VNIVERSITAT DE VALÈNCIA

Facultat de Farmàcia

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

Programa de Doctorado con Mención hacia la Excelencia en Ciencias de la Alimentación

ESTUDIO DE LA REDUCCIÓN QUÍMICA Y BIOLÓGICA DE HONGOS Y MICOTOXINAS EN PAN

STUDY OF CHEMICAL AND BIOLOGICAL REDUCTION OF FUNGI AND MYCOTOXINS IN BREAD

Tesis Doctoral

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CERTIFICAN QUE:

Dª Federica Saladino, licenciada en Ciencias de la Alimentación y Nutrición Humana, ha realizado bajo nuestra dirección el trabajo que lleva por título: "*Estudio de la reducción química y biológica de hongos y micotoxinas en pan*". Y autorizamos la presentación para optar al título de Doctora por la Universitat de València.

Y para que así conste, expiden y firman el presente certificado,

Burjassot, Julio 2017

Dra. Mónica Fernández Franzón Dr. Giuseppe Meca

La presente tesis doctoral ha dado lugar a un capítulo de libro y a 7 artículos publicados en las siguientes revistas:

1. Antimicrobial Activity of the Glucosinolates, in: Glucosinolates, Reference Series in Phytochemistry. *Springer International Publishing (2016) 1-26.*
2. Dietary exposure to mycotoxins through the consumption of commercial bread loaf in Valencia, Spain. *LWT - Food Science and Technology, 75 (2017) 697-701. Impact factor: 2,711.*
3. Bioactive compounds from mustard flours for the control of patulin production in wheat tortillas. *LWT - Food Science and Technology 66 (2016) 101-107. Impact factor: 2,711.*
4. Reduction of the aflatoxins B_1, B_2, G_1 and G_2 in Italian piadina by isothiocyanates. *LWT - Food Science and Technology 70 (2016) 302-308. Impact factor: 2,711.*
5. Shelf life improvement of the loaf bread using allyl, phenyl and benzyl isothiocyanates against *Aspergillus parasiticus*. *LWT - Food Science and Technology 78 (2017) 208-214. Impact factor: 2,711.*
6. Reaction of zearalenone and α-zearalenol with allyl isothiocyanate, characterization of reaction products, their bioaccessibility and bioavailability *in vitro*. *Food Chemistry 217 (2017) 648-654. Impact factor: 4,052.*
7. *In vitro* antifungal activity of lactic acid bacteria against mycotoxigenic fungi and their application in loaf bread shelf life improvement. *Food Control 67 (2016) 273-277. Impact factor: 3,388.*
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Para la realización de la presente Tesis Doctoral, la estudiante de Doctorado Federica Saladino:

- Ha disfrutado de una beca predoctoral orientada a la formación de personal investigador extranjero en organismos de investigación de la Comunitat Valenciana, dentro del programa Santiago Grisolía, concedida por la Conselleria d' Educació, Investigació, Cultura i Esport de la Generalitat Valenciana (2014-2017).

"Investigar es ver lo que todo el mundo ha visto, y pensar lo que nadie más ha pensado."

Albert Szent (1893-1986)

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LIST OF ABBREVIATIONS

AAM	Allylamine
AC	Allyl cyanide
ADI	Acceptable daily intake
AFB_1	Aflatoxin B_1
AFB_2	Aflatoxin B_2
AFG_1	Aflatoxin G_1
AFG_2	Aflatoxin G_2
AFM_1	Aflatoxin M_1
AFs	Aflatoxins
AIBI	Association of Plant Bakers
AITC/AIT	Allyl isothiocyanate
ALARA	As Low As Reasonably Achievable
ANS	Panel on Food Additives and Nutrient Sources added to Food
ASC	Ascorbigen
ATC	Ally thiocyanate
a_w	Activity water
BAM	Benzylamine
BC	Benzyl cyanide
BEA	Beauvericin
BITC	Benzyl isothiocyanate
CEBP	European Confederation of National Bakery and Confectionery Organisations
CECT	Spanish Type Culture Collection
CEPT	1-cyano-2,3-epithiopropane
CFS	Cell-free supernatant
COFALEC	Confederation of yeast
CUR	Curtain gas
Dc	Mycelium diameter in control dishes
DIM	3,3′-di-indolylmethane
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DON	Deoxynivalenol

