VNIVERSITAT DE VALÈNCIA Facultat de Farmàcia

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

TESIS DOCTORAL INTERNACIONAL

CARLOS LUZ MÍNGUEZ

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VNIVERSITAT DE VALÈNCIA

ACTIVIDAD ANTIFÚNGICA Y ANTITOXIGÉNICA *IN VITRO* Y EN ALIMENTOS DE BACTERIAS ÁCIDO LÁCTICAS

ANTIFUNGAL AND ANTITOXYGENIC ACTIVITY *IN VITRO* AND IN FOOD OF LACTIC ACID BACTERIA

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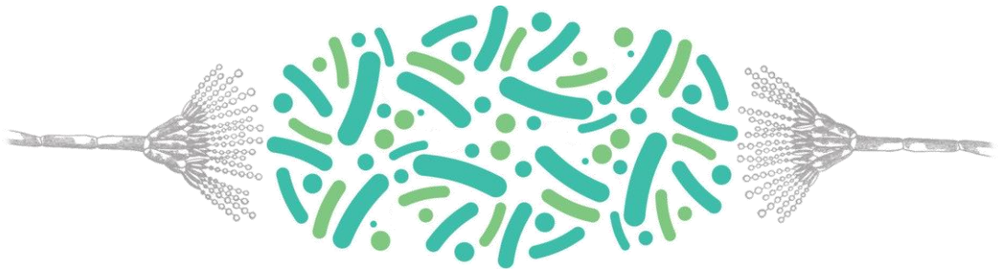

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Programa de Doctorat amb Menció cap a l'Excel·lència en Ciències de l'Alimentació

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Tesi Doctoral Internacional

València, 2020

Presentada per:
Carlos Luz Mínguez

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

VNIVERSITAT DE VALÈNCIA Facultat de Farmàcia

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

CERTIFIQUEN QUE:

El graduat en Ciència i Tecnologia dels Aliments **Carlos Luz Mínguez** ha realitzat baix la seua direcció el treball **"Actividad antifúngica y antitoxigénica *in vitro* y en alimentos de bacterias ácido lácticas"**, 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), 18 de Maig de 2020.

Dr. Jordi Mañes i Vinuesa

Dr. Giuseppe Meca

Aquesta tesi doctoral ha donat lloc a 7 articles, publicats a la següents revistes:

1. *In vitro* antifungal activity of bioactive peptides produced by *Lactobacillus plantarum* against *Aspergillus parasiticus* and *Penicillium expansum*. *LWT-Food Science and Technology, 81 (2017) 128-135. Impact factor: 3.129*

2. Evaluation of biological and antimicrobial properties of freeze-dried whey fermented by different strains of *Lactobacillus plantarum*. *Food & Function, 9 (2018) 3688. Impact factor: 3.241*

3. A natural strategy to improve the shelf life of the loaf bread against toxigenic fungi: The employment of fermented whey powder. *International Journal of Dairy Technology, 73(1) (2020) 88-97. Impact factor: 1.522*

4. Antifungal and antimycotoxigenic activity of hydrolyzed goat whey on *Penicillium* spp: An application as biopreservation agent in pita bread. *LWT-Food Science and Technology, 118 (2020) 108717. Impact factor: 3.714*

5. Antifungal activity and shelf life extension of loaf bread produced with sourdough fermented by *Lactobacillus* strains. *Journal of Food Processing and Preservation, 43(10) (2019), e14126. Impact factor: 1.288*

6. Potential Application of Lactic Acid Bacteria to Reduce Aflatoxin B1 and Fumonisin B1 Occurrence on Corn Kernels and Corn Ears. *Toxins, 12(1) (2020), 21. Impact factor: 3.895*

7. Toxicity reduction of ochratoxin A by lactic acid bacteria. *Food and Chemical Toxicology, 112 (2018) 60–66. Impact factor: 3.775*

El doctorand ha gaudit de la beca predoctoral del Programa "Atracció de talent" UV-INV_PREDOC17F1-534905 de la Universitat de València així com l'ajuda de mobilitat (2019) per la menció internacional del títol de Doctor, la qual es va realitzar a la Escuela de Agraria y Medicina Veterinaria, Universitá degli Studi di Napoli Federico II, Portici (Itàlia), baix la direcció del Dr. Raffaele Romano.

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“Nada en la vida debe temerse,
solo debe ser entendida. Ahora es
el momento de comprender más,
para que podamos temer menos”

Marie Curie (1895–1906)

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LIST OF ABBREVIATIONS

ACN	Acetonitrile
AFB_1	Aflatoxin B_1
AFB_2	Aflatoxin B_2
AFG_1	Aflatoxin G_1
AFG_2	Aflatoxin G_2
AMP	Péptidos antimicrobianos (Antimicrobial peptides)
BAL	Bacterias Ácido Lácticas
BEA	Beauvericin
CE	Comisión Europea
CECT	Colección Española de Cultivos Tipo
CFS	Sobrenadante libre de células (Cell Free Supernatant)
DAD	Diode array detector
DBO	Demanda biológica de oxígeno
DON	Deoxinivalenol
EA	Ethyl acetate
ECA	Enzima convertidora de angiotensina
EFSA	European Food Safety Authority
EPI	Product ion scan
ER	Enhanced resolution scan
FA	Formic acid
FB_1	Fumonisin B_1
FDA	Food & Drug Administration
FLD	Fluorescence detector
GRAS	Generally Recognized As Safe
HGW	Goat whey hydrolisate
IARC	International Agency for Research on Cancer
ISPA	Istituto Scienze delle Produzioni Alimentari
LC	Liquid-chromatography
Lf	Lactoferrina
LIT	Linear ion trap
MALDI	Matrix-Assisted Laser Desorption/Ionization
MC	Moisture content
MeOH	Methanol

MFC	Minimum fungicidal concentration
$MgSO_4$	Magnesium sulphate
MIC	Minimum inhibitory concentration
MRS	Man-Rogosa-Sharpe
MS/MS	Tandem mass spectrometry
MW	Molecular weight
NaCl	Sodium Chloride
OTA	Ochratoxin A
OTA-α	Ochratoxin alpha
PBS	Phosphate buffer saline
PDA	Agar de patata y dextosa (Potato Dextrose Agar)
PDB	Potato dextrose broth
PLA	Ácido feniláctico (Phenyllactic acid)
QPS	Calificación de presunta Seguridad
QqQ	Triple quadrupolo
RAS	Renin-agiotensin system
SDS-PAGE	Electroforesis en gel de poliacrilamida
TFA	Trifluoroacetic acid
TOF	Time of flight
TPC	Total fenolic content
UE	Unión Europea (European Union)
UFC	Unidad formadora de colonias
WHO	World Health Organization
WM	Whey-based medium
WSE	Water soluble extracts
ZEA	Zearalenone
α-La	α-lactalbumin
β-Lg	β-Lactoglobulin

RESUMEN

El desarrollo de hongos en materias primas, alimentos y piensos puede ser responsable de pérdidas económicas considerables por disminuir la calidad y reducir la vida media de todos ellos. Además, algunos hongos pueden producir compuestos potencialmente tóxicos, lo que supone un problema de seguridad alimentaria para los consumidores. La bioconservación es un concepto que se refiere al empleo de microorganismos o sus productos metabólicos en los alimentos, para inhibir el crecimiento o destruir microorganismos no deseados, con el objetivo de mejorar la seguridad, conservar la calidad y extender la vida útil de los productos alimenticios.

La presente Tesis Doctoral tiene por objeto el estudio de la actividad antifúngica de bacterias ácido lácticas (BAL) frente a hongos productores de micotoxinas pertenecientes a los géneros *Aspergillus, Penicillium* y *Fusarium*. Para tal efecto, se realizaron fermentaciones en caldo MRS, suero de leche y masa madre panaria, y se determinaron los valores de concentración mínima fungicida, a efectos de comparar el potencial antifúngico.

En base a estos primeros datos, se planteó la necesidad de desarrollar estrategias basadas en el uso de las BAL más activas como cultivos iniciadores de fermentación en el propio alimento o bien empleando el caldo fermentado libre o no de células como ingrediente, de manera que proporcionaran valores positivos en cuanto a el aumentar la vida útil de los alimentos y la reducción de la presencia de micotoxinas.

Los resultados obtenidos reflejan una eficacia de las BAL y los medios fermentados como conservantes de origen natural frente a *P. expansum* en pan, con valores medios equivalentes o ligeramente superiores a los que presentan los

conservantes clásicos ampliamente utilizados por la industria alimentaria, como es el caso del propianato de calcio.

Por otra parte, la pulverización del caldo MRS fermentado por *L. plantarum* CECT 749 retrasó el crecimiento de *A. flavus* en maíz y *F. verticillioides* en mazorca en varios días frente al blanco. Dicho tratamiento evidenció una muy importante reducción en la producción de AFB_1 por *A. flavus* en maíz, y por *F. verticillioides* en mazorcas de maíz durante el almacenamiento.

Como efectos beneficiosos adicionales, cabe decir que la digestión gastrointestinal de pan contaminado con OTA y previamente tratado con diversos inóculos de BAL mostraron una marcada reducción de la bioaccesibilidad de la micotoxina en los diferentes compartimentos digestivos *in vitro*.

Y que la fermentación del suero de leche con cepas de *L. plantarum* mostraron un aumento de la capacidad antioxidante, un mayor porcentaje de la actividad inhibitoria de la enzima convertidora de la angiotensina y un incremento de la capacidad de quelación del hierro.

SUMMARY

The development of fungi in raw materials, food and feed can be responsible for considerable economic losses by decreasing quality and reducing the shelf life of all of them. In addition, some fungi can produce potentially toxic compounds, posing a food safety problem for consumers. Biopreservation is a concept that refers to the use of microorganisms or their metabolic products in food, to inhibit the growth or destroy unwanted microorganisms, with the aim of improving safety, preserving quality and extending the shelf life of food products.

The purpose of this Doctoral Thesis is to study the antifungal activity of lactic acid bacteria (LAB) against fungi that produce mycotoxins belonging to the genera *Aspergillus*, *Penicillium* and *Fusarium*. For this purpose, fermentations were carried out in MRS broth, whey and panary sourdough, and the minimum fungicidal concentration values were determined, in order to compare the antifungal potential.

Based on these first data, the need to develop strategies based on the use of the most active LAB as fermentation starter cultures in the food itself or using the cell-free or non-cell-free fermented broth as an ingredient was raised, in order to provide positive values in terms of increasing the shelf life of food and reducing the presence of mycotoxins.

The results obtained reflect the efficacy of LAB and the fermented media as preservatives of natural origin against *P. expansum* in bread, with mean values equivalent to or slightly higher than those of the classic preservatives widely used by the food industry, such as case of calcium propionate.

On the other hand, the spraying of the MRS broth fermented by *L. plantarum* CECT 749 delayed the growth of *A. flavus* in corn and *F. verticillioides* on the cob for several days against white. This treatment evidenced a very important

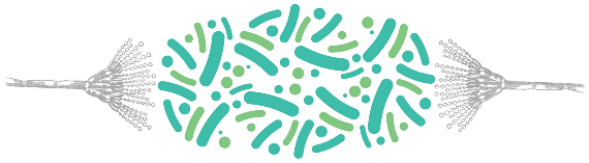
reduction in the production of AFB_1 by *A. flavus* in corn, and by *F. verticillioides* in corn cobs during storage.

As additional beneficial effects, it can be said that the gastrointestinal digestion of bread contaminated with OTA and previously treated with various LAB inoculum showed a marked reduction in the bioavailability of mycotoxin in the different digestive compartments *in vitro*.

And that the fermentation of the whey with *L. plantarum* strains showed an increase in the antioxidant capacity, a higher percentage of the inhibitory activity of the angiotensin-converting enzyme and an increase in the chelation capacity of iron.

1. INTRODUCTION

Introducción



1.1. Contaminación de alimentos por hongos toxigénicos

Los alimentos son susceptibles al deterioro fúngico debido a varios factores, estos pueden clasificarse dependiendo de; i) la naturaleza de la matriz alimentaria (líquida, sólida), su composición centesimal (proteínas, carbohidratos, lípidos, minerales), parámetros biológicos (microbiota natural), físicos y químicos (actividad de agua, pH); ii) manejo durante la cosecha (madurez) y almacenamiento postcosecha (temperatura, humedad, y tiempo); iii) procesos tecnológicos aplicados durante el procesado (tratamiento térmico, secado, procesos de fermentación, uso de aditivos alimentarios) incluido las etapas de limpieza y desinfección; iv) condiciones de almacenamiento después del procesado (tipo, grado y atmósfera de envasado, humedad relativa, temperatura) (Sarrocco y Vannacci, 2018).

En cuanto a la calidad, el impacto de las contaminaciones fúngicas puede alterar las características nutricionales y organolépticas de los alimentos. El crecimiento visible de microorganismos como hongos filamentosos, levaduras y bacterias, conduce a la eliminación de todo el producto a nivel industrial o al rechazo por parte del consumidor. Según la naturaleza del alimento y el tipo de microorganismo contaminante, el impacto visual puede ser menos evidente, en cambio, el metabolismo de estos en los alimentos puede conducir a otros defectos organolépticos como producción de gas, sabores desagradables y cambios de textura (Pitt y Hocking, 2009).

Las frutas y verduras frescas son el nicho oportuno para muchos hongos filamentosos debido a la alta actividad de agua, y periodos largos de transporte y almacenamiento. Los principales géneros descritos en la alteración de esta

tipología de alimentos son *Penicillium*, *Botrytis*, *Monilinia*, *Rhizopus*, *Alternaria*, *Aspergillus*, *Fusarium*, *Geotrichum*, *Gloeosporium*, and *Mucor* (Liu *et al*., 2013).

Los productos lácteos, incluidos los quesos y el yogurt, también son susceptibles al ataque de microorganismos. Los yogures, concretamente, son susceptibles al crecimiento de levaduras debido a su bajo pH, el almacenamiento a temperaturas de refrigeración y la presencia en ocasiones de frutas como ingredientes en su formulación. Las levaduras que alteran el queso con más frecuencia incluyen *Candida* spp., *Yarrowia lipolytica*, *Pichia* spp., *Kluyveromyces marxianus*, *Geothricum candidum* y *Debaryomyces hansenii*. Sin embargo, el deterioro del queso puede ser causado por bacterias y hongos. Los hongos más comunes en los quesos pertenecen a los géneros *Penicillium, Aspergillus*, *Cladosporium*, *Mucor*, *Fusarium* y *Alternaria* (Garnier *et al*., 2016). En los quesos frescos con un pH más alto en comparación a los más curados, las especies psicotróficas gram negativas, como *Pseudomonas* y otros géneros como *Bacillus* spp., y *Clostridium* spp., pueden causar deterioro (Johnson, 2002).

Los causantes del deterioro microbiológico del pan, odenados de menor a mayor importancia son, las levaduras, las bacterias del género *Bacillus*, y los hongos (Cauvain, 2015). Las características intrínsicas de los productos panarios como un pH (5,5-6,0) y la actividad de agua (0,95-0,98) favorecen las contaminaciones fúngicas. Los hongos alterantes de pan más habituales son los de los géneros *Penicillium*, *Aspergillus*, *Cladosporium*, *Mucor*, *Neurospora* y *Rhizopus*. La fuente de contaminación de estos productos por hongos va desde la carga fúngica presente en las materias primas a la contaminación ambiental durante el procesado, enfriado, corte y envasado (Garcia *et al*., 2019).

En lo referido a la inocuidad y seguridad de los alimentos, la presencia y el crecimiento de hongos puede conducir a riesgos para la salud asociados con la presencia de micotoxinas. Estas pueden ser producidas por gran diversidad de hongos pertenecientes a los géneros *Aspergillus*, *Penicillium*, *Fusarium* y *Alternaria*. Son compuestos procedentes del metabolismo secundario de estos microorganismos y presentan un peso molecular medio inferior a 700 Da. En la actualidad, se han descrito más de 400 micotoxinas diferentes, y muchas de ellas son de importancia por su presencia habitual en alimentos a nivel mundial. La temperatura (25-30 ^{o}C) y a actividad de agua (0,80-0,95) son parámetros importantes para el crecimiento de los hongos y la producción de las toxinas (Willey y Prescott, 2009; Bennett y Klich, 2003).

Los alimentos que pueden encontrarse contaminados por micotoxinas son principalmente los de origen vegetal, como cereales, frutos secos, verduras y frutas, aunque también pueden encontrarse en alimentos derivados de ellos como son la cerveza, el vino y los zumos de fruta. En los alimentos de origen animal también se han encontrado micotoxinas debido al consumo de piensos contaminados (Richard, 2009).

La presencia habitual de las micotoxinas en alimentos y piensos a bajas concentraciones y asociadas a casos de exposición crónica, pueden causar un amplio espectro de efectos fisiopatológicos en humanos y animales: carcinogénicos, neurotóxicos, nefrotóxicos, hepatotóxicos, lesiones cardiacas y transtornos gastrointestinales (Marín *et al*., 2013; Escrivá *et al*., 2017) (Tabla 1).

Tabla 1. Principales especies fúngicas productoras de micotoxinas y su clasificación en función de su carcinogenicidad.

Micotoxina	IARC	Principales especies fúngicas productoras
Aflatoxinas B_1, B_2, G_1 y G_2	1	*Aspergillus parasiticus* *Aspergillus flavus*
Beauvericina (BEA)	-	*Fusarium sporotrichioides* *Fusarium poa* *Fusarium langsethiae* *Fusarium sección liseola* *Fusarium avenaceum*
Deoxinivalenol (DON)	3	*Fusarium graminearum* *Fusarium culmorum* *Fusarium cerealis*
Eniatinas A_1, A_2, B_1, B_2	-	*Fusarium avenaceum* *Fusarium tricinctum*
Fumonisinas B_1 y B_2	2B	*Fusarium verticillioides*
Ocratoxina A	2B	*Penicillium verrucosum* *Aspergillus ochraceus*
Patulina	3	*Penicillium expansum,* *Aspergillus clavatus*
Toxinas T-2 y HT-2	3	*Fusarium acuminatum* *Fusarium poae* *Fusarium sporotrichioides* *Fusarium langsethiae*
Zearalenona	3	*Fusarium graminearum* *Fusarium culmorum* *Fusarium equiseti* *Fusarium cerealis* *Fusarium verticillioides* *Fusarium incarnatum*
Alternariol Alternariol metil éter Altenueno Ácido tenuazónico Altertoxinas	-	*Alternaria alternata* *Alternaria arborescens* *Alternaria brassicae* *Alternaria citri* *Alternaria solani*

1.2. Control del crecimiento de hongos toxigénicos por microorganismos

En la actualidad, observamos un aumento en el interés por parte de los consumidores en cuanto a los términos alimentación y salud. En los últimos años, se ha producido como consecuencia un incremento en el mercado de los denominados alimentos funcionales, con propiedades que promueven la salud. Por otro lado, el amplio uso de aditivos alimentarios para la conservación de los alimentos está despertando una incomodidad en un número considerable de consumidores. En cambio, el empleo de aditivos para asegurar la seguridad de los alimentos y la calidad de estos es necesario. Existe una demanda en cuanto a la reducción de uso de aditivos y empleo de técnicas de procesado de alimentos menos agresivas, con el objetivo de producir alimentos mínimamente procesados que reúnan las características organolépticas proporcionadas por el alimento fresco o tradicional (Oliveira *et al*., 2014).

La industria alimentaria presionada por las demandas del mercado, se ve sometida al desarrollo de nuevos productos y procesos de transformación de alimentos para obtener alimentos seguros y que presenten las características deseadas por los consumidores.

La bioconservación es un concepto que se refiere al empleo de microorganismos o sus productos metabólicos en los alimentos, para inhibir el crecimiento o destruir microorganismos no deseados, con el objetivo de mejorar la seguridad alimentaria y extender la vida útil de los productos alimentarios (Crowley *et al*., 2013).

En los últimos años, se han identificado diversas especies microbianas que presentan propiedades antifúngicas. Estas se han aislado de diferentes fuentes naturales, como frutas, verduras, cereales, productos lácteos y panarios, carne y

otros productos relacionados con los alimentos (Gaggia *et al*., 2011). El aislamiento de nuevos cultivos bioprotectores ha sido recientemente extendido a otros ambientes, como las aguas profundas (Wang *et al*., 2015a) y muestras de suelo antártico (Vero *et al*., 2013) para descubrir microorganismos que potencialmente producen nuevos metabolitos antifúngicos.

Numerosos estudios evidencian que la variación intragénero, intraespecie y entre cepas dentro de una misma especie, influye en en la actividad antifúngica y en el espectro de acción. Por ejemplo, un estudio en el que se determinó el espectro antifúngico de un total de 88 cepas de la misma especie (*Lactobacillus plantarum*), solo un 10 % presentaron propiedades antifúngicas frente *Aspergillus* spp. (Russo *et al*., 2017).

Las bacterias ácido lácticas (BAL) son los microorganismos más empleados en la bioconservación de alimentos, principalmente en productos lácteos, productos de panadería, productos cárnicos y vegetales fermentados, granos, semillas, frutas y bebidas alcohólicas como la cerveza y el vino. Dentro de las bacterias, *Bacillus* spp. es un género utilizado comúnmente como agente de control biológico en enfermedades de cultivos agrícolas (Sharma *et al*., 2009). Así mismo, *Trichoderma* spp. es el género fúngico más empleado en biocontrol de plantas, donde ejercen efectos beneficiosos, incluyendo la promoción del crecimiento y la inducción de defensas frente a estreses bióticos y abióticos (Adnan *et al*., 2019). También se emplean microoganismos como levaduras (*Debaryomyces hansenii*) y hongos pertenecientes a *Penicillium* spp. para combatir el deterioro de productos cárnicos fermentados por hongos (Delgado *et al*., 2019).

1.2.1. Bacterias Ácido Lácticas

Las BAL son un grupo grande y heterogéneo de bacterias Gram-positivas, catalasa-negativas, no esporuladas, y con forma cocoide o bacilar. Son homofermentativas cuando originan ácido láctico como principal producto metabolico de la fermentación, y heterofermentativas cuando producen otros metabolitos a parte del ácido láctico tales como, ácido acético, dióxido de carbono (CO_2), etanol, ácido fórmico o succínico. Pertenecen al orden de *Lactobacillales*, que incluyen 6 familias, 36 géneros y más de 200 especies (Gänzle, 2015).

Tabla 2a. Aplicaciones de la bioconservación frente a hongos contaminantes de productos panarios.

***Lactobacillus* spp.**	**Fuente de aislamiento**	**Espectro de actividad**	**Referencia**
L. brevis ITM18	Masa madre	*Aspergillus niger*	Di Biase *et al*., 2014
L. amylovorus DSM19280	Cereal	Hongos ambientales	Axel *et al*., 2015
L. plantarum CRL778	Masa de trigo	Hongos ambientales	Gerez *et al*., 2015
L. plantarum UFG 121	no especificado	*Fusarium culmorum, Penicillium chrysogenum, Penicillium expansum, Penicillium roqueforti, Aspergillus flavus*	Russo *et al*., 2017
L. amylovorus DSM19280	Cereal	*Fusarium culmorum*	Axel *et al*., 2016a
L. reuteri R29 *L. brevis* R2 *L. amylovorus* DSM19280	Humano Cerdo Cereal	Hongos ambientales	Axel *et al*., 2016b
L. sakei *L. plantarum* *L.spicheri* O15 *L. reuteri* 5529 *L. brevis* Lu35	Leche	*Cladosporium sphaerospermum, Wallemia sebi, Eurotium repens, Aspergillus niger, Penicillium corylophilum*	Le Lay *et al*., 2016a

Tabla 2b. Aplicaciones de la bioconservación frente a hongos contaminantes de productos lácteos.

***Lactobacillus* spp.**	**Fuente de aislamiento**	**Espectro de actividad**	**Referencia**
L.harbinensis K.V9.3.1Np *L. rhamnosus* K.C8.3.1I *L.paracasei* K.C8.3.1Hc1	Leche de vaca y de cabra	*Debaryomyces hansenii, Kluyveromyces lactis, Kluyveromyces marxianus, Penicillium brevicompactum, Rhodotorula mucilaginosa, Yarrowia lipolytica*	Delavenne *et al*., 2013
L. casei AST18	Productos lácteos Chinos	*Penicillium* sp.	Li *et al*., 2013
L. paracasei DCS302	no especificado	*Penicillium* sp. nov. DCS 1541 y *Penicillium solitum*	Aunsbjerg *et al*., 2015
L. harbinensis K.V9.3.1Np	Leche de vaca	*Yarrowia lipolytica*	Delavenne *et al*., 2015
L. reuteri INIA P57	Heces de cerdo	no determinado	Gómez-Torres *et al*., 2016
L. amylovorus DSM 19280	Cereal	*Penicillium expansum* y hongos ambientales	Lynch *et al*., 2014
L.plantarum	Vegetales y frutas	*Penicillium commune*	Cheong *et al*., 2014
L.rhamnosus A238 *L. rhamnosus* A119	no especificado	*Penicillium chrysogenum*	Fernandez *et al*., 2017

Se encuentran en varios biotipos como microbiota ambiental, vegetal, humana y animal. Son conmunmente utilizadas en la elaboración de un amplio número de alimentos fermentados, donde contribuyen a mejorar la vida útil, propiedades organolépticas y valor nutricional. Las principales BAL en alimentos corresponden a especies pertencientes a los géneros *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Carnobacterium*, *Enterococcus*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* y *Weissella* (Leroy y Vuyst, 2004).

Tabla 2c. Aplicaciones de la bioconservación frente a hongos contaminantes de productos vegetales fermentados y frutas.

***Lactobacillus* spp.**	**Fuente de aislamiento**	**Espectro de actividad**	**Referencia**
L. plantarum B4496 *L. brevis* 207 *L. sanfranciscensis* BB12	Cacao fermentado	*Aspergillus carbonarius, Aspergillus niger, Aspergillus ochraceus*	Ngang *et al*., 2015
L. fermentum YML014	Yuca fermentada	*Penicillium expansum, Aspergillus flavus, Aspergillus niger, Candida albicans*	Adedokun *et al*., 2016
L. helveticus KLDS 1.8701	Lácteo	*Penicillium* sp.	Bian *et al*., 2016
L. paracasei ŁOCK0921	Plantas y humanos	*Alternaria brassicicola, Alternaria alternata, Aspergillus niger, Fusarium lateritium, Geotrichum candidum, Mucor hiemalis*	Lipinska *et al*., 2016
L. plantarum TK9	Arroz fermentado Chino	*Penicillium roqueforti, Penicillium citrinum, Aspergillus fumigatus, Aspergillus flavus, Rhizopus nigricans*	Zhang *et al*., 2016

Las especies más estudiadas por sus propiedades antifúngicas pertenecen a los géneros *Lactobacillus*, *Pediococcus* y *Leuconostoc*. Entre ellas, *Lactobacillus plantarum* es la especie más estudiada y representa aproximadamente un tercio de los informes sobre las BAL. En la literatura podemos encontrar diversas aproximaciones en las que se emplea esta especie para la prevención de la contaminación de los alimentos con hongos (Tabla 2) (Salas *et al*., 2017).

Tabla 2d. Aplicaciones de la bioconservación frente a hongos contaminantes de cereales y semillas.

***Lactobacillus* spp.**	**Fuente de aislamiento**	**Espectro de actividad**	**Referencia**
L. plantarum LR/14	no especificado	*Aspergillus niger, Rhizopus stolonifer, Mucor racemosus, Penicillium chrysogenum*	Gupta y Srivastava, 2014
L.plantarum YML007	Kimchi	*Aspergillus niger*	Ahmad *et al*., 2013
L. plantarum	Granos de kefir	*Aspergillus flavus*	Gamba *et al*., 2016
L. paracasei ŁOCK0921	Plantas y humanos	*Alternaria brassicicola, Alternaria alternata, Aspergillus niger, Fusarium lateritium, Geotrichum candidum, Mucor hiemalis*	Lipinska *et al*., 2016
L. plantarum TK9	Arroz fermentado Chino	*Penicillium roqueforti, Penicillium citrinum, Aspergillus fumigatus, Aspergillus flavus, Rhizopus nigricans*	Zhang *et al*., 2016
L.brevis R2D	Cerdo	no determinado	Axel *et al*., 2014
L. brevis R2D *L. plantarum* FST1.7	Cerdo Cebada	*Fusarium culmorum*	Peyer *et al*., 2016
L. brevis R2D	Cerdo	*Fusarium culmorum*, *Fusarium graminearum*	Peyer *et al*., 2017
L. reuteri R29 *L. amylovorus* DSM19280	Humano Cereal	*Fusarium culmorum*	Oliveira *et al*., 2015

Debido a la larga historia de consumo tradicional seguro de alimentos fermentados como parte de nuestra dieta, las BAL y algunos de sus metabolitos han adquirido la aprobación como GRAS (Generally Recognized As Safe) por la FDA (Food & Drug Administration), y algunas de sus especies están incluidas en la lista de Calificación de presunta Seguridad (QPS) establecida por la EFSA (European Food Safety Authority) (Leuschner *et al*., 2010).

1.3. Factores que influencian en la actividad antifúngica de las BAL

Las BAL con propiedades antifúngicas podría reducir el problema de la contaminación de los alimentos con hongos toxigénicos. Sin embargo, la actividad antifúngica de estas está modulada por un conjunto de parámetros físico-químicos; medio de crecimiento, temperatura, tiempo de incubación, pH y factores nutricionales (Batish *et al*., 1997).

1.3.1. Temperatura y tiempo de incubación

La temperatura y el tiempo de incubación son factores esenciales que modulan el crecimiento de las BAL y afectan significativamente a la cantidad de metabolitos antifúngicos producidos durante la fermentación. Rouse *et al.* (2008) observaron un aumento de la actividad antifúngica empleando tiempo de incubación de 48 h y 72 h. Tiempos superiores de fermentación presentan en ocasiones una reducción de la actividad antifúngica, esta disminución fue asociada a una posible metabolización de los compuestos activos o a una degradación enzimática de los mismos. Muchos autores coinciden en que la temperatura de 30-37 ^{o}C es la más adecuada para el crecimiento de la mayoría de especies de BAL y la producción de metabolitos activos (Adamberg *et al*., 2003).

1.3.2. Medios de cultivo

Encontramos poca información sobre el impacto del medio de cultivo y su composición en la producción de metabolitos antifúngicos por las BAL. Algunos autores han afirmado que esta modula significativamente el metabolismo de las BAL. El caldo de cultivo más empleado para el crecimiento de BAL y el estudio de

sus propiedades antimicrobianas es el medio Man-Rogosa-Sharpe (MRS) (Muhialdin *et al*., 2016). En cambio, la variedad de medios de cultivos empleados para el crecimiento de las BAL y sus diversas modificaciones con diferentes factores nutricionales es muy amplia. El medio Eliker recomendado para el cultivo de *Estreptococcus* y *Lactobacillus*, evidenció ser el mejor para la producción de compuestos antifúngicos por *Lactobacillus lactis* subsp. *Lactis* CHD-28.3 frente a *Aspergillus flavus* IARI, en comparación con los medios M17 y MRS (Roy *et al*., 1996). Otros autores, evideciaron un incremento de la actividad antifúngica del medio de cultivo definido fermentado con BAL adicionando 6,1 mM de ácido fenilpiruvico, precursor de compuestos bioactivos como el ácido feniláctico (PLA, del inglés "*phenyllactic acid*") (Li *et al*., 2007; Valerio *et al*., 2016). Suplementación de los medios de cultivo con aminoácidos como la fenilalanina y la tirosina también mostraron un aumento significativo en la producción de PLA por las BAL durante la fermentación (Valerio *et al*., 2004).

1.3.3. pH

Las condiciones de pH del medio de cultivo modulan en gran medida la producción de compuestos antifúngicos por las BAL. Además, este efecto también está relacionado a su vez con muchos factores, como el sustrato, periodo de incubación y la temperatura. De manera similar a la temperatura, el efecto del cambio de pH en las características de crecimiento entre las diferentes especies de BAL es muy variable. Por otro lado, la tolerancia a la disminución de pH es una propiedad importante de las BAL en el proceso de fermentación, esta le permite seguir fermentando los azúcares del medio e incrementar la producción de metabolitos activos (Adamberg *et al*., 2003).

Lactobacillus lactis subsp. *diacetylactic* presentó la capacidad de producir compuestos antifúngicos en un estrecho rango de pH (5,5-7), aunque la producción máxima se observó a pH 6,8 (Batish *et al*., 1997). Resultados similares se evidenciaron con la cepa *Lactobacillus sanfrancisco* CB1, en este caso con un pH óptimo de 6 (Corsetti *et al*., 1998).

1.4. Compuestos antifúngicos producidos por BAL

Encontramos un gran número de compuestos activos que han sido aislados a partir de BAL y presentan una fuerte actividad antifúngica. La mayoría de estos compuestos identificados presentan un bajo peso molecular. Entre estos compuestos podemos destacar ácidos orgánicos, ácidos fenólicos, peróxido de hidrógeno, ácidos grasos de cadena corta, reuterina, proteínas y péptidos bioactivos (Dalié *et al*., 2010) (Figura 1) Los compuestos antifúngicos producidos por BAL y caracterizados mediante ensayos *in vitro*, así como su actividad se enumeran en la Tabla 3.

Tabla 3. Concentración Mínima Inhibitoria (MIC) de compuestos antifúngicos producidos por BAL frente a hongos contaminantes de alimentos.

Compuesto antifúngico	MIC (mM)	Especie fúngica	Referencia
Diacetilo	0,005	*Penicillium* spp.	Aunsbjerg *et al*., 2015
Peróxido de hidrógeno	200-300	*Penicillium digitatum, Penicillium italicum,* y *Geotrichum candidum*	Cerioni *et al*., 2009
Etanol	434	*Chrysonilia sitophila* y *Hyphopichia burtonii*	Berni *et al*., 2013
Ciclo(L-Phe-L-Pro)	82	*Penicillium roqueforti* y *Aspergillus fumigatus*	Ström *et al*., 2002
Reuterina	1,0-3,3	*Aspergillus* spp. y *Penicillium* spp.	Vimont *et al*., 2019

Tabla 3. Continuación.

Compuesto antifúngico	MIC (mM)	Especie fúngica	Referencia
Ácido Láctico	274-405	*Aspergillus flavus*	Peláez *et al*., 2012
Ácido Acético	38-41	*Penicillium* spp. , *Aspergillus* spp. y *Trichoderma atroviride*	Stratford *et al*., 2009
Ácido Feniláctico	45-60	*Penicillium roqueforti* y *Aspergillus fumigatus*	Ström *et al*., 2002
Ácido propiónico	8,1	*Aspergillus niger, Fusarium graminearum, Penicillium expansum* y *Monilia sitophila*	Corsetti *et al*., 1998
Ácido benzoico	0,8-8	*Penicillium roqueforti* y *Aspergillus fumigatus*	Broberg *et al*., 2007
Ácido 4-hidroxibenzoico	>7,2	*Penicillium roqueforti* y *Aspergillus fumigatus*	Broberg *et al*., 2007
Ácido ferúlico	>0,5	*Penicillium roqueforti* y *Aspergillus fumigatus*	Broberg *et al*., 2007
Vanillic acid	>0,6	*Penicillium roqueforti* y *Aspergillus fumigatus*	Broberg *et al*., 2007

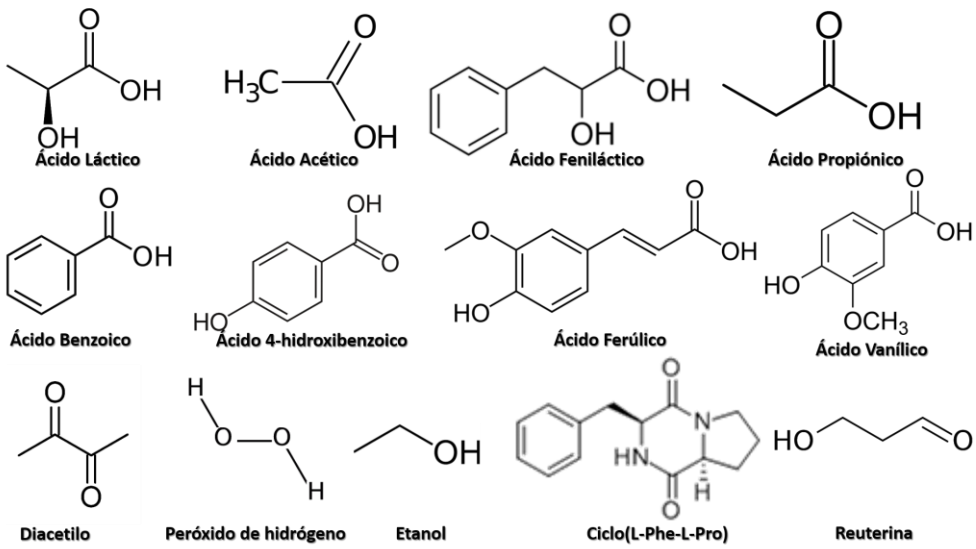


Figura 1. Estructura molecular de los compuesto antifúngicos producidos por BAL.

1.4.1. Ácidos orgánicos

Los ácidos orgánicos presentes en los alimentos son aditivos o productos finales del metabolismo de carbohidratos de las BAL. El ácido láctico y acético son los principales productos de la fermentación de carbohidratos por BAL. Estos ácidos, generalmente reconocidos como agentes seguros para la conservación de alimentos, difunden a través de la membrana de los microorganismos en su forma hidrofóbica no disociada y reducen el pH citoplasmático afectando las actividades metabólicas (Wang *et al*., 2015b) (Figura 2).

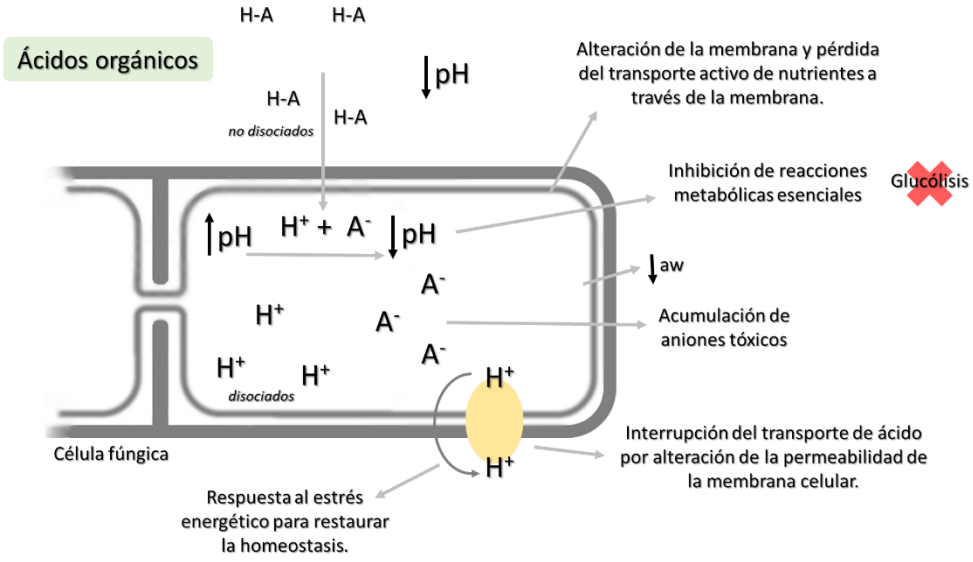


Figura 2. Mecanismo de acción de los ácidos orgánicos.

Se presume que los ácidos orgánicos actúan sobre la membrana plasmática neutralizando su potencial electroquímico y aumentando su permeabilidad, que conduce a la bacteriostasis y eventualmente a la muerte de organismos susceptibles. La misma hipótesis también podría explicar La susceptibilidad de

algunos hongos a los ácidos orgánicos. Esta propiedad fue demostrada para el ácido acético y propiónico (Eklund, 1989). El ácido acético (pKa = 4,76) se describió como más efectivo que el ácido láctico (pKa = 3,88) como inhibidor del crecimiento de hongos (Batish *et al*., 1997). Estos son autorizados como aditivos seguros empleados en la conservación de los alimentos por el Reglamento (UE) nº 1130/2011, de 11 de noviembre de 2011 por el que se establece una lista de aditivos alimentarios autorizados (CE, 2011).

1.4.2. Ácidos fenólicos

Diversos compuestos fenólicos han sido determinados en alimentos o medios fermentados con BAL. Numerosos estudios evidencian diversas propiedades como actividad antioxidante, antifúngica y antimicotoxigénica (Antognoni *et al*., 2019; Guimarães *et al*., 2018). El PLA y su derivado ácido 4-hidroxifeniláctico son los principales ácidos fenólicos producidos por esta familia. En concreto el PLA, ha sido reconocido como un metabolito que repercute en la actividad antifúngica de los medios fermentados por BAL y mejora de la vida útil de los alimentos (Guimarães *et al*., 2018; Bustos *et al*., 2018). Se han descrito otros ácidos fenólicos producidos por BAL durante la fermentación de medio modificado formulado con harina de trigo hidrolizada, tales como ácido succínico, ácido 4-hidroxibenzoico, ácido vanílico, ácido cafeico, ácido p-coumárico, ácido salicílico, ácido ferúlico, ácido benzoico, ácido hidroxicinámico, 1,2-dihidroxibenceno y ácido 3,4-dihidroxihidrocinámico (Le Lay *et al*., 2016b).

1.4.3. Péptidos antimicrobianos

Los péptidos antimicrobianos (AMP, del inglés "*antimicrobial peptides*") son compuestos de origen natural constituidos por un número variable (5-100) aminoácidos unidos a través de enlaces peptídicos (Ali y Dacheng, 2013). Han sido aislados de diversas fuentes, entre las que podemos destacar humanos, plantas, insectos, crustáceos, aves, anfibios y bacterias (Hafeez *et al*., 2014).

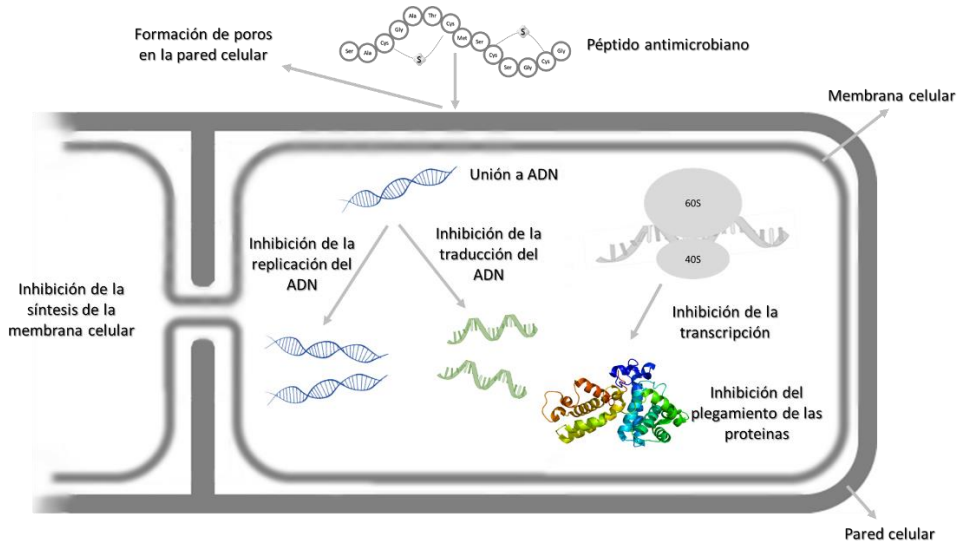


Figura 3. Mecanismo de acción de los AMP.

En general, presentan un extremo hidrófobo y otro hidrófilo otorgándole propiedades catiónicas. La mayoría de los AMP reportados hasta la fecha se pueden clasificar como uno de los siguientes tipos, en función de su estructura secundaria: α-hélice, hoja-β, extendida y bucle. Estos péptidos pueden presentar un amplio espectro de actividad antimicrobiana frente a bacterias Gram-positivas y Gram-negativas, hongos, y protozoos. El mecanismo de acción principal de los

AMP está asociado a su carácter amfipático, ejerce interacciones electroestáticas con los fosfolípidos aniónicos de membrana de los microorganismos. Esta intereacción produce la formación de poros en la pared celular que afectan a su estabilidad. Tambien se describen otros mecanismos como unión a ADN, inhibición de la replicación, transcripción, traducción, plegamiento de las proteínas, y síntesis de la membrana celular (Rai *et al*., 2016) (Figura 3).

Para el estudio de AMP, los procesos como la fermentación con BAL y la hidrólisis enzimática son fundamentales para la producción de estos a partir de matrices proteicas, como por ejemplo productos lácteos. En la Tabla 4 podemos observar algunos AMP obtenidos a partir de proteínas lácteas empleando BAL y enzimas digestivas (Akalin, 2014). En el caso de la fermentación, las BAL hacen uso de su sistema proteolítico para la formación de péptidos más pequeños a partir de proteínas o péptidos más grandes. Este es un sistema complejo que consta de tres componentes principales: proteasas que se encuentran unidas a la pared celular, transportadores específicos que transfieren los oligopéptidos al citoplasma y peptidasas intracelulares que terminan el proceso de hidrólisis de estos oligopéptidos dando lugar a péptidos de bajo peso molecular y aminoácidos libres (Thery *et al*., 2019). Además de los productos lácteos como matriz proteica para la obtención de AMP, también se pueden obtener de diversos procesos fermentativos como la producción de masas panarias (Coda *et al*., 2008).

Las BAL pueden producir péptidos que muestran actividad antifúngica. La cepa *Lactobacillus plantarum* LR/14 produce una serie de péptidos activos contra *Aspergillus niger* y *Penicillium chrysogenum* (Gupta y Srivastava, 2014). Sin embargo, su actividad solo aparece a altas concentraciones (5 mg/mL) (Muhialdin, Hassan, Bakar y Saari, 2016). La mayoría de estos péptidos presentan una buena estabilidad al calor, interesante para resistir los numerosos procesos de

producción alimentaria y ejercer su efecto como conservante después del procesado. En cambio, la degradación de estos péptidos por las proteasas del tracto gastrointestinal, cuando se usa como aditivo alimentario, limitaría su actividad contra la flora intestinal y los posibles efectos adversos para la salud (Thery *et al*., 2019). Estudios de digestión *in vitro* son necesarios para evaluar la actividad biológica de estos AMP a través del tracto gastrointestinal y a nivel celular.

Tabla 4. AMP obtenidos a partir de proteínas lácteas.

Proteína nativa	Fragmento	Hidrólisis	Secuencia peptídica
α-Lactoalbúmina	f(1-5)	Tripsina	EQLTK
α-Lactoalbúmina	f(17-31)	Tripsina	GYGGVSLPEWVCTTF
α-Lactoalbúmina	f(61-68)	Quimotripsina	CKDDQNPH
α-Lactoalbúmina	f(117-121)	Pepsina	KVGIN
β-Lactoglobulina	f(14-18)	Pepsina	KVAGT
β-Lactoglobulina	f(123-125)	Pepsina	VRT
β-Lactoglobulina	f(50-54)	Pepsina	PEGDL
β-Lactoglobulina	f(143-146)	Pepsina	LPMH
α-caseina bovina	f(1-23)	Quimosina	RPKHPIKHQGLPQEVLNENLLRF
α-caseina bovina	f(99-109)	Pepsina	LRLKKYKVPQL
α-caseina bovina	f(21-29)	*Lactobacillus* sp.	IKHQGLPQE
α-caseina bovina	f(30-37)	*Lactobacillus* sp.	VLNENLLR
α-caseina ovina	f(165-170)	Pepsina	LKKISQ
α-caseina ovina	f(165-181)	Pepsina	LKKISQYYQKFAWPQYL
α-caseina ovina	f(184-208)	Pepsina	VDQHQKAMKPWTQPKTNAIPYVRYL
α-caseina ovina	f(203-208)	Pepsina	PYVRYL
κ-caseina bovina	f(28-30)	Pepsina	IQY
κ-caseina bovina	f(162-169)	Pepsina	VQVTSTAV
κ-caseina bovina	f(141-146)	Pepsina	STVATL
κ-caseina bovina	f(18-24)	Pepsina	FSDKIAK

1.5. Disipación de micotoxinas por BAL

Cuando los microorganismos crecen juntos o coexisten en un ambiente, sus actividades fisiológicas, así como el crecimiento se ve influenciado debido a la producción de antagonistas y la competencia por el espacio y los nutrientes. Los hongos no son diferentes, y su crecimiento y potencial para producir micotoxinas están afectados por microorganismos coexistentes como resultado de varias interacciones. Las especies fúngicas producen micotoxinas en respuesta a ciertas señales ambientales o factores relacionados con el estrés, que conducen a la activación de la cascada transcripcional asociada con la modulación de genes productores de micotoxinas. Por ejemplo, el estrés oxidativo y nutricional junto con los factores ambientales como el pH, la temperatura, la actividad del agua (aw) y la luz, influyen en la producción de micotoxinas en muchas especies de hongos (Reverberi *et* al., 2010). Las BAL modulan el entorno de crecimiento de hongos y afectan su capacidad de producir micotoxinas (Briard *et al*., 2016).

1.5.1. Impacto directo en el crecimiento de hongos

La influencia directa de las BAL en la producción de toxinas por los hongos, es en muchas ocasiones debido a la reducción del crecimiento de estos como resultado de la competencia por el espacio y los nutrientes, así como la producción de metabolitos con actividad antifúngica. La producción de micotoxinas se incia al final de la fase de crecimiento fúngico, por lo tanto, la inhibición del crecimiento de los hongos toxigénicos a menudo se considera como la estrategia más efectiva para evitar la producción de las micotoxinas y la presencia de estas en los alimentos (Gomaa *et al*., 2018).

Las micotoxinas son metabolitos segundarios de los hongos que no son necesarios para el crecimiento, pero a menudo se producen en respuesta al estrés una vez están desarrollados. Por lo tanto, el crecimiento de los hongos y la producción de micotoxinas debe considerarse como dos rasgos fisiológicos independientes. Hay muchas especies de hongos donde el crecimiento y la producción de micotoxinas están regulados diferencialmente. Factores como la aw, pH, temperatura, nutrientes, humedad, nivel de inóculo, naturaleza del sustrato y estado fisiológico afectan tanto el crecimiento como la producción de micotoxinas en muchas especies de hongos, siendo así difícil establecer un solo conjunto de condiciones favorables para ambas condiciones fisiológicas (Aldars-García *et al*., 2018).

