VNIVERSITAT DE VALÈNCIA

Facultat de Farmàcia

Departament de Medicina Preventiva i Salut Pública, Ciències de l'Alimentació, Toxicologia i Medicina Legal

Programa de Doctorat amb Menció cap a l'Excel·lència en Ciències de l'Alimentació

ESTRATEGIAS DE REDUCCIÓN DEL DESARROLLO DE HONGOS TOXIGÉNICOS EN ALIMENTOS MEDIANTE COMPUESTOS NATURALES

STRATEGIES TO REDUCE TOXIGENIC FUNGAL GROWTH IN FOODS USING NATURAL COMPOUNDS

Tesi Doctoral Internacional

València, 2022

Presentada per:
Raquel Torrijos Caparrós

Dirigida per:
Dr. Jordi Mañes Vinuesa
Dr. Giuseppe Meca

VNIVERSITAT DE VALÈNCIA Facultat de Farmàcia

Departament de Medicina Preventiva i Salut Pública, Ciències de l'Alimentació, Toxicologia i Medicina Legal

El **Dr. Jordi Mañes Vinuesa**, Catedràtic d'Universitat de l'àrea de Nutrició i Bromatologia i el **Dr. Giuseppe Meca**, Catedràtic d'Universitat de l'àrea de Nutrició i Bromatologia,

CERTIFIQUEN QUE:

La graduada en Ciència i Tecnologia dels Aliments **Raquel Torrijos Caparrós** ha realitzat baix la seua direcció el treball **"Estratègies de reducció del desenvolupament de fongs toxigènics en aliments mitjançant compostos naturals"**, i autoritzen la seua presentació per a optar al títol de Doctor per l'Universitat de València.

I, perquè així conste, expedeixen i signen el present certificat.

Burjassot (València), 9 de juny de 2022.

Dr. Jordi Mañes i Vinuesa

Dr. Giuseppe Meca

Aquesta tesi doctoral ha donat lloc a 5 articles, publicats o que es publicaran en les següents revistes:

1. Development of a bioactive sauce based on oriental mustard flour with antifungal properties for pita bread shelf life improvement. *Molecules, 24:6 (2019), 1019. Impact factor: 3.267*

2. Application of white mustard bran and flour on bread as natural preservative agents. *Foods, 10 (2021), 431. Impact factor: 4.350*

3. Use of mustard extracts fermented by lactic acid bacteria to mitigate the production of fumonisin B_1 and B_2 by *Fusarium verticillioides* in corn ears. *Toxins, 14 (2022), 80. Impact factor: 4.546*

4. Phytochemical profiling of volatile and bioactive compounds in yellow mustard (*Sinapis alba*) and oriental mustard (*Brassica juncea*) seed flour and bran. *LWT (Under review). Impact factor: 4.952*

5. Antifungal activity of natamycin and development of an edible film based on hydroxyethylcellulose to avoid *Penicillium* spp. growth on low-moisture mozzarella cheese. *LWT, 154 (2022), 112795. Impact factor: 4.952*

La doctoranda ha gaudit de la beca predoctoral "Formació de Profesorat Universitari" (FPU 17/06104) del Ministeri d'Universitats. A més, ha gaudit d'una ajuda de mobilitat Erasmus+ per a la menció internacional del títol de Doctor, la qual es va realitzar a Parma (Itàlia) a la Università degli Studi di Parma (UNIPR), baix la direcció de la Dra. Chiara Dall'Asta.

Aixi mateix, aquesta Tesi Doctoral Internacional s'engloba dins dels següents projectes:

- Mitigación, biomarcadores y toxicidad de micotoxinas legisladas y emergentes (AGL2016-77610-R).
- Biopreservación de pan de molde con suero de leche fermentado frente a micotoxinas y hongos toxigénicos. Seguridad de uso en presencia de carotenoides (SAFEBIOBREAD) (PID2019-108070RB-100)
- The European Project (H2020-Research and Innovation Action) MycoKey "Integrated and innovative key actions for mycotoxin management in the food and feed chain" (GA 678781).
- Programa Prometeo para grupos de investigación de excelencia (Prometeo 2018/126).
- Enhancing Research and Innovation Capacity of Tubitak MAM Food Institute on Managment of Mycotoxigenic Fungi and Mycotoxins (MycoTWIN) (GA 952337).

"El mejor científico está abierto a experimentar y comienza con un romance, la idea de que todo es posible"

Ray Bradbury (1920-2012)

Agradecimientos

Llegado a este punto, quiero acordarme de todas las personas que de un modo u otro han contribuido para que este día llegase. Primero de todo, querría dar las gracias a mis directores de tesis por todo el apoyo que me han ofrecido durante estos años. Al Dr. Giuseppe Meca, por confiar en mí cuando era una estudiante de grado y por trasmitirme la ilusión por la investigación. Al Dr. Jordi Mañes, por permitirme formar parte del grupo de investigación y aconsejarme en el camino.

También quiero dar las gracias a la Dra. Guillermina Font, por sus consejos y por el tiempo dedicado para que pudiera cumplir este objetivo.

Al personal del departamento de Medicina Preventiva. Especialmente, gracias a Carmen y Amparo, por ayudarme siempre con una sonrisa con las innumerables dudas con las que os he asaltado estos años. También, a todos los profesores del Departamento que, de un modo u otro, me han ayudado a formarme y llegar a este momento.

Grazie innanziutto a la Prof.ssa Chiara Dall'Asta, per avermi gentilmente accolto nel suo laboratorio di ricerca all'Università degli Studi di Parma. Grazie anche a Laura, Martina e Luca, per avermi insegnato tantissimo e a tutte le ragazze che hanno fatto della mia permanenza un ricordo bellissimo: Sara, Francesca, Martina, Clara, Luisa e Ceci.

Gracias a todos los estudiantes de grado, máster, doctorado o erasmus con los que he podido compartir mis días en el laboratorio. De todos vosotros he podido aprender y sin vuestra ayuda no habría podido llegar hasta aquí. También agradecer a todos los compañeros de laboratorio que han convertido la rutina en algo maravilloso: Dio, Noelia, Carlos, Juanma, Alessandra, Veronica, Mercedes, Fran,

Fojan, Alfonso, Massimo, Víctor, Manu... Gracias por todos los momentos que hemos vividos juntos.

Gracias a todos los amigos que durante estos años me han animado. Especialmente a Júlia, ya que los primeros años han sido increíbles gracias a ti, y tus consejos y ánimos siempre me han ayudado durante la travesía.

A Tiago, porque sin tu apoyo incondicional desde el inicio, jamás me habría aventurado a recorrer este camino. Gracias por acompañarme en este proceso día tras días, por tu confianza, por tu amor y por tu comprensión.

Por último, pero no menos importante, quiero agradecer a mis padres y a mi hermana Ana el apoyo que me han brindado estos años. Por alimentar siempre mi curiosidad desde que era niña y por enseñarme a no rendirme nunca. Por cuidarme y por estar siempre cuando más os he necesitado. Sin vosotros, este trabajo jamás se habría hecho realidad.

Muchas gracias por todo.

Raquel

ÍNDICE

LISTA DE ABREVIATURAS

ACN	Acetonitrile
AFs	Aflatoxins (Aflatoxinas)
AITC	Allyl isothiocyanate
APPCC	Análisis de Peligros y Puntos de Control Crítico (Hazard Analysis and Critical Control Points)
a_w	Water activity
BITC	Benzyl isothiocyanate
BPA	Buenas practicas agrícolas (Good farming practices)
BPF	Buenas practices de fabricación (Good manufacturing practices)
BPW	Buffered Peptone Water
CE	Comisión Europea (European Comission)
CECT	Colección Española de Cultivos Tipo
CFS	Sobrenadante libre de células (Cell Free Supernatant)
CFU	Unidades Formadoras de Colonias (Colony Forming Units)
CID	Collision-induced dissociation
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DON	Deoxynivalenol
EA	Ethyl acetate
EFSA	European Food Safety Authority
EO	Essential oil
ESI	Electrospray ionization
FA	Formic acid
FAO	Food and Agriculture Organization of the United Nations
FB_1	Fumonisin B_1
FB_2	Fumonisin B_2
FDA	Food & Drug Administration
FID	Flame Ionization Detector
FP	Fungal Population
GAE	Gallic acid equivalents
GC	Gas Chromatography
GRAS	Generally Recognized as Safe
GS-GTS	Glucosinolates
HEC	Hydroxyethylcellulose
HS	Head space
IARC	International Agency for Research on Cancer
ISPA	Istituto di Scienze delle Produzioni Alimentari
ITCs	Isothiocyanates

LAB	Bacterias ácido lácticas (Lactic acid bacteria)
LC	Liquid-chromatography
LRI	Linear Retention Indices
MB	Mustard Bran
MCS	Mozzarella cheese slice
MetOH	Methanol
MF	Mustard Seed Flour
MFC	Minimum Fungicidal Concentration
$MgSO_4$	Magnesium sulphate
MIC	Minimum Inhibitory Concentration
MRS	Man-Rogosa-Sharpe
MS	Mass Spectrometry
MS/MS	Tandem mass spectrometry
NaCl	Sodium Chloride
NTC	Natamycin
OM-OMF	Oriental Mustard Flour
OMB	Oriental Mustard Bran
OTA	Ochratoxin A
PCA	Principal Component Analysis
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
p-HBITC	*p*-hydroxybenzyl isothiocyanate
PITC	Phenyl isothiocyanate
PLA	Ácido feniláctico (Phenyllactic acid)
ROS	Especies reactivas de oxígeno
RT	Retention time
SPME	Solid-phase microextraction
SRM	Selected Reaction Monitoring
TOF	Time of flight
TPC	Total Phenolic Content
TE	Trolox Equivalent
UE	Unión Europea (European Union)
UHPLC	Ultra High Performance Liquid Cromatography
YM-YMF	Yellow Mustard Flour
YMB	Yellow Mustard Bran
WHO	World Health Organization

RESUMEN

El desarrollo de hongos es una de las causas más habituales del deterioro de alimentos como son los cereales, sus productos derivados y el queso, por provocar cambios en las propiedades organolépticas y reducir el valor nutricional. Además, algunas especies son capaces de sintetizar micotoxinas, metabolitos altamente tóxicos que suponen un problema para la salud humana y animal. De forma tradicional se aplican sustancias fungicidas para disminuir la incidencia de mohos toxigénicos en los alimentos; sin embargo, la creciente preocupación de los consumidores por sus efectos tóxicos ha favorecido la búsqueda de alternativas que garanticen la calidad y seguridad alimentaria.

Por ello en la presente Tesis Doctoral se han desarrollado estrategias basadas en el empleo de sustancias naturales, principalmente mostaza amarilla (*Sinapis alba*), mostaza oriental (*Brassica juncea*), bacterias ácido-lácticas (LAB) y natamicina, para reducir la incidencia de hongos toxigénicos en los alimentos. En concreto, se ha evaluado la actividad antifúngica del salvado y el polvo de semilla de mostaza amarilla, los extractos fermentados de harina de mostaza amarilla y oriental con LAB, y la natamicina *in vitro*.

En base a los primeros resultados, se plantearon diferentes estrategias de aplicación en alimentos como el pan, el maíz y el queso, ya sea mediante la incorporación del ingrediente durante el proceso de producción, mediante su pulverizado en superficie, o a través de su incorporación en un sistema de envase activo. Además, se realizó la caracterización química de los fermentados de mostaza con LAB y se estudiaron los compuestos volátiles y bioactivos presentes en la harina y salvado de las mostazas amarilla y oriental.

Los resultados obtenidos mostraron la versatilidad de aplicación de la mostaza como estrategia antifúngica y antitoxigénica en alimentos. La harina de mostaza oriental se aplicó en la formulación de salsas bioactivas para liberar alil isotiocianato *in situ* en un sistema de envase activo y ejercer acción conservante en pan. Además, el salvado de mostaza amarilla se revalorizó como ingrediente con propiedades antifúngicas en el pan de molde, equiparando su uso al aditivo químico de síntesis propionato de sodio. Por otra parte, la pulverización del medio fermentado de mostaza amarilla con *Lactiplantibacillus plantarum* TR71 permitió reducir la síntesis de fumonisinas B_1 y B_2 en maíz contaminado con *Fusarium verticillioides*. La caracterización química permitió detectar compuestos con propiedades bioactivas como ácidos fenólicos, flavonoides y glucosinolatos.

Por último, la aplicación de la natamicina tanto en pulverizado directo en superficie como tras su incorporación en biopolímeros de hidroxietilcelulosa, resultaron estrategias efectivas para aumentar la vida útil del queso mozzarella contaminado con el moho *Penicillium commune*, constituyendo alternativas a la aplicación de aditivos químicos de síntesis en este tipo de productos.

RESUM

El desenvolupament de fongs és una de les causes més habituals de la deterioració d'aliments com els cereals, els seus productes derivats i el formatge, per provocar canvis en les propietats organolèptiques i reduir el valor nutricional. A més, algunes espècies són capaces de sintetitzar micotoxines, metabòlits altament tòxics que suposen un problema per a la salut humana i animal. De manera tradicional s'apliquen substàncies fungicides per a disminuir la incidència de fongs toxigènics en els aliments; no obstant això, la creixent preocupació dels consumidors pels seus efectes tòxics ha afavorit la cerca d'alternatives que garantisquen la qualitat i seguretat alimentària.

Per això, en la present Tesi Doctoral s'han desenvolupat estratègies basades en l'ús de substàncies naturals, principalment mostassa groga (*Sinapis alba*), mostassa oriental (*Brassica* juncea), bacteris àcid-làctics (LAB) i natamicina, per a reduir la incidència de fongs toxigènics en els aliments. En concret, s'ha avaluat l'activitat antifúngica del segó i la llavor de mostassa groga, els extractes fermentats de farina de mostassa groga i oriental amb LAB, i la natamicina *in vitro*.

D'acord amb els primers resultats, es van plantejar diferents estratègies d'aplicació en aliments com el pa, la dacsa i el formatge, ja siga mitjançant la incorporació de l'ingredient durant el procés de producció, mitjançant polvoritzat en superfície, o a través de la incorporació en un sistema d'envàs actiu. A més, es va realitzar la caracterització química dels fermentats de mostassa amb LAB i es van estudiar els compostos volàtils i bioactius presents en la farina i segó de les mostasses groga i oriental.

Els resultats obtinguts van mostrar la versatilitat d'aplicació de la mostassa com a estratègia antifúngica i antitoxigènica en aliments. La farina de mostassa oriental es va aplicar en la formulació de salses bioactives per a alliberar alil isotiocianato *in situ* en un sistema d'envàs actiu i exercir acció conservant en pa. A més, el segó de mostassa groga es va revaloritzar com a ingredient amb propietats antifúngiques en el pa de motle i va equiparar el seu ús a l'additiu sintètic propionat de sodi. D'altra banda, la polvorització del medi fermentat de mostassa groga amb *Lactiplantibacillus plantarum* TR71 va permetre reduir la síntesi de fumonisines B_1 i B_2 en dacsa contaminada amb *Fusarium verticillioides*. La caracterització química va permetre detectar compostos amb propietats bioactives com a àcids fenòlics, flavonoides i glucosinolats.

Finalment, l'aplicació de la natamicina tant en polvoritzat directe en superfícies com després de la seua incorporació en biopolímers d'hidroxietilcelulosa, van resultar estratègies efectives per a incrementar la vida útil del formatge mozzarella contaminat amb el fong *Penicillium commune*, i constitueix una alternativa a l'aplicació d'additius químics de síntesis en aquesta mena de productes.

SUMMARY

Fungal growth is one of the most common causes of spoilage of foods such as cereals, their by-products and cheese, as it causes changes in the organoleptic properties and reduces the nutritional value. In addition, some species can synthesize mycotoxins, highly toxic metabolites that pose a problem for human and animal health. Fungicides are traditionally applied to reduce the incidence of toxigenic moulds in food; however, the growing concern of consumers about their toxic effects has favoured the search for alternatives that guarantee food quality and safety.

Therefore, in this Doctoral Thesis, strategies based on the use of natural substances, mainly yellow mustard (*Sinapis alba*), oriental mustard (*Brassica juncea*), lactic acid bacteria (LAB) and natamycin, have been developed to reduce the incidence of toxigenic fungi in food. Specifically, the antifungal activity of yellow mustard bran and seed powder, fermented extracts of yellow and oriental mustard flour with LAB, and natamycin were evaluated *in vitro*.

Based on the first results, different application strategies were proposed in foods such as bread, maize, and cheese, either by incorporating the ingredient during the manufacturing process, spraying it on the surface, or including it in an active packaging system. In addition, the chemical characterization of mustard fermentates with LAB was carried out, and the volatile and bioactive compounds present in the flour and bran of yellow and oriental mustards were studied.

The results obtained showed the versatility of mustard application as an antifungal and antitoxigenic strategy in food. Oriental mustard flour was applied in the formulation of bioactive sauces to release allyl isothiocyanate *in situ* in an active packaging system and exert preservative action in bread. In addition, yellow mustard bran was revalued as an ingredient with antifungal properties in baking

bread, equating its use to the chemical synthesis additive sodium propionate. Moreover, the spraying of yellow mustard fermented with *Lactiplantibacillus plantarum* TR71 reduced fumonisin B_1 and B_2 synthesis in maize contaminated with *Fusarium verticillioides*. The chemical characterization allowed the detection of compounds with bioactive properties such as phenolic acids, flavonoids and glucosinolates.

Finally, the application of natamycin both by direct spraying on the surface and after its incorporation in hydroxyethylcellulose biopolymers were effective strategies to increase the shelf life of mozzarella cheese contaminated with *Penicillium commune* mould, constituting alternatives to the application of synthetic chemical additives in this type of products.

1. INTRODUCTION

Introducción

1. INTRODUCCIÓN

1.1. Contaminación fúngica de alimentos

Los alimentos, debido a su propia naturaleza, son susceptibles de alteración microbiológica por bacterias, levaduras y mohos, por lo que para garantizar la calidad e inocuidad alimentaria resulta esencial conocer los elementos asociados al desarrollo microbiológico. Principalmente, los factores que condicionan la microflora presente en el alimento se clasifican en dos grandes grupos: factores intrínsecos y factores extrínsecos (Petruzzi et al., 2017).

Los factores intrínsecos son aquellos relacionados directamente con la composición química del alimento e incluye: la estructura del alimento, la composición del alimento, la actividad de agua (a_w) y el valor de pH. La microflora del alimento puede cambiar de acuerdo a si el alimento es sólido o líquido, en función a su composición centesimal (proteínas, lípidos y carbohidratos) y a la presencia o no de sustancias inhibidoras. La actividad de agua se define como la relación entre la presión de vapor del alimento y la presión de vapor del agua pura. De forma general, las bacterias requieren de una elevada a_w para llevar a cabo sus actividades metabólicas. Sin embargo, los mohos son capaces de desarrollarse a valores de a_w comprendidos entre 0,70-0,80. El valor de pH del alimento también condiciona la microflora, ya que mientras que las bacterias requieren un pH próximo a la neutralidad para crecer (6,5-7,5), las levaduras y mohos son capaces de desarrollarse a pH más ácido (4,0-6,8) (Davidson & Critzer, 2012; Jeantet et al., 2016; Morpeth, 1995).

Los factores extrínsecos son aquellos asociados a las condiciones ambientales donde se conserva el alimento, como la temperatura de almacenamiento, la composición gaseosa de la atmósfera y la interacción con otros microorganismos,

entre otros. Los mohos y levaduras presentan un desarrollo óptimo a temperatura ambiente, aunque también son capaces de crecer a temperaturas de refrigeración. Además, la composición de la atmósfera también puede limitar la presencia de mohos en el alimento, ya que al ser aerobios estrictos requieren de O_2 para su desarrollo. De igual modo, pueden existir relaciones de competición o de sinergia entre diferentes microorganismos que colonizan un alimento, condicionando así la presencia de alterantes en el producto final (Abdel-Aziz et al., 2016; Rolfe & Daryaei, 2020).

Como consecuencia de la contaminación microbiológica se pueden producir toda una serie de modificaciones en el alimento, incluyendo la disminución de su valor nutritivo y produciendo cambios en sus propiedades organolépticas como sabor, textura, color y olor (Amit et al., 2017). Además, la contaminación fúngica presenta un peligro asociado, la síntesis de micotoxinas, la cual constituye un riesgo para la salud humana y animal. Asimismo, se asocia a importantes pérdidas económicas y se estima que un 25% de los alimentos producidos mundialmente son desechados debido a contaminaciones microbiológicas (Bondi et al., 2014).

1.1.1. Contaminación fúngica de cereales y productos derivados

Los cereales son una de las fuentes fundamentales de la alimentación desde hace milenios. Los principales cultivos de cereales producidos a nivel mundial son el trigo (*Triticum* spp.), el arroz (*Oryza* spp.), el maíz (*Zea mays* L.) y la cebada (*Hordeum vulgare* L.). Las infecciones fúngicas pueden generar numerosas enfermendades en la planta, traduciéndose en importantes pérdidas en la producción y en una disminución de la calidad de los granos destinados para consumo humano y animal (Ferrigo et al., 2016).

Los cereales están afectados en el campo generalmente por hongos filamentosos de los géneros *Alternaria*, *Aureobasidium*, *Cladosporium*, *Epicoccum*, *Fusarium*, *Helminthosporium* y *Claviceps*. En etapas de almacenamiento y procesamiento de los cereales, la microflora fúngica varía debido a la disminución del contenido de humedad y a_w, y está constituida principalmente por mohos de los géneros *Aspergillus*, *Penicillium* y *Fusarium* (Oliveira et al., 2014).

En el maíz (*Zea mays*), las principales especies contaminantes en el campo pertenecen al género *Fusarium*, las cuales producen enfermedades de gran importancia económica para los agricultores. La podredumbre roja es característica de regiones más frías y húmedas de Europa y es ocasionada por *F. graminearum*, *F. culmorum*, *F. avenaceum*, *F. cerealis*, *F. poae*, *F. equiseti* y *F. sporotrichioides*. En cambio, en regiones más cálidas y secas del continente, son predominantes las denominadas podredumbres rosas ocasionadas por *F. verticillioides* y *F. proliferatum* (Oldenburg et al., 2017).

Al igual que la materia prima, los productos derivados elaborados a partir de cereales también son susceptibles a la contaminación fúngica. Entre ellos se encuentra el pan, el cual es definido por el Real Decreto 308/2019, de 26 de abril, por el que se aprueba la norma de calidad para el pan como el "producto resultante de la cocción de una masa obtenida por la mezcla de harina y agua, con o sin adición de sal, fermentada con la ayuda de levadura de panificación o masa madre".

El pan una vez cocido no presenta formas vegetativas de hongos debido a la inactivación térmica ocasionada por la elevada temperatura de horneado. Sin embargo, durante el proceso de manipulación y envasado se puede contaminar con esporas presentes en el ambiente o en los utensilios de procesado. Principalmente, debido a sus características fisicoquímicas (pH comprendido entre 5,4-6,0 y a_w entre 0,94-0,97) es contaminado por hongos xerolíficos de los géneros *Aspergillus,*

Penicillium y *Eurotium*, siendo las colonias fúngicas perceptibles transcurridos 3-4 días de almacenamiento a temperatura ambiente (Pateras, 2007).

Las especies del género *Penicillium* que aparecen comúnmente como contaminantes en el pan son *P. commune*, *P. crustosum*, *P. chrysogenum*, *P. corylophilum*, *P. palitans*, *P. polonicum*, *P. roqueforti*, *P. brevicompactum* y *P. solitum*, mientras que del género *Aspergillus*, las especies más características aisladas son *A. versicolor*, *A. sydowii* y *A. flavus* (Nielsen & Rios, 2000; Smith et al., 2004).

1.1.2. Contaminación fúngica del queso

El Real Decreto 113/2006, de 29 de septiembre, por el que se aprueban las normas de calidad para quesos y quesos fundidos define como queso al "producto fresco o madurado, sólido o semisólido, obtenido de la leche, de la leche total o parcialmente desnatada, de la nata, del suero de mantequilla o de una mezcla de algunos o de todos estos productos, coagulados total o parcialmente por la acción del cuajo u otros coagulantes apropiados, antes del desuerado o después de la eliminación parcial de la parte acuosa, con o sin hidrólisis previa de la lactosa, siempre que la relación entre la caseína y las proteínas séricas sea igual o superior a la de la leche".

Los microorganismos desempeñan un papel fundamental durante la fabricación del queso. Generalmente, la microbiota primaria está constituida por bacterias ácido-lácticas (LAB) de los géneros *Lactobacillus* y *Streptococcus*. Desempeñan la función de disminuir el valor de pH para favorecer la actividad del cuajo, ayudan en la expulsión del suero de la cuajada y previenen el desarrollo de microorganismos patógenos. La microbiota secundaria va a proporcionar las

propiedades organolépticas del producto final (apariencia, consistencia y flavor) y está compuesta por levaduras, hongos y bacterias halófilas (Fox et al., 2004; Zheng et al., 2021). Algunas especies fúngicas como *P. camemberti*, *P. roqueforti*, *Mucor fuscus* y *M. lanceolatus* son empleadas intencionadamente durante la fabricación del queso.

La diversidad de especies de microorganismos alterantes presentes en el queso dependen fundamentalmente de la calidad microbiana de la leche empleada en el proceso productivo, la manipulación y tratamiento térmico de la leche, las condiciones de fabricación y manipulación de la cuajada, la temperatura, humedad y cantidad de sal empleada durante la maduración, así como la exposición del queso a microorganismos exógenos o esporas fúngicas durante el proceso de fabricación (Banjara et al., 2015; Lund et al., 1995; Ropars et al., 2012).

En función de la tipología de queso fabricado, los parámetros fisicoquímicos cambian considerablemente, y con ello los motivos de alteración. Las principales especies descritas como contaminantes de queso semi-duro pertenecen al género *Penicillium*, ya que se encuentran bien adaptadas al entorno de fabricación: *P. commune*, *P. crustosum*, *P. chrysogenum*, *P. griseofulvum*, *P. palitans*, *P. brevicompactum*, *P. solitum*, *P. nalgiovense, P. roqueforti* y *P. verrucosum* (Bundgaard-Nielsen & Nielsen, 1996; Garnier et al., 2017; Hermet et al., 2012). Además, algunas especies del género *Mucor* también pueden aparecer como contaminantes del queso, generando proteólisis en el producto durante el proceso de fabricación (Sengun et al., 2008).

1.1.3. Micotoxinas

Las micotoxinas son metabolitos secundarios de peso molecular inferior a 700 Da, producidos por una gran variedad de hongos filamentosos de los géneros *Aspergillus*, *Penicillium*, *Fusarium*, *Claviceps* y *Alternaria*. Se han descrito más de 400 micotoxinas diferentes, algunas de las cuales presentan importantes efectos tóxicos. Principalmente se encuentran en alimentos de origen vegetal como cereales, frutos secos, frutas y verduras, así como en alimentos derivados como el vino, los zumos de fruta y la cerveza. Además, algunas micotoxinas se han reportado en alimentos de origen animal, como leche, carne y huevo, tras la ingesta de piensos contaminados por los animales (Alshannaq & Yu, 2017; Richard, 2007).

Entre los efectos tóxicos asociados a la ingesta de micotoxinas se encuentra acción hepatotóxica, nefrotóxica, mutagénica, teratogénica, estrogénica, hemorrágica, inmunotóxica, dermatóxica y neurotóxica (Milićević et al., 2010). Además, la Agencia Internacional de Investigación sobre el Cáncer (IARC) ha clasificado algunas de las micotoxinas más comunes de acuerdo a su potencialidad de producir efectos carcinogénicos (**Tabla 1**). En concreto, de acuerdo a la evidencia científica disponible clasifica los tóxicos en cinco grupos (Grupo 1, 2A 2B, 3 y 4). Las aflatoxinas (AFs) de los grupos B y G se encuentran clasificadas en el grupo 1, confirmando que son agentes carcinogénicos con suficiente evidencia científica disponible en humanos. Las fumonisinas B_1 y B_2, así como la ocratoxina A (OTA) se encuentran englobadas en el subgrupo 2B, que implica que son agentes posiblemente carcinogénicos, ya que hay limitada evidencia de estudios en humanos y animales. Por último, el deoxinivalenol (DON), la patulina, las toxinas T-2 y HT-2 y la zearalenona se encuentran clasificadas en el grupo 3, considerados como agentes posiblemente no carcinógenos de acuerdo a la información científica disponible (IARC, 2012; Ostry et al., 2017).

Tabla 1. Principales micotoxinas, hongos productores y clasificación en función de su carcinogenicidad.

Micotoxina	Principales hongos productores	IARC
Aflatoxina B_1, B_2, G_1 y G_2	*Aspergillus flavus* *Aspergillus parasiticus*	1
Alternariol Alternariol monometil éter Altenueno Ácido tenuazónico Altertoxinas	*Alternaria alternata* *Alternaria arborescens* *Alternaria brassicae* *Alternaria citri* *Alternaria solani*	–
Beauvericina	*Fusarium sporotrichioides* *Fusarium poae* *Fusarium langsethiae* *Fusarium avenaceum*	–
Citrinina	*Penicillium citrinum* *Penicillium viridicatum* *Penicillium citreoviride* *Penicillium expansum*	–
Deoxinivalenol (DON)	*Fusarium graminearum* *Fusarium culmorum* *Fusarium cerealis*	3
Eniatinas A_1, A_2, B_1, B_2	*Fusarium avenaceum* *Fusarium tricintum*	–
Fumonisina B_1 y B_2	*Fusarium verticillioides* *Fusarium proliferatum*	2B
Ocratoxina A (OTA)	*Penicillium nordicum* *Penicillium verrucosum* *Aspergillus ochraceus* *Aspergillus carbonarius*	2B
Patulina	*Pencillium expansum* *Penicillium griseofulvum* *Aspergillus clavatus*	3
Toxinas T-2 y HT-2	*Fusarium acuminatum* *Fusarium poae* *Fusarium sporotrichioides* *Fusarium langsethiae*	3
Zearelanona	*Fusarium graminearum* *Fusarium culmorum* *Fusarium equiseti* *Fusarium verticillioides* *Fusarium incarnatum* *Fusarium cerealis*	3

Los principales factores que van a condicionar la síntesis de micotoxinas en los alimentos son la temperatura, la actividad de agua (a_w), la composición del sustrato colonizado y las interacciones con otros microorganismos. De forma general, los requerimientos necesarios de a_w y temperatura para sintetizar la micotoxina son más elevados que los necesarios para desarrollar la colonia fúngica. Por ejemplo, *P. verrucosum* es capaz de crecer en un rango de temperaturas entre 0 y 31 °C y a_w entre 0,80-0,85, pero presenta un máximo de producción de OTA a los 20 °C y a_w 0,95-0,99 (Pitt & Hocking, 1997). De forma similar, *F. verticillioides* es capaz de desarrollarse entre 4-37 °C y a partir de una a_w de 0,90, pero la síntesis de Fumonisina B_1 presenta un óptimo de producción entre 15 y 30 °C y a_w 0,97 (Marín et al., 2004).

La contaminación de la matriz alimentaria con diferentes cepas fúngicas puede ocurrir, lo que implica que se puede producir la acumulación de una o más micotoxinas en el alimento. Por otra parte, la problemática de la contaminación de los alimentos con las micotoxinas radica en que pueden estar presentes en toda la cadena de producción, ya que son resistentes a los factores ambientales y a gran parte de los tratamientos de procesado (Patriarca & Fernández Pinto, 2017).

En la actualidad, los límites máximos de algunas micotoxinas se encuentran recogidos en el Reglamento 1881/2006, de 19 de diciembre, por el que se fija el contenido máximo de determinados contaminantes en los productos alimenticios. Los criterios de selección empleados para designar los valores máximos legislados se deciden de acuerdo a diversos factores, como son los efectos toxicológicos de la micotoxina, el consumo poblacional de estos alimentos, la edad de la población a la que van destinados estos alimentos, así como su posible uso por un sector de la población con necesidades médicas especiales. Estos límites se han ido actualizando en los años posteriores con los siguientes reglamentos: Reglamento

(CE) n° 1126/2007 (toxinas de *Fusarium* en maíz y productos derivados), Reglamento (UE) n° 105/2010 y (UE) n° 594/2012 (OTA en productos alimenticios), Reglamento (UE) n° 165/2010 y (UE) n° 1058/2012 (AFs en productos alimenticios), Reglamento (UE) n° 212/2014 (citrinina en complementos alimenticios basados en arroz fermentado), y Reglamento (UE) n° 2021/1399 (esclerocios de cornezuelo de centeno en cereales). La **Tabla 2** recoge los intervalos de contenidos máximos de micotoxinas legislados en cereales y productos derivados.

Tabla 2. Principales micotoxinas legisladas en cereales y productos derivados y sus límites máximos.

Micotoxina	**Productos**	**Límites máximos (µg/kg)**
Aflatoxina B_1	Cereales y productos derivados de cereales, incluidos los alimentos para lactantes y niños de corta edad, así como alimentos dietéticos destinados a usos médicos especiales para lactantes.	0,10-2,0
Suma Aflatoxinas B_1, B_2, G_1 y G_2	Cereales y productos derivados de cereales.	4,0
Ocratoxina A	Todos los cereales y productos derivados de cereales, incluidos los alimentos para lactantes y niños de corta edad, así como alimentos dietéticos destinados a usos médicos especiales para lactantes.	0,50-5,0
Deoxinivalenol	Cereales elaborados y no elaborados, productos derivados de cereales, incluidos los alimentos para lactantes y niños de corta edad.	200-1750
Zearalenona	Cereales elaborados y no elaborados, productos derivados de cereales, incluidos los alimentos destinados para lactantes y niños de corta edad.	20-200
Suma Fumonisinas B_1 y B_2	Maíz no elaborado y procesado y alimentos a base de maíz, incluidos los alimentos destinados para lactantes y niños de corta edad.	200-2000

1.2. Control del desarrollo fúngico en alimentos

Aunque no es posible prevenir en su totalidad el desarrollo de hongos y la consecuente síntesis de micotoxinas en los alimentos, resulta crucial implementar una serie de estrategias que reduzcan su presencia y abarquen el proceso productivo en su totalidad.

En el campo es fundamental seguir unas Buenas Prácticas Agrícolas (BPA). Estas incluyen una serie de medidas que minimizan el riesgo de contaminación y abarcan desde el cultivo hasta la cosecha. Entre las BPA que se pueden aplicar para controlar el desarrollo fúngico destacan: el control de insectos y parásitos; el control de las infecciones fúngicas mediante el empleo de fungicidas; el uso de variedades resistentes a hongos y plagas; el roturado de la tierra; y el almacenamiento apropiado de los cultivos tras la cosecha (Zakhia-Rozis et al.,2007).

Además de la implementación de las BPA, resulta esencial que en la industria alimentaria se siga el mismo principio de prevención, aplicando unas buenas prácticas de fabricación (BPF). Este objetivo se puede conseguir mediante la elección de materias primas de calidad, la adecuada monitorización del proceso y la aplicación de medidas de control del desarrollo microbiológico, ya sean físicas, químicas o biológicas. En este contexto, resulta imprescindible implementar un sistema de análisis de peligros y puntos críticos de control (APPCC) siguiendo las directrices del Codex Alimentarius y el Reglamento 852/2004, de 29 de abril, relativo a la higiene de los productos alimenticios.

1.2.1. Conservantes químicos

Debido a la necesidad de establecer técnicas de control que inhiban o retarden el desarrollo de microorganismos, la UE autoriza diversos aditivos para su uso. Estos

son incorporados mediante la mezcla directa en las formulaciones alimentarias o a través de técnicas directas, como el pulverizado o el sumergido del producto en soluciones antimicrobianas. El uso en alimentos, así como sus dosis máximas de aplicación, se encuentran recogidos en el anexo II del Reglamento 1333/2008 del Parlamento Europeo y del Consejo para establecer una lista de aditivos de la Unión.

En los productos de panadería, los aditivos empleados más comunes son los propionatos, ya que apenas ejercen acción antimicrobiana frente a las levaduras, permitiendo la adecuada fermentación de la masa. Los sorbatos son menos utilizados ya que poseen una mayor efectividad frente a levaduras en comparación con el ácido propiónico, por lo que al afectar al proceso de fermentación, se observa una disminución en el volumen específico final del producto (**Tabla 3**) (Legan & Voysey, 1991; Smith et al., 2004).

Tabla 3. Dosis de uso autorizadas de conservantes en pan.

Número E	Denominación	Dosis máxima (mg/L o mg/kg)	Restricciones o excepciones
E 200-203	Ácido sórbico y sorbatos	2000	Solo rebanadas de pan envasado y pan de centeno, destinados a la venta al por menor, y pan de valor energético reducido destinado a la venta al por menor.
E 280-283	Ácido propiónico y propionatos	3000	Solo rebanadas de pan envasado y pan de centeno envasado.
E 280-283	Ácido propiónico y propionatos	2000	Solo pan de valor energético reducido, pan precocinado y envasado, *rolls*, *pitta*, *pølsebrød*, *boller* y *dansk flutes*, envasado.
E 280-283	Ácido propiónico y propionatos	1000	Solo pan envasado.

Por lo que respecta a la incorporación de aditivos en queso, de acuerdo con la clase de producto a comercializar, se señalan los autorizados en la **Tabla 4**. En queso en lonchas, el aditivo más empleado es el ácido sórbico (E-200). Presenta la ventaja de que no posee ni olor ni sabor cuando se emplea en las concentraciones apropiadas, por lo que no se modifican las propiedades organolépticas del producto final. Su espectro de actuación abarca levaduras, hongos y bacterias. Las sales del ácido sórbico presentan propiedades antimicrobianas similares, si bien presentan la ventaja de que son más solubles en agua (Silva & Lidon, 2016).

Tabla 4. Dosis de uso autorizadas de conservantes en queso.

Número E	Denominación	Dosis máxima (mg/L o mg/kg)	Restricciones o excepciones
E 200	Ácido sórbico	1000	Solo queso en lonchas cortado y envasado; queso en capas y queso con otros alimentos añadidos.
E 201-203	Sorbatos	1000	Solo queso en lochas cortado y envasado; queso en capas y queso con otros alimentos añadidos.
E 234	Nisina	12,5	Quesos curados, quesos fundidos.
E 351-252	Nitrito de sodio/potasio	150	Solos queso duro, semiduro y semiblando.
E 260	Ácido acético	*quantum satis*	Solo mozzarella.
E 270	Ácido láctico	*quantum satis*	Solo mozzarella.
E 280	Ácido propiónico	3000	Solo tratamiento de superficie.
E 281-283	Propionatos de sodio, calcio y potasio	3000	Solo tratamiento de superficie.

A pesar del extendido uso de estas sustancias para prolongar la vida útil de los alimentos, los aditivos químicos de síntesis presentan diversos inconvenientes. Marín et al. (2002) demostraron que concentraciones sub-óptimas (entre 0,003 y 0,03%) de los aditivos propionato cálcico, sorbato potásico y benzoato sódico pueden favorecer el desarrollo fúngico *in vitro*, por lo que no aplicar las dosis recomendadas puede resultar contraproducente para prevenir la contaminación fúngica. Por otra parte, el uso extendido y prolongado de estos aditivos puede llevar a cabo mutaciones en los microorganismos diana, generando resistencias a estos tratamientos (Levinskaitė, 2012).

1.2.2. Conservación de alimentos mediante sustancias naturales

La creciente demanda por parte de los consumidores de la reducción de uso de aditivos químicos de síntesis ha generado una tendencia centrada en la búsqueda de alternativas como alimentos mínimamente procesados o basados en compuestos naturales. De forma ideal, un antimicrobiano natural debe poseer un gran espectro de acción, ser activo a bajas concentraciones, estable térmicamente, no estar afectado por cambios en el pH, no aportar sabores ni olores al alimento, no presentar acción tóxica y resultar económicamente viable (Carocho et al., 2015). En este contexto, se han propuesto y evaluado múltiples compuestos que se pueden clasificar de acuerdo con su origen: vegetal, animal, o microbiológico.

Entre las estrategias basadas en compuestos de origen vegetal, se han estudiado metabolitos secundarios bioactivos como los fenoles, terpenos, alcoholes alifáticos, aldehídos, cetonas y aceites esenciales (EOs). Los EOs son ricos en moléculas como terpenos e hidrocarburos y han demostrado un gran potencial antimicrobiano *in vitro*; sin embargo, su uso se encuentra limitado debido a las

grandes dosis que son necesarias aplicar para observar el efecto antimicrobiano, así como a los flavores que transfieren al alimento (Hyldgaard et al., 2012; Sharma et al., 2021).

Entre los compuestos antimicrobianos descritos provenientes de fuente animal han sido evaluados principalmente proteínas de bajo peso molecular como la lactoferrina, ovotransferrina y la lisozima, así como algunos polisacáridos como la quitina y el quitosano (Ribes et al., 2018).

Por último, entre las estrategias de origen natural se encuentra la técnica de la biopreservación, la cual es definida como el uso de microorganismos no-patógenos o sus metabolitos para incrementar la vida útil de los alimentos. La atención de esta técnica se ha centrado principalmente en el uso de bacterias ácido-lácticas, así como en el uso de levaduras antagónicas (Siroli et al., 2015).

1.2.3. Envases activos

El envasado de alimentos tradicionalmente ha desempeñado su papel en la protección del producto alimentario de la influencia del entorno, para preservar y mantener su calidad y minimizar las pérdidas económicas. Los avances realizados en investigación han llevado al diseño y fabricación de envases activos, en donde producto y entorno interaccionan para extender su vida útil y/o mejorar sus propiedades organolépticas. De acuerdo con el Reglamento (CE) nº 1935/2004, sobre los materiales y objetos destinados a entrar en contacto con alimentos, se establece la siguiente definición de envase activo: “aquel destinado a ampliar el tiempo de conservación, o a mantener o mejorar el estado de los alimentos envasados, y que están diseñados para incorporar deliberadamente componentes que transmitan sustancias a los alimentos envasados o al entorno de éstos, o que

absorban sustancias de los alimentos envasados o del entorno de éstos". Los principales sistemas de envases activos que se han diseñado son controladores de humedad, eliminadores de oxígeno, absorbentes/emisores de etileno, absorbentes/emisores de CO_2 y sistemas de liberación de aromas, antioxidantes y antimicrobianos (**Figura 1**) (Prasad & Kochhar, 2014; Wyrwa & Barska, 2017).

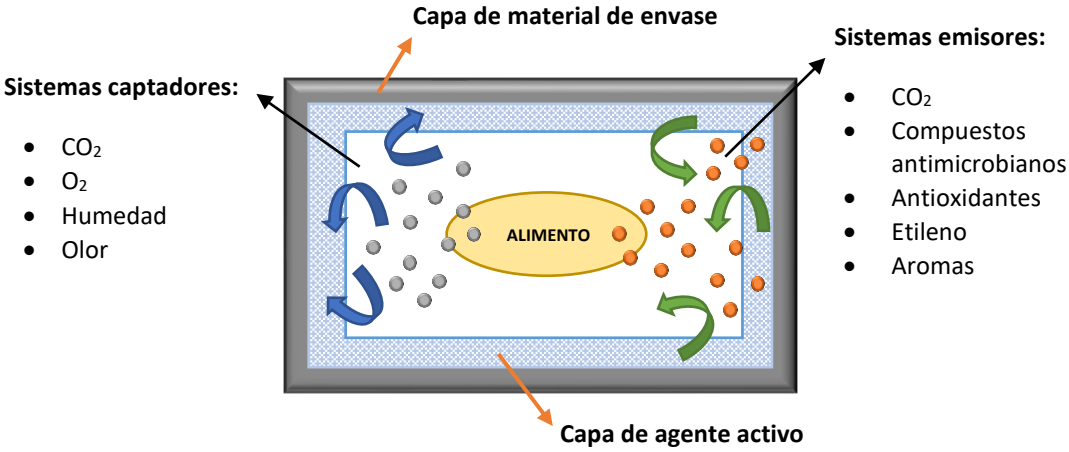


Figura 1. Funcionamiento y principales tipos de envases activos.

El uso de agentes antimicrobianos en el envasado de productos alimentarios es una estrategia de interés para prevenir el desarrollo de microorganismos, y de esta forma, incrementar su vida útil. Por ello, una gran variedad de compuestos se han propuesto para el desarrollo de envases activos, como por ejemplo, ácidos orgánicos y sus sales, alcoholes, metales, enzimas, queladores, sustancias antimicrobianas, extractos vegetales y EOs (Said & Sarbon, 2019; Sharma et al., 2021).

La incorporación de los componentes antimicrobianos en el sistema de envasado se puede abarcar mediante diferentes estrategias. Los antimicrobianos volátiles se pueden adicionar en sachets o almohadillas que estén integradas dentro del envase. Otra alternativa es la incorporación de los componentes antimicrobianos volátiles y no volátiles en polímeros. Otra opción es la inmovilización de los antimicrobianos a los polímeros mediante enlaces iónicos o covalentes. Por último, existe la posibilidad de emplear polímeros que inherentemente poseen actividad antimicrobiana, como por ejemplo el quitosano (Appendini & Hotchkiss, 2002).

1.2.3.1 Biopolímeros

Una tendencia actual en el envasado de productos alimentarios busca la sustitución de matrices poliméricas plásticas por compuestos biodegradables que sean sostenibles, permitan la reducción de emisiones de CO_2 y la dependencia de fuentes fósiles (Peelman et al., 2013). Algunos de los polímeros más estudiados están constituidos de polisacáridos, proteínas, lípidos o sus combinaciones. Entre los polisacáridos más empleados se encuentran la celulosa y sus derivados, el almidón, las pectinas, los extractos de algas (alginatos, carragenatos y agar), las gomas (acacia, tragacanto y guar), el pululano, y el quitosano. Las proteínas se emplean como agentes estructurantes y pueden provenir de una fuente animal (caseína, gelatina, albúmina de huevo) o vegetal (maíz, soja, trigo, algodón, cacahuete y arroz). Por último, entre los lípidos empleados para la fabricación de biopolímeros se encuentran grasas de origen animal y vegetal, ceras, resinas naturales, emulsificantes y agentes activos en superficie (lecitina y ácidos grasos) (Lagarón et al., 2016). La relevancia de uso de biopolímeros reside en que pueden

ser portadores de moléculas antioxidantes y/o antimicrobianas que interaccionan de forma positiva con el alimento para aumentar su vida útil (Valdés et al., 2017).

El empleo de biopolímeros antimicrobianos presenta unas ventajas respecto a la adición directa de conservantes en el producto, ya que pueden liberar de manera selectiva y gradual las sustancias antimicrobianas en la superficie del producto o en el espacio de cabeza presente entre el alimento y el envase (Soltani Firouz et al., 2021).

Por otra parte, si bien su uso es una estrategia prometedora en la conservación de alimentos, los biopolímeros presentan algunas limitaciones debido a sus bajas propiedades mecánicas y de barrera en comparación a los polímeros sintéticos (Azeredo et al., 2009). En este aspecto, se han estudiado diversas modificaciones en las estructuras poliméricas realizando mezclas de proteínas y polisacáridos, lípidos y polisacáridos o lípidos y proteínas, entre otras. Por ejemplo, se ha demostrado que la elaboración de biopolímeros con compuestos hidrofóbicos permite reducir la permeabilidad al agua (Costa et al., 2015).

1.2.3.2 Sachets

La función principal de los sachets es la liberación deseada de sustancias que ejercen un efecto positivo en el ambiente de envasado. Se pueden emplear para controlar el desarrollo microbiano al incorporar sustancias antimicrobianas volátiles (Wyrwa & Barska, 2017). Entre los compuestos activos de origen natural que han sido evaluados para su incorporación en sachets destacan principalmente los EOs y sus constituyentes, así como el alil isotiocianato (**Tabla 5**).

Tabla 5. Aplicación de sachets con efecto antimicrobiano en alimentos.

Componente activo	Alimento aplicado	Espectro actuación	Referencia
Aceite esencial canela	Pan	*Escherichia coli* *Salmonella* sp *Staphylococcus aureus* *Aspergillus* sp	Fahma et al. (2020)
Aceite esencial orégano	Pan	Levaduras y mohos	Passarinho et al. (2014)
Aceite esencial romero y tomillo	Queso mozzarella	*Listeria monocytogenes*	Han et al. (2014)
Alil isotiocianato	Hojas de espinaca	*Escherichia coli* O157:H7	Seo et al. (2012)
Citral Eugenol	Pan	*Aspergillus niger*	Ju et al. (2020)
Aceite esencial tomillo	Carne lista para comer	Levaduras	Quesada et al. (2016)
Eugenol Carvacrol	Lechuga iceberg	Coliformes	Wieczyńska & Cavoski, (2018)
Timol	Tomates cherry	*Botrytis cinerea*	Álvarez-Hernández et al. (2021)

Si bien los sachets poseen ventajas de aplicación sobre otros sistemas de envasado antimicrobiano, presentan ciertas limitaciones. La incorporación de un sachet en el envasado requiere de una operación adicional en la cadena de producción, lo que incrementa el tiempo de fabricación y se traduce en el aumento del coste del producto final (de Abreu et al., 2012). Por otra parte, los antimicrobianos empleados pueden presentar olores no deseables que afecten la percepción sensorial del consumidor cuando el envase es abierto (Otoni et al., 2016). Asimismo, Dainelli et al. (2008) han señalado que los consumidores pueden ser reacios al uso de estos dispositivos por el riesgo de una ingestión accidental al ser confundido con un elemento del alimento.

1.3. Mostaza

Por el término mostaza se designa a un grupo de plantas de la familia *Brassicaceae*, también denominadas crucíferas, perteneciente al orden Brassicales. En el mundo se cultivan principalmente tres variedades de mostaza para su uso gastronómico: la mostaza blanca o amarilla (*Sinapis alba*), la mostaza india u oriental (*Brassica juncea*) y la mostaza negra (*Brassica nigra*) (**Figura 2**).

Figura 2. Principales semillas de mostaza cultivadas. (a) Mostaza amarilla *(Sinapis alba*); (b) Mostaza oriental (*Brassica juncea*); (c) Mostaza negra (*Brassica nigra*).

Dependiendo de la especie de mostaza, la composición centesimal puede cambiar ligeramente. La semilla de mostaza posee un elevado contenido de proteínas (32,5-36,7%), grasas (31,8-36,3%) y carbohidratos (16,59-16,6%) (Abul-Fadl et al., 2011). Además, son plantas ricas en micronutrientes como sales minerales, ácido ascórbico, fenoles (principalmente ácido sinápico y sus derivados) y glucosinolatos (Tian & Deng, 2020).

La mostaza se encuentra incluida en la lista de sustancias alergénicas establecida por la Unión Europea en la Directiva 1169/2011/CE del Parlamento Europeo y del Consejo, por lo que es obligatorio informar de su presencia en el etiquetado de los productos. El principal alérgeno descrito en la mostaza amarilla (*Sinapis alba*) es Sin-a-1, mientras que en la mostaza oriental (*Brassica juncea*) es

Bra-j-1. Ambas son proteínas de bajo peso molecular resistentes a la desnaturalización por acción térmica (Assou et al., 2022; Palomares et al., 2005).

1.3.1. Glucosinolatos

Los glucosinolatos (GSs) son metabolitos secundarios característicos de las plantas de la familia *Brassicaceae*. Los GSs comparten una estructura química genérica que consta de un tiohidroximato que contiene un átomo de azufre unido a una molécula de β-D-glucopiranosa y un residuo de sulfato, con una cadena lateral variable. Dependiendo del aminoácido constituyente de la cadena variable (alanina, leucina, isoleucina, valina, fenilalanina, tirosina y triptófano), los glucosinolatos se clasifican en alifáticos, aromáticos o indólicos (Lietzow, 2021). Su síntesis se divide en tres fases: elongación de la cadena del amino ácido, formación del núcleo de la estructura y modificación de la cadena lateral secundaria (Grubb & Abel, 2006).

Los GSs poseen propiedades beneficionas como acción antioxidante, antimutagénica y antiproliferativa (Vig et al., 2009). A partir de los GSs, mediante la acción de la enzima mirosinasa (β-tioglucósido glucohidrolasa EC.3.2.1.147), se pueden sintetizar una gran variedad de metabolitos bioactivos, entre ellos los isotiocianatos (ITCs), tiocianatos, nitrilos y epitionitrilos (Ishida et al., 2014)

Se han descrito más de 120 GSs diferentes en la familia *Brassicaceae* y su distribución cambia de acuerdo con la especie de planta. En la mostaza blanca (*Sinapis alba*), el glucosinolato característico se denomina sinalbina, mientras que, en la mostaza oriental, el glucosinolato mayoritario es la sinigrina (**Figura 3b**) (Verkerk et al., 2009).

1.3.2. Isotiocianatos

En los vegetales de la familia *Brassicaceae*, los GSs se encuentran en células o compartimentos intracelulares diferentes de donde está la enzima responsable de catalizar la reacción de transformación en ITCs. Cuando la planta sufre un daño físico, enzima y sustrato entran en contacto, permitiendo la reacción de hidrólisis de los GSs. A continuación, se forma una molécula intermedia inestable (aglicona) a partir de la cual se constituyen los diferentes productos finales, entre ellos los ITCs (**Figura 3a**). Los ITCs poseen una estructura general R-N=C=S y juegan un papel fundamental en la defensa de la planta, ya que poseen elevada actividad antimicrobiana. Además, los productos de hidrólisis de los GLSs son responsables del sabor pungente característico de la mostaza (Hanschen et al., 2018).

Dependiendo del glucosinolato de origen, los ITCs sintetizados son diferentes. A partir del GS sinalbina, característico de la mostaza amarilla, el principal ITC que se produce es el *p*-hidroxibenzil isotiocianto, un compuesto poco volátil y de baja estabilidad química. En cambio, el producto de hidrólisis obtenido a partir del GS sinigrina, característico de la mostaza oriental (*Brassica juncea*), es el volátil alil isotiocianato (AITC) (**Figura 3b**) (Dubey et al., 2020; Ekanayake et al., 2016).

Los ITCs son capaces de reaccionar con diferentes moléculas intracelulares, como glutatión, sulfitos, aminoácidos, oligopéptidos y proteínas (Hyldgaard et al., 2012). Recientemente, Sun et al. (2021) han reportado que el mecanismo de acción del benzil isotiocianato se debe a la destrucción de la integridad de la membrana fúngica, la acumulación de especies reactivas de oxígeno (ROS) y además, observaron una expresión diferencial de genes relacionados con la replicación del DNA y la transcripción que conducía finalmente a la muerte celular. Liu et al., (2021) también reportaron que el AITC inducía la acumulación de ROS en la célula fúngica por la disminución del contenido de glutatión llevando a la muerte celular.

