, such as tea tree oil and betel leaf oil, have shown ergosterol reductions in *Penicillium expansum* [53,54]. The natural compounds cinnamaldehyde and citral from cinnamon and *Cymbopogon*, respectively, have also shown their effect by decreasing ergosterol in *P. expansum* [55]. Another study has suggested that the compound antofine from *Cynanchum atratum* can significantly impair ergosterol biosynthesis in *Penicillium digitatum* [56].

Therefore, the ergosterol seems to be the target site of the antifungal action of rosemary, oregano, and thyme, which could explain the reduction of OTA presence detected by Álvarez et al. [15] when testing their effects against *P. nordicum*. Accordingly, the synthesis of ergosterol has been associated with the OTA production in *A. ochraceus* and *P. verrucosum* [47,57]. Nevertheless, further studies are necessary to elucidate other modes of action that could be involved in the antifungal activities of rosemary, oregano, and thyme, since synergic effects are generally attributed to strategies against unwanted moulds.

4. Conclusions

A new methodology for the extraction and quantification of ergosterol from toxigenic moulds was developed to be used when evaluating the role of antifungal compounds applied in the meat industry. The method turned out to be precise, fast, and effective for ergosterol quantification in meat product-borne moulds. The method improved the previously developed procedures, due to the reduction of the run time up to 22 min, and consequently, the possible degradation of ergosterol in ergocalciferol. In addition, the methodology proved to be useful for determining the effect of antifungal agents related to dry-cured meat products on the ergosterol content, independent of the toxigenic mould strain. The present method could be thus used as a support tool for improving information about antifungals before their application in meat products, which would enhance their use.

Supplementary Materials: The following are available online at www.mdpi.com/2304-8158/10/2/438/s1, Table S1: Mobile phase gradient conditions evaluated in this study for detecting and quantifying ergosterol in meat product-borne toxigenic moulds by HPLC-FLD/DAD.

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IV.5. Cambios producidos por los agentes de biocontrol en el proteoma de *Penicillium nordicum*

Proteomic analyses reveal mechanisms of action of biocontrol agents on ochratoxin A repression in Penicillium nordicum

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Proteomic analyses reveal mechanisms of action of biocontrol agents on ochratoxin A repression in *Penicillium nordicum*

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A B S T R A C T

Penicillium nordicum is among the main ochratoxin A (OTA) producers in dry-cured fermented sausages. Recent strategies to prevent its development on the surface of these products include the substitution of synthetic antifungals, such as potassium sorbate, by natural compounds. However, the mechanisms of action of the latter are still unclear. The Proteomic analysis is a useful tool to elucidate the basis of the antifungal compound effects on moulds. Therefore, the aim of this study was to evaluate the efficiency of biocontrol agents on the OTA repression in *P. nordicum* and its relation to proteome changes. Seven different combinations of rosemary leaves, rosemary essential oil, acorn shell extract and the yeast *Debaryomyces hansenii* have been tested as biocontrol agents against two ochratoxigenic *P. nordicum* strains on a dry-cured fermented sausage-based medium. The rosemary essential oil and the combination of rosemary leaves with *D. hansenii* provoked significant inhibition of OTA production in both moulds ($p \leq 0.05$). The acorn shell extract and *D. hansenii* individually decreased OTA only in one of the mould strains. The Proteomic analyses revealed that rosemary essential oil reduced the abundance of proteins involved in the polyketide synthase enoylreductase domain in both moulds, which would explain the OTA reduction. However, the combination of rosemary leaves with *D. hansenii* showed inter-strain differences, reducing the abundance of proteins related to the cell wall integrity and to the purine pathway, depending on the tested mould. In conclusion, rosemary essential oil and the combination of rosemary leaves with *D. hansenii* can be proposed as efficient biocontrol agents against ochratoxigenic *P. nordicum* on dry-cured fermented sausages, although some inter-strain differences at proteome level were found regarding the mechanism of action involved in the reported OTA repression.

1. Introduction

The ripening stage of dry-cured fermented sausages offers favourable conditions for surface mould colonisation. This population provides some advantages from the technological point of view, contributing to further enhance their sensory properties (Iacumin et al., 2009; Leroy & De Vuyst, 2015). However, some moulds may produce highly toxic mycotoxins. Ochratoxin A (OTA) is one of the most frequently found mycotoxins in dry-cured fermented sausages (Pleadin et al., 2017; Zadravec et al., 2020), being mainly produced by *Penicillium* and *Aspergillus* species (Iacumin et al., 2009). Particularly, *Penicillium nordicum* is considered among the main OTA-producing moulds in dry-cured meat products (Battilani et al., 2007; Comi et al., 2013; Iacumin et al., 2009; Sonjak et al., 2011). OTA has been classified as "possibly carcinogenic to humans" (Group 2B; IARC, 1993; Ostry et al., 2017), and the animals exposed to this mycotoxin develop renal lesions and suffer neurotoxic, cytotoxic and teratogenic effects (Cimbalo et al., 2020).

A recent opinion from the European Food Safety Agency has highlighted meat and meat products as one of the main contributors to OTA exposure within the European Union (Schrenk et al., 2020). This will probably lead to a limit for OTA in this kind of foods in the European Union, so far only established in Italy (1 μg/kg; Ministero della Sanità, 1999). To limit the OTA presence in dry-cured fermented sausages, the meat industry employs chemical antifungal compounds legally permitted as additives, such as natamycin or sorbates (European Commission, 2011). Nonetheless, consumers currently demand natural products, free from chemical additives and preservatives (Román et al., 2017). Consequently, recent strategies for controlling toxigenic moulds are based on the use of biocontrol agents (BCAs) from plant or microbial

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origin (Oliveira et al., 2018; Álvarez, Andrade, García, Rondán, & Núñez, 2020). Essential oils from herbs, such as rosemary, cinnamon or oregano, have shown antifungal activity against ochratoxigenic moulds like *Aspergillus carbonarius*, *Aspergillus ochraceus* and *Penicillium verrucosum* (El Khoury et al., 2016; Hua et al., 2014; Jeršek et al., 2014). Moreover, rosemary and oregano have also demonstrated reductions in OTA production by *P. nordicum* (Álvarez, Rodríguez, Núñez, Silva, & Andrade, 2020). In addition, some by-products from Iberian pig industry of plant origin, such as acorn shells, have demonstrated antimicrobial activity (Burlacu et al., 2020). On the other hand, several microorganisms have been described as BCAs, being the yeast *Debaryomyces hansenii* widely studied against *P. nordicum* (Andrade et al., 2014; Delgado et al., 2019; Simoncini et al., 2014). However, the mechanism of action of these BCAs are not fully elucidated. The antifungal effect of rosemary essential oil as well as rosemary leaves has been associated with the downregulation of genes involved in the OTA biosynthesis in *A. carbonarius* and *P. nordicum*, respectively (El Khoury et al., 2016; Álvarez, Rodríguez, et al., 2020). Some of these genes (*acpks*, *aOTApks* and *otapks*) encode polyketide synthases (PKSs), which play an essential role in the production of secondary metabolites like mycotoxins (Wang et al., 2015). Fungal PKSs include multiple subdomains, such as β-ketoacyl reductase (KR), dehydratase (DH) and *trans*-acting enoyl reductase (ER) (Gallo et al., 2013). These subdomains act in a modular chain involved in the necessary reactions for producing the final PKSs (Gallo et al., 2013). The antagonistic effect of *D. hansenii* on toxigenic moulds has been related to the competition for nutrients and space, the production of killer toxins and volatile compounds (Andrade et al., 2014; Núñez et al., 2015; Çorbacı & Uçar, 2018), the reduction of proteins involved in cell wall integrity pathway (CWI) (Delgado et al., 2019) and the inhibition of gene expression involved in mycotoxins biosynthesis (Peromingo et al., 2019). To elucidate the mechanisms of action implied in the antifungal activity of BCAs, comparative proteomic analysis is a useful tool. Recent studies have used this technique to establish the proteome changes due to different antifungal agents, such as the protein PgAFP (Delgado et al., 2017), chitosan (Li et al., 2020) and *D. hansenii* (Delgado et al., 2019). Thus, the use of this tool to unveil the mechanisms of action of BCAs for controlling OTA-producing moulds would be of utmost interest.

The objective of this work was to elucidate the mechanisms of action of the most efficient antiochratoxigenic BCAs against *P. nordicum*. The ability to decrease *P. nordicum* OTA production by rosemary (*Rosmarinus officinalis*) and its essential oil (REO), acorn shell extract from *Quercus ilex* (ASE), and *D. hansenii,* alone or in combination were firstly tested on a dry-cured fermented sausage-based medium (FS). Quantitative proteomic analyses were then performed to evaluate the mechanisms involved in the OTA repression due to the BCAs presence.

2. Material and methods

2.1. Microorganisms

Two strains of *P. nordicum* were used in this study: *P. nordicum* FHSCC Pn15 (Pn15) from the Culture Collection of Food Hygiene and Safety (FHSCC) of the University of Extremadura (Cáceres, Spain) and *P. nordicum* BFE 856 (Pn856) kindly supplied by Rolf Geisen from Max Rubner-Institut (Karlsruhe, Germany). *D. hansenii* FHSCC 253H (Dh), also from the FHSCC, was tested as BCA. All these microorganisms had been isolated from dry-cured meat products.

2.2. Plant material

The plant-based BCAs were rosemary leaves, REO and ASE. The rosemary and the holm oak acorns were collected in the region of Extremadura, located in the southwest of Spain. In the assays, fresh rosemary was used, whilst the acorns were frozen at −20 °C until their use.

REO was extracted by Clevenger distillation based on the separation of two phases (aqueous and oily) through boiling the raw leaves with distilled water (Figueredo et al., 2012). Briefly, the raw rosemary leaves were separated from the stems and ground in a mincer model Pc123N (Jata S.A., Spain). Then, 200 g of minced leaves were introduced in the Clevenger flask with 400 mL of distilled water and brought to boil. After 2 h, a high percentage of the essential oil was separated, and kept at −20 °C. Before its use, REO was dissolved in water with 1% (v/v) of Tween 80 (Fisher Scientific, USA), achieving a final concentration of REO:water 1:1.

To obtain the ASE, the shells were separated from the pulps and minced until a fine powder was obtained. Then, 40 g of this powder were mixed with 200 mL of acetone (Scharlab, S.A., Spain) 80% (v/v) in distilled water in a Stomacher bag with filter. This mixture was homogenised for 5 min, the filtered solution was collected, and then subjected to ultrasound in a bath sonicator Ultrasons (J.P. Selecta S.A., Spain) for 15 min. After centrifuging the extract at 20854×*g*, the supernatant was evaporated in a rotavapor R210 (BÜCHI Labortechnik A.G., Switzerland) to eliminate the acetone. Finally, the ASE was transferred to 15 mL tube and the oxygen of the head space was eliminated under a flow of nitrogen to minimise oxidation. The extract was kept at −80 °C until use.

2.3. Culture media

Potato dextrose agar (PDA) was prepared following the manufacturer instructions (Scharlab, S.L.). Yeast Extract Sucrose broth (YES) was made by adding 20 g/L of yeast extract (Scharlab, S.L.) and 125 g/L of sucrose (Scharlab, S.L.) to 1 L of distilled water.

The FS, which emulates the composition of dry-cured fermented sausages, was made using 250 g/L of lyophilised Spanish dry-cured fermented sausages "salchichón" and 20 g/L of bacteriological agar (Scharlab, S.L.) as previously described (Álvarez, Rodríguez, et al., 2020). The medium FS-R was obtained by adding 2 g of rosemary leaves per kg of lyophilised "salchichón" to FS.

All the media were autoclaved for 20 min at 121 °C and plated in 9-cm Petri plates.

2.4. Inoculum and experimental settings

Mould strains were inoculated at 3-point per plate in PDA and incubated for 10 days at 25 °C. Spores were then collected by the addition of 3 mL of phosphate buffer saline (PBS) [0.32 g of NaH_2PO_4 (Scharlab, S. L.), 1.09 g of Na_2HPO_4 (Scharlab, S.L.), 9 g of NaCl (Scharlab, S.L.), 1 L of distilled water] scraping the plate surface with a glass spatula. The concentrations of spores were determined using a Thoma counting chamber Blaubrand® (Brand, Germany), visualising them in a microscope (NIKON, Japan) and adjusted to 10^6 spores/mL.

D. hansenii was incubated in 10 mL of YES for 2 days at 25 °C under stirring conditions. The culture was then centrifuged at 10640×*g* for 5 min and the pellet was resuspended in PBS. The yeast concentration was adjusted to 10^6 cells/mL as previously described for moulds.

A total of 8 treatments were applied to each *P. nordicum* strain (Table 1). Where Dh was used, 100 μL from the stock inoculum suspension of 10^6 cells/mL were inoculated in the melted agar, just before plating. The media were covered by a collagen casing (Viscofan, Spain) previously sterilised by ethanol immersion for 1 s and exposed to UV light (Telstar, Japan) for 24 h. Then, 100 μL of REO or 30 μL of ASE were spread on the casings of the corresponding treatments. Finally, 100 μL of the toxigenic mould were inoculated and spread on every casing. The plates were incubated at 12 °C for 15 days to simulate the usual temperature during ripening of dry-cured fermented sausages. The experiment was performed in triplicate.

Table 1
Biocontrol agents applied against toxigenic *Penicillium nordicum* FHSCC Pn15 and BFE 856 (Pn) in a dry-cured fermented sausage-based agar.

Treatment	Name	Concentration/cells per treatment
Non-treated *P. nordicum* control	Pn	100 μL of 10^6 spores/mL*
Rosemary leaves	Pn-R	2 g/kg
Rosemary essential oil	Pn-REO	100 μL
Acorn shell extract	Pn-ASE	30 μL
Debaryomyces hansenii	Pn+Dh	100 μL of 10^6 cells/mL
Rosemary leaves + *D. hansenii*	Pn-R+Dh	2 g/kg + 100 μL (10^6 cells/mL)
Rosemary essential oil + *D. hansenii*	Pn-REO + Dh	100 μL + 100 μL (10^6 cells/mL)
Acorn shell extract + *D. hansenii*	Pn-ASE + Dh	30 μL + 100 μL (10^6 cells/mL)

*Every treatment contains this level of *P. nordicum* spores plus the referred concentration/cells in each treatment.

2.5. OTA extraction and analysis

After the incubation period, around 1 g of sample (mycelium from the edge to the centre across one of the diameters of the plate + casing that held the mycelium) from each plate was collected with a sterile scalpel and transferred to a 45 mL tube. The samples were kept at −20 °C until their extraction using the QuEChERs procedure previously described by Delgado et al. (2018). The detection and quantification of this mycotoxin were carried out by an Agilent 1290 Infinity II uHPLC coupled with 6470 triple quadrupole (QqQ; Agilent Technologies, Inc., USA) following the methodology described by Álvarez, Rodríguez, et al. (2020). The column was a Zorbax C_{18}, 100 mm × 2.1 mm, 1.8 μm working at 45 °C. The separation used a binary gradient and OTA was detected at 6.5 min. The run time was 15 min.

2.6. Proteomic analysis

Around 200 mg of mycelium from every casing surface (n = 3 per treatment) were flash-frozen in liquid nitrogen and stored at −80 °C until extraction, following the methodology reported by Delgado et al. (2019) with some modifications. Firstly, 400 μL of lysis buffer pH 7.5 [Tris-HCL 100 mM (Sigma Aldrich, USA), NaCL 50 mM, EDTA 20 mM (Sigma Aldrich), glycerol 10% (v/v) (Fisher Scientific), PMSF 1 mM (Sigma Aldrich) and 1 μg/mL of pepstatin A (Sigma Aldrich)] were added before probe sonication in a Branson sonifier™ 250 (Emerson, Spain). Mould lysates were centrifuged to remove cell debris. The lysates were partially run in a SDS-PAGE to be excised and in-gel digested as previously described by Shevchenko et al. (2006) with some modifications. The bands were subjected to a process of reduction with dithiothreitol (Promega, USA) and iodoacetoamide-mediated alkylation (Promega). Then, the trimmed bands were digested with sequencing-grade trypsin (Promega) and ProteaseMAX surfactant (Promega) for 1h at 50 °C, following the manufacturer's instructions. A total of 5 μg from every sample were analysed using a Q-Exactive Plus (Thermo Scientific, Germany) coupled to a Dionex Ultimate 3000 RSLCnano (Thermo Scientific). The gradients used ranged from 8 to 30% B (A: 0.1% formic acid (FA), B: acetonitrile, 0.1% FA) for 4 h on an Acclaim PepMap RSLC C18, 2 μm, 100 Å, 75 μm i.d. × 50 cm, nanoViper (Thermo Scientific), thermostated at 45 °C in the oven compartment.

Data was collected using a Top15 method for MS/MS scans (Delgado et al., 2017, 2019; Dolan et al., 2014). Label-free comparative proteome abundance and data analysis were performed using MaxQuant software (v. 1.6.15.0; https://www.maxquant.org/download_asset/maxquant/latest) (Cox & Mann, 2008), with Perseus (v. 1.6.14.0) applied to organise the data and conduct statistical analysis. Carbamidomethylation of cysteines was set as a fixed modification, while oxidation of methionines and acetylation of *N*-terminals were set as variable modifications. Database searching was performed against a *P. nordicum* protein database downloaded from Uniprot (https://www.uniprot.org/). The maximum peptide/protein false discovery rates (FDR) were set to 1% based on comparison to a reverse database. The label-free quantitative algorithm (LFQ) was used to generate normalised spectral intensities and infer relative protein abundance (Luber et al., 2010). Proteins that matched with a contaminant database or the reverse database were removed, and proteins were only retained in final analysis if detected in at least two replicates from at least one treatment. Quantitative analysis was performed using a *t*-test to compare treatments to the control ($p < 0.05$). Qualitative analysis was also performed to detect proteins found in at least two replicates of a particular treatment but undetectable in the compared treatment. For enrichment analysis, the proteins were evaluated trough ClueGO (v. 2.5.6) (Bindea et al., 2009). To define term-term interrelations and functional groups based on shared genes between the terms, the Kappa score was stablished at 0.4. Three GO terms and 4% of genes covered were set as the minimum required to be selected. The *p*-value was corrected by Bonferroni step down and set as $p \leq 0.05$.

2.7. Statistical analysis

SPSS software v. 20 (IBM Corporation, USA) was used for the OTA data analysis. After testing their normality, OTA data showed a non-normal distribution. Thus, the non-parametric tests Kruskal-Wallis and Mann-Whitney U were performed. The statistical significance was established at $p \leq 0.05$.

3. Results and discussion

The ability of several plant-based BCAs alone and combined with *D. hansenii* to inhibit the OTA production by *P. nordicum* has been tested in the present study. Besides, to inquire how *P. nordicum* is affected by the most efficient BCAs regarding the OTA repression, comparative proteomic analyses were carried out. To the best of our knowledge, this is the first proteomic approach to plant-based BCAs' effect on *P. nordicum*. Notably, this study has been performed by using a model culture medium similar to dry-cured fermented sausages in composition but lacks other microorganisms from those intentionally added: *P. nordicum* and/or *D. hansenii*. This fact allows identification of putative mechanisms under highly controlled conditions, which could be hidden in natural systems (Crowther et al., 2018).

3.1. OTA production

Both tested *P. nordicum* strains were able to produce OTA on the dry-cured fermented sausage-based substrate at remarkably different levels. The non-treated control of Pn15 produced 7.78 ± 4.76 ng/g of OTA whilst that of Pn856 produced 879.49 ± 359.22 ng/g. A high intraspecific variability on mycotoxin production, including OTA in *P. nordicum,* has been previously reported (Berni et al., 2011). This feature has been attributed to isolates' geographical origin, substrate, environmental conditions, molecular characteristics, etc. (Garcia et al., 2011). In this sense, Pn15 had been isolated in Spain (Delgado et al., 2018), while Pn856 comes originally from Italy (Leggieri et al., 2020).

The effect of the different BCAs on OTA production by both tested moulds is shown in Fig. 1. Significant OTA reductions were found in Pn-REO and Pn-R + Dh for both Pn15 and Pn856 ($p \leq 0.05$). Moreover, Pn15 showed significant decreases in OTA accumulation in Pn-ASE and Pn + Dh treatments ($p \leq 0.05$). Thus, each strain of *P. nordicum* was affected in a different manner by the BCAs, with Pn15 exhibiting higher sensitivity to them than Pn856.

Rosemary leaves only showed inhibitory effect when applied in combination with Dh (Pn-R + Dh), although it had displayed a significant OTA reduction on another *P. nordicum* strain (Álvarez, Rodríguez, et al., 2020). In that study, rosemary leaves reduced OTA production as well as the relative expression of OTA biosynthetic genes (*otapks* and *otanps*) after 14 days of incubation. Regarding the antifungal effect of

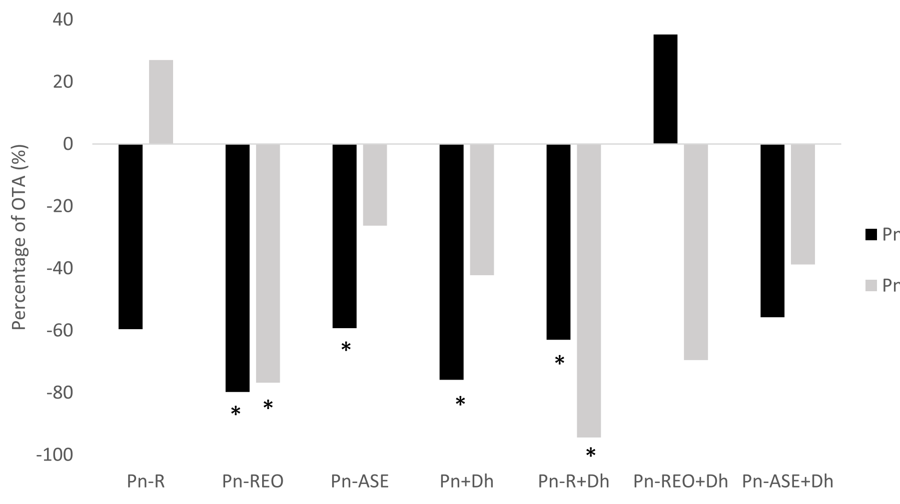

Fig. 1. Ochratoxin A (OTA) production by *Penicillium nordicum* FHSCC Pn15 and BFE 856 (Pn) using different biocontrol agents in a dry-cured fermented sausage-based agar. OTA expressed as percentage of reduction/increment compared to the non-treated control (Pn grown in the absence of biocontrol agents). Treatments: Pn-R: Rosemary; Pn-REO: Rosemary essential oil; Pn-ASE: Acorn shell extract; Pn + Dh: *Debaryomyces hansenii* FHSCC 253H (Dh); Pn-R + Dh: Rosemary + Dh; Pn-REO + Dh: Rosemary essential oil + Dh; Pn-ASE + Dh: Acorn shell extract + Dh. Pn15: *P. nordicum* FHSCC Pn15. Pn856: *P. nordicum* BFE 856. *Statistical differences ($p \leq 0.05$) between a given treatment and the non-treated control. The statistical analysis was performed using the mean concentration ± standard deviation of each treatment. The experiment was performed in triplicate.