En relación a esto, muchos trabajos reportan como la acción de los metabolitos producidos por las BAL influencian directamente sobre el crecimiento y producción de micotoxinas. Los ácidos orgánicos evidencian un retraso de estos procesos en forma dependiente de la dosis. Sin embargo, Hassan y Bullerman (2008) informaron que concentraciones subletales de ácidos orgánicos producidos por *Lactobacillus paracasei* subsp. *tolerans* mostraron una reducción significativa del crecimiento de *Fusarium* spp. con un simultáneo aumento de la producción de micotoxinas. Por otro lado, la influencia de los ácidos orgánicos en la producción de micotoxinas también puede correlacionarse con la inhibición del proceso de esporulación de los hongos, ya que, en muchos casos, la inhibición de la esporulación muestra una reducción significativa de la producción de micotoxinas (Calvo *et al*., 2002).

1.5.2. Inhibición de la producción mediante la modificación del medio

El biocontrol de hongos por las BAL a menudo implica modulación del entorno de crecimiento de una manera que dificulta la capacidad fúngica de producir toxinas, independientemente del crecimiento fúngico.

Guimarães *et al*. (2018) evidenció una reducción del 91% en la producción de aflatoxina B_1 (AFB_1) por parte de *Aspergillus flavus* con solo una reducción del crecimiento del 32% bajo la influencia del sobrenadante libre de células (CFS) de *Lactobacillus plantarum* UM55. Este efecto se revirtió cuando se neutralizó el medio, lo que muestra el papel de los ácidos orgánicos en la modulación de la producción de micotoxinas independientemente del crecimiento fúngico. Se sabe muy poco en relación al mecanismo de acción de los ácidos orgánicos sobre la producción de micotoxinas aparte de la dependencia del pH. Además, el ácido láctico puede afectar a la estabilidad de algunas micotoxinas. Aiko *et al*. (2016) informaron sobre la eficiencia de este ácido orgánico en la degradación de hasta un 85% de la AFB_1.

1.5.3. Reducción de las micotoxinas a través de la adsorción

Los componentes de la pared celular de las BAL tienen la propiedad de reducir el contenido de micotoxinas mediante la adsorción. Los grupos funcionales implicados en este proceso, se localizan en la multicapa de peptidoglicano que rodea la membrana citoplásmática, y son polisacáridos, proteínas, ácidos lipoteicoicos y ácidos teicoicos. Sin embargo, los polisacáridos y peptidoglicanos son considerados como los principales componentes de las BAL encargados de la adsorción de las micotoxinas (Chapot-Chartier y Kulakauskas, 2014).

Las diferencias existentes en la composición de la pared celular entre diferentes especies de BAL pueden explicar la variablidad en los rendimientos de adsorción de micotoxinas entre ellas. Este mecanismo de detoxificación depende de diversos factores, como el tipo de medio de crecimiento, estado bacteriano (viable o no), concentración de micotoxinas, recuento bacteriano, temperatura de incubación y pH del medio (Taheur *et al*., 2019). En cambio, no se muestra consenso en la comunidad científica sobre el papel preciso de estos factores en la capacidad de adsorción de micotoxinas por las BAL.

1.5.4. Hidrolisis de las micotoxinas

Dentro de los mecanismos de degradación biológica por BAL de micotoxinas, después de la adsorción, la hidrólisis es el segundo más conocido en alimentos y piensos.

La hidrólisis de las micotoxinas, a diferencia de la adsorción, puede generar posibles metabolitos secundarios con toxicidad variable. En ocasiones el metabolito obtenido de la reacción, como es el caso de la conversión de zearalenona (ZEA) en α-zearalenol, es más tóxico que la micotoxina (Figura 4a). En este caso, la biotransformación de ZEA en sus productos finales no puede considerarse como un proceso completo de detoxificación. No obstante, el otro producto posible de la reacción, el β-zearalenol evidencia menores efectos estrogénicos que α-zearalenol y ZEA (Keller *et al*., 2015). En cambio, algunos estudios evidencian la degradación de otras micotoxinas, como es el caso de la ocratoxina A (OTA), mediante la ruptura de su enlace amida dando lugar a dos metabolitos no tóxicos OTA-α y fenilalanina (Dobritzsch *et al*., 2014) (Figura 4b).

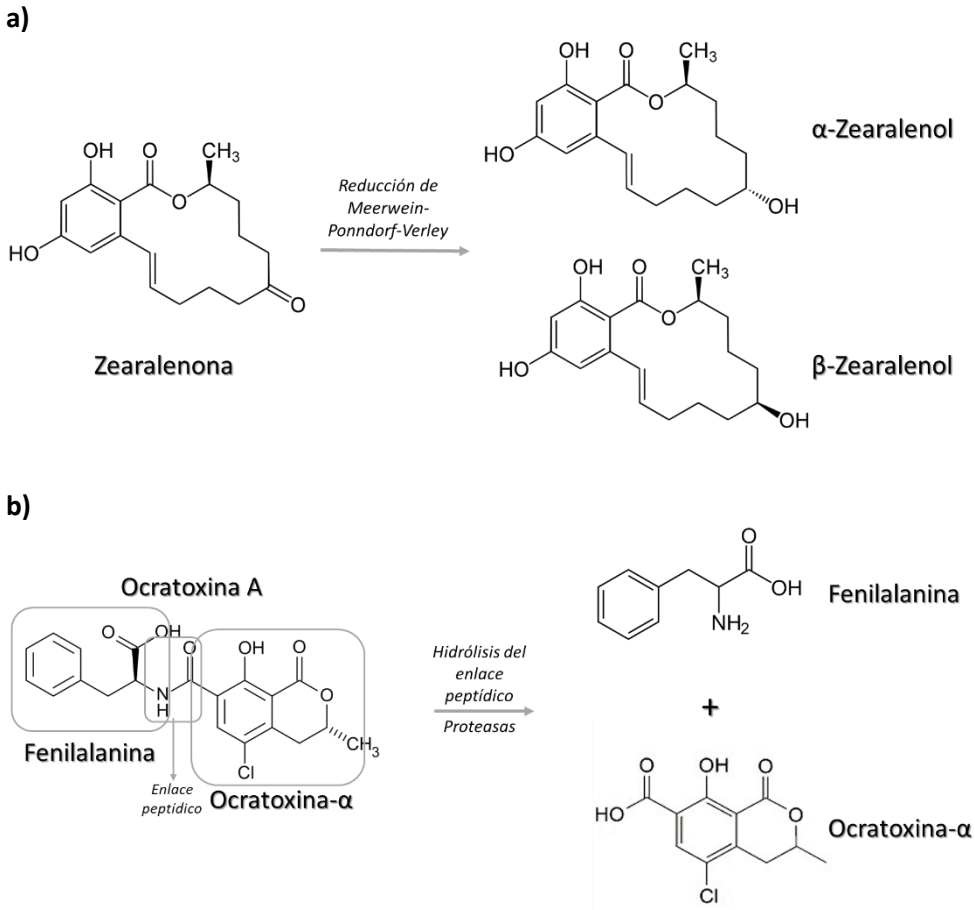


Figura 4. Reacción de biotransformación de la a) zearalenona y la b) ocratoxina A.

1.6. Potencial biotecnológico del suero de leche

El suero de leche corresponde a la fracción líquida obtenida después de la coagulación de las proteínas de la leche durante la producción de quesos. Este producto representa alrededor del 85-90% del volumen de la leche y retiene aproximadamente el 55% de los nutrientes de la misma (Smithers, 2008). Los componentes principales del suero son lactosa, proteínas, lípidos, calcio, fósforo, ácidos orgánicos y vitaminas.

El elevado contenido de materia orgánica está asociado a una alta demanda biológica de oxígeno (DBO), causando el desecho de este subproducto un gran impacto mediaoambiental. Es el contaminante más importante de la industria láctea debido a la DBO y al volumen de producción. Desde el punto de vista biotecnológico, la composición del suero permite el crecimiento de microorganismos, incluidas las BAL. Además, la fermentación del suero produce una reducción de la DBO y permite la obtención de compuestos con un alto valor agregado para la industria alimentaria (Guerra *et al*., 2001).

Otra alternativa para agregar valor a este subproducto, es la hidrólisis de sus proteínas en péptidos más pequeños con bioactividad. Estas reacciones pueden llevarse a cabo mediante el empleo de enzimas digestivas (pepsina, pancreatina, tripsina), proteasas vegetales y microoganismos proteolíticos (Korhonen y Pihlanto, 2006). Los péptidos bioactivos son el foco de varias investigaciones principalmente relacionadas con propiedades antioxidantes, antihipertensivas, y antimicrobianas (Hafeez *et al*., 2014). Los péptidos bioactivos derivados de las proteínas de leche bovina son los más estudiados. Sin embargo, recientemente encontramos una cantidad importante de trabajos que evidencia la importancia de otros tipos de proteínas de origen ovino y caprino, como fuente para la obtención de péptidos bioactivos (Panchal *et al*., 2020; Corrêa *et al*., 2019).

1.7. Aplicación de la bioconservación en alimentos

1.7.1. Cereales

La bioconservación empleando BAL es una alternativa para reducir las infecciones por hongos en cereales antes y después de la cosecha. Las infecciones pueden ocurrir durante el cultivo, la cosecha, el transporte y el almacenamiento.

Los hongos toxigénicos son un problema importante en los cultivos de cereales ya que pueden producir una multitud de metabolitos tóxicos que contaminan las plantas y productos alimenticios. Las micotoxinas son generalmente termoestables (por encima de 100 ^{o}C) y, por lo tanto, puede transferirse a los alimentos, incluso después de los pasos de estabilización microbiana, tales como calentamiento y extrusión (Raiola *et al*., 2012; Meca *et al*., 2012). En consecuencia, humanos y animales están expuestos a sus efectos tóxicos. Los hongos tienen la capacidad de crecer en una amplia gama de condiciones ambientales. Se ha estimado que del 5-10% de la producción mundial de alimentos se pierde como resultado de deterioro fúngico (Pitt y Hocking, 2009).

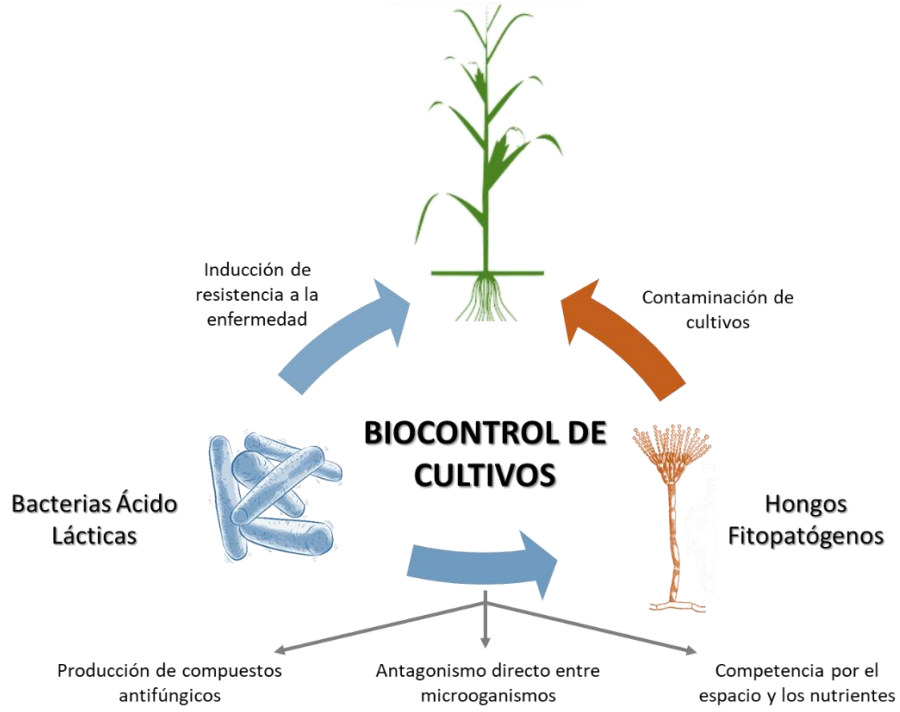


Figura 5. Mecanismos de acción de las BAL frente a hongos fitopatógenos en procesos de biocontrol de cultivos.

Aunque no es posible prevenir la introducción de patógenos en las instalaciones de procesamiento de alimentos, es crucial minimizar su presencia (Akins-Lewenthal, 2012). Las estrategias de conservación más comunes aplicadas en la industria alimentaria incluyen productos químicos o técnicas físicas. Sin embargo, estos métodos solo disminuyen las contaminaciones fúngicas y no alcanzan la eliminación de toxinas (Meca *et al*., 2013).

Los microorganismos que controlan las enfermedades de las plantas operan a través de uno o más mecanismos; la producción de compuestos antifúngicos, antagonismo directo entre microoganismos, inducción al huésped de resistencia a la enfermedad, y competencia por el espacio y los nutrientes. Las interacciones microbianas y la cooperación en la rizosfera pueden contribuir al biocontrol de cultivos de plantas. Las BAL muestran un gran potencial para ser aplicadas en programas de protección de cultivos, se han demostrado que pueden tener efectos críticos sobre la patogenicidad de los hongos (Frey-Klett *et al*., 2011), reducir los daños causandos por el desarrollo micelial (Prusky *et al*., 2006) e inhibir la producción de micotoxinas (Tsitsigiannis *et al*., 2012) (Figura 5).

La pulverización de soluciones diluidas de BAL sobre la planta y el suelo puede ayudar a mejorar el crecimiento de los cultivos. En la mayoría de estudios, se emplea medio fermentado por un cóctel de microoganismos de diferente género y especie, que incluyen BAL, levaduras, bacterias fototróficas, entre otros (Talaat, 2014). Este cocultivo de diferentes microoganismos en la rizosfera proporciona un amplio espectro de metabolitos. Esta tecnología presenta un bajo coste, que cuando se optimiza para un área geográfica específica y ecosistema, pueden presentar un impacto postivo sobre la agricultura. Se han observado beneficios tales como promoción del crecimiento y detoxificación de los suelos y cultivos, aumento del rendimiento de producción, aumentos en los procesos de

fotosíntesis, y mejora de la resistencia a las enfermerdades de las plantas (Asam y Rychlik, 2013). Este tratamiento, se puede aplicar en las diferentes etapas de crecimiento de la planta con dosis específicas para inhibir el crecimiento fúngico. Este tipo de enfoque se puede utilizar como alternativa a los productos químicos agrícolas, como herramienta para desarrollar fertilizantes orgánicos que mejoren la microbiota del suelo, y promover un ambiente saludable para las plantas (Mendes *et al*., 2011). Sin embargo, la efectividad de estas aplicaciones en el campo todavía necesita una mayor optimización.

1.7.2. Productos panarios

El pan es un producto perecedero. El deterioro microbiano causa pérdidas económicas significativas para la industria de la panadería y también para los consumidores (Melikoglu y Webb, 2013). En 2011, la empresa Novozymes encuestó a más de 4000 consumidores de pan en toda Europa y encontró evidencia de que la causa principal de tirar el pan era por observación de crecimiento fúngico en su superficie (Axel *et al*., 2017).

Debido a las características intrínsecas del pan de molde como el pH (5,5-6) y la aw (0,95-0,98) y en ausencia de conservantes, se produce un crecimiento visible de los hongos después de 3-4 días de almacenamiento a temperatura ambiente, aunque el olor característico de deteterioro por metabolitos volátiles se puede observar después de 2 días (Nielsen y Rios, 2000; García *et al*., 2019).

Penicillium spp. y *Aspergillus* spp. son los hongos más comunes que causan el deterioro del pan (Dal Bello *et al*., 2007). Las colonias de *Penicillium* en el pan evolucionan en forma de micelio y pueden ser azules o verdes. Los pigmentos producidos por *Aspergillus* pueden ser de color amarillo, amarillo rojizo o marrón

rojizo en la etapa de esporulación, o verde en la etapa conidial. Algunas especies de *Aspergillus* que contaminan el pan pueden producir aflatoxinas que son carcinógenas (Nielsen y Rios, 2000). El crecimiento de *Penicillium* y *Aspergillus* se ve favorecido en panes con valores de aw alrededor de 0,6-0,85 y temperaturas que oscilan entre 22 y 30 °C (Smith *et al*., 2004). Las micotoxinas de *Fusarium* spp. se pueden detectar en el pan porque este género contamina las materias primas utilizadas para la producción del pan.

La variedad de los panes, la receta utilizada, el procesamiento de la panadería y la localización de los productos pueden afectar el tipo y la cantidad de hongos en descomposición presentes en el pan (Pateras, 2007; Smith *et al*., 2004). *Aspergillus* y *Penicillium*, junto con *Eurotium*, son los hongos predominantes causantes del deterioro en los productos de panadería españoles (Guynot *et al*., 2005). En Irlanda del Norte, *Penicillium* spp. son los hongos de descomposición del pan más comunes, mientras que *Aspergillus* spp. es más predominante en India (Pateras, 2007).

En general, el pan contiene pocos microorganismos después de la cocción debido a la inactivación térmica de estos. Sin embargo, la contaminación del pan se debe principalmente a los procesos posteriores al horneado, como las operaciones de enfriamiento, corte y envasado, ya sea indirectamente por esporas en el aire o directamente por contacto con equipos contaminados. Los panes en rodajas tienen una mayor probabilidad de crecimiento de moho ya que las superficies internas están expuestas, condición indispensable porque los hongos son aeróbicos y crecen solo en la superficie del pan. Empacar pan caliente también puede promover el crecimiento de hongos debido a las áreas localizadas de condensado dentro del paquete. Además, la presencia de esporas en la harina y su capacidad de propagarse en todo el entorno de producción por el aire

facilitan su difusión en las panaderías (Pateras, 2007; Erkmen y Bozoglu, 2016). También debe considerarse que la reducción de las micotoxinas presentes en la masa panaria durante el horneado, puede ser relativamente baja y varía con las toxinas (Kaushik *et al*., 2015).

Un reglamento de la CE (No. 1881/2006) y una recomendación de la CE (No. 165/2013) definen los niveles máximos para algunas micotoxinas en determinados alimentos (Tabla 5). Sin embargo, para algunas micotoxinas de *Fusarium* como nivalenol, eniatinas y moniliformina todavía no se ha establecido un nivel máximo.

Tabla 5. Contenidos máximos legislados de micotoxinas en productos panarios.

Micotoxinas	Productos Alimenticios	Límites máximos (µg/kg)
AFB_1	Todos los cereales y productos derivados de cereales.	2
∑ (AFB_1+ AFB_2+ AFG_1+AFG_2)	Todos los cereales y productos derivados de cereales.	4
OTA	Todos los cereales y productos derivados de cereales.	3
Deoxinivalenol	Pan (incluidos pequeños productos de panadería), pasteles, galletas, snacks de cereales y cereales para el desayuno.	500
ZEA	Pan (incluidos pequeños productos de panadería), pasteles, galletas, snacks de cereales y cereales para el desayuno, excepto los aperitivos de maíz y los cereales para el desayuno a base de maíz	50
Toxina T-2+HT-2	Pan (incluyendo pequeños productos de panadería), pasteles, galletas, cereales y pastas	25
Fumonisina B_1+B_2	Maíz y alimentos a base maíz de consumo humano directo	1000

La fermentación microbiana es uno de los métodos ecológicos más antiguos y económicos para la conservación de los alimentos (Pawlowska *et al*., 2012). Entre los productos de panadería, los microorganismos más utilizados como cultivos iniciadores, aplicado por ejemplo en la producción de masa madre, son las BAL.

Los compuestos activos responsables de la bioconservación son producidos o liberados por las BAL *in situ*. El uso de masa madre como ingrediente en la elaboración de productos panarios puede reeplazar a los conservantes químicos, además de aportar efectos positivos, como mejora de las características sensoriales (sabor, textura), propiedades nutricionales, junto con una mayor aceptación del consumidor (Pawlowska *et al*., 2012).

En los últimos años, el interés en la biopreservación de alimentos ha aumentado drásticamente. Evidencia de esto, se muestra en que el 21% de los nuevos productos panarios lanzados al mercado en Europa en 2013-2014, se presentan como productos elaborados sin aditivos conservantes (Mintel, 2014).

Sin embargo, solo unas pocas aplicaciones de BAL en la producción de productos panarios están documentadas tanto a nivel artesanal como industrial (Corsetti, 2013). Esto, hace importante la investigación de nuevas cepas de BAL más adecuadas al proceso de producción de esta tipología de productos. Así también, el conocimiento sobre el origen del efecto antifúngico es fundamental para la mejora de la bioconservación. La producción de compuestos antifúngicos está influenciada por el crecimiento microbiano, composición del sustrato, producción de enzimas y la interacción con la microflora (Van der Meulen *et al*., 2007).

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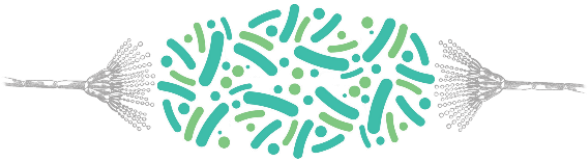
2. OBJECTIVES

Objetivos



2. OBJETIVOS

El objetivo general del presente trabajo es el estudio de la actividad antifúngica y antimicotoxigénica de las bacterias ácidos lácticas (BAL) *in vitro* y su aplicación en cereales y productos de panadería.

Para conseguir este objetivo, se han planteado los siguientes **objetivos específicos**:

1. Evaluar la actividad antifúngica del caldo MRS fermentado por BAL frente a *Aspergillus parasiticus* y *Penicillium expansum*, y purificar, aislar e identificar los compuestos activos producidos durante la fermentación.
2. Evaluar las propiedades antifúngicas del suero de leche fermentado por BAL, caracterizar los compuestos antifúngicos producidos durante la fermentación mediante CL-ESI-qTOF y utilizar el suero de leche fermentado para mejorar la vida útil de pan de molde contaminado con *Penicillium expansum*.
3. Evaluar actividad antifúngica del suero de leche hidrolizado con tripsina frente a *Penicillium* spp., caracterizar los péptidos antifúngicos producidos durante la hidrólisis e incorporar como ingrediente en la elaboración de pan de pita contaminado con *Penicillium verrucosum* para alargar la vida útil y reducir la síntesis de ocratoxina A (OTA).
4. Evaluar la actividad antifúngica de masas madre fermentadas con BAL frente a hongos toxigénicos, identificar los compuestos antifúngicos responsables de la actividad mediante CL-ESI-qTOF y aplicar la masa madre fermentada como ingrediente para mejorar la vida útil de pan de molde contaminado con *Penicillium expansum*.
5. Caracterizar el caldo MRS fermentado por BAL como agente de biocontrol para reducir el crecimiento de *Aspergillus flavus* (productor de aflatoxina

B_1) y *Fusarium verticillioides* (productor de FB_1), y evaluar la síntesis de micotoxinas en granos de maíz y mazorcas.

6. Estudiar la reducción de OTA por BAL en medio MRS y durante la digestión gastrointestinal simulada *in vitro*, y caracterizar los productos de degradación de la OTA mediante CL-MS/MS.
7. Evaluar la actividad antioxidante e inhibidora de la enzima convertidora de angiotensina, y la capacidad de quelación de metales del suero de leche fermentado por BAL.

2. OBJETIVES

The general objective of the present work is the study of the antifungal and antimycotoxigenic activity of lactic acid bacteria (LAB) *in vitro* and its application in cereals and bakery products.

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

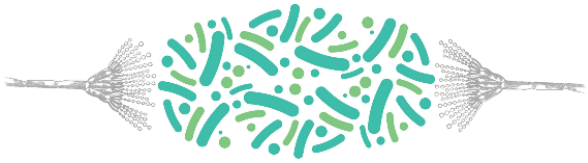
1. Evaluate the antifungal activity of the MRS broth fermented by LAB against *Aspergillus parasiticus* and *Penicillium expansum*, and purify, isolate and identify the active compounds produced during fermentation.
2. Evaluate the antifungal properties of whey fermented by LAB, characterize the antifungal compounds produced during fermentation using CL-ESI-qTOF, and use fermented whey to improve the shelf life of sliced bread contaminated with *Penicillium expansum*.
3. Evaluate antifungal activity of trypsin hydrolyzed whey against *Penicillium* spp., characterize the antifungal peptides produced during hydrolysis and incorporate as an ingredient in the production of pita bread contaminated with *Penicillium verrucosum* to extend shelf life and reduce synthesis Ochratoxin A (OTA).
4. Evaluate the antifungal activity of sourdoughs fermented by LAB against toxigenic fungi, identify the antifungal compounds responsible for the activity using CL-ESI-qTOF and apply the fermented sourdough as an ingredient to improve the shelf life of bread contaminated with *Penicillium expansum*.
5. Characterize the MRS broth fermented by LAB as a biocontrol agent to reduce the growth of *Aspergillus flavus* (producer of aflatoxin B_1) and

Fusarium verticillioides (producer of FB_1), and evaluate the synthesis of mycotoxins in corn kernels and ears.

6. Study the reduction of OTA by BAL in MRS medium and during simulated gastrointestinal digestion *in vitro*, and characterize the degradation products of OTA using CL-MS / MS.
7. To evaluate the antioxidant and inhibitory activity of angiotensin-converting enzyme, and the metal chelation capacity of whey fermented by LAB.

3. RESULTS

Resultados



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3.1. *In vitro* antifungal activity of bioactive peptides produced by *Lactobacillus plantarum* against *Aspergillus parasiticus* and *Penicillium expansum*

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1. Introduction

Mould deterioration of food, feed and other agricultural commodities can be responsible for considerable economical losses. Thirty percent of crop yields are destroyed and more than 30% of perishable crops are damaged in developing countries by lowering their quality and quantity. Furthermore, moulds produce compounds potentially toxics to the consumers called mycotoxins which can cause illness and death (Pawlowska, Zannini, Coffey & Arendt, 2012). High incidences of mould and mycotoxin contamination in food and feed are due to fungi ubiquitous nature, to their ability to colonize different substrates and to the lack of effective control measures (Hassan, Zhou & Bullerman, 2015).

Currently, food industry depends on chemical preservatives to extend the shelf life and control the growth of spoilage fungi. The consumer's awareness about the health hazards associated with chemicals has recently increased, and they are demanding for processed foods that are free of preservatives. There are many natural alternative preservatives produced by certain microorganisms, however these microorganisms have to be non-toxic, easy to grow and require simple media for cultivation. Lactic acid bacteria (LABs) are a known potential source for generating a variety of secondary metabolites such as bacteriocines, organic acids and peptides (Cizeikiene, Juodeikiene, Paskevicius & Bartkiene, 2013). In the past decade the interest for antifungal LABs has increased and different studies have showed that many LAB strains have the potential to combat the proliferation of fungi in various food and feed materials (Rouse, Harnett, Vaughan & Van Sinderen, 2008; Dalie, Deschamps & Richard-Forget, 2010; Mauch, Dal Bello, Coffey & Arendt, 2010; Gerez, Torino, Rollan & De Valdez, 2009; Dal Bello et al. 2007). Consumers are demanding the replacement of artificial chemical preservatives by natural biopreservatives to reduce fungal

contamination in foods (Crowley, Mahony & Van Sinderen, 2013; Schnurer & Magnusson, 2005; Reis, Paula, Casarotti & Penna, 2012; Brul, & Coote 1999). LABs, due to their long history of safe use in food and feed fermentations, (Stoyanova, Ustyugova & Netrusov, 2012; Hugenholtz, 2013) have received both GRAS (Generally Recognised as Safe) and QPS (Qualified Presumption of Safety) status in the EU. Therefore, LABs have good potential for future as antifungal biocontrol agents.

Bacteriocins/antimicrobial peptides produced by LABs have been reported to be involved in the preservation of many processed and natural foods (Settanni & Corsetti, 2008; Bellei, Miguel, Mere Del Aguila, Silva & Paschoalin, 2011; Udhayashree, Senbagam, Senthilkumar, Nithya & Gurusamy, 2012). Their usage in the food industry has promoted the reduction of chemical preservatives and intense heat treatments, thus, resulting in foods which are more naturally-preserved and richer in nutritional properties (Parada, Caron, Medeiros & Soccol, 2007; De Vuyst & Leroy, 2007; Coda, Cassone, Rizzello, Nionelli, Cardinali & Gobbetti, 2011). Pediocin PA-1/AcH is not allowed as a food additive but is only applied in form of protective cultures whereas nisin is the only commercially acceptable food-grade bacteriocin in use (Díez, Rojo-Bezares, Zarazaga, Rodríguez, Torres & Ruiz-Larrea, 2012; Chen & Hoover, 2003; Settanni & Corsetti, 2008). In addition to these, other bacteriocins (lacticin 3147, enterocin AS-48 or variacin) also offer promising perspectives (Gálvez, Abriouel, López & Omar, 2007). So it is meaningful and highly pertinent to screen for natural bioactive peptides to find novel ones for a specific application.

The antifungal effect by the culture supernatant of various LAB strains against few fungi including food-deteriogens has been worked out by various authors (Yang & Clausen, 2005; Sathe, Nawani, Dhakephalkar & Kapadnis, 2007;

Rouse, Canchaya & Van Sinderen, 2008; Gerez, Torino, Rollán, & de Valdez, 2009; Smaoui et al., 2009; Adebayo & Aderiye, 2010; Coda, Cassone, Rizzello, Nionelli, Cardinali & Gobbetti, 2011). Several compounds have been proposed as responsible for the antifungal activity of LAB, like organic acids, low molecular weight compounds, phenylacetic and fatty acids, cyclic dipeptides, proteinaceous compounds and other miscellaneous compounds e.g. lactones (Peyer et al., 2016). The fungistatic activity of pentocin TV35b against *Candida albicans* (Okkers, Dicks, Silvester, Joubert, & Odendaal, 1999) and of two LAB strains against *Fusarium* (isolated from cereals) has been reported (Laitila, Alakomi, Raaska, Mattila-Sandholm & Haikara, 2002). Due to their inhibitory spectrum these peptides are consider to be powerful antimicrobials to target bacteria, fungi and parasites (Mason et al., 2009).

The aims of this study were a) to evaluate the antifungal activity of cell free supernatant containing peptides obtained from different LABs against *A. parasiticus* and *P. expansum*; b) to purify, isolate and identify the active compounds produced during the fermentation by the most potential LAB and c) to test each one of them against fungal growth of *A. parasiticus* and *P. expansum*.

2. Materials and methods

2.1. Chemicals

Acetonitrile was purchased from VWR (Leuven, Bélgica). Deionized water (<18 MΩ cm resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Chromatographic solvents and water were degassed for 20 min using a Branson 5200 (Branson Ultrasonic Corp., CT, USA) ultrasonic bath. Buffered peptone water, potato dextrose agar (PDA), phosphate buffer saline (PBS, pH 7.4), potato dextrose broth (PDB) and De Man Rogosa and

Sharpe broth and agar (MRS broth and agar) were provided by Oxoid (Madrid, Spain).

2.2. Microorganisms and culture conditions

The strain of *A. parasiticus* CECT 2681 and *P. expansum* CECT 2268 were obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain). These microorganisms were maintained in sterile glycerol at -80 °C. Then, they were recovered in PDB broth at 25 ºC until they were inoculated in PDA Petri dishes.

The LABs used in this study were: *Lactobacillus rhamnosus* CECT 278T*, Lactobacillus johnsoni* CECT 289*, Lactobacillus plantarum* CECT 749*,* and *Lactobacillus delbrueckii bulgaricus* CECT 4005. These bacteria were also obtained from CECT Valencia, Spain. The LABs were preserved in sterile 18% glycerol and stored at -80 ºC before use. They were recovered in MRS broth at 37 ºC for 48 h under anaerobic conditions (Anaerocult A, Merk – Darmstadt, Germany) before experiments (Meroth, Walter, Hertel, Brandt & Hammes, 2003). Bacteria concentration was measured by optical density at 600 nm using a spectrophotometer (Shimadzu UV mini 1240 – Columbia, EE.UU.) and adjusted to 10^6 LAB/mL to obtain the same initial conditions for all the bacteria. After the incubation period, the cells were removed by centrifugation in Eppendorf 5810R centrifuge (Hamburg, Germany) at 4000 rpm for 10 min. The cell free supernatants (CFSs) were lyophilized using a lyophilizer Virtis SP SCIENTIFIC sentry 2.0 (Warminster, EE.UU) and stored at -18 °C until the day of the analysis.

2.3. Antifungal activity of the LAB cell-free supernatant in solid medium

Kirby-Bauer assay, used to evaluate the antimicrobial activity of the LAB CFSs against *A. parasiticus* and *P. expansum*, was performed according to Varsha et al. (2014) with some modifications. Lyophilized CFSs were reconstituted with 2 mL of sterile water and filtered with a 0.22 µm sterile nylon filter. For the agar well diffusion assay, 100 µL of the CFS were added to the wells (5 mm diameter) cut on PDA which was previously spread plated with *A. parasiticus* or *P. expansum* and kept at 26°C for 2 days. A control composed of MRS broth treated in the same way of the CFSs was also prepared. The microorganisms were considered positive to the antimicrobial activity of the bioactive compounds if an inhibition zone of at least 8 mm wide was observed around the well

2.4. Purification and peptides fractionation by gel filtration chromatography

The purification was carried out using the method described by Muhialdin et al. (2016) with some modifications. The lyophilized medium (400 mg) was dissolved in 2 mL deionized water and the peptides were fractionated using Sephadex G-25 size exclusion chromatography. The super fine resin can be used to separate peptides ranging between 1 and 5 KDa. The dry powder was swollen in boiling water for 3 h and the water was discarded and replaced with a separation buffer. The resin, which had been washed with 5 M HCl and degassed, was poured into the column (size 1.6 x 50 cm) and buffered with 0.2 M phosphate buffer pH 7. The flow rate was adjusted to 1 mL/min with a peristaltic pump and the end of the column was connected to a fraction collector FC 204 from Gilson (Middleton, USA) to recollect 80 fractions of 7 mL. The fractions were injected in

the analytical liquid chromatography (LC) coupled to diode array detector (DAD) to identify the range of positive fractions to the presence of the peptides.

2.5. LC analyses of peptides

Peptide mixtures were analyzed by a LC system equipped with LC-7100 pump, autosampler L-2200 (20 µL loop) and a DAD L-7455 from Hitachi (Tokyo, Japan). Elution of the peptides was performed using an Aeris peptide XB-C18 (100 x 4.6 mm; 3.6 µM of ID, Phenomenex, Madrid, Spain) at 30 °C and a flow rate of 1 mL/min. The mobile phase was composed of water (solvent A) and acetonitrile (solvent B) with 0.1% (v/v) trifluoroacetic acid (TFA). The elution gradient established was: 0 min-5% B; 10 min-35% B; 30 min-100% B; 40 min-100% B; 50 min-5% B (Brosnan et al., 2014). The eluting peptides were monitored by measuring the absorbance at 214 nm.

2.6. Peptides isolation using semi-preparative LC

The fractions positive to the presence of peptides were injected for peptides isolation in a semi-preparative LC composed by a LC system equipped with LC-7100 pump, autosampler L-2200 (200 µL loop) and a DAD L-7455 from Hitachi (Tokyo, Japan). A semi-preparative Gemini C18 column (250x10 mm, 5 µM of ID) (Phenomenex, Madrid, Spain) was used and a mobile phase with water (solvent A) and acetonitrile (solvent B) containing 0.1 % of TFA at flow rate of 3.0 mL/min. The elution gradient established was: 0 min-5% B; 10 min-35% B; 30 min-100% B; 40 min-100% B; 50 min-5% B. Peptides were detected at 214 nm. The end of the column was connected at a fraction collector FC 204 from Gilson (Middleton, USA).

The LC-purified peptides solutions were concentrated on a rotary evaporator at 35ºC, freeze-dried and stored at -80ºC before MALDI-TOF analysis.

2.7. MALDI-TOF analyses

Samples were dissolved in 0.1% (v/v) TFA and concentrated using the ziptip C18 pipette tips (Millipore, Billerica, MA). Peptide solution (1.0 µL) was mixed with the same volume of a-cyano-4-hydroxycinnamic acid matrix and spotted on a MALDI plate. MALDI-MS and MALDI-MS/MS were performed on an Applied Biosystems 4700 Proteomics Analyzer with TOF/TOF ion optics (Applied Systems, Foster City, CA). The mass spectrometer was operated in positive ion reflector mode with five spots of standard (ABI 4700 calibration mixture) for calibration. Mass spectra were obtained from each spot using 500 shots per spectrum. Tandem mass spectra were acquired by accelerating the precursor ions to 8 keV, selecting them with the timed gate set to a window of 3 Da, and performing CID at 1 keV. Gas pressure (air) in the CID cell was set at 0.2 µTorr. Fragment ions were accelerated to 14 keV before entering the reflector. The mass spectral data were submitted to a database search using the MASCOT program (Matrix Science, version 2.1).

2.8. Antifungal activity in liquid medium

The fungal strain of *A. parasitcus* and *P. expansum* were cultured in Potato Dextrose Broth (PDB) and antifungal activity was assessed with the method of Bolivar et al. (2011) with some modifications. Peptides previously isolated were dissolved in sterile water with concentrations ranging from 10 to 15000 ppm.

For the antifungal tests, 9.8 mL of PDB were added to screw-capped tubes, followed by 0.1 mL of fungal inoculum (10^6 ufc/mL counted with a Neubauer chamber). After the inoculum was added, the tubes were treated with 0.1 mL of the peptide solution obtaining final concentration ranged from 0.1 to 150 ppm. Control groups contained 9.9 mL of PDB with water and 100 µL of inoculum. Experiments were performed in triplicate. The tubes were incubated for 48h at 30 ºC for Minimum Inhibitory Concentration (MIC) calculation and for 72h for Minimum Fungicidal Concentration, (MFC) calculation at 30ºC under orbital shaking.

After that 100 µL of each tube were inoculated in a PDA Petri dishes and incubated at 30 ºC during one week for the MIC and MFC calculation that are defined as the lowest concentration of an antimicrobial compound that will inhibit the visible growth of a microorganism after 48 and 72h incubation. The cells colonies were also counted to perform the viability curve of the microorganism exposed at the different concentration of the peptides. All the experiments were performed in triplicate.

3. Results and discussion

3.1. Antifungal activity of the LAB cell-free supernatant in solid medium

The results related to the antifungal activity of the CFSs against *A. parasiticus* and *P. expansum* in solid medium of PDA are shown in Figure 1. The analysis of the data demonstrate that the CFSs of *L. rhamnosus* CECT 278T*, L. johnsoni* CECT 289 and *L. plantarum* CECT 749 possess antifungal activity against the mycotoxigenic fungi tested. *L. plantarum* was the most effective with the largest inhibition zone, while *L. delbrueckii bulgaricus* CECT 4005 did not possess any

antimicrobial activity against *A. parasiticus* and *P. expansum*. The control composed of MRS broth treated in the same way did not show evidence of any antifungal property against the mycotoxigenic fungi tested.

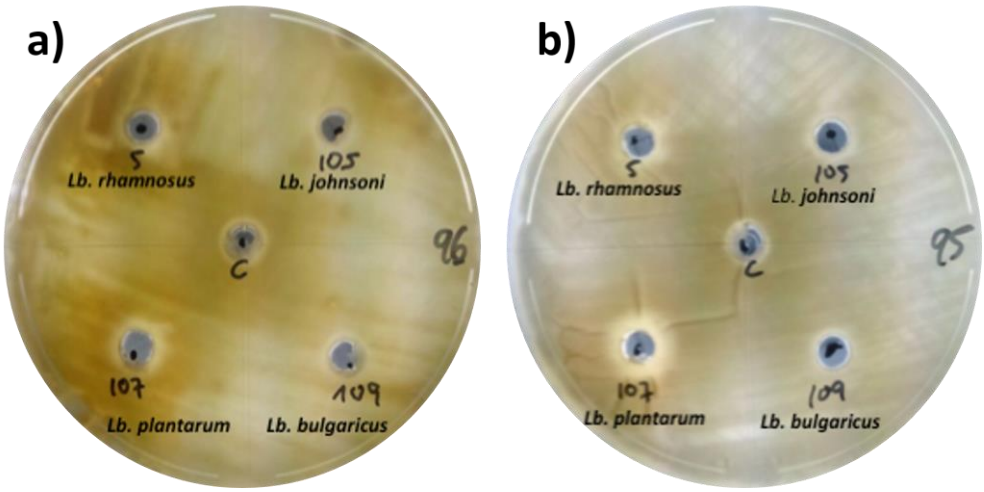

Figure 1. Antifungal activity of the lyophilized fermented medium of MRS by prebiotic strains of *L. rhamnosus, L. johnsoni, L. plantarum,* and *L. delbrueckii bulgaricus* on the mycotoxigenic fungi a) *A. parasiticus* and b) *P. expansum*.

Other authors investigated the antimicrobial properties of bioactive components produced by LABs. The results evidenced in our study are confirmed by the data obtained by Ryan et al. (2008). These authors investigated the antifungal activity of two strains of *L. plantarum* fermented sourdoughs comparing their activity to that of a sourdough fermented by *L. sanfranciscensis* LTH2581 as well as to that of a chemically acidified dough and a dough containing antibiotics. *L. plantarum* fermeted sourdough showed the highest inhibitory activity. Valerio et al. (2009) tested fermentation products of 17 LAB strains

against *Penicillium roqueforti* and *Aspergillus niger*. *Lactobacillus citreum*, *Weissella cibaria*, and *Lactobacillus rossiae* inhibited (>98%) the growth of *A. niger*. *L. plantarum* was the most effective against *P. roqueforti*. These results, in particular the data related to the antifungal activity of the bioactive compounds of *L. plantarum*, are in agreement with the data evidenced in our study. Ahmad Rather et al. (2013) screened 1400 bacteria isolated from different kimchi samples for their antifungal activity against *A. niger* by dual-culture agar plate assay. According to our study, the strain exhibiting the high antifungal activity was *L. plantarum* YML007 so that it was further screened against various pathogens showing the highest inhibition against *A. niger*, followed by *Aspergillus flavus, Aspergillus oryzae* and *Fusarium oxysporum*. Moreover, biopreservative activity of *L. plantarum* YML007 was evaluated using dried soybeans and no fungal growth was observed in the soybeans treated with fivefold concentrated cell-free supernatant of *L. plantarum* YML007. Gupta et al. (2014) studied the antifungal activity of 88 *L. plantarum* strains against *A. niger*, *A. flavus*, *Fusarium culmorum*, *Penicillium roqueforti*, *Penicillium expansum*, *Penicillium chrysogenum* and *Cladosporium* spp.. Nine of these strains that strongly inhibited at least three moulds were further screened based on the antifungal properties of their cell-free supernatant. Even if none of the *L. plantarum* strain tested was able to completely inhibit the growth of the analyzed filamentous fungi, the maximum antagonistic effect was observed after 5 days of incubation in plates supplemented with CFS (12% v/v) from *L. plantarum* UFG 108 and *L. plantarum* UFG 121, in which the growth of *P. expansum* and *F. culmorum* was reduced about 50 and 60%, respectively. Finally an oat-based beverage obtained by fermentation with *L. plantarum* UFG 121 and contaminated with *F. culmorum* showed the best biopreservative effects without no differences in terms of qualitative features between not or contaminated samples.

3.2. Isolation and identification of the antimicrobial peptides produced by L. plantarum in MRS broth

The gel filtration chromatography developed on G-25 fine sephadex was used for the fractionation of the bioactive peptides produced by *L. plantarum*. The fractions recollected by the purification process through the low pressure liquid chromatography were injected into the LC-DAD for the identification of the fractions positive to the presence of the peptides. Fraction three was particularly interesting for the presence of three peaks (Figure 2a). They were purified by the technique of the LC-DAD using a semi preparative C18 column to recollect important amounts of the bioactive compounds, and then, their antifungal activity against *A. parasiticus* and *P. expansum* was studied *(Figure 3)*. As evidenced in the Figure 2 b, c, d, each peak present in the fraction three was separated from the other compounds by the purification of the peptides (semipreparative chromatography approach) produced by the *L. plantarum* through a fermentation process in MRS medium. The analysis of the fractions purity evidenced that the semi preparative approach produced three different isolated compounds with 95, 96 and 95% of purity, respectively (Table 1).

Q-TOF-MS analysis indicated that the molecular mass of the peak 1 was 1041.26 Da. The observed molecular mass was in agreement with the calculated molecular mass of the peptide. The MS/MS analysis showed that the major sequence of the peak 1 was Ser-Gly-Ala-Asp-Thr-Thr-Phe-Leu-Thr-Lys (SGADTTFLTK). The mass spectrometry analysis associated to the peak two, detected an observed molecular weight (MW) of 1019.30 Da and the MS^2 analysis of the fragment corresponding to the MW showed that the amino acidic sequence was composed by Leu-Val-Gly-Lys-Lys-Val-Gin-Thr-Phe (LVGKKVQTF). Q-TOF-MS analysis associated to the peak three evidenced an observed MW that was in

agreement with the calculated molecular mass of 995.15 Da. The MS^2 analysis of the ion corresponding to the MW evidenced an amino acidic sequence of the peak 1 of Gly-Thr-Leu-Ile-Gly-Gin-Asp-Tyr-Lys (GTLIGQDYK).

Table 1. Purity, molecular weight and amino acid sequence of the bioactive peptides purified by a fermentation of *L. plantarum* in MRS broth.

Peak nº	Fraction purity (%)	Observed MS (Da)	Expected MS (Da)	Calculated MS	Delta	Peptide sequence
1	95%	1041.2600	1040.2527	1039.5186	0.73	SGADTTFLTK
2	96%	1019.3000	1018.2927	1018.6175	0.32	LVGKKVQTF
3	95%	995.1500	994.1427	993.5131	0.62	GTLIGQDYK

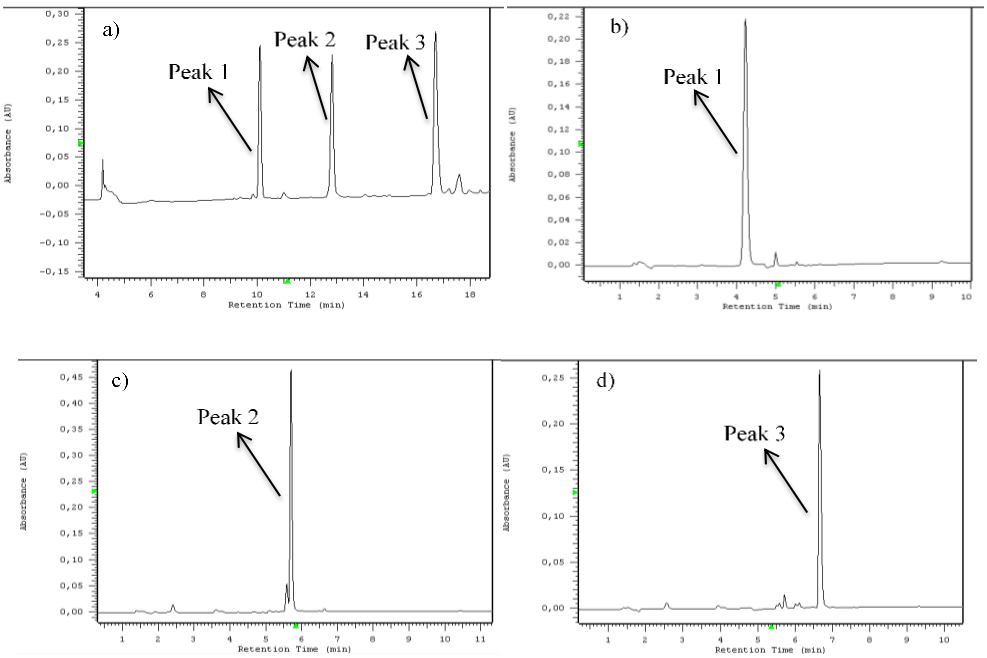

Figure 2. a) Chromatogram of bioactive peptides produced by *L. plantarum* and analyzed with LC-DAD with an analytical column and **b) c)** and **d)** chromatograms of the peaks corresponding to the bioactive peptides 1, 2 and 3 purified using a LC-DAD with a semipreparative approach column.

The sequence of the three purified peaks, using low pressure gel filtration liquid chromatography associated to the LC with the semipreparative column was referred to a novel peptides, in accordance to the database available online (http://prospector.ucsf.edu; http://www.expasy.ch). The purified peptides were used as external standards for LC-DAD quantitative analysis. A stock solution was prepared in water and dilutions were made to cover a concentration range from 10 to 300 mg/L. The *L. plantarum* produced in the MRS medium 250.16±4.7, 280.45±7.2 and 246.87±6.2 mg/L of the three bioactive peptides purified, respectively.

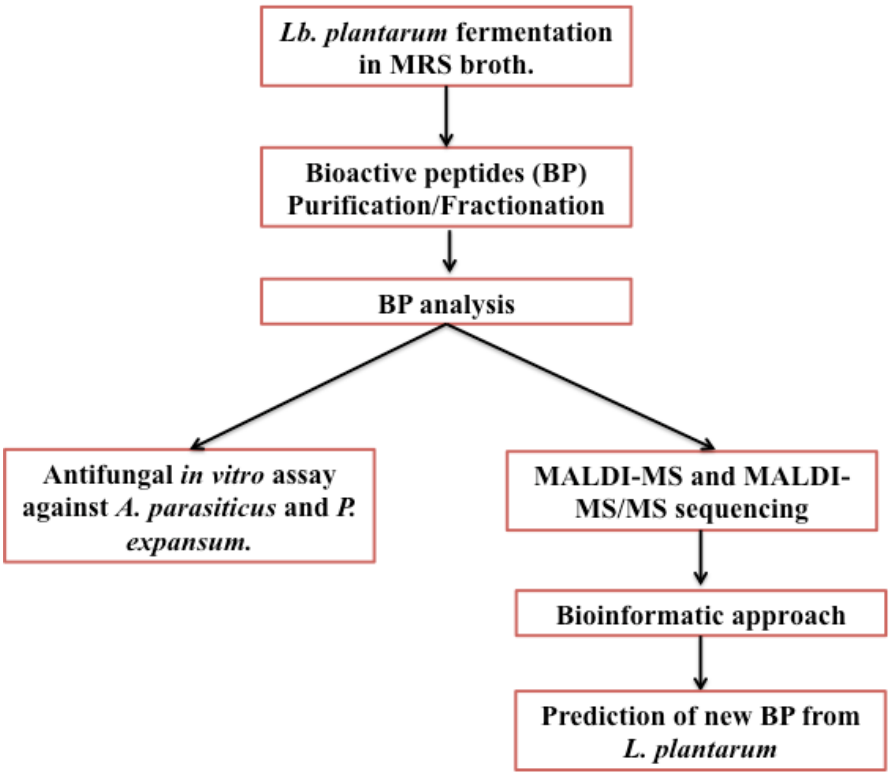

Figure 3. Schematically approach for the study of the antifungal activity of the antimicrobial peptides produced by lactobacillus strains and of the mass spectrometry characterization of the new formed compounds through the MALDI-TOF.