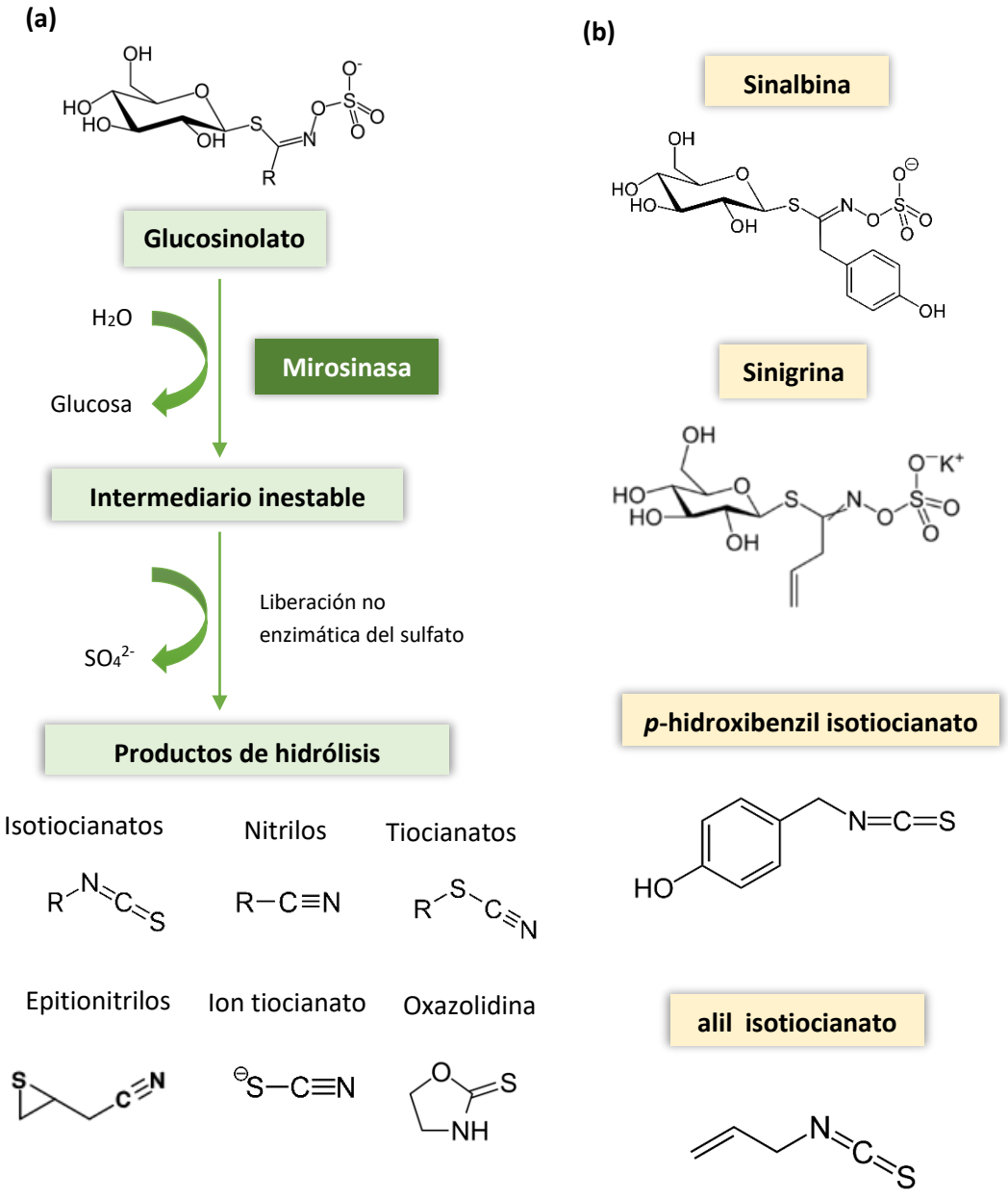


Figura 3. (a) Esquema de la degradación de glucosinolatos por acción de la enzima mirosinasa y (b) estructuras químicas de la sinalbina, sinigrina, *p*-hidroxibenzil isotiocianato y alil isotiocianato.

Debido a su amplio espectro antimicrobiano, los isotiocianatos han sido estudiados para la preservación de alimentos afectados por hongos, tal y como se recoge en la **Tabla 6**.

Tabla 6. Aplicación de los isotiocianatos frente a hongos contaminantes en alimentos.

Isotiocianato	**Alimento aplicado**	**Cepa fúngica ensayada**	**Referencia**
Alil isotiocianato	Cacahuete	*Aspergillus parasiticus*	Okano et al. (2018)
Benzil isotiocianato	Fresa	*Botrytis cinerea*	Seo et al. (2012)
Alil isotiocianato	Maíz	*Aspergillus glaucus* *Aspergillus penicillioides* *Aspergillus restrictus*	Okano et al. (2015)
Alil isotiocianato Benzil isotiocianato	Maíz Uva	*Aspergillus ochraceus* *Apergillus carbonarius* *Aspergillus niger*	Yang et al. (2021)
Alil isotiocianato	Maíz	*Aspergillus parasiticus* *Fusarium verticillioides* *Fusarium graminearum*	Evangelista et al. (2021)
Metil isotiocianato Alil isotiocianato Butil isotiocianato Etil isotiocianato Benzil isotiocianato 2-Feniletil isotiocianato	Mandarina	*Geotrichum citri-aurantii*	Kara & Soylu, (2020)
Alil isotiocianato Etil isotiocianato	Manzana	*Botrytis cinerea* *Penicillium expansum*	Wu et al. (2011)
2-Feniletil isotiocianato	Pera	*Alternaria alternata*	Zhang et al. (2020)
Alil isotiocianato	Cebada Trigo Maíz	*Penicillium verrucosum* *Aspergillus flavus*	Quiles et al. (2019)
Alil isotiocianato	Cachuete	*Aspergillus flavus*	Otoni et al. (2014)

1.3.3. Procesado de la semilla de mostaza

La semilla de mostaza se puede procesar para obtener diferentes productos como el aceite y la harina, siendo sus principales deshechos el salvado y la torta (Reungoat et al., 2021). La harina de mostaza es obtenida mediante molienda en rodillos para disminuir el tamaño de partícula y se emplea como emulsificante o saborizante de productor alimentarios. La fracción del salvado es eliminada durante este proceso y se estima que en torno al 60% de la semilla de mostaza constituye un producto de deshecho (Sehwag & Das, 2015).

Estas fracciones descartadas constituyen una oportunidad para revalorizarse o bien como ingredientes en la formulación de alimentos o para recuperar compuestos biológicamente activos de estas especies de plantas como glucosinolatos, isotiocianatos y compuestos fenólicos.

1.4. Bacterias ácido-lácticas

Las bacterias ácido-lácticas (LAB) son un grupo de bacterias pertenecientes al orden *Lactobacillales*, gram-positivas, catalasa-negativas, no esporuladas, con morfología característica en coco o bacilo, cuyo principal metabolito de la fermentación de carbohidratos es el ácido láctico. Estos microorganismos han sido empleados desde antiguo para la producción de alimentos fermentados debido a su potencialidad de incrementar la vida útil, mejorar las propiedades organolépticas e incrementar el valor nutricional (Leyva Salas et al., 2017).

La clasificación taxonómica tradicional divide el orden *Lactobacillales* en dos familias diferentes: la familia *Lactobacillae* está constituida por las especies del género *Lactobacillus*, *Paralactobacillus* y *Pediococcus*; mientras que la familia *Leuconostocaceae* incluye los géneros *Convivina*, *Fructobacillus*, *Leuconostoc*,

Oenococcus y *Weissella*. Sin embargo, Zheng et al. (2020) evaluaron las secuencias genómicas de las familias *Lactobacillaceae* y *Leuconostocaceae* y propusieron la reclasificación del género *Lactobacillus* en 25 nuevos géneros, entre los que se incluye: *Lactobacillus delbrueckii*, *Paralactobacillus*, *Holzapfelia*, *Amylolactobacillus*, *Bombilactobacillus*, *Companilactobacillus*, *Lapidilactobacillus*, *Agrilactobacillus*, *Schleiferilactobacillus*, *Loigolactobacillus*, *Lacticaseibacillus*, *Latilactobacillus*, *Dellaglioa*, *Liquorilactobacillus*, *Ligilactobacillus*, *Lactiplantibacillus*, *Furfurilactobacillus*, *Paucilactobacillus*, *Limosilactobacillus*, *Fructilactobacillus*, *Acetilactobacillus*, *Apilactobacillus*, *Levilactobacillus*, *Secundilactobacillus* y *Lentilactobacillus*. Además, propusieron modificar la descripción de la familia *Lactobacillae* para incluir los géneros separados previamente en la familia *Leuconostocaceae*. Por ello, a partir del año 2020, en la literatura científica se observa un cambio en el nombre de especies de BAL aisladas y empleadas, aunque es común encontrar referencias a la clasificación taxonómica antigua.

Entre las LAB, las especies más ampliamente estudiadas por su acción antimicrobiana pertenecen a los géneros *Lactobacillus*, *Pediococcus* y *Leuconostoc*, de acuerdo a la antigua clasificación taxonómica (Dalié et al., 2010). Generalmente, la actividad inhibitoria de las LAB se asocia a los metabolitos sintetizados en el proceso de fermentación, aunque la competición por nutrientes y espacio también es considerado como un mecanismo de acción de estos microorganismos en alimentos (Rouse et al., 2008; Oliveira et al., 2014; Siedler et al., 2019).

La aplicación de LAB como estrategia de biopreservación resulta ventajosa, ya que la Unión Europea ha incluido estos microorganismos en la lista QPS (Calificación de Presunta Seguridad). Además, en los EE. UU., las BAL son generalmente

reconocidas como seguras (GRAS) por la Food and Drug Administration (FDA) (Speranza et al., 2017).

1.4.1 Metabolitos con propiedades antimicrobianas

Las LAB son capaces de sintetizar una gran variedad de metabolitos con acción antimicrobiana como por ejemplo: ácidos orgánicos, ácidos fenólicos, ácidos volátiles, dipéptidos, dióxido de carbono, etanol, peróxido de hidrógeno, reuterina y bacteriocinas (nisina, pediocina, etc.) (Crowley et al., 2013; Santos et al., 2018) (**Figura 4**). El potencial antimicrobiano de las LAB no se debe a la síntesis de un único compuesto metabólico, sino a la probable acción sinérgica entre ellos (Ruggirello et al., 2019). En este contexto, Cortés-Zavaleta et al. (2014) demostraron que la actividad antimicrobiana producida por fermentados de BAL en comparación al uso de estándares comerciales de ácido feniláctico (PLA) no era equivalente, hipotetizando la posible sinergia existente entre los diferentes metabolitos sintetizados por las LAB. Por otra parte, no todas las cepas de BAL poseen el mismo espectro de acción, ya que dependiendo de la cepa ensayada, la producción de metabolitos puede variar considerablemente y, por consecuente, su actividad antimicrobiana (Guimarães et al., 2018).

Los antimicrobianos más importantes y caracterizados en las LAB son los ácidos orgánicos como el ácido láctico y el ácido acético. El ácido láctico es capaz de traspasar la membrana celular en su forma hidrofóbica, descomponerse en el interior y liberar protones (H^+) en el medio. Además, la forma disociada es capaz de colapsar el gradiente electroquímico de protones, llevando finalmente a la muerte celular (Chen, Yan, et al., 2021). Las bacterias heterofermentativas pueden sintetizar ácido acético, y de manera similar al ácido láctico, interacciona con las

membranas celulares neutralizando el potencial electroquímico, aunque su efecto es dependiente de la disminución de pH causado por el ácido láctico (Schnürer & Magnusson, 2005).

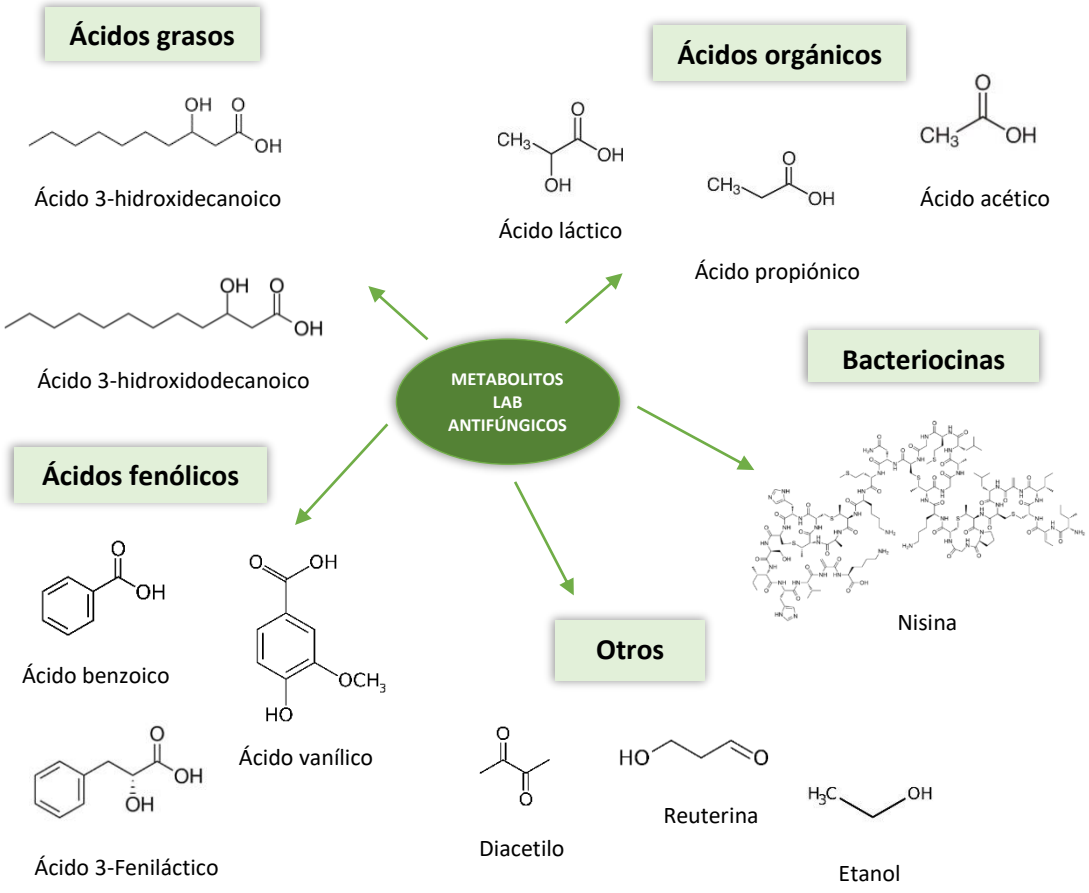


Figura 4. Estructuras químicas de diversos metabolitos antifúngicos producidos por bacterias ácido-lácticas.

Además, una gran variedad de compuestos fenólicos, como el PLA, han sido aislados en medios fermentados por LAB (Brosnan et al., 2012; Lynch et al., 2014).

Estos compuestos han evidenciado múltiples propiedades de interés como acción antioxidante, antiinflamatoria, antifúngica y antitoxigénica (Chtioui et al., 2022; Han et al., 2020; Wang et al., 2012).

Numerosos autores han referenciado que la modificación de los sustratos de fermentación pueden influenciar en las rutas metabólicas de síntesis de compuestos antimicrobianos (Schmidt, Lynch, et al., 2018; Valerio et al., 2016). De forma general, el caldo de cultivo más empleado para la producción de metabolitos antimicrobianos de las LAB es el medio Man-Rogosa-Sharpe (MRS) (Renschler et al., 2020). Sin embargo, el medio MRS no se puede aplicar para la producción de alimentos debido a que parte de sus constituyentes (extracto levadura, peptona y extracto de carne) no se encuentran autorizados como aditivos y, además, su aplicación a nivel industrial es costosa (Kawai et al., 2017). Por ello, se deben estudiar y optimizar otras formulaciones que presenten efecto antimicrobiano para su posible aplicación en los alimentos.

1.4.2 Empleo de LAB como estrategia de biocontrol

Debido al potencial de uso de estos microorganismos por su capacidad de sintetizar una amplia variedad de substancias antimicrobianas y su uso considerado como seguro, se ha estudiado y propuesto su aplicación como agentes de biocontrol en la producción de alimentos. Las LAB se pueden aplicar en los alimentos ya sea mediante la adición directa de las células, sus fermentados, el sobrenadante libre de células (CFS) o los metabolitos purificados (Leyva Salas et al., 2017).

Entre los alimentos en los que se ha evaluado recientemente su aplicación como bioconservantes están las frutas y verduras, productos de panadería y

derivados lácteos. La **Tabla 7** recoge ejemplos de aplicación de LAB en el tratamiento de productos de origen vegetal afectados por hongos.

Tabla 7. Empleo de LAB como agente de biocontrol en productos vegetales.

cepa BAL	Alimento aplicado	Cepa fúngica ensayada	Referencia
Lactobacillus plantarum	Uva	*Aspergillus carbonarius*	Lappa et al. (2018)
Lactobacillus sucicola *Weissella paramesenteroides* *Pediococcus acidilactici*	Naranjas	*Penicillium digitatum*	Ma et al. (2019)
Lactiplantibacillus plantarum	Kiwi	*Botrytis cinerea*	De Simone et al. (2021)
Lactobacillus plantarum	Uva	*Botrytis cinerea*	Marín et al. (2019)
Lactobacillus plantarum TE10	Maíz	*Aspergillus flavus*	Muhialdin et al. (2020)
Lactobacillus plantarum C10	Melón	*Trichothecium roseum*	Lv et al. (2018)
Lactobacillus plantarum CM10	Fresa	*Botrytis cinerea*	Chen et al. (2020)
Lactobacillus plantarum CWXP24 *Lactobacillus plantarum* CKZP13	Naranja	*Penicillium expansum*	Chen, Hong, et al. (2021)

1.5 Natamicina

La natamicina (también denominada pimaricina) es un fungicida perteneciente al grupo de los polienos macrólidos. Con un peso molecular de 665,725 g/mol, la molécula está constituida por un anillo de lactona de 25 átomos de carbono con cuatro enlaces conjugados (cromóforo), unido a una micosamina (m-amino-azúcar) mediante un enlace β-glicosídico (**Figura 5**). Debido a la naturaleza anfiflílica de la molécula, la natamicina es poco soluble en agua (40 µg/mL) e insoluble en

disolventes no polares. Además, el polvo es estable en la oscuridad, pero fotosensible en soluciones acuosas (Aparicio et al., 2016).

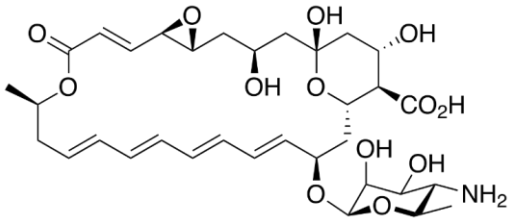


Figura 5. Estructura química de la natamicina.

Es una sustancia producida por la fermentación natural del microorganismo *Streptomyces natalensis* en medios ricos en carbono (almidón o melaza) y nitrógeno (licor de maíz fermentado, caseína) (Lule et al., 2016). Está descrita como una molécula con potente acción antifúngica, ya que es capaz de unirse específicamente con el ergosterol de la membrana celular fúngica. Debido precisamente a la especificidad que posee frente al ergosterol, el cual es un esterol constituyente de las membranas fúngicas, no posee acción frente a bacterias (Aparicio et al., 2004).

Su mecanismo de acción, a diferencia de otros polienos macrólidos, no consiste en permeabilizar la membrana fúngica, sino que interfiere en el correcto funcionamiento del ergosterol y por consecuente, afecta en múltiples funciones celulares como la endocitosis, la exocitosis, la fusión vacuolar, la polaridad y la

morfogénesis celular (Te Welscher et al., 2008; Te Welscher et al., 2010; Te Welscher et al., 2012).

1.5.1 Aplicación de la natamicina como agente antifúngico

La aplicación de la natamicina se ha estudiado tanto a nivel terapéutico como para su aplicación en alimentos. El principal uso de la natamicina en clínica es el tratamiento de micosis oftalmológicas, ya que posee un gran espectro de acción frente a hongos patógenos (Cevher et al., 2008).

En Europa, su uso como aditivo alimentario se encuentra regulado en el Reglamento 1129/2011 de la Comisión, de 11 de noviembre de 2011. Tiene asignado el número E-235, siendo su uso aprobado para el tratamiento superficial de queso semicurado, blando y embutidos curados secos a un nivel máximo de 1 mg/dm^2 en los 5 mm superficiales del producto. Tiene el estatus GRAS por la FDA y, además, en el año 2009, la Autoridad Europea de Seguridad Alimentaria (EFSA) emitió una Opinión Científica sobre el uso de natamicina como aditivo alimentario, concluyendo que a los niveles aplicados no suponía un problema de seguridad alimentaria si únicamente se empleaba para el tratamiento superficial de determinados productos (Koenig et al., 2009).

Su aplicación en alimentos presenta ciertas ventajas, ya que es una molécula que queda retenida en la superficie del alimento y no es capaz de migrar al interior. Al no poseer acción antimicrobiana frente a bacterias, se puede emplear en alimentos que requieren de una fermentación bacteriana en su proceso de fabricación, como por ejemplo en embutidos curados y derivados lácteos. Además, es una molécula que no afecta a las propiedades organolépticas del alimento (sabor, textura y color) (Delves-Broughton, 2008; Meena et al., 2021).

2. OBJECTIVES

Objetivos

2. OBJETIVOS

El **objetivo general** del presente trabajo es la aplicación de ingredientes de origen natural, principalmente mostaza y metabolitos de bacterias, para reducir la presencia de hongos toxigénicos y micotoxinas en alimentos.

Para ello, se han planteado los siguientes **objetivos específicos**:

1. Evaluar el uso de la harina de mostaza oriental integrada en una salsa para liberar AITC en un sistema de envase activo, con la finalidad de incrementar la vida útil y la reducción de síntesis de Ocratoxina A en pan de pita contaminado con *Penicillium verrucosum*, así como medir la evolución del compuesto antimicrobiano durante el almacenamiento.
2. Caracterizar la actividad antifúngica de los extractos de salvado y semilla de mostaza amarilla (*Sinapis alba*) y estudiar la aplicación del salvado de mostaza como ingrediente antifúngico en la elaboración de pan de molde contaminado con *Penicillium commune*.
3. Evaluar la actividad antifúngica de los extractos fermentados de mostaza amarilla y oriental con bacterias ácido-lácticas, analizar los compuestos antifúngicos presentes y aplicar el sobrenadante libre de células como estrategia de biocontrol en maíz contaminado con *Fusarium verticillioides*, para reducir la síntesis de Fumonisina B_1 y B_2.
4. Caracterizar la fracción volátil e identificar los compuestos con propiedades bioactivas de las harinas y salvados de mostaza amarilla (*Sinapis alba*) y oriental (*Brassica juncea*).
5. Evaluar la actividad antifúngica de la natamicina y estudiar su aplicación como agente de tratamiento del queso mozzarella contaminado con *Penicillium commune*, mediante pulverizado directo o incorporación en un

biopolímero de hidroxietulcelulosa, así como estudiar la evolución del compuesto en la superficie durante el almacenamiento.

2. OBJECTIVES

The **general objective** of the present work is the application of natural ingredients, mainly mustard and bacteria metabolites, in order to reduce toxigenic fungi and mycotoxins in food.

To achieve this, the following **specific objectives** have been established:

1. Evaluate the use of oriental mustard flour integrated into a sauce in order to produce AITC in an active packaging system, with the objective to increase shelf-life and reduce the Ochratoxin A synthesis on pita bread contaminated with *Penicillium verrucosum*, and study the antimicrobial compound evolution during storage.
2. Characterize the antifungal properties of yellow mustard (*Sinapis alba*) seed and bran extracts and study the application of bran mustard as an antifungal ingredient in the manufacture of bread contaminated with *Penicillium commune*.
3. Evaluate the antifungal activity of yellow and oriental fermented mustard extracts with lactic acid bacteria, characterize the antifungal compounds, and apply the cell-free supernatant as a biocontrol strategy in maize contaminated with *Fusarium verticillioides* to reduce Fumonisin B_1 and B_2 synthesis.
4. Characterize the volatile fraction and identify the compounds with bioactive properties from the flour and bran of yellow mustard (*Sinapis alba*) and oriental mustard (*Brassica juncea*)
5. Evaluate the antifungal activity of natamycin and study its application as a treatment on mozzarella cheese contaminated with *Penicillium commune* through direct spray or by incorporation on a hydroxyethylcellulose biopolymer and study the evolution of the compound on the food surface during storage.

3. RESULTS

Resultados

Molecules, 24, 1019 (2019)

3.1 Development of a Bioactive Sauce Based on Oriental Mustard Flour with Antifungal Properties for Pita Bread Shelf Life Improvement

1. *Introduction*

Bread is a staple food consumed around the world and, like other perishable products, is susceptible to fungal contamination. Therefore, the fungal spoilage is a concern on bakery industry, representing a significant source of economic losses and a potential risk to human health due to the production of mycotoxin by toxigenic fungi, mainly from genus *Aspergillus* and *Penicillium* (Smith et al., 2004). Bread possesses a relatively high-water activity (a_w 0.94-0.97) with a pH ranging from approximately 7 to 8.6 (Legan, 1993). These properties are favorable for the germination and growth of a wide range of molds. The loaves of bread have a higher probability of fungal growth since they are commonly sliced. This process increases the surface area for microbial contamination; moreover, the slicing machine can be a vector of spoilage agents (Saladino et al., 2017).

Microbial spoilage reduces the shelf life of food and compromises the safety of consumers with the consequent economic loss for the industry. In this context, synthetic additives are widely used in the food industry and play an important role in the preservation of food quality as well as inhibiting the growth of spoilage and pathogenic microorganisms (Tajkarimi et al., 2010). Nowadays, consumers demand a reduction of synthetic additives in food due to the concern of the effect of these substances on health. For this reason, the increment in the food of antimicrobial substances from natural sources may be an alternative to increase the shelf life and safety of products, satisfying the consumer requirements (Davidson & Harrison, 2002).

A healthy diet should include vegetables of *Brassica* genus such as broccoli, Brussels sprouts, cabbage or cauliflower. These vegetables are health-promoting due to the high concentration of ascorbic acid, selenium, soluble fiber and glucosinolates (GTS). Among these compounds, the GTS are secondary metabolites

that have been extensively studied in the past decades. Depending on their side chain can be classified in aromatic, indolic or aliphatic compounds (Fahey et al., 2001). After tissue damage, the GTS is hydrolyzed by enzymes denominated myrosinases. These enzymes are stored separately from GTS in vacuoles inside the plant cell (Kissen et al., 2009). After the tissue disruption, the myrosinase cleaves the thioglucosides releasing an unstable aglycone named thiohydroximate-O-sulphonate. This substance spontaneously rearranges in various products such as isothiocyanates (ITCs), nitriles, organic thiocyanates or epithionitriles depending on the pH of the medium, the presence of specific proteins and particular structural prerequisites (Hanschen et al., 2017).

The ITCs are unique in comparison to other essential oils, since they are formed only when the plant cell undergoes some type of injury such as insect bite, grinding, milling or fungi contamination, in addition to the presence of water (Luciano & Holley, 2009). Consequently, ITCs are not present in mustard flour, being formed slowly after the addition of water. The antifungal activity of ITCs has been widely studied in the last decade due to its biocidal activity against microorganisms, including bacteria (Borges et al., 2015), fungi (Tracz et al., 2017), nematodes (Giarratana et al., 2015) and insects (Paes et al., 2012). The mechanisms of action of ITCs are not clearly described but within the cell, ITCs react with nonspecific molecules in the plasm such as saccharides, amino acids, proteins, and lipids, essential components for maintaining life (Clemente, Aznar,& Nerín, 2016).

The main ITC generated by the hydrolysis of the glucosinolate sinigrin give rise of allyl isothiocyanate (AITC), which has shown antimicrobial activity at low concentration against pathogenic microorganisms (Clemente, Aznar, Silva, et al., 2016; Lin, 2000).

The employment of AITC as an antimicrobial compound from natural sources has been analyzed in several perishable food matrices like tuna meet (Hasegawa et al., 1999), pork sausage (Bortolotto et al., 2018), chopped beef (Chacon et al., 2006), dry-cured ham (Graumann & Holley, 2007) and cheese (Winther & Nielsen, 2006).

The purpose of this study was to elaborate an active packaging system containing a packaged sauce of oriental mustard flour (OMF) to generate allyl isothiocyanate (AITC) in order to prevent the *Penicillium verrucosum* growth and ochratoxin A production in pita bread. In addition, the kinetics of volatilization and residual concentrations of AITC in the food matrix were evaluated to know the evolution of the antimicrobial compound during the storage period.

2. Results and Discussion

2.1 Residual Concentration of AITC in Plastic Tray Headspace and Pita Bread

The addition of OMF as an ingredient in the sauce allowed the hydrolysis of sinigrin to generate AITC. Consequently, the AITC was volatilized reducing the fungal growth of the *P. verrucosum* in pita bread. The samples were studied using four different OMF concentrations: 8, 16, 33 and 50 mg/g. In particular, the concentration of AITC released by the sauce in the headspace of the plastic tray was proportional to the quantity of mustard flour used in the sauce formulation (**Figure 1**). After 1 h of incubation, the AITC detected in the headspace ranged from 1 to 7 mg/L to the treatments of 8 and 50 mg/g, respectively. These concentrations increased up to 8 h of incubation, which was the point time with a maximum concentration of AITC in the headspace, reaching values of this antifungal compound ranging from 2.3 to 12.9 mg/L for the treatments of 8 and 50 mg/g of

OMF, respectively. As showed in **Figure 1**, after 8 h, the AITC detected in the plastic tray decreased up to obtaining constant concentrations at day seven of incubation that ranged from 0.4 to 1.1 mg/L of AITC. It is important to emphasize that the concentrations of AITC detected during all the storage period of the pita bread and considering all the quantity of the oriental mustard flour used for the sauce development are close to the inhibitory concentration referenced by Lopes et al. (2018) against *P. nordicum* (0.25, 0.5, 1 and 2 mg/L).

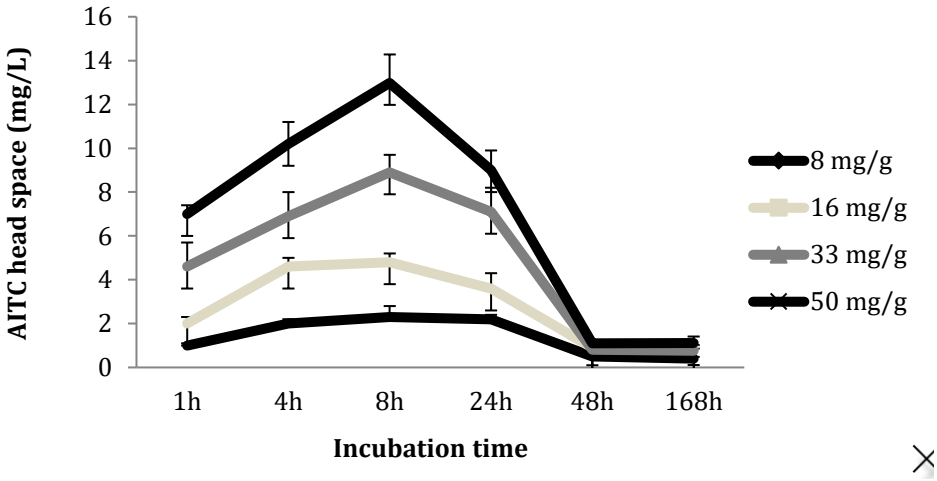


Figure 1. Volatilization kinetics of the AITC contained in the plastic trays produced through the sinigrin conversion obtained from 8, 16, 33 and 50 mg/g of oriental mustard flour incorporated in the bioactive sauce.

Related with the amount of the AITC absorbed by the pita bread during the storage period of evaluation, the results of this determination are shown in **Table 1**. In particular, at 7 days of incubation, the concentration of the AITC detected on the bread ranged from 4.3 to 59.3 mg/kg depending by the dose of the OMF used for the sauce formulation. Analyzing the amount of this antifungal compound after

the storage period stipulated in this study and in particular, after 14 days, it is possible to observe that the AITC was detected just in the bread treated with 33 and 50 mg/g of the OMF with concentrations ranging from 15.9 to 25.6 mg/kg, respectively. These concentrations observed at day 14 were lower in comparison with those detected at day 7, probably due to that the sinigrin present in the OMF could be hydrolyzed in other substances with less antimicrobial activity such as thiocyanates and nitriles (Meca et al., 2012). In addition, the AITC generated by hydrolysis of sinigrin can react with group amino of several peptides in the matrix to form a conjugate and reduce its concentration (Cejpek et al., 2000).

Table 1. AITC adsorbed (mg/kg) by the pita bread, treated with different concentration of oriental mustard flour sauce contained in a bioactive sachet and introduced inside the bread packaging.

Treatment	Day		
	0	**7**	**14**
Control	nd	nd	nd
8 mg/g	nd	4.3 ± 1.9^{a}	nd
16 mg/g	nd	9.5 ± 0.2^{a}	nd
33 mg/g	nd	48.3 ± 6.0^{b}	15.9 ± 3.0^{a}
50 mg/g	nd	59.3 ± 15.1^{b}	25.6 ± 3.1^{b}

Different letters show significant differences among treatments ($p \leq 0.05$); nd= no detected

The EFSA (European Food Safety Authority) describes the safety of AITC when used as food preservative or active packaging (EFSA, 2018). Considering the weight of our product (40 g) and the concentration of AITC adsorbed at day 7, the treatments of 33 and 50 mg/g showed a total concentration of 1.93 and 2.37 mg of AITC, respectively. These values were higher that the established by the EFSA for the acceptable daily intake of 0.02 mg/kg/BW/day. However, the exposition of AITC

by adult consumers from different sources could exceedance from 2 to 8 times the total daily exposure of AITC without toxic effects demonstrated. After day 7, the total concentration of AITC gradually decreased and all treatments showed values above the 0.02 mg/kg/BW/day.

Nielsen & Rios (2000) evaluated the sensorial effect of AITC in bread packed. The sensorial test was realized with the untrained panel and the results suggested that the judges did not identify doses above 2 mg of AITC. Therefore, after 7 days of storage, the AITC could be undetectable to the human palate.

2.2 Shelf Life Improvement of Pita Bread Contaminated with P. verrucosum and OTA Production

Related with the shelf life results of pita bread contaminated with *P. verrucosum* and treated with the bioactive sauce elaborated with different quantities of OMF, the data is showed in **Table 2**. In particular, in the control group, a visible growth of the *P. verrucosum* was detected at day three of incubation, whereas the commercial control formulated with calcium propionate presented a visible fungal growth at day five with an increase of the bread shelf life in comparison with the control of 2 days. The pita bread treated with the bioactive sauce prepared with 8 mg/g of the OMF presented a shelf life of 4 days, whereas the application of 16 mg/g of OMF in the sauce obtained the same shelf life of the pita bread with commercial control (E-282 additive). The pita bread exposed to the sauce containing 22 mg/g of OMF presented an increase of the shelf life in comparison with the commercial control and control of 3 and 1 days, respectively. Using the OMF in the quantity of 50 mg/g for the antifungal sauce formulation, any

visible *P. verrucosum* growth has been observed on the pita bread during the incubation period analyzed.

Table 2. The shelf life of pita bread, stored in a plastic tray, contaminated with *P. verrucosum* and treated with 8, 16, 33 and 50 mg/g of oriental mustard flour sauce, in comparison to the control experiment and the pita bread treated with the preservative compound E-282.

Treatment	Days				
	3	**4**	**5**	**6**	**7**
Control	+	+	+	+	+
E-282	−	−	+	+	+
8 mg/g	−	+	+	+	+
16 mg/g	−	−	+	+	+
33 mg/g	−	−	−	+	+
50 mg/g	−	−	−	−	−

(+) Positive growth; (−) Negative growth.

The results of visible fungal growth on the pita bread exposed to the AITC vapors generated by sinigrin conversion are according to the microbial population in the bread after 7 days of incubation (**Figure 2**). In particular, as shown in **Figure 3**, the control experiment presented 8.3 log CFU/g, whereas the bread produced with the E-282 additive, evidenced fungal contamination of 6.8 logs CFU/g, 1.5 logs lower that the data observed in the control experiment. The application of 8 mg/g of the OMF did not produce any significant decrease in the fungal population when compared to the control experiment and with the bread baked with E-282, whereas using 16, 33 and 50 mg/g of the OMF, the log of the CFU/g of *P. verrucosum* detected was 6.8, 5.2 and 2.6, respectively. The log reduction of *P. verrucosum* for the treatments of 16. 33 and 50 mg/g was 1.5, 3.1 and 5.7, respectively, when compared to the control group.

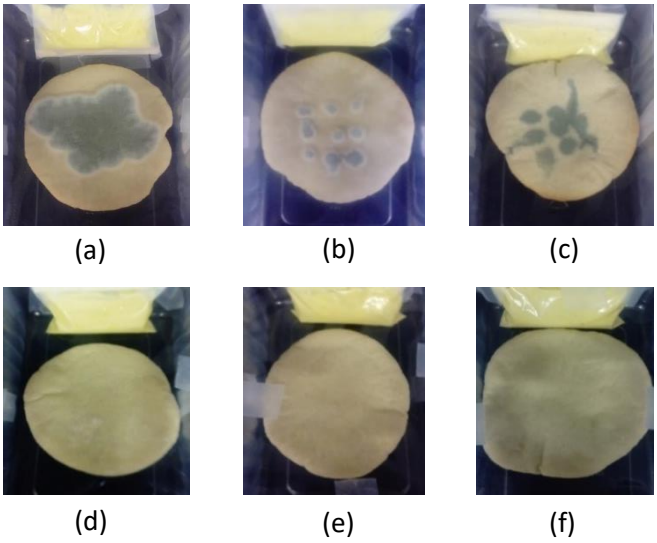


Figure 2. Pita bread inoculated with *P. verrucosum* and stored during 7 days in a plastic tray containing a small sachet sealed with (a) control experiment; (b) pita bread treated with the preservative compound E-282; (c) 8 mg/g; (d) 16 mg/g; (e) 33 mg/g; and (f) 50 mg/g of oriental mustard flour sauce.

Clemente et al. (2017) analyzed the use of benzyl isothiocyanate (BITC) incorporated in an active packaging against *A. ochraceus*. The use of BITC in vapor phase inhibited the fungal growth by promoting morphological alteration and cellular damage depending on the contact with the antifungal compound. Likewise, the use of AITC reduced the fungal growth of *A. flavus* over time. These results suggested that AITC could also generate cell damage.

Considering that the strain of the *P. verrucosum* used in this study is OTA producer, the data related to the production of this toxic compound in pita bread is plotted in **Figure 4**. In particular, after 7 days of incubation, the control experiment presented 9.1 µg/kg of OTA, whereas, on the pita bread baked with the preservative compound E-282, the concentration of this toxic compound detected

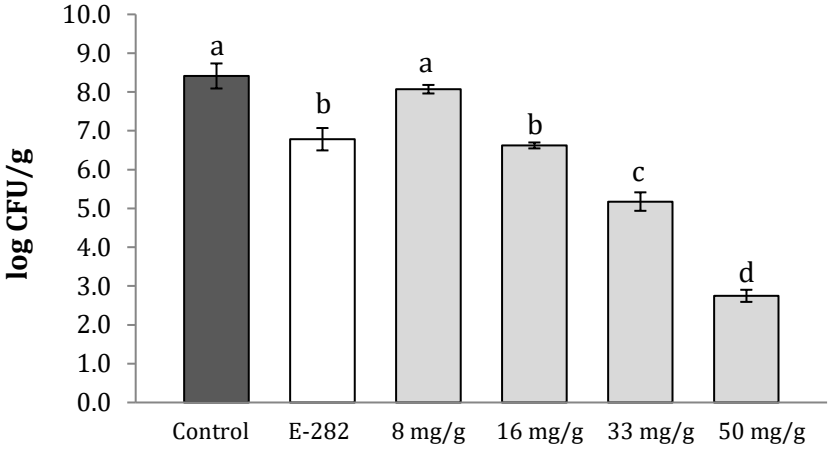


Figure 3. *P. verrucosum* growth in pita breads stored for 7 days in a plastic tray containing a small sachet sealed with 8, 16, 33, and 50 mg/g of oriental mustard flour sauce. Different letters show significant differences among treatments ($p \leq 0.05$).

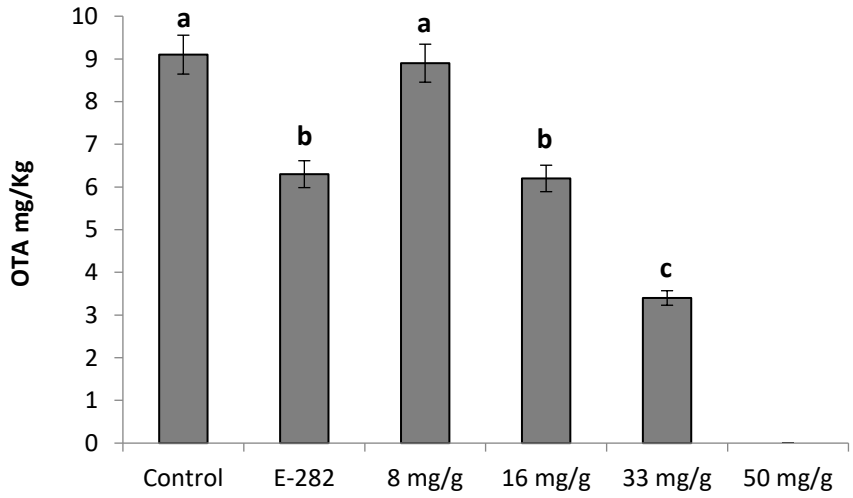


Figure 4. OTA produced by *P. verrucosum* growth in pita breads stored during 7 days in a plastic tray containing a small sachet sealed with 8, 16, 33 and 50 mg/g of oriental mustard flour. Different letters show significant differences between treatments ($p \leq 0.05$).

was 6.3 μg/kg. In the pita bread sealed in the plastic tray and treated with bioactive sauce with 8 mg/g of OMF, no statistically differences on the OTA production was detected in comparison to the control group. On the other hand, the treatments of 16 and 33 mg/g of the OMF showed 6.2 and 3.4 μg/kg of OTA in the bread on day 7, respectively. These values are significantly different from the control group, respectively.

All treatments demonstrated values of OTA higher than 3.0 μg/kg stipulated by CE (1881/2006) for processed cereal products. However, with the increment of 50 mg/g of the OMF in the sauce was able to avoid the OTA production to not detected levels, showing an antifungal and anti-mycotoxigenic potential of the OMF as an ingredient in the sauce.

In the study of Quiles et al. (2015a), active packaging containing AITC or OMF were evaluated as preservatives in pizza crust inoculated with *Aspergillus parasiticus*, an aflatoxin producer. The inhibition of *A. parasiticus* growth was detected after 30 days with the employment of gaseous AITC at 5 mL/L and 10 mL/L, sachets containing 5 mL/L and 10 mL/L of AITC and OMF sachet elaborated with 850 mg + 850 mL of water. These authors demonstrated that the application of OMF sachet at 850 mg + 850 mL of water could completely avoid the aflatoxins (AFs) production. These results corroborate with our data since the implementation of OMF in concentrations higher than 50 mg can reduce mycotoxin production by fungal inhibition.

Quiles et al. (2015b) investigated the production of AFs (B_1, B_2, G_1, G_2) by *A. parasiticus* in wheat tortillas treated with yellow and oriental mustard flour incorporated into an active packaging system. The treatment consisted of 0.1, 0.5 and 1 of yellow or oriental mustard flour mixed with 2 mL of water, with a storage period of 1 month. The AITC generated from the sinigrin hydrolyzed in OMF and the

p-hydroxybenzyl isothiocyanate (*p*-HBITC) obtained from yellow mustard flour inhibited the mycotoxin production. However, the treatments with OMF were more effective. With the employment of 1 g of OMF, a reduction of 90% in AFs B_1, B_2, G_1, and G_2 production was achieved, while *p*-HBITC demonstrated an average of 17.7 to 45.2% on mycotoxin reduction.

Saladino, Bordin, et al. (2016) evaluated the antimycotoxigenic capacity of OMF employed in piadina at concentrations ranging from 0.1 to 1 g in active packaging. The results demonstrated that OMF reduced the *A. parasiticus* growth by 12.2% to 80.6%. In addition, the ITC generated in active packaging avoided the AFs synthesis by 60.5% to 89.3%, depending on the quantity of the mustard flour used.

Saladino, Quiles, et al. (2017) studied the mycotoxin reduction and the fungal growth inhibition of *A. parasiticus* in loaf bread by the employment of allyl, benzyl, and phenyl (PITC) isothiocyanates. Treatments consisted in paper filters or small plastic bags with paper filters soaked with AITC, BITC or PITC at three different concentrations (0.5, 1 and 5 mL/L) and introduced into a plastic tray with the loaf bread. The study evidenced an increase of the shelf life with the employment of 5 mL/L of AITC. In addition, this treatment showed the highest reduction of the AFs content (above 60%).

3. Materials and Methods

3.1 Chemicals and Microorganisms

OTA (purity >99%), formic acid (analytical grade, purity >98%), ammonium formate (analytical grade, purity ≥99.0%) and calcium propionate (E-282) were purchased from Sigma Aldrich (St. Louis, MO, USA). Methanol (LC-MS grade, purity

≥99.9%) was obtained from Fisher Scientific (Hudson, NH, USA). Deionized water (<18 MΩ cm resistivity) was acquired from a Milli-Q Millipore water purification system (Massachusetts, United States). Culture media potato dextrose agar (PDA), potato dextrose for broth (PDB) and buffered peptone water were purchased from Liofilchem Bacteriology Products (Roseto, Italy). The strain of *Penicillium verrucosum* VTT D-01847 was obtained from the VTT Technical Research of Finland LTD (VTT, Otaniemi, Finland). The microorganism was maintained in sterile glycerol at −80 °C. Then, it was recovered in PDB broth at 25 °C for 48 h prior to use.

3.2 Samples Preparation and Antifungal Treatment

The pita bread recipe included 250 g of wheat flour, 2.5 g of sugar, 5 g of NaCl, 15 g of yeast bakery products (Levital, Spain), 10 mL of olive oil and 125 mL of warm water. Briefly, all ingredients were placed in a recipient mixed and kneaded manually for 15 min. Then, the dough produced was fermented for 40 min at room temperature. Posteriorly, the dough was divided into 9 portions of 40 g, flattened, and baked at 180 °C for 5 min using a deck oven (MIWE, Arnstein, Germany). Each pita bread was inoculated with 100 µL of a 0.1% buffered peptone water suspension containing 4×10^5 conidia/mL of *P. verrucosum* (VTT D-01847) in 9 equidistant points. The control group did not receive treatment and, the commercial control was prepared with 2 g/kg of calcium propionate (E-282), which is a common preservative granted as an ingredient in bakery products (EC 1333/2008).

The conidial suspension was previously determined as described by Kelly et al. (2006). The conidia were harvested from the potato dextrose agar plates with sterile water and scraping the colonies. Then, the spore concentration was

measured by optical density at 600 nm and adjusted in a buffered peptone water medium.

The sauce was elaborated with 125 g of yogurt, 10 mL of mustard sauce and 10 mL of honey. In addition, the antifungal sauce was produced adding OMF at the doses of 8, 16, 33 and 50 mg/g to the basic ingredients. To improve the conversion of the glucosinolate sinigrin into AITC, OMF was previously mixed with 2 mL of distilled water and then added to the sauce formulation. Each plastic bag contained 30 g of sauce and was closed hermetically.

A commercial control and a control group were used for each set of assays, being packaged with sauce without OMF incorporated in the formulation. The sauces were packaged with bread in polyethylene trays, closed hermetically and incubated during 7 days at room temperature. During the storage period, the visible fungal growth was evaluated to establish the effect of treatments in the shelf life of the pita breads. After the incubation period, the sampling was carried out to determine the fungal population and to quantify the OTA production. All analyses were carried out in triplicate (n=9).

3.3 Ochratoxin A Extraction

OTA extraction was carried out using the method described by Saladino, Bordin, et al. (2017) with some modification. The samples of pita bread (40 g) were finely crushed in a grinder (Oster, Valencia, Spain). Posteriorly, 5 g of the sample was weighed in Falcon tubes (50 mL) containing 25 mL of methanol and homogenized employing an Ultra Ika T18 basic Ultraturraz (Staufen, Germany) at 10000 rpm for 3 min. Then, the extracts were centrifuged at 4 °C with a rotation of 4000 rpm for 5 min, and the supernatant was recovered and evaporated using a

Büchi Rotavapor R-200 (Postfach, Switzerland). The residue obtained was resuspended in 5 mL of methanol, transferred to a 15 mL falcon tube, and evaporated with gaseous nitrogen at 35 °C in a Turbovap Evaporator (Zymark, Hopkinton, MA, USA). The dry extract obtained was recovered with 1 mL of methanol (100%) filtered through a 0.22 µm syringe filter, transferred to a glass vial, and injected into an LC-MS/MS system.

3.4 Ochratoxin A Identification and Quantification by LC-MS/MS

OTA analyses were performed using a liquid chromatograph system Agilent 1200 chromatograph (Agilent Technologies, Palo Alto, CA, USA) associated to a mass spectrometer 3200 QTRAP (AB Sciex, Foster City, CA, USA) and equipped with an interface of electrospray ionization (ESI). The software used to process the data was Analyst version 1.5.2 for Windows. The stationary phase was an analytical column of reversed phase (Gemini C18 column, 150 × 2mm × 3 µm) (Phenomenex, Madrid, Spain). The mobile phases employed as eluents consisted of water 0.1% formic acid with 5mM ammonium formate (Sigma-Aldrich, St. Louis, MI, USA) (A) and methanol with 5 mM ammonium formate and formic acid at 0.1% (B). Elution gradient was established as follows. The initial condition was 10% B increasing to 80% in 1.5 min and kept up to 2.5 min. Afterwards, eluent B was increased to 90% in 6 min and later 100% was obtained in 4 min. Posteriorly, the initial conditions (B: 10%) were reestablished for 5 min. The flow rate was set at 0.25 mL/min in all steps. The injection volume was 20 µL. MS/MS analysis was obtained applying the following parameters: the ion spray voltage at 5500 V; source temperature, 450 °C; curtain gas, 20 psi; the ion source gas 1 or sheath gas at 50 psi; the ion source gas 2 or drying gas at 55 psi. The gas used for nebulization and collision was nitrogen.

The precursor-to-product ion transition was *m/z* 404.3/102.1-404.3/239.0 and 404.3/358.1 for OTA.

A calibration curve was previously prepared with standards of OTA at concentrations ranging from 0.1 to 100 µg/L. The peak areas obtained in the standard curve were compared to the values found in the samples to quantify the OTA production.

3.5 Shelf Life and Determination of the Fungal Population

The breads contained in the polyethylene trays were examined daily to determine their shelf life. When breads showed a visible sign of fungal growth, the shelf life was closed, considering that consumers would eventually reject the product (Dal Bello et al., 2007).

The determination of the fungal population was performed after 7 days of incubation. The breads (40 g) were transferred to sterile plastic bags containing 160 mL of sterile peptone water (0.1%) and shaken with a stomacher (IUL, Barcelona, Spain) for 40 s. The mixture obtained was serially diluted in sterile 15 mL plastic tubes and then aliquots of 100 µL of each dilution were placed in PDA plates and incubated at 25 °C for 3 days.

3.6 Extraction and Analysis of AITC in Pita Bread

The AITC extraction and quantification from pita bread were carried out as described by Nazareth et al. (2018) with some modifications.

Each pita bread (40 g) was added to hermetic tubes of 150 mL containing 80 mL of methanol. The mixture was extracted for 30 min in a water bath at 40 °C and

10 min in an ultrasonic bath. Then, the extract was centrifuged at 4000 x *g* for 5 min at 20 °C. The supernatant was recovered and filtered through a nylon membrane filter (0.22 µm), and an aliquot of 10 µL was injected into a gas chromatograph.

The residual AITC absorbed by samples was quantified using a gas chromatograph (GC) coupled to a flame ionization detector (FID) (GC 6890, Agilent Technologies Inc., Santa Clara, CA, USA), equipped with a fused capillary column (CP-SIL 0.25mm x 30m) (Varian, Middelburg, Netherlands). The inlet temperature was set at 200 °C with 250 °C of detector temperature. H_2 at 5 mL/min was the carrier gas, and the FID gasses were H_2 at 40 mL/min, and purified air at 450 mL/min. The temperature program was a gradient when the initial temperature was 60 °C for 1 min, increased at 8 °C/min up to 100 °C and held for 5 min, then the temperature was raised at 15 °C/min up to 200 °C, the time of analysis was 16.6 min per sample.

Identification and quantification of AITC was carried out by comparing the sample areas with the point standards curve (1–100 mg/L).

3.7 Headspace Analysis of AITC

The AITC evaporated in the headspace of the plastic trays was determined during the storage period (7 days), injecting 100 mL of the headspace through a septum applied in the tray cover in a GC system. GC parameters employed for the identification and quantification of AITC were identical as described for the determination of AITC in the food matrix in paragraph 3.6. Three replicates were carried out for each test condition.

3.8 Statistical Analysis

GraphPad Prism version 3.0 (GraphPad Software Inc., La Jolla, CA, USA) was used for the statistical analysis of data. Differences among groups were realized using analysis of variance ANOVA followed by Tukey's multiple comparison tests. Statistical differences were considered significant if $p \leq 0.05$.

4. Conclusions

In summary, this paper showed the capacity of AITC released from a packaged bioactive sauce elaborated with OMF to reduce the growth of the toxigenic fungi *P. verrucosum* in pita bread. The results demonstrated that the employment of this ingredient reduces the production of OTA by the fungi with a dose depending effect. In conclusion, OMF could be potentially used as a precursor of antimicrobial substances against *Penicillium* species, commonly found as contaminants in bakery products. Considering the current trend of reducing the presence of synthetic chemical additives in food, the employment of natural ingredients that releases antimicrobial substances such as OMF may be an alternative to food preservation rather than traditional additives.

Foods, 10, 431 (2021)

3.2 Application of White Mustard Bran and Flour on Bread as Natural Preservative Agents

1. Introduction

Bakery products are subject to various spoilage problems, such as chemical, physical, and microbial (Smith et al., 2004). Bacteria, fungi, and yeast can cause spoilage of bread due to its relatively high-water activity and alkaline pH. These properties are suitable for the growth of a wide range of fungal species. Indeed, fungal spoilage is the primary concern in the bakery industry. Contamination occurs predominantly after baking, by fungal spores from the environment that settle on food (Axel et al., 2017). The genera *Penicillium* and *Aspergillus* are the most common fungi responsible for the deterioration of bread (Garcia et al., 2019).

In addition to the fungal spoilage, these microorganisms can also produce mycotoxins (Lee & Ryu, 2017; Russo et al., 2017; Vaclavikova et al., 2013). Mycotoxins are highly toxic metabolites produced by various fungal species in certain foods, including bread. Among them, Zearalenone, Ochratoxin A, and Aflatoxin B1 are mycotoxins with significant occurrence in wheat and wheat products worldwide (Alexa et al., 2013; Almeida-Ferreira et al., 2013; Iqbal et al., 2014; Škrbić et al., 2012; Vidal et al., 2013). These molecules are of concern to human health because of their toxicological properties and the capacity to maintain in food even after thermal processing. For instance, Aflatoxin B1, Ochratoxin A, and Zearalenone have been associated with hepatotoxicity, nephrotoxicity and hyperestrogenism (Edite Bezerra da Rocha et al., 2014; Thanushree et al., 2019). Besides, based on recent reports, mycotoxin prevalence could be as high as 60-80% in food (Lee & Ryu, 2017; Nazareth et al., 2019).