DPPH	Diphenyl-1-picrylhydrazyl
dSPE	Dispersive Solid-Phase Extraction
Dt	Mycelium diameter in treated dishes
EDI	Estimated daily intake
EITC	Ethyl isothiocyante
EN A	Enniatin A
EN A_1	Enniatin A_1
EN B	Enniatin B
EN B_1	Enniatin B_1
ENs	Enniatins
EO	Essential oil
EPI	Enhanced product ion
ER	Enhanced resolution
ESI	Electrospray ionization
ESI-MS	Electrospray ionization mass spectrometry
EU	European Union
FB_1	Fumonisin B_1
FB_2	Fumonisin B_2
FBs	Fumonisins
FCS	Fetal carf serum
Fedima	European Federation of Manufactuers and Suppliers of Ingredients to the Bakery, Confectionery and Patisserie Industries
FID	Flame ionization detector
FUS	Fusaproliferin
GC	Gas chromatography
GLSs/GL*s*	Glucosinolates
GRAS	Generally recognized as safe
HBSS	Hank's balanced salt solution
HCl	Hydrochloric acid
HCOOH	Formic acid
I3C	Indole-3-carbinol
IAN	Indole-3-acetonitrile
IARC	International Agency for Research on Cancer

IC_{50}	Inhibitory concentration 50%
IG	Growth inhibition
IS	Ion spray voltage
ITCs	Isothiocyanates
KCl	Potassium chloride
KSCN	Potassium thiocyanate
LABs	Lactic acid bacteria
LB	Lower bound
LC	Liquid-chromatography
LC-DAD	Liquid chromatography with a diode array detector
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LOAEL	Lowest observed adverse effect level
LOD	Limit of Detection
LOQ	Limit of Quantification
MAGRAMA	Spanish Ministry of Agriculture, Food and Environment
MAL	Maximum allowed levels
MAP	Modified atmosphere packaging
MCT	Medium chains triglyceride
ME	Matrix effects
MFC	Minimum fungicidal concentration
$MgSO_4$	Magnesium sulfate
MIC	Minimum inhibitory concentration
MITC	Methyl isothiocyanate
MON	Moniliformin
MRM	Multiple reactions monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MS-LIT	Linear ion trap spectrometer
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Molecular weight
Na_2SO_4	Sodium sulfate
NaCl	Sodium chloride
NaH_2PO_4	Monosodium phosphate

$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
ND	No detected levels
NE	No effect observed
NEAA	No essential aminoacids
NIV	Nivalenol
OL	Oleoresins
OM	Oriental mustard
OMF	Oriental mustard flour
OTA	Ochratoxin A
PAM	2-phenylethylamine
PAT	Patulin
PBS	Phosphate buffer saline
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PEC	2-Phenylethyl cyanide
PEITC	Phenylethyl isothiocyanate
PHBITC/ρ-HBITC	Para-hydroxybenzyl isothiocyanate
Phe	Phenylalanine
PITC	Phenyl isothiocyanate
PLA	Phenyllactic acid
PSA	Primary secondary amine
pTDI	Provisional tolerable daily intake
PTFE	Polytetrafluoroethylene
QPS	Qualified Presumption of Safety
Qq	Linear ion trap
QqQ	Triple-quadrupole
R^2	Coefficient of determination
Rh	Relative humidity
RSD	Relative standard deviation
RSDr	Repeatability relative standard deviation
RSDR	Reproducibility relative standard deviation
RT	Retention time
S/N	Signal-to-noise ratio

SA/SNB	Sinalbin
SBO	Soybean oil
SFN	Sulforaphane
SMEs	Medium-sized enterprises
SN/SNG	Sinigrin
SRM	Selected reaction monitoring
TBA	Tetrabutylammonium hydrogensulfate
TC	Thiocyanate
TCs	Thricothecenes
TDI	Tolerable daily intake
TTA	Total titratable acidity
UB	Upper bound
YM	Yellow mustard
ZEA	Zearalenone
α-ZAL	α-Zearalanol
α-ZOL	α-Zearalenol
β-ZOL	β-Zearalenol