REO, its antiochratoxigenic activity on aspergilla has been widely described (Císarová et al., 2020; El Khoury et al., 2016, 2017). Besides, this essential oil has been reported as an inhibitor of the biosynthesis of other mycotoxins, such as cyclopiazonic acid and aflatoxins (Císarová et al., 2020; Rasooli et al., 2008). With respect to those treatments including ASE, the only significant reduction in OTA production ($p \leq 0.05$) was found in Pn15 (Pn-ASE). The antifungal effect of ASE from *Q. ilex* had not been previously reported, whilst *Quercus infectoria* acorn extracts have demonstrated inhibitory effect against *Candida* spp. in eggs (Burlacu et al., 2020). Nevertheless, the antibacterial activity of acorn extracts from *Quercus* spp. has been broadly described (Burlacu et al., 2020). For instance, the acorn shells from *Quercus variabilis* have shown antibacterial activity against *Staphylococcus aureus* and *Salmonella* Paratyphi triggering a leakage of proteins through the bacterial membrane (Zhou et al., 2019). Its effect on *P. nordicum* is likely different as moulds possess unique structures, such as cell wall, and presumably alternative cellular targets are available in them. The observed drop in the OTA biosynthesis by Pn15 due to the action of Dh (Pn + Dh) had been previously described in dry-cured ham-based agar (Delgado et al., 2019), being associated with the reduction in abundance of proteins involved in CWI. Moreover, *D. hansenii* has shown antifungal effect on dry-cured meat substrates against other *P. nordicum* strains (Andrade et al., 2014; Cebrián et al., 2019) and other moulds, such as the aflatoxigenic *A. parasiticus* (Peromingo et al., 2019). On the contrary, *D. hansenii* when individually inoculated, did not significantly reduce the OTA production in Pn856 ($p > 0.05$; Fig. 1). Thus, the antiochratoxigenic activity of *D. hansenii* depended on the tested mould strain. However, the addition of *D. hansenii* and rosemary (Pn-R + Dh) significantly decreased the OTA levels in both toxigenic moulds ($p \leq 0.05$). Therefore, the combination of these two BCAs seems to have a synergistic effect, boosting the reduction of the OTA biosynthesis.

3.2. Comparative proteomic analysis

Quantitative proteomic analyses were carried out for *P. nordicum* treated with REO and rosemary leaves combined with *D. hansenii* (Pn-REO and Pn-R + Dh), due to the significant decrease of OTA production in both moulds ($p \leq 0.05$; Fig. 1). This tool has been proven to be fruitful for unravelling the mechanism of action of antifungal proteins, BCAs and chitosan on different moulds of interest from the food safety point of view (Delgado et al, 2017, 2019; Li et al., 2020).

The LFQ analysis identified a total of 1371 proteins in Pn15 and 1196 in Pn856. Quantitative ($p < 0.05.$) and qualitative (only detected in one condition) changes in protein abundance were identified (Tables S1–S4 Supplementary material). The level of 84 proteins was lowered in the presence of REO in Pn15 while only 14 were reduced in Pn856. On the other hand, 35 proteins were increased in Pn15 and 50 in Pn856. In Pn15, 569 proteins were identified only in control (Pn) whilst 5 only in Pn-REO. In contrast, in Pn856, 5 proteins were exclusively found in the control (Pn) and 125 proteins in Pn-REO.

The ClueGO software performs gene ontology analyses, allowing to group the different proteins in general “groups” that are subdivided in more specific “terms”. The analyses conducted with this bioinformatic tool indicated that the proteins decreased in the presence of REO in Pn15 belonged to 186 metabolic routes or terms, and the increased ones are only involved in 11 routes (data not shown). The pathways including decreased proteins were grouped, being necessary to highlight those found with the highest number of terms per group (about 16% each one, Fig. S1 Supplementary material) related to nucleobase-containing small molecule metabolic process, aromatic amino acid family metabolic process and positive regulation of organelle organisation. The majority of the terms for the proteins found in the highest relative abundance were classified in the polysaccharide metabolic process and the hydrolase activity compound groups (27.27% each group) (Fig. S2 Supplementary material). In contrast, the software did not show any group for Pn856 due to the few decreased proteins, but the raised proteins were included in 29 metabolic routes and 10 groups (Fig. S3 Supplementary material).

Although the impact at proteome level of REO was much higher on Pn15 than on Pn856, both strains were similarly affected regarding the OTA biosynthesis inhibition (Fig. 1). This might point to the existence of any common pathway involved in the abovementioned OTA repression. Regarding the shared pathways among the altered proteins, a lower abundance of proteins belonging to the PKS ER domain were found when both strains were treated with REO (Table 2). The PKS ER domain has been previously associated with the OTA biosynthesis in *A. ochraceus* (Wang et al., 2015). This domain has been also involved in the biosynthetic gene cluster of other fungal toxins as betaenona from *Phoma betae* and the host-selective toxins (HTS) from *Alternaria alternata* (Ajiro et al., 2009; Ugai et al., 2015). Therefore, the application of REO provoked a decrease in the proteins involved in the PKS ER domain, which would explain the inhibition of OTA biosynthesis in both moulds.

The combination of biocontrol agents consisting of *D. hansenii* and rosemary leaves also induced changes in the proteomes of both *P. nordicum* strains. A total of 66 proteins were reduced and 72 were raised in abundance in Pn15, while 36 proteins were decreased and 65 increased in Pn856. In Pn15, 88 proteins were only found in the non-treated control and 146 in R + Dh. In Pn856, 9 proteins were solely encountered in the non-treated control and 153 in R + Dh. Considering the number of altered proteins, the impact of these two BCAs was less

Table 2
Changes in protein abundance ($p < 0.05$) for *Penicillium nordicum* FHSCC Pn15 (Pn15) and *P. nordicum* BFE 856 (Pn856) by two of the applied treatments in dry-cured fermented sausage-based agar (FS).

Mould strain	Treatment[a]	Protein	ID	Fold change
Pn15	Pn-REO	PKS ER domain-containing protein	A0A0M9WBI0	Only in C*
			A0A0M8P146	Only in C
			A0A0M9WBD1	Only in C
			A0A0M8PHV1	Only in C
			A0A0M8NZI2	Only in C
			A0A0M9WA89	Only in C
			A0A0M8PC30	Only in C
			A0A0M8PC30	Only in C
			A0A0M8P7Q6	Only in C
			A0A0N0RZN3	Only in C
			A0A0M8P7I5	Only in C
			A0A0M9WIV4	Only in C
			A0A0M9WIK7	Only in C
			A0A0M9WIK6	Only in C
			A0A0M9WKW6	Only in C
			A0A0M8P8D8	Only in C
			A0A0N0RYG6	Only in C
			A0A0M8NWL5	Only in C
			A0A0M9WCE2	Only in C
			A0A0M9WC73	Only in C
			A0A0N0RXZ1	−138.38
	Pn-R + Dh	3-methyl-2-oxobutanoate hydroxymethyltransferase	A0A0M8PBL5	Only in C
		Putative metallocarboxypeptidase ECM14	A0A0M9WKJ2	−1.10051
Pn856	Pn-REO	PKS ER domain-containing protein	A0A0M8PHV1	−2.45
	Pn-R + Dh	Pyruvate dehydrogenase E1 component subunit beta	A0A0M8NSB5	2.73683
		Rhamnogalacturonate lyase	A0A0N0RZ79	3.04426

[a] Pn-REO: Rosemary essential oil added on the surface of collagen casings over FS (100 μL); Pn-R + Dh: Rosemary leaves (2 g/kg) + Debaryomyces hansenii FHSCC 253H (100 μL of 10^6 cfu/mL) added to melted FS. *Only in C: Protein detected only in the non-treated control.

powerful on the *P. nordicum* strains than that of REO.

The results obtained from ClueGO related the proteins found in the lowest abundance with 12 terms or metabolic routes (data not shown) within 4 groups in Pn15, being the 58.33% of the terms per group gathered in carboxylic acid metabolic processes (Fig. S4 Supplementary material). The increased proteins in Pn15 were included in 30 metabolic routes belonging to 4 groups, highlighting that the 66.67% of the terms were linked to translation step (Fig. S5 Supplementary material). In Pn856, 7 metabolic routes were down, being the majority of the terms from these groups (85.71%) related to the purine nucleoside binding (Fig. S6 Supplementary material). In this strain, 35 metabolic routes from proteins found in higher abundance than in the non-treated control were described belonging to 10 groups, being the most abundant the carbohydrate catabolic process (22.86%, Fig. S7 Supplementary material).

Despite the fact that in the base of the number of altered proteins no apparent dramatic effect was observed by the action of R + Dh on *P. nordicum* strains, the results showed a drop in the amount of the protein 3-methyl-2-oxobutanoate hydroxymethyltransferase in Pn15 (Table 2), which is involved in the synthesis of Acetyl-CoA. This is part of the pathway that synthesises pantothenate, reported as indispensable for the synthesis of Coenzyme A in fungi (Calder et al., 1999; Chiu et al., 2019), the precursor of Acetyl-CoA. This molecule has been described as a precursor of OTA where the PKS combines Acetyl-CoA and malonyl-CoA to synthetise 7-methylmellein, which is oxidised to ochratoxin β (Gallo et al., 2013; Wang et al., 2018). Additionally, Acetyl-CoA is crucial for multiple metabolic processes, such as biosynthesis of ergosterol (Chiu et al., 2019; Dupont et al., 2012), an essential component of mould membranes with a key role in the regulation of cell permeability (Kadakal & Tepe, 2019). Therefore, the ergosterol might be affected by the combined antifungal effect of rosemary and Dh (Pn-R + Dh) in Pn15, since this substance has been correlated with the production of OTA in *P. verrucosum* and *A. ochraceus* (Hua et al., 2014; Saxena et al., 2001). Moreover, the data showed the decrease in the abundance of a protein related to the cell wall organisation (putative metallocarboxypeptidase ECM14; Table 2). This protein has been associated with cell wall biogenesis, fungal invasion or nutrient utilization (McDonald et al., 2020). It is known that some yeast species, including *D. hansenii*, are able to produce killer toxins (Çorbacı & Uçar, 2018). They act through different mechanisms of action, affecting cell wall components and provoking cell wall damage, cell membrane permeabilization or cell-cycle perturbation (Mannazzu et al., 2019). Thus, Dh might produce extracellular proteins whose detrimental effect on *P. nordicum* could be related to the abovementioned proteins, involved in cell wall organisation as well as in ergosterol biosynthesis and, consequently, to cell membrane permeability. Cell-free supernatants from this yeast strain cultured in malt extract broth have shown antifungal effect on *P. nordicum*, suggesting the production of killer toxins or extracellular compounds (Andrade et al., 2014). Moreover, the alteration of the cell wall proteins by this strain (Dh) has been previously described for the same *P. nordicum* (Delgado et al., 2019). Notwithstanding, neither in those studies nor in the present work any killer toxin was studied, so the possible involvement of a killer toxin in the antifungal effect of this yeast strain should be considered in a future research. Furthermore, the reduction of mycotoxins by Dh could be enhanced by the presence of rosemary that decreased the OTA produced by *P. nordicum* in parallel to changes in CWI gene expression (Álvarez, Rodríguez, et al., 2020). In contrast, different proteins involved in Acetyl-CoA pathway were found in higher quantity in Pn856, such as the pyruvate dehydrogenase, which converts pyruvate into Acetyl-CoA through pyruvate decarboxylation (Gao et al., 2016), as well as the rhamnogalacturonate lyase related to CWI. Therefore, the obtained results firmly indicate inter-strain differences in the response of *P. nordicum* to R + Dh that require further investigations to shed light to this apparent controversy.

Although the mechanism of action of R + Dh in Pn856 cannot fully explained yet, the purine binding pathway is markedly affected (Fig. S6 Supplementary material), displaying the 85.71% of the decreased terms belonging to this group. It is worthy to highlight that the pathways connected to purine have been considered important targets for antimicrobial drug development due to their crucial role in DNA and RNA synthesis (Cepas et al., 2020; Lin et al., 2019). Furthermore, Yang et al.

(2019) related the downregulation of genes involved in purine metabolism in *A. flavus* treated with benzenamide to the drop of aflatoxin synthesis. Thus, the effect of R + Dh on Pn856 would be associated with the inhibition of purine non-binding processes that could lead to DNA damage and, consequently, the reduction of translational activity (Cepas et al., 2020).

4. Conclusions

REO and the combination of rosemary with *D. hansenii* could be proposed as BCAs to be implemented in the manufacturing of dry-cured fermented sausages due to their ability to reduce the OTA production, regardless of the *P. nordicum* strain. In addition, this is the first approach to study the effect of these BCAs through proteomics to unveil their antiochratoxigenic mechanism of action on *P. nordicum*. In both tested mould strains, REO provoked a drop in the abundance of proteins belonging to the PKS ER domain that are directly linked to OTA production. The combination of rosemary with *D. hansenii* affected the proteins involved in the CWI in Pn15. Although the mechanism of action of this combination of BCAs on the OTA inhibition in Pn856 was not still fully clear, the purine binding pathway was remarkably affected. Despite such lack of information, the present work has contributed to expand the existing knowledge about the physiological reaction that explains the repression of OTA in different *P. nordicum* strains by the most successful BCAs combination. The unveiled target of these BCAs' antifungal activity relies on the key PKS ER domain involved in OTA biosynthesis. This fact implies the advantage of directly impacting on the OTA biosynthesis pathway, shared by different *P. nordicum* strains, minimising thus the significance of collateral mechanisms of action individually found in every strain.

Finally, as a future perspective, it will be of utmost interest to further study the *P. nordicum*'s proteome changes when grown on dry-cured fermented sausages at industrial scale. Under this context, both the autochthonous microbiota found in the pieces and the oscillating temperature and relative humidity conditions during ripening could have also an influence on the BCAs' efficiency to reduce OTA level and on the *P. nordicum*'s proteome profiles.

CRediT authorship contribution statement

Micaela Álvarez: Investigation, Formal analysis, Writing – original draft, Visualization. **Josué Delgado:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Visualization. **Félix Núñez:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition. **Eva Cebrián:** Formal analysis, Visualization. **María J. Andrade:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2021.108232.

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IV.6. Evaluación del impacto de los agentes de biocontrol en la calidad de los embutidos curado-madurados

Effects of preservative agents on quality attributes of dry-cured fermented sausages

Article

Effects of Preservative Agents on Quality Attributes of Dry-Cured Fermented Sausages

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Abstract: *Enterococcus faecium* SE920, *Debaryomyces hansenii* FHSCC 253H, *Penicillium chrysogenum* CECT 20922, producer of the antifungal protein PgAFP, and this protein itself have previously been proposed to control toxigenic molds in dry-cured meat products. However, their effects on the usual microbial population, and the sensory characteristics of these foods, have not yet been evaluated. The aim of this study was to assess the viability of the inoculation of these protective cultures, and their impact on the quality of dry-cured fermented sausages. These microorganisms were co-inoculated with a native desirable population (*Penicillium nalgiovense*, *P. chrysogenum*, *D. hansenii*, and *Staphylococcus vitulinus*) in a dry-cured fermented sausage (salchichón)-based medium in the presence and absence of PgAFP. Macroscopically, the biocontrol candidates did not produce relevant changes in the growth of the native population, enabling their coexistence. However, PgAFP causes the alteration of the hyphae structure in desirable molds. Thus, PgAFP was discarded for use on the surface of raw dry-cured fermented sausages (salchichón) in the pilot plant. The used biocontrol agents did not negatively affect the physico-chemical parameters of the dry-cured fermented sausages (salchichón) after ripening, which showed the typical volatile profile and odor. Thus, the application of *E. faecium* SE920, *D. hansenii* FHSCC 253H, and *P. chrysogenum* CECT 20922 as protective cultures against toxigenic molds during the ripening of dry-cured fermented sausages does not modify their typical sensorial quality.

Keywords: biocontrol; dry-cured fermented sausages; mycotoxins; microbial population; physio-chemical parameters; sensorial evaluation

1. Introduction

Traditional dry-cured fermented sausages, such as "salchichón", are highly appreciated in the Southern European countries because of their sensory characteristics. Some of these attributes are linked to the activity of the microbial population, mainly lactic acid bacteria (LAB), gram positive catalase positive cocci (GCC+), yeasts, and molds [1–3]. However, other microorganisms with interest from a food safety point of view could grow during the production of dry-cured fermented sausages. One of the main safety issues consists of the uncontrolled growth of toxigenic molds on the product surface, with the consequent hazard associated with the mycotoxin presence. The most frequent mycotoxin reported on dry-cured meat products is the ochratoxin A (OTA), but aflatoxins, citrinin, and cyclopiazonic acid have also been found in these foods [4–7]. Therefore, the dry-cured fermented sausage manufacturers need to implement preventive measures for reducing the presence of the previously mentioned mycotoxins. Several physical and chemical methods, with varying effectiveness, have been proposed

for this purpose, but they may interfere with the appropriate ripening of these dry-cured meat products [8]. Moreover, consumers are increasingly demanding natural and additive-free products, which is considered a "decisive buying incentive" due to the common association of this term with healthier food [9]. This trend has involved searching for new alternative measures of natural origin to counteract mycotoxin production, such as biocontrol strategies. These preventive methods are based on the use of spices [10,11], or the inoculation of protective cultures, like *Enterococcus faecium*, *Debaryomyces hansenii*, and *Penicillium chrysogenum* CECT 20922 (producer of the antifungal protein PgAFP) [12,13]. These microorganisms and the protein PgAFP have shown their ability for implantation and their effectiveness against toxigenic molds in dry-cured fermented sausages. *E. faecium* isolated from Iberian dry-cured fermented sausages has demonstrated its antifungal impact on *P. nordicum*, reducing its OTA production [12]. The antagonistic effect of *D. hansenii* on *Aspergillus parasiticus* and *P. nordicum* has been reported, with a significant reduction of mycotoxins in dry-cured meat products [13,14]. Other studies have shown that *P. chrysogenum*, as well as its antifungal protein PgAFP, affected different toxigenic molds, including *Penicillium griseofulvum* and *Aspergillus flavus*, on dry-cured fermented sausages [15,16]. However, the presence of protective cultures could interfere with the beneficial microbial population and, consequently, cause undesirable changes in the technological and sensorial characteristics of dry-cured fermented sausages. To the best of our knowledge, this topic has not been studied yet, despite the fact that the physico-chemical, textural, and sensorial characteristics of foods are crucial aspects for consumer acceptance [17,18]. As stated above, some typical characteristics of dry-cured fermented sausages are related to the native population, or the starter cultures added to provide a standardized flavor [19]. The action of LAB is essential to decrease the pH of the product, producing lactic acid by fermentation and, consequently, limiting the development of pathogenic and spoilage microorganisms [20]. In addition, some of the LAB suggested as starter cultures have shown a probiotic effect, providing a benefit to human health [21]. The group of GCC+ contributes to the development of the appropriate color and flavor [20]. Yeasts and molds make a huge contribution to the peculiar flavor that characterizes dry-cured meat products [1,2,22].

Therefore, before using microorganisms for controlling toxigenic molds in dry-cured fermented sausages, it is necessary to assess their effects on both the usual microbial population, and the sensory characteristics of such products. Additionally, the potential antagonism between biocontrol agents and the native population of sausages should be checked by studying their growth in dual cultures on a meat substrate [23].

The objective of this study was to test the interactions between potential protective cultures and the native population of dry-cured fermented sausages. This study is the first approach to evaluating the effects of the previously proposed biocontrol candidates on the sensorial quality of dry-cured fermented sausages, by studying the changes of their physico-chemical, textural, and sensorial parameters.

2. Materials and Methods

2.1. Microorganisms

Three previously suggested biocontrol agents isolated from dry-cured meat products were tested: *P. chrysogenum* CECT 20922 (Pc), producer of the antifungal protein PgAFP [16], from the Spanish Type Culture Collection (CECT; Valencia, Spain); *D. hansenii* FHSCC 253H (Dh) from the Culture Collection of the Food Hygiene and Safety Research Group of the University of Extremadura (FHSCC; Cáceres, Spain) [14], and *E. faecium* SE920 (Ef) from the Food Quality and Microbiology Research Group of the University of Extremadura (Badajoz, Spain) [24]. The native desirable microorganisms isolated from dry-cured meat products *Penicillium nalgiovense* FHSCC Pj261 (Pn), *P. chrysogenum* FHSCC Pg222 (Pg222), *D. hansenii* FHSCC 46P (Dh46P), and *Staphylococcus vitulinus* FHSCC MSA19 (Sv) belonged to the FSHCC.

2.2. Inocula Setting

The mold inocula were prepared by growing on potato dextrose agar (PDA; Scharlab, S.L.; Barcelona, Spain) at 25 °C for 7 days. Conidia were harvested by washing the surface of the plates with 3 mL of phosphate-buffered saline (PBS) containing 0.32 g/L NaH_2PO_4 (Scharlab, S.L.), 1.09 g/L Na_2HPO_4 (Scharlab, S.L.), and 9 g/L of NaCl (Fisher Scientific S.L.; Waltham, MA, USA). Each conidia suspension was quantified using a Thoma counting chamber (Blaubrand®; Wertheim, Germany) and adjusted to 10^5 spores/mL to be used as inoculum.

D. hansenii strains were incubated in yeast extract-sucrose broth (YES; 20 g/L yeast extract (Scharlab, S.L.)) and 125 g/L sucrose (Scharlab, S.L.) at 25 °C for 24 h under stirring conditions (150 rpm). After centrifuging, the pellet was resuspended in PBS and quantified using the Thoma counting chamber before adjusting to 10^6 cfu/mL.

E. faecium and *S. vitulinus* were cultured in brain heart infusion broth (BHI, Scharlab, S.L.) and incubated for 48 h at 30 °C under stirring (150 rpm). After their centrifugation, the pellets were resuspended in PBS and turbidimetrically adjusted to 10^6 cfu/mL.

The protein PgAFP was extracted by fast protein liquid chromatography (FPLC) using the method previously described by Delgado et al. [16].

2.3. Experimental Design

2.3.1. Dual-Culture Assay

Each biocontrol agent was co-inoculated with each native microorganism in a dual-culture assay, based on Magan and Lacey [23], with some modifications [25]. The culture medium was prepared with lyophilized Spanish dry-cured fermented sausages (salchichón) (3% *w*/*v*) and 20 g/L of bacteriological agar (Scharlab, S.L.). In addition, 5% and 9% (*w*/*v*) of NaCl was added to achieve water activity values of 0.97 (a_w; DFS-0.97) and 0.94 a_w (DFS-0.94), respectively. The a_w was determined using a LabMaster a_w meter (Novasina AG; Lachen, Switzerland). The effect of the antifungal protein PgAFP on the native positive population was also tested by adding 20 µg/mL to each culture media (DFS-0.97P and DFS-0.94P).

The plates were incubated for 7 days at three different temperatures: 25, 20, and 15 °C. The interactions between microorganisms were macroscopically and microscopically visualized using a microscope, NIKON Eclipse E200 (NIKON; Tokyo, Japan). The sampling of mycelia was performing by scraping them with a sterile scalpel immediately before visualizing under the microscope.

2.3.2. Pilot Plant Assay

Raw sausages made with pork meat and backfat, salt, garlic, sugar, dextrose, and black pepper were purchased from a local industry and inoculated on their surface with different combinations of the protective cultures. Five different batches were prepared: a non-inoculated control (C), one inoculated with *E. faecium* SE920 (E), one inoculated with *D. hansenii* FSHCC 253H (D), one inoculated with *P. chrysogenum* CECT 20922 (P), and one inoculated with a mix of the three biocontrol agents (M). Each batch included 5 replicates. The sausages were ripened in pilot plants for 21 days adjusting the relative humidity (RH) and temperature to the natural process in the meat industry. The cycle of maturation started with 3 days at 4 °C and 85% RH, followed by 1 day at 13 °C and 84% RH, and, finally, 17 days at 12 °C and 84% RH.

2.4. Physico-Chemical Analysis

The pH, a_w, and moisture were measured at the end of the ripening to assure the correct processing of the sausages. The pH of the dry-cured fermented sausages was evaluated using a pH meter electrode, model FC232D (HANNA Instruments S.L.; Eibar, Spain). The a_w was measured as previously mentioned. The moisture was analyzed following the reference method 935.29 from the AOAC [26], drying at 105 °C.