The consumption of bakery products has changed in recent decades due to the growing alert of consumers about health and environmental issues that have led to an increase in demand for whole food and food products without chemical additives or preservatives (Elsanhoty et al., 2013). Accordingly, new products have

been introduced to the market, and food preservation has become increasingly complex. These products require more extended shelf life and a more generous guarantee of the absence of foodborne pathogenic organisms, as well as a reduction in the application of chemical preservatives (Gavahian et al., 2020). Such requirements offered new challenges and opportunities for those who have sought new food preservatives.

Nowadays, the overuse of chemical additives has increased the pressure on food manufacturers to remove chemical preservatives from their food products (Le Lay et al., 2016). Against this background, natural origin substances may be an excellent alternative for preserving these products since the search for new chemical substances is hampered by regulatory restrictions; hence, much time and money may be needed to develop a newly approved preservative that satisfies the public pressure against chemical additives.

White mustard (*Sinapis alba*) belongs to the *Brassicaceae* family. These plants are known for the high concentration of glucosinolates, which play an important role in plant defense. A plant injury activates the glucosinolate-myrosinase system, leading to enzymatic hydrolysis of glucosinolates by myrosinase. This reaction culminates in isothiocyanates (ITC) that have been recognized for their human nutrition benefits (Grubb & Abel, 2006). The ITC are also molecules with known fungicidal, bactericidal, and insecticidal activity that stand out for their antifungal effects (Bahmid, Heising, et al., 2020; Nazareth et al., 2019; Quiles et al., 2019; Vig et al., 2009). In white mustard, the hydrolysis of the glucosinolate sinalbin generates the ITC *p*-hydroxybenzyl isothiocyanate (*p*-HBIT), which has significant antimicrobial activity against bacteria and fungi (Ekanayake et al., 2006).

The commercial preparation of mustard seed flour (MF) produces many by-products, called mustard bran (MB). The MB is the outer husk removed from

mustard during milling. On the one hand, MB represents a valuable by-product because it is rich in proteins (16% tw), hydrophilic polysaccharides, high-molecular-weight polysaccharides, and a significant antioxidant activity provided by uronic acid (Donsì & Velikov, 2020). On the other hand, the MB is costly to eliminate and could be deleterious to the environment; therefore, using MB as a natural antifungal ingredient can be an alternative to alleviate this problem.

According to current knowledge, the application of mustard by-products as an antifungal compound was not studied. Therefore, the objective of this study was to evaluate the antifungal potential of MF and MB against toxigenic strains of the genera *Aspergillus*, *Fusarium*, and *Penicillium*. Accordingly, we proposed a method of extraction of water-soluble components to assess their antifungal activities in vitro. Furthermore, we determined the antifungal compound stability by incubating the extracts at different temperatures and storage times. Finally, MB was employed in dough formulation to increase the shelf life and to avoid the growth of *P. commune* CECT 20767 on the bread.

2. Materials and methods

2.1. Chemicals

The microbiological products such as Potato Dextrose Broth (PDB), Potato Dextrose Agar (PDA), and Buffered Peptone water were obtained from Liofilchem Bacteriology Products (Roseto, Italy). The deionized water was obtained from a Milli-Q water purification system. The TWEEN® 80 and the sodium propionate (E-281) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The white mustard seed (product code 501) and the white MB (product code 412) were purchased from G.S. Dunn (Hamilton, ON, Canada).

2.2 Fungal Strains and Culture Conditions

The toxigenic fungal strains *P. camemberti* CECT 2267, *P. expansum* CECT 2278, *P. roqueforti* CECT 2905, *P. digitatum* CECT 2954, *P. commune* CECT 20767, *P. solitum* CECT 20818, *A. parasiticus* CECT 2681, *A. ochraceus* CECT 2093, *A. lacticoffeatus* CECT 20581, *A. steynii* CECT 20510, *A. tubingensis* CECT 20543 and *A. tubingensis* CECT 20544 were purchased from the Spanish Type Culture Collection CECT (Valencia, Spain). *A. flavus* ITEM 8111, *F. verticillioides* ITEM 12052, *F. proliferatum* ITEM 12072, *F. verticillioides* ITEM 12044, *F. graminearum* ITEM 126, *F. sporotrichioides* ITEM 12168, and *F. poae* ITEM 9151 were obtained from the Institute of Sciences of Food Production ISPA (Bari, Italy). The strain *P. verrucosum* VTT D-01847 was purchased from the VTT Technical Research Centre of Finland LTD (Otaniemi, Finland). The fungal strains were preserved in liquid PDB with 30% glycerol. Then, the strains were thawed and inoculated in PDB medium for one week at 25 °C. Afterward, the mycelium was transferred to a PDA plate and incubated again for five days at 25 °C. These PDA plates were used to harvest the fungal spores employed in this study.

2.3 Extraction of Water-Soluble Mustard Components

The mustard seed was ground using an Oster classic grinder (Valencia, Spain) before the extraction step. The extraction of water-soluble components was realized as follows. MF or MB (4 g) were homogenized with 50 mL of distilled water using an Ultra Ika T18 basic UltraTurrax (Staufen, Germany) for 5 min at 12,000 ×*g*. Next, the extracts were centrifuged for 15 min at 5000 ×*g*, and the supernatant was recovered and placed in polypropylene trays. Posteriorly, the supernatant was

freeze-dried in a FreeZone 2.5 L Labconco (Kansas, MO, USA) for 72 h. The powder obtained was stored at −20 °C before use in the antifungal activity tests.

2.4 Antifungal Activity of Freeze-Dried Mustard Extracts by Agar Difussion Method

The freeze-dried powder of MF and MB was resuspended in sterile water at 100 g/L and tested against the toxigenic *Penicillium*, *Aspergillus*, and *Fusarium* strains described in item 2.2. For this, the fungal strains were sowed on PDA plates with a cotton swab soaked with sterile water TWEEN® 0.2%. Next, a 10 mm well was realized on the PDA, and 100 µL of either MF or MB suspension was placed. The plates were incubated for 48 h at 25 °C, and then, the inhibition halos were measured on a scale of mm. The inhibition halos were classified as follows: (−) means that no inhibition halo was detected; (+) means that inhibition halos of 5 mm diameter were detected on PDA plates; (++) means that inhibition halos between 5-10 mm diameter were detected on PDA plates; and (+++) means that inhibition halos were more extensive than 10 mm diameter.

2.5 Antifungal Properties of MB over Time by Agar Diffusion Method

The antifungal stability of MB was studied, suspending the freeze-dried powder in sterile water at 100 g/L and then stored at three temperatures (4, 25, and 50 °C). The assay was performed during different storage times (24, 48, 72, and 168 h) using the same methodology described in section 2.4. After 48 h of incubation at 25 °C, the inhibition halos were measured.

2.6 Determination of the Minimum Inhibitory Concentration (MIC) and the Minimum Fungicidal Concentration (MFC) of MB

The in vitro antifungal activity of MB was determined according to the CLSI document M38-A2 in 96-well microplates with minor modifications (Espinel-Ingroff et al., 2012). First, conidia concentration was measured and adjusted in PDB media to 5 x 10^4 conidia/mL with a Neubauer chamber (Marienfeld, Lauda-Königshofen, DE). Then, the 96-well microplate was filled as follows. A negative control containing only PDB medium and a positive control containing PDB with the fungal strains were realized. The following columns were filled with 100 µL of freeze-dried MB resuspended in PDB at concentration decreasing from 100 to 0.4 g/L and 100 µL of the fungal strains. The 96-well microplates were incubated at 25 °C for 72 h. After the incubation time, the MIC value was established as the lowest MB concentration that visually inhibited the fungal growth. To determine the MFC value, 10 µL of the MICs higher doses were subcultured on PDA plates and incubated again at 25 °C for 72 h. The MFC was defined as the lowest extract concentration in which the fungal growth was prevented.

2.7 Application of MB in Bread Formulation

The bread dough (1 kg of weight) was prepared by mixing the following ingredients: 600 g of wheat flour, 20 g of sugar, 10 g of NaCl, 40 g of yeast (purchased from a local supermarket), and 250 mL of water. Four different MB doses were tested: 2.5 g/kg; 5 g/kg; 7.5 g/kg; and 10 g/kg. Commercial treatment was prepared with the additive sodium propionate (E-281) at 2 g/kg concentration. The control treatment was realized following the recipe without including MB or chemical additives in its composition. The kneading was performed using a

SilverCrest Bread Maker SBB 850 A1 (Kompernass GMBH, Bochum, Germany) for 10 min. The dough obtained was fermented for 1 h at room temperature (25 °C). The dough was then transferred to a cast and baked in a MIwe deck oven (Arnstein, Germany) at 200 °C for 40 min. The bread was cooled at room temperature in a sterile cabinet before sliced (30 g for each portion). After that, each bread-loaf sample was individually inoculated in nine equidistant points with 10 µL of a *P. commune* CECT 20767 solution adjusted to 3×10^5 conidia/mL using a pipette. The loaves were placed in polyethylene bags and stored for seven days at room temperature.

2.8 Determination of the Fungal Population and Shelf Life

After seven days of incubation, the FP in bread loaves was evaluated according to Pitt & Hocking (1997). Each loaf (30 g) was placed in a sterile plastic bag containing 270 mL of sterile peptone water 0.1% and was homogenized in a Stomacher IUL (Barcelona, Spain) for 30 s. Then, an aliquot of 1 mL was tenfold diluted in Falcon tubes with sterile peptone water 0.1%, and 100 µL of each dilution was placed on PDA plates and spread with a Drigalski-hook. The plates were incubated for 48 h at 25 °C, and the fungal colonies were counted. The sampling was realized in duplicate.

The microbial shelf life was determined, according to Doulia et al. (2009). The contaminated loaves were visually analyzed daily. Shelf life ended when the growth of *P. commune* was detected on the surface because consumers would reject the product at the first sign of spoilage.

2.9 Statistical analysis

GraphPad Prism version 3.0 software was employed for statistical analysis. The differences between treatments considering $p < 0.05$ were analyzed by a one-way-ANOVA statistical test followed by the Tukey post hoc test for multiple comparisons.

3. Results and Discussion

3.1 In Vitro Antifungal Activity of MB and Antifungal Stability over Time

The antifungal properties of the aqueous MF and MB extracts at 100 g/L were evaluated on PDA plates against toxigenic fungi of the *Aspergillus*, *Penicillium*, and *Fusarium* genera (**Table 1**). The MF extract only showed antifungal activity against *F. verticillioides* ITEM 12044 and *F. poae* ITEM 9151, with inhibition halos of 5 mm diameter. In contrast, the MB extract showed antifungal activity against all the strains tested, obtaining inhibition halos larger than 10 mm diameter for the *P. camemberti* CECT 2267, *P. expansum* CECT 2278, *P. roqueforti* CECT 2905, *P. commune* CECT 20767, *P. verrucosum* VTT D-01847, *F. verticillioides* ITEM 12052, and *F. verticillioides* ITEM 12044 strains. Despite MB has shown significant antifungal potential towards *Aspergillus* spp., the *Aspergillus* strains demonstrated more resistance to the MB extract than the other genera, with inhibition halos lower than 5 mm in diameter.

The MB showed greater effectiveness in avoiding the toxigenic fungi growth in agar diffusion assay; thus, the following test was carried out to determine the stability of the antifungal compounds presents in these extracts. In other words, the extracts were reevaluated through the agar diffusion method after incubation at different temperatures and storage times. Specifically, between 4 and 50 °C for

24-168 h. The data obtained are plotted in **Table 2**. The results suggested that the storage temperature directly affects the MB extract antifungal properties since the extract stored at 50 °C lost the antifungal activity after 24 h of storage. The extract stored at 25 °C also reduced their antifungal properties; however, even after 168 h of storage showed inhibition halos of 5 mm diameter against *Penicillium* and *Fusarium* genera.

Table 1. Antifungal activity of white mustard seed flour (MF) and white mustard bran (MB) extracts in agar diffusion method against *Penicillium*, *Aspergillus*, and *Fusarium* spp.

Fungi strain	**MF**	**MB**
P. camemberti CECT 2267	−	+ + +
P. expansum CECT 2278	−	+ + +
P. roqueforti CECT 2905	−	+ + +
P. digitatum CECT 2954	−	+ +
P. commune CECT 20767	−	+ + +
P. solitum CECT 20818	−	+ +
P. verrucosum VTT D-01847	−	+ + +
A. flavus ITEM 8111	−	+
A. parasiticus CECT 2681	−	+
A. ochraceus CECT 2093	−	+
A. lacticoffeatus CECT 20581	−	+
A. steynii CECT 20510	−	+
A. tubingensis CECT 20543	−	+
A. tubingensis CECT 20544	−	+
F. proliferatum ITEM 12072	−	+ +
F. verticillioides ITEM 12052	−	+ + +
F. verticillioides ITEM 12044	+	+ + +
F. graminearum ITEM 126	−	+ +
F. sporotrichioides ITEM 12168	−	+
F. poae ITEM 9151	+	+ +

The inhibition halos were measured on a mm scale: (−) means no inhibition halo detected; (+) means inhibition halo of 5 mm diameter; (+ +) means inhibition halo ≥5 mm and <10 mm diameter; and (+ + +) means inhibition halo ≥10 mm.

Table 2. White mustard bran (MB) antifungal stability test during storage at different temperatures (4, 25, and 50 °C). The extract was prepared at 100 g/L and tested against toxigenic *Aspergillus*, *Fusarium*, and *Penicillium* strains.

Fungi strain	4 °C				25 °C				50 °C
	24 h	48 h	72 h	168 h	24 h	48 h	72 h	168 h	24-168 h
P. camemberti CECT 2267	+ + +	+ + +	+ + +	+ +	+ +	+ +	+ +	+	−
P. expansum CECT 2278	+ + +	+ + +	+ + +	+ +	+ +	+ +	+ +	+	−
P. roqueforti CECT 2905	+ + +	+ + +	+ + +	+ +	+ +	+ +	+ +	+	−
P. digitatum CECT 2954	+ + +	+ + +	+ + +	+ +	+ +	+ +	+	+	−
P. commune CECT 20767	+ + +	+ + +	+ + +	+ +	+ +	+ +	+ +	+	−
P. solitum CECT 20818	+ + +	+ + +	+ + +	+ +	+ +	+ +	+ +	+	−
P. verrucosum VTT D-01847	+ + +	+ + +	+ + +	+ +	+ +	+ +	+ +	+ +	−
A. flavus ITEM 8111	+	+	+	−	+	+	−	−	−
A. parasiticus CECT 2681	+	+	+	−	+	+	−	−	−
A. ochraceus CECT 2093	+	+	+	−	+	+	−	−	−
A. lacticoffeatus CECT 20581	+	+	+	−	+	+	−	−	−
A. steynii CECT 20510	+	+	+	−	+	+	−	−	−
A. tubingensis CECT 20543	+	+	+	−	+	+	−	−	−
A. tubingensis CECT 20544	+	+	+	−	+	+	−	−	−
F. proliferatum ITEM 12072	+ +	+ +	+ +	+	+ +	+ +	+ +	+	−
F. verticillioides ITEM 12052	+ + +	+ + +	+ + +	+ +	+ +	+ +	+ +	+	−
F. verticillioides ITEM 12044	+ + +	+ + +	+ +	+	+ +	+ +	+	+	−
F. graminearum ITEM 126	+ +	+ +	+ +	+	+	+	+	+	−
F. sporotrichioides ITEM 12168	+	+	+	+	+	+	+	+	−
F. poae ITEM 9151	+ +	+ +	+ +	+	+ +	+ +	+ +	+	−

The inhibition halos were measured on a mm scale: (−) means no inhibition halo detected; (+) means inhibition halo of 5 mm diameter; (+ +) means inhibition halo ≥5 mm and <10 mm diameter; and (+ + +) means inhibition halo ≥10 mm.

The MB extracts stored at 4 °C showed the highest antifungal activity during storage for all the fungi tested. In particular, the MB extract antifungal properties were preserved until 168 h of storage against *Penicillium* and *Fusarium* strains tested, showing inhibition halos between 5-10 mm in diameter. Thus, the temperature was a crucial factor in preserving the antifungal properties of MB extracts during storage. These findings may be explained by the myrosinase enzyme activity responsible for converting the sinalbin into *p*-HBIT. It has been reported that myrosinase activity in white mustard decreases significantly with temperatures above 60 °C for 10 min of exposure (Okunade et al., 2015; Van Eylen et al., 2006). In our study, the higher time of exposure (24 h) for the MB extract to the heat treatment (50 °C) may explain the loss of the myrosinase activity and the loss of antifungal properties. Tsao et al. (2000) also described that the stability of glucosinolates and the degradation rate of isothiocyanates are closely related to the pH of the aqueous solution, and they evidenced less stability when the medium was alkalized (pH 9.00). Therefore, it is worthy to note that the *p*-HBIT stability may differ depending on the extraction matrix because it interacts with other components that increase or decrease its stability over time.

In addition to the agar diffusion assay, we performed a quantitative test on the antifungal effectiveness of MB extracts, and the results are shown in **Table 3**. Two parameters were established: the Minimum Inhibitory Concentration (MIC) and the Minimum Fungicidal Concentration (MFC). The effectiveness of MB depended on the fungal species tested. *Penicillium* strains obtained the lower MIC values, with values ranging from 0.3 to 1.2 g/L; i.e., *Penicillium* spp. were more susceptible to MB exposure. The other fungal genera presented MIC values ranging from 0.6 to 2.3 g/L, and 0.6 to 4.7 g/L for the *Aspergillus* and *Fusarium* genera, respectively. Regarding the MFC, the *Penicillium* strains also presented lower values, ranging

from 0.6 to 4.7 g/L. It seems that MB water extract could bean alternative to control *Penicillium* spoilage in long term storage foods.

Table 3. Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of white mustard bran extract (MB) evaluated against *Penicillium*, *Aspergillus*, and *Fusarium* strains. Results are expressed in g/L.

Fungi strain	MIC	MFC
P. camemberti CECT 2267	0.3	0.6
P. expansum CECT 2278	0.6	1.2
P. roqueforti CECT 2905	0.3	0.6
P. digitatum CECT 2954	1.2	2.3
P. commune CECT 20767	0.6	1.2
P. solitum CECT 20818	1.2	4.7
P. verrucosum VTT D-01847	0.6	1.2
A. flavus ITEM 8111	1.2	4.7
A. parasiticus CECT 2681	1.2	4.7
A. ochraceus CECT 2093	1.2	4.7
A. lacticoffeatus CECT 20581	1.2	2.3
A. steynii CECT 20510	2.3	9.4
A. tubingensis CECT 20543	1.2	4.7
A. tubingensis CECT 20544	1.2	18.8
F. proliferatum ITEM 12072	0.6	9.4
F. verticillioides ITEM 12052	0.6	2.3
F. verticillioides ITEM 12044	1.2	2.3
F. graminearum ITEM 126	4.7	9.4
F. sporotrichioides ITEM 12168	4.7	9.4
F. poae ITEM 9151	2.3	4.7

The most sensitive strains exposed to MB extracts were *P. camemberti* CECT 2267 and *P. roqueforti* CECT 2905, with MFC values of 0.6 g/L. The *Fusarium* strains presented MFC values ranging from 2.3 to 9.4 g/L, and the *Aspergillus* strains MFC values ranging from 1.2 to 18.8 g/L. The strains with the most resistance properties to the MB extract was *A. tubingensis* CECT 20544, and a concentration of 18.8 g/L was needed to obtain the fungicidal effect. These results were consistent with

those obtained on the agar diffusion method, evidencing, for the first time, a significant fungicidal effect of MB.

Previous studies have shown the efficacy of yellow mustard (*Sinapis alba*) and oriental mustard (*Brassica juncea*) to control toxigenic bacteria and fungi *in vitro*. These botanical antimicrobial activities were closely related to the production of ITC, which have been described in the literature as antimicrobial and antifungal agents (Saladino, Bordin, et al., 2017; Sotelo et al., 2015). Wang et al. (2020) studied the antifungal properties of benzyl isothiocyanate (BITC) (0.312, 0.625, and 1.25 mM) against the fungal pathogen *Alternaria alternata*. The authors notices that the compound reduced the spore germination, the rate of mycelial growth, and the synthesis of *Alternaria* mycotoxins in a dose-dependent manner. Moreover, they proposed that the antifungal toxicity of BITC occurred through a cell growth hindrance and a membrane disruption. Azaiez et al. (2013) studied the antifungal activity of allyl, benzyl, and phenyl isothiocyanate standard solutions prepared at 10, 25, and 50 µg per plate against *Gibberella moniliformis*, a Fumonisin B_1 producer, and monitored the mycelial growth. The employed ITCs reduced the mycelial growth between 2.1-89.7% according to the dose and exposure time.

Even though the effectiveness of essential oils (EOs) in these previous studies was high, it is essential to emphasize that the application EOs in foods is limited due to their intense residual flavors and odors (Ribeiro-Santos et al., 2017). The main advantage of MB in comparison with EOs is -in addition to using a by-product that would contaminate the environment- the high antifungal activity in aqueous solution, which facilitates its application as an antifungal agent in bakery product formulations.

3.2 Antifungal Properties of White Mustard Bran on Bread Formulations

The MB aqueous extract demonstrated a high antifungal effect *in vitro*, inhibiting the growth of toxigenic fungi that commonly affect food. Thus, we proposed the direct application of MB in the formulation of bread to enhance the shelf life. The fungal strain selected for this test was *P. commune* CECT 20767 because it is one of the primary contaminants isolated in bakery products (Nielsen & Rios, 2000). Also, the MB extract showed a higher fungicidal effect against *Penicillium* strains. As a result, incorporating MB in the baking process increased the bread-slices shelf life in a dose-dependent manner (**Table 4**). In other words, MB reduced the *P. commune* CECT 20767 growth in a dose-dependent manner. For example, the MB dose at 2.5 g/kg did not increase the bread shelf life because the fungal growth was observant on the slice surface after four inoculation days. This result was similar to the control treatment that did not receive any preservatives. In contrast, the application of 5 and 7.5 g/kg of MB into the bread formulation expanded the shelf life by two days compared to the control treatment. The highest dose tested (10 g/kg) increased the shelf life of the bread slices up to day 7 of incubation, equaling the antifungal effect with the application of the synthetic chemical preservative sodium propionate (E-281) (**Figure 1**).

Table 4. The shelf life of bread loaves formulated with white mustard bran (MB) at 2.5, 5, 7.5, and 10 g/kg. The control did not receive treatment, and the commercial treatment was performed with sodium propionate (E-281) at 2 g/kg. The bread was contaminated with *P. commune* CECT 20767.

Treatment	Days						
	1	2	3	4	5	6	7
Control	-	-	-	+	+ +	+ +	+ +
Commercial	-	-	-	-	-	-	+
MB 2.5 g/kg	-	-	-	+	+ +	+ +	+ +
MB 5 g/kg	-	-	-	-	-	+	+ +
MB 7.5 g/kg	-	-	-	-	-	+	+ +
MB 10 g/kg	-	-	-	-	-	-	+

Results are expressed as follows: (-) means no superficial growth detected; (+) means superficial mycelium detected; (+ +) means superficial mycelium with spores detected.

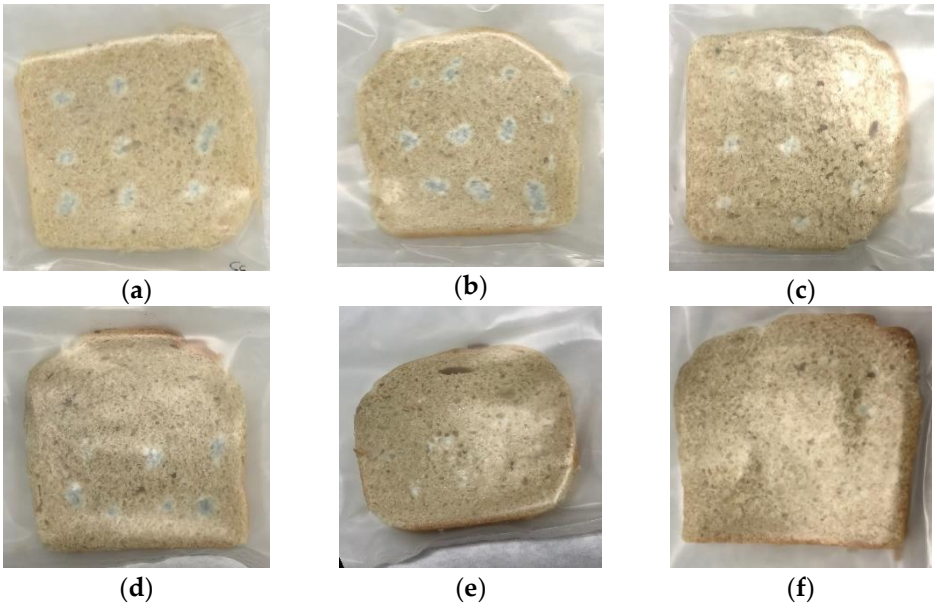


Figure 1. Bread slices contaminated with *P. commune* CECT 20767 after seven days of incubation at room temperature. Different treatments were carried out: (a) Control treatment; (b) 2.5 g/kg of white mustard bran (MB); (c) 5 g/kg of MB; (d) 7.5 g/kg of MB; (e) 10 g/kg of MB, and (f) Commercial treatment with sodium propionate (E-281).

Regarding the fungal population (FP), the shelf life results were corroborated by counting the fungal colonies of the bread slices at day 7 of incubation. Likewise, as plotted in **Figure 2**, the bread elaborated with MB at 2.5 g/kg did not significantly reduce the FP regarding the control treatment ($p < 0.05$). However, statistically, significant differences were observed for MB at 5 g/kg and 7.5 g/kg. Specifically, the FP was reduced by 1.21 and 1.37 log CFU/g compared to the control treatment, which meant a reduction of 96.1 and 97.3%, respectively. The most effective dose was 10 g/kg of MB, which reduced the FP by 4.20 log CFU/g (99.9% reduction), and it also equaled the effectiveness of the treatment with additive E-281. The data obtained showed that MB is a potential candidate for a preservative ingredient in bakery products affected by the genus *Penicillium*. Therefore, its incorporation into the bread dough could avoid fungal growth and, consequently, increase the shelf life and the safety of these foods.

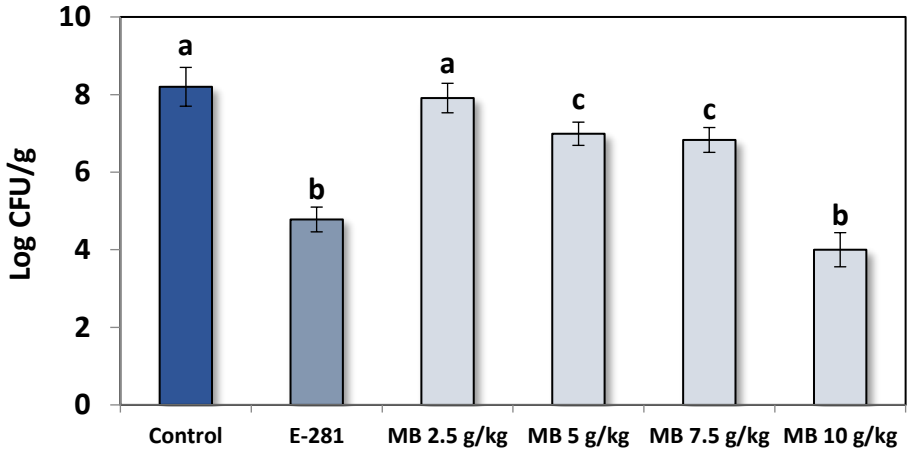


Figure 2. The fungal population of the loaf bread formulated with MB (2.5, 5, 7.5 and 10 g/kg) and contaminated with *P. commune* CECT 20767. The control group did not receive any treatment, and the commercial treatment was performed with sodium propionate (E-281) at 2 g/kg. Different letters mean significant differences between treatments ($p < 0.05$).

The use of mustard-derived isothiocyanates was previously applied to reduce the growth of pathogenic microorganisms in different food systems. These studies focused on preventing fungal growth in bakery products, avoiding mycotoxin production, and improving bread shelf life. Saladino, Manyes, et al. (2016) studied the mycotoxin reduction of patulin in wheat tortillas using volatile bioactive compounds released from oriental and white mustard flour. The results showed that 0.5, 1.0, and 2.0 g of both mustard flour released ITC in the samples, but no significant differences were detected in the fungal population. Nonetheless, the authors achieved a higher percentage of patulin reduction than 80%.

Quiles et al. (2015a) also studied the inhibition of *A. parasiticus* and aflatoxin reduction in pizza crust using allyl isothiocyanate by multiple strategies. The active packaging strategy consisted of applying allyl isothiocyanate (AITC) at final concentrations of 2, 5, and 10 μL/L through a sachet or a paper filter. The authors also evaluated the inclusion of autoclaved oriental mustard into the dough (0.7, 1.7, or 3.4% as dry ingredients). The authors showed that autoclaved oriental mustard flour did not reduce the *A. parasiticus* growth and aflatoxin production. The difference between that study and our results may be explained because the authors utilized another variety of mustard (*Brassica juncea*), and we also applied MB into the dough without previous heat treatment. Hence, the activity of the myrosinase enzyme remained intact. Notwithstanding the preceding, the active packaging strategies proposed were effective in a dose-dependent manner.

Furthermore, Clemente et al. (2019) evaluated the antifungal activity of mustard EO on commercial traditional Spanish bread contaminated with *Rhizopus stolonifer*. The bread was treated with 1 μL of EO (AITC purity > 95%) and incubated at two temperatures (25 and 4 °C). They evidenced that the antifungal activity of AITC reduced in the food matrix compared to *in vitro* studies; however, the

fungicidal effect was significant to improve the shelf life in both storage temperatures.

Despite the application of essential mustard oils, AITC and *p*-HBITC had previously been studied as effective strategies to reduce toxigenic microorganisms in food; nowadays, there are no reports on the MB application as an antifungal agent. This novel approach allowed to employ a by-product that increases the shelf life of bread. Moreover, it is worth highlighting that MB satisfies the consumer demand for natural products, either avoiding or reducing the application of synthetic chemical additives. Against this background and adding the results obtained in our study, it can be concluded that white-mustard bran could be a strategy of great interest because it delays the spoilage caused by mycotoxigenic and non-mycotoxigenic fungi and increases the shelf life of bakery products, reducing economic losses. Furthermore, the incorporation of MB into the dough could reduce environmental waste.

4. Conclusions

In the present study, the MB, a by-product of yellow mustard (*Sinapis alba*), was proposed as an antifungal agent. The evaluation of the aqueous extracts *in vitro* against the toxigenic fungi genera *Aspergillus*, *Fusarium*, and *Penicillium* confirmed the antifungal activity of the MB and its stability during storage at 4 and 25 °C. MB was also evaluated as an enhancer of the microbiological shelf life of bread contaminated with *P. commune* CECT 20767, a traditional spoilage agent of bakery products.

The application of 10 g/kg of MB resulted in an effective strategy to reduce the fungal contamination without compromising the organoleptic properties.

Moreover, 10 g/kg doses also obtained similar results to the sodium propionate regarding the bread shelf-life. For this reason, MB could be a good candidate to replace traditional preservatives as sodium propionate once MB could satisfy consumer demand by reducing the content of synthetic chemical additives in food.

Furthermore, it is important to note that MB is a cheap ingredient priced at around $1.40/kg, which could lead to cheaper bakery products.

Further research will focus on applying MB in commercial bakery products associated with different storage conditions, such as vacuum and modified atmosphere packaging. Likewise, the identification of MB antifungal compounds and the direct application of MB extracts will be studied.

Toxins, 14, 80 (2022)

3.3 Use of Mustard Extracts Fermented by Lactic Acid Bacteria to Mitigate the Production of Fumonisin B_1 and B_2 by *Fusarium verticillioides* in Corn Ears

1. Introduction

The contamination of food and feedstuffs by mycotoxins currently remains a significant concern in developed countries, and it estimated that between 5 and 10% of the world's food supply is squandered because of fungal growth (Pitt & Hocking, 2009). Moreover, depending on the mycotoxin of concern and the analytical method employed, the prevalence of mycotoxins in food grains might be 60-80% (Eskola et al., 2019). Thus, toxigenic fungi are, perchance, the most significant pathogens worldwide in term of food safety (Giorni et al., 2019; Moretti et al., 2017).

Corn (*Zea mays*) is subjected to infection by fungi such as *Fusarium verticillioides* and *Aspergillus flavus* throughout the supply chain (Palumbo et al., 2020). The contact of corn kernels with such toxigenic agents not only leads to grain quality diminishments and economic losses but also menaces the health of animals and consumers who are subject to mycotoxin ingestion through corn or derived foods (Alshannaq & Yu, 2017). *Fusarium* species are prevalent in the field and frequently invade and synthesize mycotoxins in the crop. Moreover, the inadequate pre-harvest procedures of small-holder farmers, along with favorable meteorological conditions, contribute to fungal growth and mycotoxin contamination after harvest (Agriopoulou et al., 2020; Tran et al., 2021). Fumonisin B_1 (FB_1) is the major mycotoxin generated by *F. verticillioides*, being considered probably carcinogenic to humans, according to IARC, and along with fumonisin B_2 (FB_2), has become significant contaminants in the food and feed industries (Hassan et al., 2021). These mycotoxins are frequently found as single and co-contaminants in cereals or cereal-based food and feed products (Esposito et al., 2016; Yang et al., 2019). In addition, the synergistic or additive toxic effects of mycotoxins,

established by multiple occurrences or co-occurrences, have been highlighted by several authors (Andretta et al., 2016; Kifer et al., 2020).

Synthetic antifungals have represented the most prevalent method of combating fungal spoilage due to their broad-spectrum action. However, their use presents several disadvantages (Gajbhiye & Kapadnis, 2016). Agrochemicals are associated with environmental challenges, due to their stability and toxicity since they can accumulate over time. They are hazardous to aquatic creatures, and their concentration in stream water has grown significantly in recent years (Želonková et al., 2019). Fungicides are also related to carcinogenic, teratogenic, and irritant effect in various human organs (Le Lay et al., 2016; van der Ven et al., 2020), prompting researchers to develop novel techniques of food spoilage management that assure food safety without compromising human health. Among these, biopesticides (natural pesticide compounds) are considered a promising and sustainable solution because they can remove target pests and lead to minimal environmental pollution (Sharma et al., 2020).

Yellow mustard (*Sinapis alba*) and oriental mustard (*Brassica juncea*) have been previously used as culinary seasonings. However, recently the antifungal properties of powdered mustard have been demonstrated in food (Bahmid et al., 2021; Olaimat & Holley, 2016). Both species contain a high concentration of glucosinolates, which are cleaved by myrosinase (EC 3.2.1.147) in the presence of moisture and an acidic pH, producing isothiocyanates as well as thiocyanates, nitriles, and a few other minor chemicals. The myrosinase synthesizes *p*-hydroxybenzyl isothiocyanate (*p*-HBIT) from sinalbin, the predominant glucosinolate in yellow mustard. In contrast, allyl isothiocyanate (AITC) is synthesized from sinigrin, the main glucosinolate in oriental mustard (Avato & Argentieri, 2015).

Biopreservation is a natural process that uses microbes, or their antimicrobial active metabolites, to prolong the shelf life and the safety of foods. Recently, authors have suggested the use of lactic acid bacteria (LAB) as an alternative to synthetic biocides for preventing fungal growth (Lv et al., 2018; Saladino, Luz, et al., 2016). Additionally, it is critical to mention that the majority of LAB are widely acknowledged as safe and have QPS (qualified presumption of safety), so they can be considered excellent candidate for their use as natural preservatives in food and feedstuff (Mokoena et al., 2021). However, no reports demonstrated the antifungal capacity of mustard and its by-products, fermented by LAB against toxigenic fungi, in cereal crops such as corn. Therefore, this work contributed to filling this literary gap.

Against this background, the study aimed to develop a biopesticide, based on fermented mustard with LAB, as a solution to reduce fungal contamination and mycotoxin synthesis in corn ears. For this, the antifungal properties of aqueous extracts of yellow mustard (YM) and oriental mustard (OM) fermented by LAB were investigated against toxigenic *Fusarium* strains in vitro. Besides, the Cell-Free Supernatants (CFS) were characterized by determining the main phenolic compounds and organic acids produced. Finally, a biopreservative made from yellow mustard extract, fermented by *Lactiplantibacillus plantarum* TR71 was evaluated in corn ears contaminated with *F. verticillioides* to prevent the FB_1 and FB_2 production.

2. Results and Discussion

2.1 Antifungal Activity of the Fermented Mustard Extracts

Two water extracts prepared from different varieties of mustard, YM (*Sinapis alba*) and OM (*Brassica juncea*), were fermented by nine LAB and tested against toxigenic *Fusarium* strains *in vitro*. For this purpose, a qualitative assay on PDA plates was employed to initially screen the different CFS' antifungal properties. The control group consisted of non-fermented YM and OM water extract. As plotted in **Table 1**, only the extracts fermented by *L. plantarum* TR7, *L. plantarum* TR71, *L. plantarum* TR14, and *L. plantarum* CECT 8962 evidenced antifungal properties. In particular, the YM extracts fermented by *L. plantarum* TR71 and *L. plantarum* TR14 showed inhibition halos larger than 10 mm against all *Fusarium* strains. The other LAB strains tested (*Leuconostoc pseudomesenteroides* IRK751, *Levilactobacillus brevis* IRK82, *Levilactobacillus brevis* SMF76, *Leuconostoc pseudomesenteroides* POM, and *Liquorilactobacillus ghanensis* TR2), and the control extracts did not show antifungal effect. Comparing both mustard extracts, YM fermented extracts were more effective than OM fermented extracts since some fungal strains were resistant to the latter.

Therefore, based on these results, the mustard extracts fermented by *L. plantarum* strains were selected for further analysis, which consisted of a quantitative antifungal test to determine the Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) values against the *Fusarium* strains. The results obtained from MIC and MFC trials are presented in **Table 2**.

Table 1. Antifungal activity of the Cell-Free Supernatant (CFS) at 100 g/L against toxigenic *Fusarium* strains. Two mustard varieties were employed as fermentation substrates: Yellow Mustard (YM) and Oriental Mustard (OM). Antifungal activity was considered positive (+) when the inhibition halo measurement was more extensive than 10 mm.

Fungal Strain	Control		IRK751		IRK82		SMF76		POM		TR7		TR71		TR14		TR2		CECT 8962	
	YM	OM	YM	OM	YM	OM	YM	OM	YM	OM	YM	OM	YM	OM	YM	OM	YM	OM	YM	OM
F. graminearum ITEM 126	-	-	-	-	-	-	-	-	-	-	+	-	+	-	+	+	-	-	+	-
F. graminearum ITEM 6352	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	+	+
F. graminearum ITEM 6415	-	-	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	-	+	-
F. proliferatum ITEM 12072	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	-	-	+	+
F. proliferatum ITEM 12103	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-
F. proliferatum ITEM 16031	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	-	-	+	+
F. verticillioides ITEM 12052	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	-	-	+	+
F. verticillioides ITEM 12043	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	-	-	+	+
F. verticillioides ITEM 12044	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	-	-	+	+
F. sporotrichioides ITEM 121	-	-	-	-	-	-	-	-	-	-	+	-	+	-	+	+	-	-	-	-
F. langsethiae ITEM 11031	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	+	-	-	+	+
F. poae ITEM 9131	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	+	-
F. poae ITEM 9151	-	-	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	-	+	-
F. poae ITEM 9211	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	+	-	-	+	+

Leuconostoc pseudomesenteroides IRK751*; Levilactobacillus brevis* IRK82*; Levilactobacillus brevis* SMF76*; Leuconostoc pseudomesenteroides* POM*; Lactoplantibacillus plantarum* TR7*; Lactiplantibacillus plantarum* TR71*; Lactiplantibacillus plantarum* TR14*; Liquorilactobacillus ghanensis* TR2*; Lactiplantibacillus plantarum* CECT 8962.

The MIC and MFC values varied according to the LAB strain tested and the mustard variety (yellow or oriental) employed as fermentation substrate. The YM extract fermented by *L. plantarum* TR71 obtained the lower MIC and MFC values, ranging from 7.8-15.6 g/L and 15.6-31.3 g/L, respectively, i.e., the extract fermented by *L. plantarum* TR71 needed lower doses than other fermented extracts to inhibit fungal growth. In particular, the most susceptible fungal strains to this extract were *F. graminearum* ITEM 126, *F. graminearum* ITEM 6352, *F. graminearum* ITEM 6415, *F. verticillioides* ITEM 12043, *F. sporotrichioides* ITEM 121, *F. langsethiae* ITEM 11031, and *F. poae* ITEM 9151. Although the other YM extracts fermented by LAB exhibited antifungal capacity, their MIC and MFC values were higher, ranging from 15.6-31.3 g/L abd 31.3-62.5 g/L, respectively. Therefore, the antifungal activity seemed to be lower.

In general, the MIC and MFC values for the OM fermented extracts were higher than those for the YM fermented extracts, supporting the previous qualitative test findings (**Table 1**). Similarly, MIC values varied from 15.6-62.5 g/L according to the strain used, whereas for MFC values, the concentration needed to achieve a fungal inhibition ranged from 31.3-125.0 g/L. The higher resistance to the OM extract was obtained by *F. proliferatum* ITEM 16031, *F. verticillioides* ITEM 12044, *F. poae* ITEM 9131, *F. poae* ITEM 9151, and *F. poae* ITEM 9211, with MFC values ranging from 62.5 to 125.0 g/L, depending on the *L. plantarum* strain used in the fermentation procedure. Thus, this study demonstrated the *in vitro* antifungal activity of the mustard CFS after fermentation by LAB against *Fusarium* spp.

Table 2. Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) values determined in vitro against toxigenic *Fusarium* strains of (a) Yellow Mustard fermented cell free supernatant; (b) Oriental Mustard fermented cell-free supernatant. Results were expressed as g/L.

(a)

Fungal Strain	TR7		TR71		TR14		CECT 8962	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
F. graminearum ITEM 126	15.6	31.3	7.8	15.6	7.8	15.6	31.3	62.5
F. graminearum ITEM 6352	15.6	31.3	7.8	15.6	15.6	31.3	15.6	31.3
F. graminearum ITEM 6415	7.8	15.6	7.8	15.6	7.8	15.6	7.8	15.6
F. proliferatum ITEM 12072	31.3	62.5	7.8	15.6	15.6	31.3	15.6	31.3
F. proliferatum ITEM 12103	15.6	31.3	15.6	31.3	15.6	31.3	15.6	31.3
F. proliferatum ITEM 16031	15.6	62.5	15.6	31.3	31.3	62.5	15.6	31.3
F. verticillioides ITEM 12052	15.6	31.3	15.6	31.3	31.3	62.5	15.6	31.3
F. verticillioides ITEM 12043	7.8	15.6	7.8	15.6	15.6	31.3	15.6	31.3
F. verticillioides ITEM 12044	15.6	31.3	15.6	31.3	31.3	625	15.6	31.3
F. sporotrichioides ITEM 121	31.3	62.5	7.8	15.6	7.8	15.6	15.6	31.3
F. langsethiae ITEM 11031	15.6	31.3	7.8	15.6	7.8	15.6	7.8	15.6
F. poae ITEM 9131	15.6	31.3	15.6	31.3	15.6	31.3	15.6	31.3
F. poae ITEM 9151	7.8	15.6	7.8	15.6	15.6	31.3	31.3	62.5
F. poae ITEM 9211	15.6	31.3	15.6	31.3	15.6	31.3	31.3	62.5

(b)

Fungal Strain	TR7		TR71		TR14		CECT 8962	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
F. graminearum ITEM 126	31.3	62.5	31.3	31.3	15.6	62.5	31.3	62.5
F. graminearum ITEM 6352	31.3	62.5	31.3	31.3	31.3	62.5	15.6	31.3
F. graminearum ITEM 6415	15.6	31.3	15.6	31.3	15.6	62.5	15.6	31.3
F. proliferatum ITEM 12072	31.3	62.5	31.3	62.5	31.3	62.5	31.3	62.5
F. proliferatum ITEM 12103	31.3	62.5	31.3	62.5	31.3	62.5	31.3	62.5
F. proliferatum ITEM 16031	62.5	125.0	31.3	62.5	31.3	62.5	62.5	125.0
F. verticillioides ITEM 12052	31.3	62.5	31.3	62.5	31.3	62.5	31.3	62.5
F. verticillioides ITEM 12043	31.3	62.5	31.3	62.5	31.3	62.5	31.3	62.5
F. verticillioides ITEM 12044	31.3	62.5	31.3	62.5	62.5	125.0	31.3	62.5
F. sporotrichioides ITEM 121	31.3	62.5	31.3	62.5	31.3	62.5	31.3	62.5
F. langsethiae ITEM 11031	15.6	31.3	15.6	31.3	7.8	15.6	15.6	31.3
F. poae ITEM 9131	31.3	125.0	62.5	125.0	62.5	125.0	31.3	62.5
F. poae ITEM 9151	31.3	62.5	31.3	62.5	31.3	62.5	62.5	125.0
F. poae ITEM 9211	31.3	62.5	31.3	62.5	62.5	125.0	62.5	125.0

Lactiplantibacillus plantarum TR7; *Lactiplantibacillus plantarum* TR71; *Lactiplantibacillus plantarum* TR14; *Lactiplantibacillus plantarum* CECT 8962.

Although previous studies have confirmed the efficacy of YM and OM in preventing fungal development, the use of fermented mustard extract as an antifungal treatment method has not been reported in the literature. Quiles et al. (2018) studied the antifungal properties of water extracts prepared from YM and OM flour and confirmed that YM water extract was effective against toxigenic fungi of the *Aspergillus*, *Penicillium*, and *Fusarium* genera in concentration ranging from 0.24-7.5 g/L, whereas OM water extract was not antifungal. The YM extract prepared as a control in our study was not effective after incubation at 37 °C for 72 h. This finding agrees with our previous study since the antifungal properties of YM water extracts may decrease when the extract is stored from more than 24 h at a temperature higher than 25 °C (Torrijos et al., 2021). Therefore, it seems that fermentation might yield more stable molecules and, hence, enhance the antifungal activity of YM and OM extracts. To be precise, the OM extract exhibited antifungal activity only when fermentation was applied.

Concerning antifungal effectiveness of the CFS, other authors have reported MIC and MFC values of LAB after fermentation of different food matrices. Luz et al. (2018) evaluated the antimicrobial properties of lyophilized whey, fermented by LAB, against nine toxigenic strains of the *Penicillium*, *Aspergillus*, and *Fusarium* genera. The CFS evidenced antifungal properties regarding *Fusarium* strains, with MIC and MFC values ranging from 31.3-125 and 62.5-250 g/L, respectively.

Izzo et al. (2020) determined the MIC and MFC concentration of fermented goat's sweet whey using *Lactobacillus* spp. against ten toxigenic *Fusarium* strains. The author obtained MIC values ranging from 1.5 to 31.3 g/L, whereas the mean MFC values ranged from 7.8 to 250 g/L. It is essential to underline that our results corroborate that study since similar MIC and MFC values inhibited the growth of

Fusarium spp. Our results, associated with previous studies, could confirm the possible application of CFS of YM as an antifungal agent against *Fusarium* strains.

2.2 Phenolic Acids and Organic Acids Profile of the CFS

This study characterized the main phenolic acids of the fermented mustard extracts that exhibited antifungal properties in vitro through liquid chromatography (UHPLC-qTOF/MS). There were 11 different phenolic acids identified in the CFS of the YM and OM fermented extracts. As expected, it was noted that the profile and concentration of phenolic acids differed according to the LAB strain and the mustard variety employed as substrate for the fermentation. The results are summarized in **Table 3**. In the YM extracts (**Table 3a**), 1,2-dihydroxybenzene, 3,4-dihydroxycinnamic acid, and benzoic acid were significantly increased ($p < 0.05$) after fermentation by *L. plantarum* strains compared to control extracts. In particular, *L. plantarum* TR71 produced the higher concentration of these compounds with a mean of 292.85, 44.95, and 220.12 ng/mL, respectively. Moreover, this strain synthesized 559.15 ng/mL of 3-phenyllactic acid, the highest concentration among the assessed CFS, regardless of the mustard extract tested.

Regarding OM extracts (**Table 3b**), 3,4-dihydroxicinnamic acid, benzoic acid, and 3-phenyllactic acid were identified and quantified in higher concentration in comparison with the control extract ($p< 0.05$), the concentration ranged from 146.01 to 217.67, 140.53 to 228.43, and 31.20 to 37.16 ng/mL, respectively.

Table 3. Phenolic and organic acids identify in the cell-free supernatant of the yellow mustard extract (a) and the oriental mustard extract (b). Results are expressed in ng/mL.

(a) Yellow mustard extract					
Phenolic Acid	**Control**	**TR7**	**TR71**	**TR14**	**CECT 8962**
1,2-Dihydroxybenzene	62.88 ± 10.86^{a}	259.53 ± 11.12^{b}	292.85 ± 62.97^{b}	194.99 ± 64.37^{c}	250.17 ± 5.76^{b}
3,4-Dihydroxicinnamic acid	11.45 ± 3.67^{a}	32.73 ± 4.13^{b}	44.95 ± 9.76^{c}	26.01 ± 5.26^{b}	28.76 ± 8.24^{b}
Benzoic acid	14.74 ± 2.36^{a}	134.42 ± 5.09^{b}	220.12 ± 27.12^{c}	128.19 ± 11.15^{b}	127.29 ± 10.51^{b}
3-Phenyllactic acid	13.67 ± 1.68^{a}	45.66 ± 6.87^{ab}	559.15 ± 78.51^{c}	57.48 ± 12.07^{b}	45.62 ± 8.16^{ab}
Hydoxycinnamic acid	6.84 ± 0.93	n.d	n.d	n.d	n.d
p-Coumaric acid	16.13 ± 6.07^{a}	42.66 ± 5.37^{b}	76.07 ± 15.81^{c}	65.25 ± 7.68^{cd}	62.13 ± 11.97^{d}
Protocatechuic	31.13 ± 7.60^{a}	158.68 ± 12.96^{b}	17.25 ± 2.11^{c}	8.96 ± 3.69^{c}	39.08 ± 17.86^{a}
Sinapic acid	61.72 ± 4.86^{a}	8.29 ± 2.39^{b}	16.79 ± 0.12^{c}	28.40 ± 7.78^{d}	40.50 ± 8.51^{e}
Vanillin	4.17 ± 1.56^{a}	n.d	30.28 ± 7.92^{b}	17.75 ± 6.06^{c}	20.71 ± 7.62^{c}
Syringic acid	4.30 ± 1.34	n.d	n.d	n.d	n.d
Ferulic acid	11.69 ± 4.69	n.d	n.d	n.d	n.d
Organic acid					
Lactic acid	n.d	728.00 ± 20.97^{a}	799.88 ± 27.08^{b}	591.56 ± 25.50^{c}	570.26 ± 29.64^{c}
(b) Oriental mustard extract					
Phenolic Acid	**Control**	**TR7**	**TR71**	**TR14**	**CECT 8962**
1,2-Dihydroxybenzene	7.23 ± 2.64^{a}	n.d	39.27 ± 6.70^{b}	34.62 ± 5.39^{b}	n.d
3,4-Dihydroxicinnamic acid	2.83 ± 0.98^{a}	146.01 ± 16.30^{b}	217.67 ± 35.00^{c}	190.56 ± 52.87^{cd}	157.06 ± 18.40^{bd}
Benzoic acid	76.51 ± 8.85^{a}	140.53 ± 30.09^{b}	159.10 ± 8.99^{b}	228.43 ± 16.90^{c}	143.07 ± 36.13^{b}
3-Phenyllactic acid	6.65 ± 2.87^{a}	34.68 ± 3.37^{bc}	37.16 ± 4.57^{bc}	42.43 ± 14.18^{b}	31.20 ± 7.27^{c}
Hydoxycinnamic acid	10.14 ± 4.04	n.d	n.d	n.d	n.d
p-Coumaric acid	58.09 ± 15.02^{ab}	68.29 ± 20.46^{ac}	76.50 ± 8.62^{d}	30.70 ± 13.59^{ce}	42.44 ± 10.27^{be}
Protocatechuic	20.30 ± 1.33^{a}	44.24 ± 13.22^{b}	32.23 ± 9.96^{c}	31.33 ± 2.52^{c}	33.39 ± 4.15^{c}
Sinapic acid	62.37 ± 27.75^{a}	25.09 ± 2.96^{b}	22.59 ± 6.09^{b}	48.21 ± 11.49^{a}	19.18 ± 4.12^{b}
Vanillin	9.47 ± 1.24^{a}	18.64 ± 2.57^{a}	60.14 ± 19.03^{b}	58.89 ± 10.49^{b}	59.47 ± 11.65^{b}
Syringic acid	6.81 ± 1.45	n.d	n.d	n.d	n.d
Ferulic acid	19.74 ± 1.45	n.d	n.d	n.d	n.d
Organic acid					
Lactic acid	n.d	209.04 ± 66.16^{a}	203.39 ± 6.53^{a}	89.24 ± 20.20^{b}	174.55 ± 7.26^{a}

n.d= no detected; Different letters represent statistical differences between the treatments ($p \leq 0.05$) (n = 9).
Lactiplantibacillus plantarum TR7; *Lactiplantibacillus plantarum* TR71; *Lactiplantibacillus plantarum* TR14; *Lactiplantibacillus plantarum* CECT8962.

Comparing both mustard extracts, lower values of 1,2-dihydroxybenzene were detected in the OM extracts, whereas higher values of 3,4-dihydroxycinnamic acid were quantified in this extract. Moreover, other cinnamic acid derivatives, characteristic of mustard seed, such as *p*-coumaric acid, ferulic acid, and sinapic acid, were also identified in both extracts (Nicácio et al., 2021). Although both extracts increased phenolic acid concentration after fermentation, the YM extract showed a slightly higher concentration than OM. These results suggested that the LAB fermentation could be beneficial, increasing the antifungal potential of YM extracts and generating antifungal compounds in OM extract.

Only lactic acid was identified in the fermented samples regarding the organic acids. In particular, the lactic acid content in the YM fermented extracts ranged from 570.26-799.88 ng/mL, and the highest content of this organic compound was produced by *L. plantarum* TR71. Lactic acid was also detected on the OM fermented extract. However, the concentrations quantified were lower compared to YM extracts, with values ranging from 89.24-209.44 ng/mL.

The LAB antimicrobial potential is well-known and, for this reason, have been studied for food and feed application (Lv et al., 2018; Sadeghi et al., 2019; Saladino et al., 2016). The antimicrobial properties of these microorganisms are not characteristic of one chemical compound; for instance, several metabolites, such as organic acids, phenolic acids, antimicrobial peptides, and fatty acids, can act synergistically and provide antifungal activity (Schmidt, Zannini, et al., 2018). Among the metabolites produce by LAB, organic acids are considered the main compounds responsible for the biopreservative activity of LAB. Their antifungal properties are directly related to the decrease in pH, which inhibits the fungal cell's metabolic activities and disrupts the cell membrane (Gajbhiye & Kapadnis, 2016). In this study, only lactic acid was detected in all fermented CFS, and the highest

concentration of this metabolite was detected on the YM fermented extracts, which also evidenced the highest antifungal properties in the *in vitro* studies.