LISTA DE ABREVIACIONES

AFB_1	Aflatoxina B_1
AFB_2	Aflatoxina B_2
AFG_1	Aflatoxina G_1
AFG_2	Aflatoxina G_2
AFs	Aflatoxinas
AITC	Alil isotiocianato
BALs	Bacterias lácticas
BEA	Beauvericina
BITC	Bencil isotiocianato
CG-DILL	Cromatógrafo de gases con detector de ionización de llama
CL-DAD	Cromatografia liquida acoplada a detector diodo array
CL-EM/EM	Cromatografía liquida acoplada a espectrometría de masas en tándem
DON	Deoxinivalenol
EFSA	Autoridad Europea de Seguridad Alimentaria
EM	Efecto matriz
ENA	Eniatina A
ENA_1	Eniatina A_1
ENB	Eniatina B
ENB_1	Eniatina B_1
FB_1	Fumonisina B_1
FB_2	Fumonisina B_2
FUS	Fusaproliferina
GLSs	Glucosinolatos
IDE	Ingesta diaria estimada

IDT	Ingesta diaria tolerable
ITCs	Isotiocianatos
JECFA	Comité Mixto FAO/OMS de Expertos en Aditivos Alimentarios
LD	Limite de detección
LQ	Limite de cuantificación
MAGRAMA	Ministerio de Agricultura, Alimentación y Medio Ambiente
MCF	Mínima concentración fungicida
MCI	Mínima concentración inhibitoria
OTA	Ocratoxina A
PAT	Patulina
PITC	Fenil isotiocianato
R^2	Coeficiente de correlación
SCF	Comité Científico sobre la Alimentación Humana
ZEA	Zearalenona
α-ZOL	α-Zearalenol
β-ZOL	β-Zearalenol
ρ-HBITC	ρ-Hidroxibencil Isotiocianato

SUMMARY

Food spoilage caused by molds that produce mycotoxins is of primary concern and represents an important food safety problem. Cereal grains and their processed food products, such as bread, are frequently contaminated with mycotoxigenic fungi. Therefore, in this PhD Thesis, the occurrence of 17 mycotoxins in 80 samples of bread loaf and the risk exposure of the population to these toxic compounds were determined. Aflatoxins (AFs), zearalenone (ZEA) and enniatins (ENs) were detected respectively in 20%, 65% and 96% of bread samples analyzed.

The presence of these mycotoxins and, above all, the samples in which AFs and ZEA, exceeded the maximum limits established, raised the interest of reducing these compounds. In particular, two natural alternatives to the conventional preservatives were studied: isothiocyanates (ITCs) in active packaging and lactic acid bacteria (LABs) as starter cultures. ITCs are originated from the hydrolysis of glucosinolates (GLSs), which are found in mustard, broccoli, cauliflower, cabbage, etc. Different packaging with ITCs and mustard flour were evaluated for their ability to extend the shelf life and reduce the presence of mycotoxins in cereals contaminated by *Aspergillus parasiticus* or *Penicillium expansum*. Furthermore, the potential of AITC to react with ZEA and α-zearalenol (α-ZOL) was determined; reaction products were characterized and bioaccessibility and bioavailability of the mycotoxins and adducts with AITC were studied.

LABs were added during the baking process to extend the shelf life and reduce the production of AFs in bread contaminated with *A. parasiticus*. Finally, the ability of different LABs to reduce the bioaccessibility of aflatoxins B_1 (AFB_1) and B_2 (AFB_2), through a model of *in vitro* dynamic, digestion was studied.

Among the ITCs, only AITC extended the shelf life and reduced AFs in bread contaminated with *A. parasiticus* and patulin (PAT) produced by *P. expansum*. Among the two mustard flours studied, the oriental mustard flour was more effective in reducing AFs while the yellow flour in the reduction of PAT. AITC can react directly and reduce α-ZOL and ZEA *in vitro* at levels up to 97%, forming adducts ZEA/α-ZOL-AITC. A reduction of mycotoxins and their reaction products was observed after simulated digestion. Reaction products of mycotoxins with AITC were more bioavailable compared to the isolated mycotoxins. The use of LABs in bread fermentation increased the shelf life of bread and reduced the formation of AFs. Finally, some of the bacteria studied were able to reduce the bioaccessibility of AFB_1 and AFB_2 contained in contaminated bread up to 99%.