2.5. Instrumental Texture

The texture profile analysis (TPA) was performed in slices 1 cm thick using a TA.XT plus Texture Analyser (Stable Micro Systems Ltd.; Godalming, UK). The samples were axially compressed to 50% at 2 mm/s, with a 2-cycle sequence using a flat plunger of 50 mm in diameter (P/50). The texture parameter values were obtained from the force deformation curves, previously described by Bourne [27], and the hardness, adhesiveness, springiness, cohesiveness, and chewiness were analyzed.

2.6. Instrumental Colour

The color was measured in slices 1 cm thick with a Minolta CR-300 colorimeter (Konica Minolta, Inc.; Nieuwegein, The Netherlands) using the CIE $L^*a^*b^*$ color space determined by luminosity (L^*), redness (a^*), and yellowness (b^*). The display area was 2.5 cm, with an illuminant D65, and an observer angle of 0°. All samples were measured by triplicate.

2.7. Volatile Compounds Analysis

The volatile compounds were extracted by solid phase microextraction (SPME) after heating to 37 °C for 30 min, using a divinylbenzene-carboxen-polydimethylsiloxane (DVB/CAR/PDMS) 50/30 µm fiber (Merck; Darmstadt, Germany). They were then analyzed by gas chromatography-mass spectrometry (GC-MS) in a gas chromatograph 6890 GC (Agilent Technologies; Santa Clara, CA, USA) equipped with a HP-5 column (5% phenyl–95% dimethylpolysiloxane) and coupled to a mass spectrometer detector, 5975C (Agilent Technologies). Oven temperature started at 40 °C for 5 min and was increased to 280 °C, with a rate of 7 °C/min. The desorption time was 30 min at 250 °C. The transfer line temperature was established at 280 °C. The carrier gas was helium with a flow rate of 1.2 mL/min. MS detection was performed in full scan (50–350 amu). Automated peak find and spectral deconvolution were used for data treatment, and the dentification of the volatile compounds was achieved by comparing their mass spectra with the NIST/EPA/NIH library.

2.8. Sensory Evaluation

The sensory evaluation consisted of a hedonic preference test and an ordination test using a scale of odor intensity. The analysis was carried out by untrained volunteers (22) recruited at the Faculty of Veterinary Sciences of the University of Extremadura. Each sample was presented to the panelists labelled with a three-digit random codes, and served at room temperature in covered Falcon tubes to avoid visual influences. For the hedonic test, the panelists had to order the samples corresponding to each batch from the least preferred to the most preferred. For the scale of odor intensity, the panelists had to order the samples corresponding to each batch from the least intensity to the most intensity. Re-tasting of samples was permitted to confirm the rankings. The results were analyzed through rank sums scores following the methodology described by Hein et al. [28]. Thus, the scores show the aggregate of the positions in the scale for each sample, the highest results being the most preferred or intense. Subsequently, a slice of each batch was presented to the panelists and they were asked if they would purchase it or not.

2.9. Statistical Analysis

The statistical analysis was performed using SPSS IBM v.22 software (IBM; New York, NY, USA). The non-parametric Kruskal–Wallis and Mann–Whitney tests were used, since the data failed the normality and homoscedasticity tests. The analysis of correlations between data from the parameters of color was carried out using the Spearman correlation test. Friedman's chi-square test was applied to the sensorial test results. The statistical significance was stablished at $p \leq 0.05$.

3. Results and Discussion

Since microorganisms have a great impact on the development of the sensory characteristics of dry-cured fermented sausages, the study of the interaction between the candidates for biocontrol agents and the beneficial population of this product is a key issue to avoid disturbance in the microbial population during the ripening and, consequently, in the quality of the final product [29].

3.1. In Vitro Effect of the Biocontrol Agents on the Growth of Beneficial Microorganisms

The biocontrol agents Ef, Dh, and Pc did not affect the growth of Sv nor Dh46P in dual cultures in the four media (DFS-0.97, DFS-0.94, DFS-0.97P, and DFS-0.94P) at every combination of a_w and temperature tested. In Figure 1, co-inoculated plates of Sv and Dh46P with the biocontrol agents incubated at 25 °C are shown as an example. The diameter of the colonies of Pn (Figure 2) and Pg222 (Figure 3) was slightly lower in the presence of the biocontrol agents than in their absence, but not completely impaired. Regarding the co-inoculation with Pc, mutual inhibition at contact was observed for both beneficial molds in DFS-0.97 and DFS-0.94 (Figures 2 and 3).

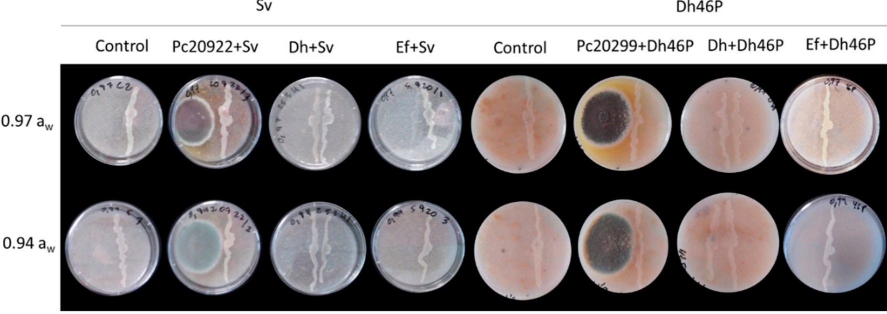

Figure 1. Interactions of three biocontrol agents (*Enterococcus faecium* SE920, *Debaryomyces hansenii* FSHCC 253H, and *Penicillium chrysogenum* CECT 20922) with native dry-cured meat product microorganisms (*Staphylococcus vitulinus* MSA19 and *D. hansenii* 46P) at 0.97 and 0.94 water activity (a_w) after 7 days at 25 °C in a dry-cured fermented sausage (salchichón)-based medium. Treatments: Control: native microorganism inoculated without biocontrol agents; Sv: *S. vitulinus* MSA19; Dh46P: *D. hansenii* 46P; Pc20299: *P. chrysogenum* CECT 20922; Dh: *D. hansenii* FSHCC 253H; Ef: *E. faecium* SE920. The biocontrol agent was inoculated on the left side of the plate and the tested microorganism on the right side of the plate.

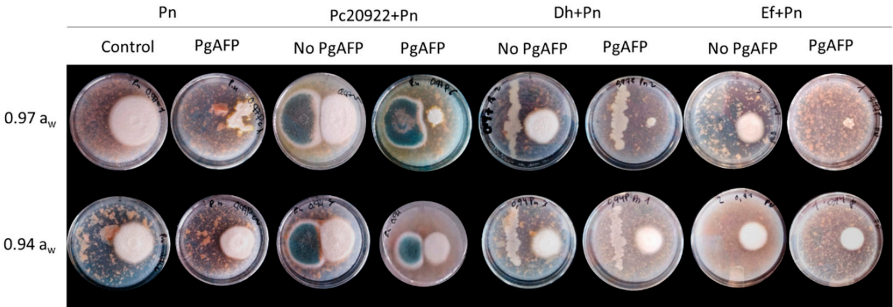

Figure 2. Interactions of the three biocontrol agents (*Enterococcus faecium* SE920, *Debaryomyces hansenii* FSHCC 253H, and *Penicillium chrysogenum* CECT 20922) with the native dry-cured meat product mold *Penicillium nalgiovense* FSHCC Pj261 at 0.97 and 0.94 water activity (a_w) after 7 days at 25 °C in the presence and absence of the antifungal protein PgAFP in a dry-cured fermented sausage (salchichón)-based medium. Treatments: Control: *P. nalgiovense* FSHCC Pj261 inoculated without biocontrol agents; Pn: *P. nalgiovense* FSHCC Pj261; Pc20922: *P. chrysogenum* CECT 20922; Dh: *D. hansenii* FSHCC 253H; Ef: *E. faecium* SE920. No PgAFP: PgAFP was not added; PgAFP: PgAFP was added. The biocontrol agent was inoculated on the left side of the plate and *P. nalgiovense* FSHCC Pj261 on the right side of the plate.

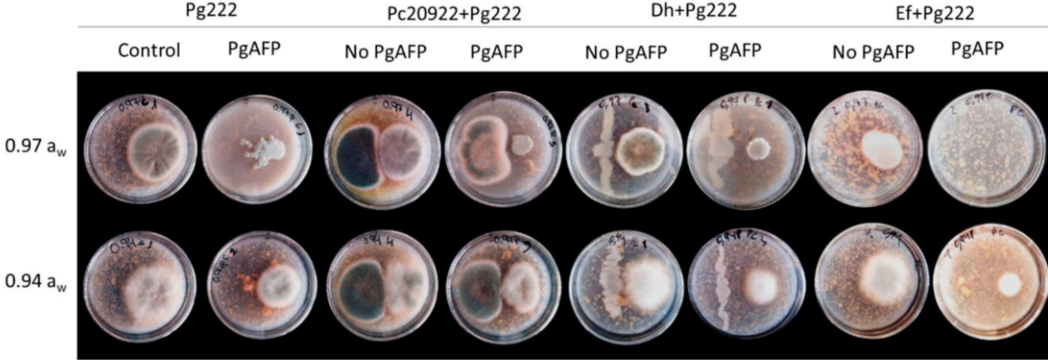

Figure 3. Interactions of the three biocontrol agents (*Enterococcus faecium* SE920, *Debaryomyces hansenii* FSHCC 253H, and *Penicillium chrysogenum* CECT 20922) with native dry-cured meat product mold *P. chrysogenum* FSHCC Pg222 at 0.97 and 0.94 water activity (a_w) after 7 days at 25 °C in the presence and absence of the antifungal protein PgAFP in dry-cured fermented sausage (salchichón)-based medium. Treatments: Control: *P. chrysogenum* FSHCC Pg222 inoculated without biocontrol agents; Pg222: *P. chysogenum* FSHCC Pg222; Pc20922: *P. chrysogenum* CECT 20922; Dh: *D. hansenii* FSHCC 253H; Ef: *E. faecium* SE920. No PgAFP: PgAFP was not added; PgAFP: PgAFP was added. The biocontrol agent was inoculated on the left side of the plate and *P. chrysogenum* FSHCC Pg222 on the right side of the plate.

The marginal impact found could be attributed to the different mechanism of action reported for the studied biocontrol agents. Thus, the antagonistic effect of *D. hansenii* Dh253H by nutritional competition, and the production of extracellular soluble and volatile antifungal compounds have previously been described [30]. The protective *P. chrysogenum* CECT 20922 antagonism against Pg222 and Pn could be related to competition for nutrients and space, as previously described against *P. nordicum* [25]. However, this limited action of the protective cultures on the growth of both non-toxigenic molds did not completely inhibit their growth. Thus, they could coexist without affecting their contribution to the development of the sensory characteristics of sausages. The three biocontrol agents were then used for manufacturing dry-cured fermented sausages in the pilot plant assay.

On the other hand, the presence of the antifungal protein PgAFP did not affect the growth of Sv nor Dh46P. However, the protein caused a decrease in the growth of Pn and Pg222, mainly at 0.97 a_w (Figures 2 and 3, respectively). In addition, at this a_w value, the colonies of Pn had an abnormal appearance in all conditions tested. The colonies lost their circular shape and seemed to be composed by multiple dots. When visualized under microscopy, the mycelia were brittle, not presenting the characteristic structure of the hyphae (Figure 4).

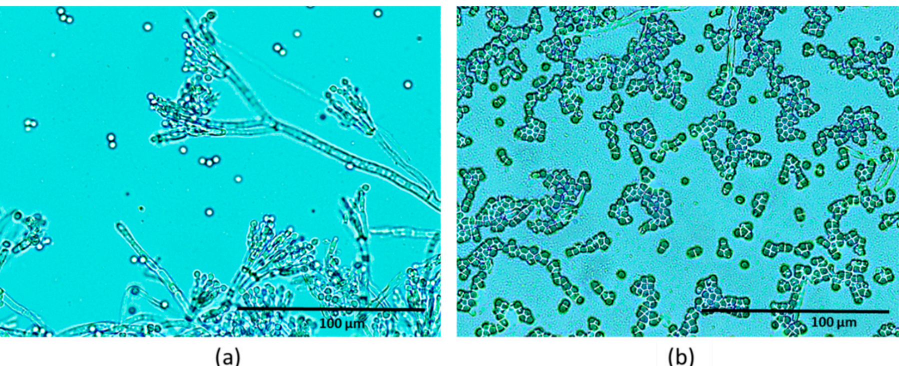

Figure 4. Microscopical image of *Penicillium nalgiovense* Pj261 in the absence (**a**) and presence (**b**) of the antifungal protein PgAFP after 15 days of incubation at 25 °C and 0.97 water activity in a dry-cured fermented (salchichón)-based medium.

These results are consistent with the antifungal effect of PgAFP, mainly related to the repression of the cell wall integrity pathway (CWI) and chitin biosynthesis in sensitive molds [31], which could entail a reduction of the aerial mycelium [32]. As a consequence, PgAFP should not be recommended to be added to the surface of dry-cured fermented sausages where desirable molds are expected to grow. Thus, PgAFP was not selected for the following experiment in the pilot plant.

3.2. Effect of Biocontrol Agents on the Dry-Cured Fermented Sausages Characteristics

Sausages inoculated with different combinations of the biocontrol agents were then ripened (Figure 5) to assess the influence of these microorganisms on the physico-chemical and sensory characteristics of the final products. After the inoculation of Ef, Dh, and Pc on the surface of sausages, and their drying and ripening, the physico-chemical parameters were measured (Table 1).

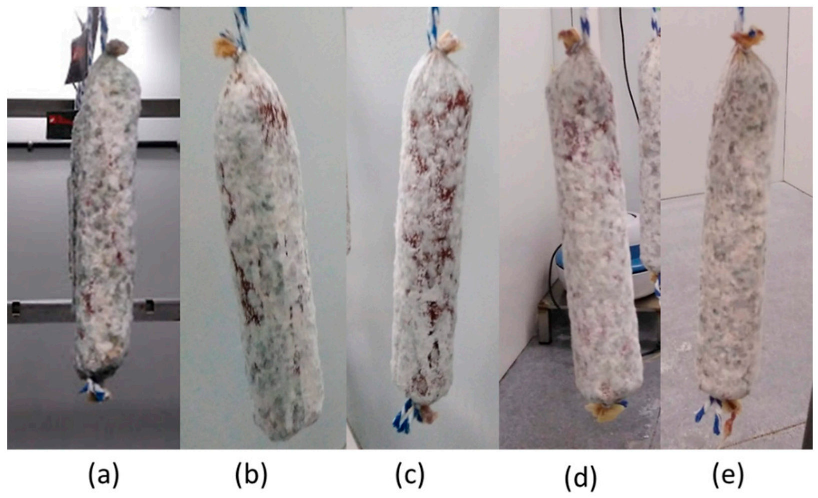

Figure 5. External appearance of dry-cured fermented sausages throughout ripening. (**a**): batch C (without biocontrol agents); (**b**): batch E (*Enterococcus faecium* SE920); (**c**): batch D (*Debaryomyces hansenii* FHSCC 253H); (**d**): batch P (*Penicillium chrysogenum* CECT 20922); (**e**): batch M (*E. faecium* SE920 + *D. hansenii* FHSCC 253H + *P. chrysogenum* CECT 20922).

Table 1. Values of pH, water activity (a_W), moisture, instrumental texture parameters (hardness, adhesiveness, springiness, cohesiveness, and chewiness), and CIE $L^*a^*b^*$ parameters of dry-cured fermented sausage (salchichón) inoculated with biocontrol agents after 21 days of ripening.

Parameters	Batches				
	C [a]	E	D	P	M
pH	5.76 ± 0.01	5.62 ± 0.11 *	6.08 ± 0.08 *	5.81 ± 0.14	5.78 ± 0.06
a_W	0.86 ± 0.00	0.85 ± 0.00 *	0.86 ± 0.00 *	0.86 ± 0.00 *	0.85 ± 0.00 *
Moisture (%)	37.99 ± 1.08	37.72 ± 1.09	38.00 ± 2.23	35.60 ± 2.34	37.18 ± 0.67
Hardness (N)	216.95 ± 27.50	203.49 ± 21.11	232.38 ± 9.21	211.69 ± 15.40	165.01 ± 40.15
Adhesiveness (N/s)	−9.85 ± 1.22	−11.41 ± 1.27	−11.61 ± 1.03	−10.97 ± 0.74	−10.80 ± 3.37
Springiness	0.74 ± 0.06	0.70 ± 0.09	0.80 ± 0.07	0.66 ± 0.03 *	0.75 ± 0.16
Cohesiveness	0.61 ± 0.00	0.62 ± 0.00	0.61 ± 0.00	0.59 ± 0.00 *	0.62 ± 0.00
Chewiness(N)	100.76 ± 15.84	89.94 ± 18.63	115.62 ± 10.42	82.70 ± 7.67	78.02 ± 26.50
L^*	42.68 ± 0.31	42.33 ± 2.95	45.24 ± 1.57 *	45.38 ± 3.08 *	45.35 ± 5.21
a^*	19.99 ± 2.39	19.21 ± 0.58	20.67 ± 1.42	18.31 ± 1.2	16.34 ± 2.0 *
b^*	6.74 ± 0.89	5.83 ± 0.21	6.88 ± 0.92	5.85 ± 0.89	4.43 ± 0.78 *

[a] C: non-inoculated control; E: *Enterococcus faecium* SE920; D: *Debaryomyces hansenii* FHSCC 253H; P: *Penicillium chrysogenum* CECT 20922; M: inoculated with the three biocontrol agents. * Indicates statistical differences with respect to the control ($p \leq 0.05$).

The biocontrol agent Pc grew properly in the inoculated batches P and M (Figure 5). Nonetheless, the surfaces of the sausages from the remaining batches were gradually covered by spontaneous molds from the native population during the ripening period (Figure 5), as happens in the traditional industries.

The pH of the sausages was not significantly affected by the inoculation of Pc and the mix of the biocontrol agents when compared with the non-inoculated batch (Batch C; Table 1). However, the use of Ef and Dh slightly changed the pH (batches E and D, respectively). It is well known that LAB acidify dry-cured fermented sausages throughout the fermentation stage, with the subsequent improvement of their safety [33]. On the contrary, Dh causes a small rise in the pH, that could be a consequence of the increased proteolytic activity or the lactic acid consumption [18,34].

The a_w in all the batches inoculated with the biocontrol agents was significantly lower with respect to batch C (Table 1). However, no differences were observed in the moisture among batches (Table 1). That is in accordance with other studies performed on dry-cured fermented sausages with the addition of *D. hansenii* [34], because it favors dehydration through proteolysis; and LAB are linked to the decrease in the water-holding capacity of denaturalized proteins, in the last case induced by the acidification [35]. The reduction of a_w in batch P could be related to the capacity for holding water by molds growing on the surface of dry-cured fermented sausages [36]. The mix of the three biocontrol agents was also significantly different with respect to the control, showing the lowest a_w value (0.85). However, no differences among batches were detected in the moisture that could indicate a uniform drying process.

Despite the fact that the inoculated batches showed some differences in physico-chemical parameters with respect to the control, the obtained values were within the usual range for dry-cured fermented (salchichón), the pH being from 5 to 6 [3,37], a_w from 0.80 to 0.87 [18,38], and moisture from 20% to 44% [3,39]. Therefore, the studied protective cultures did not lead to relevant disturbances of these parameters through the ripening process of the sausages.

Regarding the instrumental texture parameters, similar results were obtained in the five manufactured batches, their values being in the common ranges of dry-cured fermented sausages: for hardness, from 74.4 to 269.2 N [17,40]; springiness, from 0.55 to 0.8 [41,42]; cohesiveness, from 0.39 to 0.8 [3,17]; and chewiness, from 94.8 to 152.4 N [3]. However, all the sausages showed higher levels of adhesiveness than others previously reported, from −0.44 to −2.44 [17,42]. This difference could be due to a high fat content [17] that can achieve the 60% of dry matter. Significant differences ($p < 0.05$) in the springiness and cohesiveness were only found in batch P, with respect to batch C (Table 1). The significant reductions of springiness and cohesiveness in this batch could be related to an intense proteolysis [40], due to the high production of proteases by *P. chrysogenum* [43].

Concerning the CIE $L^*a^*b^*$ parameters, no differences were observed, with the exception of the parameter L^* in batches D and P that increased with respect to the control (Table 1). The increase in lightless by the inoculation of *D. hansenii* has also been reported in dry cured-fermented sausages [42]. For the parameters a^* and b^* in batch M, both values were the lowest (Table 1). When evaluating the correlation among the color parameters, a positive relation (0.854) was found between parameters a^* and b^* ($p \leq 0.01$), which indicates that both reductions are likely related. The rise of a^* in meat products has been related to the increase of lactic acid that interferes in the denaturation of myoglobin and moisture loss [44]. Nonetheless, in the present study the pH and moisture values did not show differences between batches C and M, so the changes in redness seem not to be linked to these parameters. The decrease in the parameter b^* has been attributed to the drop of oxymyoglobin, probably due to the oxygen consumption by microorganisms [44]. Therefore, the highest microbial load inoculated in batch M, due to the co-inoculation of the three biocontrol agents, could be responsible for the reduced redness and yellowness in these sausages.

A total of 42 volatile compounds were identified and quantified in the dry-cured fermented sausages after ripening (Table 2), among them being those usually found in dry-cured fermented sausages [1,45]. The volatile pattern of the different batches was the result of the addition of spices to a

complex meat matrix, combined with the reactions derived from lipid auto-oxidation and the activity of both native microorganisms and the inoculated biocontrol agents.

Table 2. Volatile compounds [a] identified and quantified from dry-cured fermented sausages (salchichón) inoculated with biocontrol agents after 21 days of ripening.

Origin/Compound	Id [b]	Batches [c]				
		C	E	D	P	M
Lipid oxidation						
1-propanol	MS	n.d. [d]	0.07 *	n.d.	n.d.	n.d
1-hexanol	MS	2.25	6.43	n.d. *	5.24	2.41
2-heptanol	MS	0.18	n.d. *	0.15	0.17	0.16
Hexanal	MS/Rf	1.63	8.25	0.45 *	3.32	1.63
Heptanal	MS/Rf	n.d.	0.71 *	n.d.	0.42 *	n.d.
Octane	MS	0.16	n.d. *	n.d. *	0.22	0.15
2-heptanone	MS	0.66	0.47	0.91	1.01	n.d. *
4-heptanone	MS	n.d.	n.d.	0.14 *	n.d.	1.52 *
2-octanone	MS	0.10	n.d. *	0.08 *	n.d. *	0.17
3-octanone	MS	0.13	0.60 *	n.d. *	0.31	0.26 *
2,3-octanedione	MS	n.d.	0.22 *	n.d.	n.d.	n.d.
Hexanoic acid	MS	0.88	2.18	0.52 *	1.10	0.40 *
Octanoic acid	MS	0.39	0.41	0.44	0.19 *	0.34
Nonanoic acid	MS	n.d.	0.28 *	0.27 *	n.d.	n.d.
1-octen-3-ol	MS	1.87	4.42	0.92 *	2.75	1.47
2-nonanone	MS	0.19	n.d. *	0.19	0.35	0.27
Carbohydrate fermentation						
Acetoin	MS	1.40	2.40	1.97	2.39	0.90
Amino acid catabolism						
2-methylpropanal	MS/Rf	0.14	n.d. *	0.08	n.d. *	n.d. *
2-methyl-1-propanol	MS	n.d.	0.17 *	0.19 *	0.17 *	0.18 *
3-methylbutanal	MS/Rf	0.14	0.22	0.34 *	0.09 *	0.23
2-methylbutanal	MS/Rf	n.d.	n.d.	0.09 *	n.d.	0.07 *
3-methyl-1-butanol	MS	1.56	1.17	2.02	1.29	1.64
2-methyl-1-butanol	MS	0.28	0.26	0.28	0.24	0.27
2-methylpropanoic acid	MS	0.34	0.29	0.40	0.16 *	0.41
2-methylbutanoic acid	MS	0.23	0.21	0.20	0.13	0.28
3-methylbutanoic acid	MS	0.46	n.d. *	0.84 *	0.29	0.49
2-ethyl-1-hexanol	MS	0.25	0.32	n.d. *	n.d. *	n.d. *
Phenylethyl alcohol	MS	0.22	n.d. *	0.25	0.22	0.20
Spices						
Thujene	MS	0.32	0.27	0.35 *	0.22 *	0.27
α-pinene	MS	2.25	2.40	2.60	1.69	1.67 *
β-pinene	MS	3.63	3.37	4.22	3.07	2.58 *
α-phellandrene	MS	0.57	0.41	0.60	0.40 *	0.46 *
β-phellandrene	MS	0.74	0.60	1.03	0.37 *	0.38
3-carene	MS	7.63	7.00	8.92	5.87	4.91 *
o-cymene	MS	1.03	1.61	1.06	1.06	0.93
D-limonene	MS	4.71	4.11	5.40	3.88	3.18 *
γ-terpinene	MS	0.15	0.26 *	0.21 *	0.11 *	0.10 *
L-terpinen-4-ol	MS	0.48	0.40 *	0.57 *	0.42	0.40 *
α-terpineol	MS	0.18	0.17	0.23 *	0.17	0.17
Safrole	MS	0.11	0.10 *	0.13 *	0.09 *	0.10
Caryophyllene	MS	0.77	0.77	0.98	0.65	0.68
Myristicin	MS	0.59	0.50	0.79	0.42	0.47

[a] Results are expressed in arbitrary area units ($\times 10^{-6}$), as means of 3 replicates of each batch. [b] Id: reliability of identification: MS: chromatogram deconvolution and identification by comparing the mass spectrum of the compounds with the NIST/EPA/NIH database; Rf: mass spectrum and retention time identical with a reference compound. [c] C: non-inoculated control; E: *Enterococcus faecium* SE920; D: *Debaryomyces hansenii* FHSCC 253H; P: *Penicillium chrysogenum* CECT 20922; M: inoculated with the three biocontrol agents. [d] n.d.: not detected. *: Indicates statistical differences in the amount of a volatile compound, with respect to the control ($p \leq 0.05$).