The identified compounds in our extract have been described previously as antifungal substances in other CFS regarding the phenolic acids. Chen, Ju, et al. (2021) reported several phenolic compounds in CFS obtained through fermentation of *L. kefiri* M4 with antifungal properties against *P. expansum* such as 1,2-dihydroxybenzene, 3,4-dihydroxycinnamic acid, benzoic acid, and 3-phenyllactic acid. Among the identified phenolic compounds, 3-phenyllactic acid has been widely studied for its antifungal potential against mycotoxigenic fungi, and some authors have established a positive correlation between PLA content and the antifungal properties (Rajanikar et al., 2021). In this context, Cortes-Zavaleta et al. (2014) screened 13 LAB for their ability to produce 3-phenyllactic acid and their antimicrobial properties against food spoilage moulds, such as *Botrytis cinerea*, *Penicillium expansum*, and *Aspergillus flavus*. They correlated the antifungal properties of the LAB regarding the 3-phenyllactic acid synthesized. However, the authors agree that further investigation should be done since the antifungal properties are not exclusively related to this phenolic compound. Therefore, the higher content of 3-phenyllactic acid synthesized in YM extracts fermented by *L. plantarum* TR71 could be related to the higher antifungal properties. Due to the higher *in vitro* antifungal activity, this extract was proposed as an antimycotoxigenic agent in corn ears contaminated with *F. verticillioides.*

2.3 Application of the CFS on Corn Ears as an Antimycotoxigenic Agent

The YM fermented extract with *L. plantarum* TR71 was selected and applied as a biopreservative agent against *F. verticillioides* (FB_1 and FB_2 producer) in corn ears. For this purpose, the fermented YM extract with *L. plantarum* TR71 was applied directly through spray technique on the corn ears, or after lyophilization and preparation, at 350 g/L in sterile water. In addition, the YM extract was also tested on the corn ears without fermentation (through direct spray or lyophilization and preparation at 350 g/L). The control group was prepared with non-treated corn ears inoculated with the fungal agent. Then, the corn ears were stored at 25 °C for 14 days (**Figure 1**), determining the mycotoxin content at times 0, 7, and 14 days (**Figure 2**) through UHPL Q-TOF/MS technique.

At the initial time (0 d), the samples did not show mycotoxins. After 7 days post-inoculation (**Figure 2a**), the control contained 0.30 mg/kg of FB_1 and 0.05 mg/kg of FB_2. Furthermore, only the administration of lyophilized extracts (fermented or unfermented) demonstrated a decrease in FB_1 levels, as compared to the control treatment ($p< 0.05$). Additionally, the lyophilized extract fermented by TR71 was the only treatment that did not evidence FB_2 production after 7 days of incubation.

After 14 days (**Figure 2b**), the FB_1 synthesized by *F. verticillioides* increased in all the treatments tested. The FB_1 content (without treatment) was raised to 14.71 mg/kg in the control group. In contrast, we noticed that the direct application of the YM extract fermented by TR71 reduced the FB_1 production (8.02 mg/kg) compared to the control (49.5% of reduction), and, similarly, the unfermented lyophilized YM significantly reduced the FB_1 concentration regarding the control group. Nevertheless, it is worth noting that the higher decrease in FB_1 was achieved by applying the lyophilized YM extract fermented by TR71. Remarkably, the average

content obtained after application of this treatment and incubation for 14 days was 1.09 mg/kg, which, compared to the control, reduced the incidence of this mycotoxin in corn ears by 92.6%.

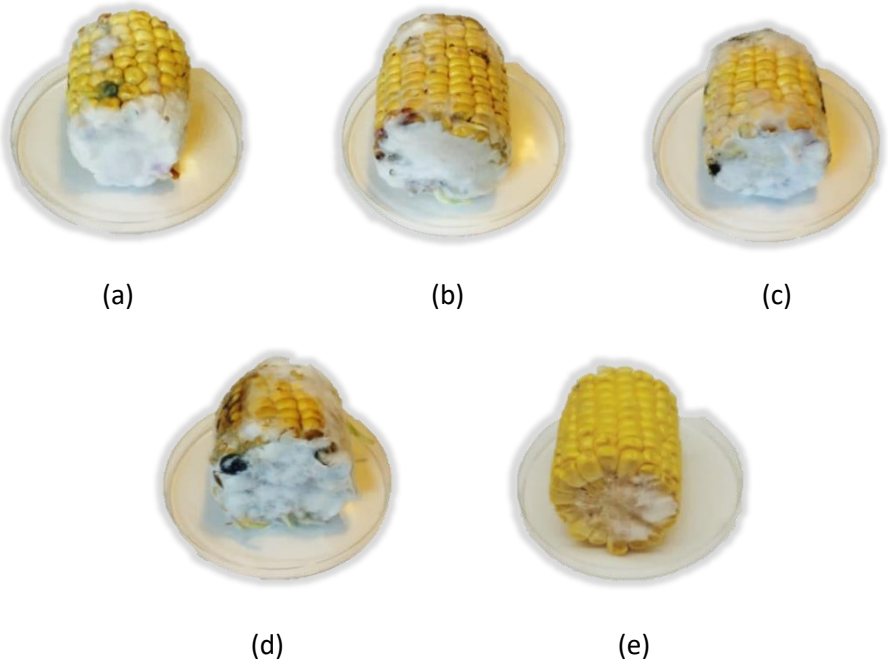


Figure 1. Corn ears contaminated with *F. verticillioides* ITEM 12052 after 14 days of storage. Treatments applied were the following: (a) control; (b) non-fermented yellow mustard extract; (c) fermented yellow mustard extract with *L. plantarum* TR71; (d) non-fermented yellow mustard extract lyophilized and prepared at 250 g/L in water; (e) fermented yellow mustard extract with *L. plantarum* TR71, lyophilized and prepared at 250 g/L in water.

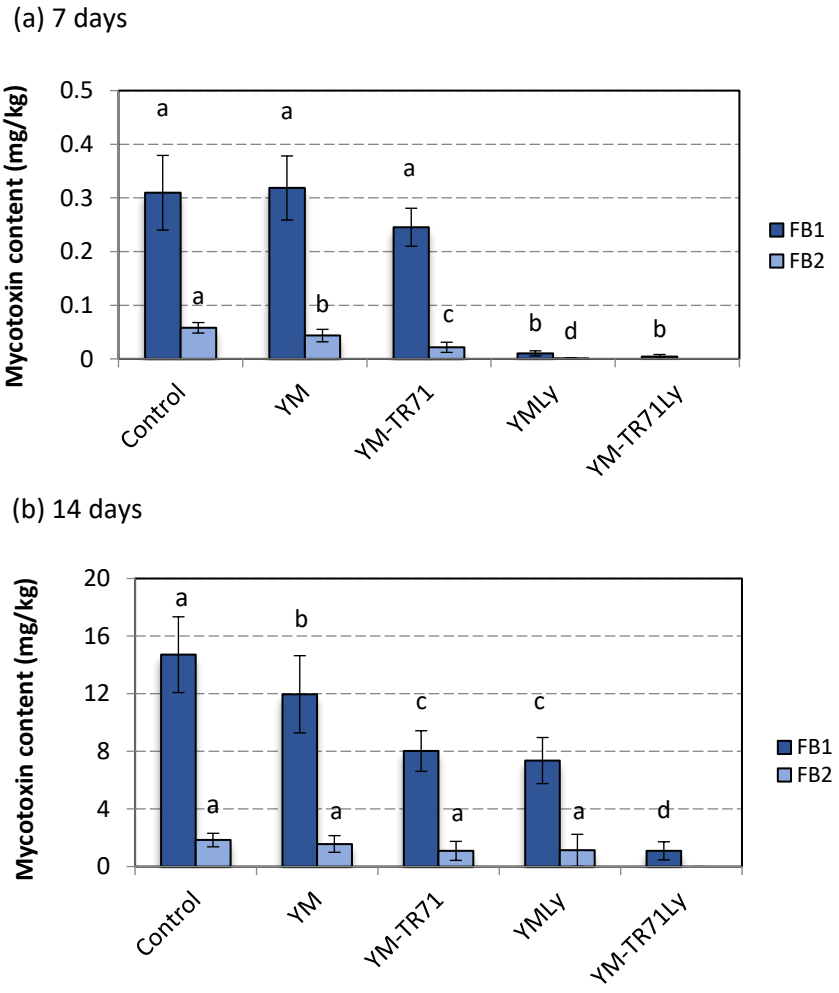


Figure 2. Fumonisin B_1 (FB_1) and Fumonisin B_2 (FB_2) determination in corn ears contaminated with *F. verticillioides* ITEM 12052 and treated with cell-free supernatant. Treatments applied were the following: YM: non-fermented yellow mustard extract; YM-TR71: fermented yellow mustard extract with *L. plantarum* TR71; YMLy: non-fermented yellow mustard lyophilized and prepared at 250 g/L; YM-TR71Ly: fermented yellow mustard with *L. plantarum* TR71 lyophilized and prepared at 250 g/L. Mycotoxin was determined on 7th (a) and 14th day (b). The different letters mean statistical differences in the mycotoxin content among the treatments applied ($p < 0.05$).

The antimycotoxigenic effect of the fermented YM extract could be explained because the application of the CFS reduced the fungal growth (**Figure 1**), and, in consequence, the secondary metabolism responsible for the mycotoxin synthesis could be retarded (Daou et al., 2021). Regarding FB_2, non-statistically differences ($p < 0.05$) were evidenced between the spray with mustard and the control group, except for the lyophilized YM fermented by TR71. This treatment completely inhibited FB_2 synthesis by *F. verticillioides* on corn ears.

The treatments applied could not wholly reduce the production of FB_1 on corn ears. However, it is essential to underline that, after 14 days of incubation, the mycotoxin content, in corn ears treated with lyophilized *L. plantarum* TR71, was below 4 mg/kg, which means below the maximum levels specified in the European legislation for the sum of FB_1 and FB_2 in unprocessed corn (EC n° 401/2006). Thus, YM fermented with TR71 was proved to be an antimycotoxigenic solution for corn ears, and we suggested its application during pre-harvest to increase food safety.

The present trend toward minimizing the use of agrochemicals in food has prompted researchers to investigate alternative strategies for lowering the occurrence of toxigenic fungal agents. Several authors have confirmed the promising employment of LAB to avoid mycotoxins production in food and feed. In this context, Nazareth et al. (2019) evaluated the application of the CFS prepared from fermented MRS broth with *L. plantarum* CECT 749 against *F. verticillioides* and *Aspergillus flavus* in corn and corn kernels, respectively. Although they did not completely reduce the incidence of FB_1, the content of this mycotoxin on corn ears decreased 90.6% after 7 days compared to the control. In corn kernels, the effect of applying the CFS reduced the incidence of aflatoxin B_1 by 99.7 and 97.5% after 25 and 40 days, respectively.

Dopazo et al. (2021) isolated and studied the use of LAB on red grapes as bio-preservative agents against *A. flavus*, *A. niger*, and *Botrytis cinerea*. They found that the use of *L. fallax* UTA 6 CFS was effective against *A. flavus* and *B. cinerea*, reducing the fungal population on red grapes by 0.4 and 0.6 log spores per gram. Additionally, they investigated the efficacy of CFS treatment in reducing mycotoxin occurrence on red grapes, and the authors observed that aflatoxin B_1 and fumonisins (B_2, B_3, and B_4) were reduced in percentages ranging from 28 to 100%.

Ben Taheur et al. (2019) applied the CFS obtained by LAB in almonds against *A. flavus* and *A. carbonarius*. The use of the CFS of *L. kefiri* FR7 reduced the incidence of aflatoxin B_1 and aflatoxin B_2, synthesized by *A. flavus* in 85.27% and 83.94%, respectively. Moreover, a similar effect was observed when the inoculant agent was *A. carbonarius*, since the Ocratoxin A content was reduced 25% compared to the control.

3. Conclusions

In this study, the fermented mustard extracts by LAB were proposed as a natural biopreservative solution in corn ears. The in vitro evaluation of the antifungal properties showed that the YM extracts fermented by *L. plantarum* strains presented the highest antifungal effect against *Fusarium* spp.

Although 11 different phenolic acids were identified, the characterization of the CFS highlighted that lactic acid and 3-phenyllactic acid were the most abundant antifungal metabolites in the YM extract fermented by *L. plantarum* TR71. Therefore, due to the higher *in vitro* antifungal activity, as well as lactic and phenolic acid production, this extract was applied on corn ears contaminated with *F. verticillioides* to reduce FB_1 and FB_2 production.

In conclusion, the fermented YM extract effectively reduced more than 90% of FB_1 and FB_2 content after 14 days of incubation. Since consumers are demanding a reduction in pesticides to preserve crops, the proposed application of YM fermented extracts by *L. plantarum* TR71 is a sustainable solution that reduces the incidence of mycotoxin contamination and, hence, increases the food safety of corn ears. Finally, we recommend its application against different fungal contaminants in the field to evaluate its capacity to avoid the production of different mycotoxins.

Further studies should be developed using this biopreservation associated with different barrier technologies such as temperature control, water activity, application of other natural compounds, or modified atmosphere packaging. Using one or several barriers would probably increase crop quality, reducing the Fumonisin production to undetectable levels.

4. Materials and Methods

4.1 Chemicals

The FB_1 standard solution (purity > 99%) was obtained from Sigma–Aldrich (St. Louis, MO, USA). The phenolic standards 1,2-dihydroxybenzene, 3,4-dihydroxicinnamic acid, benzoic acid, 3-phenyllactic acid, hydroxycinnamic acid, p-coumaric acid, protocatechuic, sinapic acid, vanillin, syringic acid, and ferulic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). Lactic acid was obtained from Sigma–Aldrich (St. Louis, MO, USA).

Acetonitrile (ACN) (LC-MS/MS grade), ethyl acetate (EA), formic acid (FA), and methanol (HPLC-MS/MS grade) were obtained from VWR Chemicals (Randor, PA, USA). The deionised water used in chromatography analysis (<18 MΩ cm resistivity) was obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA). The

salts, magnesium sulphate ($MgSO_4$) and sodium chloride (NaCl), were provided from Sigma–Aldrich (St. Louis, MO, USA).

The Potato Dextrose Agar (PDA), Potato Dextrose Broth (PDB), and Buffered peptone water (BPW) were purchased from Liofilchem Bacteriology Products (Roseto, Italy). De man Rogosa Sharpe (MRS) Broth was obtained from Oxoid (Hampshire, UK).

The Yellow Mustard Flour (YM) (code #106) and Oriental Mustard Flour (OM) (code #107) were provided by G.S. Dunn Dry Mustard Millers (Hamilton, ON, Canada).

4.2 Microorganisms and Culture Conditions

The fungal strains *Fusarium graminearum* ITEM 126, *F. graminearum* ITEM 6352, *F. graminearum* ITEM 6415, *F. proliferatum* ITEM 12072, *F. proliferatum* ITEM 12103, *F. proliferatum* ITEM 16031, *F. verticillioides* ITEM 12052, *F. verticillioides* ITEM 12043, *F. verticillioides* ITEM 12044, *F. sporotrichioides* ITEM 12168, *F. langsethiae* ITEM 11031, *F. poae* ITEM 9131, *F. poae* ITEM 9151, and *F. poae* ITEM 9211 were obtained from the Institute of Sciences of Food Production (ISPA-CNR, Bari, Italy). The fungi were preserved in sterile PDB 25% glycerol at −80 °C. Prior to their use, the strains were transferred into PDA plates and incubated for 7 d at 25 °C. The spores collected from these plates were used in the study.

The LAB strain *Leuconostoc pseudomesenteroides* IRK751, *Levilactobacillus brevis* IRK82, *Levilactobacillus brevis* SMF76, *Leuconostoc pseudomesenteroides* POM, *Lactiplantibacillus plantarum* TR7, *Lactiplantibacillus plantarum* TR71, *Lactiplantibacillus plantarum* TR14, and *Liquorilactobacillus ghanensis* TR2 were isolated from tomatoes and sourdough and identified through the 16S rNA analysis

sequence by Luz et al. (2020). The strain *Lactiplantibacillus plantarum* CECT 8962 was obtained from the Spanish Culture Type Collection CECT (Valencia, Spain). The LAB strains were recovered from MRS 25% glycerol stored at −80 °C and inoculated in fresh MRS broth for 72 h at 37 °C.

4.3 Fermentation Conditions and Preparation of CFS

The mustard extracts used for fermentation were prepared, according to Quiles et al. (2018), with minor modifications. Firstly, 10 g of YM or OM were mixed with 250 mL of distilled water and homogenised using an Ultraturrax T18 basic mixer (Ika, Staufen, Germany) and then centrifuged at 4000 x *g* for 15 min at 4 °C. The supernatant obtained was used for bacteria fermentation, as follows. Next, 1 mL of each LAB (10^7 CFU/mL) described by Section 4.2 and growth in MRS for 12 h (to achieve the exponential phase growth) was added to 9 mL of the YM or OM water extract (proportion 1:10 v/v), homogenised, and incubated for 72 h at 37 °C. Control extracts were prepared without adding LAB. Then, the fermented extracts were centrifuged at 3200 x *g* for 10 min to obtain the CFS. Part of the extract was lyophilised (FreeZone 2.5 L, Labconco, Kansas City, MO, USA) and stored at −30 °C to characterise the antifungal properties. The liquid CFS was used to determine the organic acids and phenolic acids profile.

4.4 Qualitative Antifungal Test on PDA Plates

The lyophilised CFS were prepared at a final concentration of 100 g/L with sterile water and tested on PDA plates against the *Fusarium* fungal strains described by Section 4.2. The fungal spores were collected with a cotton swab, soaked with 0.1% buffered peptone water 0.2% TWEEN® and cultivated on PDA plates. Then,

wells of 10 mm diameter were prepared in the agar, and 100 µL of the CFS was placed. The plates were incubated at 25 °C for 48 h to observe fungal inhibition. The inhibition on the fungal growth was considered positive (+) when the inhibition zone was more extensive than 10 mm in diameter. The control was realised by testing the extracts of YM and OM without fermentation.

4.5 Determination of the MIC and MFC values of the CFS

The Minimum Inhibitory Concentration (MIC) and the Minimum Fungicidal Concentration (MFC) of the mustard-fermented CFS were established, according to the CLSI document M38-A2 (2008), with modifications. The lyophilized extracts were mixed with PDB, and 100 µL were assayed in 96-well microplates at concentrations ranging from 7.8 to 200 g/L. In addition, two controls were prepared on each microplate. The first one constituted the negative control, which contained only 200 µL of PDB. The second control, the control of the microorganism, was prepared, adding to the plate the fungal strains described by Section 4.2 without the antifungal agent. The fungal spores were collected from PDA plates and, with a cotton swab, counted with a Neubauer chamber and adjusted to 5×10^4 spores/mL in PDB. Next, 100 µL of the fungal spores were added to the wells containing the antifungal agent, so the final volume was 200 µL/well. The plates were incubated 72 h at 25 °C, and the MIC was established as the smallest concentration of the antifungal agent that inhibited the fungal growth compared to the control of the microorganism.

After determination of the MIC, 10 µL of the higher doses of the MIC were subcultured on PDA plates and incubated 48 h at 25 °C. Finally, the MFC value was

considered the lowest concentration in which fungal growth was not detected on the PDA plate.

4.6 Organic Acids and Phenolic Acids Determination in the CFS

For the determination of the organic acids, the mustard CFS was diluted 1:20 (v/v) in Milli-Q water and then filtered with a 0.22 µm syringe filter. The samples were injected into an Agilent 1100 Series HPLC System (Palo Alto, CA, USA), equipped with a diode array detector and a quaternary pump. The separation was realised with a Rezez ROA-Organic Acid (140 x 7.8 mm) reverse phase column (Phenomenex, Torrance, CA, USA). The isocratic mobile phase used was water 0.1% FA (v/v) with a flow rate of 0.6 mL/min. The chromatogram was monitored at 210 nm (Khosravi et al., 2015). The results were expressed in ng/mL Three replicates (*n* = 3) of each extract were analysed and the experiment was conducted three times.

The extraction of the phenolic acids from the mustard CFS was realised following the methodology of Brosnan et al. (2014). There was 10 mL of the CFS incorporated in Falcon tubes together with 10 mL of EA 1% FA, 4 g of $MgSO_4$, and 1 g of NaCl. Then, the tubes were mixed by vortex for 1 min and kept on ice for 5 min. To separate the ethylic phase, the tubes were centrifuged. Afterwards, the ethylic phase was transferred to a new Falcon tube, containing 150 mg of C18 and 900 mg of $MgSO_4$, and the mixture was vortexed again for 1 min. Then, the samples were centrifuged, and the supernatant recovered was dried under N_2 flow. The samples were reconstituted with 1 mL of Milli-Q water: ACN (50:50 v/v) and filtered with a 0.22 µm syringe filter before the injection on the LC system.

The analysis of the phenolic acids was realised using a 6450 Agilent Ultra High-Definition Accurate Mass QTOF-MS, equipped with an Agilent Dual Jet Stream

Electrospray Ionization. The column employed for chromatographic separation was a Gemini C18 (50 mm x 2 mm, 100 Å, 3 µm particle size) (Phenomenex, Torrance, CA, USA), and the mobile phases used were Milli-Q water (phase A) and ACN (phase B), both acidified with FA 0.1%. The gradient elution was programmed as follows: 0 min, 5% B; 30 min, 95% B; 35 min, 5% B. The equilibration of the column was set at 3 min before the following analysis. There were 20 µL of the samples injected, and the flow rate was 0.3 mL/min.

The mass spectrometry analyses were conducted in negative ionisation mode with the following conditions: drying gas (N_2), 8.0 L/min; nebuliser pressure, 30 psig; gas drying temperature, 350 °C; capillary voltage, 3.5 kV; fragmentation voltage, 175 V; scan range, 20–380 m/z. Collision energies for MS/MS experiments were 10, 20, and 40 eV. The integration and data elaboration was realised with MassHunter Qualitative Analysis Software B.08.00 (Denardi-Souza et al., 2018). Results were expressed in ng/mL. Three replicates (n = 3) of each extract were analysed, and the experiment was conducted three times.

4.7 Application of the CFS in Corn Ears

The antimycotoxigenic activity of the YM CFS fermented by *L. plantarum* TR71 was studied on corn ears contaminated with *F. verticillioides* CECT 2982 (FB_1 and FB_2 producer). Samples of corn ears (*Zea mays* L. var. *rugosa*) (70 g), purchased from a local supermarket, were placed in 1L glass jars. Then, corn ears were treated by spraying 2 mL of the fermented YM extract by *L. plantarum* TR71 or 2 mL of the YM extract lyophilised, preparing the solutions at a concentration of 350 g/L in sterile water. Moreover, the non-fermented YM extract was prepared and applied in the same conditions: 2 mL of YM extract; 2 mL of the YM extract, lyophilised and

prepared at 350 g/L. A control treatment was designed by spraying 2 mL of sterile water on the corn ears. Then, 1 mL of *F. verticillioides* CECT 2982, prepared at 10^3 spores/mL in 0.1% buffered peptone water, was sprayed on the corn earns and was let dry for 1 h. Afterwards, the jars were closed and stored at 25 °C for 14 days. Nine replicates (*n* = 9) of each treatment were prepared, and the study was conducted three times.

4.8 Extraction and Determination of Mycotoxins by Q-TOF

The extraction of FB_1 and FB_2 was realised using the methodology described by Nazareth et al. (2019) with modifications. Before the extraction, the lyophilised corn portions were finely grounded with an Oester Classic grinder (Madrid, Spain). Then, 5 g were mixed with 25 mL of methanol, and the extraction was performed in an Ultraturrax at 12,000 rpm for 5 min. Next, the mixture obtained was centrifuged, and then, the supernatant was recovered, filtered through a 0.22 µm syringe filter and injected into a UHPLC (1290 Infinity LC, Agilent Technologies) coupled to a Q-TOF (Agilent 6540 LC/QTOF) Mass Spectrometer.

The chromatographic separation of the mycotoxins was realised in an Agilent Zorbax RRHD SB-C18 (2.1 x 50 mm, 1.8 µm) column. Mobile phases employed were the following: Milli-Q water 0.1% FA (Phase A); ACN 0.1% FA (Phase B). The gradient used was configured as: 0 min, 2% B; 22 min, 95% B; 25 min, 5% B. Then, the column was equilibrated 3 min before the next injection. Flow rate was established at 0.4 mL/min, and the injection volume was 5 µL. For q-TOF analysis, an Agilent Dual Jet Stream electrospray ionization (ESI) was operating in positive ionisation mode. Conditions of ESI were configured as follows: gas temperature: 325 °C; gas flow: 10 L/min; nebuliser pressure: 40 psig; sheath gas temperature: 295 °C; sheath gas flow:

12 L/min; capillary voltage: 4000 V; nozzle voltage: 500 V; skimmer: 70 V; scan range: 100–1500 Da; collision energy: 10, 20, 40 eV. For quantification, fumonisin calibration curves were prepared, with concentration ranging from 0.01 to 10 mg/L. The integration and data elaboration were realised using MassHunter Qualitative Analysis Software B.08.00.

4.9 Statistical analysis

For statistical analysis, GraphPad Prism version 3.0 software (San Diego, CA, USA) was used. The differences between groups ($p < 0.05$) were analysed by One-Way ANOVA test followed by the post-hoc Tukey test for multiple comparisons. Results were expressed as mean ± SD.

LWT– Food Science and Technology (Under Review)

3.4 Phytochemical Profiling of Volatile and Bioactive Compounds in Yellow Mustard (*Sinapis alba*) and Oriental Mustard (*Brassica juncea*) Seed Flour and Bran

1. Introduction

The term mustard refers to a group of plants of the Cruciferae family, which belongs to the genus *Brassica*. Mainly three species of mustard are cultivated worldwide for their gastronomic value: yellow or white mustard (*Sinapis alba*), oriental mustard (*Brassica juncea*), and black mustard (*Brassica nigra*). Usually, mustard plants are consumed as edible oils, condiments, sauces, fermented vegetables, or salad greens (Rahman et al., 2018). These plants have been reported for their high nutritional value and richness in bioactive compounds such as glucosinolates, polyphenols, dietary fiber, *β*-carotene, and ascorbic acid (Campbell et al., 2012; Frazie et al., 2017; Tian & Deng, 2020).

Glucosinolates are amino-acid derived compounds produced in the secondary metabolism of the *Brassicaceae* genus. The hydrolysis of glucosinolates by the myrosinase enzyme (thioglucoside glucohydrolase EC 3.2.3.2) produces a relevant number of biologically active compounds, such as isothiocyanates, thiocyanates, nitriles, and epithionitriles (Hanschen et al., 2018).These substances exert an essential effect on plant defense, and have antimicrobial, antioomycet, antihelmintic, antimutagenic and anticarcinogenic effect (Adegbeye et al., 2020; Kamal et al., 2022; Moccellin et al., 2017; Poveda et al., 2020; Traka & Mithen, 2009). Moreover, few glucosinolates as isothiocyanates are volatile and responsible for the characteristic pungent smell of Brassica plants (Bell et al., 2018). Nowadays, allyl isothiocyanate (AITC) is one of the most studied isothiocyanates due to its antimicrobial potential and possible applications as a preservative and is derived from the hydrolysis of the glucosinolate sinigrin (Araújo et al., 2018; Bahmid, Pepping, et al., 2020; Olaimat et al., 2018).

In addition to glucosinolates, mustard plants are a dietary source of polyphenolic compounds with the potential to scavenge reactive oxygen species

due to their electron-donating properties. In detail, mustard contain flavonoids (especially flavonols), hydroxybenzoic acids and hydroxycinnamic acids (Cartea et al., 2010). Among phenolic compounds, sinapic acid and sinapate esters are described as the main phenolic compounds found in the Brassicaceae family (Nguyen et al., 2021). Some biological properties have been associated with these metabolites such as antioxidant, antibacterial, anti-inflammatory, and UV-filter activities (Calabriso et al., 2020; Hussain et al., 2019; Mouterde et al., 2020; Nićiforović & Abramovič, 2014).

During mustard seed processing, different products can be obtained. The mustard flour is produced by grinding the mustard seed, and the powder is used whether as an emulsifier or flavoring ingredient in culinary preparations. During the grinding, the bran fraction is discarded, and it is estimated that 60% of the mustard seed constitutes a waste product (Sehwag & Das, 2015). This by-product can be revalorized and used to recover bioactive compounds with biological properties. Some authors have addressed this issue; for instance, Reungoat et al. (2021) recovered sinapic acid from oriental mustard bran. On another approach, the bran fraction of yellow mustard has been revalued as an ingredient with antifungal properties in bread baking (Torrijos et al., 2021).

To our knowledge, the phytochemical profile of the by-product bran fraction of mustard species has not been reported so far, and only a few reports were focused on yellow mustard seed bioactive compounds. Thus, to revalue this by-product and find new potential sources of bioactive compounds, the study aimed to comprehensively characterize the phytochemical profile of yellow mustard (*Sinapis alba*) and oriental mustard (*Brassica juncea*) seed fractions (flour and bran). For this, the volatile fraction was investigated using head space solid-phase microextraction (HS-SPME/CG-MS) approach, while the free and bound bioactive

compounds were extracted and analysed using the UHPLC-ESI-MS/MS technique. Moreover, the total phenolic content (TPC), and antioxidant properties were investigated for all the mustard samples.

2. Material and Methods

2.1 Chemicals and Reagents

HPLC-grade water, acetonitrile, n-hexane and methanol (MetOH) and ethyl acetate for analysis were purchased from VWR Chemicals (Milan, Italy). Diethyl ether stabilised with BHT for analysis was obtained from PanReac AppliChem (ITW Reagents). Formic acid (>99% purity) and Folin-Ciocalteu's reagent were purchased from VWR Chemicals. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Bidistilled water was obtained with a Milli-Q system (Millipore, Bedford, MA, USA).

Chemical standards such as p-coumaric acid, caffeic acid, sinapic acid (98% titration), ferulic acid, p-hydroxybenxoic acid, rutin hydrate and gallic acid monohydrate (purity >98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Pure Yellow Mustard Flour (YMF) (Product code: 106), Pure Oriental Mustard Flour (OMF) (Product code: 107), Fine Yellow Mustard Bran (YMB) (Product code: 412), and Oriental Mustard Bran (Product code: 403) were obtained from G.S.Dunn (Hamilton, Canada).

2.2 Characterisation of mustard sample volatile fraction by headspace solid phase microextraction coupled with gas chromatography-mass spectrometry technique (HS-SPME/CG-MS)

For the analysis of the volatile profile of the mustard samples, the headspace solid-phase microextraction (HS-SPME), coupled with gas chromatography mass spectrometry technique (GC-MS) was employed following the methodology described by Shen et al. (2018) with minor modifications.

1 g of each mustard was placed on 20 mL glass vials, and 10 µL of an aqueous toluene standard solution (350 µg/mL) was added to each vial as reference. Headspace analyses were performed with an SPME fiber coated with 50/30 of divinylbenzene-carboxen-polidimethylsiloxane (DVB/carboxen/PDMS; Supelco, Bellefonte, PA, USA). The equilibration of the mixture was programmed for 15 min at 60 °C, while the extraction of volatile components for 50 min at 60 °C.

For GC-MS analysis, a Thermo Scientific Trace 1300 gas chromatograph coupled to a Thermo Scientific ISQ single quadrupole mass spectrometer equipped with an electronic impact (EI) source was employed (Thermo Fisher Scientific,Waltham, MA, USA). The desorption was performed at 250 ºC for 3 minutes. The column used for chromatographic separation was a ZB-5MS capillary column (30m x 0.25 mm, 0.25 µm film thickness) (Phenomenex, Inc, Torrance, CA, USA). The temperature ramp was set as follows: 5 min at 60 ºC; then raised to 180 ºC at 5ºC/min and held 1 min; then raised up to 260 °C at 10 °C/min and held isothermally for 5 min. The injection was performed in splitless mode. The carrier gas used was helium at a flow of 1.4 mL/min. The detection was realised in Full Scan Acquisition Mode in a range of 40-350 *m/z*.

The chromatographic peaks were identified by the comparison of their registered mass spectra with those registered in NIST 14 library. Moreover, Linear Retention Indices (LRIs) were calculated based on the retention time of a C8-C20 alkane solution ran in the same conditions as the samples and then compared with the literature. A semi-quantification of the identified compounds was realised through the comparison with the internal standard (toluene). The experiments were conducted in duplicate.

2.3 Extraction of bioactive compounds from mustard samples

The extraction of the polyphenolic compounds was realised according to Martinović et al. (2020) with minor modifications. First, defatted samples were prepared by combining 1 g of powder with 10 mL of hexane (1:10 (w/v)) and mixing for 8 h with a reciprocating shaker at 140 strokes/min. Then, the hexane was removed by filtering through Millipore SMWP filters (5.0 µm) under vacuum, and the powders were dry overnight.

The free bioactive compounds (Fraction #1) were extracted from the defatted mustard with a mixture of methanol:acetone:water 7:7:6 (v/v) in proportion 1:10 (w/v), and then shook for 6h at room temperature. The extracts were centrifuged at 3000 rpm for 5 min, and then the powder was extracted twice with the same solvent combination but assisted with ultrasounds. The fractions were collected and extracted (liquid-liquid extraction) with a mixture of diethyl ether:ethyl acetate 1:1 (v/v) three times. The recovered ethylic phases were dried using N_2. The remaining aqueous fraction was divided into two equal parts and used to extract the bound phenolics.

Alkaline hydrolysis was realized by spiking the aqueous extract to NaOH 4M 1:2 (v/v) for 4h at 25 °C under N_2 flow and constant mixing. The extract was then acidified (pH=2), and bioactive compounds were extracted with diethyl ether:ethyl acetate 1:1 (v/v) three times. The ethylic phases were dried completely with N_2 flow. This fraction was considered the bound bioactive compounds released after alkaline hydrolysis (Fraction #2).

Acid hydrolysis of the extracts was carried out by reflux technique. For this purpose, the extract was mixed with HCl 6M at 1:1 (v/v) and hydrolyzed at 90 °C for 45 min. Then, phenolic compounds were extracted again with a mixture of diethyl ether:ethyl acetate 1:1 (v/v). The ethylic phases were dried completely under N_2, and the result obtained was considered as the bound bioactive compounds released after performing acid hydrolysis (Fraction #3).

Previous to the injection, the dried samples were resuspended in 1 mL of methanol:H_2O (0.2% FA) 1:1, and sonicated for 5 min. Then, samples were filtered (0.45 µM, nylon membrane) and stored in glass vials.

2.4 UHPLC-MS/MS analysis of bioactive compounds in mustard

The samples (Fraction #1, Fraction #2 and Fraction #3) were injected in a UHPLC Dionex Ultimate 3000 system coupled to a triple quadrupole mass spectrometer (TSQ Vantage; Thermo Fisher Scientific Inc., San Jose, CA, USA) equipped with an Electrospray source (H-ESI II). Chromatographic separation was performed using a SunShell C18 column (2.6 µm; 2.1 i.d. x 100 mm) (ChromaNik Technologies, Osaka, Japan). The mobile phases used were water (Phase A) and acetonitrile (phase B), both acidified with formic acid (0.1%). The gradient set for analysis was the following: 0 min, 5%B; 1 min, 5% B; 6 min 40%B; 7 min 90% B; 9

min 90% B; 10 min 5% B; 17 min 5% B. For Full Scan Analysis, the gradient used for chromatographic separation was the following: 0 min, 1% B; 1 min, 1%B; 10 min 80% B; 13 min, 80% B; 14 min, 1%B; 22 min, 1%B. Flow rate was set at 0.3 mL/min and injection volume was 2 µL.

The bioactive compounds were monitored in negative ionization mode, except for sinapine confirmation that was also performed in positive ionization mode. During ESI- analyses, the spray voltage was set at 3000 V, and the capillary temperature was kept at 270 °C. Vaporizer temperature was set at 200 °C, while sheath gas (N_2) was 50 units and the auxiliary gas (N_2) was 5 units. The S-Lens RF amplitude and Collision Energy (CE) values were obtained employing an automatic function of Xcalibur software (Thermo Fisher Scientific Inc., San Jose, CA, USA). Phenolic acids were monitored using the Selected Reaction Monitoring (SRM) mode. The fragment ions monitored for *p*-coumaric acid, caffeic acid, ferulic acid, sinapic acid and *p*-hydroxybenzoic acid were the same described by Righetti et al. (2019).

In addition, analysis of unknow metabolites were carried out using full scan, scanning from m/z 100 to 1500, followed by a target MS/MS analysis with a collision-induced dissociation (CID) equal to 30 V. Pure argon gas was used for CID. The identification was performed based on the retention times (RTs), mass spectrometry data, and previous published information (Engels et al., 2012; Qu et al., 2020; Sun et al., 2013). Quantification of bioactive compounds was performed with calibration curves of standards (0.1-20 µg/mL) or by using the most similar structurally similar compound, as reported in **Table 3**.

2.5 Determination of the Total Phenolic Content (TPC) and Antioxidant Activity by DPPH methodology

The Total Phenolic Content (TPC) and the antioxidant activity were evaluated in the mustard powders, extracting 1 g of mustard with 10 mL of MetOH:H_2O (70:30 v/v) and shook for 30 min in a reciprocating shaker at 120 strokes/min (Martelli et al., 2020).

The TPC was determined using Folin-Ciocalteu reagent. Firstly, 250 µL of the methanolic extract or standard (gallic acid) were combined with 1 mL of Folin-Ciocalteu diluted reagent 1:10 (v/v), and then 2 mL of Na_2CO_3 10% (w/v) was added. The samples were stored at room temperature in the darkness for 30 minutes. Then, absorbance was measured at 760 nm in a Jasco V-530 spectrophotometer (Champaign, IL, USA). A calibration curve was prepared with gallic acid (10-100 µg/mL). Results were expressed as mg/kg of gallic acid equivalents (GAE), and the experiment was realised three times (n=3).

For the determination of the antioxidant activity, the DPPH methodology was used. As a reference standard, a calibration curve with Trolox was prepared (0.1-1 mM). Then, 0.1 mL of sample or Trolox standard were mixed with 2.9 mL of DPPH solution (0.05 mM). Also, a blank was prepared with 0.1 mL of distilled water. The samples were incubated at 25 ºC for 30 min in darkness. Then, the absorbance of the sample was measured at 517 nm. The calibration curve was realized calculating the inhibition percentage (%IP) of the Trolox standard. Results were expressed as mM of Trolox Equivalent (mM TE) and the experiments were conducted in triplicate (n=3).

2.6 Statistical analysis

Statistical analysis was realised using GraphPad Prism 3.0 software was used (San Diego, Ca, USA). The differences ($p< 0.05$) were detected by ANOVA test followed by a post-hokey Tukey test.

Moreover, to classify the mustard fractions, a principal component analysis (PCA) of the quantified volatile and bioactive compounds was realised using MetaboAnalyst 5.0 software (Pang et al., 2021). The features included were log transformed and mean centered.

3. Results and Discussion

3.1 Volatile profile of the yellow and oriental mustard samples

The characterisation of the volatile profile of the mustard samples was realised by HS-SPME coupled to GC-MS technique. A total of 53 compounds belonging to different chemical classes such as isothiocyanates (1), alkanes (36), ketones (5), esters (4), alcohols (5), and miscellaneous compounds (2) were detected in the mustard samples. The full identification of the detected volatile compounds, their calculated LRIs and those reported in the literature are plotted in **Table 1**. As expected, differences in the volatile profile were observed between the mustard species and fractions analysed (flour or bran). Moreover, a semi-quantification of the peaks identified was performed using toluene as an internal standard and the results are presented in **Table 2**.

Table 1. Identification of the volatile compounds of yellow (*Sinapis alba*) and oriental (*Brassica juncea*) mustard flour and bran, with calculated LRIs, identification method, and references.

Peak	Compound	LRI calc.	LRI lit.	Identification	Reference
Isothiocyanate					
1	Allyl Isothiocyanate	880	887	MS + LRI	Engel et al. (2002)
Alkane					
2	n-Octane	802	800	MS + LRI	Adams (2007)
3	2,4-Dimethylheptane	818	822	MS + LRI	Xu et al. (2003)
4	4-Methyloctane	856	858	MS + LRI	Wang et al. (1994)
5	n-Nonane	884	899	MS + LRI	Adams (2007)
6	2-Methylnonane	964	962	MS + LRI	Zaikin & Borisov, (2002)
7	n-Decane	1000	1000	MS + LRI	Adams (2007)
8	2,5-Dimethylnonane	1013		MS	
9	2,5-Dimethylnonane like			MS	
10	2,6-Dimethylnonane	1022	1022	MS + LRI	Kotowska et al. (2012)
11	5-Methyldecane	1057	1056	MS + LRI	Kotowska et al. (2012)
12	4-Methyldecane	1061	1059	MS + LRI	
13	n-Undecane	1100	1099	MS + LRI	
14	n-Undecane like			MS	
15	3,7-Dimethyldecane	1113	1127	MS + LRI	Kotowska et al. (2012)
16	5-Methylundecane	1156	1154	MS + LRI	Zaikin & Borisov (2002)
17	4-Methylundecane	1160	1158	MS + LRI	Zaikin & Borisov (2002)
18	2-Methylundecane	1165	1165	MS + LRI	Xu et al. (2003)
19	3-Methylundecane	1171	1169	MS + LRI	Zaikin & Borisov (2002)
20	2,5-Dimethylundecane			MS	
21	n-Dodecane	1200	1200	MS + LRI	Adams (2007)
22	2,4-Dimethylundecane	1209	1213	MS + LRI	Liu et al. (2007)
23	2,6-Dimethylundecane	1213	1213	MS + LRI	Zeng et al. (2007)
24	4,8-Dimethylundecane	1222		MS	
25	6-Methyldodecane	1253	1253	MS + LRI	Rembold et al. (1989)
26	4-Methyldodecane	1259	1259	MS + LRI	Zaikin & Borisov (2002)
27	2,6,11-Trimethyldodecane	1274	1275	MS + LRI	Luo & Agnew, (2001)
28	2,6,11-Timethyldodecane like			MS	
29	2,6,11-Trimethyldodecane like			MS	
30	2,6,11-Trimethyldodecane like			MS	
31	n-Tridecane	1300	1300	MS + LRI	Adams (2007)
32	4,6-Dimethyldodecane	1321	1325	MS + LRI	Liu et al. (2007)
33	4,6-Dimethyldodecane like			MS	
34	2-Methyltridecane	1365	1365	MS + LRI	Zaikin & Borisov (2002)
35	3-Methyltridecane	1370	1371	MS + LRI	Kallio et al. (2006)
36	n-Tetradecane	1399	1399	MS + LRI	Adams (2007)
37	2,6,10-Trimethyltridecane	1460	1463	MS + LRI	Flamini et al. (2002)

Table 1. Continuation

Peak	Compound	LRI calc.	LRI lit.	Identification	Reference
Ketone					
38	6-Methyl-5-hepten-2-one	984	985	MS + LRI	Pino et al. (2011)
39	3-octen-2-one	1039	1040	MS + LRI	Fan & Qian (2006)
40	2(3H)-Furanone, 5-ethyldihydro	1051	1047	MS + LRI	Mahajan et al. (2004)
41	3,5-octadien-2-one (E,E)	1070	1072	MS + LRI	Beaulieu & Grimm (2001)
42	3,5-octadien-2-one (E,E) like			MS	
Ester					
43	Tetradecanoic acid, 1-methylethyl ester	1823	1827	MS + LRI	Skaltsa et al. (2001)
44	Palmitic acid, methyl ester	1924	1921	MS + LRI	Santos et al., (2004)
45	9-Octadecenoic acid (Z), methyl ester			MS	
46	Octadecenoic acid, methyl ester			MS	
Alcohol					
47	1-Hexanol	864	865	MS + LRI	Cho et al., (2007)
48	1-Heptanol	971	970	MS + LRI	Flamini et al. (2003)
49	6-Methyl-5-hepten-2-ol	994	993	MS + LRI	Shafi et al. (2011)
50	1-Nonanol	1173	1172	MS + LRI	Methven et al. (2007)
51	1-Nonanol like			MS	
Other					
52	D-Limonene	1029	1028	MS + LRI	Angioni et al. (2006)
53	5-Ethylthiazole	960	959	MS + LRI	Parker et al. (2000)

The mustard sample that presented the highest concentration of volatile compounds was the OMF, with a mean value of 66.97 ± 0.11 μg/g ($p < 0.05$) (**Figure 1**). This fraction of *Brassica juncea* seed was richer in volatile compounds than YMF sample, which presented an average value of 28.59 ± 4.47 μg/g of aromatic compounds. Furthermore, the analysis highlighted that the mustard bran fractions (OMB and YMB) contained lower concentration of volatile compounds when compared to their respective flours, with mean values of 27.80 ± 0.08 and 4.94 ± 0.43 μg/g, respectively.

Table 2. Concentration of the volatile compounds expressed in µg/g. The samples analysed were the following: YMF: Yellow Mustard Flour; YMB: Yellow Mustard Bran; OMF: Oriental Mustard Flour; and OMB: Oriental Mustard Bran.

Compound	YMF	YMB	OMF	OMB
Isothiocyanate				
Allyl Isothiocyanate	1.21 ± 0.62^{a}	0.23 ± 0.04^{b}	38.97 ± 1.09^{c}	6.98 ± 1.03^{d}
Alkane				
n-Octane	0.37 ± 0.10^{a}	0.18 ± 0.03^{b}	n.d	1.16 ± 0.12^{c}
2,4-Dimethylheptane	0.81 ± 0.17^{a}	n.d	n.d	0.42 ± 0.03^{b}
4-Methyloctane	1.04 ± 0.07^{a}	0.19 ± 0.00^{b}	n.d	1.52 ± 0.12^{c}
n-Nonane	0.21 ± 0.04^{a}	0.13 ± 0.04^{b}	n.d	n.d
2-Methylnonane	0.16 ± 0.06^{a}	0.06 ± 0.00^{b}	n.d	n.d
n-Decane	2.10 ± 1.41^{a}	0.23 ± 0.01^{b}	0.13 ± 0.01^{b}	1.29 ± 0.04^{c}
2,5-Dimethylnonane	0.25 ± 0.02^{a}	0.07 ± 0.00^{b}	0.36 ± 0.06^{c}	n.d
2,5-Dimethylnonane like	0.22 ± 0.03^{a}	0.11 ± 0.00^{a}	0.22 ± 0.04^{b}	n.d
2,6-Dimethylnonane	0.62 ± 0.23^{a}	0.15 ± 0.04^{b}	0.83 ± 0.10^{a}	n.d
5-Methyldecane	1.48 ± 0.61^{a}	0.06 ± 0.00^{b}	1.19 ± 0.17^{a}	0.84 ± 0.09^{ab}
4-Methyldecane	1.05 ± 0.07^{a}	0.27 ± 0.02^{b}	0.95 ± 0.04^{a}	0.50 ± 0.01^{c}
n-Undecane	2.30 ± 0.12^{a}	0.54 ± 0.01^{b}	1.58 ± 0.01^{c}	3.45 ± 0.23^{d}
n-Undecane like	0.84 ± 0.11^{a}	0.20 ± 0.01^{b}	0.51 ± 0.00^{c}	0.82 ± 0.04^{a}
3,7-Dimethyldecane	0.08 ± 0.01	n.d	n.d	n.d
5-Methylundecane	0.12 ± 0.02^{a}	0.06 ± 0.00^{b}	0.08 ± 0.00^{b}	0.17 ± 0.01^{c}
4-Methylundecane	0.29 ± 0.02^{a}	0.04 ± 0.00^{b}	0.38 ± 0.01^{c}	n.d
2-Methylundecane	0.75 ± 0.03^{a}	0.14 ± 0.00^{b}	0.98 ± 0.07^{c}	0.07 ± 0.01^{b}
3-Methylundecane	0.10 ± 0.01^{a}	n.d	0.04 ± 0.02^{b}	0.29 ± 0.03^{c}
2,5-Dimethylundecane	0.42 ± 0.03^{a}	0.10 ± 0.00^{b}	n.d	n.d
n-Dodecane	3.66 ± 1.52^{ac}	0.31 ± 0.00^{b}	1.85 ± 0.11^{ab}	5.15 ± 0.15^{c}
2,4-Dimethylundecane	0.27 ± 0.05^{a}	0.04 ± 0.00^{b}	0.48 ± 0.07^{c}	n.d
2,6-Dimethylundecane	0.77 ± 0.03^{a}	0.13 ± 0.00^{b}	1.30 ± 0.07^{c}	n.d
4,8-Dimethylundecane	0.81 ± 0.06^{a}	0.10 ± 0.01^{b}	1.49 ± 0.18^{c}	n.d
6-Methyldodecane	0.72 ± 0.02^{a}	0.10 ± 0.01^{b}	1.25 ± 0.02^{c}	n.d
4-Methyldodecane	0.21 ± 0.02^{a}	0.03 ± 0.00^{b}	n.d	n.d
2,6,11-Trimethyldodecane	2.31 ± 0.10^{a}	0.23 ± 0.00^{b}	3.85 ± 0.07^{c}	0.40 ± 0.04^{d}
2,6,11-Trimethyldodecane like	0.34 ± 0.03	n.d	n.d	n.d
2,6,11-Trimethyldodecane like	0.23 ± 0.01	n.d	n.d	n.d
2,6,11-Trimethyldodecane like	0.26 ± 0.02	n.d	n.d	n.d
n-Tridecane	0.27 ± 0.01^{a}	0.02 ± 0.00^{b}	0.26 ± 0.06^{a}	n.d
4,6-Dimethyldodecane	1.23 ± 0.02^{a}	0.14 ± 0.00^{b}	1.28 ± 0.01^{a}	0.44 ± 0.04^{c}
4,6-Dimethyldodecane like	0.44 ± 0.01^{a}	n.d	n.d	0.05 ± 0.00^{b}
2-Methyltridecane	0.18 ± 0.02^{a}	n.d	0.32 ± 0.05^{b}	0.10 ± 0.01^{c}
3-Methyltridecane	n.d	n.d	n.d	0.13 ± 0.00
n-Tetradecane	0.48 ± 0.16^{a}	0.03 ± 0.00^{b}	0.58 ± 0.00^{a}	0.45 ± 0.01^{a}
2,6,10-Trimethyltridecane	n.d	n.d	0.12 ± 0.00	n.d
Ketone				
6-Methyl-5-hepten-2-one	n.d	n.d	n.d	0.37 ± 0.02
3-octen-2-one	n.d	n.d	0.24 ± 0.01	n.d
2(3H)-Furanone, 5-ethyldihydro	n.d	n.d	0.46 ± 0.00^{a}	0.11 ± 0.01^{b}
3,5-octadien-2-one (E,E)	n.d	n.d	0.53 ± 0.01^{a}	0.45 ± 0.05^{b}
3,5-octadien-2-one (E,E) like	n.d	n.d	0.21 ± 0.06^{a}	0.44 ± 0.05^{b}

Table 2. Continuation

Compound	YMF	YMB	OMF	OMB
Ester				
Tetradecanoic acid, 1-methylethyl ester	n.d	n.d	n.d	0.13 ± 0.02
Palmitic acid, methyl ester	0.16 ± 0.02[a]	0.23 ± 0.10[a]	0.23 ± 0.04[a]	0.17 ± 0.04[a]
9-Octadecenoic acid (Z), methyl ester	0.17 ± 0.01[a]	0.33 ± 0.022[a]	0.25 ± 0.02[a]	0.13 ± 0.05[a]
Octadecenoic acid, methyl ester	0.13 ± 0.01[ab]	0.22 ± 0.10[b]	0.17 ± 0.03[ab]	0.13 ± 0.05[a]
Alcohol				
1-Hexanol	n.d	n.d	4.98 ± 0.05	n.d
1-Heptanol	n.d	n.d	0.31 ± 0.03	n.d
6-Methyl-5-hepten-2-ol	0.14 ± 0.01[a]	0.09 ± 0.00[a]	n.d	1.18 ± 0.04[b]
1-Nonanol	n.d	n.d	0.30 ± 0.03	n.d
1-Nonanol like	n.d	n.d	0.09 ± 0.01	n.d
Other				
D-Limonene	1.35 ± 0.23[a]	0.56 ± 0.02[b]	n.d	n.d
5-Ethylthiazole	0.07 ± 0.01[a]	0.02 ± 0.00[a]	0.19 ± 0.01[b]	n.d

n.d= no detected. Different letters mean statistical differences in the volatile content of each mustard ($p < 0.05$).

The main characteristic volatile detected in OMF and OMB was AITC, with a mean concentration of 38.97 ± 1.09 and 6.98 ± 1.03 µg/g, respectively. In contrast, the AITC detected in YMF and YMB was lower, with a mean value of 1.21 ± 0.62 and 0.23 ± 0.04 µg/g, respectively. This chemical compound is responsible for the sulfur and pungent odor of this spice and some beneficial properties such as antimicrobial and antifungal properties (Eib et al., 2020; Nazareth et al., 2018). Compared to the YMF and YMB samples, in which the AITC represented only 4.3% and 4.7% of the total aromatic compounds, respectively, AITC in the OMF and OMB represented 58.2% and 25.1%, respectively.

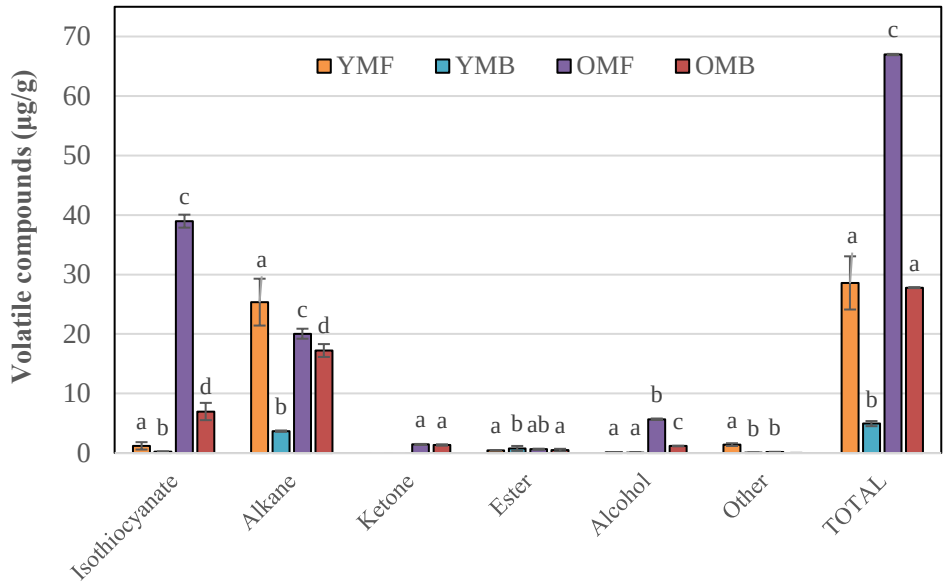


Figure 1. Volatile composition of mustard samples. The samples analyzed were the following: Yellow Mustard Flour (YMF), Yellow Mustard Bran (YMB), Oriental Mustard Flour (OMF) and Oriental Mustard Bran (OMB). The means with different letters are statistically different (p< 0.05)

The greatest variety of aromatic compounds detected in all samples were alkanes, mainly linear alkanes such as n-octane, n-nonane, n-decane, n-undecane, n-dodecane, n-tridecane and n-tetradecane, as well as multiple branched alkanes. This fraction was the most representative in the YMF and YMB samples, with a mean concentration of 25.36 ± 3.94 and 3.68 ± 0.11 µg/g, implying 88.70 and 74.45% of the total aromatic compounds detected, respectively. Although a total of 20.05 ± 0.84 and 17.23 ± 1.08 µg/g of alkanes were detected in the OMF and OMB samples, and they represented about the 30 and 62 % of the total aromatic fraction, respectively. Some linear alkanes reported in the study have been

identified previously in mustard essential oils, fresh mustard and other vegetables of the Brassica family (Wei et al., 2021; Zhao et al., 2007). Nevertheless, only a few reports refer to branched alkanes in mustard (Sharma et al., 2017). Thus, this study highlighted and identified for the first time new volatile alkanes that contribute to the aromatic fraction in mustard seeds.