RESUMEN

El deterioro de los alimentos causado por los hongos que producen micotoxinas representa un problema importante en seguridad alimentaria. Los cereales en grano y sus productos derivados, como el pan, frecuentemente están contaminados con hongos micotoxigénicos. Es por ello que en la presente Tesis Doctoral se han estudiado la presencia de 17 micotoxinas en 80 muestras de pan de molde y el riesgo de exposición de la población a estos compuestos. Aflatoxinas (AFs), zearalenona (ZEA) y eniatinas (ENs) han sido detectadas respectivamente en el 20%, 65% y 96% de las muestras de pan analizadas.

La presencia de dichas micotoxinas y, sobre todo, de las muestras en las que las AFs y la ZEA superan el límite máximo legislado, planteó la necesidad de buscar metodologías que permitieran reducir estos compuestos. En concreto, se han estudiado dos alternativas naturales a los conservantes convencionales: los isotiocianatos (ITCs) en envases activos y las bacterias ácido lácticas (BALs) como cultivos starters. Los ITCs se originan de la hidrólisis de los glucosinolatos (GLSs), que se encuentran en mostaza, brócoli, coliflor, repollo, etc. Se han evaluado diferentes envases con ITCs y harina de mostaza por sus capacidad para extender la vida útil y reducir la presencia de micotoxinas en los cereales contaminados con *Aspergillus parasiticus* o *Penicillium expansum*. Además, se ha determinado el potencial del AITC para reaccionar con ZEA y α-zearalenol (α-ZOL); se han identificado los productos de reacción y se han estudiado la bioaccesibilidad y la biodisponibilidad de las micotoxinas y de los aductos con el AITC.

Las BALs se han añadido durante el proceso de cocción para prolongar la vida útil y reducir la producción de AFs en pan contaminado con *A. parasiticus*. Finalmente, se ha estudiado la capacidad de diferentes BALs para reducir la bioaccesibilidad de las aflatoxinas B_1 (AFB_1) y B_2 (AFB_2), a través de un modelo de digestión dinámica *in vitro*.

De los ITCs, únicamente el AITC ha alargado la vida útil y reducido las AFs en pan contaminado con *A. parasiticus* y la patulina (PAT) producida por *P. expansum*. Entre las dos harinas de mostaza estudiadas, la harina de mostaza oriental ha sido más eficaz en la reducción de las AFs mientras la harina amarilla en la reducción de la PAT. El AITC puede reaccionar y reducir α-ZOL y ZEA *in vitro* a niveles de hasta el 97%, formando aductos ZEA/α-ZOL-AITC. Una reducción de las micotoxinas y de sus productos de reacción se ha observado tras el tratamiento de la digestión. Los productos de reacción de las micotoxinas con AITC fueron más biodisponibles durante el estudio de biodisponibilidad comparado con las micotoxinas aisladas.

El uso de BALs en la fermentación del pan ha aumentado la vida útil del pan y ha reducido la formación de las AFs. Por último, algunas de las bacterias estudiadas fueron capaces de reducir la bioaccesibilidad de las AFB_1 y AFB_2 contenidas en pan contaminado hasta un 99%.

1. INTRODUCTION

1. INTRODUCTION

1.1. Food spoilage

1.1.1. Fungal spoilage and mycotoxin production

Food spoilage is defined as an unpleasant change in food's normal state due to alterations in sensory characteristics (tactile, visual, olfactory, or flavor) which makes foods undesirable or unacceptable for human consumption. Bacteria, fungi, yeasts and insects can be responsible for food spoilage but microorganisms represent the main reason for food deterioration and decay. Microbial spoilage is characterized by visible mold growth, production of gas, diffusible pigment and enzymes which may cause softening and rotting (proteolysis), slime, off-odor, and off-flavor (Erkmen & Bozoglu, 2016).