The results are categorized by groups according to their possible origin, such as lipid oxidation (16), carbohydrate fermentation (1), amino acid catabolism (11), and spices (14).

A total of 35 compounds were detected in batches C, D, and M, followed by batches P (34) and E (33). Batch D showed the lowest number of compounds derived from lipid oxidation, and the highest from amino acid catabolism. The unique compound coming from carbohydrate fermentation, acetoin, and all the compounds resulting from spices were detected in every batch. The volatile compounds originating from spices showed the highest amounts. Terpenes were most of the compounds derived from spices, 3-carene and D-limonene being predominant in all batches. Among them, 3-carene derived from black pepper [46] was the volatile compound showing the highest amount, since this ingredient is one of the main spices added to "salchichón" [38,41]. The heterogeneity in the distribution of spices in the matrix, mainly the whole peppercorns, could explain the differences found among the batches.

Regarding the volatile compounds from lipid oxidation, there were no substantial differences between the batches, and no large variations in flavor notes related to these compounds, such as rancidity [47], should thus be expected. Since 1-propanol and 2,3-octanedione were only found in batch E, they were likely produced by the action of *E. faecium*. Both compounds have been previously associated with enterococci metabolism in dry-cured fermented sausages [45]. The 3-octanone was also found in higher levels in batches E (0.60) and M (0.26) than in batch C (0.13), suggesting that *E. faecium* was the main producer of this ketone. This compound is responsible for a mushroom or musty aroma, and has been ascribed to the presence of other LAB, such as *Carnobacterium maltaromaticum*, in meat [48,49]. The volatiles 4-heptanone and 2-methylbutanal were only found in batches D and M. Heptanal was exclusively found in batches E and P, and nonanoic acid in batches E and D. Among ketones derived from lipid oxidation, the level of 4-heptanone was significantly higher in batch M, and 3-octanone in batch E, compared to batch C ($p \leq 0.05$). Regarding amino acid catabolism, alcohols were mostly identified, the 3-methyl-1-butanol standing out with the highest amount in all batches. The detection of 2-methyl-1-propanol was remarkable in all inoculated batches (E, D, P, and M), but not in batch C. Moreover, batch D showed the highest amounts of 2-methyl-1-propanol, 3-methylbutanal, 2-methylbutanal, and 3-methylbutanoic acid ($p \leq 0.05$). Some of these branched-chain volatiles, such as 3-methylbutanal, 2-methyl-1-propanol, 3-methyl-1-butanol, and 2-methylpropanoic acid, have been associated with the typical aroma of dry-cured meat products [50]. 3-methylbutanal and 3-methylbutanoic acid have been related to the activity of yeasts in meat substrates [1,51], which could clarify the highest level of these compounds being found in batch D. The limited differences found in the pattern of volatile compounds between batches is consistent with the small influence that protective cultures have shown on the growth of microorganisms usually present in dry-cured fermented sausages (Figures 1–3). Therefore, although the tested microorganisms caused small changes in the generation of volatiles that contribute to the characteristic aroma of dry-cured fermented sausages in the different batches, it is not expected that such modifications can lead to significant variations in the odor of the sausages. In this sense, no significant differences were detected by the tasting panel between batches in preference ranking, intensity of odor scale, and buying preference (Table 3). Concerning the purchase preferences, 18 of 22 panelists would buy all the batches. These positive aspects support the implementation of these biocontrol agents since they did not affect the purchase intention of the panelists.

Table 3. Rank sums scores for preference ranking and the odor intensity scale of dry-cured fermented sausages (salchichón) inoculated with the biocontrol agents after 21 days of ripening. *p*-values from Friedman's chi-square test are also shown.

Test	Batch [a]	Rank Sums	*p*-Value [b]
Preference ranking	C	52	0.329
	E	70	
	D	71	
	P	67	
	M	70	
Intensity of odor	C	69	0.656
	E	66	
	D	69	
	P	56	
	M	70	

[a] C: non-inoculated control; E: *Enterococcus faecium* SE920; D: *Debaryomyces hansenii* FHSCC 253H; P: *Penicillium chrysogenum* CECT 20922; M: inoculated with the three biocontrol agents. [b] Statistical significance stablished at $p \leq 0.05$.

4. Conclusions

In conclusion, the biocontrol candidates *E. faecium* SE920, *D. hansenii* FHSCC 253H, and *P. chrysogenum* CETC 20,922 did not have a significant influence on the native population of dry-cured fermented sausages. However, the application of PgAFP on the surface of products, where desirable molds are expected to grow, could not be recommended. In addition, the superficial inoculation of these three protective microorganisms individually or combined in dry-cured fermented sausages did not significantly modify the physico-chemical parameters, nor the sensorial properties of the product. Therefore, there are no technological drawbacks against the application of these microorganisms as protective cultures for the biocontrol of toxigenic molds during the ripening of dry-cured fermented sausages.

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IV.7. Implantación de los agentes de biocontrol durante la elaboración de los embutidos curado-madurados

Debaryomyces hansenii and rosemary leaves reduce ochratoxin A by *Penicillium nordicum* during dry-cured fermented sausages industrial ripening, through disrupting proteins involved in OTA biosynthesis and the cell wall integrity pathway.

Food Control

Debaryomyces hansenii and rosemary reduce ochratoxin A by *Penicillium nordicum* during dry-cured fermented sausages industrial ripening, through disrupting proteins involved in mycotoxin biosynthesis and in cell wall integrity pathway

--Manuscript Draft--

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Abstract:	*Penicillium nordicum* is the main ochratoxin A (OTA) producer in dry-cured meat products. The aim of the study was to test *Debaryomyces hansenii* and rosemary derivatives as biocontrol agents (BCAs) against *P. nordicum* during the industrial manufacturing of dry-cured fermented sausages. Eighteen batches of sausages with different combinations of the BCAs were made. *D. hansenii* FHSCC253H was inoculated in the meat dough or on the casings. Regarding rosemary, it was used either as an ingredient, for macerating the casings or applying its essential oil. *P. nordicum* was finally added on the surface of the sausages by immersion in a solution containing 10^6 spores/mL. OTA was quantified by uHPLC-MS/MS QqQ after ripening time and comparative proteomic analyses were carried out to elucidate the involved modes of action. OTA accumulation decreased when the yeast or rosemary leaves were added in the meat dough as well as when the casings were macerated with the herb. The yeast presence provoked the reduction in the abundance of *P. nordicum* proteins involved in OTA biosynthesis, and its combination with rosemary interfered with the cell wall integrity pathway linked to mycotoxin synthesis in moulds. The addition of rosemary leaves and the casing maceration changed the abundance of proteins involved in ergosterol biosynthesis. Furthermore, the maceration affected to proteins involved in the synthesis of phenylalanine, essential for OTA synthesis. In conclusion, the yeast *D. hansenii* and the rosemary leaves, alone or in combination, could be proposed as BCAs to hamper the *P. nordicum* OTA production during the industrial elaboration of dry-cured fermented sausages.
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Highlights

Debaryomyces hansenii and rosemary, alone and combined, decreased OTA synthesis.

The maceration of the casings with rosemary leaves reduced OTA production.

D. hansenii and rosemary reduced the amount of proteins related to OTA biosynthesis.

D. hansenii and rosemary affected to cell wall integrity pathway.

Rosemary disrupted phenylalanine- and ergosterol-related proteins.

1 ***Debaryomyces hansenii* and rosemary reduce ochratoxin A by *Penicillium nordicum* during**
2 **dry-cured fermented sausages industrial ripening, through disrupting proteins involved in**
3 **mycotoxin biosynthesis and in cell wall integrity pathway**

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21

22 **Abstract**

23 *Penicillium nordicum* is the main ochratoxin A (OTA) producer in dry-cured meat products. The
24 aim of the study was to test *Debaryomyces hansenii* and rosemary derivatives as biocontrol
25 agents (BCAs) against *P. nordicum* during the industrial manufacturing of dry-cured fermented
26 sausages. Eighteen batches of sausages with different combinations of the BCAs were made. *D.*
27 *hansenii* FHSCC253H was inoculated in the meat dough or on the casings. Regarding rosemary,
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29 *nordicum* was finally added on the surface of the sausages by immersion in a solution
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31 comparative proteomic analyses were carried out to elucidate the involved modes of action.
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38 Furthermore, the maceration affected to proteins involved in the synthesis of phenylalanine,
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40 or in combination, could be proposed as BCAs to hamper the *P. nordicum* OTA production
41 during the industrial elaboration of dry-cured fermented sausages.

42

43 Keywords: Biocontrol agents, dry-cured fermented sausages, ochratoxin A, proteomics, cell
44 wall integrity pathway, ergosterol.

45 **1. Introduction**

46 Ochratoxin A (OTA) is a toxic secondary metabolite produced by several *Aspergillus* and
47 *Penicillium* species. It is a potent nephrotoxic included in the group 2B as possible human
48 carcinogen by the International Agency for Research on Cancer (Claeys et al., 2020; IARC,
49 1993). Dry-cured meat products, including dry-cured fermented sausages and dry-cured ham,
50 are regarded among the most important contributors to the chronic dietary exposure to OTA
51 in the European Union (EFSA CONTAM Panel, 2020). The production of dry-cured fermented
52 sausages is of great importance in European countries being "salchichón" one of the most
53 consumed meat products in Spain (Fernández-López et al., 2008). Its composition includes a
54 mixture of pork meat, fat, salt, additives as nitrites and spices as black pepper (Fernández-
55 López et al., 2008; Delgado et al., 2021). This composition and the usual ripening conditions of
56 temperature and relative humidity (RH) make this meat product a perfect substrate for mould
57 development. The frequent isolation of ochratoxigenic *Penicillium nordicum* strains from the
58 surface of dry-cured fermented sausages (Sonjak et al., 2011; Zadravec et al., 2020) and their
59 ability to produce OTA under the industrial ripening conditions (Delgado et al., 2021) should
60 lead to the implementation of strategies to control this hazard.

61 The application of biocontrol agents (BCAs) to hamper OTA production is on the focus of new
62 preservative strategies due to the current trend to avoid the use of synthetic compounds in
63 foods (Román et al., 2017). Microorganisms and plant derivatives seem to be the most
64 appropriate BCAs to be used in dry-cured fermented sausages. Microorganisms largely studied
65 are lactic acid bacteria, yeasts and non-toxigenic moulds, such as *Enterococcus faecium,*
66 *Debaryomyces hansenii* and *Penicillium chrysogenum*, respectively (Álvarez et al., 2019;
67 Andrade et al., 2014; Delgado et al., 2019). Selected strains could be directly applied or by
68 adding purified proteins with antifungal activity produced by them (Çorbacı & Uçar, 2018;
69 Delgado et al., 2016a). Regarding plant derivatives, a broad spectrum of antifungal activity

70 against foodborne moulds has been established for essential oils, such as those from oregano,
71 thyme, curcuma and cinnamon (Chaves-López et al., 2012; Hua et al., 2014; Prakash et al.,
72 2015). Rosemary essential oil (REO) has also antiochratoxigenic effect against *Aspergillus*
73 *carbonarius* through the downregulation of genes involved in OTA biosynthesis (El Khoury et
74 al., 2016, 2017). Moreover, REO decreased aflatoxin production by *Aspergillus flavus* and
75 interfered in the ergosterol biosynthesis (Da Silva Bomfim et al., 2020). On the other hand, the
76 *in vitro* addition of rosemary leaves or its essential oil have shown to successfully decrease the
77 OTA production by *P. nordicum* through the reduction of the expression of OTA biosynthetic
78 genes (*otapks* and *otanps*) and the quantity of proteins belonging to the PKS ER domain
79 (Álvarez et al., 2020a; 2021a). This domain has been previously linked to OTA synthesis in
80 *Aspergillus ochraceus* and to some mycotoxins produced by *Alternaria alternata* (Ajiro et al.,
81 2009; Wang et al., 2015). However, these studies collected data from *in vitro* experiments,
82 being necessary to check the effect of rosemary on OTA accumulation when the products
83 undergo industrial processing.

84 *Debaryomyces hansenii* belongs to the wild population of dry-cured fermented sausages, being
85 the most frequently isolated yeast (Aquilanti et al., 2007; García-Béjar et al., 2020). Its
86 antagonistic effect against *P. nordicum* has been widely described in dry-cured meat products
87 (Andrade et al., 2014; Delgado et al., 2019; Iacumin et al., 2017; Meftah et al., 2018; Simoncini
88 et al., 2014). The achieved inhibition rates of OTA production by using *D. hansenii* as BCA have
89 been associated with several modes of action, such as the reduction of OTA biosynthetic genes
90 in *Aspergillus westerdijkiae* and *Penicillium verrucosum* (Gil-Serna et al., 2011; Peromingo et
91 al., 2018), and to the OTA adsorption to the yeast cell wall after its production by *A.*
92 *westerdijkiae* (Gil-Serna et al., 2011). In *P. nordicum*, the OTA decrease due to *D. hansenii*
93 presence has been linked to the competition by nutrients and space, the disturbance in the cell
94 wall integrity (CWI), the production of extracellular compounds and the reduction of OTA
95 biosynthetic genes expression (Álvarez et al., 2021b; Andrade et al., 2014; Cebrián et al., 2019;

96 Delgado et al., 2019). Such differences in the mode of action state that more studies are
97 necessary to comprehend the antifungal effect of *D. hansenii* under industrial ripening.

98 The modes of action that underlie the OTA inhibition of by BCAs on toxigenic moulds deserve
99 to be further studied to a better understanding of these mechanisms that would lead to more
100 efficient treatments and BCAs combinations. Proteomics research improves the information
101 about cellular behaviour under biotic and abiotic factors and the variations that occur in the
102 proteome of toxigenic moulds. This technique can be used to elucidate the adaptive
103 physiological response of moulds that provokes secondary metabolite production (Eshelli et
104 al., 2018). Previous studies based on Proteomics reported the mechanisms of action involved
105 in the *in vitro* antifungal effect of REO, *D. hansenii* and antifungal compounds as chitosan and
106 citral (Álvarez et al., 2021a; Delgado et al., 2019; Li et al., 2020; Wang et al., 2021).

107 The aim of this study was to analyse the application of *D. hansenii*, rosemary and its essential
108 oil, alone or in combination, against *P. nordicum* under industrial ripening of dry-cured
109 fermented sausages "salchichón". Apart from evaluating the OTA contamination, comparative
110 proteomic analyses were carried out to provide information about how the toxigenic mould
111 reacts to the presence of the BCAs.

112

113 **2. Material and methods**

114 **2.1. Herb material**

115 Rosemary leaves from *Rosmarinus officinalis* were manually collected in Extremadura (Spain)
116 the day of the experiment. REO was extracted through hydrodistillation technique employing a
117 Clevenger® apparatus (Scharlab S.L., Barcelona, Spain). For that, 200 g of rosemary leaves were
118 ground using an electric mincer A320 (Moulinex, Barcelona, Spain) and mixed with 400 mL of
119 distilled water. The mix was then boiled obtaining an average yield of 0.5 % (v/w) of REO,

120 which was then diluted in distilled water with 1 % of Tween 80 (v/v) (Fisher Scientific, Madrid,
121 Spain) to reach a final concentration of REO:water 1:1. Whole rosemary leaves were used to
122 macerate the casings in water for 24 h before stuffing.

123 **2.2. Microorganisms and culture conditions**

124 *P. nordicum* FHSCC Pn15 and *D. hansenii* FHSCC Dh253H (Dh), isolated from dry-cured meat
125 products and deposited in the Culture Collection of the Food Hygiene and Safety Research
126 Group of the University of Extremadura (Cáceres, Spain), were used. *P. nordicum* inoculum was
127 prepared after its growth at 25 °C for 10 days on Potato Dextrose Agar (PDA, Scharlab S.L.).
128 The spores were harvested after adding 3 mL of phosphate buffer saline (PBS) by scrapping the
129 conidia surface with a sterile rod and then adjusted to 10^6 spores/mL using a Thoma chamber
130 Blaubrand® (Brand, Wertheim, Germany). Dh was inoculated on YES Broth (20 g/L of yeast
131 extract and 125 g/L of sucrose; Labkem, Barcelona, Spain) and incubated at 25 °C for 48 h
132 under stirring conditions. The culture was centrifuged, and the pellet resuspended in PBS. The
133 cells were adjusted to 10^6 cells/mL using a Thoma chamber.

134 **2.3. Experimental settings**

135 Dry-cured fermented sausages "salchichón" were processed in a pilot plant (Álvarez et al.,
136 2020c; Delgado et al., 2021) located at the Faculty of Veterinary Sciences of the University of
137 Extremadura. Raw meat mix to prepare "salchichón" was purchased from Mallo S.L.
138 (Cañaveral, Spain) and contained minced Iberian pork meat, Iberian pig fatback, NaCl, dextrose
139 and spices. The meat mix was supplemented with rosemary leaves, REO and/or Dh prior or
140 after being stuffed into collagen casing. An antifungal preparation (AP) composed by
141 potassium sorbate and natamycin was prepared following the manufacture instructions
142 (Taberner S.A., Valencia, Spain). The length of the sausages was *c. a.* 25 cm and the diameter
143 40 mm. Eighteen different combinations of the BCAs were made (Table 1). Dh was applied
144 together with other treatments through minced-meat inoculation at a 10^6 cells/g level

145 (batches from Dh_i to AP-Dh_i, Table 1) or after stuffing on the surface by immersion in a yeast
146 suspension that resulted in *c.a.* $8*10^2$ cfu/cm^2 (batches from Dh_s to AP+Dh_s, Table 1). Finally,
147 all sausages were superficially inoculated the next day by their immersion for 30 s in a
148 suspension containing 10^6 spores/mL of *P. nordicum*, reaching a concentration of
149 approximately 10^3 spores/cm^2 (Delgado et al., 2021). Dry-cured fermented sausages were
150 done in quintuplicate for every batch.

151 **2.4. Ripening conditions**

152 Dry-cured fermented sausages were processed in maturation chambers for 3 days at 5 °C and
153 85 % RH, then 1 day at 13 °C and 84 % RH and finally 17 days at 12 °C and 84 % RH. The a_w and
154 pH were measured at the end of ripening time using a LabMaster a_w meter (Novasina AG,
155 Lachen, Switzerland) and a pH meter electrode model FC232D (Hanna Instruments S.L., Eibar,
156 Spain), respectively.

157 **2.5. Growth assessment**

158 Samples from the meat dough from each batch before stuffing were used to count the
159 microbiological population at the beginning of the ripening (day 0). For this, 10 g of the meat
160 samples were homogenised with 90 mL of peptone water (0.1 % (p/v)) in a Stomacher® 400
161 Circulator (Seward, Worthing, UK). After preparing decimal dilutions, the aliquots were
162 inoculated on Plate Count Agar (PCA; Condalab, Madrid, Spain) for total viable counts (TVC)
163 and on Rose Bengal Chloramphenicol Agar (RBC; Condalab) for yeasts and moulds. At the end
164 of the ripening process, samples from the inner part of "salchichón" were taken to detect TVC
165 and yeasts by using the previously mentioned procedure and culture media. The casings were
166 analysed through the same methodology, sampling 1 cm^2, to count moulds and yeasts in RBC.

167

168

169 **2.6. OTA production**

170 About 1 g of sample (50 cm^2 approximately), including only mycelium and casing, was collected
171 from each "salchichón". OTA was extracted by QuEChERS and analysed by an Agilent 1290
172 Infinity II UHPLC with a 6470 triple quadrupole (Agilent Technologies, Inc., Santa Clara, USA)
173 following the methodology described by Álvarez et al. (2020a). The mobile phase was
174 composed by two eluents: milli-Q water with formic acid 0.1 % (A) and acetonitrile with formic
175 acid 0.1 % (B). The flow rate was established at 0.4 mL/min using a binary gradient: 0-3.5 min
176 20 % eluent B, 3.5-10 min linear increase from 20 to 95 % eluent B, a plateau phase after
177 eluent B raised to 100 % eluent B until 12 and 3 min at 20 % eluent B. The column of the HPLC-
178 MS was a Zorbax C_{18}, 100 mm x 2.1 mm and 1.8 µm of particle size (Agilent Technologies, Inc.)
179 OTA was eluted at 6.5 min and the run time was 15 min. The MS detector was equipped with
180 an Agilent Stream electrospray ionisation source. The Multiple Reaction Monitoring conditions
181 presented two transitions from 404 *m/z* to 358 *m/z* and from 358 *m/z* to 239 *m/z* with a
182 collision energy of 10 V and 25 V, respectively. The limit of detection (LOD) was established at
183 0.08 ng/mL and the limit of quantification (LOQ) at 0.25 ng/mL. These limits were calculated
184 following the equations LOD=3*SD and LOQ=10*SD, where SD is the standard deviation of the
185 average of the concentration obtained for the lowest point of the calibration curve.