3.3. *Antifungal activity of the peptides produced by L. plantarum against A. parasiticus and P. expansum*

The use of natural sources of antimicrobial compounds has enormous potential due to their characteristics such as low toxicity and high specificity. Their mechanism of action is different against bacterial, fungal and animal cells. Bacterial cells have a layer rich in negatively charged phospholipids pointing toward the external environment, facilitating their interactions with peptides, most of which are positively charged. In contrast, animal cells are mainly composed of uncharged lipids in the outermost layer, and the negatively charged regions are pointed toward the cytoplasm (Matsuzaki, 1999).

Antimicrobial peptides are widely distributed in nature and are essential to the immune system. They are the organism's first line of defense against colonization by exogenous microorganisms (Zasloff, 2002). More than 800 antimicrobial peptides have been described in plants and animals (Boman, 2003). Despite the great diversity in their primary structures, most antimicrobial peptides are similar as they are short amino acid chains composed primarily of cationic and hydrophobic amino acids (Dashper, Liu & Reynolds, 2007). The low molecular weights of the peptide fractions, the resulting higher exposure of the amino acids and their charges, and the formation of small channels in the lipid bilayer promote interactions between the peptide and the membrane (Gómez-Guillén et al., 2010; Patrzykat & Douglas, 2005). The exact mechanisms of action for many antimicrobial peptides have not been well established (Dashper, Liu & Reynolds, 2007). Peptides with antimicrobial activity have also been identified in several protein hydrolysates.

In this study the antifungal activity of the three isolated peptides produced by *L. plantarum* was tested. The data related to the growth inhibition of *A.*

parasiticus and *P. expansum*, after 48 and 72 h, induced by the exposure to different concentrations of the purified peptides are shown in Figure 4. MIC and MFC could not be calculated since the peptides did not show the ability to reduce fungal growth ≥99% than the control at the concentrations tested. LVGKKVQTF and GTLIGQDYK peptides did not show significant antifungal activity against *P. expansum* and *A. parasiticus* if compared to the control. On the other side, a significant reduction of *A. parasiticus* and *P. expansum* growth was observed using all the concentrations of SGADTTFLTK peptide. In particular, *A. parasiticus* viability (%) was reduced of 73% and 42% after 48 and 72 h, respectively. Slightly lower reductions were obtained for *P. expansum*: 58% and 49% after the same incubation times.

Several authors describe similar antimicrobial activity of peptides produced by LAB during fermentation. Russo et al. (2016) investigated the effect of antimicrobial peptides (AMPs LR14) produced by *L. plantarum* strain LR/14 against *A. niger*, *Rhizopus stolonifera*, *Mucor racemosus* and *Penicillium chrysogenum* by dual culture assay. All the four fungi were inhibited: the peptides inhibited both the spore germination and hyphal growth. In addition, fungal growth was inhibited in wheat seeds treated with AMPs LR14 even after a prolonged storage under laboratory conditions for 2.5 years. Muhialdin et al. (2016) reported that concentrations of 0.02 mg peptide/mL inhibited the fungal growth of *Aspergillus flavus*, *P. roqueforti* and *Eurotium rubrum* between 20-66%. Both peptide concentrations used in this study for the inhibition (%) of fungal growth resulted in the same order of magnitude as those obtained in our study. Wen et al. (2016) studied the mode of action of a bacteriocin with low molecular weight, isolated from *L. plantarum* K25. This antimicrobial peptide, named plantaricin K25, exhibited a broad spectrum of inhibitory activity against both Gram-positive and

Gram-negative bacteria. This bacteriocin showed to be a pore-forming bacteriocin capable of permeabilising the cytoplasmic membrane of targeted bacterial cells. It inhibited the growth of *B. cereus* cultivated in mul-kimchi with reduction of viable cell counts compared to the control sample. De Souza Barbosa et al. (2015) isolated two bacteriocins, produced by *Lactobacillus curvatus*, with anti-*Listeria* activity from salami samples and evaluated their effectiveness in the control of *L. monocytogenes* during the fermentation step of salami manufacture. In particular, bacteriocin MBSa2 presented activity against 22 of 23 *L. monocytogenes* strains while the bacteriocin MBSa3 inhibited all 23 strains in addition to several other Gram-positive bacteria.

Garofalo et al. (2012) studied two sourdough strains, *Lactobacillus rossiae* LD108 and *Lactobacillus paralimentarius* PB127 selected on the basis of their capacity to inhibit fungal growth, which was evidenced for the first time in their study. These authors observed that their antifungal properties against three cultures ascribed to *Aspergillus japonicus*, *Eurotium repens*, and *Penicillium roseopurpureum* were due to peptides identified as gluten proteolysis byproducts. Furthermore, the ability to prevent mold growth was demonstrated on bread for both strains, whereas *L. rossiae* LD108 also inhibited mold growth on panettone. Coda et al. (2011) investigated the antifungal activity of *Wickerhamomyces anomalus* and sourdough lactic acid bacteria to extend the shelf life of wheat flour bread. *Penicillium roqueforti* DPPMAF1 was used as the indicator fungus. Sourdough fermented by *Lactobacillus plantarum* 1A7 (S1A7) and dough fermented by *W. anomalus* LCF1695 (D1695) were selected and characterized. The water/salt-soluble extract of S1A7 was partially purified, and several novel antifungal peptides, encrypted into sequences of Oryza sativa proteins, were identified. The water/salt-soluble extract of D1695 contained ethanol and,

especially, ethyl acetate as inhibitory compounds. Both water/salt-soluble extracts showed a large inhibitory spectrum, with some differences, toward the most common fungi isolated from bakeries. Bread making at a pilot plant was carried out with S1A7, D1695, or a sourdough started with a combination of both strains (S1A7-1695). Slices of the bread manufactured with S1A7-1695 did not show contamination by fungi until 28 days of storage in polyethylene bags at room temperature, a level of protection comparable to that afforded by 0.3% (wt/wt) calcium propionate.

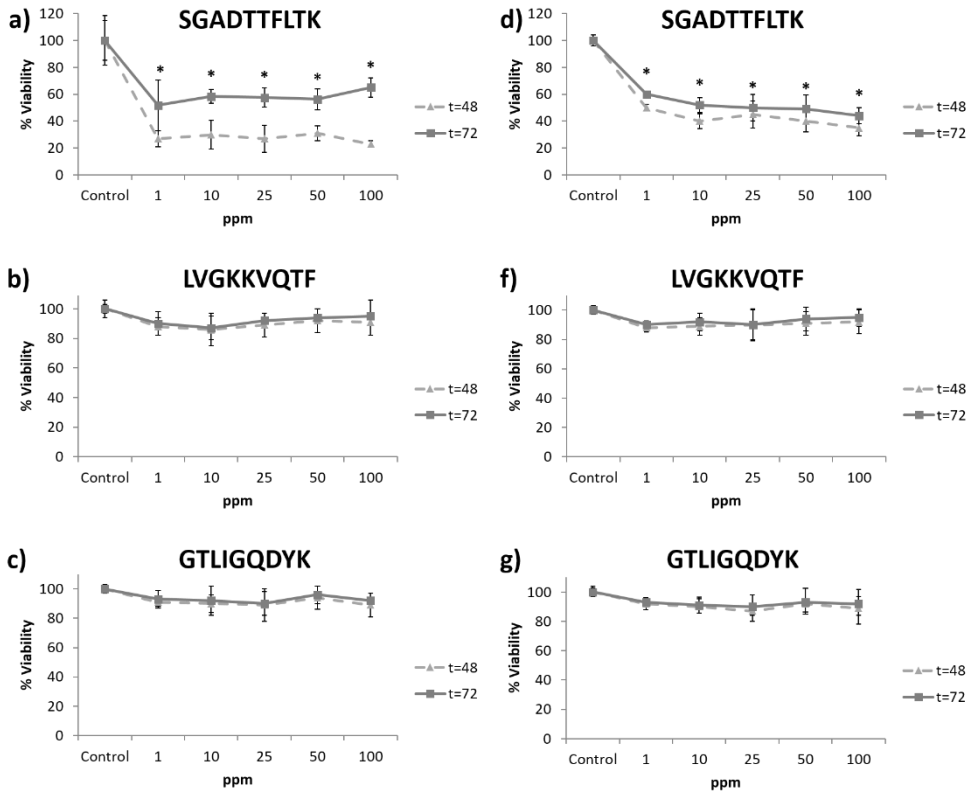

Figure 4. Percentage of viability evidenced by *A. parasiticus* (a, c, d) and *P. expansum* (b, d, f) exposed at different concentration of the purified peptides at 48 h (black line) and 72 h (cinnamon line) incubation.

4. Conclusions

In conclusion, the CFSs of *L. rhamnosus*, *L. johnsoni* and *L. plantarum* produced antifungal compounds during the fermentation against the mycotoxigenic fungi tested while *L. delbrueckii bulgaricus* was the only one that did not possess any antifungal activity against *A. parasiticus* and *P. expansum*. Liquid medium fermented by *L. plantarum* showed the highest inhibition activity, with the largest inhibition zone. Three peptides were purified from the culture medium fermented by *L. plantarum* and they were identified employing mass spectrometry MALDI-TOF: SGADTTFLTK, GTLIGQDYK and LVGKKVQTF. The LVGKKVQTF and GTLIGQDYK peptides identified have no observable antifungal activity. On the other side, the SGADTTFLTK peptide produced by *L. plantarum* during fermentation showed antifungal activity against *P. expansum* and *A. parasiticus* so that it could be a potential substitute to chemical preservatives in food.

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3.2. Evaluation of biological and antimicrobial properties of freeze-dried whey fermented by different strains of *Lactobacillus plantarum*

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1. Introduction

In recent years, environmental and economic problems associated with food waste have induced industries to reuse these materials in other preparations in order to minimize food waste. The dairy industry is the largest producer of difficult food discharges such as whey, liquid residue after cheese production.[1]

According to the statistical istat data available, the 11% of the total milk product in the world is employed for cheese production, and the 85% of this milk is transformed in whey after cheese production. Whey is considered a waste product with very important nutritional properties, because its contains 55% of milk nutrients.[2]

Several methods have been proposed for whey valorization. Lactic acid bacteria have the property of producing proteolytic enzymes that hydrolyze large milk protein producing small peptides and free amino acids, with several functional properties for human health. [3] Bioactive compounds can be produced from milk proteins through fermentation of milk, by starters employed in the manufacture of fermented milks or cheese.[4]

Depending on the amino acid sequence, the milk protein-derived compounds may exert a number of different bioactive properties including antimicrobial, mineral binding, cardioprotective, immunomodulatory, anticancer, antidiabetic, satiating, opioid and antioxidant activities.[4,5] These peptides are inactive within the sequence of the parent protein but can be liberated during human gastrointestinal digestion. [6]

Xiao *et al*. [7] studied the antioxidant activity of soy whey after fermentation with *Lactobacillus plantarum* in order to find out their nutraceutical potential. The analysis showed that fermentation of soy whey influences the content of total

phenolic content (TPC), antioxidant activities and DNA damage protection. The results showed an increase in these activities compared with unfermented whey.

2. Materials and methods

2.1. Chemicals

The compounds 2, 2-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), N-Hippuryl-His-Leu substrate, angiotensin-converting enzyme, hippuric acid, ferrozine, ferrous chloride, glutathione, magnesium sulfate, sodium chloride, caffeic acid, p-Coumaric acid, ferulic acid, hydroxibenzoic acid, sallicilic acid, hydrocinnamic acid, sinapic acid, benzoic acid, DL-3-phenyllactic acid, 1-2 dihydroxybenzene were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Methanol, ethanol, trifluoroacetic acid, ethyl acetate, formic acid and acetonitrile HPLC grade were purchased from Merck (Darmstadt, Germany). All other chemicals and reagents were of analytical grade. Deionized water (<18 MX cm resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Buffered peptone water, potato dextrose agar (PDA), potato dextrose broth (PDB) were provided by Oxoid (Madrid, Spain).

2.2. Microorganisms

L. plantarum (CECT 220; 221; 748) were obtained from Colección Espanola de Cultivos Tipo (CECT) and stored at −80°C in glycerol 50%. The cultures were defrosted under sterile conditions, diluited in sterile MRS broth and finally incubated at 37°C for 48 h.

For the evaluation of the antifungal activity, the mycotoxigenic strains used *P. camemberti CECT 2267, P. expansum CECT 2278, P. roqueforti CECT 2905, A. parasiticus CECT 2681, A. flavus ITEM 8111, A. niger CECT 2088, F. moniliformis CECT 2982, F. verticilliodes CECT 20926, and F. graminearum CECT 2049* were purchased from the Colección Espanola de Cultivos Tipo (CECT). All strains were maintained in PDA at room temperature (25 °C) until use.

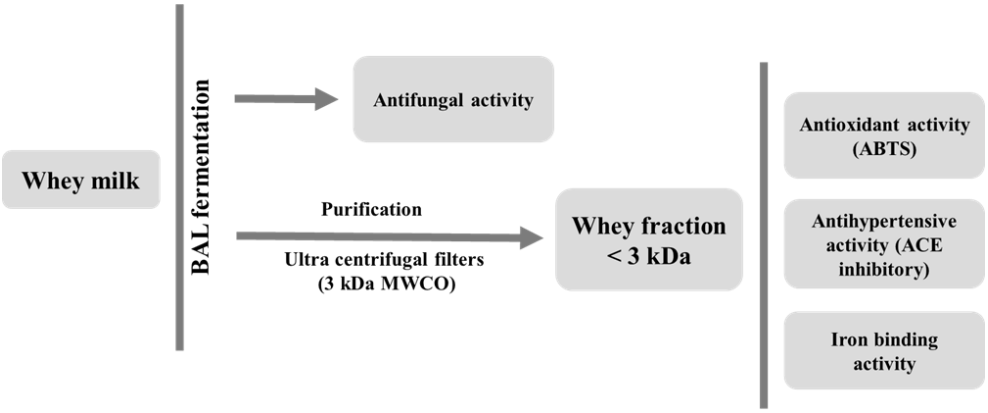

Fig 1. Whey treatments: fermentation at three different times (24, 48, 72 h), purification on centricon amicon with cut-off <3kDa and evaluation of biological and antimicrobial proprieties of this particular fraction.

2.3. Whey Fermentation

Freeze-dried whey was purchased directly from a supermarket in the city of Valencia (Spain). The sample was fermented with three different species of lactobacilli for 24, 48 and 72 hours at 37°C using the method of Pescuma et al. [13] with some modifications. Whey was reconstituted with distilled water to a 10% (w/v) final concentration. Subsequently, 30 mL were fermented with 1 mL of previously activated bacteria, *L. plantarum* (CECT 220; 221; 748). After fermentation, samples were centrifuged for 10 minutes at 4000 rpm (Eppendorf

5810R, Hamburg, Germany) to remove bacterias and 1mM of a protease inhibitor PMSF (phenylmethanesulfonyl fluoride) was added to the supernatant. The samples were lyophilized and stored at -80°C for further analysis (Fig 1).

2.4. Purification Sample

An aliquot of 250 mg of freeze-dried fermented whey was solubilized in 1 mL of 0.1% TFA aqueous solution. The sample was purified on SPE C18 columns, previously activated, and then eluted with 3 mL of a mixture of acetonitrile/0.1% formic acid in water (70:30, v/v). The eluate was further purified on Centricon Amicon with cut-off of 3 kDa to obtain a permeate consisting of small bioactive compounds as reported in the literature to have shown greater bioactivity. After purification the permeate was lyophilized and finally re-suspended in 500 µL water for subsequent analysis.[14]

The purified and diluted samples were subjected to the biological test for the evaluation of antioxidant activity (ABTS), antihypertensive activity (ACE inhibitory) and iron binding activity (Fig 1).

2.5. ABTS radical cation scavenging assay

The method reported by Xiao *et al.* [15] was used to analyze the ABTS radical cation ($ABTS^{\bullet+}$) scavenging activity. $ABTS^{\bullet+}$ was obtained by the reaction of a 7 mM aqueous solution of ABTS with 2.45 mM aqueous solution of potassium persulphate which was performed in dark conditions at room temperature for 16 h prior to use. The $ABTS^{\bullet+}$ solution was diluted with ethanol to arrive at a absorbance value of 0.70 (±0.02) at 734 nm. $ABTS^{\bullet+}$ solution (1 mL, with

absorbance of 0.700 ± 0.050) was added to the tested samples (0.1 mL, opportunely diluted) and mixed thoroughly. The reactive mixture was allowed to stand at room temperature for 2.5 min and the absorbance was immediately recorded at 734 nm.

Ethanolic solutions of known trolox concentrations were used for calibration. The results were expressed as millimoles of trolox equivalents TE kg^{-1} of dry weight.

2.6. ACE Inhibitory Activity

Evaluation of ACE inhibitory activity was assayed using the method described by Wu & Ding *et al.*[16], with slight modifications. A sample solution of 25 µL (500 mg mL^{-1}) was mixed with 50 µL of 5 mM N-Hippuryl-His-Leu substrate HHL solubilized in 0.1M borate buffer (pH 8.3) containing 0.3M NaCl, the mixture was incubated at 37 °C for 10 min. After that, 20 µL of ACE solution (100 mU mL^{-1}) was added. The mixture was then re-incubated for 60 min at 37°C. The reaction was stopped by adding 100 µL of 1M HCl, and the mixture was centrifuged at 14000 rpm (Thermo Fisher CL21R, Italy) for 10 minutes. The amount of hippuric acid (HA) in the supernatant was determined by reversed-phase HPLC (*Jasco MD-4016, Italy*) using a Kinetex C18 column (5 µm, 4.6 × 250 mm, Phennomenex, USA) and detected using an UV-VIS detector at 228 nm.

The mobile phase consisted of two solvents: solvent A was water with 0.1% trifluoroacetic acid and solvent B was acetonitrile with 0.1% trifluoroacetic acid. A linear gradient was used as follows: 0–8 min, 20%-50% B; 8–12 min, 50%–70% B; 12–14 min, 70%–20% B; 14–20 min, 20% B. The injection volume was 20 µL and the column temperature was 30°C. The flow rate of the mobile phase was 1 mL

min^{-1}. The percentage of ACE inhibition was calculated according to the following equation:

$$\% \text{ ACE inhibition activity} = \left[\frac{(\text{HA control} - \text{HA sample})}{\text{HA control}}\right] \times 100$$

where HA control and HA sample are the peak areas corresponding to HA for the blank and for an inhibitor sample, respectively.

2.7. Ferrous Ion Chelating Ability

The ferrous (Fe^{2+}) chelating ability was measured colorimetrically as described by Joshi *et al.* [17]. The solution of ferrous chloride 0.4 M (0.1 mL) was added to 1 mL (20 mg mL^{-1}) of freeze-dried fermented whey solubilized in distilled water followed by the addition of 0.2 mL of ferrozine 5mM to initiate the reaction. The mixture was vortexed and the tubes were kept at room temperature for 10 min. The absorbance values were measured at 562 nm. Glutathione 1 mg mL^{-1} was used as positive control. The ferrous ion chelating ability was calculated as follows:

$$\% \text{ chelating ability} = \left[\frac{(\text{Abs control} - \text{Abs sample})}{\text{Abs control}}\right] \times 100$$

where Abs control and Abs sample were the absorbance at 562 nm for the blank and for the sample, respectively.

2.8. Antifungal Activity Tests

Fungal inhibition was evaluated by the activity of the freeze-dried fermented whey (at 24, 48 and 72 h) against the different strains of *Penicillium*, *Aspergillus* and *Fusarium*. 70 µL of freeze-dried sample (0.5 g L^{-1}) re-suspended in potato

dextrose broth (PDB) were added into well of the plate containing PDA and fungal spores. The plates were maintained at 26 °C for 72 hours. [18] After the fungal growth was carried out the measurement of the diameter of the inhibition halo; halos larger than 8-10 mm being considered positive for antifungal activity. [19]

2.9. *Determination of Minimum Inhibitory Concentration and Minimum Fungicidal Concentration (MIC-MFC)*

The evaluation of antifungal activity of fermented whey by L*. plantarum* at 72h was determined using the method of Fothergill *et al.* [20] with some modifications. A positive control and a negative control were prepared with and without the presence of spores. A volume of 100 µL of spore ($5x10^4$ spores mL^{-1}) was added to 100 µL of sample (0.5 g L^{-1}) to obtain a final concentration range from 250 g L^{-1} to 1.95 g L^{-1}. The 96-well sterile microplates were incubated at 26 °C for 72 hours. Four replicates of each micro-assay were performed. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of freeze-dried fermented whey, in which the fungi did not show visible growth. The MFC was the lowest extract concentration in which visible growth of the subculture was prevented. The MFC was determined by planting 10 µL of each different concentration of sample in the PDA plate. The MFC value was determinated after 72 h of incubation at 25°C.[21]

2.10. *LC-ESI-qTOF-MS Separation and Identification of Phenolic Acids*

The extraction of the phenolic compounds was performed using the method described by Brosnan *et al.* [22], with some modifications. Ten milliliters of fermented whey were extracted with 10 mL of 1% of formic acid in ethyl acetate,

4 g of $MgSO_4$ and 1 g NaCl using vortex VWR international (Barcelona, Spain) for 2 minutes. The extract was centrifuged at 3000 rpm (Eppernord AG 22331, Hamburg, Germany) for 10 minutes, and the supernatant were treated and mixed using vortex for 2 minutes with 150 mg C18 and 900 mg $MgSO_4$. The samples were centrifuged at 3000 rpm (Eppernord AG 22331, Hamburg, Germany) for 10 minutes and the surnatant was completely dried using a continuous flow of nitrogen (Turbovap LV, Zymark Runcorn, UK). Finally, the sample was suspended in 1 mL of water:acetonitrile (90:10 v/v), filtered through a 0.22 µm nylon filter (Phenomenex, California, Stati Uniti) and stored for chromatographic analysis.

An Agilent 1200-LC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a vacuum degasser, auto-sampler, and binary pump, was used for the chromatographic determination. Chromatography separation was performed using a Gemini (C18 50 x 2 mm, 100 A, 3 µm) column (Phenomenex, Torrance, California, UK).

Mobile phases consisting of 1% acetic acid as solvent system A and acetonitrile as solvent system B and the following gradient elution was used: 0 min, 0.8% B; 5.5 min, 6.8% B; 16 min, 20% B; 20 min, 25% B; 25 min, 35% B; 29 min, 100% B; 32 min, 100% B; 34 min, 0.8% B; 36 min, 0.8% B. The column was equilibrated for 3 min prior to every analysis. The sample volume injected was 20 µL and the flow rate used was 0.8 mL min^{-1}.

MS analyses were carried out using a 6540 Agilent Ultra-High-Definition Accurate-Mass q-TOF-MS coupled to the HPLC, equipped with an Agilent Dual Jet Stream electrospray ionization (Dual AJS ESI) interface in negative ionization mode with the following conditions: drying gas flow (N2), 12.0 L min^{-1}; nebulizer pressure, 50 psi; gas drying temperature, 370°C; capillary voltage, 3500 V; fragmentor voltage, and scan range were 3500 V and *m/z* 50–1500, respectively.

Automatic MS/MS experiments were carried out using the following collision energy values: *m/z* 100, 30 eV; *m/z* 500, 35 eV; m/z 1000, 40 eV; and *m/z* 1500, 45 eV. Integration and data elaboration was performed using Mass Hunter Work station software (Agilent Technologies).

2.11. Statistical Analysis

Analysis of variance was carried out to assess whether the different experiments conducted to statistically different results for those variables evaluated. Statistical analysis of data was performed using the Tukey's test. Results are expressed as mean values ± s.e. mean. The chosen significance level was $P < 0.01$.

3. Results and discussion

3.1. ABTS Radical Cation Scavenging Assay

Scientific research highlights the antioxidant capacity of peptides derived from food proteins. These antioxidant compounds derived from proteins can offer a number of potential benefits and are also considered safer alternatives to synthetic antioxidants. Some of these peptides possess multiple biological activities, which can intensify their physiological efficacy and further reduce the disease risk.[23]

Moreover, peptides can act in synergy with non-peptide antioxidants improving their protective effect. The recovery of bioactive peptides obtained from whey could be useful for production of specific functional or nutraceutical foods.[24]

In this study, antioxidant activity was measured using ABTS assay and the results were expressed as millimoles of trolox equivalents TE kg^{-1} of dry weight (mmol trolox equivalent kg^{-1} of d.w.). Considering the ABTS assay, whey fermented with the strains L. *plantarum* 220 and L. *plantarum* 221 resulted in notably significant increases, in antioxidant activity, during the 72 h of treatment, from 1,384 to 2,083 mmol trolox equivalent kg^{-1} of d.w. and from 1,127 to 1,795 mmol trolox equivalent kg^{-1} of d.w., respectively (an increase of 34% and 37%, respectively). On the other hand, whey fermented with the strain L. *plantarum* 748, showed the highest activity after 24 h of fermentation (1,694 mmol trolox equivalent kg^{-1} of d.w.) showing at this fermentation time an increase of antioxidant activity of 20% compared to those of the sample at 72 h of fermentation (1,415 mmol trolox equivalent kg^{-1} of d.w.). Whey fermented by L. *plantarum* 220 and L. *plantarum* 221 demonstrated the maximum antioxidant activity compared to control at the same fermentation time. In particular, there was an increase in the activity of 27 % and 15 %, respectively for L. *plantarum* 220 and L. *plantarum* 221 at 72 h of treatment compared to control. Regarding L. *plantarum* 748, a high value of antioxidant activity was observed after 24 h of fermentation. In particular, at this time, an increase of 46 % compared to control was observed. The enhancement of radical scavenging activity may be attributed to the production of different bioactive compounds during fermentation of whey, which is reflected in the antioxidant properties especially for L. *plantarum* 220 and L. *plantarum* 221 (Fig 2).

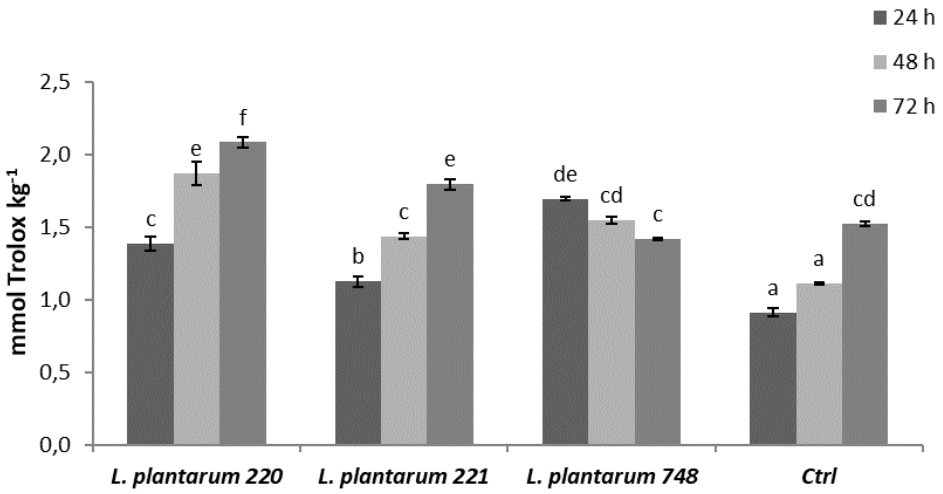

Fig 2. The radical scavenging activity of <3KDa whey fractions obtained during fermentation by lactic acid bacteria. In the figure, whey fermented with three strains of *L. plantarum* and control sample at triple fermentation (24, 48, 72 h).

In the literature it has been reported that L. *plantarum* strains are able to excrete different endopeptidases in fermentation media. The activities of various endopeptidases in medium containing proteins may result in the generation of different antioxidant compounds.[25]

Montoro *et al.* [26] analyzed the antioxidant activity of different fractions of two fermented goat milks (ultrafiltered goat milk with the classical starter bacteria or with the classical starter plus the *Lactobacillus plantarum* C4 probiotic strain) using different methods (ORAC, ABTS, DPPH and FRAP) and highlighted, according to our results, that the highest antioxidant capacity measured by ORAC

was found in the fraction of fermented milk derived from cation exchange membrane permeate with molecular weight < 3kDa.

Chen *et al.* [27] studied the antioxidant capacity of bovine milk and low molecular weight fractions (LMW) isolated from whey in order to evaluate how the pH change can influence the total antioxidant content. The result, assessed by ABTS assay, was an increase in the release of antioxidant compounds when pH increased from 6 to 7.4. Whey obtained from milk, with different percentages of fat, contains 235-554 µmol L^{-1} trolox equivalent at pH values of 6 at 7.4, respectively. The antioxidant activity of low-molecular-weight (LMW) fractions of whey was attributable to urate which was responsible for antioxidant activity using different antioxidant activity assays.

Osuntoki *et al.* [28] studied the antioxidant activity of fermented whey with *lactobacillus* isolated from Nigerian food products and demonstrated that the fermented whey showed strain-dependent DPPH radical scavenging activity. In particular, the highest level of antioxidant activity was observed in the whey fermented with the strain of *L. brevis* isolated from wara, a dairy product. This is probably due to the strain being better adapted to the milk substrate, in contrast to the other non-dairy isolates.

Aguilar-Taolá *et al.* [29] studied the antioxidant activity and various biological activities of the milk after fermentation with different strains of *Lactobacillus plantarum,* measuring antioxidative capacity from raw extracts (CE) and from peptide fractions (<3 and 3-10 kDa). In particular, the L. *plantarum* extract or their fractions showed an antioxidant activity ranging between 282.8-362.3 µmol equivalent trolox. The antioxidant capacity of fractions from fermented milks showed that <3kDa fractions gave significantly higher (234.1 µmol of trolox equivalents) capacity than 3 to 10 kDa fractions (210.3 µmol of trolox equivalents)

in the ORAC method, whereas no statistical difference was observed for the values obtained by ABTS method in both < 3kDa (108.8 µmol of trolox equivalents) and 3 to 10 kDa (111.4 µmol of trolox equivalents) fractions.

Though numerous studies have reported antioxidant activity in *lactobacillus*, a direct comparison of results is difficult because of the variety of methods used, the various ways in which the results are expressed, the use of non-standardized inoculum size and various other differences.

3.2. Ace Inibitory Assay

ACE is a central component of the renin-angiotensin system (RAS), which has an important function in cardiovascular homeostasis. In the RAS, the renin has the capability to cut the liver-derived precursor, angiotensinogen, into a decapeptide: angiotensin I. The enzyme ACE catalyzes cleavage of angiotensin I to angiotensin II (a vasoconstrictor factor). Furthermore, ACE inactivates the vasodilative peptides bradykinin and kallidin, and stimulates the release of aldosterone, which increases blood pressure.[30]

In this study, ACE inhibitory activity was measured on whey fermented by *L. plantarum* 220, *L. plantarum* 221 and L. *plantarum* 748 at the respective fermentation times (24, 48, 72h). The results obtained were expressed as the inhibition percentage compared to the control. The amount of hippuric acid released was evaluated by HPLC analysis. ACE inhibitory activity was expressed as the mean value from triplicate measurements.

As regards our results, in figure 3 the ACE inhibition activity evidenced by the fermented samples, ranged between 76 - 85% for the whey fermented by *L. plantarum* 220, 67-76 % for the whey fermented by *L. plantarum* 220 and 68-82% for the whey fermented by *L. plantarum* 748.

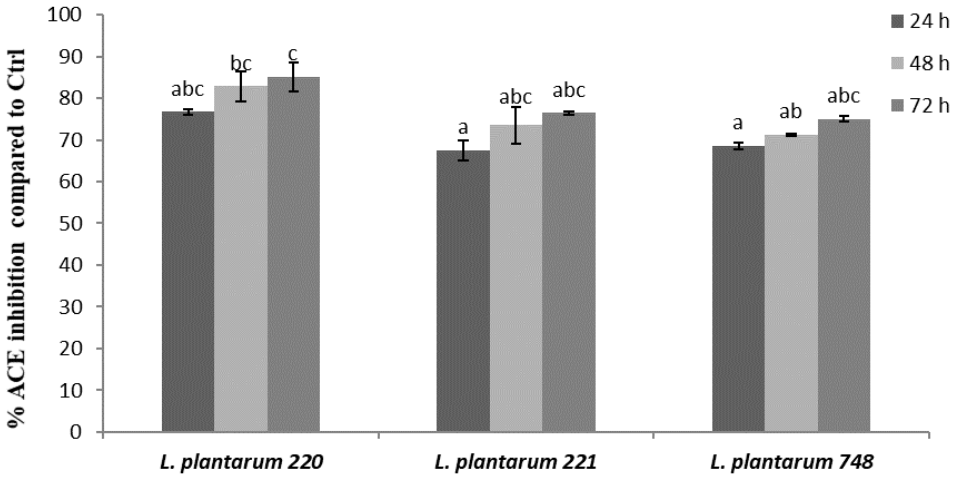

Fig 3. ACE inhibitory activity obtained from <3KDa whey fermented by three strains of *L. plantarum* at 24, 48 and 72 h. The results were expressed in percentage of inhibition compared to control.

For all samples, ACE inhibitory activity increased proportionally to the fermentation time (from 24 to 72 h), evidencing the highest activity at 72 h by the strain of L. *plantarum* 220 at 72 h of fermentation time (Fig. 3).

Pihanto *et al.* [31] studied fermented milk to test in vitro inhibitory activity towards angiotensin converting enzyme I (ACE) for 25 different LABs at different times of fermentation. The measure of ACE inhibitory activities varied from 5 to 74%, and 8 of the 25 strains did not produce measurable ACE inhibition.

Modification of fermentation conditions or pH control did not affect the ACE inhibitory activity and ACE inhibitory activity during fermentation could be closely connected to the bacterial growth and proteolysis.

Qian *et al.* [32] evaluated the antihypertensive characteristics of the skimmed milk fermented with *Lactobacillus delbrueckii ssp. bulgaricus* LB340. Four peptide fractions: 10-5 kDa, 5-3 kDa, 3-1 kDa and <1.0 kDa were investigated. The opportunely purified peptides (<1.0 k) showed the highest angiotensin I-converting enzyme inhibition activity with IC50 of 67.71 ± 7.62 mg mL^{-1}.

Hernandez-Ledesma *et al.* [33] reported ACE inhibitory activity of water-soluble extracts of fermented milk (WSEs) comparing the results with pasteurised and ultrahigh-temperature treated milks (UHT). The WSE of the fermented milk showed an inhibition percentage of 74.3%, whereas inhibition percentages of the WSEs obtained from pasteurised and UHT milk samples not fermented were below 2%. These results demostrate that fermentation plays an important role in the release of ACE-inhibitory peptides from milk proteins.

Pareira *et al.* [34] studied the ACE-inhibitory effect of a drink prepared with goat milk fermented by *Lactobacillus casei*. The drink showed no significant differences in terms of acidity compared to the control, but showed instead an increase in the inhibitory activity of ACE, due to proteolysis of the whey proteins over 24 h of fermentation. Significantly increased ACE inhibitory activity was found for the beverage with added probiotic L. *casei*. Analyzing the beverage on the first day of preparation, the results showed an ACE inhibitory activity almost comparable to captopril (ace-inhibitory drug). These results decreased after 21 days of storage.

The ACE inhibitory activity of fermented whey samples were in accordance with the reported literature. From the above, it is evident that, during

fermentation, especially with strains of *lactobacillus plantarum,* small peptides having potential biological activities, including the ACE inhibitory activity, were generated.

3.3. Ferrous Ion Chelating Ability

It has been recognized that transition metal ions are involved in many oxidation reactions in vivo. Ferrous ions (Fe^{2+}) can catalyze Haber–Weiss reaction and induce superoxide anion to form more hazardous hydroxyl radicals. Hydroxyl radicals react rapidly with the near biomolecules and cause severe damage. It has been observed that the scavenging of hydroxyl radicals by antioxidant compounds was efficacious mainly via the chelating of metal ions. In this study, the metal chelating ability was determined by measuring the reduction rate of red color, which was formed by ferrozine with ferrous ions. The iron-chelating activity was expressed as a percentage compared to the positive control represented by glutathione (1 mg/mL). Our results highlighted that in the whey fermented by L. *plantarum* 220 and L. *plantarum* 221 the iron-chelating activity increased linearly with the fermentation time, reaching a maximum percentage after 72 hours of fermentation (44.6 and 45.3%, respectively). The sample of whey fermented with L. *plantarum* 748 presented an inverse trend; in fact, the iron-chelating activity was the highest at 24 h (55.1%) and decreased by 18% with fermentation time (Fig 4).

Similar results were reported by Abubakr et al. [35] who evaluated the chelating activity of whey after fermentation with bacteria. They showed the highest iron binding capacity after 24 hours of fermentation, with a decrease from

97.6 to 41.8% in the following 48 hours, because of high number of phosphoseryl serine groups which have greater affinity for iron.

O'Loughlin *et al.* [36] investigated the iron chelating activity of two fractions of WPI at different degrees of hydrolysis (DH). They reported a 1 kDa permeate at 10% DH and a 30 kDa retentate at 5% DH. These results showed that the capability of chelating iron was 84.4 µM EDTA and 8.7 µM EDTA for 1 kDa and 30 kDa fractions, respectively. Thus, it was suggested that the lower the molecular weight of the WPI fractions, the higher iron chelating capability as shown by our experiments for the lower molecular weight fraction analysed (< 3 kDa).

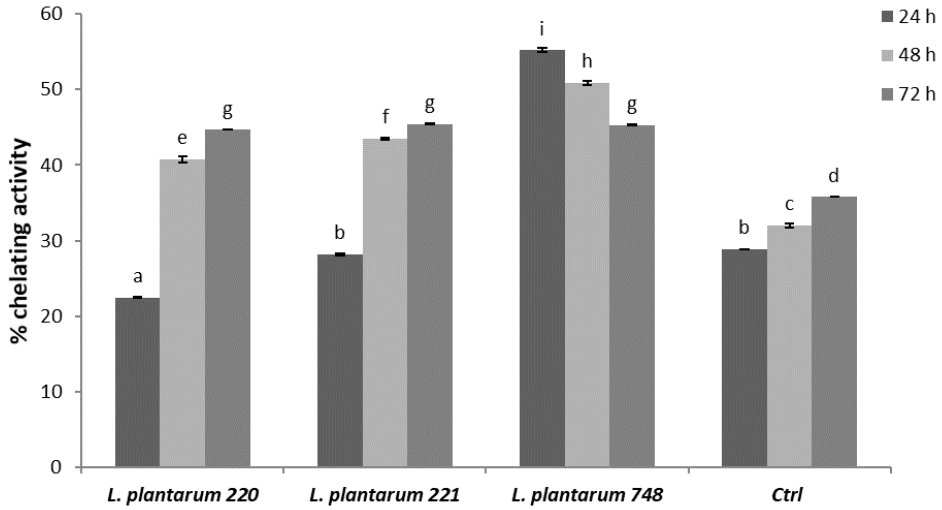

Fig 4. Iron chelating activity percentage of different samples of <3KDa whey fermented by *L. plantarum* (220, 221, 748) at three different times of fermentation 24, 48 and 72 h is illustrated in the figure.

An interesting result was also obtained by Silva *et al.* [37] who studied the iron chelation activity of whey protein hydrolized enzimatically using alcalase,

pancreatin or flavourzyme. The Fe–hydrolysate complexation reaction resulted in an increase in iron solubility at pH 7.0, from 0% to almost 100%. Fe-binding activity was attributable to peptides that were investigated by LC/MS.

Hur *et al.* [38] reported an overview of the iron chelating capability in plant-based foods and the correlation with the fermentation process. Fermentation induces the structural breakdown of plant cell walls, leading to the liberation or synthesis of various compounds responsible for this activity.

Probably, casein-derived compounds such as phosphopeptides possess amino acid residues more active in interacting and binding with metal ions. This could explain the mechanism of action in the chelation

3.4. Antifungal Activity, MIC-MFC of Freeze-dried Fermented Whey Obtained by Fermentation with Different Strains of Lactobacillus Plantarum Correlation with Organic Compounds.

The proteolytic systems of lactic acid bacteria (Gram-positive) are a considerable means of producing peptides and amino acids to milk proteins during fermentation.[39,40,41,42]

This systems contains: proteinases and peptidases. Proteinases are located in the cell wall and belong to the serine protease group, and they are able to make peptides starting caseins. Peptidases can be both extra and intra-cellular and are responsible for cleaving large peptides into small peptides.[43]

Probably, secondary metabolites products produced during fermentation by lactic acid bacteria, include organic acids, supply an unfavorable acid environment for the growth of pathogenic and deteriorating microorganisms. Acids are

generally responsible for the antimicrobial effect due by interfering with the maintenance of the cell membrane potential, reducing intracellular pH, inhibiting active transport and inhibiting a variety of metabolic functions.[44]

In Table 1, the results associated with antifungal activity on solid medium of PDA of freeze-dried fermented whey by different strains of *Lactobacillus plantarum* on 9 strains of mycotoxigenic *Aspergillus*, *Penicillium* and *Fusarium* strains, were plotted.

The results highlight that freeze-dried fermented whey by different strains of Lactobacillus *plantarum* do not have activity against mycotoxigenic Penicillium and Aspergillus strains at different fermentation time (24, 48, 72 h).

Table 1. Antifungal activity of freeze-dried whey obtained from fermentation with three different *Lactobacillus* in three different times of fermentation (24, 48, 72 h) using the antimicrobial assay on solid medium of PDA. Clearing zone (+) corresponding to 8 mm, (++) corresponding to 10 mm.

Fungi	***L. plantarum* 220**			***L .plantarum* 221**			***L. plantarum* 748**		
	24	**48**	**72**	**24**	**48**	**72**	**24**	**48**	**72**
***P. camemberti* CECT 2267**	-	-	-	-	-	-	-	-	-
***P. expansum* CECT 2278**	-	-	-	-	-	-	-	-	-
***P. roqueforti* CECT 2905**	-	-	-	-	-	-	-	-	-
***A. parasiticus* CECT 2681**	-	-	-	-	-	-	-	-	-
***A. flavus* ITEM 8111**	-	-	-	-	-	-	-	-	-
***A. niger* CECT 2088**	-	-	-	-	-	-	-	-	-
***F. moniliformis* CECT 2982**	-	++	++	-	++	++	-	++	++
***F. verticilliodes* CECT 20926**	++	++	++	++	++	++	-	++	++
***F. graminearum* CECT 20490**	+	++	++	+	++	++	+	++	++

As regards, the generum *Fusarium,* an antifungal activity was observed and, in particular, a clearing zone of 8-10 mm against mycotoxigenic fungi *F. graminearum*, *F. verticilliodes* respecively at 24 hours of fermentation time by *L. plantarum* 220 and 221 were exhibited. The sample fermented by *L. plantarum* 748 showed an antifungal activity on *F. verticilliodes* with 8 mm of clearing zone at 24 hours of fermentation time.

The antifungal activity increased with fermentation time (48, 72 h), and in particular on *F. moniliformis, F. graminearum* and *F. verticilliodes* with 10 mm of clearing zone at 72 h of fermentation time.

In Table 2, it is possible to observe the results related to the Minimum inhibitory Concentration and the Minimum Fungicidal Concentration (MIC-MFC) of freeze-dried fermented whey obtained with fermentation by different strains of *Lactobacillus plantarum* at 72 hours of fermentation time.

Table 2. Minimum inhibitory concentration and minimum fungicidal concentration expressed in g L^{-1} (MIC-MFC) evidenced by freeze-dried whey obtained by fermentation with three different *Lactobacillus* at 72 h of fermentation time, on several mycotoxigenic fungi; (a=g L^{-1}).

Fungi	*L. plantarum 220*		*L. plantarum 221*		*L. plantarum 748*	
	MIC[a]	MFC	MIC	MFC	MIC	MFC
P. camemberti	62,5	250	125	125	nd	nd
P. expansum	62,5	250	62,5	250	250	250
P. roqueforti	125	125	125	125	125	125
A. parasiticus	250	250	250	250	250	125
A. flavus	250	250	250	250	250	nd
A. niger	250	250	nd	nd	nd	nd
F. moniliformis	62,5	125	125	250	250	250
F. verticilliodes	62,5	62,5	125	125	250	250
F. graminearum	31,3	250	62,5	250	125	125

In particular, as regards the *Fusarium* strains, the MIC ranged from 31.3-250 g L^{-1}, whereas the MFC data ranged from 125-250 g L^{-1} of freeze-dried whey fermented by three types of *L. plantarum* (220, 221, 748). For *Penicillium* strains the MIC ranged from 62,5-250 g L^{-1}, while the MFC presented the value of 250 g L^{-1} necessary to obtain the minimun fungicidal concentration. Whey fermented by *L. plantarum* 748 evidenced the slightest antifungal activity, showing a MIC of 250 g L^{-1}. The highest antifungal activity was observed using the whey fermented by *L. plantarum* 220 against the *Fusarium* strains.

The antifungal activity of whey fermented with *Lactobacillus* has not yet been extensively studied. In particular, Gamba *et al.* [45] investigated the antifungal activity of permeate fermented whey with Kefir grain against *A. parasiticus* (a common fungal contaminant in food) that has the capability to produce aflatoxin B1. A fungicidal effect was obtained with 65% v/v of cell free surnatant in the culture medium at pH 4.55. In these condictions aflatoxin production was not detected.

Gamba *et al.* [46] studied the antifungal effect of whey fermented by Kefir grains (CIDCA AGK1) against three types of *Fusarium graminearum* (Fg36, Fg44, and Fg48). The percentage inhibition of the germination of *F. graminearum* at the different pHs (3.5; 4; 4.5) and different concentrations of whey-permeate cell-free supernatant showed that the maximum activity of about 70% was supplied at the lowest pH of 3.5. This confirms the hypothesis of antifungal inhibition related to pH because alkalinisation led to the gradual loss of antifungal activity.

Tulini *et al.* [47] investigated the role of lactic acid bacteria, isolated from different dairy products: milk and cheese samples from cow, buffalo and goat, in order to evaluate the correlation with the antimicrobial activity. The analyses showed that the most antifungal strain was *L. plantarum FT723* that inhibited

Penicillium expansum in a modified MRS agar and fermented milk model. Lactic acid bacteria could produce several antimicrobial compounds which make these bacteria interesting for food bio-preservation.

3.5. Identification of Phenolic Compounds in Freeze-dried Fermented Whey

Antimicrobial activity of lactic acid bacteria is associated with the production of different active molecules such as peptides, antifungal compounds (propionate, phenyl-lactate, hydroxyphenyl-lactate, cyclic dipeptides and 3-hydroxy fatty acids) and organic acids (lactic, acetic, formic, propionic and butyric acids). However, there are other mechanisms that are suspected of being involved in the inactivation or inhibition of the growth of other associated species of pathogens.[48]

The analysis of total phenolics compounds presented in the fermented whey led to the identifications of ten different compounds. In particular, in the whey fermented by *L. plantarum* 220 were detected: hydroxibenzoic acid, sinapic acid, benzoic acid and DL-3 phenillactic acid, whereas in the whey fermented by *L. plantarum* 221: ferulic acid, hydroxibenzoic acid, sinapic acid, benzoic acid and DL-3 phenillactic acid, were discovered. Finally, in the whey fermented by *L. plantarum* 748: caffeic acid, p-coumaric acid, salicilic acid, hydrocinnamic acid, DL-3 phenillactic and 1,2- dihydroxibenzene, were detected (Table 3).

The antifungal activity obtained from different types of whey fermented by *L. plantarum* was confirmed by the production of phenolic compounds. In our work of identification, we found ten different phenolic compounds. In particular, the predominant was DL-3 phenillactic (both L and D forms in a 9/1 ratio), generated during fermentation. These phenolic compounds, present in all types of

fermented whey have been widely studied by researchers for their fungal inhibition capability.[42]

Benzoic acid and sodium benzoate are antifungal agents and they are employed in industry to contribute to preservation of foods. Strains of *Lactobacillus acidophilus*, *Lactobacillus casei* and *Lactobacillus helveticus* used for milk fermentation can produce benzoic acid in fermented milk.[49,50]

Table 3. Phenolic compounds identified in freeze-dried whey obtained from fermentation with three different lactobacillus in three different times of fermentation (24, 48, 72 h) by LC-qTOF-MS.

Compound	Molecular formula	MW	*L. plantarum* 220	*L. plantarum* 221	*L. plantarum* 748
Gallic	$C_7H_6O_5$	170.1	-	-	-
Protocatechuic	$C_7H_6O_4$	154.1	-	-	-
Chlorogenic	$C_{16}H_{18}O_9$	354.3	-	+	-
Caffeic	$C_9H_8O_4$	180.2	-	-	+
Syringic	$C_9H_{10}O_5$	198.2	-	+	-
Vanillin	$C_8H_8O_3$	152.1	-	-	-
p-Coumaric	$C_9H_8O_3$	164.2	-	-	+
Ferulic	$C_{10}H_{10}O_4$	194.2	-	+	-
Hidroxibenzoic	$C_7H_6O_3$	138.1	+	+	-
Vanillic	$C_8H_8O_4$	168.2	-	-	-
Sallicilic	$C_7H_6O_3$	138.1	-	+	+
Hydrocinnamic	C_9H10O_2	150.2	-	-	+
Sinapic	$C_{11}H_{12}O_5$	224.2	+	+	-
Benzoic	$C_7H_6O_2$	122.1	+	+	-
DL-3-phenyllactic	$C_9H_{10}O_3$	166.2	+	+	+
Ácido Dihydroferulic	$C_{10}H_{12}O_4$	196.2	-	-	-
1-2 dihydroxybenzene	$C_6H_60_2$	110.1	-	-	+
3,4- dihydroxyhydrocinnamic	$C_9H_{10}O_4$	182.2	-	-	-
DL-p-hydroxyphenyllatic	$C_9H_{10}O_4$	182.2	-	+	-
3-(4-hydroxyphenyl) propionic	$C_9H_{10}O_3$	166.2	-	-	-

Lavernicocca *et al.* [40] studied the phenolic compound phenyllactic acid obtained during fermentation of wheat flour by *L. plantarum* strain 21 B. Phenyllactic acid was contained at high concentration in the bacterial culture filtrate. The results showed that this compound inhibited all the fungi tested at the concentration of 50 mg/ml^{-1} except for *P. roqueforti* IBT18687 and *P. corylophilum* IBT6978 (inhibitory concentration, 166 mg ml^{-1}).