Other volatile compounds detected in smaller proportions were ketones, alcohols, and esters. Only ketones were detected in the OMF and OMB samples (1.44 ± 0.09 and 1.35 ± 0.11 μg/g, which represents 2.15 and 4.97% of total aromatic compounds, respectively). Furthermore, except for 6-methyl-5-hepten-2-ol, which was detected in the YMF, YMB and OMB, the remaining identified alcohols (1-hexanol, 1-heptanol and 1-nonanol) were found in the OMF, with an average of 5.68 ± 0.12 μg/g (implying 8.47% of total aromatic compounds). Previously, some ketones, such as 6-methyl-5-hepten-2-one and 3,5-octadiene-2-one, as well as some alcohols (1-hexanol and 1-hepatanol) have been reported in pickled and dried oriental mustard (*Brassica juncea*, Coss.) (Shen et al., 2018; Zhao et al., 2007). Regarding esters, some of the compounds derived from fatty acids and determined in our samples have been reported by Sharma et al. (2018) in seeds and leaves of oriental mustard (*Brassica juncea*).

Among the compounds classified as miscellaneous, a terpene, D-limonene, was identified only in the YMF and YMB samples at a concentration of 1.35 ± 0.23 and 0.56 ± 0.02 μg/g, respectively. This compound has been described in yellow mustard essential oils (*Sinapis alba*), although in traces amounts (Miyazawa & Kawata, 2006).

3.2 Bioactive compound profile of mustard samples

Bioactive compounds in the mustard samples were analysed using UHPLC coupled to MS/MS detection. A total of 26 different bioactive compounds, including phenolic acids, flavonoids and glucosinolates, were identified in the mustard samples (**Table 3**). However, the distribution of these compounds changed between the mustard species and the extracted fraction analysed. Among these, 14 bioactive compounds were semi-quantified in the extracts and the results obtained are detailed in **Table 4**.

Phenolic acids in plants may exist in free, soluble-conjugated, and insoluble-bound forms. The conjugated forms are mainly linked to oligosaccharides and can be recovered after performing alkaline or acidic hydrolysis of the plant matrix (Gao et al., 2017; Kumar & Goel, 2019). So far, most studies only focused on the free fraction, while our study characterised both free and bound phenolic compounds in the mustard powders. Thus, the remaining extract obtained after performing the extraction of the free compounds was subjected to alkaline and acid hydrolysis. It was noted that the fraction analysed after performing the basic hydrolysis of the extracts (Fraction #2) had a lower quantity of phenolic acids in comparison with the free fraction (Fraction #1), except for sinapic acid. In all the samples, the phenolic acid content increased after performing the acid hydrolysis of the extracts (Fraction #3). These findings showed that acid hydrolysis is the best condition to release phenolic compounds and confirmed that these molecules in mustard are mainly esterified in the plant cell walls.

Table 3. Identification of bioactive compounds from mustard samples by ultra-high performance liquid chromatography (UHPLC-MS/MS) using different MS conditions.

Compound	RT	$[M-H]^-$ (*m/z*)	MS^2 ions (*m/z*)[a]	MS mode	Std for quantification
p-Hydroxybenzoic acid	3.20	137	93	SRM	*p*-Hydroxybenxoic acid
Salicylic acid	7.32	137	93	SRM	*p*-Hydroxybenzoic acid
p-Coumaric acid	6.12	163	119	SRM	*p*-Coumaric acid
Caffeic acid	5.00	179	135	SRM	Caffeic acid
Ferulic acid	6.60	193	134, 178, 149	SRM	Ferulic acid
Sinapic acid	6.66	223	208, 164, 121	SRM	Sinapic acid
Luteolin	7.95	285	133, 151, 175, 199, 241	Full Scan	Rutin
Quercetin	7.98	301	151, 121, 107, 179, 273	Full Scan	Rutin
Kaempferol	8.59	285	185, 187, 239, 211, 255, 171, 143, 145, 151	Full Scan	Rutin
Sinapine	5.96	294 $[M-CH4]^-$ 354 $[M+HCOO]^-$ 310 $[M]^{+}$*	264, 236, 147, 279, 119, 164, 208 251*, 175*, 207*, 119*, 147*	Full Scan	
Hydroxybenzoic acid-*O*-hexoside	4.84	299	137, 179, 151, 113, 101	Full Scan	
Sinigrin	1.15	358	97, 96, 75, 80, 116, 195, 241, 259, 275	Full Scan	
(epi)progoitrin	1.15	388	97, 96, 75, 259, 195, 128, 136, 80, 275	Full Scan	
Sinalbin	1.88	424	97, 96, 75, 182, 259, 195, 80, 275, 139, 119, 231, 241	Full Scan	
Glucobrassicin	5.89	447	97, 96, 95, 275, 254, 191, 113, 171, 137, 80	Full Scan	
Luteolin-*O*-hexoside	7.08	447	284, 285, 255, 227, 192, 175	Full Scan	Rutin
Kaempferol-O-hexoside	7.24	447	284, 285, 151	Full Scan	Rutin
Hydroxyglucobrassicin	3.29	463	97, 170, 169, 255, 275, 259, 285, 239, 189, 178, 205	Full Scan	
Quercetin-*O*-hexoside	6.83	463	300, 301	Full Scan	Rutin
Sinapoyl-vanilloyl-hexoside	7.03	535	167, 205, 152, 352, 367, 123, 108, 223	Full Scan	
Disinapoyl-hexoside I	7.62	591	223, 205, 367, 352, 164, 208, 149, 190	Full Scan	
Disinapoyl-hexoside II	7.93	591	205, 223, 190, 367, 265, 247, 164, 325, 149	Full Scan	
Disinapoyl-dihexoside	7.30	753	205, 529, 223, 247, 190, 289, 208, 179, 164	Full Scan	
Trisinapoyl-dihexoside	7.62	959	735, 529, 205, 511, 223, 289, 247, 385	Full Scan	
Kaempferol-sinapoyl-trihexoside I	6.10	977	815, 609, 447, 284, 285	Full Scan	Rutin
Kaempferol-sinapoyl-trihexoside II	6.49	977	815, 653	Full Scan	Rutin

[a]Fragment ions are reported in order of relative abundance. *MS data referred to experiment in positive ionization mode.

Regarding hydroxybenzoic acid derivatives, the yellow mustard (*Sinapis alba*) samples showed a higher content of *p*-hydroxybenzoic acid compared to oriental mustard (*Brassica juncea*). The overall content (sum of the three fractions analysed) of this phenolic acid in the YMF and YMB samples was 275.52 ± 20.82 and 226.62 ± 36.52 mg/kg, respectively, while for OMF and OMB was 34.37 ± 1.43 and 84.36 ± 17.12 mg/kg, respectively. This finding agrees with Martinović et al. (2020), who described that *p*-hydroxybenzoic acid was characteristic in *Sinapis alba* seeds. Another hydroxybenzoic acid, salicylic acid, was also identified and quantified in the mustard samples in mean values ranging from 16.12 ± 1.66 to 31.83 ± 2.68 mg/kg.

Hydroxycinnamic acid derivatives, the most common phenolic acids in the plant kingdom, such as *p*-coumaric acid, caffeic acid, ferulic acid and sinapic acid, were quantified in the four mustard samples. Sinapic acid was by far the predominant component in the four mustard samples analysed, reaching a content equal to 3060.84 ± 337.69, 1981.28 ± 195.09, 2104.72 ± 421.28 and 597.03 ± 172.83 mg/kg for YMF, YMB, OMF and OMB, respectively. The ferulic acid content in YMF and YMB samples was established at 60.02 ± 4.22 and 40.29 ± 13.08 mg/kg, while for OMF and OMB samples, it was 13.15 ± 0.59 and 4.85 ± 0.68 mg/kg. Caffeic acid and *p*-coumaric acid were the minor phenolic acids, recovered at trace levels ranged from 0.67 ± 0.07 to 3.51 ± 0.98 mg/kg and 1.88 ± 0.32 to 3.73 ± 0.54 mg/kg, respectively.

Table 4. Quantification of the main bioactive compounds of the yellow mustard flour (YMF), yellow mustard bran (YMB), oriental mustard flour (OMF) and oriental mustard bran (OMB). Results were expressed as mean ± SD in mg/kg of mustard powder.

Bioactive compound	YMF		
	Fraction #1	**Fraction #2**	**Fraction #3**
p-Hydroxybenzoic acid	25.02 ± 0.78^{a}	5.77 ± 1.06^{a}	244.73 ± 21.26^{a}
Salicylic acid	7.21 ± 0.65^{a}	5.63 ± 0.35^{a}	18.99 ± 2.53^{a}
p-Coumaric acid	0.68 ± 0.04^{a}	0.14 ± 0.03^{a}	2.45 ± 0.15^{a}
Caffeic acid	0.18 ± 0.03^{ab}	n.d	3.55 ± 0.53^{ab}
Ferulic acid	3.33 ± 0.35^{a}	1.48 ± 0.18^{ab}	60.02 ± 4.22^{a}
Sinapic acid	74.96 ± 14.61^{a}	117.67 ± 9.10^{a}	2868.21 ± 350.51^{a}
Luteolin	n.d	n.d	n.d
Quercetin	n.d	n.d	n.d
Kaempferol	n.d	n.d	n.d
Luteolin-*O*-hexoside	0.16 ± 0.09^{a}	n.d	n.d
Kaempferol-*O*-hexoside	0.66 ± 0.57^{a}	n.d	n.d
Quercetin-*O*-hexoside	n.d	n.d	n.d
Kaempferol-sinapoyl-trihexoside I	0.16 ± 0.10^{a}	0.18 ± 0.08^{a}	n.d
Kaempferol-sinapoyl-trihexoside II	n.d	n.d	n.d
Total	112.36 ±14.54^{a}	130.87 ± 9.27^{a}	3197.93 ±325.29^{a}
Bioactive compound	**YMB**		
	Fraction #1	**Fraction #2**	**Fraction #3**
p-Hydroxybenzoic acid	22.13 ± 1.90^{a}	3.05 ± 0.35^{b}	201.44 ± 35.30^{a}
Salicylic acid	6.70 ± 0.53^{a}	3.75 ± 0.16^{b}	20.71 ± 1.15^{a}
p-Coumaric acid	0.57 ± 0.03^{a}	0.13 ± 0.01^{a}	2.81 ± 0.95^{a}
Caffeic acid	0.12 ± 0.01^{a}	n.d	2.92 ± 1.32^{ab}
Ferulic acid	2.80 ± 0.29^{a}	1.26 ± 0.17^{a}	40.29 ± 13.08^{b}
Sinapic acid	32.08 ± 2.73^{b}	76.41 ± 17.81^{b}	1872.78 ± 118.28^{b}
Luteolin	n.d	n.d	0.77 ± 0.11
Quercetin	n.d	n.d	1.09 ± 0.12
Kaempferol	n.d	n.d	n.d
Luteolin-*O*-hexoside	1.51 ± 0.25^{b}	n.d	n.d
Kaempferol-*O*-hexoside	0.28 ± 0.01^{a}	n.d	n.d
Quercetin-*O*-hexoside	n.d	n.d	0.49 ± 0.05 ^{a}
Kaempferol-sinapoyl-trihexoside I	0.23 ± 0.08^{a}	n.d	n.d
Kaempferol-sinapoyl-trihexoside II	n.d	n.d	n.d
Total	66.42 ± 5.42^{b}	84.60 ± 18.21^{b}	2143.30 ± 232.04^{b}

Table 4. Continuation.

Bioactive compound	OMF		
	Fraction #1	**Fraction #2**	**Fraction #3**
p-Hydroxybenzoic acid	3.25 ± 0.31^{b}	0.40 ± 0.12^{c}	30.72 ± 1.23^{b}
Salicylic acid	7.10 ± 1.01^{a}	2.48 ± 0.93^{bc}	21.87 ± 2.72^{a}
p-Coumaric acid	0.32 ± 0.04^{b}	0.07 ± 0.01^{b}	0.95 ± 0.07^{b}
Caffeic acid	0.32 ± 0.12^{b}	n.d	4.78 ± 0.32^{a}
Ferulic acid	0.97 ± 0.10^{b}	0.10 ± 0.03^{a}	13.15 ± 0.59^{c}
Sinapic acid	132.60 ± 37.83^{c}	109.81 ± 5.84^{a}	1862.31 ± 445.11^{b}
Luteolin	n.d	n.d	n.d
Quercetin	n.d	n.d	4.44 ± 0.30
Kaempferol	n.d	n.d	15.53 ± 3.66^{a}
Luteolin-O-hexoside	0.38 ± 0.16^{ac}	3.16 ± 0.29^{a}	n.d
Kaempferol-O-hexoside	n.d	n.d	n.d
Quercetin-O-hexoside	n.d	n.d	18.44 ± 2.39^{b}
Kaempferol-sinapoyl-trihexoside I	3.84 ± 0.46^{b}	8.81 ± 0.94^{b}	n.d
Kaempferol-sinapoyl-trihexoside II	0.10 ± 0.03	0.59 ± 0.37	n.d
Total	148.87 ± 38.37^{c}	125.43 ± 4.28^{a}	1972.18 ± 448.65^{b}
Bioactive compound	**OMB**		
	Fraction #1	**Fraction #2**	**Fraction #3**
p-Hydroxybenzoic acid	2.84 ± 0.84^{b}	5.92 ± 1.19^{a}	75.60 ± 16.13^{b}
Salicylic acid	2.56 ± 0.45^{b}	2.15 ± 0.43^{c}	11.42 ± 1.68^{b}
p-Coumaric acid	0.25 ± 0.07^{b}	0.05 ± 0.02^{b}	0.37 ± 0.05^{b}
Caffeic acid	0.14 ± 0.05^{a}	n.d	1.75 ± 0.29^{b}
Ferulic acid	0.89 ± 0.29^{b}	5.28 ± 3.04^{b}	4.85 ± 0.68^{c}
Sinapic acid	8.73 ± 2.75^{b}	38.96 ± 7.86^{c}	549.33 ± 167.97^{c}
Luteolin	n.d	n.d	n.d
Quercetin	n.d	n.d	n.d
Kaempferol	n.d	n.d	1.10 ± 0.35^{b}
Luteolin-O-hexoside	0.74 ± 0.18^{c}	0.60 ± 0.11^{b}	n.d
Kaempferol-O-hexoside	n.d	n.d	n.d
Quercetin-O-hexoside	n.d	n.d	0.56 ± 0.05^{a}
Kaempferol-sinapoyl-trihexoside I	0.86 ± 0.45^{a}	0.86 ± 0.10^{a}	n.d
Kaempferol-sinapoyl-trihexoside II	n.d	n.d	n.d
Total	17.01 ± 5.04^{d}	53.82 ± 12.44^{b}	644.99 ± 185.50^{c}

Fraction #1 is referred to free bioactive compounds; Fraction #2 is referred to bioactive compounds released after alkaline hydrolysis; Fraction #3 is referred to bioactive compounds released after acid hydrolysis. Different letters mean statistical differences for each mustard powder according to the fraction analysed (p<0.05). n.d.=not detected.

Other authors have focused on phenolic compounds in different mustard plant fractions. For instance, Fang et al. (2008) quantified the main phenolic acids in potherb mustard (*Brassica juncea*, Coss.) and reported mean values of 116 ± 1.07, 211 ± 1.09, and 15.8 ± 0.25 mg/kg for ferulic, sinapic and *p*-hydroxybenzoic acid, respectively. In addition, ferulic acid, *p*-hydroxybenzoic acid and sinapic acid were identified but not quantified recently in yellow, oriental, and black mustard seeds by Boscariol Rasera et al. (2019).

Nevertheless, the values obtained previously by other authors are challenging to compare with our study because of the different extraction methodology, and the phenolic content can change considerably between plant fraction cultivars and environmental conditions (Bhandari & Kwak, 2015). Overall, the phenolic acid content was lower in the bran fractions than in the flour fraction of each mustard specie. However, it is relevant to consider that the bran is a by-product of mustard seed processing and can be used to recover these phytochemical compounds and revalue the product.

Some flavonoids, such as quercetin, luteolin, and kaempferol, were also identified in the mustard samples and quantified by comparison with a rutin standard. Luteolin was only detected in Fraction #3 of the YMB sample (0.77 ± 0.11 mg/kg). Kaempferol was exclusively found in *Brassica juncea* samples (OMB and OMF), in mean values from 1.10 ± 035 to 15.53 ± 3.66, respectively. Moreover, the ions corresponding to the loss of a hexoside (162 Da), sinapoyl-hexose (386 Da), and sinapoyl-dihexose (530 Da) allowed the identification of five glycosylated flavonoids, for instance, quercetin-*O*-hexoside (*m/z* 463), luteolin-*O*-hexoside (*m/z* 447), kaempferol-*O*-hexoside (*m/z* 447), kaempferol-sinapoyl-trihexoside I (*m/z* 977), and kaempferol-sinapoyl-trihexoside II (*m/z* 977). Flavonols such as quercetin and kaempferol are characteristic in Brassica plants and are commonly found as O-

glycoside forms (Cartea et al., 2010). These compounds were detected in a range between 0.10 and 18.44 mg/kg, according to the sample analysed, and the quercetin-*O*-hexoside was the most abundant flavonoid recovered in Fraction #3 of the OMF.

Other polyphenolic compounds were tentatively identified in the mustard samples (**Table A1**). The ions corresponding to the loss of a hexose moiety (162 Da), sinapic acid (224 Da), sinapoyl-hexose moiety (368 Da), sinapoyl-dihexose moiety (530 Da), were indicative of six additional glycosylated phenolics (hydroxybenzoic-*O*-hexoside, sinapoyl-vanilloyl-hexoside, disinapoyl-hexoside I, disinapoyl-hexoside II, disinapoyl-dihexoside and trisinapoyl-dihexoside). These compounds were mainly identified in Fraction #1 and Fraction #2 of the mustard samples. Moreover, sinapine, a sinapic acid choline ester, was also identified in the free and bound fractions of the mustard seeds. The reported values for this compound range from 4-29.2 g/kg in defatted seeds, and it is estimated that represents the 90% (w/w) of the sinapic acid derivatives (Flourat et al., 2019; Martinović et al., 2020; Mayengbam et al., 2014). The bioactive profile was in accordance with Engels et al. (2012), who identified the glycosylated esters of sinapic acid in oriental mustard (*Brassica juncea* L.) seed meal. Thus, the study showed a chemical diversity in polyphenolic composition, mainly sinapic acid derivatives in both mustard seeds.

In addition to the other bioactive compounds, up to 5 glucosinolates were identified in Fraction #1 and Fraction #2 of the mustard seeds. Sinigrin, sinalbin, progoitrin and glucobrassicin were detected in all mustard samples, while hydroxyglucobrassicin was only identified in the OMF sample. Among the glucosinolates identified, the most representative described in oriental mustard (*Brassica juncea*) is sinigrin and its main hydrolysis product by myrosinase action the AITC, an isothiocyanate that has been previously detected in the aromatic

fraction analysis (Herzallah & Holley, 2012). Likewise, in yellow mustard (*Sinapis alba*), the characteristic glucosinolate described is sinalibin, that generates *p*-hydroxybenzyl isothiocyanate, a non-volatile antimicrobial compound with low stability, which could explain its absence in the previous analysis performed (Borek & Morra, 2005; Monu et al., 2014). Furthermore, the other glucosinolates identified in our mustard seeds are precursors of isothiocyanates with beneficial properties, such as 3-indolymethyl isothiocyanate (glucobrassicin), (2R)-Hydroxybut-3-enyl isothioacyanate (progoitrin) and 4-Hydroxy-3-indolylmethyl isothiocyanate (hydroxyglucobrassicin). These compounds have been reported in mustard and other cultivars of the Brassica family, such as broccoli, cauliflower and cabbage, among others (Melrose, 2019; Qu et al., 2020).

3.3 Total phenolic content and antioxidant activity of mustard samples

The study determined the TPC in the mustard samples after extraction using the Folin-Ciocalteu reagent and the results are shown in **Figure 2a**. The YMF sample presented the higher polyphenol content (22996.97 ± 797.51 mg/kg of GAE) compared to the rest of samples analysed (p value < 0.05). It was noted that YMB also presented a high value of TPC in comparison to OMF and OMB (8601.06 ±776.59, 8174.78 ± 97.71 of mg/kg of GAE, respectively), with an average of 17136.54 ± 784.83 mg/kg of GA equivalents. Previous studies have determined the TPC of yellow and oriental mustard seeds. However, there are no reports focused exclusively on the mustard bran fraction. Depending on the solvents employed, different TPC values have been reported for mustard seeds and plant fractions. Similar results to our study were obtained by Boscariol Rasera et al. (2019) , which established a TPC content ranging from 0.82 to 20.00 mg GAE/g for *Sinapis alba* seeds, while TPC ranged from 0.65 to 12.16 mg/g for *Brassica nigra* seeds. Also, TPC

had been established in fresh *Brassica juncea* leaf extracts, for instance, Subudhi & Bhoi (2014) had reported 23.1 mg GAE/100 g.

Moreover, the antioxidant activity was measured as DPPH scavenging activity and the results obtained are plotted in **Figure 2b**. Both yellow and oriental mustard flours (YMF and OMF) showed higher antioxidant activity (119.61 ± 2.70 and 222.24 ± 14.23 mM TE/g, respectively) in comparison with their bran fractions (83.61 ± 0.18 and 78.64 ± 2.05 mM TE/g for YMB and OMB, respectively) (p<0.05). These differences between flour and bran fractions could be explained by the lower presence of bioactive compounds that was not possible to quantify in our samples but with a strong DPPH radical scavenging activity, such as sinapine or other sinapic acid derivatives (Thiyam et al., 2006).

3.4 Principal component analysis (PCA)

In order to stress the differences in the production of bioactive metabolites between the mustard fractions and species (*Sinapis alba* or *Brassica juncea*), a PCA analysis was performed with the quantification of all the compounds detected in the samples, both volatile and bioactive compounds (phenolic acids and flavonoids), and the results obtained are shown in **Figure 3**. The sum of the first two principal components (PC) reached 75.6% of the total variance, of which PC1 represented 41.4% and PC2 34.2% of the total variance. The samples analysed were distinguished in four different clusters (**Figure 3a**). The PC1 distributed the YMF, YMB and OMB on the negative axis, while OMF was positioned on the positive axis. The PC2 differenced the two species of plants analysed, *Sinapis alba* (on the negative axis) and *Brassica juncea* (positive axis) and confirmed the particularity of each mustard specie based on its bioactive compounds.

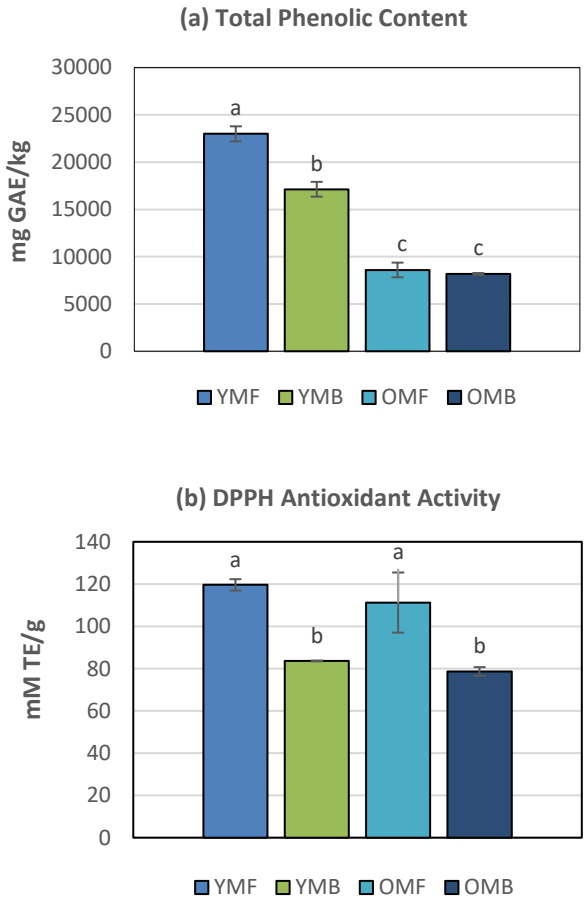


Figure 2. Total Phenolic Content (TPC) (a) and Antioxidant activity (b) of mustard samples. The samples analysed were the following: Yellow Mustard Flour (YMF), Yellow Mustard Bran (YMB), Oriental Mustad flour (OMF) and Oriental Mustard Bran (OMB). Different letters mean statistical differences ($p< 0.05$).

The loading plot is shown in **Figure 3b** and presents the relative importance of the variables analysed. The YMF and YMB fraction presented a similar profile according to the first component and were distinguished between each other based

on the second component. The phenolic acids that influenced the statistical differences among *Brassica juncea* seed fractions were the higher *p*-hydroxybenzoic (V54), ferulic (V58) and *p*-coumaric (V56) content quantified in the *Sinapis alba* seed fractions. Moreover, some volatile alkanes, such as 4-methyldodecane (V26), 2,5-dimethylundecane (V20), n-nonane (V5), and 2-methylnonane (V6), were characteristic of *Sinapis alba* seed fractions.

Regarding OMB, the variables that influenced the separation from the other samples were the presence of 6-methyl-5-hepten-2-ol (V49), tetradecanoic acid, 1-methyl ethyl ester (V43), and 6-methyl-5-hepten-2-one (38), which were characteristic of this seed fraction. Concerning OMF, higher AITC (V1) content and the identification of some glycosylated flavonoids allowed the differentiation from the OMB, YMF and YMB; for instance, kaempferol-sinapoyl-trihexoside I (V66), kaempferol (V62), quercetin-O-hexoside (V65) and kaempferol-sinapoyl-trihexoside II (V67). Moreover, other metabolites were only found in *Brassica juncea* seed fractions, such as 3,5-octadien-2-one (41), 2(3H)-furanone, ethyl dihydro (V40) and 2-methyltridecane (V34).

The samples analysed presented some particularities in aromatic compounds or their polyphenol profile, allowing the distinction between mustard species (*Sinapis alba* or *Brassica juncea*) and fractions. Hence, these differences should be considered for further uses of the mustard seeds, either in food or for recovering of the phytochemicals for pharmaceutical applications.

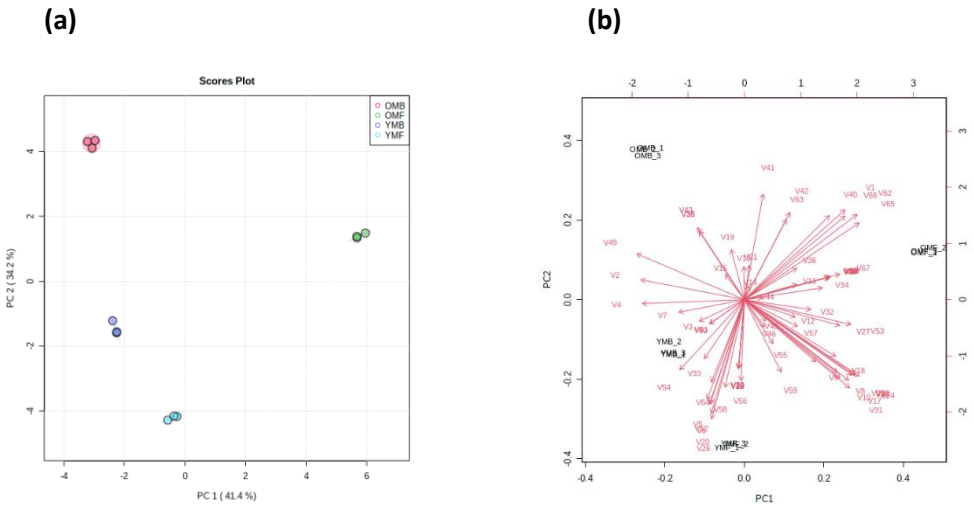


Figure 3. Scatter plot of scores from PC1 vs. PC2, obtained using the concentrations of the volatile and the bioactive compounds found in the mustard samples (a) and relative loadings of the variables used (b).

In conclusion, the information provided in the study demonstrates that mustard seed is a rich source of bioactive compounds, such as phenolic acids, flavonoids and glucosinolates. Moreover, to the best of our knowledge, yellow and oriental mustard bran was characterized for the first time, revealing high content of *p*-hydroxybenzoic acid and sinapic acid, and thus suggesting its revalorization as source of bioactive compounds with biological and health-promoting effects. Perhaps, they could be used in foods or pharmaceutical applications. However, further investigations should study strategies for the recovery of these phytochemicals from the mustard by-products, with a special focus on the bound phenolics since they represent a relevant fraction of the bioactive components in mustard.

Table A1. Distribution of non-quantified metabolites in the mustard powders. The samples analysed were the following: Yellow Mustard Flour (YMF); Yellow Mustard Bran; Oriental Mustard Flour (OMF); Oriental Mustard Bran (OMB).

Bioactive compound	YMF			YMB		
	Fraction #1	Fraction #2	Fraction #3	Fraction #1	Fraction #2	Fraction #3
Sinapine	DNQ	DNQ	DNQ	DNQ	DNQ	DNQ
Hydroxybenzoic acid-*O*-hexoside	DNQ	n.d	n.d	DNQ	n.d	n.d
Sinigrin	DNQ	DNQ	n.d	DNQ	DNQ	n.d
(epi)progoitrin	DNQ	DNQ	n.d	DNQ	n.d	n.d
Sinalbin	DNQ	DNQ	n.d	DNQ	DNQ	n.d
Glucobrassicin	DNQ	n.d	n.d	DNQ	n.d	n.d
Hydroxyglucobrassicin	n.d	n.d	n.d	n.d	n.d	n.d
Sinapoyl-vanilloyl-hexoside	n.d	n.d	n.d	n.d	n.d	n.d
Disinapoyl-hexoside I	DNQ	DNQ	n.d	DNQ	DNQ	n.d
Disinapoyl-hexoside II	DNQ	DNQ	n.d	DNQ	DNQ	n.d
Disinapoyl-dihexoside	DNQ	n.d	n.d	n.d	n.d	n.d
Trisinapoyl-dihexoside	DNQ	DNQ	n.d	DNQ	DNQ	n.d
Bioactive compound	**OMF**			**OMB**		
	Fraction #1	**Fraction #2**	**Fraction #3**	**Fraction #1**	**Fraction #2**	**Fraction #3**
Sinapine	DNQ	DNQ	DNQ	DNQ	DNQ	DNQ
Hydroxybenzoic acid-*O*-hexoside	DNQ	n.d	n.d	DNQ	n.d	n.d
Sinigrin	DNQ	DNQ	n.d	DNQ	DNQ	n.d
(epi)progoitrin	DNQ	DNQ	n.d	DNQ	DNQ	n.d
Sinalbin	DNQ	DNQ	n.d	DNQ	DNQ	n.d
Glucobrassicin	DNQ	n.d	n.d	n.d	n.d	n.d
Hydroxyglucobrassicin	DNQ	n.d	n.d	n.d	n.d	n.d
Sinapoyl-vanilloyl-hexoside	DNQ	DNQ	n.d	DNQ	DNQ	n.d
Disinapoyl-hexoside I	DNQ	DNQ	n.d	DNQ	DNQ	n.d
Disinapoyl-hexoside II	DNQ	DNQ	n.d	DNQ	DNQ	n.d
Disinapoyl-dihexoside	DNQ	DNQ	n.d	DNQ	DNQ	n.d
Trisinapoyl-dihexoside	DNQ	DNQ	n.d	DNQ	DNQ	n.d

Fraction #1 is referred to free bioactive compounds; Fraction #2 is referred to bioactive compounds released after alkaline hydrolysis; Fraction #3 is referred to bioactive compounds released after acid hydrolysis.

n.d=not detected; DNQ=detected but not quantified.

LWT – Food Science and Technology, 154, 112795 (2021)

3.5 Antifungal activity of natamycin and development of an edible film based on hydroxyethylcellulose to avoid *Penicillium* spp. growth on low-moisture mozzarella cheese

1. Introduction

Low-moisture Mozzarella is a typical Italian stretched-curd cheese made from cow's of buffalo's milk that possesses a high water activity (a_w 0.95) and a pH value slightly acid (from 5.2 to 5.6). The microflora of this product consists of the cultured used for cheese manufacture (natural or selected), mainly *Lactobacillus* and *Streptococcus* species (Ercolini et al., 2012; Marino et al., 2019). However, these physicochemical properties, in addition to he high water activity associated with high moisture content (among 45 and 52%), creates a suitable environment for microbiological spoilage by fungi, yeasts, and aerobic bacteria, even when the product is stored at low temperatures (1-5 °C) (Garnier et al., 2017; Ma et al., 2013).

Penicillium is the genus that mostly contaminates semi-hard cheese under refrigeration conditions (Banjara et al., 2015). The main species described as spoilage agents in this typology of products are *P. commune*, *P. palitans*, *P. roqueforti*, *P. camemberti*, *P. solitum*, *P. nalgiovense*, and *P. verrucosum* (Petruzzi et al., 2017). Fungal contamination is also relevant because some species may produce mycotoxins such as ochratoxin A, penillic acid, cyclopiazonic acid, and roquefortine C, some of which are highly toxic (Sengun, Yaman & Gonul, 2008).

In order to avoid fungal spoilage, antifungal compounds are incorporated into the recipe of perishable foods to minimize food spoilage and, therefore, economic losses. Specifically, the most common additives used in cheese manufacture are sorbates and benzoates (Herr, 2011). Another alternative is the direct application of antimicrobial agents by different techniques such as spraying, coating, or dipping to prevent the growth of spoilage fungi in cheese (Lucera et al., 2012). However, in the last decade, consumer have demanded new alternatives for additive-free food products or food products based on natural compounds (Leyva Salas et al., 2017;

Ribes et al., 2018). In this context, different molecules of natural origin, such as nisin, lysozyme and natamycin (NTC), were authorized for their use in cheese manufacture as preservatives by the European Union (Regulation 1333/2008/EC). Besides, the current trend in food packaging is searching for new biodegradable matrices to substitute plastic polymers that can lead to environmental problems (Costa et al., 2018; Din et al., 2020; Trajkovska Petkoska et al., 2021). Different biopolymers have been evaluated as edible packaging among these matrices, for instance, pectins, alginates, chitosan, cellulose, and its derivatives (Aziz et al., 2018; Balaguer et al., 2014; Mellinas et al., 2016).

Hydroxyethylcellulose (HEC) is a water-soluble cellulosic derivate with non-ionic properties, traditionally used in the biomedicine, paint industry, and pharmaceutical industry (Mel'nikov et al., 2020; Noreen, Zia, Tabasum, Khalid, et al., 2020). Because of its excellent film-forming capacity, safety, and low cost, current studies have described the use of HEC for the development of antimicrobial food packaging (El Fawal et al., 2020; Luz et al., 2019; Quiles et al., 2019; Silva et al., 2016). Moreover, due to its low toxicity, immunogenicity, and biocompatibility, it has been employed as a thickening or binder, as well as a protective suspension and colloid stabilizer, in a wide variety of application, including coating, biomedical, and food (Kanmani & Rhim, 2014; Noreen, Zia, Tabasum, Aftab, et al., 2020).

NTC is a macrolide polyene synthesized by *Streptomyces natalensis* bacteria without odour or tase properties (Stark & Tan, 2003). NTC has been utilized as an antifungal agent during manufacturing of cheeses such as cheddar and blue cheese. This compound is effective against fungal spoilage regardless of their fungicide resistance phenotypes and has been used as an antifungal dip or spray to prevent fungal growth on fruits such as blueberries, raspberries, and strawberries, extending their shelf life (Wang et al., 2021). For proper ripening and preserving

sausage, NTC is often used as an antifungal agent in the dipping or spraying process (Juneja et al., 2012). Previous results also demonstrated that NTC treatment significantly reduced vegetative growth in important postharvest pathogenic fungi such as *Botrytis cinerea* and *Penicillium expansum* (He et al., 2019).

NTC antifungal properties are related to the interaction with ergosterol, the principal sterol in fungal cells (Aparicio et al., 2016). Specifically, it has been described that NTC inhibits the transport of essential micronutrients across the plasma membrane due to the interaction with transport proteins dependents of ergosterol and impairs the membrane fusion of vacuoles (te Welscher et al., 2010; te Welscher et al., 2012). Moreover, NTC is considered a substance generally recognised as safe (GRAS); hence, the European Union allows its use as an additive on the surface of semi-hard and semisoft cheese rind at a maximum level of 1 mg/dm^2 (Regulation 1333/2008/EC).

Against this background, the study aimed to determine NTC's *in vitro* antifungal activity against *Penicillium* spp. and evaluate its use as a shelf-life enhancer in Mozzarella cheese slices (MCS) contaminated with *P. commune*. For these purposes, two antifungal treatments were proposed: 1) The direct application of a water solution containing NTC onto the cheese surface, and 2) The manufacture of an edible antifungal film containing HEC and NTC placed in contact with the MCS. Besides, NTC residual content was determined on the MCS during the storage to study its prevalence in the product.

2. Materials and methods

2.1 Chemicals

HPLC-grade acetonitrile (ACN), methanol, formic acid, TWEEN® 80, and glycerol were purchased from Sigma Aldrich (St. Louis, MO, USA). Deionised water for chromatography analysis was obtained from a Milli- Q water purification system (<18 MΩ). Microbiological products such as Potato Dextrose Broth (PDB), Potato Dextrose Agar (PDA), and Buffered Peptone Water were obtained from Liofilchem Bacteriology Products (Roseto, Italy). HEC was acquired from Merck Schuchardt OHG (Hohenbrunn, Germany). NTC NATAP® was purchased from Handary (Brussels, Belgium).

2.2 Fungal strains and culture conditions

The strains of *Penicillium camemberti* CECT 2267, *P. expansum* CECT 2278, *P. roqueforti* CECT 2905, *P. digitatum* CECT 2954, *P. nordicum* CECT 2320, *P. brevicompactum* CECT 2316, *P. commune* CECT 20767, and *P. solitum* CECT 20828 were obtained from the Spanish Type Culture Collection (CECT) (Valencia, Spain). *P. verrucosum* VTT D-01847 was purchased from the VTT Technical Research Centre of Finland LTD (Otaniemi, Finland). Firstly, the microorganisms were stored in sterile PDB media with glycerol 25% at −80 °C before use. After that, the strains were transferred to a PDB medium and incubated at 25 °C for 7 d. Then, aliquots of 100 µL were streaked across the surface of PDA plates and incubated at 25 °C for 5 d. Finally, these plates were used to harvest conidia and contaminate the samples.

2.3 Qualitative antifungal tests in PDA medium

The antifungal activity of aqueous NTC suspension was qualitatively evaluated against strains of the genus *Penicillium* (item 2.2). For this purpose, NTC powder was resuspended in sterile H_2O at concentrations ranging from 5 to 200 µg/mL. The fungi strains were sown on PDA plates using a sterile cotton swab, and 100 µL of NTC solution was dispensed in an 8 mm diameter well at the center of inoculated agar. The plates were incubated for 48 h at 25 °C to observe the inhibition zone measured by a scale in millimeters.

2.4 In vitro determination of the minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC)

The quantitative determination of the antifungal activity was realised according to the CLSI, 2008 document M38-A2 (2008) with some modifications.

Firstly, spore solutions were obtained from the cultivated PDA plates (previously described in section 2.2), streaking the colonies with a cotton swab soaked in sterile TWEEN water (0.2%). The recovered spores were then diluted in the PDB medium and adjusted to 5×10^4 spores/mL with the Neubauer chamber (L. Optik, Germany). Polypropylene Falcon™ 96-well microplates (Thermo Fischer Scientific, Waltham, Ma) were employed in this assay, and the solutions were dispersed into the wells as follows. The first column was filled with 200 µL of PDB as a negative control. The second column contained 100 µL of PDB and 100 µL of spore solution as a positive control. The following microplate columns contained 100 µL of spore solution and 100 µL of diluted NTC on PDB at concentrations ranging from 200 to 0.8 µg/mL. The plates were briefly incubated at 25 °C for 72 h with a final volume of 200 µL per well. After incubation, the MIC was determined

as the lowest concentration of NTC that visually inhibited the growth of the *Penicillium* strains. To establish the MFC, aliquots of 10 µL of the MIC and highest doses were inoculated onto PDA plates and incubated at 25 °C for 48 h. The MFC was defined as the lowest NTC concentration that inhibited the fungal growth on the recultured plates.

2.5 Antifungal treatment of mozzarella cheese slices

Fresh MCS free of chemical preservatives was placed in polypropylene (PP) trays and contaminated with 100 µL of the *P. commune* CECT 20767 spore suspension diluted in 0.1% buffered peptone water at a concentration of 2×10^5 spores/mL. After, the samples were treated with NTC as follows. Firstly, NTC powder was resuspended in sterile H_2O, and 2 mL were sprayed on the product surface to achieve a final concentration of 0.25, 0.5, and 1 mg/dm^2. The MCS surface was estimated by measuring the radius (r) of the cheese slices (n = 9) and applying the following equation:

$$Area = \pi r^2$$

Control treatment consisted of sterile H_2O spray. Simultaneously, the second treatment evaluated the incorporation of NTC in HEC films. These films were constituted by HEC (2%), glycerol (0.5%), and NTC. The ingredients were homogenized in sterile H_2O at 40 ◦C for 10 min with magnetic stirring. After, aliquots of 10 mL were coated in polystyrene Petri dishes (100 mm × 15 mm) and dried in an oven at 30 °C for 72 h to form the film. NTC was adjusted in the film based on the Petri dish surface (0.785 dm^2) to achieve a final concentration of 0.25, 0.5, and 1 mg/dm^2. For this, NTC was added to the homogenized ingredients in the following

proportions: 1.96 mg/100 mL, 3.92 mg/100 mL, and 7.85 mg/100 mL to form 0.25, 0.5 and 1 mg/dm^2 films, respectively. The films were placed on the surface of the MSC to observe the antifungal effect. The control groups consisted of films without NTC (0 mg/ dm^2) and slices of MCS without film. The doses studied for both spray and edible film approaches were selected according to the legislative regulations of the maximum level of NTC authorised for the surface treatment of cheese in Europe (1×, 1/2×, and 1/4× of the maximum dose allowed) (Regulation 1333/2008/EC). Lastly, PP trays were heat-sealed and incubated under refrigeration conditions for 30 days at 4 °C. All treatment doses were realised with nine replicates, and each experiment was carried out three times.

2.6 Determination of the fungal population and shelf life

After 30 days of incubation time, the fungal population (FP) was analysed on the cheese slices. Thereby, each MCS was placed into a sterile plastic bag containing 80 mL of 0.1% buffered peptone water and homogenized with a Stomacher IUL (Barcelona, Spain) for 30 s. After, 1 mL of the mixture was serially diluted in 15 mL Falcon tubes containing 9 mL of buffered peptone water (0.1%), and 100 µL of each dilution was streaked on PDA plates in triplicate. The plates were incubated at 25 °C for 48 h for colony counting.

The superficial growth of the *P. commune* was observed daily for 30 days to establish the microbiological shelf life of the MCS. The shelf life was considered to end when the fungal growth was visually detected on the product surface (Lopes et al., 2018).

2.7 Determination of residual natamycin during storage

NTC was extracted and determined from mozzarella cheese slices following the methodology described by Paseiro-Cerrato et al. (2013) with slight modifications. At different storage periods, 0.5 g of each sample (with a surface of 2.25 cm^2 and 1 mm depth) was placed in 50 mL tubes with 5 mL of methanol acidified with 0.0001% acetic acid and vortexed for 5 min. The mixture was then diluted with Milli-Q water (1:2) and stored at −18 ◦C for 12 h. Next, samples were filtered with a 0.45 µm PTFE membrane filter and injected into a chromatography system. The chromatograph was a Shimadzu LC system (Kyoto, Japan), equipped with a Kinetex EVO C18 column (4.6 × 150 mm i.d.; 5 µm; 100 Å) (Phenomenex, Palo alto, CA) and a Hitachi L-7455 Diode Array Detector (Tokyo, Japan). The mobile phases employed were ACN (Phase A) and MilliQ Water (Phase B). Elution was carried out in gradient mode at a flow rate of 0.5 mL/min, as follows: 0 min 30% A; 10 min 60% A; 15 min; 60% A; and 20 min 30% A. The injection volume was set at 20 µL. The wavelength was set at 319 nm for NTC determination.

2.8 Statistical analysis

GraphPad Prism version 3.0 software was employed for statistical analysis. The differences between groups ($p \leq 0.05$) were analysed by a one-way-ANOVA statistical test followed by the Tukey post hoc test for multiple comparisons.

3. Results and discussion

3.1 Inhibitory effect of NTC against Penicillium strains in vitro

NTC's *in vitro* antifungal activity was qualitatively evaluated on PDA medium through agar diffusion assay towards nine strains of *Penicillium* genera, most of them considered traditional spoilage agents of dairy products. As presented in **Table 1**, all tested strains were sensitive to NTC in doses ranging from 50 to 200 µg/mL, depending on the fungal strain.

In particular, NTC at 200 µg/mL evidenced inhibition halos larger than 10 mm diameter against *P. roqueforti* CECT 2905, *P. nordicum* CECT 2320, *P. commune* CECT 20767, and *P. solitum* CECT 20818 strains. Moreover, the highest dose tested also presented inhibition halos of 8 mm diameter against *P. camemberti* CECT 2267 and *P. digitatum* CECT 2954.

Table 1. In vitro antifungal activity of natamycin against Penicillium strains through agar well diffusion method. The antifungal activity was expressed as follows: (+) means 8 mm of inhibition zone; (+ +) means 8-10 mm of inhibition zone (+ + +) means > 10 mm of inhibition; (−) means no inhibition zone observed.

Fungal strain	Concentration (µg/mL)					
	5	10	25	50	100	200
P. camemberti CECT 2267	−	−	−	−	−	+
P. expansum CECT 2278	−	−	−	+	+ +	+ +
P. roqueforti CECT 2905	−	−	−	+	+ +	+ + +
P. verrucosum VTT D-01847	−	−	−	−	−	+ +
P. digitatum CECT 2954	−	−	−	−	−	+
P. nordicum CECT 2320	−	−	−	+	+ +	+ + +
P. brevicompactum CECT 2316	−	−	−	+	+ +	+ +
P. commune CECT 20767	−	−	−	+	+ +	+ + +
P. solitum CECT 20818	−	−	−	+	+ +	+ + +

NTC's MIC and MFC values were established by *in vitro* 96 well-plate assays against the same fungi qualitatively tested on PDA agar diffusion assay. The results showed that MIC values ranged from 1.6 to 3.1 µg/mL against *Penicillium* after 72 h of incubation (**Table 2**). These results suggested that the MIC values could differ depending on the fungal strain. Some authors have also reported the variability of the microbiological tests previously based on the strain used (Mouton et al., 2018). Similarly, the MFC values differed according to the fungal strain. For instance, the MFC values ranged from 12.5 to 200 µg/mL for *P. solitum* CECT 20818 and *P. camemberti* CECT 2267, respectively. These results agree with the data previously obtained in the qualitative test, evidencing NTCs' high potential to control the fungal growth of *Penicillium* strains.

Table 2. Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration of natamycin against Penicillium strains expressed in µg/mL.

Fungal strain	**MIC**	**MFC**
P. camemberti CECT 2267	3.1	200.0
P. expansum CECT 2278	1.6	100.0
P. roqueforti CECT 2905	1.6	50.0
P. verrucosum VTT D-01847	1.6	25.0
P. digitatum CECT 2954	1.6	100.0
P. nordicum CECT 2320	1.6	25.0
P. brevicompactum CECT 2316	1.6	100.0
P. commune CECT 20767	1.6	25.0
P. solitum CECT 20818	1.6	12.5

NTC has been reported as an antifungal agent and has traditionally been used as a treatment agent in human mycoses and different food applications (Ansari et al., 2013; Delves-Broughton et al., 2005). Previous studies have evaluated the *in vitro* antifungal activity of NTC against different fungal agents. Saito et al. (2020)

studied the antifungal properties of NTC against *B. cinerea* isolates from mandarin fruit. They found that MIC values of NTC for conidial germination comprised 0.7–1.0 µg/mL, whereas the MIC values for mycelial growth were in the range of 5.0–10.0 µg/mL. Brothers & Wyatt (2000) determined the MIC values of NTC against *Aspergillus* spp. that was isolated from poultry feed. In this study, the authors found MIC values of 5.08 and 40.1 µg/mL of NTC for *A. fumigatus* and *A. parasiticus*, respectively.

Similarly, Martin & Roman (2018) established the MIC50 of NTC against toxigenic strains of the *Penicillium* genera. The authors demonstrated that doses between 5 and 31 µg/mL of NTC could reduce fungal growth. Our results corroborated these findings since NTC showed an antifungal effect in amounts lower than 40.1 µg/mL and provided a better knowledge of the NTC antifungal properties against spoilage fungal agents of the *Penicillium* genera. In fact, due to the antifungal properties that NTC has shown in *in vitro* studies, several authors have recently proposed its incorporation into different polymers to increase food safety (González-Forte et al., 2019; Grafia et al., 2018; Mehyar et al., 2018). For this reason, based on our results and the results observed from previous studies, we proposed the NTC application on MCS to control the development of *P. commune*.

3.2 Antifungal effect of natamycin in low-moisture mozzarella cheese slices

The MCS were inoculated with *P. commune*, one of the most common spoilage agents contaminating dairy industry products. This species may grow at low temperatures, reducing the shelf life of cheese and related products (Pitt, 2006). This study established microbiological shelf life as the first sign of visual fungal growth on the sample surface. In particular, the control groups presented ten days

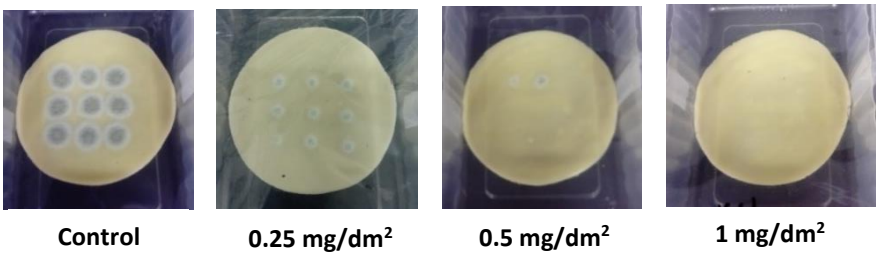
of shelf life. However, the NTC application at the dose of 0.5 mg/dm^2 by spray and film treatment increased the shelf life by 20 and 15 days, respectively (**Table 3**). The film containing NTC at 1 mg/dm^2 also extended the shelf life by 25 days compared to control; besides, the MCS sprayed with 1 mg/dm^2 of NTC did not evidence fungal growth after 30 days (**Figure 1**). Also, it is essential to note that all doses studied extended the shelf life of the samples by at least five days. These results suggested that NTC could be used as an antifungal substance to increase the safety and shelf life of MCS under refrigeration conditions, either as an aerosol through direct application or incorporated into HEC antifungal film.

Table 3. The shelf life of low moisture Mozzarella cheese contaminated with *P. commune* CECT 20767 and treated with natamycin using two methods: (a) Application of natamycin by direct spray on the surface (0.25, 0.5 and 1 mg/dm^2); (b) Incorporated in hydroxyethylcellulose antifungal films at different doses (0, 0.25, 0.5, and 1 mg/dm^2).

(a)						
Treatment	**Days**					
	5	**10**	**15**	**20**	**25**	**30**
Control	−	+	+	+	+	+
0.25 mg/dm^2	−	−	−	+	+	+
0.5 mg/dm^2	−	−	−	−	−	+
1 mg/dm^2	−	−	−	−	−	−
(b)						
Treatment	**Days**					
	5	**10**	**15**	**20**	**25**	**30**
Control	−	+	+	+	+	+
0 mg/dm^2	−	+	+	+	+	+
0.25 mg/dm^2	−	−	−	+	+	+
0.5 mg/dm^2	−	−	−	−	+	+
1 mg/dm^2	−	−	−	−	−	+

Shelf life is expressed as: (−) means non-evidenced fungal growth, and (+) means fungal growth evidenced.

(a)



(b)

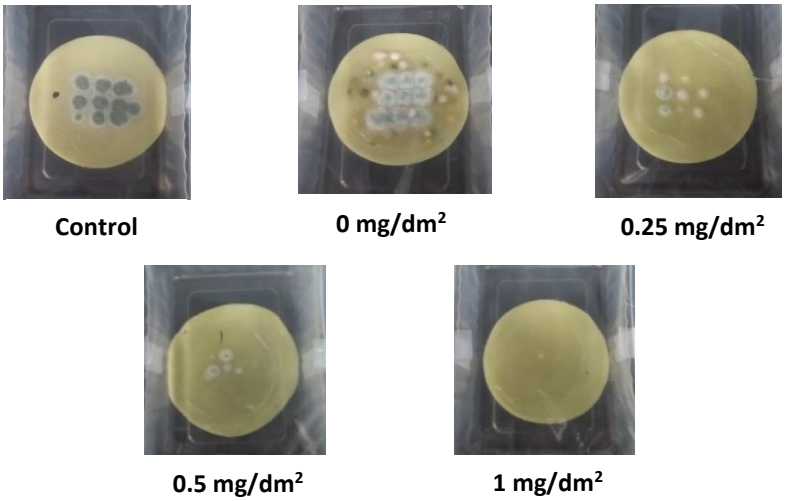


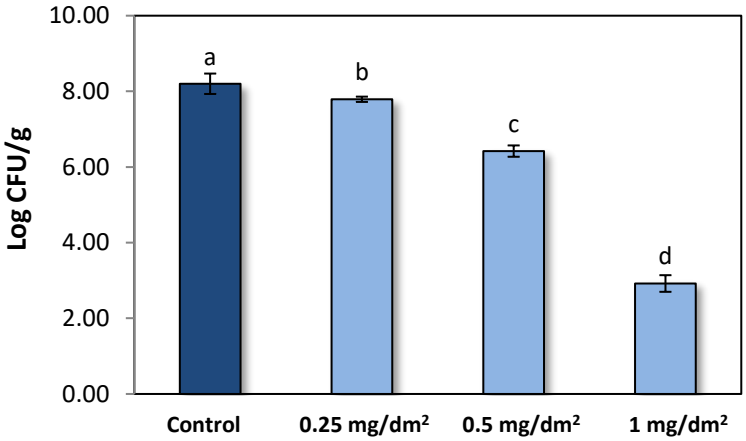
Figure 1. Low moisture Mozzarella cheese slices contaminated with *P. commune* CECT 20767 and stored at 4 °C for 30 days: (a) Direct application of natamycin by spraying at different concentrations (025, 0.5, and 1 mg/dm^2); (b) Incorporation of natamycin in hydroxyethylcellulose films at concentrations of 0, 0.25, 0.5, and 1 mg/dm^2.