Spoiled foods may not cause illness if they are free of pathogens or toxins, or they may be unsafe. In particular, spoilage caused by molds that produce toxic secondary metabolites called mycotoxins is of primary concern and represents an important food safety problem. Cereal grains and their processed food products are frequently contaminated with mycotoxigenic fungi. Mycotoxins are known to cause sickness or death in humans or animals and are produced by a number of fungal genera, primarily *Aspergillus*, *Penicillium*, *Alternaria*, *Fusarium*, and *Claviceps*. However, considering the worldwide occurrence of fungi in foods and their capability to produce mycotoxins three genera stand out: *Aspergillus*, *Fusarium* and *Penicillium*, which include the largest number of mycotoxins producer species (Soriano, 2007). *Fusarium* species are called field molds because they produce mycotoxins before, or immediately after harvest. They are plant pathogens on cereal crops and other commodities. On the otherside, *Aspergillus* and *Penicillium* are more commonly associated with commodities and foods during drying and storage so that they are considered storage molds.

1.1.1.1. Genus Aspergillus and Penicillium

Aspergillus is a large genus with more than 100 recognized species. Colony colours are those of the conidia, which may be black, yellow, brown, white, or green. *Penicillium* includes more than 200 recognized species (Pitt, 2000); among them, 50 or more are of common occurrence. They are characterized by green conidia and a slow growth (Pitt, 2012). The most significant mycotoxigenic species in *Aspergillus* genera are: *A. flavus*, *A. parasiticus* and *A. nomius*, which produce aflatoxins (AFs); *A. ochraceus* and related species, *A. carbonarius*, *A. niger* (Pitt, 2012) which produce ochratoxin A (OTA). On the other side, the most important species in *Penicillium* genera are *P. verrucosum* and *P. nordicum* (EFSA, 2006) which produce OTA and *P. expansum* that produces patulin (PAT).

AFs are difuranocoumarins that contaminate foods and feeds causing serious economic and health problem worldwide. They are often detected in cereals and their derivates, nuts and species. The main AFs are aflatoxin B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1), G_2 (AFG_2) (Baranyi et al., 2013). The International Agency for Research on Cancer (IARC) assigned all AFs to group 1 (carcinogenic to humans; IARC, 2012). They have shown to be extremely potent carcinogens in all animal species investigated, i.e. mice, rats, hamsters, fish, ducks, and monkeys, and in several organ, the liver being the primary target. Furthermore, AFs are genotoxic compounds. AFB_1 is often the most frequent in the mixture of AFs (Marin et al., 2013). It exhibits hepatocarcinogenic and hepatotoxic properties, and is considered the most potent naturally occurring carcinogen (IARC, 2012), and it is usually the major AF produced by toxigenic strains. Chronic toxicity is the most common form of aflatoxicosis in humans whereas occurrence of acute aflatoxicosis is more frequent in animals due to the high contamination of feed. Chronic consumption of AF-contaminated foods has been linked to various diseases: liver cancer, effects on the reproductive system and on the immune

system, encephalopathy and pulmonary interstitial fibrosis. Moreover, aflatoxin M_1 (AFM_1) is a hydroxylated metabolite found primarily in animal tissues and fluids (milk and urine) as a metabolic product of AFB_1 (Varga et al., 2009, 2015) and it is classified as possibly carcinogenic to humans (group 2B) (IARC, 1993, 2002). AFs are very stable and may resist quite severe processes like roasting, extrusion, baking, and cooking so that they can be a problem in processed foods, such as roasted nuts and bakery products (Marin et al., 2013).