Recently, Kwaw *et al.* [51] investigated the effect of lactic acid bacteria strains on the phenolic profile and antioxidant activities of mulberry juice. Fermentation process using *Lactobacillus plantarum* demonstrated that LABs impacted on the phenolic profile.

In addition, Valerio *et al.* [52] studied the organic acids in the lactic acid bacteria from semolina in order to find a correlation with the antifungal activity of bacterial strains. All strains isolated acidified the medium, leading to pH values ranging from 3.05 to 3.85, and they have the capability to produce organic acids. The inhibitory effect of pure lactic and acetic acids was tested. Results indicated an inhibitory effect of the medium composed of both organic acids: 99.9%, 75.5% and 97.4% inhibition against *A. niger*, *P. roqueforti* and *E. fibuliger*, respectively. Phenolic compounds showed microbicidal properties against different microorganisms.[53]

4. Conclusions

Nowadays, there is a growing demand for food safety. At the same time, the dairy industry suffers the problem of disposal of waste products such as whey. This paper represents a good starting-point as regard the valorization of the bioactivity derived from freeze-dried whey fermented by different strains of L.

plantarum in order to use it as a potential ingredient in food preparations. This process could be allow us to increase the shelf-life of food and consequently the use of natural ingredients derived as by-products from the dairy industry can be a good tool for promoting food safety.

It is, therefore, desirable to continue to expand our understanding of the effectiveness of fermented whey and lactic acid bacteria in order to estimate their efficacy for future applications in food model systems.

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3.3. A natural strategy to improve the shelf life of the loaf bread against toxigenic fungi: the employment of fermented whey powder

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1. Introduction

Whey milk obtained in the cheese production process is the main waste by-product of the dairy industry. The principal components of whey are lactose, protein, fat, calcium, phosphorus, organic acids and vitamins. Therefore, it is a good culture medium for the growth of bacteria in the laboratory. From a biotechnological point of view, the composition of whey allows the growth of microorganisms, including lactic acid bacteria (LAB), and its fermentation by them, reducing the biological demand for oxygen and obtaining products with a high added value for the food industry (Karwowska *et al.* 2014; Khem *et al.* 2016). A growing interest in whey has been noted (Figure 1).

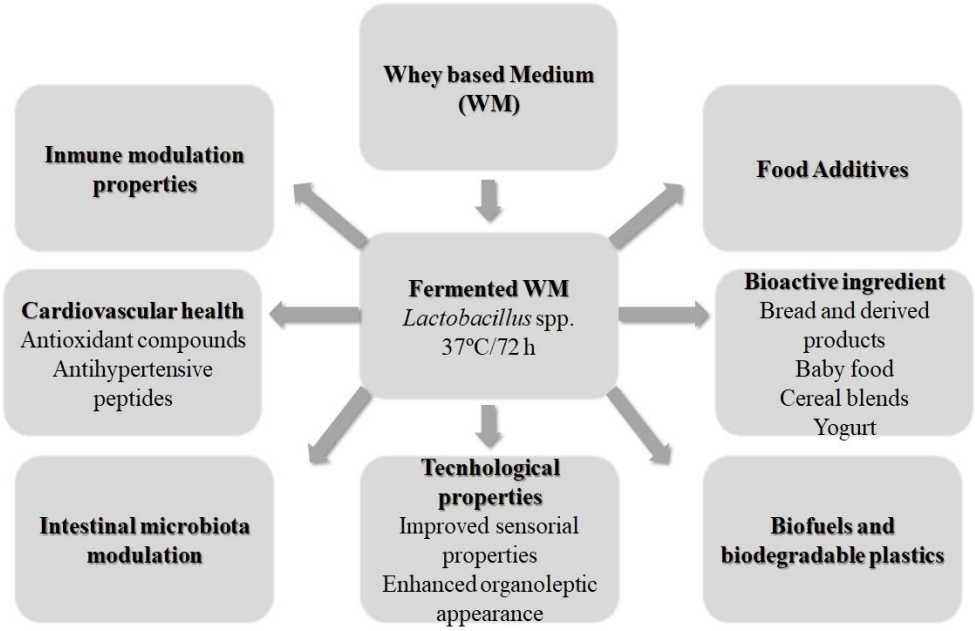

Figure 1. Technological and health promoting properties of the fermented WM with LAB.

During recent years, several studies have reported the beneficial effects of whey. On the one hand, whey protein used to supplement food improves the health of children, adults and the elderly (Stobaugh *et al.* 2016). The peptides generated from the enzymatic hydrolysis of whey proteins show biological effects *in vitro*, such as antimicrobial, antioxidant, antihypertensive and antidiabetic activity among others (Brandelli *et al.* 2015). On the other hand, recent studies evidenced that food containing LAB has beneficial effects (Alwan *et al.* 2014; Chen *et al.* 2014). The antifungal activity spectrum of LAB has been very well studied since the food industry showed interest in reducing the use of chemical preservatives to offer "additive-free" food (Arena *et al.* 2016; Russo *et al.* 2017).

Fungal growth is the most important factor limiting the shelf life of loaf bread and it is a big problem resulting in significant economic losses. The most dominant fungal species in this type of product are those of the genera *Penicillium* and *Aspergillus* (Marin *et al.* 2003; Garcia *et al.* 2019). Fungal contamination of bakery products usually is after bread processing (Suhr and Nielsen 2003; Smith *et al.* 2004). Although yeast contamination is not common, it can be observed in bakery products stored in a modified atmosphere (Deschuyffeleer *et al.* 2011).

Calcium propionate is used generally to prolong the microbiological shelf life of bread; in Europe, maximum limits of 0.1–0.3% have been established depending on the type of pre-packaged bread (European Union 2011; Belz *et al.* 2012). Instead, consumer concern about the negative effects of chemical food preservatives on health is increasing. Therefore, consumers demand of food biopreservation strategies are proliferating (Priftis *et al.* 2007; Shim *et al.* 2011).

The aims of this study were to evaluate a) the antifungal properties of whey-based medium (WM) fermented by several strains of LAB, b) characterization of the antimicrobial compounds produced during WM fermentation through LC-ESI-

qTOF mass spectrometry, and c) employment of the WM fermented by *L. plantarum* for improving the shelf life of loaf bread inoculated with *P. expansum*.

2. Materials and methods

2.1. Chemicals

Chromatographic solvents acetonitrile (ACN) and ethyl acetate (EA) were obtained from VWR Chemicals, and formic acid (FA) was obtained from Fischer Scientific UK. Dihydroferulic acid, DL-ρ-hydroxyphenyllactic acid, 3,4-dihydroxyhydrocinnamic, 1,2-dihydroxybenzene, benzoic acid, sinapic acid, hydroxycinnamic acid, vanillic acid, hydroxybenzoic acid, ρ-coumaric acid, vanillin, syringic acid, caffeic acid, chlorogenic acid and gallic acid were provided by Sigma Aldrich (Dublin, Ireland). Ferulic acid was purchased from MP Biomedicals (California, USA), protocatechuic acid from HWI Pharma Services (Ruelzheim, Germany), and DL-3-phenyllactic acid from BaChem (Weil am Rhein, Germany). All analytes had a purity of 95%.

Sodium chloride (NaCl), C18 and anhydrous magnesium sulphate ($MgSO_4$) were provided by Sigma Aldrich. The culture media potato dextrose broth (PDB), potato dextrose agar (PDA) and De Man Rogosa and Sharpe (MRS) were obtained from Liofilchem (Teramo, Italy). A Milli-Q purification system (Millipore, Bedford, MA, USA) was used to obtain Milli-Q water (< 18 MΩ cm resistivity). Gel electrophoresis equipment and molecular weight markers were provided by BioRad (California, USA).

Cow's milk whey powder was obtained from the company Nutriops S.L. (Murcia, Spain) in 440 g pot format. The nutritional composition of the whey

without chemical preservative is 71.5 g carbohydrate/100 g, 12.2 g protein/100 g, 0.9 g fat/100 g, and 2 g sodium/100 g.

2.2. Microbial strains and growth conditions

Strains of *Fusarium verticillioides* CECT 20926, *F. graminearum* CECT 20490, *F. moniliforme* CECT 2982, *Penicillium roqueforti* CECT 2905, *P. camemberti* CECT 2267, *P. expansum* CECT 2278, *Aspergillus parasiticus* CECT 2681 and *A. niger* CECT 2088 were obtained from CECT (Valencia, Spain). *A. flavus* ITEM 8111 was obtained from the ITEM microbial culture collection of the Institute of Sciences and Food Production (Bari, Italy). Fungal cryopreservation was carried out in sterile 30% glycerol at −80 °C. At the time of analysis, fungi were cultured first in PDB and later in PDA at 25 °C to obtain spores.

The LAB used in this study were also obtained from CECT: *Lactobacillus plantarum* CECT 220, *L. plantarum* CECT 221, *L. plantarum* CECT 223, *L. plantarum* CECT 224, *L. plantarum* CECT 748, *L. plantarum* CECT 749 and *L. plantarum* CECT 750. Bacterial cryopreservation was carried out in sterile 30% glycerol at −80 °C.

After the recovery period, in MRS broth at 37 °C for 48 h under anaerobic conditions, bacteria were inoculated at a concentration of 10^8 CFU/mL in WM and incubated for 24, 48 and 72 h at 37 °C. WM was constituted by 10% whey powder and 90% sterile water. After the incubation period, fermented WM was centrifuged in an Eppendorf 5810R centrifuge (Hamburg, Germany) at 4000 rpm for 10 min at 4 °C. The cell-free supernatant (CFS) of fermented WM was dried using a FreeZone 2.5 Liter Benchtop Freeze Dryer (Labconco, Missouri, USA) and stored at −19 °C.

2.3. Molecular mass estimation

The degree of protein hydrolysis of WM fermented with LAB for 24, 48 and 72 h was analyzed by SDS-PAGE using a 15% (w/v) separating gel and 4% stacking gel (El-Ghaish *et al.* 2010). For visualization of protein bands, gels were stained in a staining solution: 0.1% Brilliant Blue R-250, 50% water, 40% methanol and 10% acetic acid. Molecular mass of proteins was evaluated by comparison to the BioRad protein ladder standards Precision Plus Protein (high MW range) and Natural Polypeptide SDS-PAGE (low MW range).

2.4. Determination of phenolic acids by HPLC-ESI-Q-TOF-MS

The fermented WM was purified using the QuEChERS method before phenolic acid analysis (Brosnan *et al.* 2014). The purification method consisted of two steps. In the first, 10 mL of fermented WM was vortexed for 1 min then extracted with 10 mL EA (1% FA) and salts (4 g of $MgSO_4$ and 1 g of NaCl). In the second step, after centrifugation, 150 mg C18 and 900 mg $MgSO_4$ were added to the supernatant and the mixture was vortexed for 1 min. Finally, samples were centrifuged again, and the supernatants were evaporated under nitrogen flow. Before chromatographic analysis, samples were resuspended in 1 mL of 10% ACN and filtered with a 0.22 μm pore size filter.

Chromatographic analyses were performed by an Agilent 1200 (California, USA), which consisted of a autosampler, vacuum degasser, and binary pump. A Gemini C18 column (50 × 2 mm, 100 Å and particle size 3 μm; Phenomenex) was used for chromatographic separations. The mobile phase consisted of 0.1% FA in water (solvent A) and 0.1% FA in ACN (solvent B). For the analysis of phenolic acids, the optimized elution gradient was the following: 0 min, 5% B; 30 min, 95%

B; 35 min, 5% B. The system was re-equilibrated for 3 min; the total run-time was 37 min. Sample elution was at flow rate of 0.3 mL/min and the sample volume injected was 20 µL.

Mass spectrometry analyses were performed by using a Q-TOF-MS (6540 Agilent Ultra High Definition Accurate Mass) coupled to an Agilent Dual Jet Strem electrospray ionization (Dual AJS ESI) interface operating in the negative ion mode. The following parameters were: fragmentor voltage was 175 V; capillary coltage 3.5 kV; nebulizer pressure 30 psig; and drying gas flow (N_2) 8.0 L/min and temperature 350 ºC. Targeted MS/MS experiments were carried out using collision energy of 10, 20 and 40 eV. Integration and data elaboration were managed using Masshunter Qualitative Analysis Software B.08.00 (Denardi-Souza et al. 2018).

2.5. Determination of antifungal activity by agar diffusion method

100 µL of fermented WM for 72 h suspended in PDB at a concentration of 250 mg/mL was added to PDA plates contaminated with fungal spores. Plates were incubated at 25 °C for 3 days (Madhyastha *et al.* 1994). After that, we studied growth inhibition by measuring the diameter of inhibition halos, considering as positive only halos larger than 8 mm (Castlebury *et al.* 1999).

2.6. Determination of antifungal activity by microdilution method

100 µL of fermented WM for 72 h suspended in PDB at final concentration from 0.5 to 250 mg/mL was added to sterile microplates (96 well). After, all wells except negative control (non-contaminated PDB medium) were contaminated

with 100 µL of a 5 × 10^4 spores/mL suspension of the toxigenic fungi. The positive control consisted of contaminated medium with non-fermented WM (250 mg/mL). After that, microplates were incubated at 25 ºC for 72 h. The minimum inhibitory concentration (MIC) was considered as the concentration of fermented WM for which no visible fungal growth was observed on wells (Fothergill 2012).

After determining the MIC, concentrations equal to and higher than the MIC were cultivated on PDA plates to determine the minimum fungicidal concentration (MFC). After incubation (3 days at 25 °C), the MFC was the concentration at which no visible fungal growth was evidenced. Four replicates of each assay were carried out.

2.7. Baking and bread treatment

The loaf bread samples were elaborated by following the recipe: 300 g of wheat flour, 175 g of tap water, 20 g of yeast for bakery products (Levital, Spain), 10 g of sucrose and 5 g of NaCl. Doughs were prepared by replacing 50% (WM50) and 100% (WM100) of the total water in the bread with non-lyophilized WM fermented for 72 h by the selected strain. To compare the inhibition of fungal growth, we also made a control bread without additives, and a commercial control bread with 0.2% calcium propionate.

After that, the doughs were homogenized in a bakery machine (Silver Crest) for 15 min and fermented for 1 h at 25 °C. In total, four doughs of bread samples for each test were placed on a perforated greased plate and baked at 200 °C for 40 min in a Memmert ULE 500 muffle furnace (Madrid, Spain). After baking, bread samples were cooled to room temperature (20–22 °C, 1 h approximately). Then,

breads were cut into 30 g slices and immediately prepared to be inoculated with *P. expansum* fungi. Nine replicates for each typology of bread were inoculated in nine spots with 100 µL of a suspension containing 3×10^5 spores/mL. Finally, they were packed in sealed low-density polyethylene bags with the help of a Sammic TS-150 thermosealer (Basarte, Spain). All plastic bags were closed hermetically and incubated at room temperature for 7 days. During the preservation period, visual control of the surface of the inoculated bread was carried out daily, identifying at a glance the possible growth or not of fungus and to establish the effect of the treatment on the shelf life (Dal Bello *et al.* 2007).

2.8. Antimicrobial activity: determination of the fungal load of bread

Once the period of preservation of the bread samples was exceeded, a microbiological study was carried out. After 7 days of incubation, 40 g of bread was homogenized with 360 mL of 0.1% peptone water, previously autoclaved, in an IUL Stomacher (Barcelona, Spain) for 30 s. From that, three serial decimal dilutions were prepared in glass tubes with 9 mL of peptone water. Subsequently, 100 µL from each tube was plated out in PDA culture medium plates. The plates were incubated at 26 °C, and the number of viable colonies was counted after 72 h of incubation (Torrijos *et al.* 2019).

3. RESULTS AND DISCUSSION

3.1. Identification of phenolic compounds and protein fraction obtained by WM powder fermented by several strains of L. plantarum

The phenolic acids determined in the fermented WM are shown in Table 1. In particular, it is possible to show in the results that the strain of *L. plantarum* that produced the lowest amount of phenolic acids was strain CECT 220, considering that just four antimicrobial compounds were identified. Considering the compounds detected, we have to underline the importance from a microbiological point of view of hydroxybenzoic acid and DL-3-phenyllactic acid (Barman *et al.* 2017; Taofiq *et al.* 2017).

In particular, Barman *et al.* (2017) studied growth reduction of *Mucor* sp., *Penicillium digitatum* and *Trichophyton rubrum* by using the CFS of *L. plantarum* at a concentration of 10 mg/mL. In addition, they evidenced the presence of lactic acid, phenyllactic acid and other compounds unidentified in CFS. This experiment showed the high potential of the strain *L. plantarum* to improve the shelf life of bread contaminated by *Bacillus subtilis* and *Mucor* sp.

The strain of *L. plantarum* that showed the highest capacity to produce phenolic compounds (nine compounds, including several antimicrobial molecules like chlorogenic acid, syringic acid, benzoic acid and DL-3-phenyllactic acid) was strain CECT 221. The other strains of *L. plantarum* produced between four and six antimicrobial phenolic compounds.

Figure 2 shows the SDS-PAGE separation gel for the WM powder protein fraction after fermentation of the matrix with different strains of *L. plantarum* for 24, 48 and 72 h. In particular, it is possible to observe a decrease in the band intensity of the proteins with a molecular weight ranging from 100 to 250 kDa, and the decrease in the protein fraction observed is proportional to the incubation time. More assays have to be performed to understand the possible peptide fraction that could be responsible also for the antifungal activity.

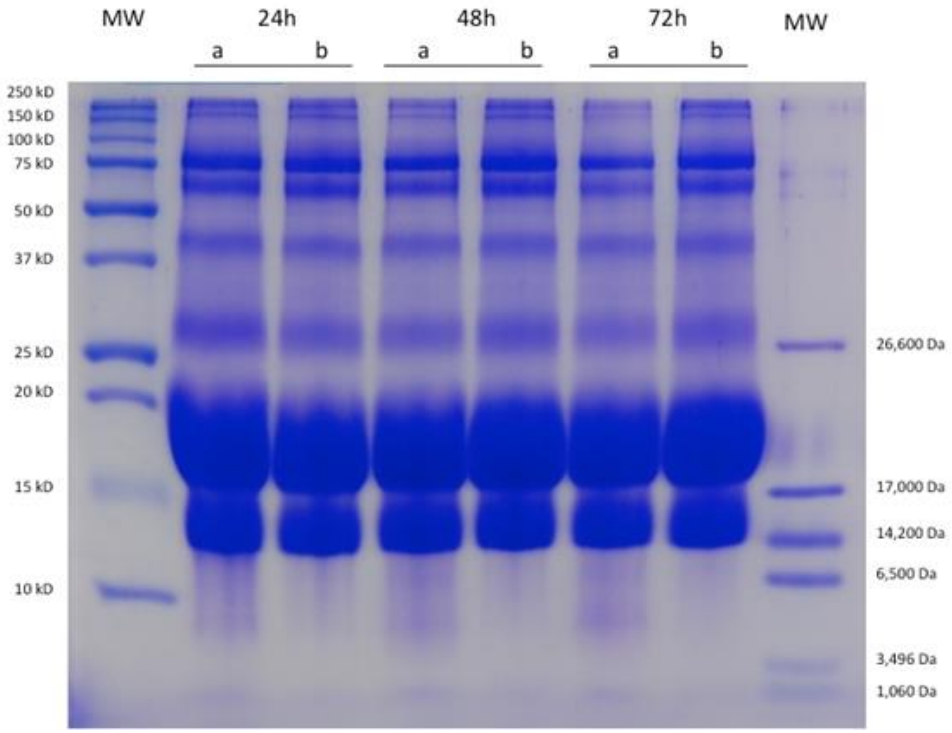

Figure 2. SDS-PAGE separation of WM proteins fermented by a) *L. plantarum* CECT 221 and b) *L. plantarum* CECT 750 at 24, 48 and 72 h incubation.

Table 1. Phenolic compounds identified in lyophilized crude extract, through LC-qTOF-MS, obtained from WM fermented by seven strains of *L. plantarum*.

Compound	Molecular formula	MW	*L. plantarum* 220	*L. plantarum* 221	*L. plantarum* 223	*L. plantarum* 224	*L. plantarum* 748	*L. plantarum* 749	*L. plantarum* 750
Gallic	$C_7H_6O_5$	170,12	-	-	-	-	-	-	-
Protocatechuic	$C_7H_6O_4$	154,12	-	-	-	-	-	-	-
Chlorogenic	$C_{16}H_{18}O_9$	354,31	-	+	-	-	-	-	-
Caffeic	$C_9H_8O_4$	180,16	-	-	-	-	+	+	-
Syringic	$C_9H_{10}O_5$	198,17	-	+	-	-	-	+	+
Vanillin	$C_8H_8O_3$	152,13	-	-	-	-	-	+	+
p-Coumaric	$C_9H_8O_3$	164,16	-	-	-	-	+	+	+
Ferulic	$C_{10}H_{10}O_4$	194,18	-	+	+	+	-	-	+
Hidroxibenzoic	$C_7H_6O_3$	138,12	+	+	+	-	-	-	-
Vanillic	$C_8H_8O_4$	168,15	-	-	-	-	-	-	+
Sallicilic	$C_7H_6O_3$	138,12	-	+	+	+	+	-	-
Hydrocinnamic	C_9H10O_2	150,17	-	-	-	-	+	-	-
Sinapic	$C_{11}H_{12}O_5$	224,21	+	+	+	+	-	-	+
Benzoic	$C_7H_6O_2$	122,12	+	+	+	+	-	-	-
DL-3-phenyllactic	$C_9H_{10}O_3$	166,17	+	+	+	-	+	-	-
Dihydroferulic	$C_{10}H_{12}O_4$	196,2	-	-	-	-	-	-	-
1-2 dihydroxybenzene	$C_6H_60_2$	110,11	-	-	-	-	+	-	-
3,4-dihydroxyhydrocinnamic	$C_9H_{10}O_4$	182,17	-	-	-	-	-	-	-
DL-p-hydroxyphenyllatic	$C_9H_{10}O_4$	182,17	-	+	-	-	-	-	-
3-(4-hydroxyphenyl) propionic	$C_9H_{10}O_3$	166,17	-	-	-	-	-	-	-

3.2. Antifungal activity and MIC/MFC of WM fermented with LAB

Table 2 shows the data related to the antimicrobial activity of the WM fermented by several bacteria on nine different toxigenic fungi of the genera *Penicillium*, *Aspergillus* and *Fusarium*, using antimicrobial tests on solid PDA medium. The fermented WM produced through a biotechnological fermentation process using *L. plantarum* strain CECT 221 presented antifungal activity against *P. camemberti* and *P. expansum* and also on the three *Fusarium* strains tested, whereas the ingredient tested did not show any antimicrobial effect on the strains of *P. roqueforti* and *Aspergillus* used. The WM fermented by *L. plantarum* CECT

220, 223, 224, 748, 749 and 750 all showed the same antifungal effect, in particular on the three *Fusarium* strains used in this study.

Table 2. Antifungal activity on solid medium of PDA of whey protein fermented with 7 strains of lactic acid bacteria and against several toxigenic fungi.

Fungi	Lactic Acid Bacteria																				
	L. plantarum 220			*L. plantarum 221*			*L. plantarum 223*			*L. plantarum 224*			*L. plantarum 748*			*L. plantarum 749*			*L. plantarum 750*		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
P. camemberti	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. expansum	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. roqueforti	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A. parasiticus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A. flavus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A. niger	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F. moniliformis	-	+	+	-	+	+	+	+	+	-	+	+	-	+	+	+	+	+	-	+	+
F. verticillioides	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	-	+	+
F. graminearum	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-	-	-

The MIC and MFC of the WM fermented by different strains of *Lactobacillus* spp. for 72 h are summarized in Table 3. In particular, for the *Penicillium* strains, the MIC and MFC of the WM ranged from 15.6 to 250 mg/mL. The highest antimicrobial activity was evidenced by the strains *L. plantarum* CECT 221, 749 and 750, with an MFC of 62.5 mg/mL, whereas the lowest activity was evidenced by the strains *L. plantarum* CECT 220, 223 and 224, for which the MFC detected was 250 mg/mL. Analyzing the results related to the antimicrobial activity of the WM fermented by LAB on *Aspergillus* strains, it is possible to observe that the activity presented by the fermented matrices is lower than for the *Penicillium* strains, due probably to some resistance factors typical of the genus *Aspergillus* against many antimicrobial compounds. In particular, for *A. parasiticus*, the MIC and MFC for all the fermented WM were 250 mg/mL, and these data were also confirmed on the strain of *A. flavus*. The fermentation matrices produced by *L.*

plantarum CECT 748 and 749 did not show any antifungal activity on the toxigenic strain tested. On the strain *A. niger*, only the fermentation matrix produced by *L. plantarum* CECT 220 showed MIC and MFC at 250 mg/mL, whereas the other fermentation matrices produced by the other strains of LAB did not show any antifungal effect on these toxigenic fungi.

Tabla 3. MIC-MFC expressed in mg/mL evidenced by the extract obtained by the whey protein fermented with 7 strains of lactic acid bacteria and against several toxigenic fungi.

Lactic acid bacteria	*P. camemberti*		*P. expansum*		*P. roqueforti*		*A. parasiticus*		*A. flavus*		*A. niger*		*F. moniliformis*		*F. verticillioides*		*F. graminearum*	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
***L. plantarum* CECT 220**	62.5	250	62.5	250	125	125	250	250	250	250	250	250	62.5	125	62.5	62,5	31.3	250
L. plantarum CECT 221	125	125	15.6	62.5	125	125	250	250	250	250	nd	nd	125	250	125	125	62.5	250
***L. plantarum* CECT 223**	62.5	250	125	250	nd	nd	250	250	250	250	nd	nd	250	250	125	125	62.5	250
***L. plantarum* CECT 224**	62.5	250	62.5	250	nd	nd	250	250	250	250	nd	nd	62.5	250	62.5	125	62.5	250
***L. plantarum* CECT 748**	nd	nd	250	nd	125	125	250	250	nd	nd	nd	nd	250	250	250	250	125	125
***L. plantarum* CECT 749**	125	125	125	250	125	125	250	250	nd	nd	nd	nd	125	250	125	125	62.5	62.5
***L. plantarum* CECT 750**	125	125	62.5	250	125	125	250	250	250	250	nd	nd	125	250	125	125	62.5	62.5

Related to the antimicrobial activity of the WM fermented by LAB on the *Fusarium* strains, the fermented matrices presented an antifungal effect similar to the activity shown for *P. camemberti* and *P. expansum*; in particular, the MIC and MFC ranged from 31.3 to 250 mg/mL. The most active fermented matrices were produced by *L. plantarum* CECT 749 and 750 which had MIC and MFC of 62.5 mg/mL for the strain *F. graminearum*. The WM fermented by *L. plantarum* CECT 748 showed the lowest antifungal activity, in particular on the strains *F. moniliformis* and *F. verticillioides*, for which the MIC and MFC was 250 mg/mL, respectively.

Gamba *et al.* (2016) reported that whey obtained from the fermentation of milk by the kefir grains CIDCA AGK1 showed antifungal activity against *F.*

graminearum, and the production of zearalenone was not detected in the treatment of higher concentrations of whey.

3.3. Shelf life improvement of bread treated with fermented WM

Whey is considered the main residue of the cheese and dairy industry. It has a high nutritional value and can be used to obtain valued products such as lactic acid, biodegradable polymers and bioethanol, among others. In the first case, the fermentative processes of whey carried out by bacteria and yeasts have been directed to obtain compounds of interest and to produce new ingredients and/or foods (Pescuma *et al.* 2015).

Related to the results for the shelf life of the breads treated with 0.2% calcium propionate (E282) and WM fermented with *L. plantarum* CECT 221 as a replacement for the water used for bread dough preparation, the data are shown in Table 4. On the one hand, the control bread without any additives showed visible fungal growth after 3 days. Nevertheless, the commercial control bread with additive E282 and WM50 bread evidenced an increment in shelf life of 1 day compared to the control bread (Figure 3). On the other hand, the WM100 bread showed fungal growth at 5 days incubation. Therefore, the replacement of all water by fermented WM provided an increment in shelf life of 1 and 2 days, respectively, in comparison with the commercial control bread and WM50 bread.

Table 4. Shelf life monitored in days, of the bread loaves contaminated with *P. expansum* and treated with whey calcium propionate at 0.2 % (E282), WM fermented with *L. plantarum* CECT 221 in replacing of the 50 and 100 % respectively of the water used for the bread dough preparation. The treatments where also compared with a control test consisting of bread loaves contaminated with *P. expansum* without any preservative ingredient.

Treatments	Days						
	1	2	3	4	5	6	7
Control	-	-	+	+	+	+	+
E282	-	-	-	+	+	+	+
WM50%	-	-	-	+	+	+	+
WM100%	-	-	-	-	+	+	+

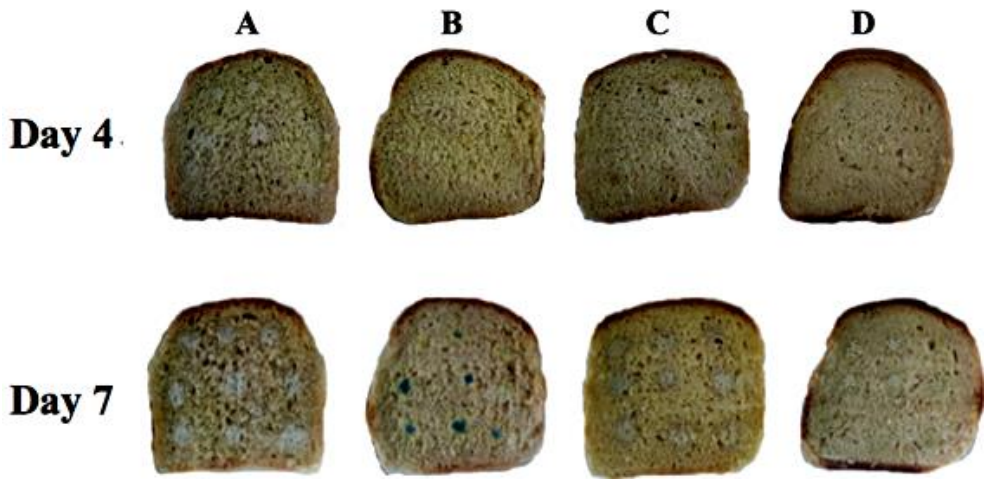

Figure 3. Bread loaves contaminated with *P. expansum* a) control and treated with b) 0.2 % of calcium propionate c) and d) WM fermented with *L. plantarum* CECT 221 in replacing of the 50 and 100 % respectively of the water used for the bread dough preparation, at 4 and 7 days of incubation.

The data shown for the visible shelf life of the bread loaves treated with WM fermented by *L. plantarum* and contaminated with *P. expansum* were also confirmed by microbiological analysis of the population of fungal contaminants in the food matrix studied. In particular, the control bread at 7 days incubation presented a fungal population of 5.1 log CFU/g, whereas for the commercial control bread it was 4.6 log CFU/g, the difference of 0.5 log CFU/g in comparison with the control bread being statistically significant. The treatment that produced the smallest fungal charge at 7 days incubation was WM100, for which the *P. expansum* population detected was 4.5 log CFU/g, a statistically significant fungal reduction of 0.2 and 0.6 log CFU/g in comparison with the WM50 bread and control bread, respectively. No significant differences were observed with respect to bread with additive (Figure 4).

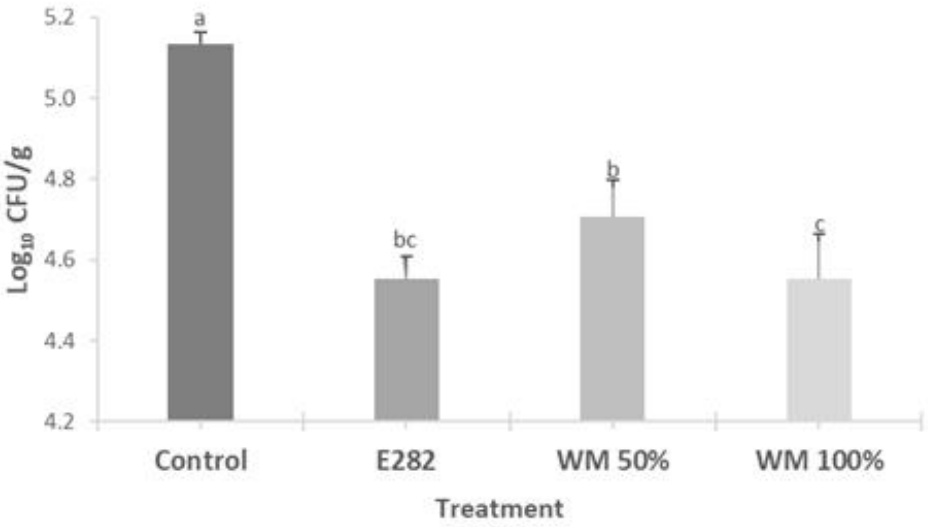

Figure 4. Population of the *P. expansum* in bread loaves treated with calcium propionate at 0.2 % (E282), WM fermented with *L. plantarum* CECT 221 in replacing of the 50 and 100 % respectively of the water used for the bread dough preparation, in comparison with the control bread.

Microbial fermentation is one of the oldest and most economically and ecologically friendly methods of preserving foods (Zannini *et al.* 2012). In this context, the use of chemical compounds is not recommended, and natural solutions are frequently proposed for food preservation. Nowadays, consumers are looking for natural products and less processed and clearly safer products. In this context, the possibility of obtaining antifungal compounds, deriving from a fermentation process, capable of reducing the percentage of fungal growth represents a growing interest as an alternative to chemical preservation. Several reviews have examined the antifungal activity spectrum of LAB and production of secondary metabolites, as well as their interactions with mycotoxins (Dalie *et al.* 2010; Crowley *et al.* 2013).

Dal Bello *et al.* (2007) showed improve the shelf life of breads using the antifungal strain *L. plantarum* FST1.7. The production of lactic acid, phenyllactic acid and two cyclic dipeptides (L-Leu-L-Pro and L-Phe-L-Pro) in the culture media and sourdough were directly related to the antifungal activity. (Ryan *et al.* 2009a, 2009b).

4. Conclusions

Whey powder fermentation using different strains of *L. plantarum* produced several phenolic acids and possible peptides; their maximum antimicrobial activity in bread against *P. expansum* was shown on the fifth day of treatment, with an increment in shelf life of the bread treated.

The whey powder fermented by strains of *L. plantarum* demonstrated a high antifungal activity against several toxigenic fungi of the genera *Aspergillus*, *Fusarium* and *Penicillium* on solid and liquid media. This study demonstrates the

importance of whey as a culture medium for *L. plantarum,* and the production of bioactive metabolites with antimicrobial activity. Further studies will be focused on identification and characterization of the peptide fraction produced during whey fermentation and its possible application as a food ingredient and for nutraceutical purposes.

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3.4. Antifungal and antimycotoxigenic activity of hydrolyzed goat whey on *Penicillium* spp: An application as biopreservation agent in pita bread

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1. Introduction

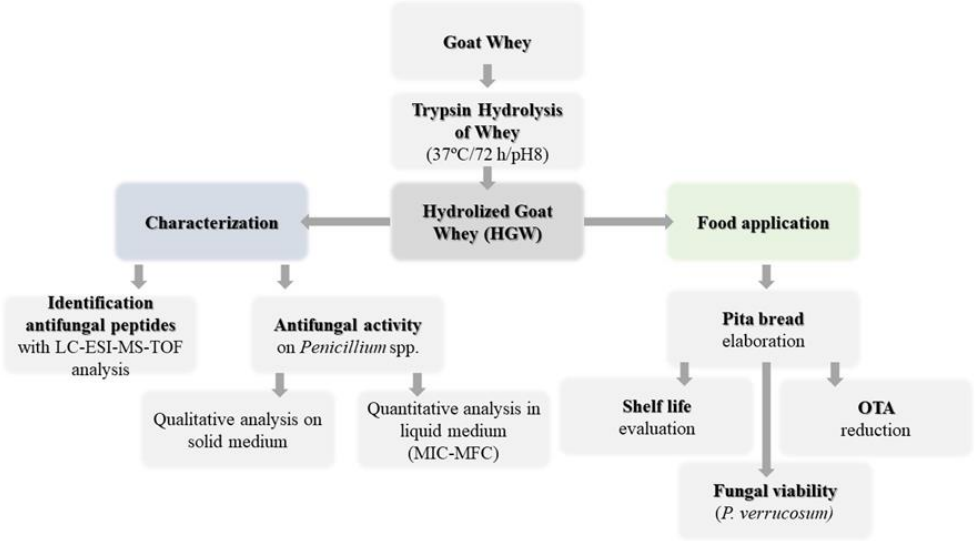
Toxigenic fungal growth in food and the production of mycotoxins pose serious health concerns and cause severe economic losses. Many foods are highly susceptible to spoilage by fungal growth, such as fruits, vegetables, dairy products, meat and bakery products, and their derived products (Adeyeye, 2016). In wheat-based bakery products, deteriorations have been attributed to several fungal genera, largely *Penicillium*, *Aspergillus*, *Cladosporium* and *Neurospora* (Ashiq et al., 2015). Some of the toxins produced by these fungi are carcinogenic, probably carcinogenic and possibly carcinogenic to humans. Ochratoxin A (OTA) is a compound produced as a secondary metabolite of moulds belonging to the genera *Penicillium* and *Aspergillus* (Molinié et al., 2005) and it is classified in Group 2B by the International Agency for Research on Cancer (IARC) because it is possibly carcinogenic to humans (IARC, 1993).

Bioactive peptides exert various health benefits, such as angiotensin-converting enzyme (ACE) inhibition, antioxidant activity, antithrombotic activity, immune system stimulation, antimicrobial and antifungal properties (Welderufael et al., 2012; Pihlanto, 2011). Hydrolysis by specific enzymes or fermentation with proteolytic strains is necessary to release the bioactive peptides from the parent proteins (Foegeding et al., 2002). Several studies have investigated the formation of antimicrobial peptides by digestion of goat whey proteins using various proteolytic enzymes (Osman et al., 2016; Atanasova & Ivanova, 2010).

Cheese manufacturing generates a high-strength biochemical oxygen demand waste stream, whey, an environmental pollutant (Ryan & Walsh, 2016). Worldwide whey production is estimated at around 190×10^6 t/year and growing. Over the last few years, there has been a growing interest in researching and developing natural antimicrobial agents derived from by-products of the food

industry to inhibit the growth of fungi in food (Ribes et al., 2018). This double-edged approach addresses the health concerns associated with synthetic food additives by replacing them with natural compounds, promoting healthier food products while, at the same time, assists in preventing and reducing food waste (Hati et al., 2018; Gamba et al., 2015).

Fig. 1. Workflow of the experiments carried out to study the possible application of the HGW as preservative agent in pita bread.



Based on reported evidence, this study aims to increase the shelf-life of bakery products using a hydrolysed whey product as a natural preservative. To achieve this objective, it (i) evaluated the antifungal activity of trypsin-catalysed whey hydrolysates against *Penicillium* spp., (ii) characterised the antifungal peptides produced during the hydrolysis of goat whey, and (iii) incorporated the goat whey hydrolysates (HGW) as a bio-preservative in bread preparation to prevent fungal growth of *Penicillium verrucosum*. The workflow of the experiments carried out in this study is depicted in Fig. 1.

2. Materials and methods

2.1. Chemicals and microorganisms

A Milli-Q purification system (Millipore, Bedford, MA, USA) was used to obtain Milli-Q water (<18 MΩ/cm resistivity). Culture media, including buffered peptone water, potato dextrose broth (PDB) and potato dextrose agar (PDA), were obtained from Liofilchem (Teramo, Italy).

All analytes had a purity of 95%, and the chromatographic solvents acetonitrile (ACN), methanol (MeOH) and formic acid (FA) were obtained from VWR Chemicals (Radnor, PA, USA). Gel electrophoresis equipment and molecular weight (MW) markers were provided by Bio-Rad (Hercules, CA, USA). β-Lactoglobulin (β-Lg), α-lactalbumin (α-La) and trypsin from porcine pancreas with an activity of 1080 BAEE U/mg protein, were obtained from Sigma–Aldrich (Dublin, Ireland).

For the evaluation of the antifungal activity, the mycotoxigenic strains used, namely, *Penicillium camemberti* CECT 2267, *Penicillium roqueforti* CECT 2905, *Penicillium digitatum* CECT 2954, *Penicillium nordicum* CECT 2320, *Penicillium pinophilum* CECT 2912, *Penicillium commune* CECT 20767, *Penicillium brevicompactum* CECT 2316 and *Penicillium solitum* CECT 20818, were purchased from the Colección Espanola de Cultivos Tipo (CECT) (Valencia, Spain). *Penicillium verrucosum* VTT D-01847 was obtained from the VTT Culture Collection (Espoo, Finland). All strains were cultured in PDA at room temperature (25 °C) until use.

Goat sweet whey was provided by the company ALCLIPOR, S. A. L. (Benassal, Spain), as a product of the cheese-making industry using goat milk without chemical preservatives. The samples were stored at -19 °C until analysis.

2.2. Pasteurisation and hydrolysis of goat whey

The standard method was simulated by heating goat sweet whey in a water bath to 63 °C for 30 min with continuous mixing, followed by rapid cooling, according to standardised guidelines. Pasteurised goat sweet whey was adjusted to pH 8 with NaOH (1 M) and pre-heated at 37 ºC. Afterwards, trypsin was added at 333 mg/L and incubated for 24, 48 and 72 h. After the addition of 1 mM PMSF protease inhibitor in MeOH, the trypsin-catalysed goat whey hydrolysate (HGW) was lyophilised, and the powder stored at -80 ºC for further analysis.

The degree of trypsin-catalysed goat whey protein hydrolysis after the three incubation times (24, 48 and 72 h) was analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 15% (w/v) separating gel and 4% stacking gel (El-Ghaish et al., 2010). For visualisation of the proteins bands, gels were stained with a solution of 0.1% Brilliant Blue R-250, 50% water, 40% MeOH and 10% acetic acid. The molecular mass of the proteins was determined by simultaneous electrophoresis of Bio-Rad Precision Plus Protein (high-MW range) and Natural Polypeptide SDS-PAGE (low-MW range), and two major whey proteins (β-Lg and α-La) as the reference standards.

2.3. Qualitative assay of antifungal activity

The effect of the HGW on fungal growth was evaluated against different strains of *Penicillium* using the diffusion agar method. Briefly, 100 µL of the 72 h HGW, suspended in PDB at a final concentration of 250 mg HGW/mL, was added to the wells made in the PDA plates. The PDA plates were previously contaminated with fungal spores (10^6 spores/plate) using sterile cotton swabs. The plates were incubated at 25 °C for 3 days. Growth inhibition was determined

by measuring the diameter (mm) of the inhibition halos, only considering positive halos larger than 8 mm (Torrijos et al., 2019). Three replicates of each experiment were undertaken.

2.4. Quantitative assay of antifungal activity

As described by Fothergill (2012) with some modifications, a 100-µL aliquot of the 72-h HGW at final concentrations of 0.5 to 250 mg/mL was added to 96-well sterile microplates. The microwells were then contaminated with 100 µL of a 5×10^4 spores/mL suspension of the toxigenic fungi. The positive control consisted of the contaminated medium with non-hydrolysed goat whey (250 mg/mL), and the negative control was the non-contaminated medium without any treatment. Inoculated microplates were incubated at 25 °C for 3 days. After incubation, the minimum inhibitory concentration (MIC) was defined as the concentration of HGW where no visible fungal growth was observed.

After determining the MIC, 10 µL of medium corresponding to the MIC and higher concentrations were cultivated on PDA plates for the determination of the minimum fungicidal concentration (MFC). After incubation (3 days at 25 ºC), the MFC values were defined as the concentration where no visible fungal growth on PDA plates was evidenced. Four replicates of each assay were completed.

2.5. Liquid chromatography–electrospray ionisation–time-of-flight-mass spectrometry (LC–ESI–TOF-MS) identification of HGW peptides

Peptide purification was achieved using the method described by Guillermo et al. (2005) with some modifications. The lyophilised HGW (100 mg) was

resuspended in 1 mL of Milli-Q water. The proteins in 200 µL of the HGW solution were precipitated with 10% trichloroacetic acid solution at the dilution ratio of 1:5. After centrifugation (Eppendorf 5810R, Hamburg, Germany) at 11,000 rpm, the peptide-containing supernatant was fractionated by centrifugation under the same conditions using an ultrafiltration membrane with a 3-kDa cut-off (Coda et al., 2008). The fraction containing peptides below 3 kDa was analysed by LC–ESI–TOF-MS.

The Agilent 1200 LC system (Palo Alto, CA, USA) was equipped with a vacuum degasser, autosampler, binary pump and a bioZen Peptide C18 column (50 × 2.1 mm, 2.6 µm; Phenomenex, Madrid, Spain). Chromatographic separation was achieved by gradient elution using 0.1% FA–water (solvent A) and 0.1% FA–ACN (solvent B) as the mobile phases: 0 min, 5% B; 30 min, 95% B; 35 min, 95% B; 37 min, 5% B. The column was equilibrated for 3 min before every analysis. The flow rate was 0.4 mL/min, and 20 µL of the sample was injected.

Mass spectrometry analysis was undertaken using an Agilent 6540 ultra-high definition accurate-mass Q-TOF mass spectrometer, equipped with an Agilent dual jet stream ESI interface in positive ionisation mode, under the following conditions: drying gas flow (N_2), 13 L/min; nebuliser pressure, 35 psig; gas drying temperature, 325 °C; capillary voltage, 4 kV; fragmentor voltage, 175 V; scan range 100–3000 *m/z*. Auto MS/MS experiments were conducted using collision energy values of 10, 20 and 40 eV. MS/MS spectrum generated were processed for de novo peptide sequencing using MassHunter Qualitative Analysis software B.08.00. Peptides and the origin protein were identified using Mascot.

2.6. Preparation of bread dough, inoculation and packaging

Bread was prepared by slight modifications of the method reported by Saladino et al. (2016). Commercial wheat flour (250 g) was mixed with 2.5 g sugar, 20 mL of extra-virgin olive oil, 5 g salt, 15 g of instant yeast and 125 mL of tap water. The resultant dough was left to rise at 30 °C for 30 min in the dark and then divided into 40-g portions and unrolled to the desired thickness of approximately 6–7 mm, and 4 cm in diameter. In total, nine bread dough samples for each test were placed on a perforated greased plate and baked at 180 °C for 7 min in a muffle furnace (Memmert ULE 500, Madrid, Spain). After baking, the bread samples were cooled to room temperature (20–22 °C, approximately 1 h) and then immediately prepared for inoculation with fungi. Five different bread types were prepared:

- Pita bread without synthetic additives (control).
- Pita bread with 0.2% calcium propionate (control propionate).
- Pita bread with 100% of the water used in bread preparation replaced with non-hydrolysed whey (control whey).
- Pita bread with 50% of the water used in bread preparation replaced with HGW (HGW 50).
- Pita bread with 100% of the water used in bread preparation replaced with HGW (HGW 100).

The impact of antifungal inhibition of HGW on bread was evaluated by following the method used by Ryan et al. (2011) with some modifications. After baking, 9 replicas for each bread type were inoculated in 9 spots with a suspension containing 1×10^8 conidia/mL of the chosen fungi. A total of 100 µL of fungal suspension was inoculated in each bread. Conidial concentration was

counted using a Neubauer chamber. For the evaluation of inhibition of fungal growth in bread, *P. verrucosum* was used. Each bread sample was hermetically sealed in a low-density polyethylene bag using a Sammic TS-150 thermo-sealer (Basarte, Spain) and incubated at room temperature for 7 days. Each day, the surface of the inoculated bread was visually examined for the possible growth or not of fungus and to establish the effect of the treatment on the shelf-life.

2.7. Determination of the fungal population of bread treated with HGW

Once the preservation period of the bread samples (7 days) was exceeded, a microbiological study was conducted. Whole bread samples were homogenised with sterile, buffered peptone water at 1:10 (w/v) in a Stomacher IUL (Barcelona, Spain) for 30 s. Three serial decimal dilutions of the homogenate were prepared in glass tubes with 9 mL of peptone water, and 100 µL of each tube was plated in PDA. The plates were incubated at 26 °C, and the number of viable colonies was counted after incubation for 72 h (Dal Bello et al*.,* 2007).

2.8. OTA identification and quantification by liquid chromatography-fluorescence detections (LC-FLD)

Mycotoxin extraction was performed as described by Serrano et al. (2013) with some modifications. Pita bread was crushed using an Oster Classic grinder (Madrid, Spain) and 5 g of the sample was placed in a 50-mL plastic tube. MeOH (25 mL) was added, and the mixture homogenised for 3 min using a T18 Basic Ultra-Turrax (IKA, Staufen, Germany). After centrifugation at 4000 rpm, 4 ºC for 15 min, the supernatant was evaporated to dryness on a Büchi Rotavapor R-200 (Postfach, Switzerland). The samples were resuspended in 5 mL of MeOH,

transferred to 15-mL plastic tubes, and the solvent evaporated using a Turbovap LV evaporator (Zymark, Hopkinton, MA, USA). Finally, the residue was reconstituted in 1 mL MeOH and filtered through a 0.22-µm filter before LC-FLD analysis.