Regarding the FP on MCS, a similar dose-depending effect was evidenced compared to the shelf-life enhancement. The direct application of NTC by spray at

0.25 and 0.5 mg/dm^2 reduced the FP by 0.41 and 1.77 Log CFU/g, respectively, which reduces the FP of 66.9 and 98.5% compared to the control treatment (**Figure 2a**). The higher dose tested (1 mg/dm^2) resulted in a reduction of 5.28 Log CFU/g (99.9% of FP). Regarding the application of HEC films, the employment of a film without NTC (0 mg/dm^2) did not affect the fungal growth of *P. commune* CECT 20767 on the surface of the MCS; however, the incorporation of NTC at different doses significantly reduced the FP (**Figure 2b**). For instance, using an edible film of HEC containing NTC at 0.25 and 0.5 mg/dm^2 decreased the FP by 0.99 and 2.52 Log CFU/g (88.8 and 99.5% of reduction), respectively, when compared to the control treatment. As with the spraying technique, the highest dose tested (1 mg/dm^2) was the most effective, showing a Log reduction of 4.15 (99.9% of FP). Comparing the two treatments, it was found that the NTC spray had a more significant antifungal effect than the HEC film, despite the fact that both were effective. These results may be explained because the gradual diffusion of NTC from the HEC film toward samples caused a lower NTC concentration on MCS, which did not occur when the NTC was directly sprayed on the product surface.

Yildirim et al. (2006) evaluated the antifungal properties of NTC through the dipping of Kashar cheese in a 0.07% (w/w) solution and stored under refrigeration for 90 days. The authors observed fungal growth after three weeks of study. Also, Ombarak & Shelaby (2017) studied the incorporation of NTC in the manufacturing process of Tallaga cheese at different concentrations (5, 10, and 20 μg/g) and stored under refrigeration for 30 d. The NTC application at 10 μg/g reduced the initial contamination by 99.7% after four weeks, and the higher dose tested (20 μg/g) reduced the fungal contamination by 100%. These studies agree with our results obtained from the direct application of the NTC and confirm its potential use as a preservative in refrigerated cheese.

(a)



(b)

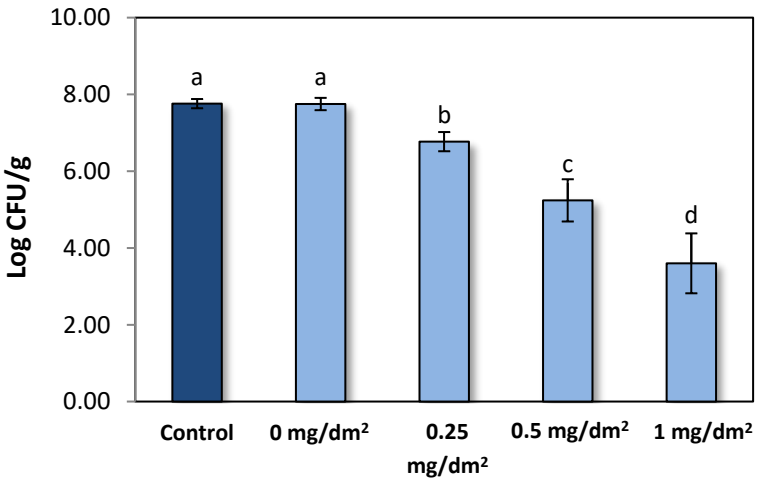


Figure 2. Antifungal effect of natamycin on low moisture Mozzarella cheese slices contaminated with *P. commune* CECT 20767 and stored at 4 °C for 30 days. (a) Direct application by spraying natamycin at concentrations of 0.25, 0.5, and 1 mg/dm^2; (b) Incorporation of natamycin in hydroxyethylcellulose films at concentrations of 0, 0.25, 0.5. and 1 mg/dm^2. The different lowercase letters represent a significant difference among the treatments ($p < 0.05$).

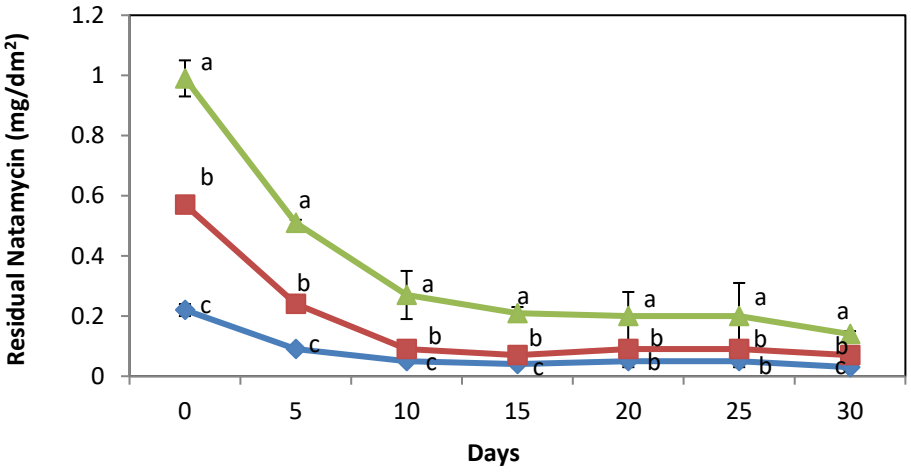
However, the interest in active packaging has grown in the last years as a promising solution to enhance the safety and quality of cheese products due to consumer demand for minimally processed foods (O'Callaghan & Kerry, 2016). In this context, some authors have evaluated the incorporation of NTC in various biopolymers to increase the shelf life of different varieties of cheese. Santonicola et al. (2017) evaluated the use of biofilms made with chitosan (1.5% w/v) and methylcellulose (3% w/v) enriched with NTC at a final concentration of 0.01% (w/v). After seven days of storage, a reduction of 7.91 Log CFU/g of yeasts and moulds was achieved in samples stored at room temperature. Romero et al. (2016) studied the application of NTC in films obtained from triticale for soft cheese packaging. The authors developed films with higher doses of NTC in their composition compared to our study: 5 mg NTC/dm^2, 10 mg NTC/dm^2, and 20 mg NTC/dm^2. After storage for 14 days at 4 °C, they observed a complete inhibition of the fungal growth. Ollé-Resa et al. (2014) incorporated the NTC in tapioca starch films at concentrations of 1.85, 3.70, and 9.15 mg/dm^2 and studied its effect against different yeasts on the surface of Port Salut cheese. Although these authors have required higher doses of NTC to inhibit tested microorganisms than our experimental results, the use of edible films incorporated with NTC has been shown an effective strategy to reduce the development of spoilage microorganisms in cheese. This technique could also satisfy consumer demands for additive-free and ecologically sustainable foods, reducing the direct application of antifungal substances, or preventing their employment altogether.

3.3 Residual content of natamycin on the cheese surface during storage

During the storage period, we evaluated the residual content of NTC on the surface of MCS to determine its stability and compare the spray and film treatments. As plotted in **Figure 3**, the spray application evidenced the highest residual content of NTC in the samples. The analysis carried out at T_0 confirmed the homogeneity of application of the NTC through the spray treatment. The residual concentration of NTC decreased up for the three doses tested over time. After 5 days, the content of NTC was reduced to 0.09, 0.24, and 0.51 mg/dm^2 compared to initial doses of 0.25, 0.5, and 1 mg/dm^2, respectively. The concentration of NTC continued decreasing up to the 10th day and remained constant until the 30th day. At this point, the concentration of NTC on the surface of the product was 0.03, 0.07, and 0.14 mg/dm^2 for 0.25, 0.5 and 1 mg/dm^2 treatments, respectively. As expected, it seems that NTC concentration decreased during the storage period. Some authors have explained that different factors such as ultraviolet light, extreme pH, exposure to heat, and moisture could degrade polyene antibiotics in the matrix (Koontz et al., 2003; Reps et al., 2002; Teixeira et al., 2019).

Regarding the HEC film application, we could notice major differences from the spray technique. The concentration of NTC increased at day 0 to the maximum content at day 10. The concentration of NTC was 0.03, 0.05, and 0.08 mg/dm^2 for the treatments of 0.25, 0.5, and 1 mg/ dm^2, respectively. Then, NTC content was slowly reduced in the samples to the end of the storage. At day 30, the remaining content of NTC was 0.01, 0.02, and 0.03 mg/dm^2 for 0.25, 0.5, and 1 mg/dm^2, respectively. Overall, our results suggest that NTC was gradually released from the HEC film into the samples, increasing the shelf life and reducing the growth of *P. commune* CECT 20767.

(a)



(b)

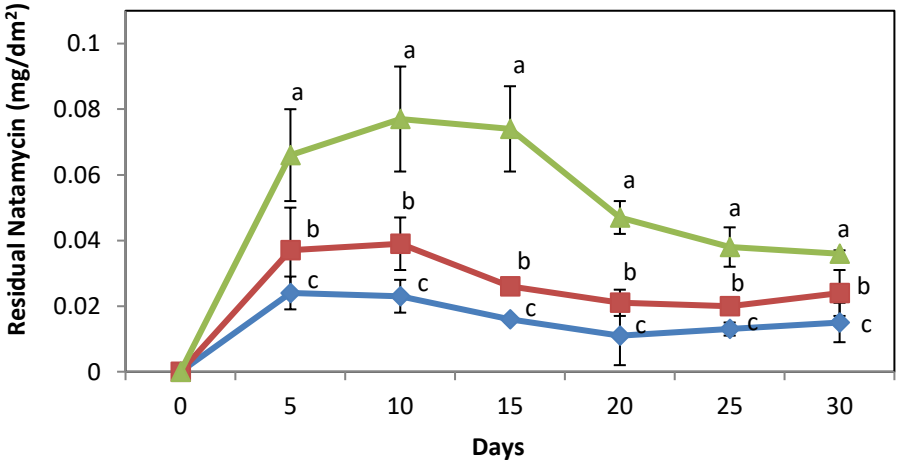


Figure 3. The residual content of natamycin on low moisture Mozzarella cheese slices treated at concentration of 0.25 (blue line), 0.5 (red line), and 1 mg/dm^2 (green line) by (a) Direct application of natamycin by spraying; (b) Incorporation of natamycin in hydroxyethylcellulose antifungal films. The samples were stored at 4 °C for 30 days. The different lowercase letters represent a significant difference between treatments at each day (p< 0.05) (n=9).

Besides, both applications were significant as preservative techniques complying with the current legislation, which establishes a maximum NTC level at 1 mg/dm^2 on the surface of cheese products (Regulation 1333/2008/EC). However, it is essential to emphasize that the lower residual content of NTC observed on MCS after the contact with HEC film in comparison with the spray application turn this treatment into a better preservative strategy, precisely because it decreases the contribution of NTC to the acceptable daily intake (ADI) established by JECFA at 0.3 mg/kg body weight (Joint FAO/WHO Expert Committee on Food Additives & World Health Organization, 2002). Moreover, cellulosic derivate may be more efficient than direct application since the HEC film delays the migration of the active compound and contributes to maintaining a functional concentration of NTC on the product (Almasi et al., 2020; Malhotra et al., 2015).

4. Conclusion

The present study demonstrated NTC's *in vitro* antifungal activity against *Penicillium* strains primarily responsible for cheese spoilage. Besides, two distinct methods were proposed as strategies to extend the shelf life of MCS: the application of the antifungal compound directly onto the surface by spraying and manufacturing an edible film packaging containing HEC and NTC.

Both strategies reduced the *P. commune* population regardless of the dose assayed (0.25, 0.5, and 1 mg/dm^2) and increased MCS shelf life. A lower NTC residual content was evidenced on the MCS surface when HEC film was applied, which constitute an advantage because it reduces NTC's contribution to the acceptable daily intake without compromising its antifungal effect. Since the current food packaging trend is searching for alternatives to avoid plastic polymers

that potentially harm the environment, the proposed HEC film containing NTC is a potential candidate in cheese manufacturing as a new preservative strategy. In addition, it satisfies the consumer's demand for the reduction of synthetic chemical additives in food composition. Future research should study the mechanical properties of HEC films containing NTC, develop packaging, and expand their application to other foods affected by fungal spoilage.

4. GENERAL DISCUSSION

Discusión general

4. DISCUSIÓN GENERAL

El trabajo de investigación llevado a cabo en esta tesis doctoral se ha centrado en el estudio de nuevas estrategias de conservación de alimentos mediante el empleo de ingredientes naturales; principalmente mostaza amarilla (*Sinapis alba*), mostaza oriental (*Brassica juncea*), compuestos derivados de la fermentación de bacterias ácido-lácticas (LAB) y la natamicina, un polieno sintetizado por la bacteria *Streptomyces natalensis*.

Inicialmente se evaluó las propiedades antifúngicas *in vitro* de la semilla y salvado de mostaza amarilla frente a cepas toxigénicas de los géneros *Aspergillus*, *Penicillium* y *Fusarium*. También se estudió la actividad antifúngica de los extractos acuosos de mostaza amarilla (YM) y oriental (OM) fermentados con LAB frente a cepas del género *Fusarium*. Por último, se estudió las propiedades antifúngicas de la natamicina (NTC) frente a mohos del género *Penicillium*.

De acuerdo con la naturaleza de cada ingrediente, se plantearon diferentes estrategias de aplicación, ya sea mediante la adición directa en la formulación del alimento, mediante pulverización directa en superficie, o a través del diseño y elaboración de un envase activo. La harina de mostaza oriental (OMF) se estudió para ser incorporada en una salsa envasada conjuntamente a un pan de pita con funcionalidad de envase activo para emitir el compuesto antimicrobiano alil isotiocianato (AITC) *in situ*. El salvado de mostaza amarilla (MB) se adicionó como ingrediente conservante en el proceso de panificación. Los extractos fermentados de YM con LAB se estudiaron como bioconservante tras pulverizarse sobre maíz. Por último, la NTC se estudió mediante aplicación directa en superficie del queso mozzarella o a través de su incorporación en un biopolímero.

Las estrategias planteadas fueron desarrolladas con la finalidad de incrementar la vida útil de los alimentos, reducir la incidencia de mohos toxigénicos, así como disminuir la síntesis de micotoxinas. Además, se estudió la evolución de los compuestos antifúngicos incorporados en los sistemas de envase activo. Por una parte, se estudió la liberación del AITC en el espacio de cabeza, así como la cantidad de substancia absorbida por el pan de pita durante el período de almacenamiento. Asimismo, la NTC fue evaluada en la superficie del queso mozzarella durante el período de almacenamiento.

Finalmente, dada la efectividad evidenciada en los ensayos, se realizó una caracterización química de los extractos fermentados de YM y OM. Además, se estudió el perfil de componentes volátiles y los principales compuestos bioactivos presentes en las harinas y salvados de la mostaza amarilla y oriental.

4.1 Ensayos *in vitro* de actividad antifúngica

4.1.1 Actividad antifúngica de la semilla y salvado de mostaza blanca (MB)

Previo a la realización del ensayo de actividad antifúngica, se elaboró un extracto acuoso de semilla y salvado de mostaza blanca. El polvo obtenido tras la liofilización fue resuspendido a una concentración de 100 g/L en agua estéril y ensayado mediante el método de difusión en agar frente a hongos de los géneros *Aspergillus*, *Penicillium* y *Fusarium*. Los resultados evidenciaron una elevada actividad antifúngica del MB frente a todas las cepas fúngicas ensayadas, con halos de inhibición mayores de 10 mm para 5 cepas del género *Penicillium* y 2 cepas del género *Fusarium*. La semilla de mostaza amarilla únicamente fue efectiva únicamente frente a dos mohos del género *Fusarium*, *F. verticillioides* ITEM 12044 y *F. poae* ITEM 9151. Debido a la efectividad antifúngica del MB, se estudió la

estabilidad del extracto a diferentes temperaturas (4, 25 y 50°C) y tiempos (24, 48, 72 y 168 h) de almacenamiento mediante ensayo de difusión de agar. Los resultados obtenidos evidenciaron que la temperatura afecta directamente a las propiedades antifúngicas del extracto de MB, ya que tras ser almacenado a 50°C durante 24 h perdió completamente su actividad. Las propiedades antifúngicas se conservaban en almacenamiento a temperatura de refrigeración (4°C), si bien transcurridas 168 h de estudio el diámetro de los halos de inhibición se redujo en todas las cepas ensayadas.

Debido a su efectividad, se estableció la Concentración Mínima Inhibitoria (MIC) y la Concentración Fungicida Mínima (MFC) del MB mediante ensayo en placas de 96 pocillos. Las especies del género *Penicillium* fueron más sensibles al extracto, con valores de MIC comprendidos entre 0,3 y 1,2 g/L y valores de MFC comprendidos entre 0,6 y 4,7 g/L. En concreto, las cepas más sensibles al extracto fueron *P. camemberti* CECT 2267 y *P. roqueforti* CECT 2905, presentando valores de MFC de 0,6 g/L. Los hongos de los géneros *Fusarium* y *Aspergillus* presentaron valores de MIC y MFC superiores, estando comprendidos entre 0,6-9,4 y 1,2-18,8 g/L, respectivamente.

Estudios previos han evidenciado la eficacia de la mostaza amarilla (*Sinapis alba*) y la mostaza oriental (*Brassica juncea*) y su actividad antifúngica se asocia estrechamente a la producción isotiocianatos (Sotelo et al., 2015). Anteriormente, se había confirmado la pérdida de actividad del extracto almacenado a 50°C. Esto puede ser debido por la disminución de actividad de la enzima mirosinasa para transformar la sinalbina en *p*-hidroxibenzil isotiocianato, ya que se ha reportado que la acción de esta enzima se reduce significativamente al ser almacenada a 60°C durante 10 min (Van Eylen et al., 2007).

Wang et al. (2020) estudiaron la actividad antifúngica del benzil isotiocianato (0,312, 0,625 y 1,25 mM) frente al hongo *Alternaria alternata*. Los autores comprobaron que el isotiocianato, de acuerdo con la dosis empleada, reducía la germinación de esporas, el crecimiento micelar y la síntesis de micotoxinas. Azaiez et al. (2013) evaluaron la actividad antifúngica del alil, benzil y fenil isotiocianatos frente a *Gibberella moniliformis*, observando que los ITCs reducían en un rango comprendido entre 2,1-89,7% el desarrollo micelar del hongo.

4.1.2 Actividad antifúngica de los fermentados de mostaza amarilla y oriental con bacterias ácido-lácticas (LAB)

Se estudió la potencialidad antifúngica de los extractos fermentados de mostaza con LAB. Para ello, se elaboró un extracto acuoso a partir de YM o de OM y se fermentó con 9 especies de LAB durante 72h a 37°C. El fermentado resultante se centrifugó para obtener el sobrenadante libre de células (CFS) y se liofilizó. Antes de su ensayo en placas de PDA, los diferentes CFS se resuspendieron en agua estéril a una dosis de 100 g/L.

Se realizó una evaluación de la actividad antifúngica frente a cepas del género *Fusarium*. El ensayo preliminar en placas de PDA mostró que solo las cepas bacterianas de *Lactiplantibacillus plantarum* (TR7, TR71, TR14 y CECT 8962) fermentadas tanto con YM y OM generaron halos de inhibición. Por ello, estas bacterias se seleccionaron para establecer los valores de MIC y MFC mediante microdiluciones en placas de 96 pocillos.

Estos valores variaron en función de la cepa bacteriana y el sustrato de fermentación empleado. El extracto más efectivo fue el obtenido a partir de YM fermentado con *L. plantarum* TR71, presentando unos valores de MIC

comprendidos entre 7,8-15,6 g/L, y valores de MFC comprendidos entre 15,6 y 31,3 g/L. Aunque los otros fermentados de YM presentaron actividad antifúngica, los valores de MIC y MFC fueron más elevados, oscilando entre 15,6-31,3 y 31,3-62,5 g/L, respectivamente. En general, los valores determinados de MIC y MFC para los extractos fermentados de OM fueron más elevados en comparación a los extractos de YM. Dependiendo de la cepa bacteriana empleada, los valores de MIC variaron entre 15,6-62,5 g/L, mientras que los valores de MFC se encontraban comprendido entre 31,3-125,0 g/L.

Otros autores han evaluado el potencial antifúngico de los CFS de las LAB, si bien el uso de extractos fermentados de mostaza no ha sido previamente reportado en la literatura. Luz et al. (2018) evaluaron las propiedades antifúngicas del suero liofilizado fermentado con LAB frente a mohos de los géneros *Penicillium*, *Aspergillus* y *Fusarium*. Los CFS presentaron actividad antifúngica frente a las cepas de *Fusarium*, con valores de MIC y MFC que oscilaron entre 31,3-125 y 62,5-250 g/L, respectivamente. De forma similar, Izzo et al. (2020) determinaron los valores de MIC y MFC de suero fermentado de cabra con cepas de *Lactobacillus* frente a hongos del género *Fusarium*, obteniendo valores de MIC que variaron entre 1,5 y 31,2 g/L, mientras que los valores de MFC oscilaron entre 7,8-250 g/L.

4.1.3 Actividad antifúngica de la natamicina

La NTC se resuspendió en agua estéril a unas dosis comprendidas entre 5 y 200 µg/mL y fue ensayada frente a hongos del género *Penicillium*. Al igual que en los procedimientos descritos previamente, se realizó una evaluación en medio sólido, mediante la técnica de difusión en agar, y la cuantificación en microplacas de 96 pocillos de la MIC y MFC.

Todas las cepas ensayadas fueron sensibles a la NTC a dosis comprendidas entre 50 y 200 μg/mL. El uso de NTC a 200 μg/mL evidenció halos de inhibición superiores a 10 mm de diámetro frete a *P. roqueforti* CECT 2905, *P. solitum* CECT 20818*, P. nordicum* CECT2320 y *P. commune* CECT 20767. Los valores de MIC oscilaron entre 1,6 y 3,1 μg/mL, mientras que los valores de MFC variaron entre 12,5 y 200 μg/mL.

Saito et al. (2020) estudiaron las propiedades antifúngicas de la NTC frente a aislados de *Botrytis cinerea* de mandarina. Establecieron un valor de MIC para inhibir la germinación conidial comprendido entre 0.7-1,0 μg/mL, mientras que, para inhibir el crecimiento micelar, los valores de MIC aumentaban y se encontraban en el rango entre 5,0-10,0 μg/mL. Asímismo, Žabka & Pavela (2018) establecieron los valores de MIC50 de la NTC frente a cepas toxigénicas del género *Penicillium*, y demostraron que dosis comprendidas entre 5 y 31 μg/mL de NTC inhibían el desarrollo fúngico.

4.2 Aplicación de los compuestos antifúngicos naturales en alimentos

4.2.1 Estudios de vida útil y reducción de la población fúngica

4.2.1.1 Desarrollo de una salsa bioactiva con mostaza oriental con propiedades antifúngicas

La mostaza oriental se caracteriza por poseer como glucosinolato mayoritario la sinigrina. Este glucosinolato, en presencia de agua, es capaz de hidrolizarse al compuesto antifúngico volátil AITC mediante acción de la enzima mirosinasa (Tsao et al., 2000). Por este motivo, se estudió la introducción de la harina de mostaza oriental como ingrediente de una salsa envasada para liberar de forma controlada el AITC y ejercer acción antifúngica *in situ*. Esta salsa, elaborada con 4 formulaciones

diferentes (8, 16, 30 y 50 mg OMF/g), se envasó conjuntamente a un pan de pita contaminado con el hongo toxigénico *P. verrucosum* VTT D-01847, y se comparó la efectividad del tratamiento frente al conservante químico de síntesis propionato cálcico (E-282).

La estrategia diseñada resultó efectiva para reducir la incidencia de *P. verrucosum*. En el grupo control se observó desarrollo fúngico visual a los 3 días de incubación. El pan de pita tratado con 8 mg/g de OMF incrementó su vida útil hasta los 4 días, mientras que la aplicación de 16 mg/g de OMF equiparó la vida útil con el pan de pita tratado con E-282 (5 días). El tratamiento de 33 mg/g evidenció crecimiento fúngico a los 6 días, mientras que con el uso de 50 mg/g de OMF no se observó desarrollo de *P. verrucosum* a los 7 días de incubación.

Respecto al recuento de las unidades formadoras de colonias (CFU), realizado tras 7 días de incubación, el pan control presentó 8,3 Log CFU/g, mientras que el pan con E-282 contuvo 6,8 Log CFU/g. La aplicación de 8 mg/g no presentó reducción fúngica en comparación al control mientras que, con el uso de 16, 33 y 50 mg/g de OMF, la población fúngica detectada fue de 6,8, 5,2 y 2,6 Log CFU/g, reduciendo en comparación con el grupo control 1,5, 3,1 y 5,7 Log, respectivamente.

Clemente et al. (2017) analizaron el uso de benzil isotiocianato incorporado en un sistema de envase activo frente al moho *Aspergillus ochraceus*. Los resultados mostraron alteraciones morfológicas en las células en función de la proximidad al agente antifúngico e inhibieron la reproducción sexual del moho. Estas modificaciones afectaron a la síntesis de ocratoxina A, resultando la aplicación de isotiocianatos una estrategia efectiva para su incorporación en envases activos con potencial antifúngico y antitoxigénico.

4.2.1.2 Aplicación del salvado de mostaza blanca en la producción del pan de molde

El MB fue estudiado para ser incorporado como ingrediente antifúngico en el proceso de panificación frente al hongo contaminante *P. commune*. Para ello, se adicionaron diferentes concentraciones de MB en la formulación de pan de molde: 2,5, 5, 7,5, y 10 g/kg.

El pan control y la dosis de 2,5 g MB/kg presentaron una vida útil de 4 días. La aplicación de 5 y 7,5 g/kg de MB en la formulación incrementaron la vida útil hasta el sexto día. La dosis más alta ensayada (10 g/kg) aumentó la vida útil del pan hasta el día 7 de incubación, equiparando su efectividad con el pan tratado con E-281. Estos resultados fueron corroborados mediante el recuento de la población fúngica. La dosis de 2,5 g/kg no presentó diferencias estadísticamente significativas en comparación al control. Con la adición de MB a 5 y 7,5 g/kg se obtuvo una reducción de 1,21 y 1,37 log CFU/g en comparación al control, implicando una reducción en la población fúngica del 96,1 y 97,3%, respectivamente. La dosis de 10 g/kg redujo la población fúngica en 4,20 Log CFU/g (99,9% de reducción).

Previamente se ha estudiado la aplicación de los isotiocianatos para incrementar la vida útil de productos de panadería y reducir la incidencia de micotoxinas. Saladino, Manyes, et al. (2016) estudiaron la reducción de la micotoxina patulina en tortas de trigo mediante el empleo de compuestos volátiles liberados por las harinas de mostaza amarilla y oriental (0,5, 1,0 y 2,0 g). La reducción media de patulina obtenida fue del 92,58%. Además, los autores no observaron diferencias entre el uso de harina de mostaza amarilla u oriental a 0,5 y 1,0 g.

Clemente et al. (2019) evaluaron la aplicación del aceite esencial de mostaza en pan contaminado con el moho *Rhizopus stolonifer*. Los panes fueron tratados

con 1 µL de AITC e incubados a 25 y 4°C y se puso de manifiesto que la efectividad del AITC se reducía al aplicarse en la matriz alimentaria en comparación con los *ensayos in vitro*. Sin embargo, el efecto fungicida permitía incrementar la vida útil a ambas temperaturas de almacenamiento.

4.2.1.3 Aplicación de la natamicina en el tratamiento del queso mozzarella

Para comprobar la efectividad de la NTC como agente antifúngico en el queso mozzarella se idearon dos estrategias de aplicación. La primera consistió en la pulverización directa de una suspensión de NTC en agua estéril para alcanzar una concentración final en la superficie de 0,25, 0,5 y 1 mg/dm^2. La segunda estrategia se basó en el desarrollo de un biopolímero a partir de hidroxietilcelulosa (HEC) y glicerol (0,2%) con NTC a concentración final de 0,25, 0,5 y 1 mg/dm^2.

Ambas estrategias desarrolladas resultaron efectivas para aumentar la vida útil y reducir la contaminación microbiológica de *P. commune*, uno de los microorganismos alterantes más común en queso refrigerado. La aplicación del pulverizado de NTC con 0,25, 0,5 y 1 mg/dm^2 aumentó la vida útil en 10, 15 y 20 días, respectivamente, en comparación al control. La aplicación en superficie del biopolímero de HEC con NTC a 0,25, 0,5 y 1 mg/dm^2 incrementó la vida útil en comparación al control en 5, 10 y 15 días, respectivamente. Transcurridos 30 días de almacenamiento a 4°C del queso mozzarella, el pulverizado redujo en 0,41, 1,77 y 5,28 los Log CFU/g, respectivamente, lo que implica una reducción de la población fúngica del 66,9, 98,5 y 99,9%, respectivamente en comparación al control. En cuanto a la aplicación del biopolímero de HEC conteniendo NTC, las dosis de 0,25, 0,5 y 1 mg/dm^2 redujeron la población fúngica en 0,99. 2,52 y 4,15 Log UFC/g, lo

que supuso la reducción de *P. commune* en superficie del 88,8, 99,5 y 99,9%, respectivamente.

La estrategia de envasado activo se ha incrementado en los últimos años como respuesta de los consumidores frente a la demanda de alimentos mínimamente procesados (O'Callaghan & Kerry, 2016). En este contexto, diversos autores han evaluado la incorporación de NTC en diferentes biopolímeros. Santonicola et al. (2017) estudiaron las propiedades biopolímeros elaborados con quitosano (1,5 % p/v) y metilcelulosa (3% p/v) enriquecidos con NTC al 0,01% (p/v). Después de 7 días de almacenamiento, observaron una reducción de 7,91 Log CFU/g en la población de mohos y levaduras. Ollé Resa et al. (2014) estudiaron la incorporación de NTC en biopolímeros de almidón de tapioca (1,85, 3,70 y 9,15 mg/dm^2) frente a levaduras de superficie en queso Port Salut, obteniendo una inhibición completa frente a los microorganismos ensayados.

Aunque el pulverizado de NTC en superficie del queso Mozzarella evidenció una mayor efectividad antifúngica, la incorporación de NTC en biopolímeros degradables también redujo significativamente la población fúngica, por lo que constituye una estrategia efectiva para reducir el desarrollo de microorganismos en queso y, además satisface la demanda de los consumidores evitando la aplicación directa de aditivos en superficie para prevenir el desarrollo microbiológico.

4.2.2 Evaluación de la reducción de micotoxinas

4.2.2.1 Reducción de OTA en panes de pita tratados con la salsa bioactiva a partir de harina de mostaza oriental

Los panes de pita fueron contaminados con *P. verrucosum*, una cepa capaz de sintetizar ocratoxina A (OTA). Después de 7 días de incubación, el control presentó

9,1 µg/kg de OTA, mientras que en el pan de pita con E-282, la concentración de este tóxico fue de 6,3 µg/kg. El pan de pita envasado con la salsa formulada con 8 mg/g de OMF no presentó diferencias estadísticamente significativas en el contenido de OTA en comparación al control. Los tratamientos con 16 y 33 mg/g de OMF presentaron 6,2 y 3,4 µg/kg de OTA en el pan, respectivamente. Todos estos tratamientos mostraron valores de OTA superiores a 3,0 µg/kg, contenido máximo estipulado por la Comisión Europea (1881/2006) para este tipo de producto. Con el uso de 50 mg/g de OMF no se detectó la OTA en el pan de pita a los 7 días de incubación, confirmando el potencial antifúngico y antitoxigénico de la OMF.

Otros autores han demostrado la efectividad de aplicar el AITC en sistemas de envase activo con la finalidad de prevenir el desarrollo de hongos toxigénicos y la aparición de micotoxinas. El estudio de Quiles et al. (2015a) diseñaron envases activos conteniendo AITC o OMF para reducir la síntesis de aflatoxinas por *A. parasiticus* en masas de pizza. La aplicación de un sachet con 850 mg de OMF y 850 mL de agua inhibió completamente la síntesis de aflatoxinas en el producto.

Saladino, Quiles, et al. (2017) estudiaron la reducción de síntesis de micotoxinas de *A. parasiticus* en pan de molde mediante el uso de alil, benzil y fenil isotiocianatos aplicados en filtros de papel o en pequeñas bolsas conteniendo los filtros de papel a dosis de 0,5, 1 y 5 mL/L. El estudio evidenció un aumento de la vida útil del pan con el uso de 5 mL/L de AITC. Además, este tratamiento mostró una reducción del contenido de aflatoxinas del 60%.

4.2.2.2 Reducción de FB_1 y FB_2 en maíz tratado con extractos de mostaza fermentados con bacterias ácido-lácticas

Las mazorcas de maíz fueron contaminadas con *F. verticillioides*, un hongo capaz de producir FB_1 y FB_2. El producto fue tratado con el extracto fermentado de YM con *L. plantarum* TR71, mediante pulverizado directo sobre la mazorca del CFS, o tras liofilizarse y resuspenderse a 250 g/L. Además, las mazorcas fueron tratadas con un extracto de YM sin fermentar (tanto por pulverizado directo o tras liofilización) y un control que consistió en agua estéril. Estas mazorcas se almacenaron y el contenido de micotoxinas se determinó a tiempo inicial y tras 7 y 14 días de almacenamiento.

A tiempo inicial las mazorcas no contenían micotoxinas. Tras 7 días de la inoculación, el control presentó 0,30 mg/kg de FB_1 y 0,05 mg/kg de FB_2. Únicamente la administración de los extractos liofilizados (tanto fermentados o no) evidenciaron un descenso en el contenido de FB_1 en comparación al control. Además, solo el extracto liofilizado y fermentado con TR71 no evidenció la presencia de FB_2 tras 7 días de incubación.

A los 14 días, el contenido de FB_1 se incrementó en todos los tratamientos aplicados. la aplicación directa del extracto de YM fermentado con TR71 redujo la producción de FB_1 (8,02 mg/kg) en comparación al control en un 49,5%. La mayor reducción de FB_1 se obtuvo con el extracto liofilizado y fermentado de YM, con un contenido de 1,09 mg/kg, lo que en comparación al control implica una reducción de la presencia de esta toxina en un 92,6%. Respecto a la FB_2, no se evidenciaron diferencias estadísticamente significativas entre los tratamientos y el control, a excepción del extracto liofilizado y fermentado de YM por TR71, que inhibió completamente la síntesis de esta toxina en la matriz alimentaria.

Aunque los tratamientos aplicados no fueron capaces de reducir por completo la incidencia de FB_1, es importante remarcar que después de 14 días, el contenido en las mazorcas tratadas con el extracto liofilizado fermentado era inferior a 4 mg/kg, por lo que cumplía con los niveles máximos especificados en la legislación europea para la suma de FB_1 y FB_2 en maíz no procesado (Directiva 401/2006/CE). Por este motivo, la aplicación de los extractos fermentados de YM con TR71 son una posible solución a la incidencia de micotoxinas en las mazorcas y se pueden aplicar durante la cosecha para incrementar la seguridad alimentaria.

Otros autores han evaluado el uso de CFS para reducir la incidencia de micotoxinas en productos vegetales. Dopazo et al. (2021) aisló y estudió LAB de uvas como agentes bioconservantes frente a *Aspergillus flavus*, *A. niger* y *Botrytis cinerea*. Los CFS aplicados redujeron el contenido de aflatoxina B_1 y fumonisinas (B_2, B_3 y B_4) en porcentajes comprendidos entre el 28 y 100%. Ben Taheur et al. (2019) evidenció la reducción de la incidencia de aflatoxinas B_1 y B_2 en un 85,27% y un 83,94%, respectivamente, tras la aplicación de CFS sobre almendras contaminadas con *A. flavus* y *A. carbonarius*.

4.2.3 Evaluación de los compuestos antimicrobianos durante el almacenamiento

4.2.3.1 Evolución del AITC en el espacio de cabeza y en el pan de pita

Durante el período de almacenamiento de los panes de pita, se determinó el contenido de AITC liberado por las salsas bioactivas elaboradas con OMF. Después de 1 h de incubación, el AITC detectado en el espacio de cabeza varió entre 1 y 7 mg/L dependiendo de la dosis de OMF empleada. La concentración se incrementó hasta las 8 h de incubación, con valores comprendidos entre 2,3 y 12,9 mg/L.

Después de 8 h de incubación, el contenido de AITC disminuyó hasta obtener una concentración constante en el día 7 que varió entre 0,4 y 1,1 mg/L de AITC.

Paralelamente al análisis de espacio de cabeza, se cuantificó la cantidad de AITC absorbido por el pan de pita durante el período de almacenamiento. A los 7 días de incubación. la concentración de AITC detectada en el pan varió entre 4,3 y 59,3 mg/kg en función de la dosis de OMF empleada en la formulación de la salsa. A los 14 días de almacenamiento solo se detectó AITC en los panes tratados con la salsa conteniendo 33 y 50 mg/g de OMF, en concreto, 15,9 y 25,6 mg/kg, respectivamente.

Considerando el peso del producto (40 g) y la concentración detectada a día 7, los tratamientos de 33 y 50 mg/g transfirieron al pan una cantidad total de 1,93 y 2,37 mg de AITC, respectivamente. Estos valores son superiores a los establecidos por la EFSA para la dosis diaria admisible del AITC, establecida en 0,02 mg/kg peso corporal. Sin embargo, se ha evidenciado que los consumidores adultos pueden llegar a exceder entre 2 y 8 veces el valor de exposición de AITC (EFSA, 2010).

4.2.3.2 Evolución de la natamicina durante el almacenamiento

Durante el almacenamiento se evaluó periódicamente el contenido de NTC en la superficie del queso mozzarella para conocer su estabilidad y evolución. Los análisis llevados a cabo a T_0 confirmaron la homogeneización en la aplicación del pulverizado en la superficie del queso mozzarella. A partir de 5 días, el contenido de NTC se redujo en la superficie a 0,09, 0,24 y 0,51 mg/dm^2 para los tratamientos de 0,25, 0,5 y 1 mg/dm^2, respectivamente. La concentración de NTC continuó disminuyendo y a día 30 el contenido final en la superficie fue de 0,03, 0,07 y 0,14 mg/dm^2. Algunos autores han reportado que diferentes factores como la luz

ultravioleta, la exposición al calor y la humedad pueden degradar la NTC en la matriz alimentaria (Koontz et al., 2003; Teixeira et al., 2019), lo que permite explicar la reducción de NTC durante el período de almacenamiento con el tratamiento de pulverizado.

La evolución del contenido de NTC en superficie con la aplicación del biopolímero presentó grandes diferencias en comparación al tratamiento de pulverizado. La concentración de NTC se incrementó hasta el día 10, donde se observó un máximo en la superficie del queso mozzarella de 0,03, 0,05 y 0,08 mg/dm^2 para los tratamientos de 0,25, 0,5 y 1 mg/dm^2, respectivamente. A partir de este momento, la concentración de NTC en superficie disminuyó y a día 30, solo se detectó 0,01, 0,02 y 0,03 mg/dm^2, respectivamente.

Ambas aplicaciones cumplen la legislación vigente, que establece un máximo de 1 mg/dm^2 en la superficie de estos productos (Reglamento 1333/2008). Sin embargo, el menor contenido residual observado con la aplicación del biopolímero lo convierte en una mejor estrategia de preservación ya que, en comparación al pulverizado, reduce la contribución de la NTC a la ingesta diaria admisible (IDA) establecida por la JECFA en 0,3 mg/kg de peso corporal (Koenig et al., 2009).

4.3 Caracterización química de los ingredientes antifúngicos

4.3.1 Determinación de compuestos fenólicos y ácidos orgánicos en los fermentados de mostaza

En el estudio se analizaron los principales compuestos fenólicos y ácidos orgánicos de los fermentados de mostaza que evidenciaron actividad antifúngica en los ensayos *in vitro*. Un total de 11 ácidos fenólicos diferentes fueron identificados en los CFS. En comparación con el extracto control, los fermentados

de YM tenían un mayor contenido de 1,2-dihidroxibenceno, 3,4-dihidroxicinámico y ácido benzoico. La cepa *L. plantarum* TR71 produjo la mayor concentración de estos metabolitos, con un promedio de 293, 45 y 220 ng/mL, respectivamente. Además, esta cepa sintetizó la mayor proporción de ácido 3-feniláctico, en concreto, 559 ng/mL. Por lo que respecta a los extractos de OM, los ácidos 3,4-dihidroxicinámico, benzoico y 3-feniláctico se obtuvieron en mayor proporción tras la fermentación en comparación al control. Los valores oscilaron de 146 a 218 ng/mL, de 140 a 228 ng/mL, y de 31 a 37 ng/mL, respectivamente.

Respecto al contenido de ácidos orgánicos, únicamente se detectó ácido láctico. El contenido en los fermentados de YM varió entre 570-800 ng/mL, siendo la cepa que más produjo *L. plantarum* TR71. Este ácido también se detectó en el extracto fermentado de OM, aunque en cantidad menor (89-209 ng/mL) en comparación al extracto fermentado de YM.

Las propiedades antifúngicas de las LAB no son debidas a un único compuesto químico, sino que la presencia de varios metabolitos como, por ejemplo, ácidos orgánicos, ácidos fenólicos, péptidos antimicrobianos y ácidos grasos pueden actuar sinérgicamente y proporcionar la acción antimicrobiana (Schmidt, Zannini, et al., 2018). Los compuestos identificados en los fermentados de mostaza han sido descritos previamente como componentes antimicrobianos en la literatura. En este contexto, Chen, Ju, et al. (2021) reportaron diversos compuestos fenólicos obtenidos en los CFS tras la fermentación de *Lactobacillus kefiri* M4 con propiedades antifúngicas frente al hongo *Penicillium expansum*, entre ellos: ácido 1,2-dihidroxibenceno, ácido 3,4-dihidroxicinámico, ácido benzoico y ácido 3-feniláctico. Además, reportó que ambos microorganismos al ser cultivados juntos evidenciaban competición por los nutrientes. Por otra parte, el contenido de ácido 3-feniláctico se correlaciona positivamente con la actividad antifúngica (Rajanikar

et al., 2021). En el estudio realizado, la cepa de *L. plantarum* TR71 fermentada en YM sintetizó una mayor proporción de ácido láctico y ácido 3-feniláctico, por lo que puede asociarse a las mayores propiedades antifúngicas de este extracto en ensayos *in vitro*.

4.3.2 Caracterización de la mostaza amarilla y oriental

4.3.2.1 Perfil de componentes volátiles

El perfil de volátiles de las harinas y salvado de mostaza amarilla y oriental se analizó mediante microextracción en fase sólida del espacio de cabeza (HS-SPME) por cromatografía de gases acoplada a espectrometría de masas (GC-MS). La identificación de los compuestos se realizó comparando los espectros obtenidos con la librería NIST14 y calculando los índices de retención linear (LRI). Un total de 53 compuestos volátiles, entre los que se incluyen isotiocianatos, alcanos, aldehídos, cetonas, ésteres y compuestos varios, se identificaron en las muestras de mostaza, si bien la concentración y distribución varió en función de la especie de mostaza y la fracción de la semilla analizada.

La fracción de mostaza que presentó un mayor contenido de volátiles fue la OMF (67,0 μg/g), seguido de la harina de mostaza amarilla (YMF) (28,6 μg/g). Las fracciones de salvado contuvieron un menor contenido de compuestos volátiles en comparación a sus fracciones de harina. En concreto, el contenido de volátiles en el salvado de mostaza oriental (OMB) fue de 27,8 μg/g, mientras que en el salvado de mostaza amarilla (YMB) fue de 4,9 μg/g.

El AITC fue el compuesto más característico detectado en la OMF y la OMB (39,0 y 7,0 μg/g, respectivamente), representando un 58 y 25% de los compuestos aromáticos totales. En cambio, en la YMF y YMB el contenido fue inferior, 1,21 ± 0,62 y 0,23 ± 0,04 μg/g, respectivamente (4,3 y 4,7%).

La mayor variedad de compuestos aromáticos detectados perteneció a la familia de los alcanos. Esta fracción fue la más representativa en la YMF (25,4 μg/g) y el YMB (3,7 μg/g) e implicó el 89 y 74% de todos los compuestos aromáticos, respectivamente. En cambio, en las fracciones de OMF y OMB representaron en torno a un 30 y 62%, respectivamente. Parte de los alcanos lineales detectados en el estudio han sido reportados previamente en aceites esenciales de mostaza y otros vegetales pertenecientes a la misma familia (Wei et al., 2021; Zhao et al., 2007). Sin embargo, gran parte de los alcanos ramificados detectados han sido identificados por primera vez como constituyentes de la fracción aromática en las semillas de mostaza.

Otros compuestos detectados en menor proporción fueron cetonas, alcoholes y ésteres. Las cetonas fueron exclusivas de la OMF y OMB, representando un 2,1 y 5,0% del total de la fracción aromática, respectivamente. Además, en la muestra de OMF, un 9,0% de los compuestos aromáticos fueron alcoholes como el 1-hexanol, 1-heptanol y el 1-nonanol. Estos componentes se habían identificado previamente en encurtidos realizados a partir de mostaza oriental y en la planta empleada para consumo (*Brassica juncea*) (Shen et al., 2018; Zhao et al., 2007). Por otra parte, las muestras de YMF y YMB presentaron en su composición el terpeno D-limoneno, en concentraciones de 1,3 y 0,6 μg/g, respectivamente. Este compuesto se había descrito en aceites esenciales de mostaza amarilla (*Sinapis alba*), aunque en contenidos traza (Miyazawa & Kawata, 2006).

4.3.2.2 Caracterización de compuestos bioactivos de la mostaza

Se realizó una extracción de los compuestos bioactivos libres presentes en las muestras de mostaza, así como de los compuestos liberados tras realizar una

hidrólisis alcalina y una hidrólisis ácida de los extractos. Los análisis realizados en UHPLC MS/MS permitieron identificar 26 compuestos bioactivos diferentes, entre los que se incluyen ácidos fenólicos, flavonoides y glucosinolatos. Además, se realizó la cuantificación de aquellos compuestos bioactivos de los que se poseía un estándar comercial.

Las muestras de YMF y YMB presentaron un mayor contenido de ácido *p*-hidroxibenzoico (275 y 227 mg/kg, respectivamente) en comparación a las muestras de OMF y OMB (34 y 84 mg/kg, respectivamente). Martinović et al. (2020) confirmaron previamente que el ácido *p*-hidroxibenzoico es característico de la mostaza amarilla (*Sinapis alba*). Otros ácidos fenólicos como el ácido salicílico, el ácido *p*-cumárico y el ácido cafeico también fueron identificados y cuantificados en las diferentes muestras de mostaza. El ácido ferúlico también fue el de mayor proporción en las muestras de YMF y YMB (60 y 40 mg/kg, respectivamente) en comparación a las muestras de OMF y OMB (13,1 y 4,8 mg/kg, respectivamente). El ácido fenólico mayoritario fue el ácido sinápico, presentando valores medios de 3061, 1981, 2105 y 597 mg/kg para las muestras de YMF, YMB, OMF y OMB, respectivamente.

En el estudio se evaluó la fracción libre obtenida mediante extracción con disolventes y asistida con ultrasonidos y la fracción conjugada. La extracción alcalina de forma general evidenció un bajo contenido de ácidos fenólicos mientras que la hidrólisis ácida resultó efectiva para liberar estos constituyentes esterificados en la pared celular vegetal. Otros autores han estudiado el contenido de fenoles en la mostaza, si bien la comparación resulta dificultosa porque el contenido de ácidos fenólicos puede cambiar considerablemente en función del método de extracción empleado y la fracción de la planta analizada (Bhandari & Kwak, 2015). En este contexto, Fang et al. (2008) reportaron valores medios de 116,

211 y 15,8 mg/kg de ácido ferúlico, ácido sinápico y ácido *p*-hidroxibenzoico en hojas de mostaza.

Algunos flavonoides como quercetina, luteolina y kaempferol se identificaron en las muestras de mostaza y se cuantificaron frente a un estándar. El kaempferol únicamente se detectó en las muestras de *Brassica juncea* (1,1-15,5 mg/kg), mientras que la luteolina se identificó solamente en la muestra de YMB (0,77 mg/kg). Además, se identificaron diferentes flavonoides glicosilados como sinapoil-hexósido, sinapoil-dihexósido, quercetina-*O*-hexósido, luteolina-*O*-hexósido, kaempferol-*O*-hexósido, kaempferol-sinapoil-trihexósido I y kaempferol-sinapoil-trihexósido II. Entre estos, la quercetina-*O*-hexósido fue semicuantificada en mayor concentración en la muestra de OMF (18,4 mg/kg).

Otros compuestos bioactivos como ácido hidroxibenzoico-*O*-hexósido, sinapoil-vanilloil-hexósido, disinapoil-hexósido I, disinapoil-hexósido II, disinapoil-dihexósido, trisinapoil-dihexósido y sinapina fueron identificados en la fracción libre y tras realizar la hidrólisis alcalina de las semillas de mostaza. El estudio realizado mostró una gran diversidad química de flavonoides, principalmente derivados del ácido sinápico en ambas semillas de mostaza. Adicionalmente, 5 glucosinolatos (sinigrina, sinalbina, progoitrina, glucobrasicina e hidroxiglucobrasicina) se identificaron en las semillas de mostaza. Estos compuestos se han reportado previamente en semillas de mostaza, así como en otros cultivos de la misma familia (Melrose, 2019; Qu et al., 2020).

4.3.2.3 Determinación de polifenoles totales y actividad antioxidante

El contenido de fenoles totales (TPC) se analizó mediante el método Folin-Ciocalteu y los resultados fueron expresados en mg/kg de equivalentes de ácido

gálico (GAE). La muestra con mayor contenido de fenoles fue la YMF (22997 mg/kg GAE). La fracción del salvado de la mostaza amarilla (YMB) también presentó un mayor contenido de fenoles (17136 mg/kg GAE) en comparación a la OMF y OMB (8601 y 8175 mg/kg GAE, respectivamente). Resultados similares obtuvieron Boscariol Rasera et al. (2019), en concreto un contenido de TPC comprendido entre 0,82 y 20,0 mg GAE/g para las semillas de *Sinapis alba*, y entre 0,65 y 12,16 mg GAE/g para las semillas de *Brassica nigra*.

La actividad antioxidante se midió con el radical DPPH y los resultados se expresaron en mM de equivalentes de Trolox (TE)/g. Las fracciones de las harinas (YMF y OMF) mostraron mayor actividad antioxidante (120 y 222 mM TE/g, respectivamente) en comparación a sus fracciones de salvado (84 y 79 mM TE/g para el YMB y OMB, respectivamente). Esto puede ser debido a la diferencia en el contenido de compuestos bioactivos que no fue posible cuantificar en las muestras de mostaza, como la sinapina o los derivados del ácido sinápico, y que influyen en la actividad antioxidante (Thiyam et al., 2006).

4.3.2.4 Análisis de Componentes Principales (PCA)

Se realizó un análisis de componentes principales (PCA) con la cuantificación realizada de los compuestos volátiles y bioactivos presentes en las muestras de mostazas. La suma de las dos primeras componentes explicó un 75,6% de la varianza total, de donde la componente 1 (PC1) explicó un 41,4% de la varianza total y la componente 2 (PC2) un 31,2% de la varianza total. La PC1 distribuyó en el eje negativo las muestras de YMF, YMB y OMB, mientras que la muestra de OMF fue posicionada en el eje positivo. La PC2 permitió diferenciar entre las dos especies de mostaza. *Sinapis alba* se distribuyó en el eje negativo y *Brassica juncea* en el

positivo, confirmando la particularidad de cada especie de mostaza de acuerdo con sus componentes bioactivos.

Las muestras de *Sinapis alba* (YMF y YMB) se diferenciaron estadísticamente de la semilla de *Brassica juncea* principalmente por el mayor contenido de ácido *p*-hidroxibenzoico, ácido ferúlico y ácido *p*-cumárico. Además, algunos compuestos volátiles como el 4-metildodecano, 2,5-dimetildodecano, n-nonano y 2-metilnonano únicamente se identificaron en las semillas de *Sinapis alba*.

La fracción de OMB presentó algunas particularidades en sus metabolitos, como la presencia de 6-metil-5-heptan-2-ol, ácido tetradecanoico 1-metiletil éster y 6-metil-5-heptan-2-ona que no presentaron las otras fracciones. Por lo que respecta la OMF, el mayor contenido de AITC cuantificado, así como la presencia de flavonoides como el kaempferol-sinapoil-trihexósido, el kaempferol, la quercetina-O-hexósido y el kaempferol-sinapoil-trihexósido II permitió la distinción estadística de esta fracción en comparación al OMB, YMF y YMB.

5. CONCLUSIONS

Conclusiones

5. CONCLUSIONES

1. El salvado de mostaza amarilla (MB) evidenció actividad antifúngica frente a hongos de los géneros *Aspergillus*, *Penicillium* y *Fusarium in vitro*.
2. Los fermentados de mostaza amarilla y oriental con las cepas de *Lactiplantibacillus plantarum* TR71 presentaron acción inhibitoria frente a mohos del género *Fusarium*, siendo el fermentado de mostaza amarilla el de mayor actividad antifúngica.
3. La natamicina (NTC) fue efectiva frente a todas las cepas de *Penicillium* ensayadas *in vitro*.
4. El uso de harina de mostaza oriental (OMF) como ingrediente liberador de alil isotiocianato (AITC) en la salsa bioactiva permitió ejercer acción conservante en los panes de pita. El pan tratado con la salsa de 50 mg/g no presentó crecimiento fúngico visual tras 7 días de inoculación.
5. El MB se puede revalorizar como ingrediente antifúngico en la formulación de pan de molde. El pan elaborado con 10 g/kg evidenció una vida útil de 7 días, siendo equiparable al empleo del aditivo químico de síntesis propionato sódico (E-281).
6. Tanto el pulverizado directo como la incorporación de la NTC en biopolímeros incrementaron la vida útil del queso Mozarella, si bien el uso de los biopolímeros antifúngicos presenta ciertas ventajas.
7. La dosis de 50 mg/g de OMF en la salsa bioactiva inhibió completamente la síntesis de ocratoxina A por *P. verrucosum* en el pan de pita tras 7 días de almacenamiento.
8. La aplicación del fermentado de mostaza amarilla con *L. plantarum* TR71 a 250 g/L sobre mazorcas redujo la incidencia de la fumonisina B_1 y B_2 en prácticamente su totalidad.

9. El mayor contenido de AITC en el espacio de cabeza del envase se detectó a las 8 horas del envasado. Asímismo, el pan de pita absorbió el AITC de manera creciente hasta los 7 días de almacenamiento.
10. La NTC se fue degradando durante el período de almacenamiento en los quesos tratados mediante pulverizado. El menor contenido de NTC en la superficie del producto se obtuvo mediante el uso de los biopolímeros.
11. Un total de 11 ácidos fenólicos diferentes fueron identificados en los extractos fermentados de mostaza, siendo el ácido 3-feniláctico el mayoritario, mientras que el único ácido orgánico detectado fue el ácido láctico.
12. Se identificaron un total de 53 compuestos volátiles en las fracciones de salvado y harina de *Sinapis alba* y *Brassica juncea*. El AITC fue el compuesto más característico de *Brassica juncea*, mientras que los alcanos fueron la fracción más abundante en las semillas de *Sinapis alba*.
13. Se identificaron un total de 26 compuestos bioactivos en las fracciones de salvado y harina de *Sinapis alba* y *Brassica juncea*, entre ellos ácidos fenólicos, flavonoides y glucosinolatos. El ácido sinápico y sus derivados fueron los compuestos más característicos en ambas variedades de mostaza.