186 **2.7. Comparative proteomic analyses**

187 For the analyses of comparative proteomics 400 mg of mycelia were removed from the surface
188 of the casing of each "salchichón" (n=5 per batch), deposited in 2 mL plastic tubes and flash
189 frozen in liquid nitrogen and stored at -80 °C. The analysis was performed on the samples
190 which did not include the yeast on the surface (C; CMR; Dh_i; R+Dh_i; CMR+Dh_i), due to the
191 interferences between the biomasses of the fungal species (*D. hansenii* and *P. nordicum*) that
192 would entail non-viable results. The extraction and the proteomic analyses were carried out
193 following the methodology described by Álvarez et al. (2021a). Firstly, 400 µL of lysis buffer,

which included PMSF (Sigma Aldrich, San Luis, USA) and pepstatin A (Sigma Aldrich), were added to the samples before sonicating. The lysates were partially run in an SDS-PAGE and in-gel digested with trypsin (Promega, Madison, USA) and ProteaseMAX (Promega) after a reduction-alkylation process. Two µg of all samples were analysed in a Q-Executive Plus coupled to a Dionex Ultimate 3000 RSLCnano (Thermo Scientific, Munich, Germany). Data was obtained from a Top15 method for MS/MS scans (Delgado et al., 2019; Dolan et al., 2014). The label-free quantitative algorithm (LFQ) was used for normalising spectral intensities and calculate relative protein abundance using MaxQuant software (v.1.6.15.9; https://www.maxquant.org/) (Cox & Mann, 2008). Perseus software (v.1.6.14.0) was applied to organise the data and statistical analysis. The maximum peptide/protein false discovery rates (FDR) were set to 1 % comparing to a reverse database. Proteins were selected if they were detected in at least three of the five replicates for each batch. A t-test was used for the quantitative analysis to compare the different batches with the control batch. Statistical differences were set at $p < 0.05$. A *P. nordicum* protein database from Uniprot (https://www.uniprot.org/) was used to accomplish the searching. Qualitative analysis was performed by detecting proteins in at least three replicates of the same batch but in none of the compared batch. ClueGO software (Bindea et al., 2009) was used for the gene ontology enrichment analysis as described by Álvarez et al. (2021a).

2.8. Statistical analysis

IBM SPSS statistics v.20 was used for the statistical analysis of the results of the growth and OTA production assessments. Since the data presented a non-normal distribution (Kolmogorov Smirnov test), non-parametric tests were performed (Kruskal-Wallis and Mann-Whitney U). The statistical significance was established at $p \leq 0.05$.

219 **3. Results and Discussion**

220 Since OTA is a matter of concern for consumers´ health and meat products are one of the main
221 contributors to OTA exposure to European citizens (EFSA CONTAM Panel, 2020), the meat
222 industry requires new and successful strategies to hamper the mycotoxin levels in their
223 products. Different BCAs have efficiently reduced the OTA synthesis by *P. nordicum* on dry-
224 fermented sausage-based medium, but it is required to evaluate their biocontrol potential
225 under real conditions. Additionally, more information about the modes of action involved in
226 the OTA repression is essential for optimising their application in the meat industry.

227 **3.1. Physicochemical and microbial parameters**

228 The pH values ranged between 5.22 and 5.63 and the a_w between 0.85 and 0.88, which are
229 within the expected values for manufactured "salchichón" (Álvarez et al., 2020c; Delgado et
230 al., 2021). These outcomes indicated a proper ripening stage that facilitated the growth of the
231 desirable microorganisms, indispensable for the development of the typical sensorial
232 characteristics (Flores et al., 2015; Iacumin et al., 2020).

233 An expected increase of the TVC and yeasts from the meat dough were observed (Table 2)
234 respect to those values recorded for the day 0 (6.8 and 4.66 log cfu/g, respectively). The values
235 for TVC at the end of ripening were similar to those described for "salchichón" and other
236 Mediterranean dry-cured fermented sausages, being the counts between 7 and 8 log cfu/g
237 (Fernández-López et al., 2008; Iacumin et al., 2020; Rubio et al., 2007). Regarding the yeast
238 counts, significant increases ($p \leq 0.05$) in the meat were detected in almost all batches respect
239 to batch C (only treated with *P. nordicum*). *D. hansenii* is often the predominant yeast in
240 fermented sausages (Encinas et al., 2000) and can be found up to 8 log cfu/g in inoculated
241 "salchichón" (Cano-García et al., 2014) as occurred in the present study. Batches combining
242 rosemary in the different formats and *D. hansenii* on the surface (batches from R+Dh_s to

243 REO+Dh_s, Table 2) showed the highest yeast levels. The addition of such herb seems to
244 enhance the implantation of the yeast.

245 Concerning the influence of BCAs on the mould growth*,* batches R and R+REO boosted its
246 growth, while batches AP+Dh_i, Dh_s, CMR+Dh_s, R+REO+Dh_s and AP+Dh_s reduced it (Table 2).
247 Therefore, rosemary seemed to enhance the mould growth, but the presence of the
248 commercial antifungal preparation (batches AP+Dh_i and AP+Dh_s) and *D. hansenii* on the
249 surface (batches AP+Dh_i, Dh_s, CMR+Dh_s and R+REO+Dh_s) limited its development. In previous
250 studies this yeast species showed its ability to affect the normal growth of toxigenic *P.*
251 *nordicum* (Andrade et al., 2014; Iacumin et al., 2017) which could be consequence of the
252 disruption of the CWI (Álvarez et al, 2021a; Delgado et al., 2019). However, the effect of *D.*
253 *hansenii* on mould growth depends on the mould strain and the culture conditions (Andrade et
254 al., 2014; Cebrián et al., 2019). For instance, the counts of *P. nordicum* FHSCC IB4 were not
255 disturbed by the presence of *D. hansenii* FHSCC 253H in dry-cured ham (Cebrián et al., 2019).

256 **3.2. OTA production**

257 The application of most of the treatments reached to significantly reduce the OTA
258 accumulation (Figure 1). It should be noted that the amount of OTA in several batches was
259 below the LOQ. The maximum mycotoxin concentrations were found in batches REO and
260 R+REO (1.20 ± 0.31 and 2.18 ± 1.87 ng/g, respectively), which exceeded the legal limit (1 ng/g)
261 set by the Italian legislation for OTA in dry-cured meat products (Ministero della Sanità, 1999).
262 Similarly the amount found in the control batch (0.85 ±0.23 ng/g) was above such limit.
263 Delgado et al. (2021) have recently described analogous OTA levels to the control batch when
264 the same *P. nordicum* strain was inoculated in dry-cured fermented sausages undergoing the
265 same process, reaching similar pH and a_w values that those obtained in the present study.
266 Nonetheless, the OTA levels in the control batch were lower than those found in “salami”, a
267 similar Italian meat product, also inoculated with *P. nordicum* (Ferrara et al., 2016). These

268 discrepancies could be due to the different ripening process, ingredients, casing and *P.*
269 *nordicum* strains used in each study.

270 When examining the results for each BCA, relevant differences were obtained for the effect of
271 rosemary derivatives when solely applied, since the highest effectiveness for reducing OTA was
272 observed for direct addition of rosemary leaves and when the casings were macerated with
273 them before stuffing (batches R and CMR; Figure 1). The *in vitro* antifungal effect of rosemary
274 leaves has been previously described on a meat substrate for another *P. nordicum* strain, being
275 associated with a reduction in the expression of the OTA biosynthetic genes *otapks* and *otanps*
276 (Álvarez et al., 2020a). On the contrary, most of the batches that included REO did not reduce
277 the OTA levels (batches REO, R+REO, R+REO+Dh_i and REO+Dh_s), despite the fact that previous
278 studies demonstrated their *in vitro* capability to reduce OTA by *P. nordicum* and *A. carbonarius*
279 (Álvarez et al., 2021a; El Khoury et al., 2016). Thus, the *in vitro* results for this essential oil
280 cannot be extrapolated to “salchichón” under industrial processing where biotic and abiotic
281 factors could have interfered. On the other hand, the antiochratoxigenic effect of rosemary
282 seems to depend on the way of preparation, which has been described to affect the presence
283 of some active antifungal compounds. Concretely, phenolic and volatile compounds with
284 antifungal properties could be removed through the REO extraction by Clevenger
285 hydrodistillation (Figueredo et al., 2012; Sadeh et al., 2019). In this sense, the phenolic
286 compounds from fenugreek plants have demonstrated antiochratoxigenic activity against *A.*
287 *carbonarius* but its essential oil had no activity against this mould (El Khoury et al., 2017).
288 Furthermore, a maceration step has demonstrated a better dissolution of phenolic compounds
289 in water (Psarrou et al., 2020). Based on our results, the most effective antiochratoxigenic
290 activity of rosemary in dry-cured fermented sausages should be reached through the addition
291 of the fresh herb or macerating the casings in order to hold the most active compounds.

292 Regarding the application of *D. hansenii*, the highest effectiveness was observed when added
293 to the meat dough than when inoculated on the surface. This may be due to the fact that *P.*
294 *nordicum* is nutritionally dominant over the yeast (Álvarez et al., 2020b) when they are
295 simultaneously present on the “salchichón”. Given that higher levels of the yeast were added
296 on the meat dough than in the surface, it is likely that a higher competence by the yeast
297 against *P. nordicum* occurred. Thus, when *D. hansenii* was inoculated in the dough, individually
298 or in combination with the other BCAs, the OTA production was generally decreased (Figure 1).
299 Considering the results from applying rosemary in different formats, the successful results
300 when combining with *D. hansenii* could be mainly attributed to the presence of the yeast in
301 the meat dough, since no additional OTA reduction was observed by the addition of these
302 plant derivatives. Therefore, it should be highlighted that, despite the interactions with the
303 native microbial population and the physicochemical changes during industrial ripening, the
304 rosemary leaves, the casings macerated and *D. hansenii* showed a key effect in dry-cured
305 fermented sausages “salchichón” from the food safety point of view.

306 **3.3. Comparative proteomic analysis**

307 The comparative proteomic analysis was firstly carried out in the batch Dh_i, using the control
308 batch (C) to compare, owing to the fact that the reduction of OTA production below the LOQ
309 when only *D. hansenii* was inoculated in the meat dough. After examining the obtained results,
310 batches CMR, $R+Dh_i$ and $CMR+Dh_i$, in which OTA was also reduced below the LOQ by different
311 BCAs combinations (Figure 1), were also analysed to elucidate the impact on the *P. nordicum*
312 proteome that provokes the OTA inhibition.

313 A total of 2072 proteins were identified in the LFQ analysis of the batch that included only *D.*
314 *hansenii* as BCA (batch Dh_i), being compared to batch C (Table S1). Quantitative and qualitative
315 (only found in one batch) changes in protein abundance were detected. A total of 396 proteins
316 were decreased in abundance and 420 were risen in *P. nordicum* when *D. hansenii* was

317 inoculated in the dough (batch Dh_i). Forty-two proteins were only detected in this batch, whilst
318 37 or them were only found in the batch C.

319 The gene ontology enrichment analysis classified the proteins in “groups” which were
320 subdivided in more specific “terms”. ClueGo divided the decreased proteins identified in 109
321 metabolic routes or terms whilst the increased proteins were included in 83 terms. Most of the
322 terms found decreased (33.94 %) were included in the “small molecule biosynthetic process”
323 group, followed by the “organic acid metabolic process” group (15.60 %) (Figure S1). The terms
324 for proteins found in higher relative abundance were mainly grouped in “purine ribonucleoside
325 triphosphate binding” (27.71 %) and “ribosome” (26.51 %) groups (Figure S2).

326 Regarding the proteins found in lower abundance because of *D. hansenii* (Table 3), some of
327 them were directly related to the OTA biosynthesis pathway (Figure 2). The protein chorismate
328 synthase is involved in the synthesis of chorismate, a precursor of phenylalanine (Woodin &
329 Nishioka, 1973), which is essential for forming the molecular structure of OTA. Other proteins
330 whose abundance was reduced due to the yeast are linked to the formation of Acetyl CoA,
331 such as the pyruvate dehydrogenase E1 component subunit alpha and the probable acetate
332 kinase. The Acetyl CoA intervenes in numerous routes, being necessary for the synthesis of
333 ochratoxin β, and a component of mould membranes, ergosterol (Dupont et al., 2012; Mille-
334 Lindblom et al., 2004). The results also showed a lower abundance of numerous proteins
335 belonging to the PKS ER domain (Table 3), involved in mycotoxin biosynthesis, such as OTA
336 from *Penicillium thymicola* and *Aspergillus niger* or fumonisins from *Fusarium* spp. (Brown et
337 al., 2012; Ferracin et al., 2012; Huffman et al., 2010; Nguyen et al., 2016). This effect had been
338 previously reported in an *in vitro* assay on meat model using the same strains of *D. hansenii*
339 and *P. nordicum* than in the present study (Álvarez et al., 2021a). This finding thus
340 demonstrates that the activity of *D. hansenii* on PKS ER domain is not limited to *in vitro*
341 context, being not modified by other microbial population present in dry-cured fermented

342 sausages, neither the dynamic physicochemical properties of such meat product during its
343 ripening. The drop of the abundance of proteins from this domain, in comparison with the
344 batch non-treated with the BCAs (batch C), would confirm the previously pointed key role of
345 the PKS ER domain to explain the OTA reduction by *D. hansenii* (Álvarez,et al., 2021a).

346 Some proteins related to the CWI pathway were also decreased in abundance in the presence
347 of *D. hansenii*, as the actin-related protein 2/3 complex subunit 4, the actin-related protein 2/3
348 complex subunit 5, the cytoskeletal adapter protein sagA, the adenylyl cyclase-associated
349 protein and the rhamnogalacturonate lyase. These proteins are essential for the normal hyphal
350 growth through the modulation of actin binding and the cell wall organization. Likewise the
351 interferences in the CWI pathway have been linked to a reduction in the production of
352 secondary metabolites, such as mycotoxins (Valiante, 2017).

353 Changes on proteins from CWI pathway have been previously reported for this *P. nordicum*
354 strain when co-cultured with the same *D. hansenii* strain on two culture media elaborated
355 from dry-cured ham and dry-cured fermented sausages (Álvarez et al., 2021a; Delgado et al.,
356 2019). Though the proteins from the CWI found in our study do not exactly match with those
357 found in *P. nordicum* Pn15 treated with *D. hansenii* in these previous works, the tendency to
358 lower CWI pathway metabolism is maintained under the real industrial ripening conditions of
359 the dry-cured fermented sausages.

360 Furthermore, the presence of *D. hansenii* seems to enhance the ribosome activity translating
361 new proteins involved in ribosome structure as the ribosomal_ L28e domain-containing
362 protein and the Ribos_L4_asso_C domain- containing protein (Table 3), consequently
363 increasing the abundance of transporter and folding proteins (Table S1). Although the reason
364 of this activation of ribosome activity is not still clear, previous studies in fungi suggest that
365 ribosome biogenesis can be intensified as a mechanism against antifungals, such as rapamycin
366 and sorbic acid (Lai et al., 2016; Víglaš & Olejníková, 2020). This response has been also

367 observed in *Aspergillus flavus* and *Penicillium polonicum* when they were treated with the
368 antifungal protein PgAFP, regardless to their sensitivity to this protein (Delgado et al., 2015,
369 2016b). Thus, it could be considered as an unspecific reaction against antifungal treatments.

370 When *D. hansenii* was combined with rosemary (batches R+Dh_i and CMR+Dh_i; Tables S3 and
371 S4), the comparative proteomic analysis revealed that the CWI pathway was affected too,
372 being more noticeable when macerated casings were used (batch CMR). Besides,
373 phosphomevalonate kinase was reduced in abundance in batch R+Dh_i (Table 3). This protein is
374 involved in ergosterol biosynthesis in fungi (Jordá & Puig, 2020; Thykaer et al., 2009), an
375 essential constituent of the cell membranes that regulates the permeability and maintains the
376 membrane integrity. This is in accordance with a previous study where rosemary leaves
377 provoked a fall in the ergosterol content in toxigenic moulds (Álvarez et al., 2021b). On the
378 other hand, the rhamnogalacturonate lyase in batch CMR+Dh_i was even more diminished in
379 abundance (-2.04) than in batch Dh_i (-0.77), in both cases when compared to the control batch
380 (C), so the combination between *D. hansenii* and the macerated casings in rosemary enhance
381 their reduction, although it did not entail a further OTA inhibition. In these two batches, the
382 phenylalanine biosynthesis was also influenced by the reduction in abundance of chorismate
383 synthase as well as of the proteins phospho-2-dehydro-3-deoxyheptonate aldolase and
384 prephenate dehydratase (Woodin & Nishioka, 1973) (Table 3). It deserves to be highlighted
385 that the chorismate synthase and the phospho-2-dehydro-3-deoxyheptonate aldolase take
386 part of chorismate synthesis (Figure 2).

387 Moreover, the protein prephenate dehydratase was decreased in batch CMR (Tables 3 and S5)
388 but not in the batch Dh_i, so this alteration is linked to the macerated casings with rosemary
389 leaves. The lowest level of this protein was found in batch CMR+Dh_i. Hence, the
390 coadministration of *D. hansenii* and the casings macerated with rosemary showed a
391 cumulative inhibition of this protein that was not translated to lower OTA levels.

392 As previously mentioned for batches Dh_i, R+Dh_i and CMR+Dh_i, the CWI was modified in batch
393 CMR by the reduction of F-actin-capping protein subunit beta, related to the actin
394 cytoskeleton organization, and the NADPH-cytochrome P450 reductase involved in ergosterol
395 biosynthesis pathway (batches CMR and CMR+Dh_i). The disruption of the gene that codified
396 for the capping proteins in *Saccharomyces cerevisae* and *Fusarium graminearum* resulted in
397 viable cells but with a heterogeneous slower growth and a reduction of the production of
398 deoxynivalenol by the mould (Amatruda et al., 1990; Tang et al., 2020).

399 Summarising, *D. hansenii* affected proteins involved in the OTA production, which implies an
400 advantage for the direct inhibition of the biosynthesis and the CWI pathway. Both routes were
401 also altered when macerating the casings with rosemary. The combination of *D. hansenii* with
402 the casings macerated (batches CMR+Dh_i) boosted the reduction of some common proteins,
403 such as the rhamnagalacturonate lyase and the chorismate synthase. Since this combination
404 boosts the inhibition of key enzymes involved in OTA biosynthesis, it is likely that the lack of
405 apparent additive or synergic effect on OTA reduction relies on the relative low amount of OTA
406 levels found in our dry-cured fermented sausages, that could hide this plausible summatory
407 effect on OTA inhibition when these levels are below the LOQ. Additionally, the presence of
408 rosemary in batches CMR, R+Dh_i and CMR+ Dh_i decreased the abundance of proteins related
409 to ergosterol biosynthesis, being in accordance with previous studies about the mode of action
410 of this herb on toxigenic moulds (Álvarez et al., 2021b ; Da Silva Bomfim et al., 2020).

411

412 **4. Conclusions**

413 The inoculated BCAs allowed the normal growth of native population of dry-cured fermented
414 sausages "salchichón" undergone an industrial ripening with usual values for physicochemical
415 parameters. Since the addition of *D. hansenii* or rosemary leaves in the meat dough or the
416 maceration of casings with the herb successfully decreased the OTA production, these

417 treatments could be proposed as natural and safe alternatives to synthetic antifungals in the
418 meat industry. The proteomic results validated the modes of action previously obtained in *in*
419 *vitro* tests. The reduction of OTA by *D. hansenii* suggested to be directly related with the
420 decrease of proteins involved in OTA biosynthesis, and its combination with rosemary altered
421 the CWI, linked to a reduction of mycotoxins in toxigenic moulds. The batches with rosemary
422 reduced the abundance of proteins related to the synthesis of phenylalanine and ergosterol.
423 These findings could be set in the framework of HACCP as optimal preventative measures to
424 reduce the hazard posed by *P. nordicum* and its ability to contaminate the meat products with
425 OTA.

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668

669 Table 1. Batches of dry-cured fermented sausages "salchichón" manufactured in this study.

	Treatments[a]						
Batch designation	*Penicillium nordicum*	R	Casing MR	REO	AP	Dh_i	Dh_s
C	X						
R	X	X					
CMR	X		X				
REO	X			X			
R+REO	X	X		X			
AP	X				X		
Dh_i	X					X	
R+Dh_i	X	X				X	
CMR+Dh_i	X		X			X	
REO+Dh_i	X			X		X	
R+REO+Dh_i	X	X		X		X	
AP+Dh_i	X				X	X	
Dh_s	X						X
R+Dh_s	X	X					X
CMR+Dh_s	X		X				X
REO+Dh_s	X			X			X
R+REO+Dh_s	X	X		X			X
AP+Dh_s	X				X		X

670 [a]*Penicillium nordicum: P. nordicum* FHSCC Pn15 applied on the surface by immersion in a
671 solution containing 10^6 spores/mL; R: rosemary leaves added to the meat dough as ingredient
672 (2 g/kg); Casing MR: casings macerated with rosemary leaves; REO: rosemary essential oil
673 applied by spraying on the surface of dry-cured fermented sausages after stuffing; AP:
674 antifungal preparation (potassium sorbate + natamycin) applied on the surface of dry-cured
675 fermented sausages by immersion after stuffing; Dh_i: *Debaryomyces hansenii* FHSCC 253H (Dh)
676 inoculated at 10^6 cells/g in the meat dough before stuffing; Dh_s: Dh inoculated on the surface
677 by immersion in a suspension containing 10^6 cells/mL after stuffing.

678 Table 2. Mean values of microorganisms' levels in the dry-cured fermented sausages
679 "salchichón" manufactured in this study at the end of the ripening.

Batch[a]	Total viable counts (log cfu/g)	Yeasts in the dough (log cfu/g)	Yeasts in the casing (log cfu/cm^2)	Moulds in the casing (log cfu/cm^2)
C	7.44	6.30	8.09	8.52
R	7.28	7.20*	7.91	9.60*
CMR	7.41	7.19*	7.97	8.40
REO	7.75	7.57*	8.54*	8.91
R+REO	7.93*	7.59*	8.22	9.45*
AP	7.20	6.63	8.23	7.93
Dh_i	7.82*	7.48*	8.27	8.02
R+Dh_i	7.49	7.13	8.53*	8.16
CMR+Dh_i	7.34	7.39*	8.36*	7.98
REO+Dh_i	8.06*	7.64*	8.34	8.29
R+REO+Dh_i	7.82	8.01*	8.66*	8.37
AP+Dh_i	7.71	7.26*	8.62	7.92*
Dh_s	7.42	7.92*	8.96*	7.46*
R+Dh_s	7.25	7.46*	9.26*	8.04
CMR+Dh_s	7.40	7.49*	9.45*	7.60*
REO+Dh_s	7.40	7.93*	9.20*	8.29
R+REO+Dh_s	7.34	6.93	8.19	7.68*
AP+Dh_s	6.56*	7.28*	8.23	7.23*

680 [a]C: batch non-treated with biocontrol agents; R: rosemary leaves added to the meat dough as
681 ingredient (2 g/kg); CMR: casings macerated with rosemary leaves; REO: rosemary essential oil
682 applied on the surface by spraying of dry-cured fermented sausages after stuffing; AP:
683 antifungal preparation (potassium sorbate + natamycin) applied on the surface of dry-cured
684 fermented sausages by immersion after stuffing; Dh_i: *Debaryomyces hansenii* FHSCC 253H (Dh)
685 inoculated at 10^6 cells/g in the meat dough before stuffing; Dh_s: Dh inoculated on the surface
686 by immersion in a suspension of 10^6 cells/mL after stuffing. *Penicillium nordicum* FHSCC Pn15
687 was applied on the surface of all batches by immersion in a solution containing 10^6 spores/mL.
688 *Statistical differences ($p \leq 0.05$) between each batch containing any biocontrol agent respect
689 to C. The experiment was performed in quintuplicate.

690

691 Table 3. Changes in protein abundance ($p < 0.05$) for *Penicillium nordicum* FHSCC Pn15 (Pn15)
692 in the presence of different biocontrol agents (BCAs) in dry-cured fermented sausages
693 “salchichón”.