OTA was analysed using a Shimadzu HPLC system (Kyoto, Japan) with two binary pumps (LC-10ACvp), a fluorescence detector (RF-10Axl), a system controller (SCL-10Avp) and a Rheodyne 7725 valve injector with a 20-µL loop (Rohnert Park, CA, USA). Chromatographic separation was performed under gradient conditions at a flow rate of 1.2 mL/min. The fluorescence detector was set at λ_{ex} 333 nm and λ_{em} 460 nm. The instrument was equipped with a Hamilton syringe (Reno, NV, USA) and a Kinetex C18 column (150 × 4.6 mm, 5 µm; Phenomenex). The mobile phases were water–1% acetic acid (eluent A) and ACN–1% acetic acid eluent (eluent B). The elution gradient was established initially with 30% eluent B, increased to 70% in 5 min. Eluent B was reduced to 30% in 5 min, and then the initial conditions were maintained for 5 min (Luz et al., 2018a).

3. Results and discussion

3.1. Antifungal activity of HGW and peptide characterisation

As the antimicrobial activity data of the HGW against 10 different strains of *Penicillium* on solid PDA showed (Table 1), HGW was active against 5 of the 10 strains tested, most notably *P. camemberti*, *P. verrucosum*, *P. commune*, *P. brevicompactum* and *P. expansum*. All these fungal strains are considered important contaminants of bread and bread-derived products. The MIC data showed a range of inhibition from 3.9 to 62.5 mg/mL.

Table 1. Antifungal activity evaluated through the assay on solid medium of PDA of HGW, against several toxigenic fungi. Calculation of antifungal activity: 8 mm diameter clearing zone (+), 10 mm diameter clearing zone (++), and more than 10 mm diameter clearing zone (+++). Minimum inhibitory concentration and minimum fungicidal concentration expressed in mg/mL (MIC-MFC) evidenced by the HGW on several fungal bread contaminants.

Fungi	Qualitative	Quantitative	
		MIC	MFC
Penicillium camemberti	+	7.8	62.5
Penicillium verrucosum	+	15.8	31.2
Penicillium roqueforti	-	nd	nd
Penicillium digitatum	-	nd	nd
Penicillium nordicum	-	nd	nd
Penicillium commune	+	7.8	31.2
Penicillium solitum	-	nd	nd
Penicillium brevicopactum	+	3.9	15.8
Penicillium expansum	+	62.5	250
Penicillium pinophilum	-	nd	nd

The *P. brevicompactum* strains were the most sensitive to the bio-complex, with a MIC of 3.9 mg/mL, whereas *P. expansum* exhibited the least sensitivity towards HGW, with a MIC of 62.5 mg/mL. The strains of *P. camemberti* and *P. commune* also presented a noticeable sensitivity to the antimicrobial ingredient used, with a MIC of 7.8 mg/mL, respectively. In addition, the HGW had an MFC of 15.8–250 mg/mL.

In Fig. 2, the SDS-PAGE of the HGW obtained by trypsin-catalysed hydrolysis of goat whey at 37 °C for different incubation times (24, 48 and 72 h) is compared with the non-hydrolysed sample and the reference markers (Bio-Rad standard solutions, and α-La and β-Lg). It can be seen from the gel bands that the control whey is characterised by substantial amounts of the proteins lactoferrin (Lf) (80 kDa), α-La (14 kDa) and β-Lg (18 kDa), respectively, whereas all three protein

bands are markedly less intense in the HGW, proportional to the incubation time. These electrophoretic findings agree with the LC–ESI–TOF-MS data of the peptides generated by the whey hydrolysis. In particular, the trypsin-catalysed hydrolysis of goat whey produced 27 peptides from the proteins Lf, α-La and β-Lg. Sequence alignments with known antimicrobial peptides were performed using the milk AMP database.

The analysis of the protein fragments sequences showed that five peptides, covering the MW range of 618.3457–1064.5047 Da, were derived by the hydrolysis of α-La, and three peptides, with MW values in the range of 674.4236–696.3352 Da, originated from β-Lg, whereas just one peptide, with a MW of 1202.4103 Da, was formed by the hydrolysis of κ-casein.

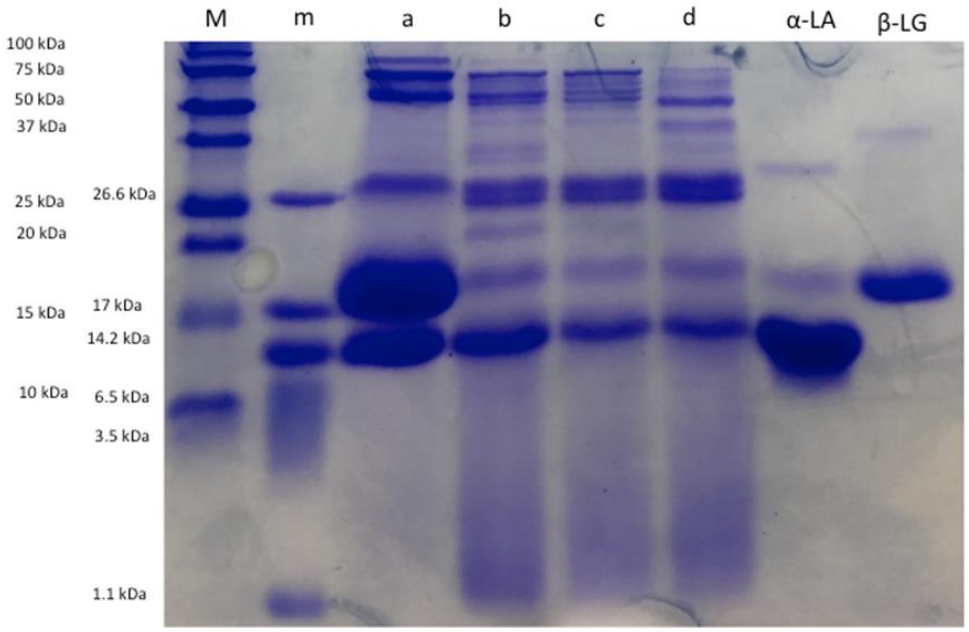


Fig. 2. SDS PAGE of HGW at 37°C at different incubation times; not hydrolyzed control (a), 24 h (b), 48 h (c) y 72 h (d). Standard of high (M) and low (m) molecular weight, α-La and β-Lg.

It was found that Lf produced the highest quantity of bioactive peptides, generating 18 different hydrolysates covering the MW range of 618.3682–1496.7514 Da. In general, the Lf-derived peptides had a higher MW relative to the hydrolysates from the other proteins. The amino acid sequence and the MW of each identified peptide are provided in Table 2.

Table 2. Molecular weight, native protein and amino acid sequence of the bioactive peptides obtained by the hydrolysis of the goat whey with trypsin.

nº	Native protein	Observed MS (Da)	Expected MS (Da)	Delta	Peptide sequence
1	α-Lactalbumin	618.7119	618.3457	0.36	EQLTK
2	α-Lactalbumin	1065.1777	1064.5047	0.67	GYGGVSLPEW
3	α-Lactalbumin	570.6892	570.2592	0.43	VCTTF
4	α-Lactalbumin	650.7763	650.3178	0.45	ALCSEK
5	α-Lactalbumin	957.0154	956.3891	0.61	CKDDQNPH
6	β-Lactoglobulin	696.7854	696.3352	0.45	VAGTWY
7	β-Lactoglobulin	976.0777	975.4993	0.57	AASDISLLDA
8	β-Lactoglobulin	674.8664	674.4236	0.44	IPAVFK
9	K-Casein	1202.4103	1201.6800	0.73	YQRRPAIAIN
10	Lactoferrin	1304.5316	1303.6913	0.84	GRRRRSVQWC
11	Lactoferrin	1397.6768	1396.6725	1.00	KCFQWQRNMR
12	Lactoferrin	1056.3234	1055.6030	0.72	KVRGPPVSCI
13	Lactoferrin	1302.5999	1301.7372	0.86	WQRRMRKLGA
14	Lactoferrin	1231.4740	1230.6524	0.82	APRKNVRWCT
15	Lactoferrin	1355.5238	1354.5885	0.93	PEWSKCYQWQ
16	Lactoferrin	1172.4498	1171.6841	0.76	RRMRKLGAPS
17	Lactoferrin	1007.2070	1006.5462	0.66	ITCVRRTSA
18	Lactoferrin	1497.8442	1496.7514	1.09	FKCRRWQWRM
19	Lactoferrin	1018.2711	1017.5761	0.69	KKLGAPSITC
20	Lactoferrin	1046.2846	1045.5823	0.70	RKLGAPSITC
21	Lactoferrin	618.7178	618.3682	0.34	VRRTS
22	Lactoferrin	1110.2818	1109.5296	1.05	VSQPEATKCF
23	Lactoferrin	1072.2791	1071.5615	0.71	GPPVSCIKRD
24	Lactoferrin	1238.4160	1237.5670	0.84	PEATKCFQWQ
25	Lactoferrin	960.1995	959.5680	0.63	RNMRKVR
26	Lactoferrin	1386.6504	1385.6565	0.99	SKCYQWQRRM
27	Lactoferrin	1046.2846	1045.5823	0.70	RKLGAPSITC

This article can be considered the first to test the antifungal activity of an HGW against several toxigenic fungi, whereas, many authors have studied the antimicrobial activity, mostly the antibacterial and anti-yeast properties, of fragments originated by Lf hydrolysis. Bovine and human Lf are the main sources for obtaining antifungal peptides. Lupetti et al. (2008) reported the antifungal activity of the human Lf peptide (1–11) against *Aspergillus fumigatus* at 29 μM. Another study evidenced MIC values ranging from 4–16 and 8–32 μM of two synthetic peptides derived from bovine Lf (17–31 and 20–25, respectively) against different *Penicillium* spp. (Muñoz & Marcos, 2006).

According to Shu et al. (2018), the hydrolysis of goat milk by proteinases from *Lactobacillus plantarum* LP69 displayed 83% ACE inhibition and 65% DPPH radical scavenging activity. In addition, peptides derived from proteolysis of Lf show several protective activities, such as immunomodulatory activity, development of the neonatal immune system, anti-inflammatory effects and anticancer activity (Giansanti et al., 2016; de Mejia & Dia, 2010; Teng et al., 2004).

3.2. Shelf-life improvement of pita bread contaminated with Penicillium verrucosum and treated with HGW

A study of the shelf-life of pita bread contaminated with *P. verrucosum* (Table 3) showed visible fungal growth of *P. verrucosum* at 3 days of incubation in the control experiment, whereas the shelf-life was extended by 3 days when using calcium propionate (E-282). For the control whey (not treated), the visible shelf-life of the pita bread was increased by just 1 day in comparison to the control experiment, and the same data was observed for the bread with HGW 50. It is important to comment that the results of the visible fungal growth observed in

the experiment where 100% of the baking water was replaced with the HGW were very similar to those observed with E-282, presenting only 1 day less in the shelf-life (Fig. 3).

Table 3. Shelf life expressed in days of pita bread contaminated with *P. verrucosum*. (a) Control pita bread without any preservative compound, b) Pita bread baked with 2 g/Kg of the calcium propionate, c) Control Pita bread produced with no hydrolyzed whey, d) and e) Pita bread produced with the HGW in replacing of the 50 and 100% of the water used for baking.

Pita Bread	Shelf life (Days)						
	1	2	3	4	5	6	7
Control	-	-	+	+	+	+	+
Control Propianate	-	-	-	-	-	+	+
Control Whey	-	-	-	+	+	+	+
HGW 50	-	-	-	+	+	+	+
HGW 100	-	-	-	-	+	+	+

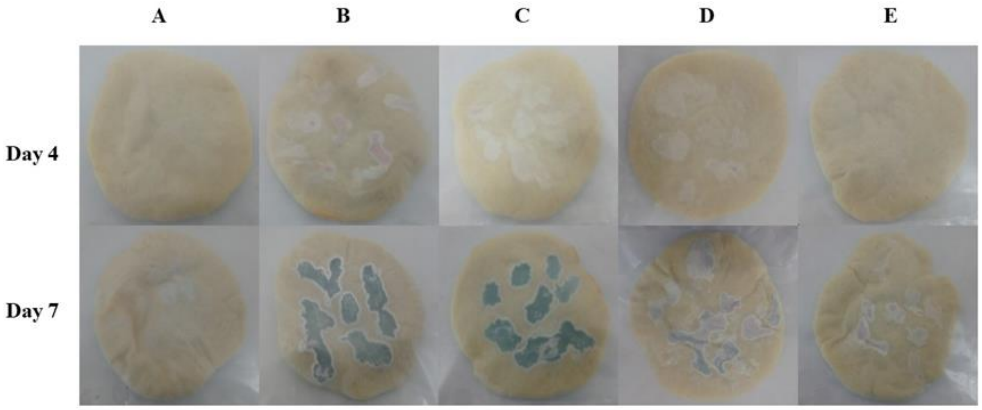


Fig. 3. Pita bread contaminated with *P. verrucosum* and in the following experiments: (a) Pita bread baked with 2 g/Kg of the calcium propionate, b) Control pita bread without any preservative compound, c) Control Pita bread produced with no hydrolyzed whey, d) and e) Pita bread produced with the HGW in replacing of the 50 and 100% of the water used for baking.

The data of the visible shelf-life of pita treated with the HGW and contaminated with *P. verrucosum* were also confirmed by the microbiological analysis of the population of the fungal contaminant in the food matrix (Table 4a). Whereas the control experiments at 7 days incubation presented a fungal population of 3.99 log CFU/g, the pita bread treated with calcium propionate had a fungal growth of 1.57 log CFU/g, equivalent to a significant 98% reduction in comparison to the control. In the experiment carried out using the control whey, the *P. verrucosum* growth was 3.90 log CFU/g and was not statistically different from the control experiment. For the bread elaborated by HGW 50 and 100, the *P. verrucosum* growth was 3.59 and 2.95 log CFU/g, equating to a statistical reduction of 59 and 83%, respectively, relative to the control experiment.

Several studies have utilised cheese whey in bread manufacture, but there are very few reports on the utilisation of HGW as a bio-preservative in bread-making to partially replace the water with whey for dough preparation (Brar et al., 2002). Nevertheless, in the bakery industry, whey is considered as a bioactive ingredient due to its nutritive value and functional characteristics (Kumar et al., 2018). As an ingredient in the bread formulation, whey confers interesting technological qualities, such as a good crumb structure, bread yield, flavour and keeping quality (Puranik, 2003). In this context, Gamba et al. (2016) investigated the antifungal activity of whey obtained from milk fermented with kefir grains (CIDCA AGK1) against *Aspergillus flavus* in corn *arepas*. Corn *arepas* inoculated with *A. flavus* (10^2 spores/g) and treated with 10% whey exhibited a longer shelf-life than the control without additive during storage at 14 ºC for 10 days.

Table 4. a) Fungal growth and b) OTA reduction in pita bread contaminated with *P. verrucosum*. Control pita bread without any preservative compound (Control), Pita bread baked with 2 g/Kg of the calcium propionate (Control propionate), Control Pita bread produced with no hydrolyzed whey (Control Whey), and Pita bread produced with the HGW in replacing of the 50 (HGW 50) and 100% (HGW 100) of the water used for baking.

a)

Pita Bread	log_{10} CFU/g	% Reduction viability
Control	3.99 ± 0.33^{a}	-
Control Propianate	1.57 ± 0.42^{c}	98 ± 3
Control Whey	3.90 ± 0.31^{a}	22 ± 18
HGW 50	3.59 ± 0.37^{ab}	59 ± 21
HGW 100	2.95 ± 0.33^{b}	83 ± 11

b)

Pita Bread	OTA (µg/Kg)	% OTA Reduction
Control	24.0 ± 0.5	-
Control Propianate	nd	100 ± 0^{c}
Control Whey	10.8 ± 0.2	55 ± 3^{a}
HGW 50	3.5 ± 0.1	85 ± 3^{b}
HGW 100	nd	100 ± 0^{c}

Considering that the strain of the *P. verrucosum* used in this study is an OTA producer, the data associated with the production of this toxic compound in the evaluated pita bread are given in Table 4b. At 7 days of incubation, 24.0 µg/kg OTA was detected in the control experiment, whereas no OTA was detected during this time when calcium propionate and the HGW 100 were used as preservative agents, demonstrating a 100% reduction in the synthesis of this metabolite by *P. verrucosum* by both treatments, respectively. The use of the

control whey produced a 55% reduction in the OTA synthesised by the *P. verrucosum* in pita bread relative to the control produced without any preservative treatment. Using the HGW 50, OTA was detected at 3.5 µg/kg in the pita bread, evidencing a significant 85% and 30% reduction of this metabolite in comparison to the control experiment and control whey, respectively.

While many articles are available on the mycotoxin reduction attributed to various functional ingredients, this article is the first to describe the anti-mycotoxigenic activity of an HGW against an OTA-producing mycotoxigenic fungus.

4. Conclusions

The results produced and analysed in this study demonstrated that the HGW obtained by controlled hydrolysis with trypsin presented broad-spectrum antifungal activity against several fungi of the genus *Penicillium*. The characterisation of the bioactive peptides by LC–ESI–TOF-MS analysis evidenced that this treatment applied on the goat whey generated 27 peptides from the native proteins Lf, α-La and β-Lg, and all with described antimicrobial properties. Furthermore, the application of the HGW as an ingredient in the formulation of pita bread improved the shelf-life of this product contaminated with the mycotoxigenic strain *P. verrucosum*, not only based on the fungal growth but also the production of the mycotoxin OTA.

Considering the results obtained in our study, HGW could be useful as a natural alternative to avoid the growth of mycotoxigenic fungi and inhibit the production of OTA in different food products in the substitution of commercial synthetic preservatives.

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3.5. Antifungal activity and shelf life extension of loaf bread produced with sourdough fermented by *Lactobacillus* strains

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1. Introduction

Mycotoxins are a naturally occurring, chemically and structurally diverse group of more than 300 secondary metabolites produced by the mycelial structure of filamentous fungi, which are characterized by their potential to elicit undesirable effects in humans and animals following consumption of contaminated foods or feedstuffs (Hussein & Brasel, 2001; Rodríguez-Carrasco, Moltó, Mañes, & Berrada, 2014). Mycotoxin contamination resulting from fungal infection of agricultural crops and commodities is closely related to environmental conditions, particularly temperature and moisture, and may occur at various stages of food life "from the farm to the fork" (Magan, Medina, & Aldred, 2011).

In the bakery industry, spoilage of products by moulds is a big problem resulting in significant economics losses (Koe & Juodeikiene, 2012; Stanciu et al., 2017). Fungal spores are normally not viable in bakery products immediately after baking. Nevertheless, in some processing environments there are around 10^3 fungal spores/m^3 of air. Therefore, bakery products are usually contaminated in post-bake processing (Legan, 1993; Magan, Aldred, & Arroyo, 2012).

The technological functions of sourdough mainly depend on the metabolism of Lactic Acid Bacteria (LAB) that can be influenced by the flour and other ingredients. To date, several different LAB species of *Lactobacillus*, *Leuconostoc*, *Weissella* and *Pediococcus* species were shown to be present in sourdoughs from different origins including wheat and rye, *Lactobacillus* is the dominant LAB species in sourdough (Vuyst & Neysens, 2005). The technological functions of the LAB species include the production of secondary metabolites that can improve the safety and shelf life of final sourdough bread (Corsetti & Settanni, 2007). The antifungal activity of LAB species is a complex process that can be originated from different compounds produced by these species (Schnürer & Magnusson, 2005).

Like organic acids, a range of other secondary metabolites has been identified with antifungal activity, antimicrobial peptides, bacteriocins and phenolic acids (Crowley, Mahony, & Van Sinderen, 2013). The increasing consumer concern about synthetic additives has led to their substitution by natural compounds in foods, search reasons for new strains of lactic acid bacteria as sourdough starter (Inetianbor, Yakubu, & Ezeonu, 2015).

The purpose of this study is to test the activity in sourdough by different LAB against several toxigenic fungi, the identification for antifungal compounds responsible of activity using LC-ESI-qTOF mass spectrometry, and novel application of the sourdough for the shelf life improvement of bread.

2. Materials and methods

2.1 Chemicals

The phenolic compounds gallic acid, chlorogenic acid, caffeic acid, syringic acid, vanillin, ρ-coumaric, hydroxybenzoic acid, vanillic acid, hydroxylcinnamic acid, sinapic acid, benzoic acid, DL-3-phenyllactic acid, 1,2-dihydroxybenzene, 3,4-dihydroxyhydrocinnamic, DL-ρ-hydroxyphenyllatic acid were provided from Sigma-Aldrich (Dublin, Ireland). Phenyllactic acid (PLA) was obtained from BaChem (Weil am Rhein, Germany). Ferulic acid was purchased from MP Biomedicals and protocatechuic acid from HWI pharma services (Rheinzaberner, Germany). All analytes had a purity of 95%.

LC grade solvents as acetonitrile (ACN), methanol, ethyl acetate (EA) and formic acid (FA) (99%) were obtained from VWR Chemicals (Radnor, USA). Magnesium sulphate (MgSO4), C18, sodium chloride (NaCl), were obtained from Sigma-Aldrich (Dublin, Ireland). Potato Dextrose Broth (PDB), Potato Dextrose

Agar (PDA), Man Rogosa Sharpe (MRS) Broth and MRS Agar was obtained from Liofilchem (Roseto degli Abruzzi, Italy). Deionized water (<18 Megaohm-cm resistivity) was obtained from a Milli-Q purification system (Bedford, USA).

2.2 Microorganisms and culture conditions

Strains of *Fusarium verticillioides* CECT 20926, *Fusarium graminearum* CECT 20490, *Fusarium moniliformis* CECT 2982, *Penicillium roqueforti* CECT 2905, *Penicillium camemberti* CECT 2267, *Penicillium expansum* CECT 2278, *Aspergillus parasiticus* CECT 2681 and *Aspergillus niger* CECT 2088 were obtained from CECT (Valencia, Spain). *Aspergillus flavus* ITEM 8111 was obtained from ITEM microbial culture collection of the Institute of Sciences and Food Production (Bari, Italy). These microrganisms were maintained in sterile 30% glycerol at -80 °C. Then they were recovered in PDB broth at 25 ºC until they were inoculated in PDA Petri dishes and the spores were obtained at the time of analysis.

The LAB used in this study were: *Lactobacillus plantarum* CECT 749 and *Lactobacillus delbrueckii bulgaricus* CECT 4005. These bacteria were also obtained from CECT. The LAB was preserved in sterile 30% glycerol and stored at -80 ºC before use. Before fermentation experiments, LAB recovery was in culture medium MRS Broth at 37 ºC for 48 h under anaerobic conditions.

2.3 Sourdough fermentation

After a recovery period, LAB were cultivated in MRS at 37 ºC until the exponential phase of growth (10 h). The microorganisms were separated by centrifugation at 10000 rpm for 10 min in Eppendorf tubes and washed with

Phosphate Buffered Saline (PBS) 0.01 M pH 7.4. Strains were individually used for sourdough fermentation. Two different sourdoughs were prepared using the ingredients detailed below:

- Fifty grams of wheat flour and 50 mL of sterile tap water denominated wheat dough.
- Forty-five grams of wheat flour, 5 g of milk whey powder and 50 mL of sterile tap water, denominated wheat-whey dough.

Both sourdoughs preparations were inoculated with LAB at a concentration of $2 \cdot 10^8$ CFU/mL. Sourdoughs were incubated for 48 h at 37 ºC. After fermentation, the water-soluble extracts (WSE) were obtained by centrifugation at 4000 rpm for 10 min at 4 ºC. These WSE were lyophilized and were used for antifungal activity test (Coda et al., 2008). The control experiment was sourdough non inoculated and incubated under the same conditions.

2.4 Qualitative assay of antifungal activity

The effect of the WSE fermented on fungal growth was evaluated for different strains of *Fusarium*, *Aspergillus* and *Penicillium*. PDA plates were inoculated with fungal spores using sterile cotton swabs. Then, the wells were made using sterile pipette tips and each well was loaded with fifty microliters of WSE lyophilized and suspended in potato dextrose broth (PDB) with a concentration of 400 mg/mL. After, plates were incubated at 25°C for 3 days. Finally, the measurement of the inhibition halo diameter was carried out, being considered positive for antifungal activity halos larger than 8 mm (Varsha, Priya, Devendra, & Nampoothiri, 2014).

2.5 Quantitative assay of antifungal activity

The assay was performed as described by Fothergill (2012) with some modifications. A volume of 100 µL of WSE fermented with at final concentrations from 0.8 to 400 mg/mL was added to 96-well sterile microplates. After, microwells were inoculated with 100 µL of a $5{\cdot}10^4$ spores/mL suspension in PDB of the toxigenic fungi. The positive control consisted of inoculated medium with WSE non inoculated (400 mg/mL) and the negative control was of non inoculated medium without any treatment. Inoculated microplates were incubated at 25°C for 3 days. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of the WM fermented, where the fungi did not show any visible growth. Four replicates of each assay were carried out.

After determining the MIC, the concentration corresponding to the inhibitory and to higher concentrations were subcultured on PDA plates for the determination of the Minimum Fungicidal Concentration (MFC). After 72 h of the plates incubation at 25 ºC, MFC results were the lowest extract concentration in which a visible growth of the subculture was prevented.

2.6 Extraction, and identification of phenolic acids by LC-ESI-qTOF-MS

The WSE were purified using by the QuEChERS method to remove possible interferents before the chromatographic analysis (Brosnan, Coffey, Arendt, & Furey, 2014). Ten milliliters of WSE fermented was extracted with 10 mL ethyl acetate 1% formic acid, 4 g of $MgSO_4$, and 1 g of NaCl, then vortexed for 1 min. The extract was centrifuged and to the supernatant were added 150 mg C18 and 900 mg $MgSO_4$ and vortexing for 1 min. The extract was centrifuged again and the supernatant was evaporated under nitrogen flow. At the time of the

chromatographic analysis the purified extract was resuspended in 1 mL of H_2O: ACN (90:10).

The total amount of phenols of the lyophilized WSE resuspended in water were quantified using Folin Ciocalteu reagent at 750 nm using a standard curve of gallic acid (8.5 to 25.5 µg mL^{-1}) (Souza, Prietto, Ribeiro, Denardi-Souza, & Badiale-Furlong, 2011).

LC system used for the chromatographic determination was an Agilent 1200 (California, USA) equipped with a vacuum degasser, autosampler, and binary pump. The column was a Gemini C18 50 x 2 mm, 100 Å and particle size 3 µm (Phenomenex). Mobile phases consisting of water as solvent A, ACN as solvent B both acidified (0.1% formic acid) and the following gradient elution: 0 min, 5% B; 30 min 95% B; 35 min, 5% B. The column was equilibrated for 3 min prior to every analysis. The flow rate used was 0.3 mL/min and the sample volume injected was 20 µL.

Mass spectrometry analysis was carried out using a Q-TOF-MS (6540 Agilent Ultra High Definition Accurate Mass), equipped with an Agilent Dual Jet Stream electrospray ionization (Dual AJS ESI) interface in negative ionization mode at the following conditions: drying gas flow (N_2), 8.0 L/min; nebulizer pressure, 30 psig; gas drying temperature, 350°C; capillary voltage, 3.5 kV; fragmentor voltage, and scan range were 175 V and m/z 20-380, respectively. Targeted MS/MS experiments were carried out using collision energy 10, 20 and 40 eV values. Integration and data elaboration were managed using Masshunter Qualitative Analysis Software B.08.00 (Denardi-Souza, Luz, Mañes, Badiale-Furlong, & Meca, 2018).

2.7 Bread preparation

The wheat flour doughs fermented by LAB were used for the production of bread. Doughs for the bread were prepared by replacing 20% of the total weight ingredients in the form of sourdough. Also, a control sourdough naturally fermented during 48 h at 37 ºC, in the same way, that doughs are fermented by LAB, and a control dough spiked with 0.2% calcium propionate dough were prepared. The final compositions of the wheat dough formulations are shown in Table 1.

Table 1. Bread dough formulation.

Ingredients	**Preservative control**	**Sourdough control**	***L. plantarum* CECT 749**	***L. bulgaricus* CECT 4005**
Wheat flour	299	250	250	250
Water	160	110	110	110
Salt	5	5	5	5
Sugar	10	10	10	10
Calcium propionate	1	-	-	-
Baker's yeast	25	25	25	25
Sourdough	-	100	-	-
Sourdough LAB	-	-	100	100
Total (g)	500	500	500	500

After, the doughs were homogenized in a bakery machine (Silver Crest) for 15 min and the fermented for 1 h at 25 ºC. Baking of the doughs was carried out at 200 °C for 40 min. The loaf bread was cooled for 1 h at room temperature. Then, breads were cut in slices of 30 g and inoculated with 100 µL of a fungal suspension at a concentration of $3 \cdot 10^5$ spores of *P. expansum*/mL. This strain is one of the most prevalent in bread contamination. Samples were stored in plastic bags and incubated at 25 ºC for 7 days.

2.8 Shelf life and determination of the fungal population

The plastic bags containing the slices were examined daily during incubation to determine visible fungal growth and evaluation of shelf life. When bread showed visible signs of fungal growth, the shelf life was closed, considering that consumers would eventually reject this bread (Dal Bello et al., 2007).

The determination of the fungal population was performed after 7 days of incubation. Each slice was mixed in a stomacher (IUL, Barcelona, Spain) in proportion 1:10 bread to sterile peptone water 0.1% for 30 s. The extract was serially 1:10 diluted with peptone water and 100 µL were plated on PDA and the plates were incubated at 25 ºC for 72 h for the fungal counting (Luz et al., 2017).

2.9 Statistical analysis of data

The statistical analysis was performed using the software *InfoStat* version 2008. The differences between the groups were analyzed with one-way ANOVA followed by the Tukey HSD post-hoc test for multiple comparisons. The level of significance considered was $p \leq 0.01$.

3. Results and discussion

3.1 Antifungal activity of WSEs fermented

In Table 2 are described the results of the antifungal activity of the WSEs produced through the fermentation of wheat dough and a wheat-whey dough by *L. plantarum* and *L. bulgaricus* on different strains of mycotoxigenic fungi employing the antimicrobial test on the solid medium of PDA. Using the wheat-WSE (W-WSE) form the dough, the strain of the *L. plantarum* was active against

eight of the nine-mycotoxigenic fungi tested producing inhibition zone from 8 to 10 mm. The only strain that was resistant to the *L. plantarum* W-WSE was the toxigenic fungi *A. flavus* ITEM 8111, an aflatoxin producer. Comparing these results with those obtained by *L. bulgaricus*, the W-WSE of this strain was active against *P. expansum*, *F. moniliformis*, *F. verticillioides* and *F. graminearum* (with inhibition zone ranged from 8 to 10 mm), whereas the other mycotoxigenic fungi tested presented a resistance.

Table 2. Antifungal activity in solid medium of the WSE produced through bacterial fermentation of a wheat and wheat-whey dough against toxigenic fungi. Calculation of antifungal activity: 8 mm diameter clearing zone (+), 10 mm diameter clearing zone (++), and more than 10 mm diameter clearing zone (+++).

Fungi	W-WSE		WW-WSE	
	L. plantarum	***L. bulgaricus***	***L. plantarum***	***L. bulgaricus***
***P. camemberti* CECT 2267**	+	-	++	-
***P. expansum* CECT 2278**	++	++	+	+
***P. roqueforti* CECT 2905**	++	-	++	-
***A. parasiticus* CECT 2681**	+	-	+	-
***A. flavus* ITEM 8111**	-	-	+	+
***A. niger* CECT 2088**	+	-	+	-
***F. moniliformis* CECT 2982**	++	++	+++	++
***F. verticillioides* CECT 20926**	+	+	+	+
***F. graminearum* CECT 20490**	+	+	++	+

Comparing the results obtained on the antimicrobial activity of W-WSE on the fungi studied, using the wheat-whey WSE (WW-WSE) from the dough, the strain of the *L. plantarum* was active against all the fungi tested. The WW-WSE

obtained by this LAB produced an inhibition zone ranging from 8 to 10 mm on eight fungi tested, whereas on the strain of *F. moniliformis* the inhibition zone detected was more than 10 mm. The WW-WSE produced by *L. bulgaricus* presented a similar activity to the W-WSE, but the WW-WSE was also an antifungal activity against *A. flavus*.

To quantify the antimicrobial activity of the WSEs of the *L. plantarum* and *L. bulgaricus* on the mycotoxigenic fungi (Table 3), the MIC and the MFC have been calculated using the antimicrobial assay performed in 96-well sterile microplates. In particular, using the WW-WSE of *L. plantarum* the MIC data observed ranged from 25 to 200 mg/mL, whereas the MFC detected resulted variable from 100 to 400 mg/mL. The fungal strains that presented the highest sensitivity to the WW-WSE were *P. camemberti*, *P. roqueforti, F. moniliformis* and *F. graminearum* whereas the lowest sensitivity to the WW-WSE were observed with the strains of *P. expansum*, *A. parasiticus*, *A. niger* and *F. verticillioides*. No MFC data were detected on the strains of *A. flavus* and *A. niger* using the WW-WSE produced by the strain of *L. plantarum*.

Related with the results obtained using the WW-WSE produced through the fermentation by *L. bulgaricus*, the MIC and the MFC data ranged from 1.6 to 200 and from 1.6 to 400 mg/mL, respectively. The fungi that were more sensitive to the antifungal complexes tested were *P.* expansum, *P. roqueforti*, *F. moniliformis*, *F. verticillioides* and *F. graminearum*, whereas the lowest antifungal activity of the WW-WSE was observed on the strains of *P. camemberti* and *A. niger*, where the MFC observed was of 400 mg/mL. No MIC and MFC data were detected on the strains of *A. parasiticus* and *A. flavus*.

Table 3. Minimum inhibitory concentration and minimum fungicidal concentration (MIC-MFC) evidenced by *L. plantarum* and *L. bulgaricus* WSE (mg/mL) produced through bacterial fermentation of a wheat and wheat-whey dough on the mycotoxigenic fungi tested.

Fungi	W-WSE				WW-WSE			
	L. plantarum		*L. bulgaricus*		*L. plantarum*		*L. bulgaricus*	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
P. camemberti	200	400	200	400	100	200	200	400
P. expansum	50	100	25	100	200	400	100	200
P. roqueforti	6.3	50	6.3	25	100	200	100	200
A. parasiticus	200	400	nd	nd	200	400	nd	nd
A. flavus	nd	nd	nd	nd	200	nd	nd	nd
A. niger	200	400	nd	nd	200	nd	200	400
F. moniliformis	100	200	6.3	25	25	100	25	100
F. verticillioides	100	200	1.6	3.1	100	200	6.3	12.5
F. graminearum	100	100	1.6	1.6	50	100	1.6	1.6

Related with the antifungal activity studied using the W-WSE produced through the fermentation by *L. plantarum*, the MIC data were variable from 6.3 to 200 mg/mL, whereas the MFC data ranged from 50 to 400 mg/mL. Four fungal strains and in particular *P. camemberti*, *A. parasiticus*, *A. flavus* and *A. niger* there was little sensitivity and some resistance to the antifungal complex contained in the W-WSE produced by *L. plantarum*. These data are different from those obtained using the W-WSE produced by *L. bulgaricus*. In particular, MIC data that ranged from 1.6 to 200 mg/mL, whereas the MFC observed resulted variable from 1.6 to 400 mg/mL. The fungal strains that presented the highest sensitivity to the W-WSE were *P. expansum*, *P.* roqueforti, F. *moniliformis*, *F. verticillioides* and *F. graminearum*, with very low MIC and MFC data, whereas the lowest sensitivity of the W-WSE were observed on the *P. camemberti* and *Aspergillus* spp.

Analyzing the data, the WSEs produced by *L. bulgaricus* using the two different doughs tested presented antifungal activity against *Fusarium* spp. in comparison with the WSEs produced by *L. plantarum* that presented a lower activity. Against *Aspergillus* spp. both WSE showed very comparable results with high MIC and MFC values. The W-WSE in comparison with WW-WSE, generally, presented a higher antifungal activity against *Penicillium* spp. especially the strains *P. expansum* and *P. roqueforti*.

Previous studies have reported the antifungal activity of sourdough fermented by LAB (*L. plantarum* and *Lactobacillus rossiae*) (Rizzello, Cassone, Coda, & Gobbetti, 2011). Methanol and water-soluble extracts from sourdough fermentation showed antifungal activity in solid medium against important toxigenic fungi in bakery products. A mixture of organic acids, phenolic compounds and peptides produced during fermentation were related to the antifungal activity. The MIC determined ranged from 2.5 to 15.2 mg/mL. Bread loaves produced with 4% (wt/wt) of freeze-dried sourdough fermented did not show fungal growth during 20 days of storage at room temperature.

Ouiddir et al. (2019) tested the antifungal activity of *L. plantarum* and *Leuconostoc mesenteroides* in bakery and dairy products. The sourdough breads containing this LAB inoculated by *A. flavus* and *Aspergillus tubingensis* showed improve shelf life of 1-2 days compared to the positive control (bread without LAB).

3.2 Identification and quantification of phenolic compounds obtained by the WSE

In this study, a total of 7 compounds were tentatively identified in WSEs fermented by the two probiotic strains used. Peak identification was performed

on the basis of their relative retention time values, and mass spectra obtained using qTOF-MS together with information previously reported in the literature. The identified compounds evidenced in the fermented WSEs are summarized in Table 4 including molecular formula, experimental and calculated m/z, and the fragments that characterize the compound structure. The strain of *L. plantarum*, during the fermentation in wheat dough, produced the antimicrobial compounds gallic, chlorogenic, caffeic and syringic acids, whereas in the wheat-whey dough the compounds detected were the gallic and caffeic acids and also the vanillin.

Table 4. Phenolic compounds identified in *L. plantarum* and *L. bulgaricus* WSE produced through bacterial fermentation of a wheat and wheat-whey dough by LC-qTOF-MS.

Compound	m/z calculated	m/z experimental	W-WSE		WW-WSE	
	$(M-H)^-$	$(M-H)^-$	*L. plantarum*	*L. bulgaricus*	*L. plantarum*	*L. bulgaricus*
Gallic	169.1243	169.0117	+		+	
Chlorogenic	353.3124	353.0653	+			+
Caffeic	179.1629	179.0323	+		+	
Syringic	197.1728	197.0427	+			
Vanillin	151.1322	151.0381			+	+
Sinapic	223.2156	223.0568		+		+
DL-3-phenyllactic	165.175	165.0532		+		

The strain of *L. bulgaricus* in the wheat dough produced phenyllactic and sinapic acids, whereas changing the fermenting conditions using the more complex medium composed by wheat-whey the compounds detected were the chlorogenic and the sinapic acids and also the bioactive compound vanillin.

The quantification of the phenolic compounds presents in the W-WSE and WW-WSE of fermented doughs by *L. plantarum* were of 40.2 and 35.1 µg of gallic acid g^{-1}, respectively, and W-WSE and WW-WSE obtained by *L. bulgaricus* were of 38.0 and 32.4 µg of gallic acid g^{-1}, respectively.

Axel, Brosnan, and Zannini (2016a) reported the detection of antimicrobial compounds in sourdoughs fermented by *Lactobacillus* spp. In particular, phenolic acids such as 3-phenyllactic acid, 4-hydrophenyllactic acid and 2-hydroxyisocaproic acid were found with concentrations in the range of 0.1 to 360 mg/Kg. *Lactobacillus reuteri* was the biggest producer, however using the sourdough as an ingredient did not improve shelf life of the bread.

3.3 Shelf life improvement

In Table 5 are evidenced by the data related shelf life monitored in days. The bread loaves contaminated with *P. expansum* produced with 4 different preservatives treatments and in particular with sourdough control non inoculated, 0.2% of calcium propionate, and two sourdough treatments with *L. plantarum* and *L. bulgaricus* during 48 h at 37 ºC. On the one hand, the sourdough control presented visible growth of the *P. expansum* at 3 days of incubation, whereas in the breads treated with calcium propionate at 0.2%, two days of the shelf life improvement has been detected in comparison with the sourdough control. On the other hand, the breads with a sourdough by *L. plantarum* and *L. bulgaricus*, showed fungal growth at 4 and 5 days of incubation, respectively, evidences by an improved the shelf life of two days (Figure 1).

Table 5. Shelf life monitored in days, of the bread loaves contaminated with *P. expansum* produced with 4 different preservatives treatments and in particular with sourdough control non inoculated, 0.2% of calcium propionate, and two sourdough treatments produced fermenting wheat dough with *L. plantarum* and *L. bulgaricus* during 48 h at 37 ºC.

Treatment	Days						
	1	2	3	4	5	6	7
Sourdough control	-	-	+	+	+	+	+
Preservative control	-	-	-	-	+	+	+
***L. plantarum* CECT 749**	-	-	-	+	+	+	+
***L. bulgaricus* CECT 4005**	-	-	-	-	+	+	+

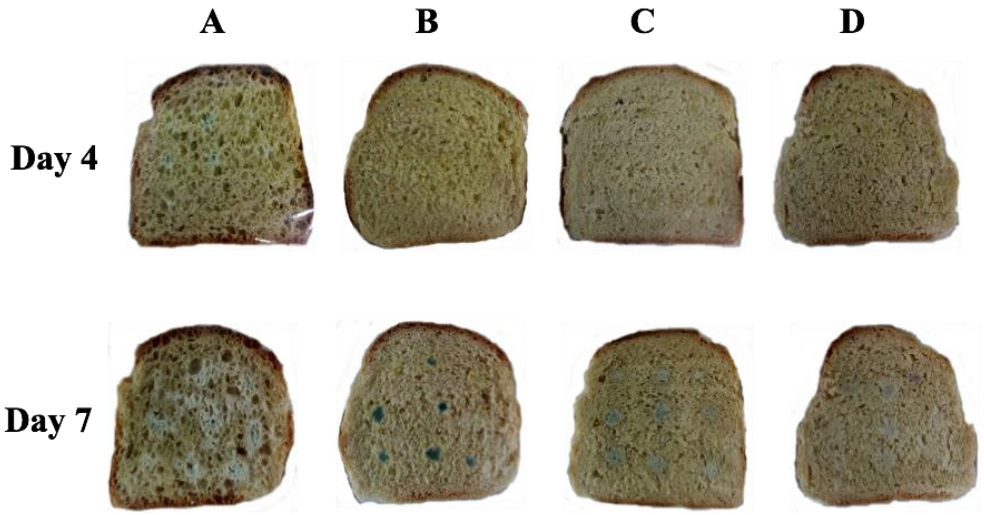

Figure 1. Bread loaves contaminated with *P. expansum* and treated with a) Sourdough control non inoculated b) 0.2% of calcium propionate c) Sourdough fermented with *L. plantarum* d) Sourdough fermented with *L. bulgaricus* at 4 and 7 days of incubation.

Shelf life of bread was improved by all treatments, the microbiological counting of bread evidenced a reduction in growth. Sourdough inoculate with *L. plantarum* was able to significantly reduce the population of *P. expansum* (0.42 log CFU/g), and sourdough *L. bulgaricus* and preservative control presented a reduction of 0.58 log CFU/g in comparisons with the sourdough control after 7 days of storage (Figure 2).

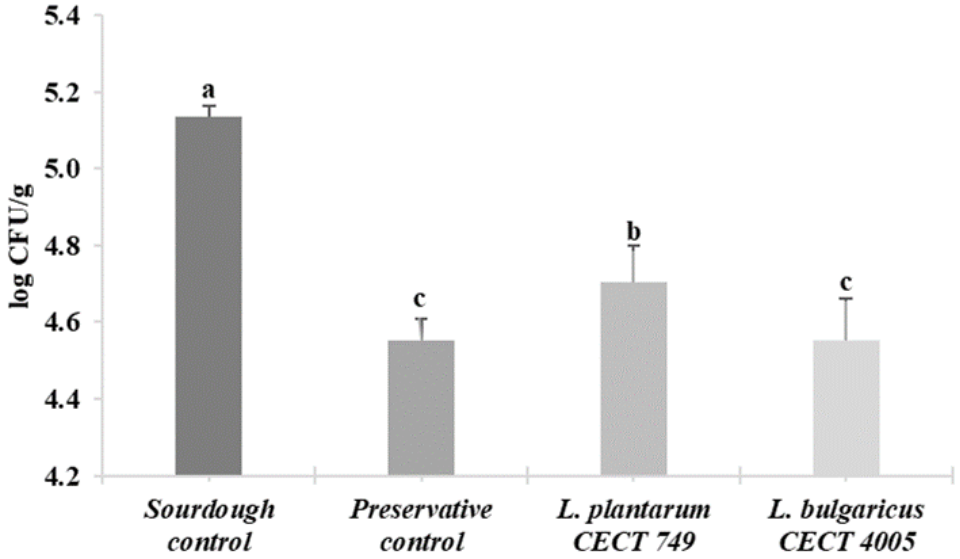

Figure 2. Population *P. expansum* loaves treated in bread sourdough non inoculated (Sourdough control), calcium propionate 0.2 % (Preservative control), and sourdough fermented by *L. plantarum* and *L. bulgaricus*. Different letters show significant difference ($P < 0.01$) among treatments.

The use of sourdough ingredient with LAB as a novel application in bakery products preservation against fungal growth can reduce use of chemical additives. Axel et al. (2016b) reported also antifungal phenolic compounds in sourdough of quinoa and rice employed strains of *Lactobacillus* spp. These compounds were

identified using a QuEChERS method extraction and detection by LC-MS/MS. The shelf life of bread with sourdough fermented by *L. reuteri* and *L. brevis* improved by 2 and 4 days when compared to the control of bread without additives, respectively.

In another study, developed by Garofalo et al. (2012), reported antifungal activity of many LABs isolated from sourdoughs against fungal isolated from bakery environment belonging to genus *Aspergillus, Penicillium* and *Eurotium*. The structure of the antifungal compounds were antimicrobial peptides corresponding to α-gliadin proteolysis and they were identified by MALDI-TOF MS analysis.

4. Conclusion

Sourdough using different strains of *Lactobacillus* spp evidenced a big broad antifungal activity against several toxigenic fungi of the genera *Aspergillus, Fusarium,* and *Pencillium*. This study demonstrated the importance of sourdoughs with LAB as an ingredient in bakery products to improve the shelf life could permit the end users a reduction in the number of chemical preservatives employed to assure the microbiological safety of bread. The results of the study are according to the demand the reduction chemical preservative in food, increasing natural alternatives, as the biopreservation by the consumers.

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3.6. Potential application of lactic acid bacteria to reduce Aflatoxin B_1 and Fumonisin B_1 occurrence on corn kernels and corn ears

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1. Introduction

Filamentous fungi contaminate several types of food such as cereals, fruits and vegetables, dried fruits, feed products, dried spices, dried cured meats, and bread, among others [1–9].

Aspergillus and *Fusarium* are the major fungal genera associated with corn contamination [10]. Indeed, many *Aspergillus* and *Fusarium* species are considered common contaminants of food and feed due to their ability to colonize and grow in a wide range of environmental conditions. Furthermore, these species are also known to produce mycotoxins.

Mycotoxins are secondary metabolites produced by the mycelial structure of filamentous fungi that have no biochemical significance in fungal growth but have the potential to elicit undesirable effects on human and animal health, following consumption of contaminated food or feedstuffs [11–13]. Therefore, mycotoxin contaminated crops result in illness and economic losses once these grains cannot be ingested or marketed.

Several factors can influence the presence of mycotoxin in foods and feed such as fungal strain specificity, fungal strain variation, and toxin stability [14–16]. However, its occurrence is closely related to environmental conditions favorable to fungal growth, in particular, the hot and humid climate. i.e., mycotoxins are most frequent in tropical areas but they can also be found in a temperate climate [17,18]. Besides that, the mycotoxin contamination may also occur at various stages of the food production chain, e.g., in the storage, in the field, during harvest, processing, and distribution [19]. Currently, the worldwide contamination of food and feedstuffs with mycotoxins is a significant problem. Based on the recent data on food grains, the prevalence of mycotoxins could be as high as 60-

80%, depending on the mycotoxin of concern, the analytical method used, and the equipment detection limit [20,21].

Aflatoxins are toxic compounds produced mainly by *A. flavus* and *A. parasiticus*. Among them, aflatoxin B_1 (AFB_1) is the most relevant for a health concern, which has been classified as Group 1A by the International Agency for Research on Cancer (IARC) owing to its carcinogenic effect (IARC, 2012). This mycotoxin is closely associated, in long-term exposure, to the hepatocellular carcinoma in humans, modulation of the immune system and malnutrition [22].

Fumonisins are a heterogeneous group of toxins synthesized by *Fusarium* species [23]. Fumonisins of group B have been widely studied due to their prevalence and toxicological effects. In particular, fumonisin B_1 (FB_1) is related to chronic effects in human health e.g., hepatocarcinoma, nephrotoxicity, suppression of the immune system and defects in the neural-tubes [24]. Also, this mycotoxin has been classified by the IARC as a group 2B (possible carcinogen for humans) [25].

The growing consumer interest in nutritional aspects and food quality has contributed to the increased consumption of organically produced foods [26,27]. However, naturally produced and highly nutritious foods such as corn and its byproducts are also prone to fungal spoilage and mycotoxin production.

Biopreservation is a natural tool to extend shelf life and to increase the safety of foods by applying microorganisms or their antimicrobial active metabolites [28]. Recently, authors have described the use of Lactic Acid Bacteria (LAB) to avoid the fungal growth and prolong the shelf life of food as an alternative to synthetic biocides. However, there are no reports on the use of LAB to prevent fungal growth in corn kernels and corn ears.

Previously, it was believed that the organic acids produced by the LAB were the main antifungal agents because of the lowering of the pH. However, authors have shown that the antifungal activity of these compounds alone was too low to fully explain the antifungal activity observed [29,30]. Thus, other metabolites have been studied as the source of antifungal activity such as proteinaceous compounds, fatty acids, phenolic acid, hydrogen peroxide, and bacteriocins [31–34].