5. CONCLUSIONS

1. The yellow mustard bran (MB) evidenced antifungal activity against fungal strains of the *Aspergillus*, *Penicillium* and *Fusarium* genera *in vitro*.
2. The yellow and oriental mustard fermented extracts with *Lactiplantibacillus plantarum* TR71 showed inhibitory action against *Fusarium* species, being the yellow mustard fermentate the one with the highest antifungal activity.
3. Natamycin (NTC) was effective against all *Penicillium* strains tested *in vitro*.
4. The use of oriental mustard flour (OMF) as an allyl isothiocyanate (AITC) releasing ingredient in the bioactive sauce exerted preservative action on pita bread. The bread treated with the 50 mg/g sauce showed no visual fungal growth after 7 days of inoculation.
5. The MB can be revalued as an antifungal ingredient in bread formulation. Bread made with 10 g/kg showed a shelf life of 7 days, being comparable to the use of the chemical additive sodium propionate (E-281).
6. Both direct spraying and incorporation of NTC in biopolymers increased Mozzarella cheese shelf life, although the use of antifungal biopolymers has certains advantages.
7. The dose of 50 mg/g of OMF in the bioactive sauce completely inhibited the synthesis of ochratoxin A by *P. verrucosum* in pita bread after 7 days of storage.
8. The application of yellow mustard fermentate with *L. plantarum* TR71 at 250 g/L on corn ears reduced the incidence of fumonisin B_1 and B_2 by almost completely.
9. The highest AITC content in the headspace was detected after 8 hours of packaging. Likewise, the pita bread absorbed the AITC in an increasing manner up to 7 days of storage.

10. The NTC degraded during the storage period in the spray-treated cheeses. The lowest NTC content on the product surface was obtained by the use of biopolymers.
11. A total of 11 different phenolic acids were identified in the fermented mustard extracts, with 3-phenhyllactic acid being the major one, while the only organic acid detected was lactic acid.
12. A total of 53 volatile compounds were identified in the bran and flour fractions of *Sinapis alba* and *Brassica juncea*. AITC was the most characteristic compound of *Brassica juncea*, whereas alkanes were the most abundant fraction in *Sinapis alba* seeds.
13. A total of 26 bioactive compounds were identified in the bran and flour fractions of *Sinapis alba* and *Brassica juncea*, including phenolic acids, flavonoids, and glucosinolates. Sinapic acid and its derivatives were the most characteristic compounds in both mustard varieties.

6. REFERENCES

Referencias

6. REFERENCIAS

Abdel-Aziz, S. M., Asker, M. M. S., Keera, A. A., & Mahmoud, M. G. (2016). Microbial food spoilage: Control strategies for shelf life extension. *Microbes in Food and Health*, 239–264.

Abdel-Aziz, M. S., Salama, H. E., & Sabaa, M. W. (2018). Biobased alginate/castor oil edible films for active food packaging. *LWT*, *96*, 455–460.

Abul-Fadl, M. M., El-Badry, N., & Ammar, M. S. (2011). Nutritional and Chemical Evaluation for Two Different Varieties of Mustard Seeds. *World Applied Sciences Journal*, *15*(9), 1225–1233.

Adams, R. P. (2007). Identification of Essential Oil Components by Gas Chromatograpy/Mass Spectrometry. *Illinois USA: Allured Publishing Corporation, Carol Stream*, 0–804.

Adegbeye, M. J., Elghandour, M. M. M. Y., Faniyi, T. O., Rivero Perez, N., Barbabosa-Pilego, A., Zaragoza-Bastida, A., & Salem, A. Z. M. (2020). Antimicrobial and antihelminthic impacts of black cumin, pawpaw and mustard seeds in livestock production and health. *Agroforestry Systems*, *94*(4), 1255–1268.

Agriopoulou, S., Stamatelopoulou, E., & Varzakas, T. (2020). Advances in Occurrence, Importance, and Mycotoxin Control Strategies: Prevention and Detoxification in Foods. *Foods*, *9*(2), 137.

Alexa, E., Dehelean, C. A., Poiana, M. A., Radulov, I., Cimpean, A. M., Bordean, D. M., Tulcan, C., & Pop, G. (2013). The occurrence of mycotoxins in wheat from western Romania and histopathological impact as effect of feed intake. *Chemistry Central Journal*, *7*(1), 1–11.

Almasi, H., Jahanbakhsh Oskouie, M., & Saleh, A. (2020). A review on techniques utilized for design of controlled release food active packaging. *Critical Reviews in Food Science and Nutrition*, *61*(15), 2601–2621.

Almeida-Ferreira, G. C., Barbosa-Tessmann, I. P., Sega, R., & Machinski Junior, M. (2013). Occurrence of zearalenone in wheat- and corn-based products commercialized in the State of Paraná, Brazil. *Brazilian Journal of Microbiology*, *44*(2), 371–375.

Alshannaq, A., & Yu, J. H. (2017). Occurrence, Toxicity, and Analysis of Major Mycotoxins in Food. *International Journal of Environmental Research and Public Health*, *14*(6).

Álvarez-Hernández, M. H., Martínez-Hernández, G. B., Castillejo, N., Martínez, J. A., & Artés-Hernández, F. (2021). Development of an antifungal active packaging containing thymol and an ethylene scavenger. Validation during storage of cherry tomatoes. *Food Packaging and Shelf Life*, *29*.

Amit, S. K., Uddin, M. M., Rahman, R., Islam, S. M. R., & Khan, M. S. (2017). A review on mechanisms and commercial aspects of food preservation and processing. *Agriculture and Food Security*, *6*(1), 1–22.

Andretta, I., Kipper, M., Hauschild, L., Lehnen, C. R., Remus, A., & Melchior, R. (2016). Meta-analysis of individual and combined effects of mycotoxins on growing pigs. *Scientia Agricola*, *73*(4), 328–331.

Angioni, A., Barra, A., Coroneo, V., Dessi, S., & Cabras, P. (2006). Chemical composition, seasonal variability, and antifungal activity of Lavandula stoechas L. ssp. stoechas essential oils from stem/leaves and Flowers. *Journal of Agricultural and Food Chemistry*, *54*(12), 4364–4370.

Ansari, Z., Miller, D., & Galor, A. (2013). Current thoughts in fungal keratitis: Diagnosis and treatment. *Current Fungal Infection Reports*, *7*(3), 209–218.

Aparicio, J. F., Barreales, E. G., Payero, T. D., Vicente, C. M., de Pedro, A., & Santos-Aberturas, J. (2016). Biotechnological production and application of the antibiotic pimaricin: biosynthesis and its regulation. *Applied Microbiology and Biotechnology*, *100*(1), 61–78.

Aparicio, J., Mendes, M., Anton, N., Recio, E., & Martin, J. (2004). Polyene macrolide antibiotic biosynthesis. *Current Medicinal Chemistry*, *11*(12), 1643–1656.

Appendini, P., & Hotchkiss, J. H. (2002). Review of antimicrobial food packaging. *Innovative Food Science and Emerging Technologies*, *3*(2), 113–126.

Araújo, M. K., Gumiela, A. M., Bordin, K., Luciano, F. B., & Macedo, R. E. F. de. (2018). Combination of garlic essential oil, allyl isothiocyanate, and nisin Z as bio-preservatives in fresh sausage. *Meat Science*, *143*, 177–183.

Assou, J., Zhang, D., Roth, K. D. R., Steinke, S., Hust, M., Reinard, T., Winkelmann, T., & Boch, J. (2022). Removing the major allergen Bra j I from brown mustard (Brassica juncea) by CRISPR/Cas9. *The Plant Journal*, *109*(3), 649–663.

Avato, P., & Argentieri, M. P. (2015). Brassicaceae: a rich source of health improving phytochemicals. *Phytochemistry Reviews 2015 14:6*, *14*(6), 1019–1033.

Axel, C., Zannini, E., & Arendt, E. K. (2017). Mold spoilage of bread and its biopreservation: A review of current strategies for bread shelf life extension. *Critical Reviews in Food Science and Nutrition*, *57*(16), 3528–3542.

Azaiez, I., Meca, G., Manyes, L., & Fernández-Franzón, M. (2013). Antifungal activity of gaseous allyl, benzyl and phenyl isothiocyanate in vitro and their use for fumonisins reduction in bread. *Food Control*, *32*(2), 428–434.

Azeredo, H. M. C., Mattoso, L. H. C., Wood, D., Williams, T. G., Avena-Bustillos, R. J., & McHugh, T. H. (2009). Nanocomposite edible films from mango puree reinforced with cellulose nanofibers. *Journal of Food Science*, *74*(5).

Brosnan, B., Aidan, C., Elke K, A., & Ambrose, F. (2014). The QuEChERS approach in a novel application for the identification of antifungal compounds produced by lactic acid bacteria cultures. *Talanta*, *129*, 364–373.

Bahmid, N. A., Dekker, M., Fogliano, V., & Heising, J. (2021). Development of a moisture-activated antimicrobial film containing ground mustard seeds and its application on meat in active packaging system. *Food Packaging and Shelf Life*, *30*, 100753.

Bahmid, N. A., Heising, J., Fogliano, V., & Dekker, M. (2020). Packaging Design Using Mustard Seeds as a Natural Antimicrobial: A Study on Inhibition of Pseudomonas fragi in Liquid Medium. *Foods 2020, Vol. 9, Page 789*, *9*(6), 789.

Bahmid, N. A., Pepping, L., Dekker, M., Fogliano, V., & Heising, J. (2020). Using particle size and fat content to control the release of Allyl isothiocyanate from ground mustard seeds for its application in antimicrobial packaging. *Food Chemistry*, *308*, 125573.

Balaguer, M. P., Fajardo, P., Gartner, H., Gomez-Estaca, J., Gavara, R., Almenar, E., & Hernandez-Munoz, P. (2014). Functional properties and antifungal activity of films based on gliadins containing cinnamaldehyde and natamycin. *International Journal of Food Microbiology*, *173*, 62–71.

Banjara, N., Suhr, M. J., & Hallen-Adams, H. E. (2015). Diversity of Yeast and Mold Species from a Variety of Cheese Types. *Current Microbiology*, *70*(6), 792–800.

Beaulieu, J. C., & Grimm, C. C. (2001). Identification of Volatile Compounds in Cantaloupe at Various Developmental Stages Using Solid Phase Microextraction. *Journal of Agricultural and Food Chemistry*, *49*(3), 1345–1352.

Bell, L., Oloyede, O. O., Lignou, S., Wagstaff, C., & Methven, L. (2018). Taste and Flavor Perceptions of Glucosinolates, Isothiocyanates, and Related Compounds. *Molecular Nutrition & Food Research*, *62*(18), 1700990.

Ben Taheur, F., Mansour, C., Kouidhi, B., & Chaieb, K. (2019). Use of lactic acid bacteria for the inhibition of Aspergillus flavus and Aspergillus carbonarius growth and mycotoxin production. *Toxicon*, *166*, 15–23.

Bhandari, S. R., & Kwak, J. H. (2015). Chemical Composition and Antioxidant Activity in Different Tissues of Brassica Vegetables. *Molecules 2015, Vol. 20, Pages 1228-1243*, *20*(1), 1228–1243.

Bondi, M., Messi, P., Halami, P. M., Papadopoulou, C., & De Niederhausern, S. (2014). Emerging microbial concerns in food safety and new control measures. *BioMed Research International*, *251512.*

Borek, V., & Morra, M. J. (2005). Ionic Thiocyanate (SCN-) Production from 4-Hydroxybenzyl Glucosinolate Contained in Sinapis alba Seed Meal. *Journal of Agricultural and Food Chemistry*, *53*(22), 8650–8654.

Borges, A., Abreu, A. C., Ferreira, C., Saavedra, M. J., Simões, L. C., & Simões, M. (2015). Antibacterial activity and mode of action of selected glucosinolate hydrolysis products against bacterial pathogens. *Journal of Food Science and Technology*, *52*(8), 4737–4748.

Bortolotto, F. C. K., Ceccoti, S. P. C., Orso, P. B., Wolupeck, H. L., Holley, R. A., Luciano, F. B., & de Macedo, R. E. F. (2018). Combined effects of garlic essential oil and allyl isothiocyanate against *Escherichia coli* O157:H7 *in vitro* and in pork sausage. *Ciência Rural*, *48*(10).

Boscariol Rasera, G., Hilkner, M. H., de Alencar, S. M., & de Castro, R. J. S. (2019). Biologically active compounds from white and black mustard grains: An optimization study for recovery and identification of phenolic antioxidants. *Industrial Crops and Products*, *135*, 294–300.

Brosnan, B., Coffey, A., Arendt, E. K., & Furey, A. (2012). Rapid identification, by use of the LTQ Orbitrap hybrid FT mass spectrometer, of antifungal compounds produced by lactic acid bacteria. *Analytical and Bioanalytical Chemistry*, *403*(10), 2983–2995.

Brothers, A. M., & Wyatt, R. D. (2000). The antifungal activity of natamycin toward molds isolated from commercially manufactured poultry feed. *Avian Diseases*, *44*(3), 490–497.

Bundgaard-Nielsen, K., & Nielsen, P. V. (1996). Fungicidal Effect of 15 Disinfectants

against 25 Fungal Contaminants Commonly Found in Bread and Cheese Manufacturing. *Journal of Food Protection*, *59*(3), 268–275.

Calabriso, N., Massaro, M., Scoditti, E., Pasqualone, A., Laddomada, B., & Carluccio, M. A. (2020). Phenolic extracts from whole wheat biofortified bread dampen overwhelming inflammatory response in human endothelial cells and monocytes: major role of VCAM-1 and CXCL-10. *European Journal of Nutrition*, *59*(6), 2603–2615.

Campbell, B., Han, D. Y., Triggs, C. M., Fraser, A. G., & Ferguson, L. R. (2012). Brassicaceae: Nutrient analysis and investigation of tolerability in people with Crohn's disease in a New Zealand study. *Functional Foods in Health and Disease*, *2*(11), 460–486.

Carocho, M., Morales, P., & Ferreira, I. C. F. R. (2015). Natural food additives: Quo vadis? *Trends in Food Science & Technology*, *45*(2), 284–295.

Cartea, M. E., Francisco, M., Soengas, P., & Velasco, P. (2010). Phenolic Compounds in Brassica Vegetables. *Molecules, 16*(1), 251–280.

CE (2004). Reglamento (CE) n° 852/2004 del Parlamento Europeo y del Consejo, de 29 de abril de 2004, relativo a la higiene de los productos alimenticios. DOUE, L 139/1.

CE (2004). Reglamento (CE) n° 1935/2004 del Parlamento Europeo y del Consejo, de 27 de octubre de 2004, sobre los materiales y objetos destinados a entrar en contacto con alimentos. DOUE, L 338/4.

CE (2006). Reglamento (CE) n° 1881/2006 de la Comisión, de 19 de diciembre de 2016, por el que se fija el contenido máximo de determinados contaminantes en los productos alimenticios. DOUE, L 364/5.

CE (2007). Reglamento (CE) n° 1126/2007 de la Comisión, de 28 de septiembre de 2007, que modifica el Reglamento (CE) n° 1881/2006 por el que se fija el contenido máximo de determinados contaminantes en los productos alimenticios por lo que se refiere a las toxinas de *Fusarium* en el maíz y los productos del maíz. DOUE, L 255/14.

CE (2008). Reglamento (CE) n° 133/2008 del Parlamento Europeo y del Consejo, de 16 de diciembre de 2008, sobre aditivos alimentarios. DOUE, L 354/16.

CE (2010). Reglamento (UE) n° 105/2012 de la Comisión, de 5 de febrero de 2012, que modifica el Reglamento (CE) n° 1881/2006. por el que se fija el contenido máximo de determinados contaminantes en los productos alimenticios por lo que se refiere a la ocratoxina A. DOUE, L 35/7.

CE (2010). Reglamento (EU) n° 165/2010 de la Comisión, de 26 de febrero de 2010, que modifica, en lo que respecta a las aflatoxinas, el Reglamento (CE) n° 1881/2006 por el que se fija el contenido máximo de determinados contaminantes en los productos alimenticios. DOUE, L 50/8.

CE (2011). Reglamento (UE) n° 1129/2011 de la Comisión, de 11 de noviembre de 2011, por el que se modifica el anexo II del Reglamento (CE) n° 1333/2008 del Parlamento Europeo y del Consejo para establecer una lista de aditivos alimentarios de la Unión. DOUE, L 295/1.

CE (2011). Reglamento (UE) n° 1169/2011 del Parlamento Europeo y del Consejo, de 25 de octubre de 2011, sobre la información facilitada al consumidor. DOUE, L 304/18.

CE (2012). Reglamento (UE) n° 594/2012 de la Comisión, de 5 de julio de 2012, por el que se modifica el Reglamento (CE) n° 1881/2066 de la Comisión, por el que se fija el contenido máximo de determinados contaminantes en los productos alimenticios, en lo concerniente a los contenidos máximos de los contaminantes ocratoxina A, PCBs no similares a las dioxinas y melamina en los productos alimenticios. DOUE, L 176/43.

CE (2012). Reglamento (UE) n° 1058/2012 de la Comisión, de 12 de noviembre de 2012, por el que se modifica el Reglamento (CE) n° 1881/2006 en lo que respecta al contenido máximo de aflatoxinas en los higos secos. DOUE, L 313/14.

CE (2014). Reglamento (UE) n° 212/2014 de la Comisión, de 6 de marzo de 2014, por el que se modifica el Reglamento (CE) n° 1881/2006 en lo que concierne a los contenidos mázimos del contaminante citrinina en complementos alimenticios basados en arroz fermentado con levadura roja *Monascus purpureus*. DOUE, L 67/3.

CE (2021). Reglamento (UE) n° 2021/1399 de la Comisión, de 24 de agosto de 2021, por el que se modifica el Reglamento (CE) n° 1881/2006 en lo que respecta al contenido máximo de esclerocios de cornezuelo y alcaloides de cornezuelo en determinados productos alimenticios. DOUE, L 301/1.

Cejpek, K., Valusek, J., & Velisek, J. (2000). Reactions of allyl isothiocyanate with alanine, glycine, and several peptides in model systems. *Journal of Agricultural and Food Chemistry*, *48*(8), 3560–3565.

Cevher, E., Şensoy, D., Zloh, M., & Mülazimoğlu, L. (2008). Preparation and characterisation of natamycin: γ-cyclodextrin inclusion complex and its evaluation in vaginal mucoadhesive formulations. *Journal of Pharmaceutical*

Sciences, *97*(10), 4319–4335.

Chacon, P. A., Buffo, R. A., & Holley, R. A. (2006). Inhibitory effects of microencapsulated allyl isothiocyanate (AIT) against Escherichia coli O157:H7 in refrigerated, nitrogen packed, finely chopped beef. *International Journal of Food Microbiology*, *107*(3), 231–237.

Chen, C., Cao, Z., Li, J., Tao, C., Feng, Y., & Han, Y. (2020). A novel endophytic strain of Lactobacillus plantarum CM-3 with antagonistic activity against Botrytis cinerea on strawberry fruit. *Biological Control*, *148*.

Chen, H., Ju, H., Wang, Y., Du, G., Yan, X., Cui, Y., Yuan, Y., & Yue, T. (2021). Antifungal activity and mode of action of lactic acid bacteria isolated from kefir against Penicillium expansum. *Food Control*, *130*, 108274.

Chen, H., Yan, X., Du, G., Guo, Q., Shi, Y., Chang, J., Wang, X., Yuan, Y., & Yue, T. (2021). Recent developments in antifungal lactic acid bacteria: Application, screening methods, separation, purification of antifungal compounds and antifungal mechanisms. *Critical Reviews in Food Science and Nutrition*. DOI: 10.1080/10408398.2021.1977610

Chen, O., Hong, Y., Ma, J., Deng, L., Yi, L., & Zeng, K. (2021). Screening lactic acid bacteria from pickle and cured meat as biocontrol agents of Penicillium digitatum on citrus fruit. *Biological Control*, *158*, 104606

Chtioui, W., Balmas, V., Delogu, G., Migheli, Q., & Oufensou, S. (2022). Bioprospecting Phenols as Inhibitors of Trichothecene-Producing Fusarium: Sustainable Approaches to the Management of Wheat Pathogens. *Toxins*, *14*(2), 72.

Clemente, I., Aznar, M., & Nerín, C. (2016). Raman Imaging Spectroscopy as a Tool to Investigate the Cell Damage on Aspergillus ochraceus Caused by an Antimicrobial Packaging Containing Benzyl Isothiocyanate. *Analytical Chemistry*, *88*(9), 4772–4779.

Clemente, I., Aznar, M., & Nerín, C. (2017). Effect of an active label based on benzyl isothiocyanate on the morphology and ochratoxins production of Aspergillus ochraceus. *Food Research International*, *101*, 61–72.

Clemente, I., Aznar, M., & Nerín, C. (2019). Synergistic properties of mustard and cinnamon essential oils for the inactivation of foodborne moulds in vitro and on Spanish bread. *International Journal of Food Microbiology*, *298*, 44–50.

Clemente, I., Aznar, M., Silva, F., & Nerín, C. (2016). Antimicrobial properties and mode of action of mustard and cinnamon essential oils and their

combination against foodborne bacteria. *Innovative Food Science & Emerging Technologies*, *36*, 26–33.

CLSI. (2008). *Reference method for Broth Dilution antifungal Susceptibility testing of Filamentous fungi; approved Standard* (2nd ed.). Wayne, PA: CLSI document M38-A2.

Cortés-Zavaleta, O., López-Malo, A., Hernández-Mendoza, A., & García, H. S. (2014). Antifungal activity of lactobacilli and its relationship with 3-phenyllactic acid production. *International Journal of Food Microbiology*, *173*, 30–35.

Costa, M. J., Cerqueira, M. A., Ruiz, H. A., Fougnies, C., Richel, A., Vicente, A. A., Teixeira, J. A., & Aguedo, M. (2015). Use of wheat bran arabinoxylans in chitosan-based films: Effect on physicochemical properties. *Industrial Crops and Products*, *66*, 305–311.

Costa, M. J., Maciel, L. C., Teixeira, J. A., Vicente, A. A., & Cerqueira, M. A. (2018). Use of edible films and coatings in cheese preservation: Opportunities and challenges. *Food Research International*, *107*, 84–92.

Crowley, S., Mahony, J., & Van Sinderen, D. (2013). Current perspectives on antifungal lactic acid bacteria as natural bio-preservatives. *Trends in Food Science and Technology*, *33*(2), 93–109.

Dainelli, D., Gontard, N., Spyropoulos, D., Zondervan-van den Beuken, E., & Tobback, P. (2008). Active and intelligent food packaging: legal aspects and safety concerns. *Trends in Food Science and Technology*, *19*, S103–S112.

Dal Bello, F., Clarke, C. I., Ryan, L. A. M., Ulmer, H., Schober, T. J., Ström, K., Sjögren, J., van Sinderen, D., Schnürer, J., & Arendt, E. K. (2007). Improvement of the quality and shelf life of wheat bread by fermentation with the antifungal strain Lactobacillus plantarum FST 1.7. *Journal of Cereal Science*, *45*(3), 309–318.

Dalié, D. K. D., Deschamps, A. M., & Richard-Forget, F. (2010). Lactic acid bacteria – Potential for control of mould growth and mycotoxins: A review. *Food Control*, *21*(4), 370–380.

Daou, R., Joubrane, K., Maroun, R. G., Khabbaz, L. R., Ismail, A., Khoury, A. El, Daou, R., Joubrane, K., Maroun, R. G., Khabbaz, L. R., Ismail, A., & Khoury, A. El. (2021). Mycotoxins: Factors influencing production and control strategies. *AIMS Agriculture and Food, 6*(1), 416–447.

Davidson, P. M., & Critzer, F. M. (2012). Interventions to Inhibit or Inactivate

Bacterial Pathogens in Foods. In: Oyarzabal, O., Backert, S. (Eds.) *Microbial Food Safety* (pp. 189-202). Food Science Text Series. Springer, New York, NY.

Davidson, P. M., & Harrison, M. A. (2002). Resistance and adaptation to food antimicrobials, sanitizers, and other process controls. *Food Technology*, *56*(11), 69-78.

de Abreu, D. A. P., Cruz, J. M., & Losada, P. P. (2012). Active and Intelligent Packaging for the Food Industry. *Food Reviews International*, *28*(2), 146–187.

de Melo Nazareth, T., Luz, C., Torrijos, R., Quiles, J. M., Luciano, F. B., Mañes, J., & Meca, G. (2019). Potential application of lactic acid bacteria to reduce aflatoxin B1 and fumonisin B1 occurrence on corn kernels and corn ears. *Toxins*, *12*(1), 1–16.

De Simone, N., Capozzi, V., de Chiara, M. L. V., Amodio, M. L., Brahimi, S., Colelli, G., Drider, D., Spano, G., & Russo, P. (2021). Screening of Lactic Acid Bacteria for the Bio-Control of Botrytis cinerea and the Potential of Lactiplantibacillus plantarum for Eco-Friendly Preservation of Fresh-Cut Kiwifruit. *Microorganisms, 9*(4), 773.

Delves-Broughton, J. (2008). Use of the natural food preservatives, nisin and natamycin, to reduce detrimental thermal impact on product quality. *In-Pack Processed Foods: Improving Quality*, 319–337.

Delves-Broughton, Joss, Thomas, L. V., Doan, C. H., & Davidson, P. M. (2005). Natamycin. *Antimicrobials in Food, Third Edition*, 275–289.

Denardi-Souza, T., Luz, C., Mañes, J., Badiale-Furlong, E., & Meca, G. (2018). Antifungal effect of phenolic extract of fermented rice bran with Rhizopus oryzae and its potential use in loaf bread shelf life extension. *Journal of the Science of Food and Agriculture*, *98*(13), 5011–5018.

Din, M. I., Ghaffar, T., Najeeb, J., Hussain, Z., Khalid, R., & Zahid, H. (2020). Potential perspectives of biodegradable plastics for food packaging application-review of properties and recent developments. *Food Additives & Contaminants: Part A*, *37*(4), 665–680.

Donsì, F., & Velikov, K. P. (2020). Mechanical cell disruption of mustard bran suspensions for improved dispersion properties and protein release. *Food & Function*, *11*(7), 6273–6284.

Dopazo, V., Luz, C., Mañes, J., Quiles, J. M., Carbonell, R., Calpe, J., & Meca, G. (2021). Bio-preservative potential of microorganisms isolated from red grape against food contaminant fungi. *Toxins*, *13*(6), 412.

Doulia, D., Katsinis, G., & Mougin, B. (2009). Prolongation of the microbial shelf life of wrapped part baked baguettes. *International Journal of Food Properties*, *3*(3), 447–457.

Dubey, S., Guignard, F., Pellaud, S., Pedrazzetti, M., Van Der Schuren, A., Gaume, A., Schnee, Sylvain S., Gindro, K., & Dubey, O. (2020). Isothiocyanate Derivatives of Glucosinolates as Efficient Natural Fungicides. *PhytoFrontiers*, *1*(1), 40–50.

Edite Bezerra da Rocha, M., Freire, F. da C. O., Erlan Feitosa Maia, F., Izabel Florindo Guedes, M., & Rondina, D. (2014). Mycotoxins and their effects on human and animal health. *Food Control*, *36*(1), 159–165.

EFSA (2018). Scientific Opinion on the safety of allyl isothiocyanate for the proposed uses as a food additive. *EFSA Journal*, *8*(12).

Eib, S., Schneider, D. J., Hensel, O., & Seuß-Baum, I. (2020). Relationship between mustard pungency and allyl-isothiocyanate content: A comparison of sensory and chemical evaluations. *Journal of Food Science*, *85*(9), 2728–2736.

Ekanayake, A., Kester, J. J., Li, J. J., Zehentbauer, G. N., Bunke, P. R., & Zent, J. B. (2006). IsoGardTM: A natural antimicrobial agent derived from white mustard seed. *Acta Horticulturae*, *709*, 101-108.

Ekanayake, A., Strife, R. J., Zehentbauer, G. N., & David, J. R. D. (2016). Yellow or White Mustard (Sinapis alba L.) Oils. *Essential Oils in Food Preservation, Flavor and Safety*, 857–863.

El Fawal, G., Hong, H., Song, X., Wu, J., Sun, M., He, C., Mo, X., Jiang, Y., & Wang, H. (2020). Fabrication of antimicrobial films based on hydroxyethylcellulose and ZnO for food packaging application. *Food Packaging and Shelf Life*, *23*, 100462.

Elsanhoty, R. M., Ramadan, M. F., El-Gohery, S. S., Abol-Ela, M. F., & Azeke, M. A. (2013). Ability of selected microorganisms for removing aflatoxins in vitro and fate of aflatoxins in contaminated wheat during baladi bread baking. *Food Control*, *33*(1), 287–292.

Engel, E., Baty, C., Le Corre, D., Souchon, I., & Martin, N. (2002). Flavor-active compounds potentially implicated in cooked cauliflower acceptance. *Journal of Agricultural and Food Chemistry*, *50*(22), 6459–6467.

Engels, C., Schieber, A., & Gänzle, M. G. (2012). Sinapic acid derivatives in defatted Oriental mustard (Brassica juncea L.) seed meal extracts using UHPLC-DAD-

ESI-MS and identification of compounds with antibacterial activity. *European Food Research and Technology*, *234*(3), 535–542.

Ercolini, D., De Filippis, F., La Storia, A., & Iacono, M. (2012). "Remake" by high-throughput sequencing of the microbiota involved in the production of water Buffalo mozzarella cheese. *Applied and Environmental Microbiology*, *78*(22), 8142–8145.

Eskola, M., Kos, G., Elliott, C. T., Hajšlová, J., Mayar, S., & Krska, R. (2019). Worldwide contamination of food-crops with mycotoxins: Validity of the widely cited 'FAO estimate' of 25%. *Critical Reviews in Food Science and Nutrition*, *0*(0), 1–17.

Espinel-Ingroff, A., Cantón, E., & Pemán, J. (2012). Antifungal susceptibility testing of filamentous fungi. *Current Fungal Infection Reports*, *6*(1), 41–50.

Esposito, F., Fasano, E., Scognamiglio, G., Nardone, A., Triassi, M., & Cirillo, T. (2016). Exposure assessment to fumonisins B 1, B 2 and B 3 through consumption of gluten-free foodstuffs intended for people affected by celiac disease. *Food and Chemical Toxicology*, *97*, 395–401.

Evangelista, A. G., Bocate, K. C. P., Meca, G., & Luciano, F. B. (2021). Combination of allyl isothiocyanate and cinnamaldehyde against the growth of mycotoxigenic fungi and aflatoxin production in corn. *Journal of Food Processing and Preservation*, *45*(9).

Fahey, J. W., Zalcmann, A. T., & Talalay, P. (2001). The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry*, *56*(1), 5–51.

Fahma, F., Fauzan, R. M., Sunarti, T., Sugiarto, S., Halim, A., Lin, K.-H., Hu, D., Enomae, T., Fateta, G., & Dramaga, K. I. P. B. (2020). Production of silica-alginate-nanocellulose composite beads with cinnamon essential oil for antimicrobial sachet. *Journal of Nanostructures, 10*(4), 779-792.

Fan, W., & Qian, M. C. (2006). Identification of aroma compounds in Chinese 'Yanghe Daqu' liquor by normal phase chromatography fractionation followed by gas chromatography[sol]olfactometry. *Flavour and Fragrance Journal*, *21*(2), 333–342.

Fang, Z., Hu, Y., Liu, D., Chen, J., & Ye, X. (2008). Changes of phenolic acids and antioxidant activities during potherb mustard (Brassica juncea, Coss.) pickling. *Food Chemistry*, *108*(3), 811–817.

Ferrigo, D., Raiola, A., & Causin, R. (2016). Fusarium Toxins in Cereals: Occurrence,

Legislation, Factors Promoting the Appearance and Their Management. *Molecules*, *21*(5), 627.

Flamini, G., & Cioni I Morelli, P. L. (2002). Analysis of the essential oil of the aerial parts of Viola etrusca from Monte Labbro (South Tuscany, Italy) and in vivo analysis of flower volatiles using SPME. *Flavour and Fragrance Journal*, *17*(2), 147–149.

Flamini, G., Cioni, P. L., & Morelli, I. (2003). Volatiles from leaves, fruits, and virgin oil from Olea europaea cv. Olivastra Seggianese from Italy. *Journal of Agricultural and Food Chemistry*, *51*(5), 1382–1386.

Flourat, A. L., Willig, G., Teixeira, A. R. S., & Allais, F. (2019). Eco-Friendly Extraction of Sinapine From Residues of Mustard Production. *Frontiers in Sustainable Food Systems*, *3*, 12.

Fox, P. F., McSweeney, P. L., Cogan, T. M., & Guinee, T. P. (Eds.). (2004). *Cheese: Chemistry, Physics and Microbiology. Volume 1, General aspects.* Elsevier.

Frazie, M. D., Kim, M. J., & Ku, K. M. (2017). Health-Promoting Phytochemicals from 11 Mustard Cultivars at Baby Leaf and Mature Stages. *Molecules : A Journal of Synthetic Chemistry and Natural Product Chemistry*, *22*(10).

Gajbhiye, M. H., & Kapadnis, B. P. (2016). Antifungal-activity-producing lactic acid bacteria as biocontrol agents in plants. *Biocontrol Science and Technology*, *26*(11), 1451–1470.

Gao, Y., Ma, S., Wang, M., & Feng, X. Y. (2017). Characterization of Free, Conjugated, and Bound Phenolic Acids in Seven Commonly Consumed Vegetables. *Molecules*, *22*(11), 1878.

Garcia, M. V., Bernardi, A. O., & Copetti, M. V. (2019). The fungal problem in bread production: insights of causes, consequences, and control methods. *Current Opinion in Food Science*, *29*, 1–6.

Garnier, L., Valence, F., & Mounier, J. (2017). Diversity and Control of Spoilage Fungi in Dairy Products: An Update. *Microorganisms*, *5*(3), 42.

Gavahian, M., Chu, Y. H., Lorenzo, J. M., Mousavi Khaneghah, A., & Barba, F. J. (2018). Essential oils as natural preservatives for bakery products: Understanding the mechanisms of action, recent findings, and applications. *Critical Reviews in Food Science and Nutrition*, *60*(2), 310–321.

Giarratana, F., Panebianco, F., Muscolino, D., Beninati, C., Ziino, G., & Giuffrida, A. (2015). Effect of Allyl Isothiocyanate against Anisakis Larvae during the Anchovy Marinating Process. *Journal of Food Protection*, *78*(4), 767–771.

Giorni, P., Bertuzzi, T., & Battilani, P. (2019). Impact of fungi co-occurrence on mycotoxin contamination in maize during the growing season. *Frontiers in Microbiology*, *10*, 1265.

González-Forte, L. del S., Amalvy, J. I., & Bertola, N. (2018). Effect of natamycin on the physicochemical properties of corn starch based films and their effect on Penicillium spp. activity. *International Journal of Polymer Analysis and Characterization*, *24*(1), 63–74.

Grafia, A. L., Vázquez, M. B., Bianchinotti, M. V., & Barbosa, S. E. (2018). Development of an antifungal film by polyethylene surface modification with natamycin. *Food Packaging and Shelf Life*, *18*, 191–200.

Graumann, G. H., & Holley, R. A. (2007). Survival of Escherichia coli O157:H7 in needle-tenderized dry cured Westphalian ham. *International Journal of Food Microbiology*, *118*(2), 173–179.

Grubb, C. D., & Abel, S. (2006). Glucosinolate metabolism and its control. *Trends in Plant Science*, *11*(2), 89–100.

Guimarães, A., Venancio, A., & Abrunhosa, L. (2018). Antifungal effect of organic acids from lactic acid bacteria on Penicillium nordicum. *Food Additives & Contaminants. Part A, Chemistry, Analysis, Control, Exposure & Risk Assessment*, *35*(9), 1803–1818.

Han, J. H., Patel, D., Kim, J. E., & Min, S. C. (2014). Retardation of listeria monocytogenes growth in mozzarella cheese using antimicrobial sachets containing rosemary oil and thyme oil. *Journal of Food Science*, *79*(11), E2272–E2278.

Han, K. J., Lee, J. E., Lee, N. K., & Paik, H. D. (2020). Antioxidant and Anti-inflammatory effect of probiotic lactobacillus plantarum KU15149 derived from Korean homemade Diced-radish kimchi. *Journal of Microbiology and Biotechnology*, *30*(4), 591–598.

Hanschen, F. S., Kühn, C., Nickel, M., Rohn, S., & Dekker, M. (2018). Leaching and degradation kinetics of glucosinolates during boiling of Brassica oleracea vegetables and the formation of their breakdown products. *Food Chemistry*, *263*, 240–250.

Hanschen, F. S., & Schreiner, M. (2017). Isothiocyanates, nitriles, and epithionitriles from glucosinolates are affected by genotype and developmental stage in Brassica oleracea varieties. *Frontiers in Plant Science*, *8*, 1095.

Hasegawa, N., Matsumoto, Y., Hoshino, A., & Iwashita, K. (1999). Comparison of effects of Wasabia japonica and allyl isothiocyanate on the growth of four strains of Vibrio parahaemolyticus in lean and fatty tuna meat suspensions. *International Journal of Food Microbiology*, *49*(1–2), 27–34.

Hassan, Z. U., Al Thani, R., Alsafran, M., Migheli, Q., & Jaoua, S. (2021). Selection of Bacillus spp. with decontamination potential on multiple Fusarium mycotoxins. *Food Control*, *127*, 108119.

He, C., Zhang, Z., Li, B., Xu, Y., & Tian, S. (2019). Effect of natamycin on Botrytis cinerea and Penicillium expansum—Postharvest pathogens of grape berries and jujube fruit. *Postharvest Biology and Technology*, *151*, 134–141.

Hermet, A., Méheust, D., Mounier, J., Barbier, G., & Jany, J. L. (2012). Molecular systematics in the genus Mucor with special regards to species encountered in cheese. *Fungal Biology*, *116*(6), 692–705.

Herzallah, S., & Holley, R. (2012). Determination of sinigrin, sinalbin, allyl- and benzyl isothiocyanates by RP-HPLC in mustard powder extracts. *LWT*, *47*(2), 293–299.

Hussain, S., Rehman, A. U., Luckett, D. J., Blanchard, C. L., Obied, H. K., & Strappe, P. (2019). Phenolic Compounds with Antioxidant Properties from Canola Meal Extracts Inhibit Adipogenesis. *International Journal of Molecular Sciences*, *21*(1).

Hyldgaard, M., Mygind, T., & Meyer, R. L. (2012). Essential oils in food preservation: Mode of action, synergies, and interactions with food matrix components. *Frontiers in Microbiology*, 25(3), 12.

IARC. (2012). Monographs on the evaluation of caricinogenic risks to humans: chemical agents and related occupations. A review of human carcinogens. International Agency for Research on Cancer, 100F, 224-248.

In, H. C., Soh, M. L., Se, Y. K., Choi, H. K., Kim, K. O., & Kim, Y. S. (2007). Differentiation of aroma characteristics of pine-mushrooms (Tricholoma matsutake Sing.) of different grades using gas chromatography-olfactometry and sensory analysis. *Journal of Agricultural and Food Chemistry*, *55*(6), 2323–2328.

Iqbal, S. Z., Asi, M. R., Jinap, S., & Rashid, U. (2014). Detection of aflatoxins and zearalenone contamination in wheat derived products. *Food Control*, *35*(1), 223–226.

Ishida, M., Hara, M., Fukino, N., Kakizaki, T., & Morimitsu, Y. (2014). Glucosinolate

metabolism, functionality and breeding for the improvement of Brassicaceae vegetables. *Breeding Science*, *64*(1), 48.

Izzo, L., Luz, C., Ritieni, A., Beses, J. Q., Mañes, J., & Meca, G. (2020). Inhibitory effect of sweet whey fermented by Lactobacillus plantarum strains against fungal growth: A potential application as an antifungal agent. *Journal of Food Science*, *85*(11), 3920–3926.

Jeantet, R., Croguennec, T., Schuck, P., & Brulé, G. (2016). Microbial Spoilage. *Handbook of Food Science and Technology 1*, 53–97.

Joint FAO/WHO Expert Committee on Food Additives, & World Health Organization. (2002). *Evaluation of Certain Additives and Contaminants: Fifty-seventh Report of the Joint FAO/WHO Expert Committee on Food Additives, 57*. World Health Organization.

Ju, J., Xie, Y., Yu, H., Guo, Y., Cheng, Y., Zhang, R., & Yao, W. (2020). Synergistic inhibition effect of citral and eugenol against Aspergillus niger and their application in bread preservation. *Food Chemistry*, *310*.

Juneja, V. K., Dwivedi, H. P., & Yan, X. (2012). Novel Natural Food Antimicrobials. *Annual Review of Food Science and Technology*, *3*(1), 381–403.

Kallio, M., Jussila, M., Rissanen, T., Anttila, P., Hartonen, K., Reissell, A., Vreuls, R., Adahchour, M., & Hyötyläinen, T. (2006). Comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry in the identification of organic compounds in atmospheric aerosols from coniferous forest. *Journal of Chromatography A*, *1125*(2), 234–243.

Kamal, R. M., Razis, A. F. A., Sukri, N. S. M., Perimal, E. K., Ahmad, H., Patrick, R., Djedaini-Pilard, F., Mazzon, E., & Rigaud, S. (2022). Beneficial Health Effects of Glucosinolates-Derived Isothiocyanates on Cardiovascular and Neurodegenerative Diseases. *Molecules*, *27*(3), 624.

Kanmani, P., & Rhim, J. W. (2014). Properties and characterization of bionanocomposite films prepared with various biopolymers and ZnO nanoparticles. *Carbohydrate Polymers*, *106*(1), 190–199.

Kara, M., & Soylu, E. M. (2020). Assessment of glucosinolate-derived isothiocyanates as potential natural antifungal compounds against citrus sour rot disease agent Geotrichum citri-aurantii. *Journal of Phytopathology*, *168*(5), 279–289.

Kawai, Y., Kasuga, G., Togashi, F., Abe, M., Tanaka, M., Arakawa, K., Kawai, Y., Miyamoto, T., & Masuda, T. (2017). Development of an MRS broth-based

complete food-grade medium for Lactobacillus gasseri cultivation using food-grade yeast extract and bacteriocins produced by the bacteria. *Milk Science*, *66*(3), 195-204.

Kelly, S., Grimm, L. H., Bendig, C., Hempel, D. C., & Krull, R. (2006). Effects of fluid dynamic induced shear stress on fungal growth and morphology. *Process Biochemistry*, *41*(10), 2113–2117.

Khosravi, F., Rastakhiz, N., Iranmanesh, B., & Jafari Olia, S. S. S. (2015). Determination of Organic Acids in Fruit juices by UPLC. *International Journal of Life Sciences*, *9*(5), 41–44.

Kifer, D., Jakšić, D., & Šegvić Klarić, M. (2020). Assessing the Effect of Mycotoxin Combinations: Which Mathematical Model Is (the Most) Appropriate? *Toxins*, *12*(3), 153.

Kissen, R., Rossiter, J. T., & Bones, A. M. (2009). The "mustard oil bomb": Not so easy to assemble?! Localization, expression and distribution of the components of the myrosinase enzyme system. *Phytochemistry Reviews*, *8*(1), 69–86.

Koenig, C., Lambré, J.-C., Larsen, J.-C., Leblanc, A., Mortensen, D., Parent-Massin, I., Pratt, I. M. C. M., Rietjens, I., Stankovic, P., Tobback, T., & Verguieva, R. A. (2009). Scientific Opinion on the use of natamycin (E 235) as a food additive. *EFSA Journal*, *7*(12), 1412.

Koontz, J. L., Marcy, J. E., Barbeau, W. E., & Duncan, S. E. (2003). Stability of Natamycin and Its Cyclodextrin Inclusion Complexes in Aqueous Solution. *Journal of Agricultural and Food Chemistry*, *51*(24), 7111–7114.

Kotowska, U., Żalikowski, M., & Isidorov, V. A. (2012). HS-SPME/GC-MS analysis of volatile and semi-volatile organic compounds emitted from municipal sewage sludge. *Environmental Monitoring and Assessment*, *184*(5), 2893–2907.

Kumar, N., & Goel, N. (2019). Phenolic acids: Natural versatile molecules with promising therapeutic applications. *Biotechnology Reports*, *24*.

Lagarón, J. M., López-Rubio, A., Fabra, M. J., Appl Polym Sci, J., Mellinas, C., Valdés, A., Ramos, M., Burgos, N., C Garrigós, M. D., Jiménz, A., Appl, J., Martínez-Abad, A., Ruthes, A. C., Vilaplana, F., Pardo, P., Martínez-Sanz, M., Lopez-Rubio, A., Villano, M., Oliveira, C. S., … Fabra, M. (2016). Active edible films: Current state and future trends. *Journal of Applied Polymer Science*, *133*(2).

Lappa, I. K., Mparampouti, S., Lanza, B., & Panagou, E. Z. (2018). Control of Aspergillus carbonarius in grape berries by Lactobacillus plantarum: A phenotypic and gene transcription study. *International Journal of Food Microbiology*, *275*, 56–65.

Le Lay, C., Mounier, J., Vasseur, V., Weill, A., Le Blay, G., Barbier, G., & Coton, E. (2016a). In vitro and in situ screening of lactic acid bacteria and propionibacteria antifungal activities against bakery product spoilage molds. *Food Control*, *60*, 247–255.

Lee, H. J., & Ryu, D. (2017). Worldwide Occurrence of Mycotoxins in Cereals and Cereal-Derived Food Products: Public Health Perspectives of Their Co-occurrence. *Journal of Agricultural and Food Chemistry*, *65*(33), 7034–7051.

Legan, J. D., & Voysey, P. A. (1991). Yeast spoilage of bakery products and ingredients. *Journal of Applied Bacteriology*, *70*(5), 361–371.

Levinskaitė, L. (2012). Susceptibility of food-contaminating Penicillium genus fungi to some preservatives and disinfectants. *Annals of Agricultural and Environmental Medicine*, *19*(1), 85–89.

Leyva Salas, M., Mounier, J., Valence, F., Coton, M., Thierry, A., & Coton, E. (2017). Antifungal microbial agents for food biopreservation—a review. *Microorganisms*, *5*(3).

Lietzow, J. (2021). Biologically Active Compounds in Mustard Seeds: A Toxicological Perspective. *Foods*, *10*(9).

Lin, C. M., Preston, J. F., & Wei, C. I. (2000). Antibacterial Mechanism of Allyl Isothiocyanate. *Journal of Food Protection*, *63*(6), 727–734.

Liu, H., Wu, J., Su, Y., Li, Y., Zuo, D., Liu, H., Liu, Y., Mei, X., Huang, H., Yang, M., & Zhu, S. (2021). Allyl Isothiocyanate in the Volatiles of Brassica juncea Inhibits the Growth of Root Rot Pathogens of Panax notoginseng by Inducing the Accumulation of ROS. *Journal of Agricultural and Food Chemistry*, *69*(46), 13713–13723.

Liu, Y., Xu, X. L., & Zhou, G. H. (2007). Comparative study of volatile compounds in traditional Chinese Nanjing marinated duck by different extraction techniques. *International Journal of Food Science and Technology*, *42*(5), 543–550.

Lopes, L. F., Meca, G., Bocate, K. C. P., Nazareth, T. M., Bordin, K., & Luciano, F. B. (2018). Development of food packaging system containing allyl isothiocyanate against Penicillium nordicum in chilled pizza: Preliminary

study. *Journal of Food Processing and Preservation*, *42*(2), e13436.

Lucera, A., Costa, C., Conte, A., & Del Nobile, M. A. (2012). Food applications of natural antimicrobial compounds. *Frontiers in Microbiology*, *3*, 287.

Luciano, F. B., & Holley, R. A. (2009). Enzymatic inhibition by allyl isothiocyanate and factors affecting its antimicrobial action against Escherichia coli O157:H7. *International Journal of Food Microbiology*, *131*(2–3), 240–245.

Lule, V. K., Garg, S., Gosewade, S. C., & Khedkar, C. D. (2016). Natamycin. *Encyclopedia of Food and Health*, 56–62. Elsevier.

Lund, F., Filtenborg, O., & Frisvad, J. C. (1995). Associated mycoflora of cheese. *Food Microbiology*, *12*(C), 173–180.

Luo, J., & Agnew, M. P. (2001). Gas characteristics before and after biofiltration treating odorous emissions from animal rendering processes. *Environmental Technology*, *22*(9), 1091–1103.

Luz, C., D'Opazo, V., Quiles, J. M., Romano, R., Mañes, J., & Meca, G. (2020). Biopreservation of tomatoes using fermented media by lactic acid bacteria. *LWT*, *130*, 109618.

Luz, C., Izzo, L., Graziani, G., Gaspari, A., Ritieni, A., Mañes, J., & Meca, G. (2018). Evaluation of biological and antimicrobial properties of freeze-dried whey fermented by different strains of Lactobacillus plantarum. *Food and Function*, *9*(7), 3688–3697.

Luz, C., Torrijos, R., Quiles, J. M., Mañes, J., & Meca, G. (2019). Shelf life extension of mozzarella cheese contaminated with Penicillium spp. using the antifungal compound ε-polylysine. *Food Science and Technology International*, *25*(4), 295–302.

Lv, X., Ma, H., Lin, Y., Bai, F., Ge, Y., Zhang, D., & Li, J. (2018a). Antifungal activity of Lactobacillus plantarum C10 against Trichothecium roseum and its application in promotion of defense responses in muskmelon (Cucumis melo L.) fruit. *Journal of Food Science and Technology*, *55*(9), 3703.

Lynch, K. M., Pawlowska, A. M., Brosnan, B., Coffey, A., Zannini, E., Furey, A., McSweeney, P. L. H., Waters, D. M., & Arendt, E. K. (2014). Application of Lactobacillus amylovorus as an antifungal adjunct toextend the shelf-life of Cheddar cheese. *International Dairy Journal*, *34*(1), 167–173.

Ma, J., Hong, Y., Deng, L., Yi, L., & Zeng, K. (2019). Screening and characterization of lactic acid bacteria with antifungal activity against Penicillium digitatum on citrus. *Biological Control*, *138*, 104044.

Ma, X., James, B., Balaban, M. O., Zhang, L., & Emanuelsson-Patterson, E. A. C. (2013). Quantifying blistering and browning properties of Mozzarella cheese. Part II: Cheese with different salt and moisture contents. *Food Research International*, *54*(1), 917–921.

Mahajan, S. S., Goddik, L., & Qian, M. C. (2004). Aroma compounds in sweet whey powder. *Journal of Dairy Science*, *87*(12), 4057–4063.

Malhotra, B., Keshwani, A., & Kharkwal, H. (2015). Antimicrobial food packaging: Potential and pitfalls. *Frontiers in Microbiology*, *6*, 611.

Marín, A., Plotto, A., Atarés, L., & Chiralt, A. (2019). Lactic acid bacteria incorporated into edible coatings to control fungal growth and maintain postharvest quality of grapes. *HortScience*, *54*(2), 337–343.

Marín, S., Guynot, M. E., Neira, P., Bernadó, M., Sanchis, V., & Ramos, A. J. (2002). Risk assessment of the use of sub-optimal levels of weak-acid preservatives in the control of mould growth on bakery products. *International Journal of Food Microbiology*, *79*(3), 203–211.

Marín, S., Magan, N., Ramos, A. J., Sanchis, V. (2004). Fumonisin-producing strains of Fusarium: a review of their ecophysiology. *Journal of Food Protection 67*(8), 1792-805.

Marino, M., Dubsky de Wittenau, G., Saccà, E., Cattonaro, F., Spadotto, A., Innocente, N., Radovic, S., Piasentier, E., & Marroni, F. (2019). Metagenomic profiles of different types of Italian high-moisture Mozzarella cheese. *Food Microbiology*, *79*, 123–131.

Martelli, F., Cirlini, M., Lazzi, C., Neviani, E., & Bernini, V. (2020). Edible Seaweeds and Spirulina Extracts for Food Application: In Vitro and In Situ Evaluation of Antimicrobial Activity towards Foodborne Pathogenic Bacteria. *Foods*, *9*(10).

Martinović, N., Polak, T., Ulrih, N. P., & Abramovič, H. (2020). Mustard Seed: Phenolic Composition and Effects on Lipid Oxidation in Oil, Oil-in-Water Emulsion and Oleogel. *Industrial Crops and Products*, *156*, 112851.

Mayengbam, S., Aachary, A., & Thiyam-Holländer, U. (2014). Endogenous Phenolics in Hulls and Cotyledons of Mustard and Canola: A Comparative Study on Its Sinapates and Antioxidant Capacity. *Antioxidants*, *3*(3), 544–558.

Meca, G., Luciano, F. B., Zhou, T., Tsao, R., & Mañes, J. (2012). Chemical reduction of the mycotoxin beauvericin using allyl isothiocyanate. *Food and Chemical Toxicology*, *50*(5), 1755–1762.

Meena, M., Prajapati, P., Ravichandran, C., & Sehrawat, R. (2021). Natamycin: a natural preservative for food applications—a review. *Food Science and Biotechnology*, *30*(12), 1481–1496.

Mehyar, G. F., Al Nabulsi, A. A., Saleh, M., Olaimat, A. N., & Holley, R. A. (2018). Effects of chitosan coating containing lysozyme or natamycin on shelf-life, microbial quality, and sensory properties of Halloumi cheese brined in normal and reduced salt solutions. *Journal of Food Processing and Preservation*, *42*(1), e13324.

Mel'nikov, I. S., Sotnikova, Y. S., Demina, T. S., Goncharuk, G. P., Svidchenko, E. A., Veselov, V. I., Akopova, T. A., & Babaevskii, P. G. (2020). Deformation-Strength Properties of Films Derived from Hydroxyethylcellulose Filled with Micro- and Nanocrystalline Cellulose. *Fibre Chemistry*, *51*(5), 340–345.

Melrose, J. (2019). The Glucosinolates: A Sulphur Glucoside Family of Mustard Anti-Tumour and Antimicrobial Phytochemicals of Potential Therapeutic Application. *Biomedicines*, *7*(3).

Methven, L., Tsoukka, M., Oruna-Concha, M. J., Parker, J. K., & Mottram, D. S. (2007). Influence of sulfur amino acids on the volatile and nonvolatile components of cooked salmon (Salmo salar). *Journal of Agricultural and Food Chemistry*, *55*(4), 1427–1436.

Milićević, D. R., Škrinjar, M., & Baltić, T. (2010). Real and Perceived Risks for Mycotoxin Contamination in Foods and Feeds: Challenges for Food Safety Control. *Toxins, 2*(4), 572–592.

Miyazawa, M., & Kawata, J. (2006). Identification of the main aroma compounds in dried seeds of Brassica hirta. *Journal of Natural Medicines*, *60*(1), 89–92.

Moccellin, R., dos Santos, I., Heck, D. W., Malagi, G., & Dallemole-Giaretta, R. (2017). Control of cucumber damping-off caused by Pythium aphanidermatum using canola residues. *Tropical Plant Pathology*, *42*(4), 291–297.