Batch[a]	Protein	Identification	Fold change
CMR	Prephenate dehydratase	A0A0M9WCM5	-0.510
	Chorismate synthase	A0A0M9WE56	-1.124
	F-actin-capping protein subunit beta	A0A0M9WDR3	-0.778
	NADPH-cytochrome P450 reductase	A0A0M8P305	-0.933
Dh_i	PKS ER domain-containing protein	A0A0M9WBI0	-0.71
		A0A0M9WBD1	-1.23
		A0A0M8PHV1	-0.56
		A0A0M8NY36	-0.48
		A0A0M9WIK6	-1.10
		A0A0M9WKW6	-0.62
		A0A0M8P7V8	Only in C[b]
		A0A0M8P7D0	-1.09
		A0A0N0RYG6	-0.88
	Chorismate synthase	A0A0M9WE56	-1.17
	Pyruvate dehydrogenase E1 component subunit alpha	A0A0M9WCM4	-0.41
	Probable acetate kinase	A0A0M8NYD5	-1.32
	Actin-related protein 2/3 complex subunit 4	A0A0M9WA57	-0.45
	Actin-related protein 2/3 complex subunit 5	A0A0M9WGD1	-1.35
	Actin-related protein 2/3 complex subunit	A0A0M9WKC7	-0.83
	Cytoskeletal adapter protein sagA	A0A0M9WHT9	Only in C
	Adenylyl cyclase-associated protein	A0A0M8P5B1	-0.87
	Rhamnogalacturonate lyase	A0A0N0RZ79	-0.77
	Ribosomal_L28e domain-containing protein	A0A0M8NVL1	3.55
	Ribos_L4_asso_C domain-containing protein	A0A0M8NRI1	2.68
R+Dh_i	Phosphomevalonate kinase	A0A0M8NZR3	Only in C
CMR+Dh_i	Rhamnogalacturonate lyase	A0A0N0RZ79	-2.04
	Prephenate dehydratase	A0A0M9WCM5	-0.31
	Phospho-2-dehydro-3-deoxyheptonate aldolase	A0A0M8NXZ6	-0.86
	Chorismate synthase	A0A0M9WE56	-1.50

NADPH-cytochrome P450 reductase	A0A0M8P305	-1.05

694 [a]CMR: casings macerated with rosemary leaves; Dh_i: *Debaryomyces hansenii* FHSCC 253H
695 inoculated at 10^6 ufc/g in the meat dough; R+Dh_i: rosemary leaves added in the meat dough
696 (2g/kg) + Dh_i; CMR+Dh_i: CMR and Dh_i. [b]Only in C: Only in batch non-treated with BCAs (batch
697 C). *P. nordicum* was applied on the surface of all batches by immersion in a solution containing
698 10^6 spores/mL. The experiment was performed in quintuplicate.

699

700

701 **Figure legends**

702 Figure 1. Ochratoxin A (OTA) production by *Penicillium nordicum* FHSCC Pn15 in the presence
703 of different biocontrol agents (BCAs) in dry-cured fermented sausages “salchichón”. OTA
704 expressed as percentage of reduction/increment compared to the batch only inoculated with
705 *P. nordicum* (batch C base line).

706 R: rosemary leaves added in the meat dough as ingredient (2g/kg); CMR: casings macerated
707 with rosemary leaves; REO: rosemary essential oil applied by spraying on the surface of dry-
708 cured fermented sausages after stuffing; AP: antifungal preparation (potassium sorbate +
709 natamycin) applied on the surface of dry-cured fermented sausages by immersion after
710 stuffing; Dh_i: *Debaryomyces hansenii* FHSCC 253H (Dh) inoculated at 10^6 cells/g in the meat
711 dough before stuffing; Dh_s: Dh inoculated on the surface by immersion in a suspension of 10^6
712 cells/mL after stuffing. *P. nordicum* was applied on the surface of all batches by immersion in a
713 solution of 10^6 spores/mL. *Statistical differences ($p \leq 0.05$) between each batch containing the
714 BCAs respect to batch C. Batches with a reduction of 100 % showed OTA levels under the limit
715 of quantification (0.25 ng/mL). The experiment was performed in quintuplicate.

716 Figure 2. Schematic representation of the ochratoxin A biosynthesis pathway adapted from
717 Gallo et al. (2017).

718

719 **SUPPLEMENTARY MATERIAL**

720 Table S1. *Penicillium nordicum* FHSCC Pn15 proteins identified in this study along with fold
721 change ($2^{\text{Student's T-test Difference}}$) and significance values (*p*) of the batch containing *Debaryomyces*
722 *hansenii* FHSCC 253H (Dh_i) in comparison to the non-treated control (C). Control samples in
723 blue, samples with *D. hansenii* in orange and statistical differences in green.

724 Table S2. *Penicillium nordicum* FHSCC Pn15 proteins identified in this study along with fold
725 change ($2^{\text{Student's T-test Difference}}$) and significance values (*p*) of the batch containing rosemary
726 leaves plus *Debaryomyces hansenii* FHSCC 253H ($R+Dh_i$) in comparison to the non-treated
727 control (C). Control samples in blue, samples with rosemary leaves plus *D. hansenii* in orange
728 and statistical differences in green.

729 Table S3. *Penicillium nordicum* FHSCC Pn15 proteins identified in this study along with fold
730 change ($2^{\text{Student's T-test Difference}}$) and significance values (*p*) of the batch containing casings
731 macerated in rosemary leaves plus *Debaryomyces hansenii* 253H ($CMR+Dh_i$) in comparison to
732 the non-treated control (C). Control samples in blue, samples with *D. hansenii* plus casings
733 macerated in rosemary leaves in grey and statistical differences in green.

734 Table S4. *Penicillium nordicum* FHSCC Pn15 proteins identified in this study along with fold
735 change ($2^{\text{Student's T-test Difference}}$) and significance values (*p*) of the batch containing casings
736 macerated in rosemary leaves (CMR) in comparison to the non-treated control (C). Control
737 samples in blue, samples with casings macerated in rosemary leaves in gray and statistical
738 differences in green.

739 Figure S1. Protein reduction in relative abundance in *Penicillium nordicum* FSHCC Pn15 in the
740 presence of *Debaryomyces hansenii* FSHCC 253H (Dh_i) classified in groups by ClueGO.
741 Percentage calculated from the number of terms (more specific protein classification) per
742 group (more general protein classification). Data lower than 2.75 % are not named.
743 **Statistical differences ($p \leq 0.01$).

744 Figure S2. Protein increment in relative abundance in *Penicillium nordicum* FSHCC Pn15 in the
745 presence of *Debaryomyces hansenii* FSHCC 253H classified in groups by ClueGO. Percentage
746 calculated from the number of terms (more specific protein classification) per group (more
747 general protein classification). **Statistical differences ($p \leq 0.01$).

748

749

Figure 1

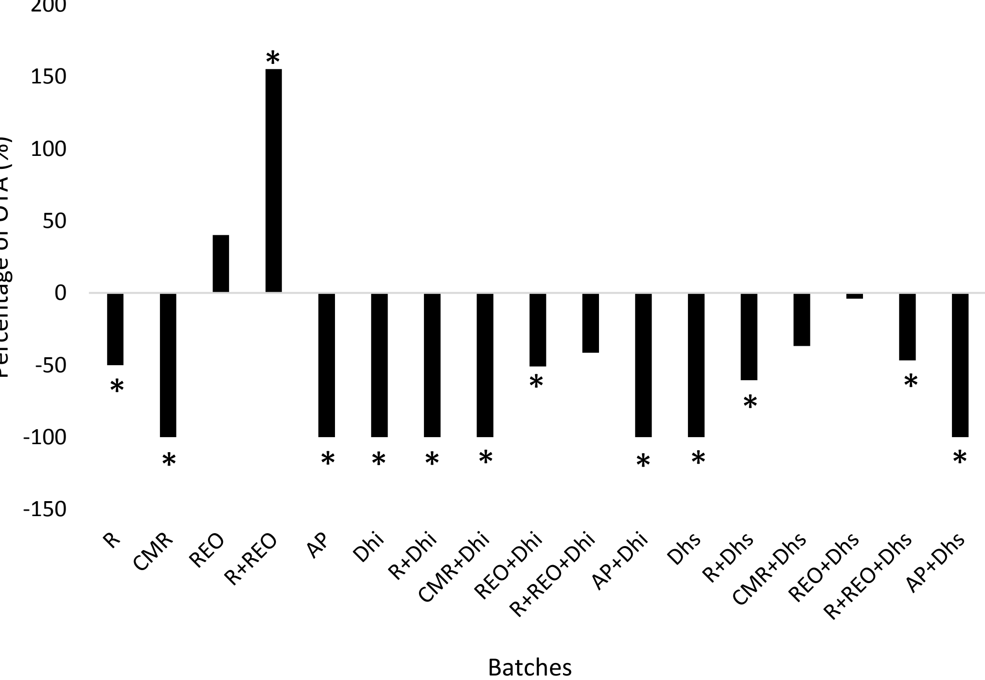


Figure 2

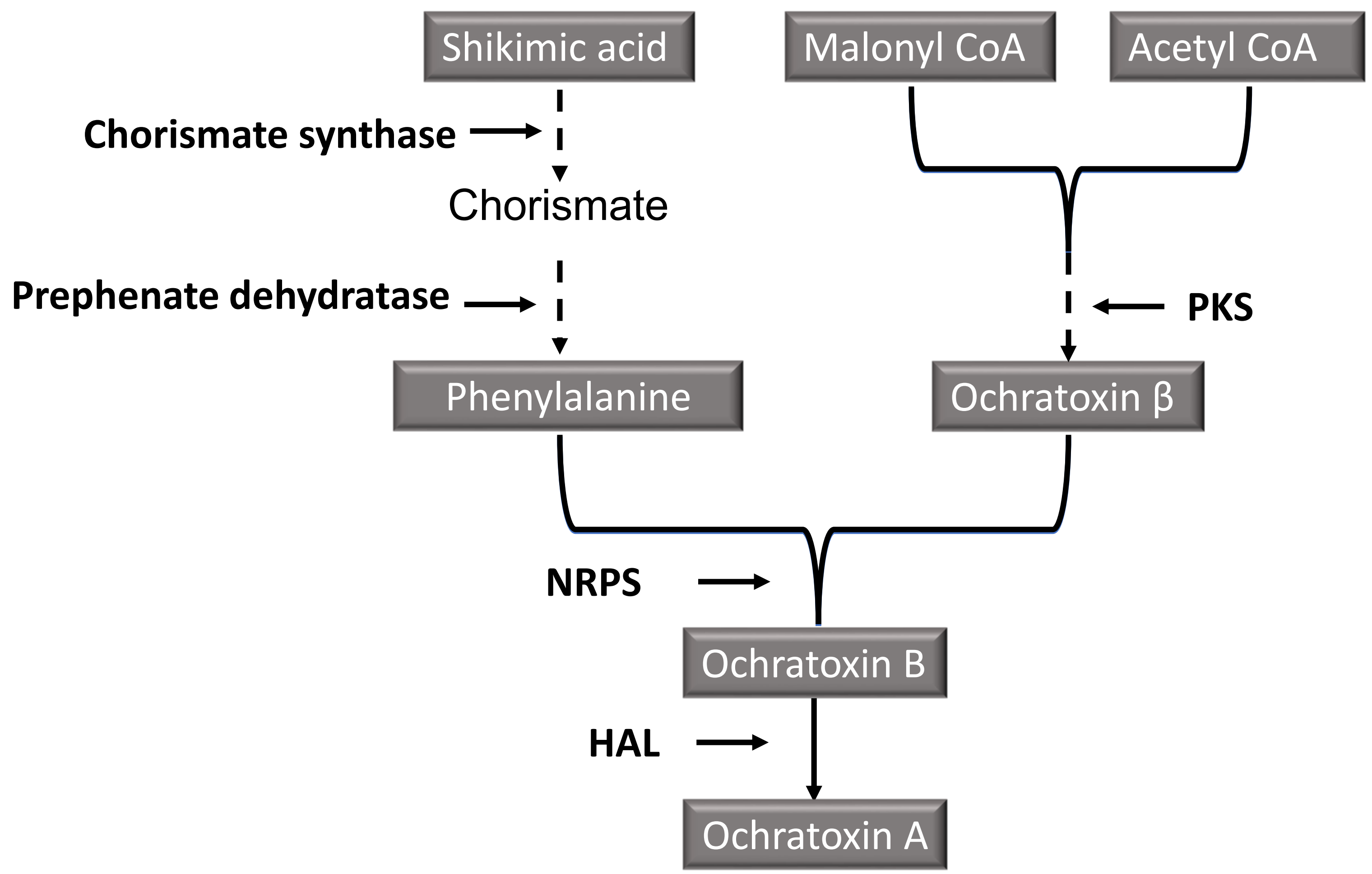


Figure S1

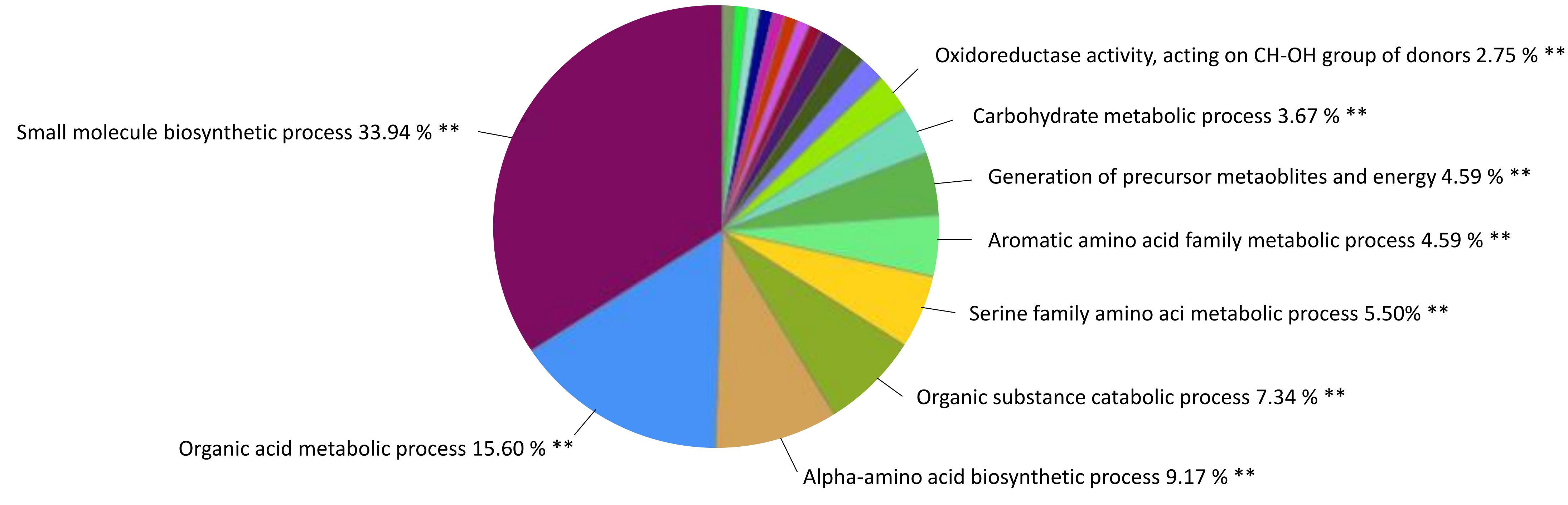
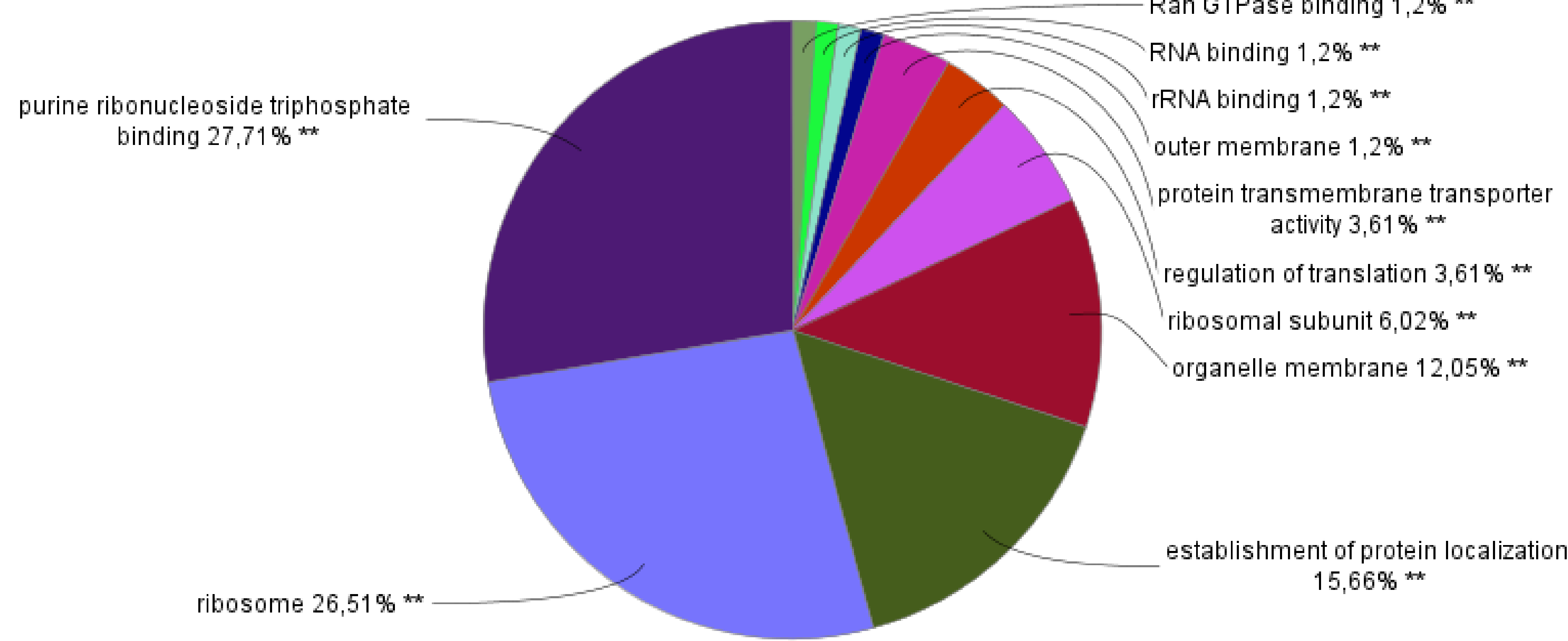


Figure S2



IV.8. Evaluación y efecto de los agentes de biocontrol seleccionados para *P. nordicum* sobre *Aspergillus westerdijkiae*

Biocontrol agents reduce the OTA production by Aspergillus westerdijkiae *in dry-cured fermented sausage-based media*

Biocontrol agents reduce the OTA production by *Aspergillus westerdijkiae* in dry-cured fermented sausage-based media

1. Introduction

Dry-cured fermented sausages are generally produced from pork meat and fat, salt and condiments as aromatic herbs or pepper. In the Iberian Peninsula these foods are part of the traditional diet and represent one of the economic pillars of the meat industry in some regions. The fermentation stage by the action of bacteria and yeasts is an ancient preserving method which together with a drying-ripening stage extends their shelf life (Oliveira et al., 2018) and confers the characteristic sensory properties. However, during this phase undesirable moulds can growth on their surface and produce mycotoxins, harmful to consumers health. The most important mycotoxin found in dry-cured fermented sausages is the ochratoxin A (OTA), mainly produced by *Penicillium nordicum*. However, recently *Aspergillus westerdijkiae* has been isolated from dry-cured meat products, being able to produce high levels of OTA (Merla et al., 2018; Parussolo et al., 2019; Vipotnik et al., 2017). This mycotoxin has been related to the Balkan endemic nephropathy by its nephrotoxic effect and is associated with urinary tract tumors in animals (Khoury & Atoui, 2010), being included into Group 2B as possibly carcinogenic to humans (IARC, 1993), although a new classification in the Group 2A as probably carcinogenic to humans has been suggested (Ostry et al., 2017).

Currently, consumers are aware of the use of food synthetic additives being more attractive for them the use of natural biocontrol agents (BCAs) such as microorganisms and spices (Balciunas et al., 2013; Oliveira et al., 2018). These BCAs should show antagonistic effects against toxigenic moulds without adverse characteristics on the sensorial profile of the product. Different microorganisms have been described as potential BCAs against ochratoxigenic moulds, such as *Lactobacillus plantarum* and *Lactobacillus graminis* against *Aspergillus carbonarius* in wheat (Belkacem-Hanfi et al., 2014b) and *Lactobacillus buchneri* against *P. nordicum* in YES media (Guimarães et al., 2018).

In dry-cured meat products, autochthonous yeasts have been studied as BCAs due to their proper implantation and their contribution to the typical physico-chemical properties (Corral et al., 2017; Flores et al., 2015). *Debaryomyces hansenii*, the predominant yeast in dry-cured fermented sausages (Cocolin et al., 2006; Flores et al., 2015), has been proposed as a possible BCA against ochratoxigenic moulds such as *P. nordicum* (Álvarez et al., 2021a; Andrade et al., 2014; Simoncini et al., 2014), *Penicillium verrucosum* (Peromingo et al., 2018), and *Aspergillus ochraceus* (Iacumin et al., 2017). Their effect against ochratoxigenic moulds in meat substrates

has been attributed to the repression of otanps gen (Peromingo et al., 2018, Cebrián et al., 2019), and the decreases in the abundance of proteins involved in OTA biosynthesis and in the CWI pathway (Delgado et al., 2019; Álvarez et al., 2021b). The ability of *D. hansenii* to decrease OTA production by *A. westerdijkiae* in standard culture medium has been associated with the regulation at transcriptional level of OTA biosynthesis and the adsorption to yeast cell wall (Gil-Serna et al., 2011). However, the effectiveness of *D. hansenii* against *A. westerdijkiae* and their modes of action could differ in dry-cured meat substrates due to the different composition of the medium and the environmental conditions of ripening process.

Plants and their derivatives as essential oils (EOs) and other extracts have also been studied as BCAs against toxigenic moulds. Rosemary leaves have been demonstrated to reduce OTA concentration by *P. nordicum* in dry-cured fermented sausages (Álvarez et al., 2021a) and rosemary EO the OTA amounts produced by *A. ochraceus* and *A. westerdijkiae* in culture medium (Císarová et al., 2020). Other EOs from basil and sage totally inhibited the OTA in *A. westerdijkiae* (Císarová et al., 2020). However, *Oreganum vulgare* EO stimulated its production in yeast extract broth by *A. westerdijkiae* but successfully reduced it in *P. verrucosum* (Schlösser & Prange, 2019).

The aim of the study was to evaluate the potential use of *D. hansenii* FHSCC 253H, rosemary leaves (RL), rosemary EO (REO) and their combinations as BCAs against *A. westerdijkiae* in a dry-cured fermented sausages-based medium (FS).

2. Material and methods

2.1. Microorganisms and plant material

The fungal strain *A. westerdijkiae* MUM. 16.142 (Aw) was kindly provided by Dra. Paula Rodrigues, from the *Micoteca da Universidade do Minho* (Braga, Portugal). The yeast *D. hansenii* FHSCC 253H (Dh) belongs to the Culture Collection of Food Hygiene and Safety of the University of Extremadura (Cáceres, Spain). Both strains have been isolated from dry-cured ham.

Fresh RL were collected in the region of Extremadura (Spain). REO was extracted by Clevenger hydrodistillation following the methodology used in (Álvarez et al., 2021b). For that procedure, 200 g of RL were boiled with 400 mL of distilled water for 2 h. The oily phase was separated and kept at -20 °C. REO was then dissolved in water with 1 % of Tween 80 (Fisher Scientific, Waltham, Massachusetts, USA) up to a final concentration of REO:water 1:1.

2.2. Culture media

A. westerdijkiae was growth on Potato Dextrose Agar (PDA) following the manufacturer instructions (Biolife, Milán, Italy). Yeast Extract Sucrose browth (YES) for *D. hansenii* growth was made by adding 20 g/L of yeast extract (Biolife) and 125 g/L of sucrose (Thermo Fisher Scientific, Waltham, Massachusetts, USA) to 1 L of distilled water.

FS was prepared as described by Álvarez et al. (2020) containing 25 % of lyophilised "salchichón" and 2 % of bacteriological agar (VWR Chemicals, Radnor, Pennsylvania, USA). This medium was supplemented with 2 g of RL per kg of lyophilised "salchichón" to obtain the FS-R medium.

All the media were autoclaved for 15 min at 121 °C, cooled at 50 °C and poured into Petri plates.