It seems that the LAB possess a large potential for biopreservation due to the production of antimicrobial compounds. Moreover, it is important to emphasize that most LAB are generally recognized as safe and QPS (Qualified Presumption of Safety), which promote them as very favorable candidates for integration as natural preservatives in food and feedstuffs [35,36]. Indeed, they are already used as starter cultures in the food industry, in dairy and dry-fermented meat products [37].

Taking into account the growing interest in the use of natural antimicrobial compounds as an alternative to synthetic chemical control and the potential of LAB, the objective of this study was to evaluate the employment of *Lactobacillus plantarum* cell-free supernatant as a biocontrol agent in order to reduce the growth of *A. flavus* ISPA 8111 (AFB_1 producer) and *F. verticillioides* CECT 2982 (FB_1 producer) as well as mycotoxin production on corn kernels and corn ears.

2. Results and Discussion

2.1. In vitro antifungal activity of L. plantarum spp

The qualitative antifungal effect of cell-free supernatant (CFS) obtained by *L. plantarum* spp fermentation was determined by halo inhibition test on the solid

medium of Potato Dextrose Agar (PDA). The analyses of results demonstrated that all evaluated strains of *L. plantarum* fermented MRS, and their CFS possessed antifungal effect (Table 1). Regarding the *L. plantarum* CECT 749, its CFS showed the highest halo inhibition zone against the strain of *F. graminearum* ITEM 126*, F. cerealis* CECT 20489, *F. verticillioides* CECT 2152, *F. verticillioides* CECT 2982, and *A. flavus* ITEM 8111 in comparison to others. Moreover, the strains of *F. verticillioides* showed higher sensitivity towards *L. plantarum* CECT 749 CFS related to other fungi.

In general, the quantitative results evidenced that the *Fusarium* strains were more sensitive to CFS than *Aspergillus* strains. Moreover, agreeing to qualitative results, all CFS showed antifungal effect, presenting minimum inhibitory concentration (MIC) values ranging from 4 to 125 g/L. Specifically, the *L. plantarum* CECT 748 and *L. plantarum* CECT 749 CFS evidenced the highest antifungal activity against *Fusarium* and *Aspergillus* strains (Table 2). Furthermore, these CFS were the only ones that demonstrated fungicidal doses against *Aspergillus* strains, more precisely, the minimum fungicidal concentration (MFC) values were 125 and 62 g of CFS/L, respectively.

Analyzing the results, it is important to emphasize that the *L. plantarum* CECT 749 CFS was more efficient than others, showing the lowest concentration of MIC and MFC against all evaluated strains. Therefore, based on results, this CFS was employed in the biocontrol assays in order to avoid the growth of *A. flavus* ITEM 8111 on corn kernels and *F. verticillioides* CECT 2982 on corn ears.

Table 1. Antifungal activity evaluated in PDA solid medium and treated with *Lactobacillus plantarum* cell-free supernatant (CFS) at 250 g/L against toxigenic fungi of *Aspergillus* and *Fusarium* genera. The antifungal activity was expressed as follows: (+) means 8 mm of inhibition zone between the well and fungal growth, (+ +) means 8-10 mm of inhibition zone between the well and fungal growth, (+ + +) means >10 mm of inhibition zone between the well and fungal growth.

Fungal strain	*L. plantarum*						
	CECT 220	**CECT 221**	**CECT 223**	**CECT 224**	**CECT 748**	**CECT 749**	**CECT 750**
F. graminearum ITEM 126	+	+	+	+	+	+ + +	+
F. graminearum ITEM 6415	+	+	+	+	+	+	+
F. cerealis CECT 20488	+	+	+	+	+	+	+
F. cerealis CECT 20489	+	+	+ +	+	+	+	+
F. verticillioides CECT 20926	+	+	+	+	+ +	+ + +	+
F. verticillioides CECT 2152	+ +	+	+ +	+	+ +	+ + +	+ +
F. verticillioides CECT 2982	+ +	+ +	+ +	+	+ +	+ + +	+
F. mesoamericanum CECT 20490	+	+	+	+	+	+	+
F. poae CECT 20165	+	+	+	+	+	+	+
A. flavus ITEM 8111	+	+	+	+	+	+ +	+
A. parasiticus CECT 2681	-	-	-	-	+	+	-
A. niger CECT 2088	-	+	-	-	+	+	+

Table 2. Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) expressed in g/L of *Lactobacillus plantarum* cell-free supernatants (CFS) against *Aspergillus* and *Fusarium* strains.

Fungal strain	*L. plantarum*													
	CECT 220		CECT 221		CECT 223		CECT 224		CECT 748		CECT 749		CECT 750	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
F. graminearum ITEM 126	8	8	8	16	8	16	8	16	8	8	8	16	8	16
F. graminearum ITEM 6415	8	16	8	13	8	13	8	13	4	16	4	16	4	16
F. cerealis CECT 20488	8	16	8	31	8	16	8	16	8	8	8	16	8	16
F. cerealis CECT 20489	8	16	16	16	16	31	4	16	4	16	4	16	4	31
F. verticillioides CECT 20926	16	31	16	31	16	31	16	31	8	16	8	31	16	31
F. verticillioides CECT 2152	4	31	4	31	4	31	4	31	4	31	4	31	4	31
F. verticillioides CECT 2982	16	31	16	31	16	31	16	31	16	31	16	31	16	31
F. mesoamericanum CECT 20490	8	16	8	31	8	16	8	16	8	16	8	16	8	16
F. poae CECT 20165	16	31	8	16	8	16	8	16	8	16	8	16	8	16
A. flavus ITEM 8111	250	nd[1]	250	nd[1]	250	nd[1]	250	nd[1]	125	250	62	125	250	nd[1]
A. Parasiticus CECT 2681	nd[1]	nd[1]	nd[1]	nd[1]	nd[1]	nd[1]	nd[1]	nd[1]	250	nd[1]	125	250	250	nd[1]
A. niger CECT 2088	nd[1]	nd[1]	250	nd[1]	nd[1]	nd[1]	nd[1]	nd[1]	250	nd[1]	250	nd[1]	250	nd[1]

[1] nd= non-detected

2.2. Phenolic acids produced by L. plantarum spp

In this study, nine phenolic acids were identified in MRS broth fermented by *L. plantarum* (Figure 1). These compounds were determined according to the retention time peak and molecular mass spectra obtained from the injected standard solution in LC-ESI-qTOF-MS and reported information in the literature. The phenolic acids produced by strains of *L. plantarum* are summarized in Table 3 along with their molecular formula and mass spectrum. Between the samples

with the highest antifungal effect, the *L. plantarum* CECT 749 produced DL-3-phenyllactic acid, salicylic acid, and vanillin, whereas the same compounds were generated by *L. plantarum* CECT 748, in addition to chlorogenic acid, sinapic acid, and 1,2-dihydroxybenzene, and with the exception of vanillin.

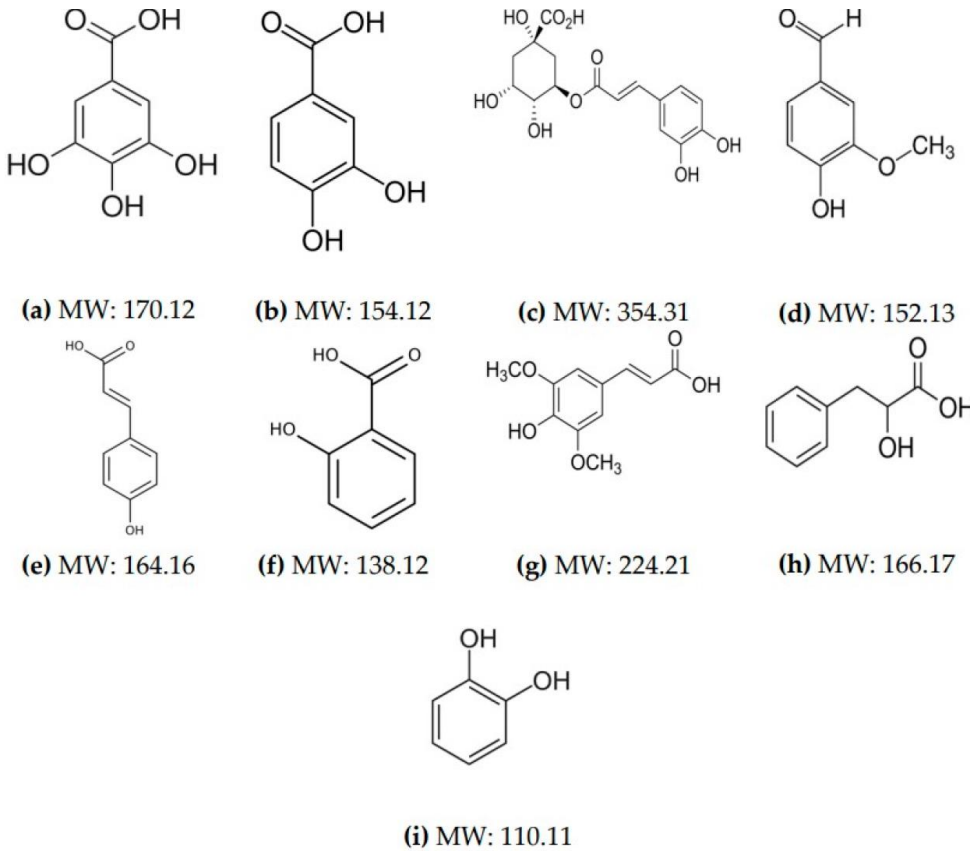

Figure 1. Chemical structures and molecular weight of the phenolic acids identified in the *L. plantarum* cell-free supernatant (CFS): (**a**) Gallic acid; (**b**)Protocatechuic; (**c**) Chlorogenic acid; (**d**) Vanillin; (**e**) p-coumaric acid; (**f**) Salicillic acid; (**g**) Sinapic acid; (**h**) Phenyllactic acid; (**i**) 1,2-Dihydroxybenzene; MW, molecular weight in g/mol.

Previous studies also identified phenolic acids produced by LAB in MRS broth. Rodrígues et al. noticed the presence of 1,2-dihydroxybenzene and other hydroxybenzoic acids such as vanillic acid, syringic acid, and gallic acid generated after hydrolyzation and methylation of hydroxybenzoic acid by enzymes of *L. plantarum* [38]. Brosnan et al. also identified phenolic acids for all the strains evaluated, more specifically, vanillic acid, 2-hydroxyisocapric acid, azelaic acid, decanoic acid, propanoic acid, 3-hydroxydecanoic acid, benzoic acid, hydrocinnamic acid, DL-p-hydroxyphenyllactic acid, phenyllactic acid, DL-β-hydroxylauric acid, 4-hydroxybenzoic acid, 3-4-hydroxy-3-methoxyphenyl, and 1,2-dihydroxybenzene [39]. Indeed, it is known that *L. plantarum* synthesizes phenolic acids by decarboxylation and/or reduction [40]. These compounds can act as chatrophic stressors, causing a disorder and cellular stress with the consequent lysis of the plasma membrane [41].

Table 3. Phenolic compounds quantified (mg/L) in the cell-free supernatant (CFS) of *Lactobacillus plantarum* strains by LC-ESI-qTOF-MS.

Compound	Molecular formula	*L. plantarum*						
		CECT 220	CECT 221	CECT 223	CECT 224	CECT 748	CECT 749	CECT 750
Gallic acid	$C_7H_6O_5$	0.7 ± 0.2	nd[1]	nd[1]	nd[1]	nd[1]	nd[1]	nd[1]
Protocatechuic	$C_7H_6O_4$	nd[1]	nd[1]	0.4 ± 0.1	nd[1]	nd[1]	nd[1]	nd[1]
Chlorogenic acid	$C_{16}H_{18}O_9$	0.6 ± 0.1	nd[1]	nd[1]	nd[1]	0.5 ± 0.1	nd[1]	nd[1]
Vanillin	$C_8H_8O_3$	nd[1]	0.3 ± 0.1	nd[1]	nd[1]	nd[1]	0.5 ± 0.2	nd[1]
p-coumaric acid	$C_9H_8O_3$	nd[1]	0.3 ± 0.1	nd[1]	0.6 ± 0.1	nd[1]	nd[1]	nd[1]
Salicylic acid	$C_7H_6O_3$	0.4 ± 0.1	0.3 ± 0.1	0.5 ± 0.2	0.3 ± 0.1	0.6 ± 0.2	0.5 ± 0.1	0.6 ± 0.1
Sinapic acid	$C_{11}H_{12}O_5$	0.7 ± 0.1	nd[1]	nd[1]	nd[1]	1.0 ± 0.2	nd[1]	0.4 ± 0.1
Phenyllactic acid	$C_9H_{10}O_3$	1.0 ± 0.2	1.1 ± 0.1	0.9 ± 0.1	1.4 ± 0.2	3.9 ± 0.1	5.3 ± 0.3	2.8 ± 0.2
1,2-Dihydroxybenzene	$C_6H_6O_2$	nd[1]	0.5 ± 0.1	nd[1]	0.6 ± 0.2	1.1 ± 0.2	nd[1]	0.9 ± 0.2

[1] nd= non-detected. Mean ± standard deviation (n=6).

Some phenolic acids -above described- were earlier studied as antifungal compounds. Among them, the 1,2-dihydroxybenzene -often known as pyrocatechol or catechol- is commonly found in its bound form in foods as part of complex structures such as lignins and hydrolyzable tannins. In addition, this phenolic acid was previously described as an antifungal compound [38,42].

Silva et al. assessed the correlations between phenolic compounds and their ability to avoiding aflatoxin production on soybeans. They demonstrated that phenolic compounds such as vanillin were able to contribute to a defense mechanism against fungal spoilage and AFB_1 production depending on the level of contamination [43]. Also, Romero-Cortes et al. evaluated the antifungal activity of vanillin against *Alternaria alternata* isolated from sorghum and barley. The vanillin showed a fungicidal effect in a dose-dependent manner; moreover, doses lower than 750 mg/L of vanillin reduced the fungal growth, increasing the lag phase from 50 to 112 h [44].

Saladino et al. evaluated the antifungal activity of LAB strains against *A. parasiticus*. The authors described that the fermentation of bread dough with LAB reduced the fungal growth as well as the content of aflatoxins. They also attributed the effectiveness of LAB by phenolic compounds and bioactive peptides presented in the media and the lowering of pH [28]. Our results pointed out that the *L. plantarum* CECT 220, CECT 221 and CECT 748 showed the highest capacity to produce phenolic compounds. And, the other strains of *L*. *plantarum* produced at least two phenolic compounds. Also, it is important to highlight that the phenolic acids identified in this study were in agreement with those that have been detected by other authors.

Despite the phenolic acids -found in the CFS- have previously been reported as antifungal compounds, the antifungal and antimycotoxigenic effects -evidenced

in our study- could be attributed to the bio-complex obtained by *L. plantarum* MRS fermentation. This bio-complex could contain other metabolites such as proteins, peptides, and organic acids that are widely known as antifungal compounds but have not been identified in our study [28,33,45]. In other words, it seems that the presence of phenolic acids in the CFS only contributed to enhancing the antifungal activity of the bio-complex.

2.3. L. plantarum CECT 749 CFS characteristics and composition

L. plantarum CECT 749 was incubated on MRS Broth for 72 h at 37 °C. After that, the bacteria cells were separated from the liquid fraction by centrifugation and, the CFS was recovered as described in section 4.3. The methodology and composition of *L. plantarum* CECT 749 CFS are plotted in Table 4. The organic acids and the final pH of the solution play an important role in mycelial growth[46]. Gerez et al. evaluated the antifungal activity of lactic acid, acetic acid, phenyllactic acid, and Propionic acid against *Aspergillus niger, Fusarium graminearum*, and *Penicillium* spp. under pH 3.5 and 6.0. These authors reported that the lowest concentration of organic acid -necessary to produce a 50% inhibition of conidia germination after 48 h of incubation- was significantly reduced in pH 3.5. For instance, the MIC of lactic acid at pH 3.5 ranged from 2.5 to 180 mM whereas in pH 6.0 the MIC ranged from 50 to 300 mM [46]. Our results corroborate with those since the pH of 3.3 ± 0.3, as well as the high content of lactic acid (15 g/L) could also contribute to the antifungal activity of the CFS.

Table 4. Characteristics and composition of cell-free supernatant (CFS) produced by *L. plantarum* CECT 749.

Parameters	*L. plantarum* CECT 749 CFS (%)	MRS (%)
Moisture[47]	94.5 ± 2.10	94.3 ± 2.40
Proteins[46]	0.3 ± 0.07	2.5 ± 0.30
Ash[48]	0.2 ± 0.06	0.2 ± 0.01
Carbohydrates[49]	3.2 ± 0.31.	2.7± 0.5
pH	3.3 ± 0.21	6.2 ± 0.22
Lactic acid[50]	1.5 ± 0.50	nd[1]

[1] nd= non-detected. Mean ± standard deviation (n=6).

2.4. Biopreservation and shelf-life improvement by L. plantarum CECT 749 CFS

In addition to evaluating the qualitative and quantitative antifungal activity of CFS, the ability to enhance shelf life was investigated on corn kernels and corn ears. The shelf life was determined by the first sign of visual fungal growth in the matrices.

The fungal growth of the control group under similar storage conditions was noticed on corn kernels after 7 days, whereas on corn ears the shelf life was determined on the fifth day onwards. Compared to control, the spray of *L. plantarum* CECT 749 CFS delayed the growth of both *A. flavus* and *F. verticillioides* CECT 2982 in 8 and 2 days, respectively (Table 5). Also, it is noteworthy that the samples of corn kernels and corn ears were treated, dried, and contaminated with final moisture content (MC) of 18 and 20%, respectively. Thus, the lower reduction in shelf life noticed on corn ears could be explained by the higher MC.

These results suggested that the CFS may be used as a biocontrol to reduce *F. verticillioides* growth on corn ears and *A. flavus* growth on corn kernels during storage, even under inappropriate MC condition.

Table 5. Fungal growth monitored in corn kernels contaminated with *Aspergillus flavus* ITEM 8111 and corn ears contaminated with *Fusarium verticillioides* CECT 2982 during the storage period. Fungal growth is expressed as (+) and absence of fungal growth as (-).

Samples	***A. flavus* ITEM 8111**				***F. verticillioides* CECT 2982**			
	Days							
	0	7	15	40	0	5	7	15
Control	-	+	+	+	-	+	+	+
CFS	-	-	+	+	-	-	+	+

Corn is a rich substrate for aflatoxin production and earlier reports demonstrated the effective use of antifungal properties of bioactive compounds produced by LAB for inhibiting AFB_1 in various crops [51]. In our study, the *L. plantarum* CECT 749 CFS reduced the fungal growth, hence, the AFB_1 production by 100% at day 5. In addition, applying the CFS spray on the surface of corn before contamination reduced the AFB_1 production by 99.7 and 97.5% at days 25 and 40, respectively (Figure 2). According to results, the *L. plantarum* CECT 749 CFS did not avoid the AFB_1 production, but this preventive treatment inhibited its production by 10 d.

The data related to FB_1 production are plotted in Figure 2. The results showed that *L. plantarum* CECT 749 CFS possess antimycotoxigenic activity against *F. verticillioides* CECT 2982, reducing the FB_1 production by 90.6% at day 7. However, it was realized that the antimycotoxigenic effect provoked by treatment

decreased over time, reaching to 73.7% of reduction after 15 days of storage. The mycotoxin reduction, evidenced in this study, could be explained perhaps because the treatment with CFS delayed the fungal growth, hence, the fungi need more time to achieve the secondary metabolism.

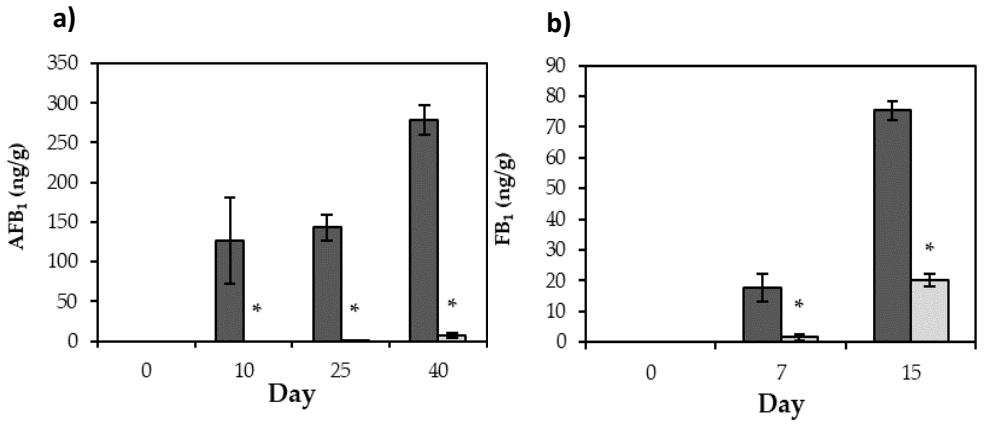

Figure 2. Effect of *L. plantarum* CECT 749 CFS on mycotoxin production: (**a**) AFB_1 content (ng/g) in corn kernels contaminated with *A. flavus* ITEM 8111 at different storage times (0, 10, 25 and 40 days). Control samples (dark grey) and treated samples (clear grey); (**b**) FB_1 content (ng/g) in corn ears contaminated with *Fusarium verticillioides* CECT 2982 at different storage times (0, 7 and 15 days). Control samples (dark grey) and treated samples (clear grey). **(*)** represents a significant difference among the treatments ($p \leq 0.05$). The experiment was carried out twice with analyses in triplicate (n=6).

Authors have noticed that LAB have the potential to either bind and mitigate mycotoxin or, to some extent, inhibit the fungal growth [52–55]. Although the LAB antimycotoxigenic mechanisms of action have not yet been explained, analyzing our data, it seems that they may be closely linked to the antifungal effects evidenced in this work. Damayanti et al. isolated twenty-eight LAB from cassava

and bean fermentation. The LAB with the highest activities was *L. plantarum* G7 [56]. Its CFS extract showed inhibition of mycelial growth between 39-63.1%. Consequently, the aflatoxin content was reduced. Also, Russo et al. investigated the antifungal capacity of eighty-eight *L. plantarum* strains [57]. They concluded that the employment of LAB increased shelf life. Although they had investigated the antifungal activity of CFS against toxigenic fungi, the mycotoxin content was not evaluated in the food samples.

Overall, the treatment did not completely avoid AFB_1 and FB_1 production, however, in both corn kernels and corn ears, the final concentration of mycotoxin was significantly reduced after fumigation of *L. plantarum* CECT 749 CFS compared to the control group. For instance, after incubation time, the final AFB_1 and FB_1 concentration in the treatment group was 6.9 and 20.1 ng/g, whereas the concentration of AFB_1 and FB_1 in the control group reached values of 278.4 and 75.4 ng/g, respectively.

On the one hand, despite the large reduction of AFB_1 provoked by CFS application, the levels of AFB_1 were higher than those set by the European Union (5 ng/g). On the other hand, the levels of AFB_1 and FB_1 were lower than those established by the FDA (20 ng/g of AFB_1 and 2000 ng/g of FB_1) to non-processed corn for human consumption [58–60].

The present results suggested that metabolism products of LAB, due to their potential to reduce the growth of the mycotoxigenic fungi and the biosynthesis of the mycotoxins, could be promising for the biocontrol of grains such as corn.

Corn grains are constantly stored for a long time before commercialization, further studies could be performed, increasing samples analysis time and setting the MC at a percentage slightly over 14% since this percentage is adequate for

grain storage [61]. In addition, we expect to isolate the antifungal compounds produced by the *L*. *plantarum* CECT 749 and evaluate the purified fraction activity as well as to compare them to the whole CFS extract. Also, it might be worthwhile to increase the experiment from a laboratory scale to an industrial scale in order to produce more compounds and to evaluate their constancy of production.

3. Conclusions

In the past, LAB have shown potential as antifungal agents to avoid fungal spoilage and the related mycotoxin production in foods such as dairy and fermented products. In this study, *in vitro* analyses have shown that *Fusarium* strains were more susceptibility to CFS treatment than *Aspergillus* spp. In terms of fungal growth and mycotoxin production in both corn kernel and corn ears, the CFS treatment did not completely inhibit either the fungal growth or mycotoxin production. Probably, high levels of antifungal compounds associated with lower MC might be required to completely avoid the growth of *A. flavus* and *F. verticillioides* on corn kernels and corn ears. In contrast, even when the mycotoxin production occurred, the content of FB_1 and AFB_1 was significantly reduced. Therefore, these results provide new insights for the biotechnological employment of *L. plantarum* CECT 749 CFS for biopreservation of corn kernels and corn ears.

4. Materials and methods

4.1. Chemicals

AFB_1 and FB_1 standard solutions (purity >99%) were purchased from Sigma-Aldrich (St. Louis, MO). The phenolic compounds gallic acid, sinapic acid, chlorogenic acid, caffeic acid, syringic acid, vanillin, vanillic acid, hydroxybenzoic acid, hydroxylcinnamic acid, p-coumaric acid, benzoic acid, DL-3-phenyllactic acid, 1,2-dihydroxybenzene, 3,4-dihydroxyhydrocinnamic, and DL-p-hydroxyphenyllatic acid were provided from Sigma-Aldrich (St. Louis, MO). Phenyllactic acid was obtained from BaChem (Weil am Rhein, Germany). Ferulic acid was purchased from MP Biomedicals (Santa Ana, CA) and protocatechuic acid from HWI pharma services (Ruelzheim, Germany). Acetonitrile (ACN) (99%), methanol (99%), ethyl acetate (EA) (99%) and formic acid (FA) (99%) used for liquid chromatography were of HPLC-grade and obtained from VWR Chemicals (Radnor, PA). Magnesium sulfate ($MgSO_4$), ammonium formate and sodium chloride (NaCl) were obtained from Sigma-Aldrich (St. Louis, MO). Sepra™ C18-E (50 µm, 65 Å) was purchased from Phenomenex (Madrid, Spain). Microbiological media such as Potato Dextrose Broth (PDB), Potato Dextrose Agar (PDA), Man Rogosa Sharpe (MRS) Broth and MRS Agar were obtained from Liofilchem Bacteriology Products (Roseto degli Abruzzi, Italy). Deionized water (<18 MΩ cm resistivity) was obtained from a Milli-Q purification system (Millipore, Bedford, MA).

4.2. Microorganisms and culture conditions

Fungal strains *A. parasiticus* CECT 2681, *A. niger* CECT 2088*, F. verticillioides* CECT 20926, *F. verticillioides* CECT 2152, *F. verticillioides* CECT 2982, *F. cerealis* CECT 20488, *F. cerealis* CECT 20489, *F. mesoamericanum* CECT 20490, and *F. poae*

CECT 20165 were obtained from the Spanish Type Culture Collection CECT (Valencia, Spain*). F. graminearum* ITEM 126, *F. graminearum* ITEM 6415 and *A. flavus* ITEM 8111 were obtained from the microbial culture collection of the Institute of Sciences of Food Production, ISPA (Bari, Italy). These microorganisms were maintained in sterile glycerol (30%) at -80 °C prior to analysis. The fungal strains were cultivated on PDB, transferring 1 mL of the glycerinated solution to 9 mL of PDB medium. The contaminated PDB was incubated for 72 h at 25 °C, and then, aliquots were plated on PDA Petri dishes in order to obtain spores. These spores were used to contaminate the corn samples.

LAB strains *Lactobacillus plantarum* CECT 220, *L. plantarum* CECT 221*, L. plantarum* CECT 223*, L. plantarum* CECT 224, *L. plantarum* CECT 748, *L. plantarum* CECT 749 and *L. plantarum* CECT 750 were obtained from the Spanish Type Culture Collection CECT (Valencia, Spain). The LAB were kept in frozen glycerol (3%) stocks at -80 °C until analysis. Prior to fermentation assays, 1 mL of LAB glycerinated was homogenized with 9 mL of MRS Broth culture medium and incubated under anaerobic conditions for 48 h at 37 °C.

4.3. LAB growth and preparation of cell-free supernatant

LAB were incubated on MRS Broth for 12 h at 37 °C in order to reach the exponential growth phase. Then, 10 mL of incubated MRS Broth at a concentration of 10^7 CFU/mL were placed in other Falcon tubes containing 40 mL of sterile MRS Broth. Subsequently, the tubes were incubated under anaerobic conditions for 72 h at 37 °C to allow MRS fermentation. After that, LAB cells were separated from the fermented solution by centrifugation at 4000 *g* for 10 min. Lastly, Cell-Free Supernatants (CFS) were recovered and freeze at -80 °C for 24 h

before drying with a lyophilizer FreeZone 2.5 L Labconco (Misuri, USA). The lyophilized CFS was stored at -18 °C prior to antifungal analyses.

4.4. Qualitative antifungal activity in solid medium

The antifungal effect of lyophilized CFS was evaluated towards strains of *Aspergillus* and *Fusarium* genera.

First, Petri dishes containing PDA were contaminated with harvested fungal conidia previously cultivated on PDA plates, as described by section 5.2. The conidia were scraped from agar and dispersed on another PDA plate, using sterile cotton swabs. Briefly, 10 mm diameter wells were made using sterile pipette tips, whereas each well was filled with 50 µL of lyophilized CFS which was previously resuspended in sterile water at the concentration of 250 g/L. A sterile water solution containing lyophilized MRS Broth was used as negative control. After that, the PDA plates were incubated for 72 h at 25 °C to allow the fungal growth and diffusion of the CFS through agar. Finally, the antifungal effect was determined by measuring the inhibition zone around the well. The treatments which showed an inhibition zone larger than 7 mm between the well and fungal growth were considered positive for antifungal activity [62].

4.5. Quantitative antifungal activity in 96-well microplates

The quantitative assay was performed as described by the guidelines in CLSI document M38-A2 with slight modifications [63]. First, 100 µL of lyophilized CFS at concentrations from 0.48 to 500 g/L was added to 96-well sterile microplates. Then, microwells were contaminated with 100 µL of PDB containing toxigenic

fungi at a concentration of 5 x 10^4 conidia/mL. The positive control consisted of contaminated PDB medium with lyophilized non-fermented CFS (250 g/L) and the negative control was prepared with a non-contaminated PDB medium without any treatment. Posteriorly, the microplates were incubated at 25 °C for 3 days.

The MIC was defined as the lowest concentration of the fermented CFS that did not show any visible growth. Four replicates of each treatment were carried out. After that, 10 µL of the concentration corresponding to the MIC and higher concentrations evaluated were recultivated on PDA plates for the determination of the MFC. Plates were incubated at 25 °C for 72 h and the MFC was determined as the concentration that avoided any visible fungal growth.

4.6. Extraction and identification of phenolic acids by LC-ESI-qTOF-MS

The CFS extracts were cleared by QuEChERS methodology prior to chromatographic analysis as described by Brosnan et al.[64]. Thus, ten milliliters of fermented CFS were extracted with 10 mL ethyl acetate 1% formic acid, 4 g of $MgSO_4$, 1 g of NaCl and then vortexed for 1 min. The extract was centrifuged at 4000 *g* for 10 min, and the supernatant was recovered in a Falcon tube containing 150 mg of C18 and 900 mg $MgSO_4$. The extraction was realized twice and the supernatant was evaporated under nitrogen flow. Afterward, the dried supernatant was resuspended in 1 mL of H_2O: ACN (90:10) prior to chromatographic analysis.

LC system used for the chromatographic determination was an Agilent 1200 (California, USA) equipped with a vacuum degasser, autosampler, and binary pump. The stationary phase was a Phenomenex column (Gemini C18 50 x 2 mm, 100 Å and particle size of 3 µm). Mobile phases consisted of water as solvent A,

ACN as solvent B both acidified (0.1% formic acid) and the gradient elution was established as follows: 0 min, 5% B; 30 min 95% B; 35 min, 5% B. The column was equilibrated for 3 min prior to analyses. The flow rate used was 0.3 mL/min and the sample volume injected was 20 µL.

Mass spectrometry analysis was carried out using a Q-TOF-MS (6540 Agilent Ultra High Definition Accurate Mass) equipped with an Agilent Dual Jet Stream electrospray ionization (Dual AJS ESI) with interface in negative ionization mode at the following conditions: drying gas flow (N_2), 8.0 L/min; nebulizer pressure at 30 psig; gas drying temperature at 350 °C; capillary voltage at 3.5 kV; fragmentor voltage and scan range were 175 V and m/z 20-380, respectively. Targeted MS/MS experiments were carried out using collision energy of 10, 20 and 40 eV. Integration and data elaboration were realized using Masshunter Qualitative Analysis Software B.08.00 [65].

4.7. Biopreservation of corn kernels and corn ears

To evaluate the antifungal and antimycotoxigenic activity of the CFS by *L. plantarum* CECT 749, the study was carried out using corn kernels contaminated with *A. flavus* ISPA 8111 (AFB_1 producer) and corn ears contaminated with *F. verticillioides* CECT 2982 (FB_1 producer). These strains were selected according to the fungal sensitivity to fermented CFS – demonstrated by qualitative and quantitative assays (5.4. and 5.5.); the main steps of food production chain in which the fungal contamination occurs; and the mycotoxin production – previously determined in corn and corn ears.

The biopreservation test was performed in laboratory scale silos as described by Nazareth et al. with modifications [60].

Samples of corn kernels (50 g) were divided into two groups: a group was sprayed with 10 mL of lyophilized CFS in order to obtain a final concentration of 12.5 mg of CFS/g of corn, and a control group was sprayed with 10 mL of sterile water (Figure 3). Next, the corn kernels were dried in an incubator (Memmert D06061 Modell 500; Memmert GmbH + Co. KG, Schwabach, Germany) at 50 °C for 30 min. Then, both groups were inoculated with suspensions containing 1 mL of *A. flavus* ISPA 8111 at 10^3 conidia/g. Lastly, the sampling was performed on days 0, 7, 15, 40 to determine the AFB_1 production.

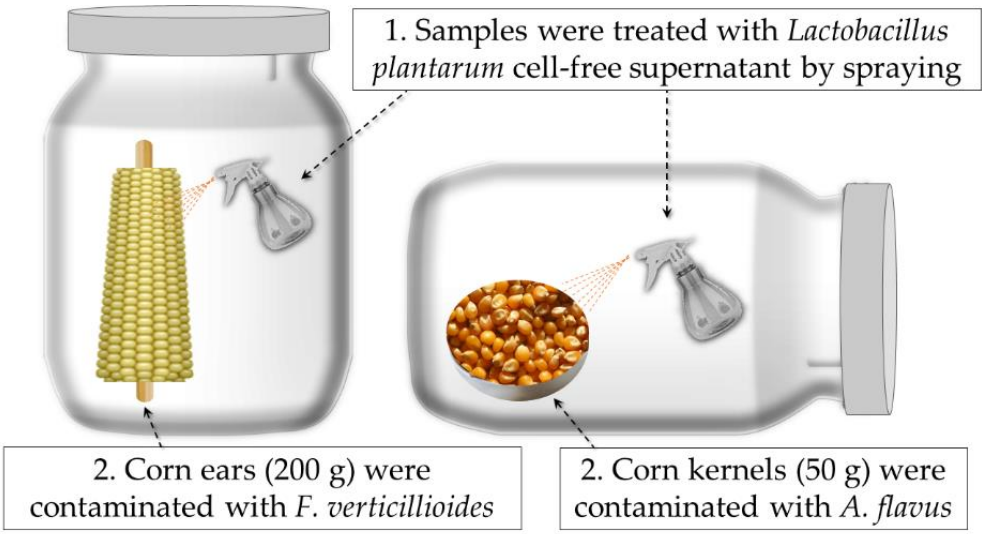

Figure 3. Biopreservation test of corn ears and corn kernels in a lab system. The samples were treated with *Lactobacillus plantarum* CECT 749 cell-free supernatant. After drying, corn kernels were contaminated with *Aspergillus flavus* ISPA 8111 and corn ears were contaminated with *Fusarium verticillioides* CECT 2982.

Similarly, 200 g of corn ears were treated with 6 mL of CFS equivalent to a concentration of 1.6 mg of lyophilized CFS /g of corn ears. In this case, corn ears were contaminated with 1 mL of *F. verticillioides* CECT 2982 suspension at a concentration of 10^3 conidia/g. And, the FB_1 production was determined on days 0, 7, and 15. Moreover, visual fungal growth was monitored daily in both studies. Also, samples were placed in the incubator at 105 °C for 24 h and the initial MC was measured by gravimetric methodology [66].

4.8. Determination of mycotoxins by LC-MS/MS spectrometry

AFB_1 and FB_1 mycotoxins were extracted using the method described and validated by Quiles et al.[67]. Corn kernels were ground with an Oester Classic grinder (Madrid, Spain) in order to reduce particle size. Corn ears were frozen at -80°C and lyophilized before grinding. The samples were weighed in 5 g, placed in a Falcon tube with 25 mL of MeOH, and homogenized for 3 min using an Ultra Ika T18 basic ultraturrax (Staufen, Germany). After, the extract was centrifuged at 4000 *g* for 15 min at 4°C, and the supernatant was evaporated to dryness with Büchi Rotavapor R-200 (Postfach, Switzerland). Finally, the dried extracts were resuspended in 2 mL of methanol and filtered in 0.22 µm before LC-MS/MS analysis.

The liquid-chromatography system consisted of an LC-20AD pump coupled to a 3200QTRAP mass spectrometer (Applied Biosystems, Foster City, CA, USA) using an ESI interface in positive ion mode. The mycotoxins were separated on a Gemini NX C18 column 150 x 2 mm x 3 µm Phenomenex (Palo Alto, CA, USA). The mobile phases were the solvent A (5 mM ammonium formate and 0.1% formic acid in water) and solvent B (5 mM ammonium formate and 0.1% formic acid in

methanol) at a flow rate of 0.25 mL/min. The elution was carried out using a linear gradient from 0 to 14 min. The injection volume set was 20 µL, the nebulizer, the auxiliary and the auxiliary gas were set at 55, 50, and 15 psi respectively. The capillary temperature and the ion spray voltage were 550°C and 5500 V, respectively. The ions transitions used for the mycotoxins identification and quantification were: m/z 313.1, 241.3, and 284.9 for AFB_1, m/z 352.4 and 334.4 for FB_1.

4.9. Statistical analysis

The software Prism version 3.0 (GraphPad, La Jolla, CA, USA) for Windows was used for the statistical analysis of data. The experiments were realized in triplicates and the difference between control and treated group was analyzed by Student's t-test. The level of significance was considered as $p \leq 0.05$.

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3.7. Toxicity reduction of ochratoxin A by lactic acid bacteria

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1. Introduction

Ochratoxin A (Fig. 1) (OTA) is a mycotoxin produced by secondary metabolism of many filamentous species belonging to the genera *Aspergillus* and *Penicillium*.

OTA is a potent nephrotoxic mycotoxin in nature and also displays other adverse effects such as hepatotoxicity, teratogenicity, and immunosuppression (Pfohl-Leszkowicz, Pinelli et al., 1998). OTA has been proven to be carcinogenic in kidney and liver. It has been classified as a group 2B human carcinogen by the International Agency for Research on Cancer (IARC), and World Health Organization (WHO) (IARC, 1993). OTA has been putatively implicated in the etiology of Balkan endemic nephropathy (BEN) and recognized to be related to urinary tract tumors in animals (Paul et al., 2002; Pfohl-Leszkowicz et al., 2002).

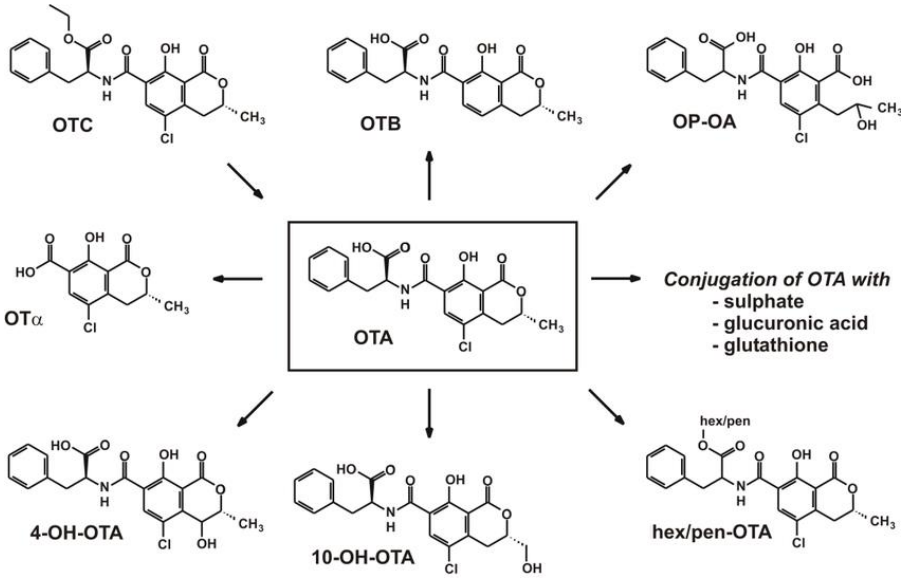

Fig. 1. Chemical structure of the OTA and of the main conjugation and degradation products.

Regarding the alimentary aspect, OTA is a common contaminant of grains such as barley, corn, rye, wheat, and oats, with cereal-based products typically accounting for 50–80% of the average consumer intake of the mycotoxin (Jorgensen and Jacobsen, 2002). OTA has also been reported in other plant products including coffee beans, spices, nuts, olives, grapes, beans, and figs (O'Callaghan et al., 2006). In addition, OTA can survive many typical food-processing procedures, and has been reported in bread made from contaminated wheat (Scudamore et al., 2003), in beer and wine (Odhav and Naicker, 2002).

Many countries and international organizations have regulated the OTA content in several commodities. The European Commission, 2006 has enforced the limits of OTA in cereals and cereal products with the following levels: 5.0 ng/g for raw cereal grains, 3.0 ng/g for cereals and cereal products intended for human consumption, 0.5 ng/g for baby food and cereal-based food intended for young children. For the dried vine fruits, soluble coffee and some dried fruits, the European commission has set a maximal permissible limit for OTA at 10.0 ng/g.

OTA is a moderately stable molecule, which can survive most food processings, such as roasting, brewing and baking, to some extent (Scott, 1996). Several chemical and physical methods such as hypochlorite treatment (Refai et al., 1996), ammoniation and heat treatment have been developed to detoxify OTA in animal feed or alcoholic beverages. Other detoxification methods suggest the use of ozone, alkaline hydrogen peroxide treatment and gamma irradiation in cereals and derivates (Janos et al., 2000).

Yeast, including *Saccharomyces* sp., *Rhodotorula* sp., *Cryptococcus* sp., and *Trichosporon mycotoxinivorans*, can hydrolyze the amide bond of OTA to produce nontoxic products phenylalanine (Phe) and ochratoxin alpha (OTA-α) (Abrunhosa et al., 2010).

A bacterium, *Phenylobacterium immobile*, which can use Phe as a sole carbon source, converted OTA to OTA-α and three other metabolites resulting from the breakdown of the phenyl moiety of Phe (Wegst and Lingens 1983).

The aims of the study were to evaluate a) the OTA elimination by different strains of lactic acid bacteria in MRS medium, b) the OTA reduction during a simulated gastrointestinal digestion in presence of lactic acid bacteria, using an *in vitro* dynamic model and c) the OTA degradation products formed during the fermentations through the technique of the Liquid chromatography coupled to the mass spectrometry with linear ion trap.

2. Materials and methods

2.1. Chemicals

A stock standard solution of OTA (Sigma–Aldrich, St. Luis, USA) was prepared by dissolving 1 mg of OTA standard in 1 mL of pure methanol, obtaining a 1 mg OTA/mL (1000 µg/mL) solution. This stock solution was diluted with methanol in order to obtain the appropriated work solutions (1, 10 and 100 mg/L). OTA solutions were stored in amber vials at 4 ºC until the liquid-chromatography coupled a fluorescence detector (LC-FLD) analysis. Acetonitrile, methanol, water, ethyl acetate (all of LC grade) and acetic acid were purchased from Merck (Whitehouse Station, NJ, USA).

2.2. Assay for proteolytic activity

Proteolytic activity was assessed by an agar diffusion method. Milk agar plates containing 1.6% (w/v) skim milk and 1.5% (w/v) agar were prepared. Ten

microliter of each strain at a final concentration of 10^8 CFU/mL was inoculated onto plates. After incubation at 37°C for 48h, absence of an inhibitory zone was observed. Each plate was examined for clear zones (Cho et al., 2015).

2.3. Strains and methodology

Twenty-seven commercial probiotic strains were used in the *in vitro* tests to evaluate their capacity to reduce OTA in MRS. In particular, *Bf. adolescentis* CECT 5781T, *Bf. bifidum* CECT 870T, *Bf. breve* CECT 4839T, *Bf. longum* CECT 4551, *Lb. casei* CECT 475T, *Lb. casei* CECT 4040, *Lb. casei*-CECT 4045, *Lb. delbrueckii bulgaricus* CECT 4005, *Lb. fermentum* CECT 562, *Lb. johnsonii* CECT 289, *Lb. paracasei* CECT 4022, *Lb. plantarum* CECT 220, *Lb. plantarum* CECT 221, *Lb. plantarum* CECT 222, *Lb. plantarum* CECT 223, *Lb. plantarum* CECT 224, *Lb. plantarum* CECT 225, *Lb. plantarum* CECT 226, *Lb. plantarum* CECT 748, *Lb. plantarum* CECT 749, *Lb. reuteri* CECT 725, *Lb. rhamnosus* CECT 278T, *Lb. rhamnosus* CECT 288, *Lb. sakei* CECT 906T, *Lb. salivarius* CECT 4062, *Lb. salivarius* CECT 4305, *Lc. mesenteroides* CECT 219T, *Lc. mesenteroides* CECT 215 and *Lc. mesenteroides* CECT 394 were obtained from the Spanish Type Culture Collection (CECT Valencia, Spain), in sterile 18% glycerol.

The microbes were cultured in 15 mL sterile plastic centrifuge tubes utilizing as growth medium 10 mL of De Man-Rogosa-Sharpe (MRS broth, Oxoid Madrid, Spain) for *Bifidobacterium* and *Lactobacillus*. The tubes were incubated at 37 ºC under anaerobic conditions (Anaerocult A, Merk-Darmstadt, Germany) before experiments. Then, the suspensions of each strain at concentrations of 10^8 CFU/mL were added to a fresh 10 mL of MRS adjusted to pH 3.5 and 6.5 contaminated with 0.6 µg/mL of OTA and incubated at 37 ºC during 24 h.

The mediums were analyzed in order to determinate the residual concentrations of OTA present in the growth medium and also to identify the possible degradation products.

2.4. OTA extraction and analysis from MRS medium

The fermentation tubes were centrifuged at 4000 rpm (Centrifuge 5810R, Eppendorf, Germany) for 5 min at 4 ºC in order to separate the fermented medium from the cells. OTA in the fermented medium was extracted as follows (Abrunhosa et al., 2014). Five milliliters of fermented MRS were putted in a 20 mL test tube, and extracted three times with 5 mL of ethyl acetate utilizing a vortex (VWR International, Barcelona, Spain) for 1 min. The resulting extracts were centrifuged (Centrifuge 5810R, Eppendorf, Germany) at 4000 rpm for 10 min at 4 ºC. The organic phases were completely evaporated using a rotary evaporator (Buchi, Switzerland) operating at 30 ºC and 30 mbar pressure; the remaining was resuspended in 1 mL of methanol, filtered with 0.22 µm filters (Pheomenex, Madrid, Spain) and analyzed by LC–FLD (Shimadzu, Kyoto, Japan). Specifically, chromatographic separation was performed under gradient conditions at flow rate of 1.2 mL/min. The fluorescence detector was set up at λex 333 nm and λem 460 nm. The instrument was equipped with a Hamilton syringe (Reno, Nev.) and a conventional C18 column was used. The mobile phases were composed of two eluents, both containing 1% acetic acid, the eluent A was water and the eluent B acetonitrile. The elution gradient was established initially with 30 % eluent B, increased to 70 % in 5 min. The eluent B is reduced to 30% in 5 min, afterwards, the initial conditions were maintained for 5 min (Tafuri et al., 2008).

2.5. OTA extraction from cell wall

For the analysis of OTA adsorbed on cell walls, extraction of the OTA from the bacteria was performed using 1 mL of methanol. The extraction was executed by agitation with an orbital shaker (IKA Ks 260 basic, Stanfen, Germany) for 12 h, then the samples were centrifuged at 4000 rpm (Centrifuge 5810R, Eppendorf, Germany) for 5 min at 4 ºC, and the supernatant was used for direct LC-FLD analysis of the toxin (Tafuri et al., 2008).

2.6 LC-MS-LIT identification of the OTA degradation products

An Applied Biosystems MDS SCIEX Q TRAP TM linear ion trap mass spectrometer (Concord, Ontario, Canada), coupled with a Turbo Ion Spray source was used. This instrument is based on a triple-quadrupole path (QqQ) in which the third quadrupole can also be operated as a linear ion trap (QqLIT) with improved performance. In the QqLIT configuration the Q TRAP TM can also operate in enhanced resolution scan (ER) and in enhanced product ion scan (EPI) modes. Applied Biosystem/MDS SCIEX Analyst software version 1.3.2 was used for data acquisition and processing.

A Gemini (150x2.0 mm, 5 mm) Phenomenex column was used. LC was set using a constant flow of 0.2 mL/min of acetonitrile/water (70:30, v/v with 0.1% of HCOOH) isocratically.

The instrument was operated in the positive-ion electrospray mode using the following parameters: cone voltage 40 V, capillary voltage 3.80 kV, source temperature 350 ºC, desolvation temperature 270 ºC and collision gas energy 5eV. Characterization of the newly formed compounds was performed with the modality of ER, utilizing the mass range from 50 to 600 Da.

Characterizations of the fragments obtained in the ER scan with the modality EPI scan to obtain a MS2 scan of a fragment of the molecule. Study of the fragments obtained in the EPI modality utilizing the modality MS/MS/MS that permits us to know the MS3 of a fragment selected in the ER scan.

2.7 In vitro dynamic digestion model

Gastrointestinal digestion in the in vitro dynamic model was carried out using 5 L bioreactors Infors (Bottmingen, Switzerland) with a working volume of 4 L. For agitation, two Rushton turbines (Ø=45 mm) were used. The agitation rate during all the gastrointestinal digestion steps was set at 260 rpm. The incubation temperature was maintained at 37°C.