Mokoena, M. P., Omatola, C. A., & Olaniran, A. O. (2021). Applications of Lactic Acid Bacteria and Their Bacteriocins against Food Spoilage Microorganisms and Foodborne Pathogens. *Molecules*, *26*(22), 7055.

Monu, E. A., David, J. R. D., Schmidt, M., & Davidson, P. M. (2014). Effect of white mustard essential oil on the growth of foodborne pathogens and spoilage microorganisms and the effect of food components on its efficacy. *Journal of Food Protection*, *77*(12), 2062–2068.

Moretti, A., Logrieco, A. F., & Susca, A. (2017). *Mycotoxins: An Underhand Food Problem BT - Mycotoxigenic Fungi: Methods and Protocols* (A. Moretti & A. Susca (Eds.); pp. 3–12. Springer New York.

Morpeth, F. F. (1995). An introduction to microbial spoilage. *Preservation of Surfactant Formulations*, 1–5.

Mouterde, L. M. M., Peru, A. A. M., Mention, M. M., Brunissen, F., & Allais, F. (2020). Sustainable Straightforward Synthesis and Evaluation of the Antioxidant and Antimicrobial Activity of Sinapine and Analogues. *Journal of Agricultural and Food Chemistry*, *68*(26), 6998–7004.

Mouton, J. W., Meletiadis, J., Voss, A., & Turnidge, J. (2018). Variation of MIC measurements: the contribution of strain and laboratory variability to measurement precision. *Journal of Antimicrobial Chemotherapy*, *73*(9), 2374-2379.

Muhialdin, B. J., Algboory, H. L., Kadum, H., Mohammed, N. K., Saari, N., Hassan, Z., & Meor Hussin, A. S. (2020). Antifungal activity determination for the peptides generated by Lactobacillus plantarum TE10 against Aspergillus flavus in maize seeds. *Food Control*, *109*.

Nazareth, T. M., Quiles, J. M., Torrijos, R., Luciano, F. B., Mañes, J., & Meca, G. (2019). Antifungal and antimycotoxigenic activity of allyl isothiocyanate on barley under different storage conditions. *LWT*, *112*, 108237.

Nazareth, T. M., Corrêa, J. A. F., Pinto, A. C. S. M., Palma, J. B., Meca, G., Bordin, K., & Luciano, F. B. (2018). Evaluation of gaseous allyl isothiocyanate against the growth of mycotoxigenic fungi and mycotoxin production in corn stored for 6 months. *Journal of the Science of Food and Agriculture*, *98*(14), 5235–5241.

Nguyen, V. P. T., Stewart, J. D., Ioannou, I., & Allais, F. (2021). Sinapic Acid and Sinapate Esters in Brassica: Innate Accumulation, Biosynthesis, Accessibility via Chemical Synthesis or Recovery From Biomass, and Biological Activities. *Frontiers in Chemistry*, *9*, 350.

Nicácio, A. E., Rodrigues, C. A., Visentainer, J. V., & Maldaner, L. (2021). Evaluation of the QuEChERS method for the determination of phenolic compounds in yellow (Brassica alba), brown (Brassica juncea), and black (Brassica nigra) mustard seeds. *Food Chemistry*, *340*, 128162.

Nićiforović, N., & Abramovič, H. (2014). Sinapic Acid and Its Derivatives: Natural Sources and Bioactivity. *Comprehensive Reviews in Food Science and Food Safety*, *13*(1), 34–51.

Nielsen, P. V., & Rios, R. (2000). Inhibition of fungal growth on bread by volatile components from spices and herbs, and the possible application in active packaging, with special emphasis on mustard essential oil. *International Journal of Food Microbiology*, *60*(2–3), 219–229.

Noreen, A., Zia, K. M., Tabasum, S., Aftab, W., Shahid, M., & Zuber, M. (2020). Hydroxyethylcellulose-g-poly(lactic acid) blended polyurethanes: Preparation, characterization and biological studies. *International Journal of Biological Macromolecules*, *151*, 993–1003.

Noreen, A., Zia, K. M., Tabasum, S., Khalid, S., & Shareef, R. (2020). A review on grafting of hydroxyethylcellulose for versatile applications. *International Journal of Biological Macromolecules*, *150*, 289–303.

O' Callaghan, K. A. M., & Kerry, J. P. (2016). Consumer attitudes towards the application of smart packaging technologies to cheese products. *Food Packaging and Shelf Life*, *9*, 1–9.

Okano, K., Ayaka, O. S. E., Takai, M., Kaneko, M., Nishioka, C., Ohzu, Y., Odano, M., Sekiyama, Y., Mizukami, Y., Nakamura, N., & Ichinoe, M. (2015). Inhibition of aflatoxin production and fungal growth on stored corn by allyl isothiocyanate vapor. *Journal of the Food Hygienic Society of Japan*, *56*(1), 1-7

Okano, K., Nishioka, C., Iida, T., Ozu, Y., Kaneko, M., Watanabe, Y., Mizukami, Y., & Ichinoe, M. (2018). Inhibition of growth of seed-borne fungi and aflatoxin production on stored peanuts by allyl isothiocyanate vapor. *Journal of the Food Hygienic Society of Japan*, *59*(1), 45-50.

Okunade, O. A., Ghawi, S. K., Methven, L., & Niranjan, K. (2015). Thermal and pressure stability of myrosinase enzymes from black mustard (Brassica nigra L. W.D.J. Koch. var. nigra), brown mustard (Brassica juncea L. Czern. var. juncea) and yellow mustard (Sinapsis alba L. subsp. maire) seeds. *Food Chemistry*, *187*, 485–490.

Olaimat, A. N., Al-Holy, M. A., Abu Ghoush, M., Al-Nabulsi, A. A., & Holley, R. A. (2018). Control of Salmonella enterica and Listeria monocytogenes in hummus using allyl isothiocyanate. *International Journal of Food Microbiology*, *278*, 73–80.

Olaimat, A. N., & Holley, R. A. (2016). Inhibition of Listeria monocytogenes on cooked cured chicken breasts by acidified coating containing allyl isothiocyanate or deodorized Oriental mustard extract. *Food Microbiology*, *57*, 90–95.

Oldenburg, E., Höppner, F., Ellner, F., & Weinert, J. (2017). Fusarium diseases of maize associated with mycotoxin contamination of agricultural products intended to be used for food and feed. *Mycotoxin Research 2017 33:3*, *33*(3), 167–182.

Oliveira, P. M., Zannini, E., & Arendt, E. K. (2014). Cereal fungal infection, mycotoxins, and lactic acid bacteria mediated bioprotection: From crop farming to cereal products. *Food Microbiology*, *37*, 78–95.

Ollé Resa, C. P., Gerschenson, L. N., & Jagus, R. J. (2014). Natamycin and nisin supported on starch edible films for controlling mixed culture growth on model systems and Port Salut cheese. *Food Control*, *44*, 146–151.

Ombarak, R. A., & Shelaby, H. H. (2017). The inhibitory Effect of Natamycin and Potassium Sorbate on Mold Growth in Egyptian Fresh Soft Cheese (Tallaga Cheese). *Alexandria Journal of Veterinary Sciences*, *53*(2), 33–37.

Ostry, V., Malir, F., Toman, J., & Grosse, Y. (2017). Mycotoxins as human carcinogens—the IARC Monographs classification. *Mycotoxin Research*, *33*(1), 65–73.

Otoni, Caio G., Espitia, P. J. P., Avena-Bustillos, R. J., & McHugh, T. H. (2016). Trends in antimicrobial food packaging systems: Emitting sachets and absorbent pads. *Food Research International*, *83*, 60–73.

Otoni, Caio Gomide, Soares, N. D. F. F., Da Silva, W. A., Medeiros, E. A. A., & Baffa Junior, J. C. (2014). Use of allyl isothiocyanate-containing sachets to reduce aspergillus flavus sporulation in peanuts. *Packaging Technology and Science*,

Paes, J. L., Faroni, L. R. D. A., Dhingra, O. D., Cecon, P. R., & Silva, T. A. (2012). Insecticidal fumigant action of mustard essential oil against Sitophilus zeamais in maize grains. *Crop Protection*, *34*, 56–58.

Palomares, O., Cuesta-Herranz, J., Rodríguez, R., & Villalba, M. (2005). A Recombinant Precursor of the Mustard Allergen Sin a 1 Retains the Biochemical and Immunological Features of the Heterodimeric Native Protein. *International Archives of Allergy and Immunology*, *137*(1), 18–26.

Palumbo, R., Gonçalves, A., Gkrillas, A., Logrieco, A., Dorne, J., Dall, C., Venâncio, A., Battilani, P., Palumbo, R., Gonçalves, A., Gkrillas, A., Logrieco, A., Dorne, J.-L., Dall, C., Venâncio, A., & Battilani, P. (2020). Phytopathologia Mediterranea Mycotoxins in maize: mitigation actions, with a chain management approach. *Phytopathologia Mediterranea*, *59*(1), 5–28.

Pang, Z., Chong, J., Zhou, G., De Lima Morais, D. A., Chang, L., Barrette, M.,

Gauthier, C., Jacques, P. É., Li, S., & Xia, J. (2021). MetaboAnalyst 5.0: narrowing the gap between raw spectra and functional insights. *Nucleic Acids Research*, *49*(W1), W388–W396.

Parker, J. K., Hassell, G. M. E., Mottram, D. S., & Guy, R. C. E. (2000). Sensory and instrumental analyses of volatiles generated during the extrusion cooking of oat flours. *Journal of Agricultural and Food Chemistry*, *48*(8), 3497–3506.

Paseiro-Cerrato, R., Otero-Pazos, P., Rodriguez-Bernaldo de Quirós, A., Sendón, R., Angulo, I., & Paseiro-Losada, P. (2013). Rapid method to determine natamycin by HPLC-DAD in food samples for compliance with EU food legislation. *Food Control*, *33*(1), 262–267.

Passarinho, A. T. P., Dias, N. F., Camilloto, G. P., Cruz, R. S., Otoni, C. G., Moraes, A. R. F., & Soares, N. D. F. F. (2014). Sliced bread preservation through oregano essential oil-containing sachet. *Journal of Food Process Engineering*, *37*(1), 53–62.

Pateras, I. M. C. (2007). Bread Spoilage and Staling. In *Technology of Breadmaking*, (pp. 275–298). Springer, Boston, MA.

Patriarca, A., & Fernández Pinto, V. (2017). Prevalence of mycotoxins in foods and decontamination. *Current Opinion in Food Science*, *14*, 50–60.

Peelman, N., Ragaert, P., De Meulenaer, B., Adons, D., Peeters, R., Cardon, L., Van Impe, F., & Devlieghere, F. (2013). Application of bioplastics for food packaging. *Trends in Food Science & Technology*, *32*(2), 128–141.

Petruzzi, L., Corbo, M. R., Sinigaglia, M., & Bevilacqua, A. (2017). Microbial Spoilage of Foods: Fundamentals. *The Microbiological Quality of Food: Foodborne Spoilers*, 1–21.

Pino, J. A., Marbot, R., Payo, A., Chao, D., & Herrera, P. (2011). Aromatic Plants from Western Cuba VII. Composition of the Leaf Oils of Psidium wrightii Krug et Urb., Lantana involucrata L., Cinnamomum montanum (Sw.) Berchtold et J. Persl. And Caesalpinia violacea (Mill.) Standley. *Journal of Essential Oil Research*, *18*(2), 170–174.

Pitt, J. I. (2006). Penicillium and related genera. *Food Spoilage Microorganisms*, 437–450.

Pitt, J. I., & Hocking, A. D. (2009). The Ecology of Fungal Food Spoilage. In *Fungi and Food Spoilage* (pp. 3–9). Springer US.

Poveda, J., Eugui, D., & Velasco, P. (2020). Natural control of plant pathogens through glucosinolates: an effective strategy against fungi and oomycetes.

Phytochemistry Reviews 2020 19:4, *19*(4), 1045–1059.

Prasad, P., & Kochhar, A. (2014). Active Packaging in Food Industry: A Review. *IOSR Journal of Environmental Science*, *8*(5), 1–07.

Qu, C., Yin, N., Chen, S., Wang, S., Chen, X., Zhao, H., Shen, S., Fu, F., Zhou, B., Xu, X., Liu, L., Lu, K., & Li, J. (2020). Comparative Analysis of the Metabolic Profiles of Yellow- versus Black-Seeded Rapeseed Using UPLC-HESI-MS/MS and Transcriptome Analysis. *Journal of Agricultural and Food Chemistry*, *68*(10), 3033–3049.

Quesada, J., Sendra, E., Navarro, C., & Sayas-Barberá, E. (2016). Antimicrobial active packaging including chitosan films with thymus vulgaris l. Essential oil for ready-to-eat meat. *Foods*, *5*(3), 1–13.

Quiles, J. M., Manyes, L., Luciano, F. B., Mañes, J., & Meca, G. (2015a). Influence of the antimicrobial compound allyl isothiocyanate against the Aspergillus parasiticus growth and its aflatoxins production in pizza crust. *Food and Chemical Toxicology, 83*, 222–228.

Quiles, J. M., Manyes, L., Luciano, F. B., Mañes, J., & Meca, G. (2015b). Effect of the oriental and yellow mustard flours as natural preservative against aflatoxins B1, B2, G1 and G2 production in wheat tortillas. *Journal of Food Science and Technology*, *52*(12), 8315–8321.

Quiles, J. M., Nazareth, T. de M., Luz, C., Luciano, F. B., Mañes, J., & Meca, G. (2019). Development of an antifungal and antimycotoxigenic device containing allyl isothiocyanate for silo fumigation. *Toxins*, *11*(3).

Quiles, J. M., Torrijos, R., Luciano, F. B., Mañes, J., & Meca, G. (2018). Aflatoxins and A. flavus Reduction in Loaf Bread through the Use of Natural Ingredients. *Molecules*, *23*(7), 1638.

Rahman, M., Khatun, A., Liu, L., & Barkla, B. J. (2018). Brassicaceae Mustards: Traditional and Agronomic Uses in Australia and New Zealand. *Molecules : A Journal of Synthetic Chemistry and Natural Product Chemistry*, *23*(1).

Rajanikar, R. V., Nataraj, B. H., Naithani, H., Ali, S. A., Panjagari, N. R., & Behare, P. V. (2021). Phenyllactic acid: A green compound for food biopreservation. *Food Control*, *128*, 108184.

RD (2006). Real Decreto 113/2006, de 29 de septiembre, por el que se aprueban las normas de calidad para quesos y quesos fundidos. BOE, 239, 34717.

RD (2019). Real Decreto 308/2019, de 26 de abril, por el que se aprueba la norma de calidad para el pan. BOE, 113, 50168.

Rembold, H., Wallner, P., Nitz, S., Kollmannsberger, H., & Drawert, F. (1989). Volatile Components of Chickpea (Cicer arietinum L:) Seed. *Journal of Agricultural and Food Chemistry*, *37*(3), 659–662.

Renschler, M. A., Wyatt, A., Anene, N., Robinson-Hill, R., Pickerill, E. S., Fox, N. E., Griffith, J. A., & McKillip, J. L. (2020). Using nitrous acid-modified de Man, Rogosa, and Sharpe medium to selectively isolate and culture lactic acid bacteria from dairy foods. *Journal of Dairy Science*, *103*(2), 1215–1222.

Reps, A., Drychowski, L. J., Tomasik, J., & Winiewska, K. (2002). Natamycin in ripening cheeses. *Pakistan Journal of Nutrition*, *1*(5), 243–247.

Reungoat, V., Mouterde, L. M. M., Chadni, M., Couvreur, J., Isidore, E., Allais, F., Ducatel, H., & Ioannou, I. (2021). Simultaneous extraction and enzymatic hydrolysis of mustard bran for the recovery of sinapic acid. *Food and Bioproducts Processing*, *130*, 68–78.

Ribeiro-Santos, R., Andrade, M., Melo, N. R. de, & Sanches-Silva, A. (2017). Use of essential oils in active food packaging: Recent advances and future trends. *Trends in Food Science & Technology*, *61*, 132–140.

Ribes, S., Fuentes, A., Talens, P., & Barat, J. M. (2018). Prevention of fungal spoilage in food products using natural compounds: A review. *Critical Reviews in Food Science and Nutrition*, *58*(12), 2002–2016.

Richard, J. L. (2007). Some major mycotoxins and their mycotoxicoses—An overview. *International Journal of Food Microbiology*, *119*(1–2), 3–10.

Righetti, L., Cirlini, M., Folloni, S., Ranieri, R., Galaverna, G., Bertuzzi, T., Dall'Asta, C., Battilani, P., & Giorni, P. (2019). 5-n-alkylresorcinols but not hydroxycinnamic acids are directly related to a lower accumulation of deoxynivalenol and its glucoside in Triticum spp. Genotypes with different ploidity levels. *Journal of Cereal Science*, *85*, 214–220.

Rolfe, C., & Daryaei, H. (2020). Intrinsic and Extrinsic Factors Affecting Microbial Growth in Food Systems. *Food Engineering Series*, 3–24.

Romero, V., Borneo, R., Passalacqua, N., & Aguirre, A. (2016). Biodegradable films obtained from triticale (x Triticosecale Wittmack) flour activated with natamycin for cheese packaging. *Food Packaging and Shelf Life*, *10*, 54–59.

Ropars, J., Cruaud, C., Lacoste, S., & Dupont, J. (2012). A taxonomic and ecological overview of cheese fungi. *International Journal of Food Microbiology*, *155*(3), 199–210.

Rouse, S., Harnett, D., Vaughan, A., & Sinderen, D. Van. (2008). Lactic acid bacteria

with potential to eliminate fungal spoilage in foods. *Journal of Applied Microbiology*, *104*(3), 915–923.

Ruggirello, M., Nucera, D., Cannoni, M., Peraino, A., Rosso, F., Fontana, M., Cocolin, L., & Dolci, P. (2019). Antifungal activity of yeasts and lactic acid bacteria isolated from cocoa bean fermentations. *Food Research International*, *115*, 519–525.

Russo, P., Fares, C., Longo, A., Spano, G., & Capozzi, V. (2017). Lactobacillus plantarum with Broad Antifungal Activity as a Protective Starter Culture for Bread Production. *Foods 2017, Vol. 6, Page 110*, *6*(12), 110.

Sadeghi, A., Ebrahimi, M., Mortazavi, S. A., & Abedfar, A. (2019). Application of the selected antifungal LAB isolate as a protective starter culture in pan whole-wheat sourdough bread. *Food Control*, *95*, 298–307.

Said, N. S., & Sarbon, N. M. (2019). Protein-Based Active Film as Antimicrobial Food Packaging: A Review. In I. Var, & S. Uzunlu (Eds.), *Active Antimicrobial Food Packaging*. IntechOpen.

Saito, S., Wang, F., & Xiao, C. L. (2020). Efficacy of natamycin against gray mold of stored Mandarin fruit caused by isolates of botrytis cinerea with multiple fungicide resistance. *Plant Disease*, *104*(3), 787–792.

Saladino, F., Bordin, K., Luciano, F. B., Fernández Franzón, M., Mañes, J., Meca, G., Saladino, F., Franzón, M. F., Mañes, J., Meca, G., Bordin, K., & Luciano, F. B. (2017). Antimicrobial Activity of the Glucosinolates. *Reference Series in Phytochemistry*, 249–274.

Saladino, F., Bordin, K., Manyes, L., Luciano, F. B., Mañes, J., Fernández-Franzón, M., & Meca, G. (2016). Reduction of the aflatoxins B1, B2, G1 and G2 in Italian piadina by isothiocyanates. *LWT*, *70*, 302–308.

Saladino, F., Luz, C., Manyes, L., Fernández-Franzón, M., & Meca, G. (2016). In vitro antifungal activity of lactic acid bacteria against mycotoxigenic fungi and their application in loaf bread shelf life improvement. *Food Control*, *67*, 273–277.

Saladino, F., Manyes, L., Luciano, F. B., Mañes, J., Fernandez-Franzon, M., & Meca, G. (2016). Bioactive compounds from mustard flours for the control of patulin production in wheat tortillas. *LWT - Food Science and Technology*, *66*, 101–107.

Saladino, F., Quiles, J. M., Luciano, F. B., Mañes, J., Fernández-Franzón, M., & Meca, G. (2017). Shelf life improvement of the loaf bread using allyl, phenyl

and benzyl isothiocyanates against Aspergillus parasiticus. *LWT*, *78*, 208–214.

Santonicola, S., Ibarra, V. G., Sendón, R., Mercogliano, R., & de Quirós, A. R. B. (2017). Antimicrobial films based on chitosan and methylcellulose containing natamycin for active packaging applications. *Coatings*, *7*(10).

Santos, A. P., Lopes, M. C., Limberger, R. P., Apel, M. A., Henriques, A. T., & Moreno, P. R. H. (2004). Analysis of the volatile oil from Pilocarpus pennatifolius Lemmaire (Rutaceae) leaves by GC-MS. *Flavour and Fragrance Journal*, *19*(4), 325–326.

Santos, J. C. P., Sousa, R. C. S., Otoni, C. G., Moraes, A. R. F., Souza, V. G. L., Medeiros, E. A. A., Espitia, P. J. P., Pires, A. C. S., Coimbra, J. S. R., & Soares, N. F. F. (2018). Nisin and other antimicrobial peptides: Production, mechanisms of action, and application in active food packaging. *Innovative Food Science & Emerging Technologies*, *48*, 179–194.

Schmidt, M., Lynch, K. M., Zannini, E., & Arendt, E. K. (2018). Fundamental study on the improvement of the antifungal activity of Lactobacillus reuteri R29 through increased production of phenyllactic acid and reuterin. *Food Control*, *88*, 139–148.

Schmidt, M., Zannini, E., Lynch, K. M., & Arendt, E. K. (2018). Novel approaches for chemical and microbiological shelf life extension of cereal crops. *Critical Reviews in Food Science and Nutrition*, *59*(21), 3395–3419.

Schnürer, J., & Magnusson, J. (2005). Antifungal lactic acid bacteria as biopreservatives. *Trends in Food Science and Technology*, *16*(1–3), 70–78.

Sehwag, S., & Das, M. (2015). A brief overview: Present status on utilization of mustard oil and cake. *Indian Journal of Traditional Knowledge*, *14*(2), 244–250.

Sengun, I., Yaman, D., & Gonul, S. (2008). Mycotoxins and mould contamination in cheese: a review. *World Mycotoxin Journal*, *1*(3), 291–298.

Seo, H. S., Bang, J., Kim, H., Beuchat, L. R., Cho, S. Y., & Ryu, J. H. (2012). Development of an antimicrobial sachet containing encapsulated allyl isothiocyanate to inactivate Escherichia coli O157:H7 on spinach leaves. *International Journal of Food Microbiology*, *159*(2), 136–143.

Shafi, P. M., Nambiar, G. M. K., Clery, R. A., Sarma, Y. R., & Veena, S. S. (2011). Composition and Antifungal Activity of the Oil of Artemisia nilagirica (Clarke) Pamp. *Journal of Essential Oil Research*, *16*(4), 377–379.

Sharma, A, Kumar, V., Kanwar, M. K., Thukral, & Bhardwaj, R. (2017). Phytochemical profiling of the leaves of Brassica juncea L. using GC-MS. *International Food Research Journal*, *24*(2), 547–551.

Sharma, A., Shukla, A., Attri, K., Kumar, M., Kumar, P., Suttee, A., Singh, G., Barnwal, R. P., & Singla, N. (2020). Global trends in pesticides: A looming threat and viable alternatives. *Ecotoxicology and Environmental Safety*, *201*, 110812.

Sharma, A., Rai, P. K., & Prasad, S. (2018). GC–MS detection and determination of major volatile compounds in Brassica juncea L. leaves and seeds. *Microchemical Journal*, *138*, 488–493.

Sharma, S., Barkauskaite, S., Jaiswal, A. K., & Jaiswal, S. (2021). Essential oils as additives in active food packaging. *Food Chemistry*, *343*, 128403.

Shen, Q., Cheng, H., Pu, Y., Re, S., Hu, L., Chen, J., Ye, X., & Liu, D. (2018). Characterization of volatile compounds in pickled and dried mustard (Brassica juncea, Coss.) using optimal HS-SPME-GC-MS. *CyTA - Journal of Food*, *16*(1), 331–339.

Siedler, S., Balti, R., & Neves, A. R. (2019). Bioprotective mechanisms of lactic acid bacteria against fungal spoilage of food. *Current Opinion in Biotechnology*, *56*, 138–146.

Silva, M. M., & Lidon, F. C. (2016). Food preservatives - An overview on applications and side effects. *Emirates Journal of Food and Agriculture*, *28*(6), 366–373.

Siroli, L., Patrignani, F., Serrazanetti, D. I., Gardini, F., & Lanciotti, R. (2015). Innovative strategies based on the use of bio-control agents to improve the safety, shelf-life and quality of minimally processed fruits and vegetables. *Trends in Food Science & Technology*, *46*(2), 302–310.

Skaltsa, H. D., Mavrommati, A., & Constantinidis, T. (2001). A chemotaxonomic investigation of volatile constituents in Stachys subsect. Swainsonianeae (Labiatae). *Phytochemistry*, *57*(2), 235–244.

Škrbić, B., Živančev, J., Durišić-Mladenović, N., & Godula, M. (2012). Principal mycotoxins in wheat flour from the Serbian market: Levels and assessment of the exposure by wheat-based products. *Food Control*, *25*(1), 389–396.

Smith, J. P., Daifas, D. P., El-Khoury, W., Koukoutsis, J., & El-Khoury, A. (2004). Shelf life and safety concerns of bakery products--a review. *Critical Reviews in Food Science and Nutrition*, *44*(1), 19–55.

Soltani Firouz, M., Mohi-Alden, K., & Omid, M. (2021). A critical review on intelligent and active packaging in the food industry: Research and development. *Food Research International*, *141*, 110113.

Sotelo, T., Lema, M., Soengas, P., Cartea, M. E., & Velasco, P. (2015). In vitro activity of Glucosinolates and their degradation products against Brassica-pathogenic bacteria and fungi. *Applied and Environmental Microbiology*, *81*(1), 432–440.

Speranza, B., Bevilacqua, A., Corbo, M. R., & Sinigaglia, M. (2017). Safety evaluation of starter cultures. *Starter Cultures in Food Production*, 101–128.

Stark, J., & Tan, H. S. (2003). Natamycin. *Food Preservatives*, 179–195.

Subudhi, B. B., & Bhoi, A. (2014). Antioxidative effects of Brassica juncea and Moringa oliefera prepared by different processing methods. *Journal of Food Science and Technology*, *51*(4), 790.

Sun, J., Xiao, Z., Lin, L. Z., Lester, G. E., Wang, Q., Harnly, J. M., & Chen, P. (2013). Profiling Polyphenols in Five Brassica species Microgreens by UHPLC-PDA-ESI/HRMSn. *Journal of Agricultural and Food Chemistry*, *61*(46), 10960.

Sun, Y., Wang, Y., Xu, Y., Chen, T., Li, B., Zhang, Z., & Tian, S. (2021). Application and mechanism of benzyl-isothiocyanate, a natural antimicrobial agent from cruciferous vegetables, in controlling postharvest decay of strawberry. *Postharvest Biology and Technology*, *180*, 111604.

Tajkarimi, M. M., Ibrahim, S. A., & Cliver, D. O. (2010). Antimicrobial herb and spice compounds in food. *Food Control*, *21*(9), 1199–1218.

Te Welscher, Yvonne M., Ten Napel, H. H., Balagué, M. M., Souza, C. M., Riezman, H., De Kruijff, B., & Breukink, E. (2008). Natamycin blocks fungal growth by binding specifically to ergosterol without permeabilizing the membrane. *Journal of Biological Chemistry*, *283*(10), 6393–6401.

Te Welscher, Yvonne M., Van Leeuwen, M. R., Dijksterhuis, J., De Kruijff, B., Eitzen, G., & Breukink, E. (2010). Natamycin inhibits vacuole fusion at the priming phase via a specific interaction with ergosterol. *Antimicrobial Agents and Chemotherapy*, *54*(6), 2618–2625.

Te Welscher, Yvonne Maria, Van Leeuwen, M. R., De Kruijff, B., Dijksterhuis, J., & Breukink, E. (2012). Polyene antibiotic that inhibits membrane transport proteins. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(28), 11156–11159.

Teixeira, G. H. de A., Ma, S., Stewart, A. C., Wang, H., & O'Keefe, S. F. (2019).

Natamycin photostability in acidified green tea beverage is dependent on mycosporine-like amino acids and epigallocatechin gallate interaction. *LWT*, *116*, 108572

Thanushree, M. P., Sailendri, D., Yoha, K. S., Moses, J. A., & Anandharamakrishnan, C. (2019). Mycotoxin contamination in food: An exposition on spices. *Trends in Food Science & Technology*, *93*, 69–80.

Thiyam, U., Stöckmann, H., Zum Felde, T., & Schwarz, K. (2006). Antioxidative effect of the main sinapic acid derivatives from rapeseed and mustard oil by-products. *European Journal of Lipid Science and Technology*, *108*(3), 239–248.

Tian, Y., & Deng, F. (2020). Phytochemistry and biological activity of mustard (Brassica juncea): a review. *CYTA - Journal of Food*, *18*(1), 704–718.

Torrijos, R., Nazareth, T. de M., Quiles, J. M., Mañes, J., & Meca, G. (2021). Application of White Mustard Bran and Flour on Bread as Natural Preservative Agents. *Foods*, *10*(2), 431.

Tracz, B. L., Bordin, K., Nazareth, T. de M., Costa, L. B., Macedo, R. E. F. de, Meca, G., & Luciano, F. B. (2017). Assessment of allyl isothiocyanate as a fumigant to avoid mycotoxin production during corn storage. *LWT*, *75*, 692–696.

Trajkovska Petkoska, A., Daniloski, D., D'Cunha, N. M., Naumovski, N., & Broach, A. T. (2021). Edible packaging: Sustainable solutions and novel trends in food packaging. *Food Research International*, *140*, 109981.

Traka, M., & Mithen, R. (2009). Glucosinolates, isothiocyanates and human health. *Phytochemistry Reviews*, *8*(1), 269–282.

Tran, T. M., Ameye, M., Phan, L. T.-K., Devlieghere, F., De Saeger, S., Eeckhout, M., & Audenaert, K. (2021). Post-harvest contamination of maize by Fusarium verticillioides and fumonisins linked to traditional harvest and post-harvest practices: A case study of small-holder farms in Vietnam. *International Journal of Food Microbiology*, *339*, 109022.

Tsao, R., Yu, Q., Friesen, I., Potter, J., & Chiba, M. (2000). Factors affecting the dissolution and degradation of oriental mustard-derived sinigrin and allyl isothiocyanate in aqueous media. *Journal of Agricultural and Food Chemistry*, *48*(5), 1898–1902.

Vaclavikova, M., Malachova, A., Veprikova, Z., Dzuman, Z., Zachariasova, M., & Hajslova, J. (2013). 'Emerging' mycotoxins in cereals processing chains: Changes of enniatins during beer and bread making. *Food Chemistry*, *136*(2),

750–757.

Valdés, A., Ramos, M., Beltrán, A., Jiménez, A., & Garrigós, M. C. (2017). State of the Art of Antimicrobial Edible Coatings for Food Packaging Applications. *Coatings*, *7*(4), 56.

Valerio, F., Di Biase, M., Lattanzio, V. M. T., & Lavermicocca, P. (2016). Improvement of the antifungal activity of lactic acid bacteria by addition to the growth medium of phenylpyruvic acid, a precursor of phenyllactic acid. *International Journal of Food Microbiology*, *222*, 1–7.

van der Ven, L. T. M., Rorije, E., Sprong, R. C., Zink, D., Derr, R., Hendriks, G., Loo, L.-H., & Luijten, M. (2020). A Case Study with Triazole Fungicides to Explore Practical Application of Next-Generation Hazard Assessment Methods for Human Health. *Chemical Research in Toxicology*, *33*(3), 834–848.

Van Eylen, D., Indrawati, Hendrickx, M., & Van Loey, A. (2006). Temperature and pressure stability of mustard seed (Sinapis alba L.) myrosinase. *Food Chemistry*, *97*(2), 263–271.

Van Eylen, D., Oey, I., Hendrickx, M., & Van Loey, A. (2007). Kinetics of the stability of broccoli (Brassica oleracea Cv. Italica) myrosinase and isothiocyanates in broccoli juice during pressure/temperature treatments. *Journal of Agricultural and Food Chemistry*, *55*(6), 2163–2170.

Verkerk, R., Schreiner, M., Krumbein, A., Ciska, E., Holst, B., Rowland, I., de Schrijver, R., Hansen, M., Gerhäuser, C., Mithen, R., & Dekker, M. (2009). Glucosinolates in Brassica vegetables: The influence of the food supply chain on intake, bioavailability and human health. *Molecular Nutrition and Food Research*, *53*(S2), S219–S265.

Vidal, A., Marín, S., Ramos, A. J., Cano-Sancho, G., & Sanchis, V. (2013). Determination of aflatoxins, deoxynivalenol, ochratoxin A and zearalenone in wheat and oat based bran supplements sold in the Spanish market. *Food and Chemical Toxicology*, *53*, 133–138.

Vig, A. P., Rampal, G., Thind, T. S., & Arora, S. (2009). Bio-protective effects of glucosinolates – A review. *LWT - Food Science and Technology*, *42*(10), 1561–1572.

Wang, F., Saito, S., Michailides, T. J., & Xiao, C. L. (2021). Postharvest use of natamycin to control Alternaria rot on blueberry fruit caused by Alternaria alternata and A. arborescens. *Postharvest Biology and Technology*, *172*, 111383.

Wang, H. K., Yan, Y. H., Wang, J. M., Zhang, H. P., & Qi, W. (2012). Production and characterization of antifungal compounds produced by Lactobacillus plantarum IMAU10014. *PLoS ONE*, *7*(1).

Wang, T., Li, Y., Bi, Y., Zhang, M., Zhang, T., Zheng, X., Dong, Y., & Huang, Y. (2020). Benzyl isothiocyanate fumigation inhibits growth, membrane integrity and mycotoxin production in: Alternaria alternata. *RSC Advances*, *10*(3), 1829–1837.

Wang, Z., Fingas, M., & Li, K. (1994). Fractionation of a Light Crude Oil and Identification and Quantitation of Aliphatic, Aromatic, and Biomarker Compounds by GC-FID and GC-MS, Part II. *Journal of Chromatographic Science*, *32*(9), 367–382.

Wei, S., Xiao, X., Wei, L., Li, L., Li, G., Liu, F., Xie, J., Yu, J., & Zhong, Y. (2021). Development and comprehensive HS-SPME/GC–MS analysis optimization, comparison, and evaluation of different cabbage cultivars (Brassica oleracea L. var. capitata L.) volatile components. *Food Chemistry*, *340*, 128166.

Wieczyńska, J., & Cavoski, I. (2018). Antimicrobial, antioxidant and sensory features of eugenol, carvacrol and trans-anethole in active packaging for organic ready-to-eat iceberg lettuce. *Food Chemistry*, *259*, 251–260.

Winther, M., & Nielsen, P. V. (2006). Active Packaging of Cheese with Allyl Isothiocyanate, an Alternative to Modified Atmosphere Packaging. *Journal of Food Protection*, *69*(10), 2430–2435.

Wu, H., Zhang, X., Zhang, G. A., Zeng, S. Y., & Lin, K. C. (2011). Antifungal Vapour-phase Activity of a Combination of Allyl Isothiocyanate and Ethyl Isothiocyanate Against Botrytis cinerea and Penicillium expansum Infection on Apples. *Journal of Phytopathology*, *159*(6), 450–455.

Wyrwa, J., & Barska, A. (2017). Innovations in the food packaging market: active packaging. *European Food Research and Technology*, *243*(10), 1681–1692.

Xu, X., Van Stee, L. L. P., Williams, J., Beens, J., Adahchour, M., Vreuls, R. J. J., Brinkman, U. A. T., & Lelieveld, J. (2003). Comprehensive two-dimensional gas chromatography (GC×GC) measurements of volatile organic compounds in the atmosphere. *Atmospheric Chemistry and Physics*, *3*(3), 665–682.

Yang, B., Li, L., Geng, H., Zhang, C., Wang, G., Yang, S., Gao, S., Zhao, Y., & Xing, F. (2021). Inhibitory effect of allyl and benzyl isothiocyanates on ochratoxin a producing fungi in grape and maize. *Food Microbiology*, *100*.

Yang, X., Gao, J., Liu, Q., & Yang, D. (2019). Co-occurrence of mycotoxins in maize

and maize-derived food in China and estimation of dietary intake. *Food Additives & Contaminants: Part B*, *12*(2), 124–134.

Yildrim, M., Güleç, F., Bayram, M., & Yildrim, Z. (2006). Properties of kashar cheese coated with casein as a carrier of natamycin. *Italian Journal of Food Science, 18*(2).

Žabka, M., & Pavela, R. (2018). Effectiveness of environmentally safe food additives and food supplements in an in vitro growth inhibition of significant Fusarium, Aspergillus and Penicillium species. *Plant Protection Science*, *54*(3), 163–173.

Zaikin, V. G., & Borisov, R. S. (2002). Chromatographic-mass spectrometric analysis of fischer-tropsch synthesis products. *Journal of Analytical Chemistry*, *57*(6), 544–551.

Zakhia-Rhozis, N., Catalá, A., Soriano, J. M. (2007). Micotoxinas en alimentos. Trazabilidad y descontaminación/detoxificación de las micotoxinas. In: J. M. Soriano (Ed.) *Micotoxinas en alimentos*. Díaz de Santos, Spain.

Želonková, K., Havadej, S., Verebová, V., Holečková, B., Uličný, J., & Staničová, J. (2019). Fungicide Tebuconazole Influences the Structure of Human Serum Albumin Molecule. *Molecules*, *24*(17), 3190.

Zeng, Y. X., Zhao, C. X., Liang, Y. Z., Yang, H., Fang, H. Z., Yi, L. Z., & Zeng, Z. Da. (2007). Comparative analysis of volatile components from Clematis species growing in China. *Analytica Chimica Acta*, *595*(1-2), 328–339.

Zhang, M., Li, Y., Bi, Y., Wang, T., Dong, Y., Yang, Q., & Zhang, T. (2020). 2-Phenylethyl Isothiocyanate Exerts Antifungal Activity against Alternaria alternata by Affecting Membrane Integrity and Mycotoxin Production. *Toxins*, *12*(2).

Zhao, D., Tang, J., & Ding, X. (2007). Analysis of volatile components during potherb mustard (Brassica juncea, Coss.) pickle fermentation using SPME–GC-MS. *LWT - Food Science and Technology*, *40*(3), 439–447.

Zheng, J., Wittouck, S., Salvetti, E., Franz, C. M. A. P., Harris, H. M. B., Mattarelli, P., O'toole, P. W., Pot, B., Vandamme, P., Walter, J., Watanabe, K., Wuyts, S., Felis, G. E., Gänzle, M. G., & Lebeer, S. (2020). A taxonomic note on the genus Lactobacillus: Description of 23 novel genera, emended description of the genus Lactobacillus beijerinck 1901, and union of Lactobacillaceae and Leuconostocaceae. *International Journal of Systematic and Evolutionary Microbiology*, *70*(4), 2782–2858.

Zheng, X., Shi, X., & Wang, B. (2021). A Review on the General Cheese Processing Technology, Flavor Biochemical Pathways and the Influence of Yeasts in Cheese. *Frontiers in Microbiology*, *12*, 2186.

Annex

Anexo

molecules

MDPI

Article

Development of a Bioactive Sauce Based on Oriental Mustard Flour with Antifungal Properties for Pita Bread Shelf Life Improvement

Raquel Torrijos †, Tiago M. Nazareth †, Julia Pérez, Jordi Mañes and Giuseppe Meca *

Laboratory of Food Chemistry and Toxicology, Faculty of Pharmacy, University of Valencia, Av. Vicent Andrés Estellés s/n, 46100 Burjassot, Spain; raquel.torrijos@uv.es (R.T.); demena@alumni.uv.es (T.M.N.); jupea2@alumni.uv.es (J.P.); jorge.manes@uv.es (J.M.)

* Correspondence: giuseppe.meca@uv.es; Tel.: +34963544959; Fax: +34963544954

† Authors have contributed equally to the study and should be considered co-first authors.

Academic Editor: Chiara Dall'Asta

Received: 11 February 2019; Accepted: 12 March 2019; Published: 14 March 2019

check for updates

Abstract: Ochratoxin A (OTA) is a mycotoxin produced in the secondary metabolism of fungus belonging to the genus *Aspergillus* and *Penicillium*. In this study, the employment of oriental mustard flour (OMF) as an ingredient in a packaged sauce was evaluated for the generation in situ of the antimicrobial compound allyl isothiocyanate (AITC) in order to preserve pita bread contaminated with *Penicillium verrucosum* VTT D-01847, an OTA producer, in an active packaging system. Four different concentrations (8, 16, 33 and 50 mg/g) were tested. Mycelium formation, mycotoxin production, AITC absorbed by the food matrix, and volatilization kinetics were studied for each concentration. The results obtained were compared with bread treated with the commercial additive calcium propionate (E-282). The results showed a shelf life increase of two and three days with the employment of 33 and 50 mg/g of OMF, with a significant reduction of the fungal population (3.1 and 5.7 logs, respectively) in comparison with the control experiment. The use of 16 and 33 mg/g of OMF in the sauce formulation decreased the concentration of OTA in the bread samples while no OTA production was detected employing 50 mg/g of OMF.

Keywords: active packaging; antifungal properties; AITC; bread; shelf life; OTA

1. Introduction

Bread is a staple food consumed around the world and, like other perishable products, is susceptible to fungal contamination. Therefore, the fungal spoilage is a concern on bakery industry, representing a significant source of economic losses and a potential risk to human health due to the production of mycotoxin by toxigenic fungi, mainly from genus *Aspergillus* and *Penicillium* [1]. Bread possess a relatively high water activity (a_w 0.94–0.97) with a pH ranging from approximately 7 to 8.6 [2]. These properties are favorable for the germination and growth of a wide range of molds. The loaves of bread have a higher probability of fungal growth since they are commonly sliced. This process increases the surface area for microbial contamination; moreover, the slicing machine can be a vector of spoilage agents [3].

Microbial spoilage reduces the shelf life of food and compromises the safety of consumers with the consequent economic loss for the industry. In this context, synthetic additives are widely used in the food industry and play an important role in the preservation of food quality as well as inhibiting the growth of spoilage and pathogenic microorganisms [4]. Nowadays, consumers demand a reduction of synthetic additives in food due to the concern of the effect of these substances on health. For this reason, the increment in the food of antimicrobial substances from natural sources may be an alternative to increase the shelf life and safety of products, satisfying the consumer requirements [5].

foods

MDPI

Article

Application of White Mustard Bran and Flour on Bread as Natural Preservative Agents

Raquel Torrijos, Tiago de Melo Nazareth *, Juan Manuel Quiles, Jordi Mañes and Giuseppe Meca

Department of Food Chemistry and Toxicology, Faculty of Pharmacy, University of Valencia, Ave. Vicent Andrés Estellés s/n, 46100 Burjassot, Spain; raquel.torrijos@uves (R.T.); juan.quiles@uves (J.M.Q.); jorge.manes@uves (J.M.); giuseppe.meca@uves (G.M.)

* Correspondence: tiago@uv.es; Tel: +34-963-544-959; Fax: +34-963-549-54

Abstract: In this study, the antifungal activity of white mustard bran (MB), a by-product of mustard (*Sinapis alba*) milling, and white mustard seed flour (MF) was tested against mycotoxigenic fungi in the agar diffusion method. The results obtained were posteriorly confirmed in a quantitative test, determining the minimum concentration of extract that inhibits the fungal growth (MIC) and the minimum concentration with fungicidal activity (MFC). Since MF demonstrated no antifungal activity, the MB was stored under different temperature conditions and storage time to determine its antifungal stability. Finally, an in situ assay was carried out, applying the MB as a natural ingredient into the dough to avoid *P. commune* CECT 20767 growth and increase the bread shelf life. The results demonstrated that the antifungal activity of MB was dose-dependent. The higher assayed dose of MB (10 g/kg) reduced the fungal population in 4.20 Log CFU/g regarding the control group. Moreover, the shelf life was extended four days compared to the control, equaling its effectiveness with the synthetic preservative sodium propionate (E-281). Therefore, MB could be an alternative to chemical additives in bread formulations since it satisfies consumer requirements. Also, the formulation of bread with MB valorizes this by-product generated during mustard seed milling, thereby helping the industry move forward sustainably by reducing environmental impact.

Keywords: mustard bran; by-product; antifungal; bakery products; shelf-life; *Sinapis alba*; mustard flour; food safety; mycotoxigenic fungi; mycotoxins

Citation: Torrijos, R.; Nazareth, T.d.M.; Quiles, J.M.; Mañes, J.; Meca, G. Application of White Mustard Bran and Flour on Bread as Natural Preservative Agents. *Foods* **2021**, *10*, 431. https://doi.org/10.3390/foods10020431

Academic Editor: Severino Zara and Giacomo Zara

Received: 30 December 2020
Accepted: 9 February 2021
Published: 16 February 2021

1. Introduction

Bakery products are subject to various spoilage problems, such as chemical, physical, and microbial [1]. Bacteria, fungi, and yeast can cause spoilage of bread due to its relatively high-water activity and alkaline pH. These properties are suitable for the growth of a wide range of fungal species. Indeed, fungal spoilage is the primary concern in the bakery industry. Contamination occurs predominantly after baking, by fungal spores from the environment that settle on food [2]. The genera *Penicillium* and *Aspergillus* are the most common fungi responsible for the deterioration of bread [3].

In addition to the fungal spoilage, these microorganisms can also produce mycotoxins [4–6]. Mycotoxins are highly toxic metabolites produced by various fungal species in certain foods, including bread. Among them, Zearalenone, Ochratoxin A, and Aflatoxin B1 are mycotoxin with significant occurrence in wheat and wheat products worldwide [7–11]. These molecules are of concern to human health because of their toxicological properties and the capacity to maintain food even after thermal processing. For instance, Aflatoxin B1, Ochratoxin A, and Zearalenone have been associated with hepatotoxicity, nephrotoxicity, and hyperestrogenism [12,13]. Besides, based on recent reports, mycotoxin prevalence could be as high as 60–80% in food [4,14].

The consumption of bakery products has changed in recent decades due to the growing alert of consumers about health and environmental issues that have led to an increase in demand for whole food and food products without chemical additives or preservatives [15].

toxins

MDPI

Article

Use of Mustard Extracts Fermented by Lactic Acid Bacteria to Mitigate the Production of Fumonisin B_1 and B_2 by *Fusarium verticillioides* in Corn Ears

Raquel Torrijos, Tiago de Melo Nazareth *, Pilar Vila-Donat, Jordi Mañes and Giuseppe Meca

Laboratory of Food Chemistry and Toxicology, Faculty of Pharmacy, University of Valencia, Ave. Vicent Andrés Estellés s/n, 46100 Burjassot, Spain; raquel.torrijos@uv.es (R.T.); pilar.vila@uv.es (P.V.-D.); jorge.manes@uv.es (J.M.); giuseppe.meca@uv.es (G.M.)
* Correspondence: tiago@uv.es; Tel.: +34-963544959

Abstract: Corn (*Zea mays*) is a worldwide crop subjected to infection by toxigenic fungi such as *Fusarium verticillioides* during the pre-harvest stage. *Fusarium* contamination can lead to the synthesis of highly toxic mycotoxins, such as Fumonisin B_1 (FB_1) and Fumonisin B_2 (FB_2), which compromises human and animal health. The work aimed to study the antifungal properties of fermented yellow and oriental mustard extracts using nine lactic acid bacteria (LAB) in vitro. Moreover, a chemical characterization of the main phenolic compounds and organic acids were carried out in the extracts. The results highlighted that the yellow mustard, fermented by *Lactiplantibacillus plantarum* strains, avoided the growth of *Fusarium* spp. in vitro, showing Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) values, ranging from 7.8 to 15.6 g/L and 15.6 to 31.3 g/L, respectively. Then, the lyophilized yellow mustard fermented extract by *L. plantarum* TR71 was applied through spray-on corn ears contaminated with *F. verticillioides* to study the antimycotoxigenic activity. After 14 days of incubation, the control contained 14.71 mg/kg of FB_1, while the treatment reduced the content to 1.09 mg/kg (92.6% reduction). Moreover, no FB_2 was observed in the treated samples. The chemical characterization showed that lactic acid, 3-phenyllactic acid, and benzoic acid were the antifungal metabolites quantified in higher concentrations in the yellow mustard fermented extract with *L. plantarum* TR71. The results obtained confirmed the potential application of fermented mustard extracts as a solution to reduce the incidence of mycotoxins in corn ears.

Keywords: yellow mustard; oriental mustard; *Lactiplantibacillus plantarum*; biopreservation; mycotoxin; antifungal activity; *Brassica juncea*; *Sinapis alba*; fungi; antimycotoxigenic

Key Contribution: The yellow mustard extracts fermented by *L. plantarum* strains showed in vitro antifungal activity against toxigenic *Fusarium* strains. Moreover, the employment of yellow mustard fermented extract by *L. plantarum* TR71 was an effective strategy to reduce the incidence of FB_1 and FB_2 in corn ears contaminated by *F. verticillioides* after 14 days of incubation.

check for updates

Citation: Torrijos, R.; de Melo Nazareth, T.; Vila-Donat, P.; Mañes, J.; Meca, G. Use of Mustard Extracts Fermented by Lactic Acid Bacteria to Mitigate the Production of Fumonisin B_1 and B_2 by *Fusarium verticillioides* in Corn Ears. *Toxins* **2022**, *14*, 80. https://doi.org/10.3390/toxins14020080

Received: 21 December 2021
Accepted: 18 January 2022
Published: 21 January 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

1. Introduction

The contamination of food and feedstuffs by mycotoxins currently remains a significant concern in developed countries, and it is estimated that between 5 and 10% of the world's food supply is squandered because of fungal growth [1]. Moreover, depending on the mycotoxin of concern and the analytical method employed, the prevalence of mycotoxins in food grains might be 60–80% [2]. Thus, toxigenic fungi are, perchance, the most significant pathogens worldwide in terms of food safety [3,4].

Corn (*Zea mays*) is subjected to infection by fungi such as *Fusarium verticillioides* and *Aspergillus flavus* throughout the supply chain [5]. The contact of corn kernels with such toxigenic agents not only leads to grain quality diminishment and economic losses but also menaces the health of animals and consumers who are subject to mycotoxin

LWT - Food Science and Technology 154 (2022) 112795

Contents lists available at ScienceDirect

LWT

journal homepage: www.elsevier.com/locate/lwt

Antifungal activity of natamycin and development of an edible film based on hydroxyethylcellulose to avoid *Penicillium* spp. growth on low-moisture mozzarella cheese

Raquel Torrijos, Tiago Melo Nazareth *, Jorge Calpe, Juan Manuel Quiles, Jordi Mañes, Giuseppe Meca

Department of Food Science and Toxicology, Faculty of Pharmacy, University of Valencia, Ave. Vicent Andrés Estellés S/n, 46100, Burjassot, Spain

ARTICLE INFO

Keywords:
Fungal spoilage
Natamycin
Edible film
Hydroxyethylcellulose
Food spoilage

ABSTRACT

This study aimed to investigate natamycin's *in vitro* antifungal activity against *Penicillium* strains and determine its antifungal effect on Mozzarella cheese slices contaminated with *P. commune*. Besides, the residual content of natamycin in the samples was assessed during the storage. Natamycin was either sprayed on the cheese surface or incorporated into an edible hydroxyethylcellulose film at concentrations of 0, 0.25, 0.5, and 1 mg/dm^2. Natamycin showed an antifungal effect against all *Penicillium* strains with MIC values ranging from 1.6 to 3.1 μg/mL and MFC values ranging from 12.5 to 200.0 μg/mL. Moreover, both applied techniques significantly ($p \leq 0.05$) reduced the population of *P. commune* and increased the shelf life of Mozzarella cheese slices in a dose-dependent manner. The higher dose tested (1 mg/dm^2) reduced the fungal population in 5.28 Log CFU/g and 4.15 Log CFU/g for spray and film treatment compared to control, respectively. Furthermore, the samples treated with hydroxyethylcellulose antifungal film showed a lower residual concentration of natamycin on the surface than spray treatment. These findings indicate that natamycin-containing hydroxyethylcellulose films may be used on Mozzarella cheese rather than direct applications, as a biodegradable antifungal system, without sacrificing food safety.

1. Introduction

Low-moisture Mozzarella is a typical Italian stretched-curd cheese made from cow's or buffalo's milk that possesses a high water activity (a_w 0.95) and a pH value slightly acid (from 5.2 to 5.6). The microflora of this product consists of the cultures used for cheese manufacture (natural or selected), mainly *Lactobacillus* and *Streptococcus* species (Ercolini et al., 2012; Marino et al., 2019). However, these physicochemical properties, in addition to the high water activity associated with high moisture content (among 45 and 52%), creates a suitable environment for microbiological spoilage by fungi, yeasts, and aerobic bacteria, even when the product is stored at low temperatures (1–5 °C) (Garnier et al., 2017; Ma et al., 2013).

Penicillium is the genus that mostly contaminates semi-hard cheese under refrigeration conditions (Banjara et al., 2015). The main species described as spoilage agents in this typology of products are *P. commune*, *P. palitans*, *P. roqueforti*, *P. camemberti*, *P. solitum*, *P. nalgiovense*, and *P. verrucosum* (Petruzzi et al., 2017). Fungal contamination is also relevant because some species may produce mycotoxins such as ochratoxin A, penicillic acid, cyclopiazonic acid, and roquefortine C, some of which are highly toxic (Sengun, Yaman, & Gonul, 2008).

In order to avoid fungal spoilage, antifungal compounds are incorporated into the recipe of perishable foods to minimise food spoilage and, therefore, economic losses. Specifically, the most common additives used in cheese manufacture are sorbates and benzoates (Herr, 2011). Another alternative is the direct application of antimicrobial agents by different techniques such as spraying, coating, or dipping to prevent the growth of spoilage fungi in cheese (Lucera et al., 2012).

Abbreviations: ACN, Acetonitrile; FP, Fungal Population; HEC, Hydroxyethylcellulose; MFC, Minimum Fungicidal Concentration; MIC, Minimum Inhibitory Concentration; MCS, Mozzarella Cheese Slices; NTC, Natamycin; PDA, Potato Dextrose Agar; PDB, Potato Dextrose Broth.

* Corresponding author. Department of Food Science and Toxicology, Faculty of Pharmacy, University of València, Ave. Vicent Andrés Estellés s/n, 46100, Burjassot, Valencia, Spain

E-mail addresses: raquel.torrijos@uv.es (R. Torrijos), tiago@uv.es (T.M. Nazareth), jorge.calpe@uv.es (J. Calpe), juan.quiles@uv.es (J.M. Quiles), jorgev.manes@uv.es (J. Mañes), giuseppe.meca@uv.es (G. Meca).

https://doi.org/10.1016/j.lwt.2021.112795
Received 25 June 2021; Received in revised form 11 October 2021; Accepted 7 November 2021
Available online 10 November 2021