2.3. Inoculation and experimental settings

Suspension of *A. westerdijkiae* was prepared from cultures in PDA and incubated for 7 days at 25 °C. The spores were counted in a Neubauer chamber (Labor Optik, Lancing, UK) using a microscope model LaborLux 12 (Leitz, Stuttgart, Germany) and adjusted to a final concentration of 10^6 spores/mL of phosphate buffer saline (VWR Chemicals). Dh was incubated in YES for 2 days at 25 °C and the inoculum concentration stablished at 10^6 cells/mL following the methodology described for Aw.

Ten different treatments based on the combinations of the BCAs were studied (Table 1). One hundred µL of Dh were added to the melted agar at 50 °C before plating. A collagen casing, previously sterilised by immersion in alcohol and exposed to UV for 1 day, was placed on the medium surface. One hundred µL or REO or 25 µL of antifungal preparation (AP) composed by potassium sorbate and natamycin were spread with a sterile spatula on the surface of the casing. Finally, 2 µL of *A. westerdijkiae* were added in the centre of each plate. The plates were incubated for 3 days at 22 °C and 90 % relative humidity (RH) and 12 days at 15 °C and 85 % RH to simulate the industrial ripening of dry-cured fermented sausages. The treatments were performed in triplicate.

Table 1. Composition of the treatments using biocontrol agents against *Aspergillus westerdijkiae* MUM 16.142 in a dry-cured fermented sausage-based medium.

Treatment	Denomination	Inoculum concentration
Non-treated control	C	2 µL of 10^6 spores/mL
Rosemary leaves	R	2 g/kg
Rosemary essential oil	REO	100 µL
Rosemary leaves + rosemary essential oil	R-REO	2 g/kg + 100 µL
Antifungal preparation	AP	25 µL
Debaryomyces hansenii	Dh	100 µL of 10^6 cells/mL
Rosemary + *D. hansenii*	R + Dh	2 g/kg +100 µL of 10^6 cells/mL
Rosemary essential oil + *D. hansenii*	REO + Dh	100 µL + 100 µL of 10^6 cells/mL
Rosemary leaves + rosemary essential oil + *D. hansenii*	R-REO + Dh	2 g/kg +100 µL + 100 µL of 10^6 cells/mL

2.4. OTA extraction and analysis

After the incubation period, a half of the mycelium was collected with a sterile scalpel and transferred into 45 mL plastic tube. The samples were stored at -20 °C until their extraction using QuEChERs (Delgado et al., 2019). Two mL of water acidified with 0.1 % (v/v) of acetic acid and 2 mL of acetonitrile acidified with 0.1 % (v/v) of acetic acid were mixed with the samples. Then, 0.4 g of NaCl and 1.6 g of anhydrous magnesium sulphate were added to the tubes and immediately shaken and centrifuged for 5 min at 3500 g in a Multifuge X1R (Thermo Fisher Scientific). One mL from every supernatant was stored at 4 °C until analysis for a maximum of 1 day. The supernatants were filtered through a 0.22 µm polytetra-fluorethylene membrane (FiltraTech, Saint Jean de Braye, France) before analysis.

OTA was analysed using a High-Performance Liquid Chromatography (HPLC) Smartline pump 1000 (Knauer, Berlin, Germany) coupled with a fluorescence detector FP-2020 (Jasco, USA). The column was a C18 reverse-phase column PLRP-S 300 Å (250 x 4.6 mm, 8µm, Polymer Laboratories, Church Stretton, UK) at 35 °C. The mobile phase composed by water:acetonitrile: acetic acid (29.5:70:0.5) was used in an isocratic mode and pump at 0.8 mL/min. The injection volume was 20 µL. The OTA was detected at 330 nm and 463 nm excitation and emission wavelengths, respectively. The total run time was 15 min. The limit of detection (LOD) and the limit of quatification (LOQ) were calculated from the following equations: LOD= 3 x (sa/b) and LOQ= 10 x (sa/b) where “sa” is the standard deviation of the regression line obtained from the

calibration curve and b is the slope of the line. The LOD and LOQ were 3 and 9 ng/mL, respectively.

2.5. Synergistic effect

The synergia between treatments was analysed through the following equations using the Abbot formula described by Moreno et al. (2003).

$$Ie = X + Y - (XY/100)$$

Ie is the expected percent inhibition of a combination treatment, *X* and *Y* are the percent inhibition for each treatment separately and XY the percent inhibition for the combination treatment. The nature of this interaction was determined by the ratio (IR) where IR= I_o/I_e. I_o is the observed percent inhibition. A ration up to 1.5 shows a synergistic interaction. This formula was only calculated for the potential synergistic combinations.

2.6. Statistical analysis

The statistical analysis was carried out using IBM SPSS v. 20 (Armonk, New York, USA). Non-parametric tests (Kruskal-Wallis and Mann-Whitney U) were performed due to the non-normal distribution of the data. The statistical significance was established at $p \leq 0.05$.

3. Results and discussion

The percentage of OTA variation provoked by the different BCAs on *A. westerdijkiae* are indicated in Figure 1. The RL and the REO only reduced 73.87 % of the OTA production when both BCAs were combined showing a synergistic effect (IR =3.76). Therefore, the combination of both BCAs suggests the need to increase the concentration of active compounds which may be insufficient using each individual treatment. However, other ochratoxigenic moulds as *Aspergillus carbonarius* and *Penicillium nordicum* have shown to be more sensible to rosemary derivatives in culture medium (Álvarez et al., 2020; El Khoury et al., 2016). The antifungal effect of rosemary could be attributed to phenolic compounds (El Khoury et al., 2017) which could be reduced in the oily fraction during the extraction process by hydrodistillation in Clevenger (Figueredo et al., 2012; Sadeh et al., 2019). Anyhow, the implication of other synergistic mechanisms in their joint action cannot be ruled out.

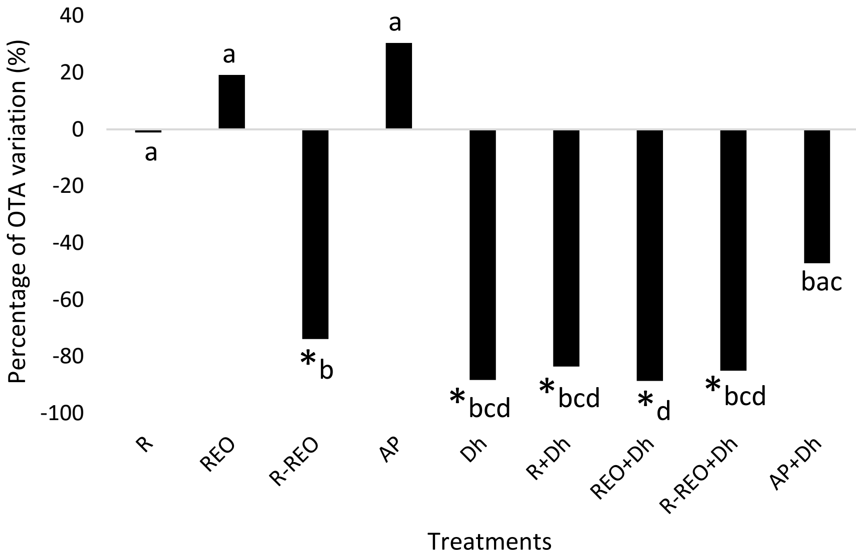


Figure 1. Percentage of OTA variation (%) using biocontrol agents against *Aspergillus westerdijkiae* MUM 16.142 in a dry-cured fermented sausage-based medium.

Treatments: R: Rosemary; REO: Rosemary essential oil; AP: antifungal preparation (potassium sorbate + natamycin); Dh: *Debaryomyces hansenii* FHSCC 253H. Statistical differences ($p \leq 0.05$) between a given treatment and the non-treated control (base line) were indicated with an asterisk and between the rest of the treatments with different letters ($p \leq 0.05$). The experiment was performed in triplicate.

No OTA reductions were found in the treatments with AP possibly due to the use of sub-inhibitory doses (Alcano et al., 2016; Álvarez et al., 2020a). Sub-inhibitory amounts of potassium sorbate have been related with the stimulation of OTA in *P. verrucosum* due to the stress produced by the antifungal compound (Schmidt-Heydt et al., 2007). This relation between stress and mycotoxin production has been associated with the adaptative response of moulds to stressful environments (Álvarez et al., 2020). The treatments where *D. hansenii* was inoculated alone or in combination with other BCAs showed percentages of inhibition higher than 80 % in all cases. No synergistic effect was found between R-REO+Dh and Dh. This strain of *D. hansenii* has previously hampered the OTA production in *P. nordicum* in dry-cured fermented sausages-based medium (Álvarez et al., 2021b; Álvarez et al., 2021c) and dry-cured meat products (Andrade et al., 2014; Cebrián et al., 2019). In *A. westerdijkiae* other strains of *D. hansenii* have also shown their ability to reduce OTA in culture medium and dry-cured ham (Gil-Serna et al., 2011; Iacumin, et al., 2020). Moreover, the inoculation of this yeast in “speck”, a dry-cured meat product, inhibited the OTA production *by P. nordicum* and *A. westerdijkiae* below the limit of quantification (Iacumin et al., 2017). Their modes of action have been related to the production of extracellular compounds (Andrade et al., 2014), the inhibition of OTA biosynthesis (Cebrián

et al., 2019) and the disruption of proteins involved in the cell wall integrity pathway (Delgado et al., 2019). This study suggested the competition by nutrients and space as the possible mode of action.

4. Conclusions

In conclusion, the combination of rosemary leaves and EO showed a synergistic effect against the OTA production by A*. westerdijkiae* and *D. hansenii* FHSCC 253H also reduced the presence of this mycotoxin. Therefore, these treatments can be proposed as BCA against *A. westerdijkiae* although its effect on the real product should be tested.

5. Bibliography

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V. DISCUSIÓN

V.1. Evaluación y selección de agentes de biocontrol de origen microbiano

V.1.1. *E. faecium*

Las bacterias lácticas (LAB) como *E. faecium*, *L. sakei*, *L. plantarum* y *P. pentosaceus* forman parte de la población habitual de los derivados cárnicos curado-madurados (Casquete et al., 2012; Fraqueza, 2015; Lorenzo et al., 2010). La actividad de las BAL frente a mohos toxigénicos ha sido ampliamente descrita (Bianchini, 2015; Dalié et al., 2010), aunque hay pocos estudios sobre su potencial antifúngico en matrices cárnicas (Luchese et al., 1992; Meftah et al., 2018). En este sentido, a pesar de que *E. faecium* ha sido frecuentemente aislado de embutidos curado-madurados (Barbosa et al., 2009; Lorenzo et al., 2007; Ruiz-Moyano et al., 2009), su interacción con mohos ocratoxigénicos no se había estudiado hasta ahora.

La cepa seleccionada de *E. faecium* CAMIALI SE920 presentó una buena adaptación a las condiciones habituales del procesado de los embutidos, siendo capaz de crecer a 15 °C, temperatura frecuente durante las fermentaciones lentas en el periodo de secado-maduración. Esto puede estar relacionado con su origen, dado que fue aislada de embutidos. Además, *E. faecium* CAMIALI SE920 disminuyó un 82,01 % la producción de OTA en *P. nordicum* tras 21 días de incubación en condiciones de maduración de los embutidos curado-madurados, aunque el crecimiento del moho no se redujo paralelamente (apartado IV.1. (Álvarez et al., 2019). Resultados similares se han descrito en diferentes mohos toxigénicos cuando interaccionan con BCAs como el romero y compuestos antifúngicos como el 2-feniletanol o el trans-cinamaldehído a concentraciones subinhibitorias (Álvarez et al., 2020; Chang et al., 2015; Schlösser & Prange, 2019), no siendo el grado de crecimiento un buen parámetro para predecir la producción de OTA (Fodil et al., 2018).

Sin embargo, la misma cepa de *E. faecium* no afectó a la producción de OTA de *P. verrucosum* por lo que su efecto antifúngico depende de la especie del moho diana como se ha descrito para otros BCAs como *D. hansenii* y *S. xylosus* frente a mohos toxigénicos (Andrade et al., 2014; Cebrián et al., 2020). Por lo tanto, *E. faecium* CAMIALI SE920 debería ser evaluado para su aplicación conjunta con otros BCAs para controlar el peligro de OTA en embutidos.

La reducción de OTA de *P. nordicum* por *E. faecium* CAMIALI SE920 parece no estar relacionada con la competición por nutrientes y espacio ya que *P. nordicum* presentó una clara dominancia nutricional frente a esta bacteria en las condiciones estudiadas (apartado IV.3, Álvarez et al., 2021a). En este sentido, el cultivo en aerobiosis y las temperaturas empleadas, entre 15 y 25 °C (apartado IV.1.) resultaron favorables para el crecimiento de *P. nordicum* y pueden limitar el desarrollo de *E. faecium*, que crece mejor en microaerofilia con una temperatura óptima

alrededor de 37 °C (Yang et al., 2018). El modo de acción de *E. faecium* puede estar relacionado con la producción de compuestos antifúngicos, bacteriocinas o la adsorción de micotoxinas. Entre los compuestos antifúngicos producidos por las BAL se han descrito ácidos orgánicos, peróxido de hidrógeno y proteínas como bacteriocinas y péptidos no ribosomales (Belkacem-Hanfi et al., 2014a; Chen et al., 2021). Otras cepas de *E. faecium* han mostrado capacidad de adsorber patulina en zumo de naranja sin afectar a la calidad del producto (Hatab et al., 2012). Otras BAL detoxifican toxinas de *Fusarium in vitro* y en granos de trigo (Franco et al., 2011; Juodeikiene et al., 2018). Por lo tanto, es necesario realizar nuevos estudios que determinen el modo de acción de la cepa de *E. faecium* utilizada.

Por otra parte, *E. faecium* CAMIALI SE920 no afectó al crecimiento de la población beneficiosa nautóctona de embutidos cárnicos curado-madurados (*P. nalgiovense* FHSCC Pj262, *P. chrysogenum* FHSCC Pg222, *D. hansenii* FHSCC 46P y *S. vitulinus* FHSCC MSA19) permitiendo su coexistencia (apartado IV.6) y contribución al desarrollo de las características sensoriales de estos productos. Además, en los salchichones, inoculados con *E. faecium* se observó una reducción significativa de los valores de pH y a_w, como consecuencia de su actividad fermentativa en este tipo de alimentos (Lücke, 2000). El descenso del pH provoca la desnaturalización de proteínas cárnicas, lo que conlleva una disminución de la capacidad de retención de agua y el descenso de la a_w (Hu et al., 2019). A pesar de los cambios en el pH y la a_w respecto a salchichones no inoculados, los resultados obtenidos se encuentran dentro de los rangos normales de estos parámetros físico-químicos para el salchichón, estando el pH entre 5 y 6 (Benito et al., 2007; Casquete et al., 2011), la a_w entre 0,80 y 0,87 (Martín-Sánchez et al., 2011; Mendonça et al., 2013) y la humedad entre el 20 y el 44 % (Cano-García et al., 2014; Casquete et al., 2011). Los parámetros de color y textura no presentaron diferencias respecto a los salchichones sin inocular estando los valores dentro de lo esperado para este tipo de producto. La dureza entre 74,4 y 269,2 N, la elasticidad entre 0,55 y 0,8, la cohesividad entre 0,39 y 0,8 y la masticabilidad entre 94, 8 y 152,4 N (Bruna et al., 2000; Casquete et al., 2011; Corral et al., 2017; Herrero et al., 2007; Rubio et al., 2007). Por consiguient, *E. faecium* CAMIALI SE920 no alteró de forma relevante estos parámetros durante la maduración de los embutidos. En cuanto a los compuestos volátiles, esta BAL fue responsable de la aparición de 1-propanol y 2,3, octanodiona, que se han asociado con el metabolismo de los enterococos en los embutidos (Latorre-Moratalla et al., 2011). *E. faecium* también aumentó la presencia de 3-octanona relacionada con el aroma a champiñón o a rancio que es generada en carne por BALs como *Carnobacterium maltaromaticum* (Pothakos et al., 2015). Sin embargo, estas modificaciones en el perfil de compuestos volátiles no afectaron al análisis sensorial al no detectarse diferencias

entre los salchichones inoculados y los no inoculados en la prueba de preferencia, en la escala de intensidad de olor, ni modificar la intención de compra de los panelistas.

Asimismo, en estudios realizados paralelamente a esta Tesis Doctoral se ha comprobado que la inoculación de este microorganismo a una concentración de 10^6 ufc/g en la masa de salchichón antes de embutir provoca una reducción significativa en la producción de OTA por *P. nordicum* durante la maduración (resultados no mostrados). Además, la cepa de *E. faecium* seleccionada es considerada segura debido a su bajo potencial aminogénico, poca resistencia a antibióticos y ausencia de genes de virulencia (Ruiz-Moyano et al., 2009) por lo que podría ser usada en la fabricación de embutidos.

V.1.2. *P. chrysogenum*

P. chrysogenum CECT 20922 ha probado en diferentes estudios su capacidad para limitar la producción de OTA en distintas cepas de *P. nordicum* en medio de cultivo a base de jamón y en jamón curado (Cebrián et al., 2019; Delgado et al., 2019; Rodríguez et al., 2015). Su efecto se ha atribuido a la producción de la proteína antifúngica PgAFP (Delgado et al., 2015) y al bloqueo en la expresión de genes de la ruta biosintética de la OTA (Peromingo, et al., 2018; Cebrián et al., 2019). Adicionalmente, teniendo en cuenta el NOI (apartado IV.3), *P. chrysogenum* parece limitar el crecimiento de *P. nordicum* al dominar nutricionalmente o coexistir en rangos de a_w y temperaturas habituales durante la elaboración de los embutidos. También se ha observado un antagonismo mutuo por contacto entre *P. chrysogenum* y *P. nordicum*, lo que indica que ambos mohos compiten por nutrientes y espacio (apartado IV.3). Estos resultados concuerdan con las modificaciones provocadas por esta cepa de *P. chrysogenum* en el proteoma de *P. nordicum* relacionadas con el metabolismo de carbohidratos y la represión del catabolismo de diferentes fuentes de carbono (Delgado et al., 2019).

P. chrysogenum CECT 20922 presentó una acción limitada sobre el crecimiento de la población autóctona, permitiendo su coexistencia (apartado IV.6.), por lo que no es esperable que se produzcan alteraciones importantes en la actividad de la población microbiana que participa en el desarrollo de las características sensoriales de los embutidos curado-madurados. De hecho, la inoculación de *P. chrysogenum* en la superficie de embutidos al inicio de su procesado no afectó negativamente a la calidad del mismo. Los parámetros-físico químicos de los embutidos inoculados se encontraron dentro de los rangos normales para este producto. Las reducciones significativas en la elasticidad y la cohesividad en el lote con *P. chrysogenum* podrían relacionarse con la intensa proteólisis inducida por la fuerte actividad enzimática de la población fúngica que coloniza superficialmente los embutidos (Benito et al., 2003; Bruna et al., 2000). A

pesar de que este moho provocó cambios ligeros en el perfil de compuestos volátiles respecto al de embutidos no inoculados, no se encontraron diferencias significativas ($p \leq 0.05$) en el la escala de preferencia, la escala de intensidad de olor o las intención de compra (apartado IV.6, Álvarez et al., 2020). De acuerdo con estos resultados y los obtenidos en ensayos previos que han demostrado su capacidad para reducir la producción de OTA en derivados cárnicos (Cebrián et al., 2019; Rodríguez et al., 2015), la cepa *P. chrysogenum* CECT 20922 puede ser considerada como un potencial cultivo protector para reducir la presencia de esta micotoxina en embutidos curado-madurados.

Sin embargo, la proteína antifúngica PgAFP, purificada a partir de cultivos de *P. chrysogenum* CECT 20922 redujo el crecimiento de mohos beneficiosos para la maduración de productos cárnicos, como *P. nalgiovense* y *P. chyrsogenum*. En el caso de *P. nalgiovense* las colonias se desarrollaron con una apariencia anómala, presentando al microscopio un micelio quebradizo sin la estructura característica de las hifas (apartado IV.6.). Este resultado es consecuente con el efecto descrito de PgAFP en mohos sensibles, en los que se observa una disrupción de las proteínas implicadas en la CWI y la consecuente reducción y alteración del micelio aéreo (a Cruz Cabral et al., 2018; Delgado et al., 2016). Debido a esta posible interferencia con la población fúngica beneficiosa, no puede recomendarse la utilización de PgAFP como BCA en la superficie de los embutidos curado-madurados porque podría impedir el crecimiento de algunos mohos importantes para el desarrollo de las características sensoriales de estos alimentos.

V.1.3. *D. hansenii*

Diferentes cepas de *D. hansenii* han mostrado su capacidad para reducir la producción de OTA por parte de *P. nordicum* y *A. westerdijkiae* en distintos sustratos (Andrade et al., 2014; Cebrián et al., 2019; Delgado et al., 2019; Gil-Serna et al., 2011; Iacumin et al., 2020a).

Como se describe en el apartado IV.3, *D. hansenii* FHSCC 253H provoca una disminución del crecimiento de *P. nordicum* en medios de cultivo elaborados con sustratos cárnicos en condiciones de temperatura y a_w similares a las alcanzadas durante el procesado de embutidos curado-madurados. Aunque se considera que la competición por nutrientes y espacio es el modo de acción más común de las levaduras (Asensio et al., 2014; Spadaro & Droby, 2016), no puede afirmarse que sea el principal factor al que se debe la capacidad inhibidora de la cepa de *D. hansenii* utilizada, dado que *P. nordicum* mostró una dominancia nutricional en la mayoría de las condiciones testadas (apartado IV. 3). Este hecho puede explicar por qué la eficacia de esta levadura es mayor cuando se inocula en el interior de los salchichones (apartado IV.7.) en

comparación a cuando se hacía en la superficie por un mejor acceso a los nutrientes que *P. nordicum*.

Para tratar de elucidar el efecto de la levadura en el metabolismo de *P. nordicum* se llevó a cabo un estudio de proteómica comparativa, en el cual se observó que *D. hansenii* provocó en *P. nordicum* una reducción de la abundancia de proteínas relacionadas directamente con la biosíntesis de OTA (apartado IV.7). Entre ellas, la corismato sintasa interviene en la síntesis de corismato (Woodin & Nishioka, 1973), un precursor de la fenilalanina, indispensable para formar la estructura molecular de la OTA (Figura 5). Las proteínas piruvato deshidrogenasa E1 subunidad Alpha y la *probable acetate kinase* están relacionadas con la formación del Acetil CoA, necesario para la síntesis de ochratoxina β o del ergosterol, un componente esencial de las membranas fúngicas (Chiu et al., 2019; Dupont et al., 2012; Mille-Lindblom et al., 2004), que, a su vez, ha sido correlacionado con la producción de OTA en *P. verrucosum* y *A. ochraceus* (Hua et al., 2014; Saxena et al., 2001). Además, disminuyó la abundancia de numerosas proteínas pertenecientes al dominio PKS ER, relacionado con la síntesis de micotoxinas como la OTA en *Penicillium thymicola* y *A. niger* o las fumonisinas de *Fusarium* spp. (Brown et al., 2012; Ferracin et al., 2012; Huffman et al., 2010; Nguyen et al., 2016). La disminución en la abundancia de proteínas de este dominio en comparación con las encontradas en el lote control es consecuente con el potencial papel que desempeñaría este dominio en la producción de OTA en *P. nordicum*. Estos resultados son compatibles con la alteración de la ruta biosintética de OTA provocada por *D. hansenii* mediante la disminución en la expresión de los genes *pks* y *p450* en *A. westerdijkiae* y del gen *otapks* en *P. nordicum* (Cebrián et al., 2019; Gil-Serna et al., 2011).