Twenty grams of spiked loaf bread inoculated with OTA at a final concentration of 10 µg/g were mixed with 60 mL of artificial saliva (composed of: 10 mL of KCl (89.6 g/L), 10 mL of KSCN (20 g/L), 10 mL of NaH2PO4 (88.8 g/L), 10 mL of Na2SO4 (57 g/L), 1.7 mL of NaCl (175.3 g/L), 20 mL NaHCO3 (84.7 g/L), 8 mL of urea (25 g/L), and 290 mg of α-amylase). The bacterial strains were also added individually at 10^5 CFU/mL to simulate the intake of a food enriched with probiotic microorganisms and consumed at the same time as contaminated loaf bread. The pH of this solution was increased to 6.8 with a 0.1 N NaOH solution. The mixture was placed in a plastic bag containing 1 L of water at 37°C, homogenized with a Stomacher IUL Instrument (Barcelona, Spain) for 30 s and introduced in the fermenter vessel. Five g of pepsin (14800 U) dissolved in 250 mL of 0.1 N HCl was introduced into this mixture, through a fermenter insert. The pH of the mixture was decreased to 2 with the addition of 0.5 N HCl contained in a glass bottle, by means of a peristaltic pump. The incubation temperature was set at 37°C, by

transferring the mixture to the fermenter vessel through a heater plate. All fermentation parameters were regulated through the software Iris 5.0 (Infors AG CH-4103, Bottmingen, Switzerland). The total incubation time was 2h. An aliquot of 20 mL of gastric fluid was sampled for the determination of the mycotoxins gastric bioaccessibility. After gastric digestion, pancreatic digestion was simulated by increasing the pH to 6.5 with NaHCO3 (0.5 N), which was contained in a glass bottle and introduced into the fermenter vessel through a peristaltic pump. Thereafter, 25 mL of pancreatin (8 mg/mL) and 25 mL of bile salts (50 mg/mL) dissolved in 200 mL of water, were introduced into the fermenter vessel and incubated at 2 g at 37°C for 2 h. An aliquot of 20 mL of the duodenal fluid was sampled for the determination of the mycotoxins duodenal bioaccessibility (Manzini et al., 2015).

2.8. OTA extraction from the simulated gastrointestinal fluids

OTA contained in gastric and duodenal fluids were extracted as follows (Tafuri et al., 2008). Five milliliters of each mixture were placed in a 14 mL plastic test tube, and extracted three times with 5 mL of ethyl acetate using a vortex VWR International (Barcelona, Spain) for 1 min. The mixtures were then centrifuged (Centrifuge 5810R, Eppendorf, Germany) at 2880 g for 10 min. at 4°C. The organic phases were completely evaporated with a rotary evaporator (Buchi, Switzerland) at 30 °C and 30 mbar pressure, resuspended in 1 mL of methanol and filtered with a 0.22 µM filter (Phenomenex, Madrid, Spain) before being analyzed by LC-FLD.

2.9. Statistical analysis of data

Graphpad Prism version 6.0 (Graphpad Software Inc., La Jolla, CA, USA) was used for the statistical analysis of data. Differences between groups were carried out using analysis of variance (ANOVA) followed by Dunnet's multiple comparison tests. Differences were considered significant if $p \leq 0.05$.

3. Results and discussion

3.1. Method performance

Mean recoveries were operated on the fortified MRS (n=3) at levels of OTA (0.1 and 1000 µg/L) and also on the cell wall model, and the mean recoveries detected for OTA in the two matrices used were of 88.3±3.4% and 83.6±4.2%, respectively. Intra-day (n=3) and interday (3 different days) variation values ranged between 2.6 and 4.2%. The detection limit (LOD) and the limit of quantification (LOQ) values were calculated according to s/n=3 and s/n=10, respectively. The LODs and the LOQs of OTA were 0.1 and 1 µg/L. The matrix effects (% ME) evidenced for OTA in the medium used for the screening on the OTA reduction by the isolated microorganism was of 88%, whereas in cells the percentage of the ME detected was of 75%.

3.2. Proteolytic activity of the lactic acid bacteria

In Table 1 are showed the results related to the proteolytic activity evidenced by LAB using skim milk agar plate. This test is specifically to detect the capacity of the LAB to hydrolyze the caseins presents in the skim milk and to know the proteolytic potential of the bacteria used in the study. The analysis of the results

evidenced that from the 27 LAB tested, only 17 showed a proteolytic activity evidencing a hydrolysis zone in the milk plate. The LAB that evidenced the highest proteolytic activity, with hydrolysis zone higher than 10 mm, were the strains of *Lb. delbrueckii bulgaricus* CECT 4005 (Fig. 2), *Lb. paracasei* CECT 4022, *Lb. plantarum* CECT 221, *Lb. plantarum* CECT 223 and *Lb. plantarum* CECT 748. Twelve trains showed a positive proteolytic activity but with hydrolysis zones lower than 10 mm. Ten strains of the 27 employed did not show any proteolytic activity using the method employed in this study.

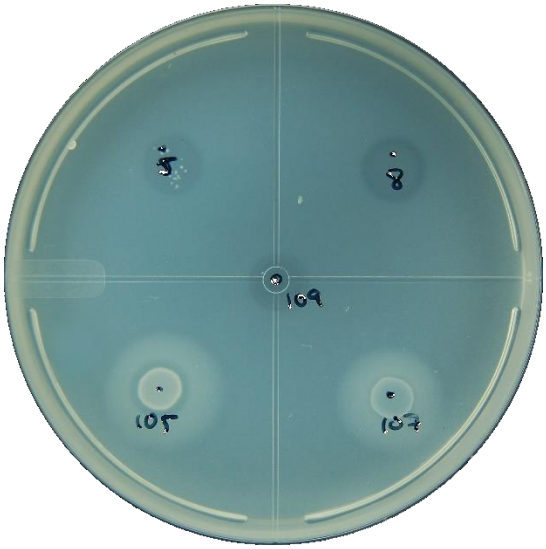

Fig. 2. Proteolytic inhibition zones evidenced by *Lb. delbrueckii bulgaricus* CECT 4005 and *Lb. paracasei* CECT 4022 due to the caseins hydrolysis, through the milk agar diffusion method.

Table 1. Comparisons of proteolytic activity using skim milk agar plate of LAB used in this study for the experiments of OTA reduction in MRS medium. The plates were incubated at 37°C for 24 (+=<10 mm and ++=>10 mm of inhibition zone respectively).

Strain	Proteolytic activity
Bf. adolescentis CECT 5781T	-
Bf. bifidum CECT 870T	+
Bf. breve CECT 4839T	+
Bf. longum CECT 4551	-
Lb. casei CECT 475T	-
Lb. casei CECT 4040	+
Lb. casei CECT 4045	+
Lb. delbrueckii bulgaricus CECT 4005	++
Lb. fermentum CECT 562	-
Lb. johnsonii CECT 289	+
Lb. paracasei CECT 4022	++
Lb. plantarum CECT 220	+
Lb. plantarum CECT 221	++
Lb. plantarum CECT 222	+
Lb. plantarum CECT 223	++
Lb. plantarum CECT 224	-
Lb. plantarum CECT 748	++
Lb. plantarum CECT 749	+
Lb. reuteri CECT 725	-
Lb. rhamnosus CECT 278T	+
Lb. rhamnosus CECT 288	+
Lb. sakei CECT 906T	-
Lb. salivarius CECT 4062	+
Lb. salivarius CECT 4305	-
Lc. mesenteroides CECT 219T	-
Lc. mesenteroides CECT 215	+
Lc. mesenteroides CECT 394	-

The hydrolysis of the protein components by LAB can due to the proteolytic systems of that microorganisms that can be divided into three groups on the basis of their function: (i) proteinases that breakdown caseins to peptides, (ii) peptidases that degrade peptides, and (iii) transport systems that translocate the breakdown products across the cyto- plasmic membrane.

3.3. OTA reduction in MRS medium

In Table 2 are reported the data related to the degradation of the OTA reduction evidenced by the lactic acid bacteria in MRS medium, adsorbed by LAB cell wall and hydrolyzed by LAB extracellular proteolytic enzymes. Finally, the total reduction of OTA was calculated taking into account the two mechanisms studied in this work as the enzymatic one and also the adsorption on the microbial cell walls.

Considering the part related to the OTA quantified in the medium (at pH 3.5) after the microbial concentration, in the control experiment the concentrations evidenced at pHs 3.5 and 6.5 were 286±3.3 and 630±6.6 ppb respectively evidencing a lower stability of the OTA in the acidified medium. The lower concentrations of the OTA were detected in the mediums fermented by *Lb. rhamnosus* CECT 278T, *Lb. casei* CECT 4045 and *Lb. plantarum* CECT 749 with 8.2±2.2, 20.6±3.6 and 15.4±2.5 ppb respectively. The highest amounts of the OTA were detected in the MRS mediums fermented with *Lb. plantarum* CECT 221 and *Lc. mesenteroides* CECT 215 with 200.0±3.7 and 285.3±4.6 ppb respectively.

Considering the OTA adsorbed on the LAB cell wall at the acidic pH the strains that demonstrated a big adsorption activity were *Lb. johnsonii* CECT 289, *Lb. bulgaricus* CECT 4005 and *Lb. salivarius* CECT 4062 with 45.1±2.5, 47.4±3.5 and

45.3±3.7 ppb respectively whereas the lowest adsorption capacity was evidenced by the strain of *Lc. mesenteroides* CECT 215. At pH 6.5 the strains that showed the highest OTA binding capacity were *Lb. plantarum* CECT 220, *Lb. plantarum* CECT 222 and *Lb. plantarum* CECT 748 with 45.1±2.3, 32.3±4.1 and 32.7±0.6 ppb respectively, and also at that pH the lowest OTA adsorption activity was showed by the strain of *Lc. mesenteroides* CECT 215.

Table 2. OTA reduction evidenced by the lactic acid bacteria in MRS medium, adsorbed by LAB cell wall and hydrolyzed.

Strain	OTA CFS (ppb)		OTA on LAB (ppb)		Hydro. Reduction (%)		Ads. Reduction (%)		Total Reduction (%)	
	pH 3.5	pH 6.5	pH 3.5	pH 6.5	pH 3.5	pH 6.5	pH 3.5	pH 6.5	pH 3.5	pH 6.5
Control	286.2±3.3	630.2±6.6								
***Bf. bifidum* CECT 870T**	31.2±3.9	144.4±4.9	27.3±3.3	21.5±2.2	80.4±3.2	74.1±3.3	9.1±0.3	3.1±0.2	89.8±.3.3	77.3±3.3
***Bf. breve* CECT 4839T**	27.7±3.2	31.9±5.1	10.0±4.5	8.1±1.0	87.2±4.1	94.1±3.5	4.2±0.1	1.4±0.1	91.4±4.1	95.1±4.3
***Lb. bulgaricus* CECT 4005**	29.0±3.6	20.1±3.1	47.4±3.5	6.3±0.4	73.9±3.2	96.4±3.4	16.0±1.3	1.6±0.1	90.3±4.3	97.1±3.1
***Lb. casei* CECT 4040**	39.9±4.5	58.7±4.9	15.9±2.2	17.4±1.1	81.4±5.2	88.5±2.5	5.3±0.8	3.5±0.3	86.6±3.2	91.4±4.1
***Lb. casei* CECT 4045**	20.6±3.6	18.1±2.2	17.9±3.1	14.7±2.1	87.1±4.1	95.7±3.6	6.2±1.2	2.2±0.6	93.2±3.3	97.6±3.3
***Lb. johnsonii* CECT 289**	25.3±1.1	18.3±1.5	45.1±2.5	26.1±0.5	76.4±2.5	93.1±2.9	16.3±1.5	4.0±0.3	91.6±2.1	97.2±1.5
***Lb. paracasei* CECT 4022**	77.5±5.4	55.4±4.6	26.1±2.2	16.0±0.3	64.2±4.1	89.9±4.1	9.4±1.0	3.3±0.3	73.1±2.5	91.7±2.2
***Lb. plantarum* CECT 220**	194.7±.1.1	179.4±6.8	5.7±0.3	45.1±2.3	31.1±2.4	64.6±3.3	2.1±0.1	7.0±0.3	32.3±2.5	72.9±3.7
***Lb. plantarum* CECT 221**	200.0±3.7	203.0±5.5	2.4±0.7	26.1±2.2	29.6±3.1	64.4±3.6	1.1±0.3	4.8±0.3	30.4±3.7	68.0±2.4
***Lb. plantarum* CECT 222**	69.9±2.1	197.1±5.6	11.6±1.4	32.3±4.1	72.6±.2.2	64.8±2.5	4.6±0.3	5.4±0.6	76.0±4.1	69.0±1.5
***Lb. plantarum* CECT 223**	103.1±2.2	206.0±6.3	2.3±0.4	7.1±1.0	63.7±3.3	66.3±3.9	1.2±0.1	1.2±0.6	64.1±4.6	67.2±1.3
***Lb plantarum* CECT 748**	194.3±2.1	232.4±4.7	7.1±0.6	32.7±0.6	30.1±2.1	58.4±3.1	3.0±0.2	5.6±0.3	32.4±3.2	63.2±3.2
***Lb. plantarum* CECT 749**	15.4±2.5	13.0±1.1	14.3±1.1	8.9±1.0	90.5±4.1	97.1±4.1	5.3±0.3	1.7±0.1	95.0±4.7	98.6±2.4
***Lb. rhamnosus* CECT 278T**	8.2±2.2	46.3±6.3	16.5±4.1	16.3±1.1	92.1±4.4	90.3±2.5	5.6±0.3	3.3±0.3	97.5±4.6	93.3±4.5
***Lb. rhamnosus* CECT 288**	26.5±2.1	20.8±2.0	15.2±1.5	13.8±0.7	86.0±3.2	95.0±2.6	5.1±0.4	2.1±0.2	91.1±4.1	97.3±5.2
***Lb. salivarius* CECT 4062**	38.4±2.5	55.9±4.8	45.3±3.7	23.0±1.5	71.4±3.6	87.7±3.5	16.1±1.1	4.4±0.4	87.0±3.3	91.1±1.7
***Lc. mesenteroides* CECT 215**	285.3±4.6	301.7±7.1	nd	nd	nd	52.4±3.1	nd	nd	nd	52.4±2.2

Considering the OTA reduction expressed by the microorganisms tested through an enzymatic mechanisms (pH 3.5) the highest reductions were evidenced by *Bf. breve* CECT 4839T, *Lb. rhamnosus* CECT 278T and *Lb. casei* CECT

4045 with 87.2±4.1, 92.1±4.4 and 87.1±4.1% respectively, whereas the lowest OTA reduction activity was showed by the strains of *Lb. plantarum* CECT 221 and *Lb. plantarum* CECT 748 with 29.6±3.1 and 30.1±2.1 % respectively. At the pH of 6.5 the reduction activity evidenced by the LAB tested, ranged from 52 to 97% respectively evidenced and higher OTA reduction performance at this pH. The highest OTA reduction data were showed by the strains of *Lb. plantarum* CECT 749 and *Lb. bulgaricus* CECT 4005 with the 97.1±4.1 and 96.4±3.4% respectively, whereas the lowest reduction data were observed in the fermentations carried out by the strains of *Lc. mesenteroides* CECT 215 and *Lb. plantarum* CECT 748.

Considering the data related with the total reduction of the OTA, including both mechanisms investigated in our work, the strain that at pH 3.5 produced the highest amount of the OTA reduction was *Lb. rhamnosus* CECT 278T with a reduction of 97.5±4.6%. A good reduction of the OTA was also observed by the strain of *Lb plantarum* CECT 749 with the 95.0±4.7%. The range of the reduction at that pH was variable from 30 to 97%, where the lowest OTA reduction activity was observed by the *Lb. plantarum* CECT 221 with the 30.4±3.7%.

At pH 6.5 the OTA reduction detected was higher than the 50% for all the strains tested. At that pH the highest reduction activity was observed by *Lb. plantarum* CECT 749 with 98.6±2.4%, and 10 strains of the 17 tested evidence and OTA reduction higher than 90%.

One of the first reports that studied the elimination of OTA from milk used the *Streptococcus salivarius subsp. thermophilus*, the *Lactobacillus delbrueckii subsp. bulgaricus* and *Bifidobacterium bifidum* (Skrinjar et al., 1996). OTA was completely eliminated at low levels (0.05 and 0.1 µg/mL), but no biodegradation products were observed and no mechanisms for degradation were described.

Del Prete et al. (2007) determined the *in vitro* interaction between OTA and wine LAB of fifteen strains belonging to five relevant oenological LAB species in liquid synthetic culture medium containing OTA. The portion of OTA removed during the bacterial growth was 8 to 28%. The OTA removed from the supernatants was partially recovered (31 to 57%) from the bacterial pellet. OTA was not degraded by cell-free extracts of wine LAB strains, and no degradation products of OTA were detected in the high-performance liquid chromatograms of the methanol extract of the bacterial pellet.

Abrunhosa et al. (2014) studied the ability of *Pediococcus parvulus* strains that were isolated from Douro wines that spontaneously underwent malolactic fermentation to detoxify OTA. The presence of OTA-α was confirmed using LC–MS/MS and the conversion of OTA into OTA-α indicates that the OTA amide bond was hydrolysed by a putative peptidase. Adsorption assays with dead *P. parvulus* cells showed that approximately 1.3% ± 1.0 of the OTA was adsorbed onto cells wall, which excludes this mechanism in the elimination of OTA by strains that degrades OTA. Under optimum conditions, 50% and 90% of OTA were degraded in 6 and 19 h, respectively. OTA biodegradation by *P. parvulus* UTAD 473 was also observed in grape must.

Belkacem-Hanfi et al. (2014) isolated 54 LAB from stored wheat samples sourced from grain silos in North Tunisia and fifteen representative isolates. These isolates were screened for antifungal activity in dual culture agar plate assay against eight post-harvest moulds. All LAB showed inhibitory activity against moulds, especially strains of *L. plantarum*, which exhibited a large antifungal spectrum. Moreover, LAB species such as *L. plantarum* LabN10, *L. graminis* LabN11 and *P. pentosaceus* LabN12 showed high inhibitory effects against the ochratoxigenic strain *A. carbonarius* ANC89. These LAB were also investigated for

their ability to reduce *A. carbonarius* ANC89 biomass and its OTA production on liquid medium at 28 and 37 ºC and varied pH conditions. High percentage of OTA reduction was obtained by *L. plantarum* and *L. graminis* (>97%) followed by *P. pentosaceus* (>81.5%). These findings suggest that in addition to *L. plantarum, L. graminis* and *P. pentosaceus* strains may be exploited as a potential OTA detoxifying agent to protect humans and animals health against this toxic metabolite.

3.4. OTA reduction during in vitro gastrointestinal digestion

In Table 3 are described the data related with gastric and duodenal bioaccessibility of the OTA during gastrointestinal digestion carried out with the LAB that showed the highest potential of reduction as evidenced in the paragraph 3.2. In particular, observing the results of the control experiments (digestion of the OTA without any bacterial fermentation) the gastric and duodenal bioaccessibility detected we of 79.7 and 38.1% respectively, with a mean general bioaccessibility of this toxic compound of 58.9%. In the experiments carried out digesting the OTA in MRS medium with LAB, the highest gastric bioaccessibility reduction was observed by the strain of *Lb. johnsonii* CECT 289 with 98.1%, also very interesting results were observed in the experiments carried out with *Bf. breve* CECT 4839T and *Lb. plantarum* CECT 749 with OTA bioaccessibility reduction at gastric level of 73.7 and 82.1% respectively. The lowest OTA bioaccessibility reduction was observed in the experiment carried out using the strain of *Lb. bulgaricus* CECT 4005 with 15.4%.

At duodenal level the strain of *Lb. johnsonii* CECT 289, confirmed the bioaccessibility OTA reduction with a data of 96.7% and showing a mean

reduction around all the gastrointestinal digestion process of 97.4%. Other interesting results were showed in the experiments carried out using *Lb. rhamnosus* CECT 278T and *Lb. rhamnosus* CECT 288 with a reduction of the OTA bioaccessibility of 50.0 and 63.9% respectively. The mean OTA bioaccessibility reduction detected considering both compartments (stomach and duodenal) ranged from 27.0 to 97.4%. Considering the data produced in our study, the strain of *Lb. johnsonii* CECT 289 could be a very important candidate to be used as a starter culture for the bio control of the mycotoxin OTA during fermentative processes and could be important to reduce the intake of this toxic compound at intestinal level.

This article can be considered one of the first that studies the influence of LAB on OTA bioaccessibility using a dynamic simulated gastrointestinal digestion model system. In the scientific literature there are available just few articles that related the reduction of OTA bioaccessibility during a gastrointestinal digestion in presence of fermentative microorganisms.

In particular González-Arias et al. (2015) studied the bioaccessibility of OTA in a spiked red wine sample treated with commercial yeast under human fasting conditions using an *in vitro* dynamic digestion model that includes a continuous flow dialysis system to simulate intestinal passage. A liquid-liquid method was used to extract the OTA and OTA-α from gastrointestinal juices, and the extracts were analysed by HPLC with a fluorescence detector. The bioaccessibility of OTA from the spiked red wine (1, 2 and 4 μg/L) was high in the gastric compartment (102.8, 128.3 and 122.3%, respectively), whereas in the simulated intestine, it did not exceed 26%, and the amount of OTA that crossed the dialysis membrane was very low (<3.3%). The amount of OTA-α in gastric chyme ranged from 5.1 to 19.1% of the spiked OTA, whereas in the intestinal compartment it did not exceed 5%.

Table 3. Gastric and duodenal bioaccessibility reduction of OTA present bread, subjected to digestion with probiotic microorganisms. Significantly different from the control, $p \leq 0.05$ (*), $p \leq 0.001$ (**), $p \leq 0.0001$ (***). The control consisted of bread contaminated by OTA digested without probiotic strains. S=Stomach, D=Duodenum.

Samples	OTA (ppm)	Bioacc. (%)	Mean	Bioacc. Red. (%)	Mean Bioacc. Red. (%)
Control S	8.0±1.0	79.7	58.9		
Control D	3.8±0.3	38.1			
***Bf. breve CECT* 4839T S**	3.2±0.5	31.8	34.0	60.1***	32.5
***Bf. breve CECT* 4839T D**	3.6±0.6	36.3		4.8	
***Lb. bulgaricus* CECT 4005 S**	6.7±0.4	67.4	45.4	15.4**	27.0
***Lb. bulgaricus* CECT 4005 D**	2.3±0.2	23.4		38.7***	
***Lb. casei* CECT 4045 S**	2.4±0.1	24.4	28.0	69.4***	43.1
***Lb. casei* CECT 4045 D**	3.2±0.3	31.7		16.8*	
***Lb. johnsonii* CECT 289 S**	0.2±0.04	1.5	1.4	98.1***	97.4
***Lb. johnsonii* CECT 289 D**	0.1±0.01	1.2		96.7***	
***Lb. Plantarum* CECT 749 S**	1.4±0.2	14.3	19.8	82.1***	58.0
***Lb. plantarum* CECT 749 D**	2.5±0.4	25.2		33.8***	
***Lb. rhamnosus* CECT 278T S**	2.1±0.2	21.0	20.0	73.7***	61.8
***Lb. rhamnosus* CECT 278T D**	1.9±0.1	19.1		50.0***	
***Lb. rhamnosus* CECT 288 S**	3.4±0.4	34.5	24.1	56.7***	60.3
***Lb. rhamnosus* CECT 288 D**	1.4±0.1	13.8		63.9***	
***Lb. salivarius* CECT 4062 S**	5.7±0.4	56.9	41.7	28.6***	29.6
***Lb. salivarius* CECT 4062 D**	2.6±0.2	26.4		30.6***	

As evidenced in our study, in the *in vitro* system assayed, OTA exhibited a high bioaccessibility in the simulated stomach, but it decreased after the intestinal digestion and passage through the dialysis membrane.

3.5. Identification of OTA degradation products

The last part of the study was focused on the identification of the OTA reduction products produced by LAB through the fermentation processes developed in this study considering the fermentation of the toxin in the MRS medium and also the reduction during the gastrointestinal digestion.

a)

b)

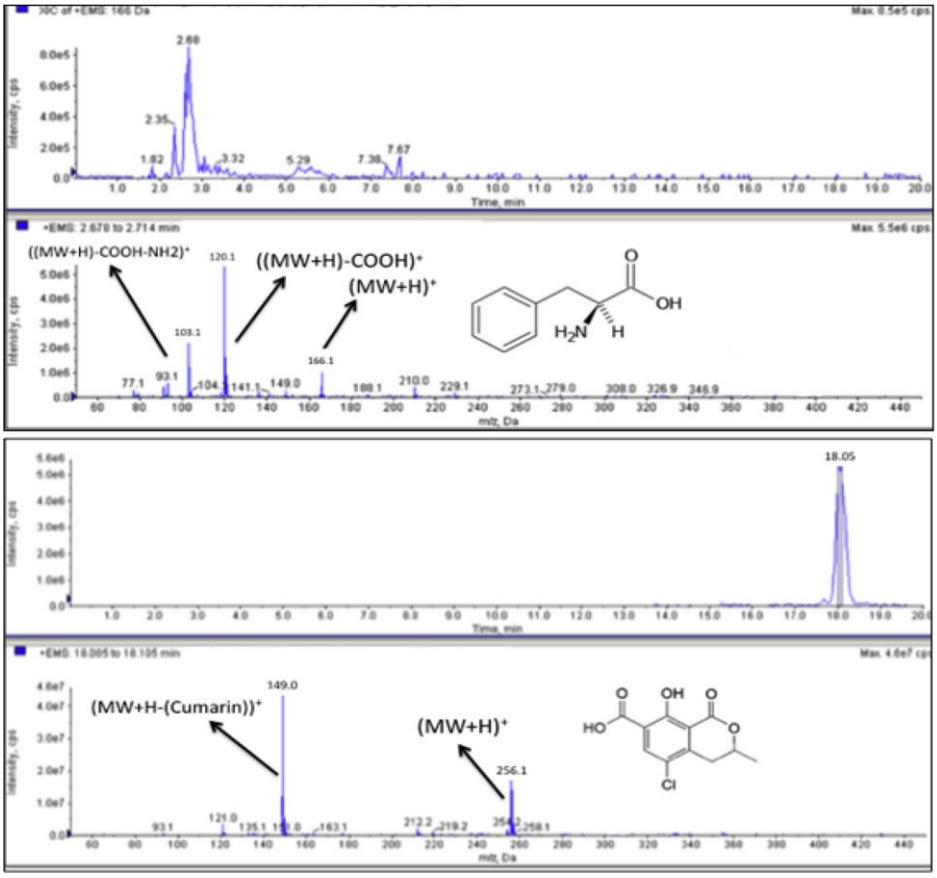

Fig. 3. LC-MS-LIT spectra of the OTA degradation products a) Phe and b) OTA-α, produced through the OTA microbial fermentation in MRS medium of the lactic acid bacteria employed in the study.

Using the identification approach of the mass spectrometry associated to the linear ion trap working in the modality full scan, in all the fermentation trials, the principal products of the OTA degradation have been identified as the OTA-α and the Phe. In particular, in the Fig. 3a is plotted the chromatographic peak of the Phe and the related MS-LIT mass spectra that identified this product. In the spectra are evidenced several diagnostic fragments that confirm the identification of this compound as the ion with a *m/z*=166.1 that correspond to the Phe molecular weight (MW), the fragments with a *m/z*=120.1 corresponding to the MW of the Phe with a loss of a carboxylic group, and the fragment with a *m/z*=103.1that represent the Phe MW with the loss of a carboxylic group and of an aminic group.

In the Fig. 3b is evidenced the chromatographic peak corresponding to the OTA-α produced by the OTA hydrolysis. In the MS-LIT chromatogram are evidenced two diagnostic molecular fragments that confirm the identification of this OTA degradation product that are the ion with *m/z*=256.1 that represents the OTA-α MW and the fragment with *m/z*=149.0 that represents the OTA-α with a loss of the cumarinic group. The data obtained in our study were confirmed by the data produced by Abrunhosa et al. (2010) that studied the pathway of OTA biodegradation used by the *P. parvulus* strains isolated from the Douro wines is the same pathway that has been identified in other microorganisms. Specifically, OTA is degraded via the hydrolysis of the OTA amide group and the subsequent release of the OTA-α and L-β-Phe moieties. It is known that this hydrolysis is mediated at varying degrees of efficiency by some peptidases. The carboxypeptidase A enzyme from the bovine pancreas (Pitout, 1969), a commercial lipase (Stander et al., 2000), several commercial proteases and an OTA-hydrolytic enzyme isolated from *Aspergillus niger* (Abrunhosa et al., 2006),

and carboxypeptidase Y from *Saccharomyces cerevisiae* (Abrunhosa et al., 2010), all can mediate the hydrolysis reaction. The hydrolysis of the amide bond of OTA is thought to mediate the detoxification of OTA because OTA-α is nontoxic.

4. Conclusions

The results proposed in this study showed the LAB proteolytic ability to degrade OTA with two different mechanisms and also during the *in vitro* gastrointestinal digestion was assessed, demonstrating their potential for the biotechnological application to reduce the health hazards associated with the intake of food contaminated with mycotoxins. The strains that evidence the highest OTA biodegradation could be used in the silage industry as inoculants or as feed additives to reduce the problem of the intake of OTA in swine production, considering that these animals are the most susceptible livestock to the toxic effects of OTA. In winemaking, these lactic acid bacteria could be used, as starter culture during alcoholic or malolactic fermentation as a biological strategy for the reduction of OTA in the final product.

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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 la actividad antifúngica y antitoxigénica de las bacterias ácidos lácticas (BAL) *in vitro*; así como la aplicación de estas y sus productos de la fermentación para reducir el crecimiento de hongos toxigénicos y micotoxinas en cereales y productos de panadería.

Inicialmente se ha realizado la selección de BAL obtenidas todas ellas de la Colección Española de Cultivos Tipo (CECT) y posterior evaluación de las propiedades antifúngicas del caldo MRS fermentado *in vitro* frente a una selección de hongos toxigénicos pertenecientes a los géneros *Aspergillus*, *Penicillium* y *Fusarium*. Por otra parte, se empleó suero de leche, desecho de la industria quesera, como medio de cultivo para ser fermentado por las BAL. También se utilizaron enzimas digestivos, como la tripsina, para la hidrólisis de las proteínas del suero de leche, con objeto de obtener péptidos de bajo peso molecular. Las propiedades antifúngicas tanto del suero fermentado como el hidrolizado fueron evaluadas *in vitro*.

La evidencia de la inhibición del crecimiento fúngico de algunos hongos por los medios fermentados por algunas especies de BAL, confirmó la necesidad de caracterizar los compuestos producidos responsables de la actividad antifúngica. Los compuestos antifúngicos se caracterizaron por CL-ESI-qTOF y MALDI-TOF.

Paralelamente, se realizaron estudios para evaluar la capacidad de las BAL para reducir la ocratoxina A (OTA) en caldo MRS y durante los procesos digestivos humanos *in vitro* de pan contaminado con OTA, así como la caracterización de sus productos de degradación. También se evaluaron las propiedades biológicas del suero de leche fermentado por BAL mediante estudios de actividad antioxidante, actividad antihipertensiva y actividad quelante de hierro.

Finalmente, las BAL, el MRS fermentado, el suero de leche fermentado y el suero de leche hidrolizado fueron aplicados en diferentes alimentos como una nueva estrategia para alargar la vida útil e incrementar la seguridad de estos mediante la inhibición del crecimiento de hongos toxigénicos y la síntesis de micotoxinas. Las BAL fueron empleadas para la elaboración de masas madre y su uso como ingrediente en la elaboración de pan de molde. Así mismo, tanto el suero de leche fermentado como hidrolizado fueron empleados como ingredientes en la elaboración de pan de molde y pan de pita. Por último, el MRS fermentado se utilizó como agente de bioconservación de maíz y mazorcas.

4.1. Actividad antifúngica y antitoxigénica *in vitro*

4.1.1. Evaluación de la actividad antifúngica del caldo MRS fermentado por BAL

El efecto antifúngico del sobrenadante libre de células (CFS) obtenido por la fermentación del medio de cultivo MRS por las BAL, se evaluó mediante el ensayo de difusión en agar empleando medio sólido Potato Dextrose Agar (PDA). Los CFS evidenciaron halos de inhibición del crecimiento micelial en hongos toxigénicos pertenecientes a los géneros *Aspergillus*, *Penicillium* y *Fusarium*. El análisis de los datos puso de manifiesto que los medios fermentados por *Lactobacillus rhamnosus* CECT 278T, *Lactobacillus johnsoni* CECT 289, *Lactobacillus plantarum* CECT 748 y *Lactobacillus plantarum* CECT 749 presentaron los mayores halos de inhibición. Concretamente, la cepa *Lactobacillus plantarum* CECT 749 fue la más efectiva frente a *Fusarium graminearum* ITEM 126, *Fusarium cerealis* CECT 20489, *Fusarium verticillioides* CECT 2152, *Fusarium verticillioides* CECT 2982 y *Aspergillus flavus* ITEM 8111, mientras que *Lactobacillus delbrueckii bulgaricus* CECT 4005 no mostró inhibición del crecimiento fúngico.

La cuantificación de la actividad antifúngica en medio líquido PDB evidenció que *Fusarium* spp. eran más sensibles al CFS que *Aspergillus* spp. Además, de acuerdo con los resultados cualitativos todos los CFS mostraron efecto antifúngico, presentando valores de MIC que varían de 4-125 g de CFS liofilizado/L. Específicamente, el CFS de *L. plantarum* CECT 748 y *L. plantarum* CECT 749 presentaron la mayor actividad antifúngica frente a las cepas de *Fusarium* y *Aspergillus*. En concreto frenta a *Aspergillus*, los valores de MFC de *L. plantarum* CECT 748 y *L. plantarum* CECT 749 fueron 125 g/L y 62 g/L, respectivamente. Analizando los resultados, es importante enfatizar que el CFS de *L. plantarum* CECT 749 fue más eficiente que el resto, mostrando la concentración más baja de MIC y MFC frente a todas las cepas fúngicas evaluadas.

Gupta *et al.* (2014) estudiaron la actividad antifúngica de 88 cepas de *L. plantarum* frente a *Aspergillus niger*, *Aspergillus flavus*, *Fusarium culmorum*, *Penicillium roqueforti*, *Penicillium expansum*, *Penicillium chrysogenum* y *Cladosporium* spp. Ninguna de las cepas de *L. plantarum* probadas fue capaz de inhibir completamente el crecimiento de los hongos filamentosos analizados, el efecto antagonista máximo se observó después de 5 días de incubación en placas de PDA suplementadas con CFS (12% v/v) de *L. plantarum* UFG 108 y *L. plantarum* UFG 121, en el que el crecimiento de *P. expansum* y *F. culmorum* se redujo un 50 y 60%, respectivamente. Otro trabajo describió la actividad antifúngica del CFS fermentado por BAL frente a *A. flavus* y *Aspergillus carbonarius*, para lo cual los valores de MIC se basaron en un reemplazo del 10-70% del medio de cultivo por CFS fermentado (Taheur *et al*., 2019).

4.1.2. Evaluación de la actividad antifúngica de suero de leche fermentado por BAL

El suero de leche fermentado empleando la cepa *L. plantarum* CECT 221 evidenció actividad antifúngica frente a *P. camemberti*, *P. expansum*, y *Fusarium* spp. Por lo contrario, no mostró ningún efecto inhibitorio en *Aspergillus* spp. y *P. roqueforti*. Los sueros fermentados por otras seis cepas de *L. plantarum* también mostraron el mismo efecto antifúngico, en particular frente a *Fusarium* spp.

En cuanto a los resultados de MIC y MFC obtenidos por parte de los sueros fermentados, frente a una selección de hongos pertenecientes a los géneros *Aspergillus*, *Penicillium* y *Fusarium*, mostraron valores de 250, 15,6-250 y 31,2-250 g/L, respectivamente.

En general, *Aspergillus* spp. mostraron resistencia a los sueros de leche fermentados, mostrando valores de MIC y MFC elevados. En cambio, los géneros *Penicillium* y *Fusarium* mostraron ser más sensibles a los compuestos presentes en el suero fermentado por BAL. Las cepas *L. plantarum* CECT 221, 749 y 750, evidenciaron la mayor actividad antifúngica, con valores de MFC de 62,5 g/L frente a *Penicillium* spp. Los mismos valores de MFC se observaron para los sueros fermentados por *L. plantarum* CECT 749 y 750 frente a *F. graminearum*.

Gamba *et al*. (2016) informaron que el suero obtenido de la fermentación de la leche por los granos de kéfir compuestos por un biocomplejo de BAL y levaduras mostró actividad antifúngica frente a *F. graminearum*.

4.1.3. Evaluación de la actividad antifúngica de suero de leche hidrolizado con tripsina

Como mostraron los datos de actividad antifúngica del suero de leche hidrolizado frente a diez cepas de *Penicillium* considerados como contaminantes

de productos panarios en medio sólido PDA, se produjo inhibición del crecimiento de *P. camemberti*, *P. verrucosum*, *P. commune*, *P. brevicompactum* y *P. expansum*. Los valores de MIC mostraron un rango de 3,9-62,5 g/L, siendo *P. brevicompactum* la especie más sensible. La especie *P. expansum* es la que mayor resistencia presentó, con una MFC de 62,5-250 g/L.

El estudio del perfil proteico mediante electroforesis SDS-PAGE evidenció una reducción correlativa con el aumento del tiempo de hidrólisis de Lactoferrina (Lf) (80 kDa), α-Lactoalbúmina (14 kDa) y β-Lactoglobulina (18 kDa). Estos hallazgos electroforéticos concuerdan con los datos obtenidos mediante la identificación de los péptidos generados por la hidrólisis del suero mediante CL-ESI-MS-TOF. En particular, la hidrólisis catalizada por tripsina del suero de cabra produjo 27 péptidos a partir de las proteínas Lf, α-Lactoalbúmina y β-Lactoglobulina. Se realizaron alineamientos de las secuencias obtenidas con péptidos antimicrobianos conocidos utilizando la base de datos péptidos antimicrobianos de leche.

El presente trabajo se puede considerar el primero en probar la actividad antifúngica de suero de leche de cabra hidrolizada frente varios hongos toxigénicos, mientras que muchos autores han estudiado la actividad antimicrobiana, principalmente las propiedades antibacterianas y anti-levaduras, de los fragmentos originados por la hidrólisis de Lf bovina y humana (Kenya *et al*., 2017). Lupetti *et al*. (2008) informaron de la actividad antifúngica del péptido Lf humano (1–11) frente a *Aspergillus fumigatus* a una concentración de 29 µM. Otro estudio evidenció valores de MIC para dos péptidos sintéticos derivados de Lf bovino (Lf 17-31 y Lf 20-25) que oscilaron entre 4-16 y 8-32 µM respectivamente, frente a diferentes *Penicillium* spp. (Muñoz y Marcos, 2006).

4.1.4. Determinación de la actividad antifúngica de extractos de masa madre fermentada por BAL

La actividad antifúngica de los extractos acuosos de las masas madre fermentadas con *L. plantarum* y *L. bulgaricus* se estudió mediante el ensayo de difusión en agar empleando medio sólido PDA. Los extractos de las masas fermentadas con *L. plantarum* evidenciaron inhibición del crecimiento fúngico de los nueve hongos toxigénicos ensayados. Si bien, los extractos de masa madre fermentada por *L. bulgaricus*, solo mostraron actividad frente a *P. expansum*, *F. moniliformis*, *F. verticillioides* y *F. graminearum*.

Para cuantificar la actividad antifúngica de los extractos de masa madre fermentada por ambas cepas, se calculó la MIC y MFC en medio líquido PDB. Los valores de MIC obtenidos por *L. plantarum* evidenciaron un rango de 6,3-200 g/L, mientras que la MFC fue de 50-400 g/L. Los valores de MIC y MFC obtenidos por *L. bulgaricus* presentaron un rango de 1,3-200 g/L y 1,6-400 g/L, respectivamente. Analizando los datos, los extractos de *L. bulgaricus* presentaron mayor actividad antifúngica frente a *Fusarium* spp. en comparación con los de *L. plantarum*. Ambos extractos frente a *Aspergillus* spp. mostraron resultados muy comparables, con altos valores de MIC y MFC.

Estudios anteriores han informado sobre la actividad antifúngica de masas fermentadas por BAL (*L. plantarum* y *Lactobacillus rossiae*) (Rizzello *et al*., 2011). Los extractos metanólicos y solubles en agua de las masas madre fermentadas mostraron actividad antifúngica en medio sólido frente a hongos toxigénicos presentes en productos de panadería. Una mezcla de ácidos orgánicos, compuestos fenólicos y péptidos producidos durante la fermentación estaban relacionados con la actividad antifúngica. La MIC osciló entre 2,5 a 15,2 g/L. Las hogazas de pan producidas con un 4% (peso/peso) de masa madre fermentada

liofilizada no mostraron crecimiento de hongos durante 20 días de almacenamiento a temperatura ambiente.

Ouiddir *et al*. (2019) estudiaron la actividad antifúngica de *L. plantarum* y *Leuconostoc mesenteroides* en panadería y productos lácteos. Los panes de masa fermentada que contienen estas BAL e inoculados con *A. flavus* y *Aspergillus tubingensis* mostraron una mejor vida útil de 1-2 días en comparación con el control positivo (pan sin BAL).

4.1.5. Caracterización de los compuestos antifúngicos producidos por BAL

Según la literatura, la actividad antifúngica de las BAL no solo se debe a un tipo de compuesto, sino que también depende de la acción sinérgica entre todos los compuestos presentes en los medios fermentados (Crowley *et al*., 2013). Una vez comprobada la capacidad antifúngica de los diferentes medios fermentados, se procedió a la caracterización de los posibles compuestos responsables de la actividad. Por un lado, se realizó una purificación, aislamiento e identificación de los péptidos producidos por BAL durante la fermentación, y por otro, se purificaron y cuantificaron los ácidos fenólicos producidos.

La cromatografía de permeación sobre gel con Sephadex G-25 se empleó para la purificación y fraccionamiento de los péptidos producidos por *L. plantarum* CECT 749 durante la fermentación del caldo MRS. Las fracciones recogidas por el proceso de purificación a través de la cromatografía líquida de baja presión se inyectaron en CL-DAD para la identificación de las fracciones positivas a la presencia de los péptidos. La fracción número tres fue particularmente interesante por la presencia de tres picos, que fueron aislados por CL sobre una columna C18 semipreparativa con una pureza ≥95%.

El análisis MALDI-TOF mostró que el peso molecular (MW) del pico 1 era 1041,26 Da. El análisis MS/MS mostró que la secuencia peptídica era Ser-Gly-Ala-

Asp-Thr-Thr-Phe-Leu-Thr-Lys (SGADTTFLTK). La espectrometría de masas del pico 2, presentó un MW de 1019,30 Da y el análisis MS/MS del fragmento correspondiente al MW mostró que la secuencia de aminoácidos estaba compuesta por Leu-Val-Gly-Lys-Lys-Val-Gin-Thr-Phe (LVGKKVQTF). El pico número 3 evidenció un MW que estaba de acuerdo con la masa molecular calculada de 995,15 Da. El análisis MS/MS del ion correspondiente al MW evidenció una secuencia de aminoácidos compuesta por Gly-Thr-Leu-Ile-Gly-Gin-Asp-Tyr-Lys (GTLIGQDYK). La secuencia de los tres picos purificados, se refirió a nuevos péptidos, de acuerdo con la base de datos disponible en línea (http://prospector.ucsf.edu; http: //www.expasy.ch).

Los péptidos purificados se utilizaron como patrones externos para el análisis cuantitativo mediante LC-DAD. Se preparó una solución madre en agua y se realizaron diluciones para cubrir un rango de concentración de 10 a 300 mg/L. La cepa *L. plantarum* CECT 749 produjo en el medio MRS un rango de valores comprendido entre 246-280 mg/L para los tres péptidos purificados.

Ensayos de actividad antifúngica de los péptidos purificados en medio líquido frente a *A. parasiticus* y *P. expasum* fueron realizados. Los péptidos LVGKKVQTF y GTLIGQDYK no mostraron actividad antifúngica significativa. En cambio, el péptido SGADTTFLTK sí evidenció una reducción significativa del crecimiento de *A. parasiticus* y *P. expansum* idependientemente de las concrentraciones ensayadas (1-100 mg/L). En particular, la reducción de la viabilidad de *A. parasiticus* tras 48 y 72 h de exposición al péptido fueron del 73 y 42%, respectivamente. Se obtuvieron reducciones ligeramente menores para *P. expansum*: 58 y 49% después de los mismos tiempos de incubación.

Varios autores describen una actividad antimicrobiana similar de péptidos producidos por BAL durante la fermentación. Russo *et al*. (2016) investigaron el efecto de los péptidos antimicrobianos (AMP LR14) producidos por la cepa LR/14

de *L. plantarum* contra *A. niger*, *Rhizopus stolonifera*, *Mucor racemosus* y *P. chrysogenum* mediante un ensayo de cultivo dual. Los péptidos inhibieron tanto la germinación de esporas como el crecimiento de hifas de los cuatro hongos.

Muhialdin *et al*. (2016) informaron que el péptido FPSHTGMSVPPP producido por *L. plantarum* IS10 en caldo MRS a la concentración de 5000 mg/L inhibió el crecimiento fúngico de *Aspergillus flavus*, *P. roqueforti* y *Eurotium rubrum* entre un 43-60%.

Los ácidos fenólicos producidos durante la fermentación de los diferentes medios fueron analizados posteriormente a la purificación mediante el método QuEChERS. Se estudiaron tres medios fermentados por BAL: medio de cutivo MRS, suero de leche y masa madre.

El análisis del MRS fermentado por BAL evidenció la presencia de un total de nueve ácidos fenólicos. Estos compuestos se investigaron y determinaron de acuerdo con el tiempo de retención del pico y los espectros de masa molecular obtenidos a partir de la solución estándar inyectada en LC-ESI-qTOF-MS e información disponible en la literatura. Entre las muestras con el mayor efecto antifúngico, la cepa *L. plantarum* CECT 749 produjo PLA, ácido salicílico y vainillina, mientras que *L. plantarum* CECT 748 generó los mismos compuestos, además del ácido clorogénico, ácido sinápico y 1,2-dihidroxibenceno, con la excepción de la vainillina. La concentración de PLA en los CFS presentaron un rango de 0,9-5,3 mg/L, siendo mayor la concentración en el medio fermentado por *L. plantarum* CECT 749.

Estudios previos también identificaron ácidos fenólicos producidos por BAL en caldo MRS. Rodríguez *et al.* (2008) describió la presencia de 1,2-dihidroxibenceno y otros ácidos hidroxibenzoicos como el ácido vanílico, el ácido siríngico y el ácido gálico generados después de la hidrólisis y metilación del ácido hidroxibenzoico por enzimas de *L. plan*tarum. De hecho, se sabe que *L. plantarum*

sintetiza ácidos fenólicos por descarboxilación y/o reducción (Sánchez-Maldonado *et al.* 2011).

Algunos ácidos fenólicos, como se describió anteriormente, se estudiaron como compuestos antifúngicos. Entre ellos, el 1,2-dihidroxibenceno, a menudo conocido como pirocatecol o catecol, se encuentra comúnmente en su forma unida en los alimentos como parte de estructuras complejas como las ligninas y los taninos hidrolizables (Rodríguez *et al.* 2008; Le Lay *et al*. 2016).

A pesar de que los ácidos fenólicos, encontrados en el CFS, se han informado previamente como compuestos antifúngicos, los efectos antifúngicos también podrían atribuirse al biocomplejo obtenido por la fermentación del caldo MRS por *L. plantarum* spp. Teniendo en cuenta los compuestos detectados, debemos subrayar igualmente la importancia desde el punto de vista microbiológico el ácido hidroxibenzoico y el PLA (Barman *et al*.2017; Taofiq *et al*.2017).

El suero de leche fermetado por *L. plantarum* spp. evidenció un total de 14 ácidos fenólicos. La cepa *L. plantarum* CECT 220, fue la que produjo la menor cantidad de estos compuestos. La cepa de *L. plantarum* CECT 221 mostró la mayor capacidad para producir compuestos fenólicos durante la fermentación, un total de nueve compuestos incluyendo compuestos antimicrobianos conocidos como el ácido clorogénico, ácido siríngico, ácido benzoico y PLA. Las otras cepas de *L. plantarum* produjeron entre cuatro y seis ácidos fenólicos.

En el caso de la masa madre fermentada por *L. plantarum* CECT 749 y *L. delbrueckii bulgaricus* CECT 4005 se indentificaron un total de 7 ácidos fenólicos. La cepa de *L. plantarum*, durante la fermentación en la masa madre de trigo, produjo los compuestos antimicrobianos ácidos gálico, clorogénico, cafeico y siringico, mientras que en la masa de trigo suplementada con suero de leche los compuestos detectados fueron los ácidos gálico y cafeico y también la vainillina. La cepa de *L. bulgaricus* en la masa madre de trigo produjo PLA y ácido sináptico,

mientras que al cambiar las condiciones de fermentación utilizando el medio más complejo compuesto por masa madre y suero de leche los compuestos detectados fueron ácido clorogénico, ácido sináptico y también el compuesto bioactivo vainillina.

Axel *et al*. (2016a) informaron la detección de compuestos antimicrobianos en masas fermentadas por *Lactobacillus* spp. En particular, se encontraron ácidos fenólicos tales como PLA, ácido 4-hidrofeniláctico y ácido 2-hidroxiisocaproico con concentraciones en el rango de 0,1-360 mg/Kg.

4.1.6. Reducción de OTA por BAL *in vitro* y durante la digestión gastrointestinal

El estudio de reducción de OTA en medio MRS Broth por BAL evidenció dos mecanismos diferenciados de acción. Por un lado, las BAL mostraron capacidad de adsorción de OTA en la pared celular. Los polisacáridos y peptidoglicanos de la pared celular son considerados como los principales componentes de las BAL encargados de la adsorción de las micotoxinas (Chapot-Chartier y Kulakauskas, 2014). Por otro lado, se puso de manifiesto la hidrólisis de OTA durante el periodo de incubación de la toxina con las BAL, dando lugar a OTA-α y fenilalanina. Estos metabolitos fueron identificados mediante análisis de CL-MS/MS.

En general, se observaron reducciones de OTA *in vitro* a pH 3,5 y 6,5 del 30-97% y 52-99%, respectivamente. La menor concentración de OTA detectada a pH 3,5 en los medios fue para la cepa *Lactobacillus rhamnosus* CECT 278T, siendo *Leuconostoc mesenteroides* CECT 215 la que menor reducción de OTA evidenció. A pH 6,5, fue *L. plantarum* CECT 749 la que mostró el mayor porcentaje de reducción de OTA (99%). A pH ácido se mostraron unos valores medios superiores de adsorción de OTA en las paredes celulares de las BAL.

Abrunhosa *et al*. (2014) estudiaron la capacidad de las cepas de *Pediococcus parvulus* aislados de vino para hidrolizar OTA. La presencia del metabolito OTA-α también fue confirmado mediante CL-MS/MS. En condiciones óptimas, evidenciaron reducciones del 50 y 90% de OTA tras 6 y 19 h de incubación, respectivamente. La capacidad de hidrólisis de la toxina por *P. parvulus* UTAD 473 también fue evidenciada en mosto de uva.

Belkacem-Hanfi *et al*. (2014) aislaron 54 cepas de BAL de trigo y estudiaron la capacidad de reducir la OTA producida por *A. carbonarius* ANC89 en medio líquido a varios pH. Las especies *L. plantarum* y *L. graminis* mostraron más del 97% de reducción.

En relación a la digestión gastrointestinal de pan contaminado con OTA empleando un inóculo de BAL durante la digestión *in vitro*, se evidenció una reducción media de la bioaccesibilidad considerando conjuntamente ambos compartimentos (estómago y dodeno) del 27-97% en comparación a la digestión sin inoculación de BAL. La cepa *Lactobacillus johnsoni* CECT 289 mostró los mejores valores medios de reducción de bioaccesibilidad de OTA durante la digestión.

González-Arias *et al*. (2015) estudiaron la bioaccesibilidad de la OTA de vino tinto fermentado con levadura comercial en un modelo de digestión dinámica *in vitro*. La bioaccesibilidad de la OTA a nivel gástrico fue superior al 100%, mientras que en el intestino no superó el 26%.

4.2. Reducción del crecimiento fúngico en alimentos

4.2.1. Empleo de suero de leche fermentado en pan

El suero de leche se considera el principal residuo de la industria del queso. Tiene un alto valor nutricional y se puede utilizar para obtener productos valiosos como ácido láctico, polímeros biodegradables y bioetanol, entre otros. En el primer caso, los procesos fermentativos de suero de leche realizados por bacterias y levaduras se han dirigido a obtener compuestos de interés y producir nuevos ingredientes (Pescuma *et al*. 2015).

El estudio compartivo de vida útil del pan control, tratado con 0.2% de propionato de calcio (E282) y tratado con suero de leche fermentado por *L. plantarum* CECT 221 como reemplazo del agua utilizada para la preparación de la masa de pan, mostró en el caso del control un crecimiento fúngico visible después de 3 días; el pan con aditivo E282 y el pan con la sustitución del 50 % del agua por suero fermentado evidenció un incremento en la vida útil de 1 día en comparación con el pan control. Por otro lado, al sustituir el 100% del agua por suero fermentado, el pan mostró crecimiento fúngico a los 5 días de incubación. Por lo tanto, el reemplazo de toda el agua por suero fermentado proporcionó un incremento en la vida útil de 1 día, en comparación con el pan control con aditivos y el pan elaborado con la concetración menor de suero.

Los datos mostrados para la vida útil visible de los panes tratados con suero fermentado por *L. plantarum* y contaminados con *P. expansum* también se confirmaron mediante análisis microbiológico de la población de contaminantes fúngicos en la matriz alimentaria estudiada. En particular, el pan control a los 7 días de incubación presentó una población fúngica de 5,1 log UFC/g, mientras que para el pan con la sustitución del 50% del agua por suero fermentado fue de 4,7 log UFC/g. El tratamiento que produjo la menor carga fúngica a los 7 días de incubación fue la sustitución del 100% del agua por suero fermentado, para el

cual la población de *P. expansum* detectada fue de 4,5 log UFC/g, una reducción fúngica estadísticamente significativa de 0,6 y 0,2 log UFC/g, respectivamente. No se observaron diferencias significativas con respecto al pan con aditivo (4,5 log UFC/g).

La fermentación microbiana es uno de los métodos más antiguos, económicos y ecológicos para conservar los alimentos (Zannini *et al*. 2012). En este contexto, no se recomienda el uso de compuestos químicos, y con frecuencia se proponen soluciones naturales para la conservación de alimentos. Hoy en día, los consumidores buscan productos naturales, menos procesados y más seguros. En este contexto, la posibilidad de obtener compuestos antifúngicos, derivados de un proceso de fermentación sobre alimentos, capaces de reducir el porcentaje de crecimiento de hongos representa un interés creciente como alternativa a la preservación química. Varias revisiones han examinado el espectro de actividad antifúngica de las BAL y la producción de metabolitos secundarios, así como sus interacciones con micotoxinas (Dalie *et al*., 2010; Crowley *et al*., 2013).

En este contexto, Gamba *et al*. (2016) investigaron la actividad antifúngica del suero obtenido de leche fermentada con granos de kéfir (CIDCA AGK1) frente a *A. flavus* en arepas de maíz. Las arepas de maíz inoculadas con *A. flavus* (10^2 esporas/g) y tratadas con suero al 10% exhibieron una vida útil más larga que el control sin aditivo durante el almacenamiento a 14 ºC durante 10 días.

4.2.2. Utilización de suero de leche hidrolizado con tripsina en pan

En el estudio de la vida útil del pan de pita contaminado con *P. verrucosum* se evidenció crecimiento fúngico visible a los 3 días de incubación en el pan control, mientras que el pan con aditivo E-282 al 0,2% se observó a día 6. Se realizó un pan empleando la sustitución del 100% del agua por suero no hidrolizado, y la vida útil

de este se incrementó en 1 día en comparación con el pan control sin aditivos. No se observaron diferencias entre los panes con la sustitución del 50% del agua por suero hidrolizado y los panes con suero no hidrolizado. La sustitución del 100% del agua por suero de leche hidrolizado presentó una vida útil de 5 días, un día menos en comparación con el pan con aditivo E282.

Los datos de la vida útil visible del pan de pita también fueron confirmados por el análisis microbiológico de la población del contaminante fúngico. Mientras que el pan control a los 7 días de incubación presentó una población fúngica de 3,99 log UFC/g, el pan con aditivo E282 evidenció un recuento fúngico de 1,57 log UFC/g, equivalente a una reducción significativa del 98% en comparación con el control. En el experimento llevado a cabo empleando el suero no hidrolizado, el crecimiento de *P. verrucosum* fue de 3,90 log UFC/g y no fue estadísticamente diferente al experimento control. Para el pan elaborado con la sustitución del 50 y 100% del agua por suero hidrolizado, el crecimiento de *P. verrucosum* fue de 3,59 y 2,95 log UFC/g, lo que equivale a una reducción significativa de 59 y 83%, respectivamente, en relación con el pan control.

Varios estudios han utilizado el suero de leche en la fabricación de pan, pero hay muy pocos informes sobre la utilización de suero hidrolizado como bioconservante en la fabricación de pan (Brar *et al*., 2002). Sin embargo, en la industria de la panadería, el suero de leche se considera un ingrediente bioactivo debido a su valor nutritivo y características funcionales (Kumar *et al*., 2018). Como ingrediente en la formulación del pan, el suero confiere cualidades tecnológicas interesantes, como una buena estructura de miga, mayor rendimiento del pan (cantidad de pan producido a partir de un determinado peso de la harina), sabor y mejora de la vida útil (Puranik, 2003).

4.2.3. Uso de masa madre como ingrediente en pan

El estudio de la vida útil del pan de molde elaborado masa madre control, con aditivo E-282, y masa madre fermentada con *L. plantarum* CECT 749 y *L. bulgaricus* CECT 4005 mostraron diferencias significativas. El pan con masa madre control y el pan con aditivo presentaron una vida útil de 3 y 5 días, respectivamente, mientras que el empleo de *L. plantarum* y *L. bulgaricus* para la fermentación de la masa madre incrementaron la vida útil en 1 y 2 días, respectivamente, en comparación con el pan control.

Del mismo modo, los panes tratados con BAL tras el periodo de conservación evidenció una reducción del crecimiento fúngico. El empleo de la masa madre inoculada con *L. plantarum* y con *L. bulgaricus* presentaron una reducción significativa de 0,42 y 0,58 log UFC/g en comparación al pan elaborado con masa madre control, respectivamente.

El empleo de masa madre inoculada con BAL como ingrediente en la elaboración de pan de molde, puede ser una aplicación frente al crecimiento de hongos y la reducción del uso de aditivos. Axel *et al*. (2016b) informaron de un incremento de la vida útil de pan elaborado con masa madre fermentada por *Lactobacillus reuteri* de 2 días en comparación con el pan control sin aditivos.

Dal Bello *et al*. (2007) mostraron mejorar la vida útil de los panes utilizando la cepa antimicótica *L. plantarum* FST1.7. La producción de ácido láctico, PLA y dos dipéptidos cíclicos (L-Leu-L-Pro y L-Phe-L-Pro) en los medios de cultivo y en la masa madre estaban directamente relacionados con la actividad antifúngica.

4.2.4. Bioconservación de maíz empleando caldo MRS fermentado

Además de evaluar la actividad antifúngica cuantitativa del CFS, se estudió la capacidad de mejorar la vida útil de granos de maíz y mazorcas de maíz.

El crecimiento fúngico del grupo control bajo condiciones de almacenamiento similares se observó en los granos de maíz después de 7 días, mientras que en las mazorcas de maíz se observó a día 5 de conservación. En comparación con el control, la pulverización del CFS de *L. plantarum* CECT 749 retrasó el crecimiento de *A. flavus* ITEM 8111 en maíz y *F. verticillioides* CECT 2982 en mazorcas en 8 y 2 días, respectivamente. Las muestras de granos de maíz y mazorcas de maíz fueron tratadas, secadas y contaminadas, presentando un contenido de humedad final de 18 y 20%, respectivamente.

Estos resultados sugirieron que el CFS puede usarse como un biocontrol para reducir el crecimiento de *F. verticillioides* en las mazorcas de maíz y el crecimiento de *A. flavus* en los granos de maíz durante el almacenamiento, incluso en condiciones inapropiadas de humedad.

4.3. Reducción de micotoxinas en alimentos

4.3.1. Empleo de suero de leche hidrolizado en pan

La cepa *P. verrucosum* VTT D-01847 empleada en el estudio de pan elaborado con suero de leche hidrolizada con tripina es productora de OTA. Una vez finalizado el estudio de vida útil la concentración final de OTA presente en los panes fue analizada, para evaluar la influencia en la producción de micotoxinas.

A los 7 días de incubación, se encontraron 24,0 µg OTA/Kg en el experimento control, mientras que no se detectó OTA cuando se empleó como agentes conservantes propionato de calcio y la sustitución del 100% del agua por suero hidrolizado, lo que demuestra una reducción del 100% en la síntesis de este metabolito por *P. verrucosum* en ambos tratamientos. El uso del suero no hidrolizado como ingrendiente evidenció también una reducción del 55% en la concentración de OTA en relación con el pan control sin aditivos. En cambio, la

sustitución del 50% del agua por suero hidrolizado evidenció una concentración de OTA en pan de 3,5 µg/Kg, mostrando una reducción en comparación con el pan con suero no hidrolizado y el pan sin aditivos del 30 y 85%, respectivamente.

Si bien hay muchos artículos disponibles sobre la reducción de micotoxinas atribuidas a varios ingredientes funcionales, este artículo es el primero en describir la actividad antimicotoxigénica de suero de leche hidrolizado frente a un hongo toxigénico productor de OTA.

4.3.2. Utilización de caldo MRS fermentado en maíz

El maíz es un sutrato idóneo para la producción de aflatoxinas y numeroros informes demostraron el uso efectivo de las propiedades antifúngicas de los compuestos bioactivos producidos por BAL para inhibir la producción de AFB_1. En nuestro estudio, el CFS de *L. plantarum* CECT 749 evidenció una reducción del 100% en la producción de AFB_1 por *A. flavus* a día 5 de almacenamiento, asociado a la inhibición del crecimiento fúngico. A día 25 y 40 de almacenamiento, donde el crecimiento fúngico visual en el maíz era marcado, la reducción de la producción de AFB_1 fue del 99,7 y 97,5%, respectivamente.

Los datos relacionados con la producción de fumonisina B_1 (FB_1) por *F. verticillioides* CECT 2982 en mazorcas de maíz evidenciaron que el uso de CFS de *L. plantarum* CECT 749 redujo la producción de FB_1 en un 90,6% a día 7 de almacenamiento. Sin embargo, este efecto antimicotoxigénico se redujo ligeramente con el tiempo de almacenamiento, alcanzando una reducción del 73% a día 15 de conservación.

En general, el tratamiento no evitó por completo la producción de AFB_1 y FB_1, sin embargo, tanto en los granos de maíz como en las mazorcas de maíz, la concentración final de micotoxina se redujo significativamente después de la aplicación de CFS de *L. plantarum* CECT 749 CFS en comparación con el grupo

control. Por ejemplo, después del tiempo de incubación, la concentración final de AFB_1 y FB_1 en el grupo de tratamiento fue de 6,9 y 20,1 ng/g, mientras que la concentración de AFB_1 y FB_1 en el grupo de control alcanzó valores de 278,4 y 75,4 ng/g, respectivamente.

Los resultados actuales sugieren que los productos del metabolismo de las BAL, debido a su potencial para reducir el crecimiento de los hongos micotoxigénicos y la biosíntesis de las micotoxinas, podrían ser prometedores para el biocontrol de granos como el maíz.

En relación a esto, muchos trabajos reportan como la acción de los metabolitos producidos por las BAL influencian directamente sobre el crecimiento y producción de micotoxinas. Los ácidos orgánicos producen un retraso de estos procesos en forma dependiente de la dosis. Sin embargo, Hassan y Bullerman (2008) informaron que concentraciones subletales de ácidos orgánicos producidos por *Lactobacillus paracasei* subsp. *tolerans* mostraron una reducción significativa del crecimiento de *Fusarium* spp. con un simultáneo aumento de la producción de micotoxinas.

Guimarães *et al*. (2018) evidenció una reducción del 91% en la producción de AFB_1 por parte de *A. flavus* con solo una reducción del crecimiento del 32% bajo la influencia del CFS de *Lactobacillus plantarum* UM55. Este efecto revertió cuando se neutralizó el medio, lo que muestra el papel de la forma química de los ácidos orgánicos en la modulación de la producción de micotoxinas, independientemente del crecimiento fúngico. Se sabe muy poco en relación al mecanismo de acción de los ácidos orgánicos sobre la producción de micotoxinas aparte de la dependencia del pH. Además, el ácido láctico puede afectar a la estabilidad de algunas micotoxinas. Aiko *et al*. (2016) informaron sobre la eficiencia de este ácido orgánico en la degradación de hasta un 85% de la AFB_1.

4.4. Evaluación de las propiedades biológicas del suero de leche fermentado por BAL

El estudio de la actividad antioxidante mediante el ensayo ABTS, la actividad inhibitoria de la enzima convertidora de angiotensina (ECA) y la capacidad de quelación de metales, evidenciaron que la fermentación del suero con BAL incrementa estas propiedades.

El tiempo de fermentación del suero (24, 48 y 72 h) fue un factor determinante en la capacidad antioxidante. Los sueros fermentados con *L. plantarum* CECT 220 y *L. plantarum* CECT 221 evidenciaron un aumento de la capacidad antioxidante a medida que aumentaba el tiempo de fermentación. En cambio, el suero fermentado por *L. plantarum* CECT 748, mostró una tendencia inversa, a mayor tiempo de fermentación menor actividad antioxidante. Sin embargo, indepedientemente del tiempo de fermentación los valores de actividad antioxidante fueron significativamente superiores en comparación al suero control no fermentado.

En la literatura se ha informado que las cepas de *L. plantarum* pueden producir diferentes endopeptidasas durante la fermentación. Estas enzimas pueden hidrolizar las proteínas del medio dando lugar a péptidos de bajo peso molecular con actividad antioxidante.

Montoro *et al*. (2017) analizaron la actividad antioxidante de leche de cabra fermentada con *L. plantarum* empleando diferentes métodos de cuantificación (ORAC, ABTS, DPPH y FRAP). Según los resultados, la mayor capacidad antioxidante se observó en el permeado resultante de la ultrafiltración (<3kDa) de las leches fermentadas.

Para todas las muestras, la actividad inhibitoria ECA aumentó proporcionalmente al tiempo de fermentación del suero de leche (de 24 a 72 h). La cepa cepa *L. plantarum* CECT 220 proporcionó a las 72 h de fermentación el mayor porcentaje de inhibición ECA en comparación con el suero control (85%).

Hernández-Ledesma *et al*. (2005) encontraron actividad inhibitoria de la ECA de extractos solubles en agua de leche fermentada, comparando los resultados con leches pasteurizadas y UHT. El extracto de la leche fermentada mostró un porcentaje de inhibición del 74%, mientras que los porcentajes de inhibición para los extractos de leche pasteurizada y UHT no fermentadas fueron inferiores al 2%. Estos resultados demuestran que la fermentación juega un papel importante en la liberación de péptidos inhibidores de la ECA procedentes de las proteínas de la leche.

La actividad quelante de hierro se expresó como un porcentaje en comparación con el control positivo representado por glutatión (1 mg/ml). Nuestros resultados pusieron de manifiesto que en el suero fermentado por *L. plantarum* CECT 220 *y L. plantarum* CECT 221 aumenta la actividad quelante de hierro de manera directamente proporcional al tiempo de fermentación, alcanzando un porcentaje máximo después de 72 h de fermentación (45%). La muestra de suero fermentado con *L. plantarum* CECT 748 presentó una tendencia inversa; si bien, la actividad quelante de hierro fue la más alta a las 24 h (55%).

Resultados similares fueron reportados por Abubakr *et al*. (2012) al estudiar la actividad quelante del suero después de la fermentación con bacterias; y encontraron la mayor capacidad de unión al hierro se producía después de 24 h de fermentación, con una disminución del 98 al 42% en las siguientes 48 h.

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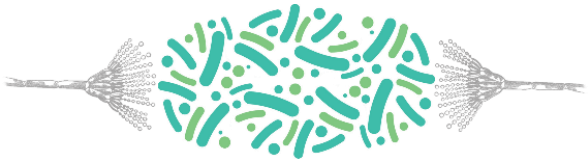
5. CONCLUSIONS

Conclusiones



5. CONCLUSIONES

1. Los caldos MRS fermentados por *Lactobacillus rhamnosus* CECT 278T, *L. johnsoni* CECT 289, y *L. plantarum* CECT 749 evidenciaron actividad antifúngica frente a *Aspergillus parasiticus* CECT 2681 y *Penicillum expansum* CECT 2278.
2. Se identificaron mediante espectrometría de masas MALDI-TOF tres péptidos de bajo peso molecular en el caldo MRS fermentado por *L. plantarum* CECT 749. Los péptidos LVGKKVQTF y GTLIGQDYK no mostraron actividad antifúngica, en cambio, el péptido SGADTTFLTK evidenció una reducción significativa del crecimiento fúngico de *A. parasiticus* y *P. expansum*.
3. El suero de leche fermentado por 5 de las 7 cepas de *L. plantarum* presentaron inhibición del crecimiento de hongos pertenecientes a los géneros *Aspergillus, Penicillium* y *Fusarium*. Los valores de MFC para estos tres géneros fúngicos fueron de 250 g/L para *Aspergillus* y comprendidas entre 62,5 y 250 g/L para *Penicillum* y *Fusarium*.
4. El empleo de suero de leche fermentado por *L. plantarum* CECT 221 como ingrediente en la elaboración de pan de molde contaminado con *P. expansum* incrementó la vida útil en 2 días en comparación con el pan control. El tratamiento también evidenció una reducción de la población fúngica de 0,6 log UFC/g.
5. El suero de leche hidrolizado con tripsina produjo una reducción del crecimiento fúngico de *P. camemberti* CECT 2267, *P. verrucosum* VTT D-01847, *P. commune* CECT 20767, *P. brevicompactum* CECT 2316 y *P. expansum,* y mostró valores de MFC comprendidos entre 15,8 y 250 g/L.
6. El empleo de suero de leche hidrolizado con tripsina como ingrediente en la elaboración de pan de pita contaminado con *P. verrucosum* incrementó la

vida útil en 2 días en comparación con el pan control y puso de manifiesto una reducción de la población fúngica de 1 log UFC/g. Este tratamiento también proporcionó una reducción del 100% de la síntesis de OTA por *P. verrucosum*.

7. El extracto acuoso de la masa madre fermentada por *L. plantarum* CECT 749 evidenció actividad antifúngica frente a nueve hongos toxigénicos pertenecientes a los géneros *Aspergillus, Penicillium* y *Fusarium*. En cambio, *L. bulgaricus* CECT 4005 únicamente inhibió el crecimiento de *P. expansum*, *F. moniliformis* CECT 2982, *F. verticillioides* CECT 20926 y *F. graminearum* CECT 20490. Los valores de MFC para los extractos fueron de 1,6-400 (*L. bulgaricus*) y 50-400 g/L (*L. plantarum*) frente a todos los hongos estudiados, a excepción de *A. flavus* y *A. niger* los cuales mostraron valores de MFC > 400 g/L.
8. El uso de las masas madre fermentadas por *L. plantarum* CECT 749 y *L. bulgaricus* CECT 4005 como ingrediente en la elaboración de pan de molde contaminado con *P. expansum* presentaron un incremento de la vida útil de 1 y 2 días, respectivamente, en comparación con el pan control.
9. La pulverización del caldo MRS fermentado por *L. plantarum* CECT 749 retrasó el crecimiento de *A. flavus* ITEM 8111 en maíz y *F. verticillioides* CECT 2982 en mazorca en 8 y 2 días, respectivamente. Además, el tratamiento evidenció una reducción del 98% en la producción de AFB_1 por *A. flavus* en maíz tras 40 días de almacenamiento, y una reducción del 90% en la síntesis de FB_1 por *F. verticillioides* en mazorcas de maíz tras 7 días de conservación.
10. Las BAL en caldo MRS evidenciaron la capacidad de adsorber la OTA en la pared celular e hidrolizarla en OTA-α y fenilalanina. La cepa *L. plantarum* CECT 749 mostró una reducción de OTA *in vitro* a pH 6,5 del 99%.
11. La digestión gastrointestinal de pan contaminado con OTA y previamente tratado con diversos inóculos de BAL mostraron una reducción de la

bioaccesibilidad comprendida entre el 27 y el 97%. La cepa *L. johnsoni* es la que presentó mayor reducción de bioaccesibilidad de OTA.

12. La fermentación del suero de leche con cepas de *L. plantarum* pusieron de manifiesto un aumento de la capacidad antioxidante, un mayor porcentaje de la actividad inhibitoria de la enzima convertidora de la angiotensina y un incremento de la capacidad de quelación del hierro.

CONCLUSIÓN FINAL

La revalorización de los subproductos de la industria alimentaria por la bioconservación por BAL, ha dado lugar a la aparición de sinergias que permiten el uso de los subproductos como caldo de cultivo fermentable, y su posterior uso como agente de conservación mediante el aumento de la vida media de alimentos y agente detoxificante mediante la disminución de la presencia de micotoxinas.

En consecuencia, las BAL podrían constituir una alternativa al empleo de conservantes alimentarios frente a hongos toxigénicos y la producción de micotoxinas en diferentes alimentos. La aplicación industrial podría realizarse mediante el uso de las BAL como cultivos iniciadores de fermentación en el propio alimento o bien empleando el caldo fermentado libre o no de células como ingrediente.

5. CONCLUSIONS

1. The fermented MRS by *Lactobacillus rhamnosus* CECT 278T, *Lactobacillus johnsoni* CECT 289, and *Lactobacillus plantarum* CECT 749 evidenced antifungal activity against *Aspergillus parasiticus* CECT 2681 and *Penicillium expansum* CECT 2278.
2. Three low molecular weight peptide were identified by mass spectrometry MALDI-TOF in fermented media by *L. plantarum*. The LVGKKVQTF and GTLIGQDYK peptides not evidenced a significant fungal growth reduction of *A. parasiticus* y *P. expansum*.
3. The whey fermented by 5 of the 7 *L. plantarum* strains showed growth inhibition of fungi belonging to the genera *Aspergillus*, *Penicillium* and *Fusarium*. The MFC values for these three fungal genera were 250 g/L, 62.5-250 g/L and 62.5-250 g/L, respectively.
4. The use of whey fermented by *L. plantarum* CECT 221 as an ingredient in the production of bread bread contaminated with *P. expansum* increased in 2 days the shelf life compared to control bread. The treatment also showed a significant reduction of 0.6 log CFU/g in the fungal population.
5. Whey hydrolyzed with trypsin reduced the fungal growth of *P. camemberti* CECT 2267, *P. verrucosum* VTT D-01847, *P. commune* CECT 20767, *P. brevicompactum* CECT 2316 and *P. expansum*, and showed MFC values between 15.8 and 250 g/L.
6. The use of trypsin hydrolyzed whey as an ingredient in the manufacture of pita bread contaminated with *P. verrucosum* increased the shelf life by 2 days compared to control bread and showed a reduction in the fungal population of 1 log CFU/g. This treatment also provided a 100% reduction in OTA synthesis by *P. verrucosum*.

7. The aqueous extract of the sourdough fermented by *L. plantarum* CECT 749 showed antifungal activity against nine toxigenic fungi belonging to the genus *Aspergillus*, *Penicillium* and *Fusarium*. In contrast, *L. bulgaricus* CECT 4005 only inhibited the growth of *P. expansum*, *F. moniliformis* CECT 2982, *F. verticillioides* CECT 20926 and *F. graminearum* CECT 20490. The MFC values for the extracts of both strains against all fungi were 50-400 g/L, with the exception of *A. flavus* and *A. niger,* which showed MFC values > 400 g/L.
8. The use of the sourdoughs fermented by *L. plantarum* CECT 749 *and L. bulgaricus* CECT 4005 as an ingredient in the production of loaf bread contaminated with *P. expansum* showed improve shelf life of 1 and 2 days, respectively, compared to bread control.
9. Spraying of the MRS fermented by *L. plantarum* CECT 749 delayed the growth of *A. flavus* ITEM 8111 on corn and *F. verticillioides* CECT 2982 on ear in 8 and 2 days, respectively. In addition, the treatment showed a 98% reduction in the production of AFB_1 by *A. flavus* in corn after 40 days of storage, and a 90% reduction in the synthesis of FB_1 by *F. verticillioides* in corn ears after 7 days of conservation.
10. The LAB in MRS Broth showed OTA adsorption ability on the cell wall and capacity of hydrolyzed the OTA in OTA-α and phenylalanine. The *L. plantarum* CECT 749 strain showed the highest percentage reduction of OTA *in vitro* at pH 6.5 (99%).
11. The gastrointestinal digestion of bread contaminated with OTA using a LAB inoculum before digestion, showed an average reduction in bioavailability of 27-97%. The *L. johnsoni* strain showed the best mean values of OTA bioavailability reduction during digestion.

12. Whey fermented by *L. plantarum* strains showed an increase in antioxidant capacity, a higher percentage of ACE inhibitory activity, and an increase in the iron chelation capacity.

FINAL CONCLUSION

The revaluation of the by-products of the food industry through biopreservation by LAB, has given rise to synergies that allow the use of by-products as a fermentable broth, and their subsequent use as a conservation agent by increasing shelf life of food and detoxifying agent by reducing the presence of mycotoxins.

Consequently, LAB could be an alternative to the use of food preservatives against toxigenic fungi and the production of mycotoxins in different foods. The industrial application could be carried out by using the LAB as starter cultures of fermentation in the food itself or by using the fermentation broth free or not of cells as an ingredient.

ANNEX

Anexo

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In vitro antifungal activity of bioactive peptides produced by *Lactobacillus plantarum* against *Aspergillus parasiticus* and *Penicillium expansum*

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ABSTRACT

Food spoilage caused by mycotoxigenic moulds represents an important problem in food security. The antimicrobial peptides are compounds of natural origin constituted by a variable number (5–100) of amino acids held together through peptide bonds. In this work, the cell free supernatants (CFSs) containing peptides obtained from four strains of LAB were lyophilized, filtered and tested to determine the antifungal activity against *Aspergillus Parasiticus* and *Penicillium expansum*. CFS obtained by *Lactobacillus plantarum* showed the highest inhibition activity. CFS was fractionated by size exclusion chromatography and injected into the liquid chromatography coupled to diode array detector. One of the recollected fractions resulted interesting for the presence of three peaks that were purified by the technique of the LC-DAD using a semi preparative C18 column. Finally, the antifungal activity of the purified peptides was studied against *A. Parasiticus* and *P. expansum* in liquid medium. The MALDI-TOF/TOF mass spectrometry was used for the peptides identification. The three purified peptides presented an amino acidic sequence identified by a bioinformatics program of SGADTTFLTK, LVGKKVQTF, and GTLIGQDYK. The first peptide purified reduced 58% and 73% the growth of *P. expansum* and *A. parasiticus*, respectively, in liquid medium after 48 h incubation.

1. Introduction

Mould deterioration of food, feed and other agricultural commodities can be responsible for considerable economical losses. Thirty percent of crop yields are destroyed and more than 30% of perishable crops are damaged in developing countries by lowering their quality and quantity. Furthermore, moulds produce compounds potentially toxics to the consumers called mycotoxins which can cause illness and death (Pawlowska, Zannini, Coffey, & Arendt, 2012). High incidences of mould and mycotoxin contamination in food and feed are due to fungi ubiquitous nature, to their ability to colonize different substrates and to the lack of effective control measures (Hassan, Zhou, & Bullerman, 2015).

Currently, food industry depends on chemical preservatives to extend the shelf life and control the growth of spoilage fungi. The consumer's awareness about the health hazards associated with chemicals has recently increased, and they are demanding for processed foods that are free of preservatives. There are many natural alternative preservatives produced by certain microorganisms, however these microorganisms have to be non-toxic, easy to grow and require simple media for cultivation. Lactic acid bacteria (LABs) are a known potential source for generating a variety of secondary metabolites such as bacteriocines, organic acids and peptides (Cizeikiene, Juodeikiene, Paskevicius, & Bartkiene, 2013). In the past decade the interest for antifungal LABs has increased and different studies have showed that many LAB strains have the potential to combat the proliferation of fungi in various food and feed materials (Rouse, Harnett, Vaughan, & Van Sinderen, 2008; Dalie, Deschamps, & Richard-Forget, 2010; Mauch, Dal Bello, Coffey, & Arendt, 2010; Gerez, Torino, Rollan & De Valdez, 2009; Dal Bello et al., 2007). Consumers are demanding the replacement of artificial chemical preservatives by natural biopreservatives to reduce fungal contamination in foods (Brul & Coote, 1999; Crowley, Mahony, & Van Sinderen, 2013; Reis, Paula, Casarotti, & Penna, 2012; Schnurer & Magnusson, 2005). LABs, due to their long history of safe use in food and feed fermentations (Hugenholtz, 2013;

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Evaluation of biological and antimicrobial properties of freeze-dried whey fermented by different strains of *Lactobacillus plantarum*

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The aim of this study was to evaluate the biological and antimicrobial activities of commercial freeze-dried whey fermented by lactic acid bacteria in order to valorize this high polluting liquid waste of the dairy industry. Freeze-dried whey was fermented by different strains of *Lactobacillus plantarum* (CECT 220, 221, 748) at three different times of fermentation (24, 48, 72 h). Afterwards, the extract was purified on centricon amicon with a cut-off of 3 kDa to obtain a permeate consisting of small bioactive compounds reported in the literature to show greater bioactivity. The purified and diluted samples were subjected to the biological and antimicrobial tests for the evaluation of antioxidant, antihypertensive, iron binding, and antifungal activities and identification of phenolic compounds. The results highlighted a radical cation scavenging activity ranging from 1.415 to 2.083 mmol trolox equivalents TE per kg of dry weight, a percentage of iron binding capacity ranging between 23–55% and a percentage of ACE inhibitory activity ranging between 67–85%. The optimal biological activity was obtained from whey fermented by *L. plantarum* 220 for all the assays performed, except for the iron chelating activity. Furthermore, the antifungal analysis showed a good activity against the mycotoxigenic fungi belonging to *Fusarium* generum (*F. moniliformis*, *F. graminearum* and *F. verticillioides*), while a slight activity was obtained for *Aspergillus* and *Penicillium* generum. This antifungal activity could be correlated to the production of phenolic compounds during fermentation. The obtained results support the hypothesis of using whey as a functional ingredient to improve food preservation.

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Introduction

In recent years, environmental and economic problems associated with food waste have induced industries to reuse these materials in other preparations in order to minimize food waste. The dairy industry is the largest producer of difficult food discharges such as whey, the liquid residue after cheese production.[1]

According to the statistical istat data available, 11% of the total milk product in the world is employed for cheese production, and 85% of this milk is transformed into whey after cheese production. Whey is considered a waste product with very important nutritional properties, because it contains 55% milk nutrients.[2]

Several methods have been proposed for whey valorization. Lactic acid bacteria (LAB) have the property of producing proteolytic enzymes that hydrolyze large milk protein producing small peptides and free amino acids, with several functional properties for human health.[3] Bioactive compounds can be produced from milk proteins through fermentation of milk, by starters employed in the manufacture of fermented milk or cheese.[4]

Depending on the amino acid sequence, the milk protein-derived compounds may exert a number of different bioactive properties including antimicrobial, mineral binding, cardioprotective, immunomodulatory, anticancer, antidiabetic, satiating, opioid and antioxidant activities.[4,5] These peptides are inactive within the sequence of the parent protein but can be liberated during human gastrointestinal digestion.[6]

Xiao *et al.*[7] studied the antioxidant activity of soy whey after fermentation by *L. plantarum* in order to find out their nutraceutical potential. The analysis showed that fermentation of soy whey has an effect on the total phenolic content (TPC), antioxidant activity and DNA damage protection. The results showed an increase in these activities compared with unfermented whey.

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ORIGINAL RESEARCH

A natural strategy to improve the shelf life of the loaf bread against toxigenic fungi: The employment of fermented whey powder

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Whey powders are used as food ingredients in many applications, from bakery goods, soups and sauces to baby food. The objective of the study was to evaluate the antifungal property of a whey-based medium (WM) fermented by lactic acid bacteria. The antifungal activity of the WM was evaluated using antifungal tests on solid and liquid media. MIC and MFC ranged from 15.6 to 250 mg/mL and 62.5 to 250 mg/mL, respectively. Using fermented WM for dough preparation produced a reduction of Penicillium expansum *growth of 0.5–0.6 log CFU/g and an improvement in shelf life of 1–2 days in relation to control bread.*

Keywords Fermentation, Whey, Antifungal activity, Shelf life, Lactic acid bacteria.

INTRODUCTION

Whey obtained in the cheese production process is the main waste by-product of the dairy industry. The principal components of whey are lactose, protein, fat, calcium, phosphorus, organic acids and vitamins. Therefore, it is a good culture medium for the growth of bacteria in the laboratory. From a biotechnological point of view, the composition of whey allows the growth of microorganisms, including lactic acid bacteria (LAB), and its fermentation, reducing the biological demand for oxygen and obtaining products with a high added value for the food industry (Karwowska *et al.* 2014; Khem *et al.* 2016). A growing interest in whey has been noted (Figure 1).

During recent years, several studies have reported the beneficial effects of whey. On the one hand, whey protein used to supplement food improves the health of children, adults and the elderly (Stobaugh *et al.* 2016). The peptides generated from the enzymatic hydrolysis of whey proteins show biological effects *in vitro*, such as antimicrobial, antioxidant, antihypertensive and antidiabetic activity among others (Brandelli *et al.* 2015). On the other hand, recent studies evidenced that food containing lactic acid bacteria (LAB) has beneficial effects (Alwan *et al.* 2014; Chen *et al.* 2014). The antifungal activity spectrum of LAB has been very well studied since the food industry showed interest in reducing the use of chemical preservatives to offer 'additive-free' food (Arena *et al.* 2016; Russo *et al.* 2017).

Fungal growth is the most important factor limiting the shelf life of loaf bread, and it is a big problem resulting in significant economic losses. The most dominant fungal species in this type of product are those of the genera *Penicillium* and *Aspergillus* (Marin *et al.* 2003; Garcia *et al.* 2019). Fungal contamination of bakery products usually is after bread processing (Suhr and Nielsen 2003; Smith *et al.* 2004). Although yeast contamination is not common, it can be observed in bakery products stored in a modified atmosphere (Deschuyffeleer *et al.* 2011).

Calcium propionate is used generally to prolong the microbiological shelf life of bread; in Europe, maximum limits of 0.1–0.3% have been established depending on the type of pre-packaged bread (European Union 2011; Belz *et al.*

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Antifungal and antimycotoxigenic activity of hydrolyzed goat whey on *Penicillium spp*: An application as biopreservation agent in pita bread

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ABSTRACT

Whey is a by-product of the cheese industry, yet it contains proteins that have a high nutritional value and are an important source of antifungal peptides. Food deterioration caused by toxigenic fungi is one of the challenges of food safety. In this context, trypsin was used to hydrolyse goat milk whey at 37. The resultant peptides were characterised by LC–ESI–TOF-MS. Antifungal activity of the goat milk whey hydrolysate (HGW) was determined against 10 toxigenic fungi from the genus *Penicillium*, in solid and liquid media. Furthermore, HGW was used as an ingredient for bread elaboration. Bread elaborated with HGW and inoculated with toxigenic fungi was included in a shelf-life study of the reduction of fungal growth, mycotoxin production and the use of the additive calcium propionate in bread. A total of 27 peptides from α-lactalbumin, β-lactoglobulin, κ-casein and lactoferrin were identified. HGW evidenced fungal growth inhibition and presented minimum inhibitory concentration and minimum fungicidal concentration ranges of 3.9–62.5 and 15.8–250 g HGW/L, respectively. Bread with HGW displayed a 1-log reduction of fungal growth, 85–100% mycotoxin production, and extended the shelf-life by 2 days.

1. Introduction

Toxigenic fungal growth in food and the production of mycotoxins pose serious health concerns and cause severe economic losses. Many foods are highly susceptible to spoilage by fungal growth, such as fruits, vegetables, dairy products, meat and bakery products, and their derived products (Adeyeye, 2016). In wheat-based bakery products, deteriorations have been attributed to several fungal genera, largely *Penicillium*, *Aspergillus*, *Cladosporium* and *Neurospora* (Ashiq, 2015). Some of the toxins produced by these fungi are carcinogenic, probably carcinogenic and possibly carcinogenic to humans. Ochratoxin A (OTA) is a compound produced as a secondary metabolite of moulds belonging to the genera *Penicillium* and *Aspergillus* (Molinié, Faucet, Castegnaro, & Pfohl-Leszkowicz, 2005) and it is classified in Group 2B by the International Agency for Research on Cancer (IARC) because it is possibly carcinogenic to humans (IARC, 1993).

Bioactive peptides exert various health benefits, such as angiotensin-converting enzyme (ACE) inhibition, antioxidant activity, antithrombotic activity, immune system stimulation, antimicrobial and antifungal properties (Welderufael, Gibson, & Jauregi, 2012; Pihlanto, 2011). Hydrolysis by specific enzymes or fermentation with proteolytic strains is necessary to release the bioactive peptides from the parent proteins (https://www.sciencedirect.com/science/article/pii/S0308814605000944, Foegeding, Davis, Doucet, & McGuffey, 2002). Several studies have investigated the formation of antimicrobial peptides by digestion of goat whey proteins using various proteolytic enzymes (Atanasova & Ivanova, 2010; Osman, Goda, Abdel-Hamid, Badran, & Otte, 2016).

Cheese manufacturing generates a high-strength biochemical oxygen demand waste stream, whey, an environmental pollutant (Ryan & Walsh, 2016). Worldwide whey production is estimated at around 190×10^6 t/year and growing. Over the last few years, there has been a growing interest in researching and developing natural antimicrobial agents derived from by-products of the food industry to inhibit the growth of fungi in food (Ribes, Fuentes, Talens, & Barat, 2018). This double-edged approach addresses the health concerns associated with synthetic food additives by replacing them with natural compounds, promoting healthier food products while, at the same time, assists in preventing and reducing food waste (Gamba et al., 2015; Hati, Patel, Sakure, & Mandal, 2018).

Based on reported evidence, this study aims to increase the shelf-life of bakery products using a hydrolyzed whey product as a natural preservative. To achieve this objective, it (i) evaluated the antifungal

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Antifungal activity and shelf life extension of loaf bread produced with sourdough fermented by *Lactobacillus* strains

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Abstract

The objective of this study was to evaluate a water-soluble extract from sourdoughs fermented with the lactic acid bacteria (LAB) for antifungal reflect on loaf bread. The extracts produced by *Lactobacillus plantarum* CECT 749 and *L. bulgaricus* CECT 4005 were effective against strains of *Fusarium* spp., *Penicillium* spp., and *Aspergillus* spp., with minimum inhibitory concentration ranging from 1.6 to 200 mg/ml and minimum fungicidal concentration from 1.6 to 400 mg/ml. LC-ESI-MS-TOF was used to analyze the antimicrobial compounds. Several antimicrobial phenolic acids were found. Fermented sourdoughs used for the production of loaf bread produced a reduction of fungal growth in relation on the sourdough control and improved to the shelf life of 1 to 2 days compared to control bread with 0.2% calcium propionate. Therefore, LAB are an alternative to synthetic compounds for decreasing fungal contaminants and prolonging the shelf life of food.

Practical applications

This study presents the importance of sourdough fermented by *Lactobacillus plantarum* spp. and the production during the fermentation of bioactive metabolites with antifungal activity. When applied in the preparation of bread, it is able to increase the useful life of the bread and its efficiency can be compared to that of calcium propionate. It is thus an alternative to synthetic compounds for decreasing fungal contaminants and prolonging the shelf life of food.

1 | INTRODUCTION

Mycotoxins are a naturally occurring, chemically and structurally diverse group of more than 300 secondary metabolites produced by the mycelial structure of filamentous fungi, which are characterized by their potential to elicit undesirable effects in humans and animals following consumption of contaminated foods or feedstuffs (Hussein & Brasel, 2001; Rodríguez-Carrasco, Moltó, Mañes, & Berrada, 2014). Mycotoxin contamination resulting from fungal infection of agricultural crops and commodities is closely related to environmental conditions, particularly temperature and moisture, and may occur at various stages of food life "from the farm to the fork" (Magan, Medina, & Aldred, 2011).

In the bakery industry, spoilage of products by molds is a big problem resulting in significant economic losses (Koe & Juodeikiene, 2012; Stanciu et al., 2017). Fungal spores are normally not viable in bakery products immediately after baking. Nevertheless, in some processing environments, there are around 10^3 fungal spores/m^3 of air. Therefore, bakery products are usually contaminated in post-bake processing (Legan, 1993; Magan, Aldred, & Arroyo, 2012).

The technological functions of sourdough mainly depend on the metabolism of lactic acid bacteria (LAB) that can be influenced by the flour and other ingredients. To date, several different LAB species of *Lactobacillus*, *Leuconostoc*, *Weissella*, and *Pediococcus* species were shown to be present in sourdoughs from different origins including wheat and rye, *Lactobacillus* is the dominant LAB species in sourdough (Vuyst & Neysens, 2005). The technological functions of the LAB species include the production of secondary metabolites that can improve the safety and shelf life of final sourdough bread (Corsetti & Settanni, 2007). The antifungal activity of LAB

toxins

MDPI

Article

Potential Application of Lactic Acid Bacteria to Reduce Aflatoxin B_1 and Fumonisin B_1 Occurrence on Corn Kernels and Corn Ears

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Abstract: Fungal spoilage is an important issue for the food industry, leading to food sensory defects, food waste, economic losses and public health concern through the production of mycotoxins. Concomitantly, the search for safer natural products has gained importance since consumers began to look for less processed and chemically treated foods. In this context, the aim of this study was to evaluate the antifungal and antimycotoxigenic effect of seven strains of *Lactobacillus plantarum*. Lactic acid bacteria (LAB) were grown on Man Rogosa Sharpe (MRS) broth at 37 ºC in anaerobic conditions. After that, the cell-free supernatant (CFS) were recovered to determine its antifungal activity by halo diffusion agar test. In addition, minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) was determined for each *L. plantarum* CFS by 96-well microplates method. Additionally, CFS was used as a natural biocontrol agent on corn kernels and corn ears contaminated with *Aspergillus flavus* and *Fusarium verticillioides*, respectively. The *L. plantarum* CECT 749 CFS showed the highest antifungal effect against all essayed strains. Moreover, the employment of this CFS in food reduced the mycotoxin production at a percentage ranging from 73.7 to 99.7%. These results suggest that the *L. plantarum* CECT 749 CFS could be promising for the biocontrol of corn.

Keywords: *Lactobacillus plantarum*; aflatoxin B_1; fumonisin B_1; biopreservation

Key Contribution: In vitro analyses showed that *Fusarium* spp. was more susceptible to *L. plantarum* spp. CFS treatment than *Aspergillus* spp. In situ, the CFS treatment did not completely avoid the fungal growth but in both cases, on corn kernel and corn ears, the content of mycotoxin was significantly reduced.

1. Introduction

Filamentous fungi contaminate several types of food such as cereals, fruits and vegetables, dried fruits, feed products, dried spices, dried cured meats, and bread, among others [1–9].

Aspergillus and *Fusarium* are the major fungal genera associated with corn contamination [10]. Indeed, many *Aspergillus* and *Fusarium* species are considered common contaminants of food and

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Toxicity reduction of ochratoxin A by lactic acid bacteria

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ABSTRACT

Ochratoxin A (OTA) is a mycotoxin produced by the metabolism of fungus belonging to the genus *Aspergillus* and *Penicillium*. In this paper we report, the capacity of different cultures of lactic acid bacteria (LAB) to degrade OTA present in MRS broth at both pH 3.5 and 6.5. A study of OTA reduction during gastrointestinal digestion carried out with the LAB was also performed. Taking into account the two reduction mechanisms of OTA studied in this work as the enzymatic one and the adsorption on the cell wall, as well as at pH 3.5 and 6.5 the reduction values of OTA were in a range of 30–99%, being the strains with greater reduction (97% and 95%) *Lb. rhamnosus* CECT 278T and *Lb. plantarum* CECT 749 respectively. In the experiments carried out digesting the OTA in MRS medium with LAB, the highest bioaccessibility reduction was observed by the strain of *Lb. johnsonii* CECT 289, showing a mean reduction around all the gastrointestinal digestion process of 97.4%. The mass spectrometry associated to the linear ion trap method identified ochratoxin alpha (OTα) m/z = 256.1 and phenylalanine (Phe) m/z = 166.1 as the major metabolites of OTA degradation in LAB cultures.

1. Introduction

Ochratoxin A (Fig. 1) (OTA) is a mycotoxin produced by secondary metabolism of many filamentous species belonging to the genera *Aspergillus* and *Penicillium*.

OTA is a potent nephrotoxic mycotoxin in nature and also displays other adverse effects such as hepatotoxicity, teratogenicity, and immunosuppression (Pfohl-Leszkowicz et al., 1998). OTA has been proven to be carcinogenic in kidney and liver. It has been classified as a group 2B human carcinogen by the International Agency for Research on Cancer (IARC), and World Health Organization (WHO) (IARC, 1993). OTA has been putatively implicated in the etiology of Balkan endemic nephropathy (BEN) and recognized to be related to urinary tract tumors in animals (Paul et al., 2002; Pfohl-Leszkowicz et al., 2002).

Regarding the alimentary aspect, OTA is a common contaminant of grains such as barley, corn, rye, wheat, and oats, with cereal-based products typically accounting for 50–80% of the average consumer intake of the mycotoxin (Jorgensen and Jacobsen, 2002). OTA has also been reported in other plant products including coffee beans, spices, nuts, olives, grapes, beans, and figs (O'Callaghan et al., 2006). In addition, OTA can survive many typical food-processing procedures, and has been reported in bread made from contaminated wheat (Scudamore et al., 2003), in beer and wine (Odhav and Naicker, 2002).

Many countries and international organizations have regulated the OTA content in several commodities. The European Commission, 2006 has enforced the limits of OTA in cereals and cereal products with the following levels: 5.0 ng/g for raw cereal grains, 3.0 ng/g for cereals and cereal products intended for human consumption, 0.5 ng/g for baby food and cereal-based food intended for young children. For the dried vine fruits, soluble coffee and some dried fruits, the European commission has set a maximal permissible limit for OTA at 10.0 ng/g.

OTA is a moderately stable molecule, which can survive most food processings, such as roasting, brewing and baking, to some extent (Scott, 1996). Several chemical and physical methods such as hypochlorite treatment (Refai et al., 1996), ammoniation and heat treatment have been developed to detoxify OTA in animal feed or alcoholic beverages. Other detoxification methods suggest the use of ozone, alkaline hydrogen peroxide treatment and gamma irradiation in cereals and derivates (Janos et al., 2000).

Yeast, including *Saccharomyces* sp., *Rhodotorula* sp., *Cryptococcus* sp., and *Trichosporon mycotoxinivorans*, can hydrolyze the amide bond of OTA to produce nontoxic products phenylalanine (Phe) and ochratoxin alpha (OTα) (Abrunhosa et al., 2010).

A bacterium, *Phenylobacterium immobile*, which can use Phe as a sole carbon source, converted OTA to OTα and three other metabolites resulting from the breakdown of the phenyl moiety of Phe (Wegst and Ligens, 1983).

The aims of the study were to evaluate a) the OTA elimination by different strains of lactic acid bacteria in MRS medium, b) the OTA reduction during a simulated gastrointestinal digestion in presence of lactic acid bacteria, using an *in vitro* dynamic model and c) the OTA degradation products formed during the fermentations through the

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