También se redujo la abundancia en presencia de *D. hansenii* de algunas proteínas relacionadas con la ruta CWI (apartado IV.7.). Estas proteínas son esenciales para el desarrollo normal de las hifas a través de la unión de la actina y de la organización de la pared celular. Por otro lado, las interferencias en esta ruta se han asociado a la disminución de metabolitos secundarios como las micotoxinas (Valiante, 2017). Estos cambios en la ruta CWI han sido detectados previamente en medios de cultivo a base de jamón y salchichón empleando la misma cepa (Álvarez et al., 2021b; Delgado et al., 2019) Aunque algunas de las proteínas no coinciden con las encontradas en estos estudios, la tendencia de disminuir el metabolismo de la ruta CWI se mantiene durante la elaboración industrial de los embutidos curado-madurados.

La proteómica comparativa también reveló el aumento de la actividad ribosomal de *P. nordicum* en presencia de *D. hansenii*, detectándose proteínas relacionadas con la estructura del ribosoma y, aumentando proteínas transportadoras o proteínas que intervienen en el plegamiento

proteico. Aunque la causa de la activación de la actividad ribosomal no ha sido establecida, se ha sugerido que la biogénesis de los ribosomas se intensifica como mecanismo contra antifúngicos como la rapamicina o el ácido sórbico (Lai et al., 2016; Víglaš & Olejníková, 2020). Esta respuesta también se ha observado en *A. flavus* y *P. polonicum* tratados con la proteína antifúngica PgAFP independientemente del grado de sensibilidad a esta proteína (Delgado et al., 1015b; 2016), por lo que podría tratarse de una reacción inespecífica frente a la acción antifúngica de *D. hansenii*.

Por otra parte, la inoculación de *D. hansenii* en los embutidos permitió el crecimiento de la población autóctona (apartados IV.6 y IV.7) y no alteró de forma relevante los parámetros físico-químicos del producto final (apartado IV.6.). Concretamente *D. hansenii* causó un ligero incremento de pH respecto al lote control que podría ser consecuencia de la actividad proteolítica de la levadura o de su consumo de ácido láctico (Mendonça et al., 2013; Patrignani et al., 2007), y redujo la a_w durante la fermentación, debido probablemente también a su actividad proteolítica (Patrignani et al., 2007). En cuanto a los volátiles, algunos de los compuestos de cadena ramificada aumentados en presencia de *D. hansenii* como el 3-metil-1-butanal, 2-metil-1-propanol, 3-metil-1-butanol o el ácido 2-metil-1-propanoico, derivan del catabolismo de los aminoácidos y se asocian al aroma típico de los productos cárnicos curado-madurados (Ruiz et al., 1999). En concreto, el 3-metil-1-butanal y el ácido 3-metilbutanoico se relacionan con la actividad de las levaduras en sustratos cárnicos (Andrade et al., 2010; Corral et al., 2015), lo que explica los altos niveles encontrados en el *lote con D. hansenii*. A pesar de las diferencias en el perfil de volátiles como consecuencia de la actividad de esta levadura, no se encontraron diferencias significativas ($p \leq 0.05$) en el análisis sensorial, y no se produjeron cambios en la intención de compra de los panelistas (apartado IV.6).

Por lo tanto, teniendo en cuenta la actividad antifúngica de *D. hansenii* FHSCC 253H y su capacidad de implantarse y de coexistir con otros microorganismos beneficiosos sin alterar las características sensoriales de los embutidos curado-madurados, esta cepa podría ser considerada para su utilización como BCA para el control del peligro de OTA durante el procesado de este alimento.

V.2. Evaluación y selección de agentes de biocontrol de origen vegetal

V.2.1. Orégano

La incorporación del orégano provocó un descenso en la velocidad de crecimiento de *P. nordicum* CBS 323.92 tras 14 días de incubación, y una reducción de la concentración de OTA en el micelio del moho y en la tripa sobre un sustrato cárnico tras 14 días (apartado IV.2; Álvarez et al., 2020a).

Cuando se evaluó el modo de acción del orégano sobre *P.* nordicum, se observó que la pared celular es una de las dianas del efecto antifúngico de esta planta, al detectarse una disminución en la concentración de ergosterol (apartado IV.4) y un aumento de la expresión del gen *Rho1* (apartado IV.2). La activación de la proteína Rho1 inicia la señal que estimula a la proteína quinasa C, desencadenando la cascada de las proteínas quinasas asociadas a mitógenos (MAPK) responsables de mantener la CWI (Levin, 2011; Víglaš & Olejníková, 2020). La inhibición de la síntesis de ergosterol puede provocar un aumento en la expresión de *Rho1* en situaciones de estrés debido a compuestos que afectan a la integridad de la pared celular (Hayes et al., 2014). Compuestos como el carvacrol o el timol que se encuentran en el AE de orégano han mostrado una disminución del ergosterol en mohos toxigénicos como *A. flavus* y *Fusarium graminearum* (Gao et al., 2016; Tian et al., 2018). Este estrés puede ser la causa del aumento de OTA tras una semana de incubación ya que las condiciones estresantes pueden inducir la producción de micotoxinas (Schmidt-Heydt et al., 2013; Yun et al., 2014) y *Rho1* presentó una fuerte correlación con la acumulación de OTA. Este gen se ha relacionado con la producción de OTA en *A. carbonarius* en presencia de la proteína antifúngica PgAFP y la producción de toxinas de *Alternaria tenuissima* (a Cruz Cabral et al., 2019; Fodil et al., 2018). Al final del experimento se encontró una mayor correlación entre la producción de OTA y la expresión del gen *otapks*, lo que indicaría que este gen es el principal responsable de su producción tras la adaptación del moho al estrés. Esta relación también ha sido descrita en *P. nordicum* (Bernáldez et al., 2018; Geisen et al., 2006) y la inactivación de este gen suprime la capacidad de producir OTA en *P. nordicum* (Karolewiez & Geisen, 2005).

Por lo tanto, el orégano podría emplearse como BCA en combinación con otras medidas para eliminar totalmente la presencia de OTA y tendría dos modos de acción claramente diferenciados afectando en primer lugar a la síntesis de ergosterol y posteriormente inhibiendo la síntesis de OTA a nivel transcriptómico. Además, otros mecanismos podrían estar implicados en la reducción de OTA aplicando orégano debido a la menor acumulación de esta micotoxina en la etapa final del experimento respecto a la inicial. Esto concordaría con estudios que

muestran la capacidad de los AE y otros extractos vegetales de detoxificar micotoxinas *in vitro* (Perczak et al., 2016; Sun et al., 2015; Xing et al., 2014).

V.2.2. Tomillo

Al contrario que el orégano, el tomillo no disminuyó la producción de OTA respecto al control en ninguna de las condiciones evaluadas (apartado IV.2), no observándose inhibición a nivel transcriptómico de los genes *otapks* y *otanrps*. A pesar de que el tomillo provocó una disminución de la síntesis de ergosterol (apartado IV.4), ésta no se tradujo en ningún efecto significativo en *P. nordicum*. Por lo tanto, existe una variabilidad en la respuesta de *P. nordicum* a la inhibición de la síntesis de ergosterol que puede ser atribuida a los diferentes efectos de los distintos compuestos presentes en las plantas. Por consiguiente, el tomillo no puede ser propuesto como BCA frente a mohos productores de OTA en embutidos curado-madurados.

V.2.3. Romero

V.2.3.1. Hojas

Las hojas de romero presentaron las mayores reducciones de OTA en *P. nordicum* CBS 323.92 que el orégano y el tomillo (apartado V.2) debido, posiblemente, a la inhibición de los genes pertenecientes al clúster de la síntesis de OTA (*otapks* y *otanrps*). La variabilidad en los resultados de expresión génica obtenidos en *P. nordicum* al aplicar distintos tratamientos con derivados de plantas concuerda con otros estudios donde se muestra la compleja regulación de la expresión génica relacionada con la OTA en distintas especies productoras (Gil-Serna et al., 2020b; Lappa et al., 2017; Schmidt-Heydt et al., 2008). Aunque no hay estudios previos de la influencia de las especias en la respuesta transcripcional de *P. nordicum*, algunos aceites esenciales han mostrado la capacidad de regular la producción de OTA en *A. carbonarius*. El de romero o hinojo actúan inhibiendo genes reguladores (*laeA* y *veA*) y reduciendo consecuentemente la expresión de los genes *otapks* y *otanrps* , mientras que el de anís disminuye directamente su expresión (El Khoury et al., 2016). En consecuencia, la producción de metabolitos secundarios puede estar influida por numerosas señales que provocan efectos inhibitorios o estimulantes dentro de los sistemas de regulación génica.

Debido a la disminución de OTA observada en los estudios *in vitro* (apartado IV.2), se seleccionó el romero como BCA para los siguientes ensayos (Figura 6). Sin embargo, las hojas de romero no tuvieron el mismo efecto *in vitro* sobre la producción de esta micotoxina en *P. nordicum* FHSCC Pn15 y BFE 856 y *A. westerdijkiae* MUM 16.142 al encontrarse diferencias intra-especie e inter-especie (apartados IV.5 y IV.8). Teniendo en cuenta estos resultados dependientes de la cepa,

se estudió su actividad frente a *P. nordicum* durante la elaboración de los embutidos curado-madurados (apartado IV.7.). En los embutidos se encuentra otra microbiota y unas condiciones ambientales definidas que pueden influir en dicho efecto, y los mecanismos de acción implicados en la represión de OTA pueden variar bajo estas circunstancias. La incorporación de las hojas de romero en la masa provocaron un estímulo en el crecimiento de *P. nordicum* FHSCC Pn15, pero disminuyeron la biosíntesis de OTA respecto al lote control sin BCAs. Estos resultados indican, de nuevo, que el crecimiento es un parámetro que puede no estar directamente relacionado con la producción de micotoxinas y apoyan la necesidad de evaluar los BCAs durante la elaboración del producto real.

Por otra parte, el efecto antiocratoxigénico del romero parece depender del modo de aplicación, encontrándose diferencias en el resultado de los lotes en los que se adicionaron las hojas en la masa y en el que se maceraron las tripas antes del embutido. Dado que el proceso de maceración conlleva una mejor disolución de los compuestos fenólicos en agua (Psarrou et al., 2020), podría ser el responsable del mayor efecto antiocratoxigénico del romero. Los compuestos fenólicos de otras especias como el fenugreco han mostrado actividad antiocratoxigénica frente a *A. carbonarius* (El Khoury et al., 2017). El extracto de anís, principalmente compuesto por ácido rosmarínico, presente mayoritariamente en el romero (Gonçalves et al., 2019), reduce la producción de aflatoxinas en *A. flavus* y zearalenona en *Fusarium culmorum* (Sabry et al., 2021). Cuando se evaluó el efecto del romero sobre el proteoma de *P. nordicum* en los lotes con el macerado en romero disminuyeron proteínas como la prepenato deshidratasa y la corismato sintasa (apartado IV.7.), relacionadas con la ruta biosintética de la OTA desde el ácido shikímico hasta la formación de fenilalanina (Figura 5). Esto indicaría que la ruta de biosíntesis de la micotoxina se ve afectada en diversos puntos por la acción del romero.

Además de la influencia a nivel transcriptómico, las hojas de romero redujeron *in vitro* la concentración de ergosterol en *P. nordicum CBS 323.92* siendo uno de los modos de acción de esta especia (apartado IV.2). Esto fue corroborado con el estudio proteómico de *P. nordicum* FHSCC Pn15 en el lote de tripas maceradas con romero (apartado IV.7) en el cual se encontraron disminuidas en abundancia proteínas de la CWI como la *actin-capping subunit beta,* relacionada con la organización de la actina en el citoesqueleto y la NADPH-citocromo P450 reductasa que interviene directamente en la biosíntesis de ergosterol. La disrupción del gen que codifica las proteínas *capping* en *Saccharomyces cerevisae* o *F. graminearum* produce células viables pero con un crecimiento lento y heterogéneo y una menor producción de DON el caso del moho (Amatruda et al., 1990; Tang et al., 2020). Teniendo en cuenta los efectos sobre la producción de OTA de *P. nordicum* las hojas de romero pueden ser empleadas como BCA.

V.2.3.2. Aceite esencial

El AE de romero provocó en agar salchichón la disminución significativa de la concentración de OTA en las dos cepas de *P. nordicum* evaluadas. Su capacidad antiocratoxigénica ha sido ampliamente descrita frente a *Aspergillus* spp. (Císarová et al., 2020; El Khoury et al., 2016, 2017) y ha mostrado ser un buen inhibidor de la síntesis de ácido ciclopiazónico o de aflatoxinas en otras especies (Císarová et al., 2020; Rasooli et al., 2008). Sin embargo, para inhibir la producción de OTA en *A. westerdijkiae* (apartado IV.8) fue necesario el efecto sinérgico de la combinación del AE de romero con las hojas de la misma planta sugiriendo que puede ser necesario aumentar los compuestos activos y que *P. nordicum* es más sensible que *A. westerdijkiae* a la acción del romero.

El empleo del AE de romero tuvo mayor impacto en el proteoma de *P. nordicum* FHSCC Pn15 que en el de BFE 856, pero dado que la reducción de OTA fue similar, se puede deducir que ambas actúan en alguna ruta común relacionada con la síntesis de esta micotoxina. Dentro de las rutas que comparten, se encontró una menor abundancia de las proteínas que pertenecían al dominio PKS ER, asociado previamente con la biosíntesis de OTA en *A. ochraceus* (Wang et al., 2015) y relacionado con los clústeres de otras toxinas fúngicas como la betaenona de *Phoma betae* o las toxinas *host-selective de Alternaria alternata* (Ajiro et al., 2009; Ugai et al., 2015). Además, las proteínas del dominio PKS ER se encontraron disminuidas en *Trichoderma aggresivum* en presencia de un extracto antifúngico de *Bacillus velezensis* (Kosanovic et al., 2021). Por lo tanto, la disminución de las proteínas del dominio PKS ER explicaría la disminución de la biosíntesis de OTA en ambos mohos.

En cambio, la aplicación del AE en la superficie de los embutidos durante el procesado tuvo el efecto contrario, observándose un aumento en la producción de OTA. Estas diferencias pueden ser debidas a las interacciones con la población nativa, las condiciones ambientales o a la evaporación de compuestos volátiles con actividad antifúngica que podrían haber permanecido dentro de las placas de Petri en el estudio *in vitro* y no en la superficie del embutido. Esto se relaciona con la capacidad de los compuestos volátiles de unirse a los componentes del alimento siendo necesario aplicar concentraciones más altas para alcanzar el mismo efecto (Cruz Cabral et al., 2013). La capacidad antifúngica del AE de romero ha sido atribuida a compuestos volátiles como el cineol, el alcanfor o el α-pineno (Císarová et al., 2015; Da Silva Bomfim et al., 2015). La microencapsulación del AE de romero y su adición a la masa del embutido podría mantener su efecto antiocratoxigénico (Prakash et al., 2015b), pero sería imprescindible realizar estudios de aceptabilidad por parte del consumidor antes de proponer su aplicación como BCA.

V.2.4. Extracto de cáscara de bellota

Este estudio fue el primero en abordar la capacidad de reducir la OTA de un extracto de cáscara de bellota, un subproducto de la industria del cerdo ibérico. Este extracto consiguió disminuir la producción de OTA en *P. nordicum* FHSCC Pn15 pero no en *P. nordicum* BFE 856 (apartado IV.5.) mostrando diferencias dentro de la misma especie. Los extractos de cáscaras de bellotas han presentado actividad antimicrobiana y extractos de *Quercus infectoria* y de *Quercus ilex* inhiben el crecimiento de distintas especies de levaduras (Boy et al., 2021; Burlacu et al., 2020). El efecto antifúngico de extractos de *Quercus* spp. frente a *A. flavus*, *Penicillium funiculosum*, y *Penicillium ochrochloron* se atribuye a polifenoles como el ácido elágico o el ácido cafeico (Elansary et al., 2019). Un extracto de *Quercus infectoria* redujo la concentración de mohos, levaduras y bacterias en la cáscara del huevo asociándose a la alta concentración de compuestos fenólicos y la presencia de taninos (Tayel et al., 2018). Esta Tesis Doctoral muestra el posible aprovechamiento de un subproducto como BCA aunque es necesaria la acción conjunta con otras estrategias antifúngicas para reducir completamente la producción de OTA en *P. nordicum* debido a las diferencias intra-especie (apartado IV.5.).

V.3. Evaluación y selección de combinaciones de agentes de biocontrol

Debido a que aplicados individualmente ninguno de los BCAs estudiados pudo garantizar la inhibición total de la producción de OTA, se estudió la combinación de los que presentaron una mayor actividad antifúngica, es decir, *D. hansenii* y romero. La adición conjunta de las hojas de romero con *D. hansenii in vitro* logró disminuir la OTA en *P. nordicum* FHSCC Pn15, BFE 856 y *A. westerdijkiae* MUM. 16.142.En *P. nordicum BFE 856*, esta combinación parece tener un efecto acumulativo.

El efecto de la combinación del romero con *D. hansenii* parece ser complejo y depende de la cepa evaluada. Así, los resultados del análisis proteómico de *P. nordicum* FHSCC Pn15 en medio de cultivo mostraron una disminución de la proteína 3-metil-2-oxobutanoato hidroximetil-transferasa, relacionada con la síntesis de acetil-CoA a través de la ruta del pantotenato (Calder et al., 1999; Chiu et al., 2019). El acetil-CoA ha sido descrito como un precursor de la OTA al combinarse con malonil-CoA a través de una PKS para formar 7-metilmelleína la cual se oxida a ocratoxina β (Gallo et al., 2017; Wang et al., 2018). Así mismo, el acetil-CoA interviene en múltiples procesos metabólicos como la síntesis de ergosterol. Se ha atribuido el efecto de diferentes AE sobre la producción de OTA a la reducción de ergosterol, que podría ser afectado por la presencia de romero en las muestras (Hua et al., 2014). Además, en esta Tesis Doctoral se

ha observado una reducción de la metalocarbopeptidasa ECM14, relacionada con la biogénesis de la pared celular, la invasión fúngica o la utilización de nutrientes (McDonald et al., 2020).

Paralelamente, el efecto de la levadura parece potenciado por la presencia de romero que provocó cambios en el gen *Rho1* relacionado con la CWI (apartado IV.2.). Por el contrario, se ha observado un aumento en las proteínas relacionadas con la síntesis de acetil-CoA a partir de piruvato y con la CWI en *P. nordicum* BFE Pn856 con esta combinación de BCAs. Sin embargo, en este moho se produce una disminución de numerosas proteínas de la ruta de la unión de la purina. Las rutas conectadas con la purina han sido consideradas como dianas antimicrobianas debido a su papel en la síntesis de ADN y ARN (Cepas et al., 2020; Leonard et al., 2020). Además, la inhibición de genes relacionados con el metabolismo de la purina se ha vinculado con la disminución de la síntesis de aflatoxinas en *A. flavus* (Yang et al., 2019). Por lo tanto, el efecto combinado del romero y la levadura sobre Pn856 parece estar asociado a la supresión de la unión de la purina que puede provocar daño en el ADN y consecuentemente menor actividad translacional (Cepas et al., 2020).

Por otra parte, cuando *D. hansenii* se combinó con las hojas de romero, especialmente en los lotes con la tripa macerada, pudo constatarse que en *P. nordicum* la ruta CWI estaba afectada (apartado IV.7.), con un resultado similar al descrito en los estudios previos en medios de cultivo (apartado IV.5). La adición de la levadura junto a las hojas de romero provocó la disminución de la proteína fosfomevalonato quinasa, relacionada con la síntesis de ergosterol en los hongos, compuesto cuya concentración se redujo en el estudio *in vitro* con hojas de romero (apartado IV.4; Álvarez et al., 2021c). La disminución de la proteína ramnogalacturonato liasa, relacionada con la organización de la pared celular, fue más pronunciada en el tratamiento combinado con *D. hansenii* y las tripas maceradas que en el que incluyó la levadura individualmente (apartado IV.7), por lo que esta combinación potenció su reducción. Además, la combinación de *D. hansenii* con las tripas maceradas en romero redujo la abundancia de proteínas relacionadas con la síntesis de fenilalanina (apartado IV.7.), precursora de la OTA (Figura 5). La proteína prepenato dehidratasa, afectada por esta combinación, también se encontró reducida en el lote que solo incluyó las tripas maceradas en romero, por lo que puede deducirse que esta alteración se debe a la acción del macerado. En cuanto a la proteína corismato sintasa, se vio afectada en los tratamientos con la levadura o el macerado, siendo mayor su descenso al combinar ambos BCAs por lo que se observa un efecto acumulativo sobre la inhibición de esta proteína.

La combinación de *D. hansenii* con las hojas de romero durante la fabricación de salchichón en el interior y tras macerar las tripas, presentó un efecto similar al estudio *in vitro* sobre la

producción de OTA en *P. nordicum* FHSCC Pn15. Por lo tanto, el efecto antiocratoxigénico de estos BCAs continúa durante el procesado de los embutidos, a pesar de las interacciones con la población microbiana y los cambios físico-químicos ocurridos durante la maduración. Por ello, ambos BCAs pueden recomendarse para el control de *P. nordicum* durante la elaboración de los embutidos curado-madurados con el objetivo de reducir la contaminación con OTA y disminuir el uso de antifúngicos sintéticos.

VI. CONCLUSIONES/CONCLUSIONS

Conclusiones

1. *E. faecium* CAMIALI SE920 es un buen candidato combinado con otros cultivos protectores para reducir la producción de OTA en embutidos curado-madurados.

2. Las hojas de romero son efectivas *in vitro* frente a *P. nordicum* al disminuir el contenido de ergosterol y bloquear a nivel transcripcional la biosíntesis de OTA.

3. La adición *in vitro* de hojas de romero redujo el contenido en ergosterol, mientras que las tripas maceradas con romero causaron la disrupción de la ruta CWI, la biosíntesis de ergosterol y la de OTA en *P. nordicum*.

4. *D. hansenii*, el BCA más efectivo frente a mohos productores de OTA, disminuye la abundancia de proteínas relacionadas con la biosíntesis de OTA y la ruta CWI.

5. *P. chrysogenum* CECT 20922, *D. hansenii* FHSCC 253H y *E. faecium* CAMIALI SE920 pueden ser propuestos como BCAs para controlar la OTA al no producir cambios relevantes en el crecimiento de la población autóctona, los parámetros físico-químicos y la calidad sensorial típica de los embutidos curado-madurados.

6. La adición de hojas de romero como ingrediente o en el macerado de las tripas, junto a la inoculación de *D. hansenii* en la masa cárnica es una estrategia prometedora para controlar la OTA en los embutidos curado-madurados debido a que su combinación potencia la reducción en la abundancia de proteínas relacionadas con la CWI y la biosíntesis de OTA.

Conclusions

1. *E. faecium* CAMIALI SE920 is a good candidate to be combined with other protective cultures for reducing OTA production in dry-fermented sausages.

2. The oregano leaves are *in vitro* effective *in vitro* against *P. nordicum* by decreasing the ergosterol content and blocking the OTA biosynthesis at transcriptional level.

3. The *in vitro* addition of rosemary leaves reduced ergosterol content, while the casings macerated with them caused the disruption of the CWI pathway, ergosterol biosynthesis and OTA biosynthesis in *P. nordicum*.

4. *D. hansenii*, the most effective BCA against ochratoxigenic moulds, decreases the abundance of proteins involved in OTA biosynthesis and in the CWI pathway.

5. *P. chrysogenum* CECT 20922, *D. hansenii* FHSCC 253H and *E. faecium* CAMIALI SE920 can be proposed as BCAs to control the OTA hazard since they did not produce relevant changes in either the growth of the native population, the physico-chemical parameters and the typical sensorial quality of dry-cured fermented sausages.

6. The addition of rosemary leaves as ingredient or by macerating the casings, together with the inoculation of *D. hansenii* in the sausage mix consists of a promising strategy to control OTA in dry-cured fermented sausages since their combination enhanced the reduction in abundance of proteins related to CWI pathway and OTA biosynthesis.

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