OTA is a phenylalanyl derivative of a substituted isocumarin. OTA is structurally similar to the amino acid phenylalanine (Phe). For this reason it has an inhibitory effect on a number of enzymes that use Phe as a substrate such as Phe-tRNA synthetase which can result in the inhibition of protein synthesis. Moreover, it can cause mitochondrial damage, oxidative burst, lipid peroxidation and interferes with oxidative phosphorylation and it can also increase apoptosis in several cell types (Kuiper-Goodman & Scott, 1989). It is classified as possibly carcinogenic to humans (group 2B) (IARC, 1993). OTA can cause nephropathies in humans because kidney results the major target organ; in animals it increases the incidence of testicular cancer. Furthermore, OTA is recognized as teratogenic, genotoxic, carcinogenic, and immunotoxic but its neurotoxic effect remains unconfirmed. Contamination with OTA was reported in dried fruits, cacao and derived products, wine, cereals and spices. It is not destroyed by common food preparation procedures; temperatures above 250°C for several minutes are necessary to reduce the concentration of this toxin (Boudra et al., 1995; Marin et al., 2013).

PAT is produced by a wide range of fungal species of the *Penicillium* and *Aspergillus* genera but *P. expansum* is the most important (Morales et al., 2007). PAT has been found as a contaminant in many moldy fruits, vegetables, cereals and other foods. However, the major sources of contamination are apples and

apple products, which are also the most important source of PAT in the human diet (Baert et al., 2007; Murillo-Arbizu et al., 2009; Reddy et al., 2010). It is included in a group of compounds known as toxic lactones and it has affinity for sulfhydryl groups, thus can inhibits several enzymes. In long-term studies with animals, PAT has been reported to be mutagenic and to cause neurotoxic, immunotoxic, genotoxic and gastrointestinal effects in rodents. Moreover, it was demonstrated that it can alter the intestinal barrier function (Mahfoud et al., 2002). PAT is classified by IARC in group 3 (IARC, 1993) due to the inadequate evidence for its carcinogenicity in experimental animals (Wright, 2015).

1.1.1.2. Genus Fusarium

Infection of *Fusarium* species may occur during seedling, especially in cereals, and also in maturing fruits and vegetables. The very important role of *Fusarium* species as mycotoxins producers has remained unknown until the 1970s. The main *Fusarium* toxins reported in foods are fumonisins B_1 and B_2 (FB_1 and B_2), thricothecenes (TCs), zearalenone (ZEA) and the emerging mycotoxins that include fusaproliferin (FUS), moniliformin (MON), beauvericin (BEA), and enniatins (ENs).

At least 12 fumonisins are known but the B series (FBs) result to be the most important and in particular FB_1 and FB_2. FBs are mostly found in maize. *F. verticillioides* (syn. *F. moniliforme*) and *F. proliferatum* are the main producing species. They present a structural similarity to sphinganine which is the precursor of sphingolipids. The main reason for their toxicity and possibly carcinogenicity is their capacity to inhibit the enzyme ceramide synthase causing an increase of the intracellular sphinganine and other sphingoid bases which are highly cytotoxic compounds (Solfrizzo et al., 2004). The IARC has classified FB_1 as possibly carcinogenic to humans (group 2B) (IARC, 2002). FBs content is reduced only

during processes in which the temperature exceeds 150°C and they can be little degradated during fermentation (EFSA, 2005).

TCs are compounds characterized by a common tetracyclic sesquiterpenoid 12,13-epoxytrichothec-9-ene ring system. They are divided into four types A-B-C-D according to variations in the functional hydroxyl and acetoxy side groups (Bennett & Klich, 2003). Types A represented by HT-2 and T-2 toxins and type B by deoxynivalenol (DON) and nivalenol (NIV) are the most important whereas C and D groups include some TCs of lesser importance. *F. sporotrichioides*, *F. langsethiae*, *F. acuminatum*, and *F. poae* are the main producers of T-2 and HT-2 and *F. graminearum*, *F. culmorum*, and *F. cerealis* the most important producing species of DON. These mycotoxins can inhibit eukaryotic protein synthesis by binding to the 60S ribosomal subunit and by interacting with the enzyme peptidyltransferase. HT-2, T-2 toxin and DON are classified by the IARC in group 3 (not classifiable as to its carcinogenicity to humans) (Marin et al., 2013). Even if TCs are in general very stable during storage/milling and the processing/cooking of food and they can resist high temperatures, it was demonstrated that baking of bread, cookies and biscuits can lead to a DON reduction up to 71% (Bullerman & Bianchini, 2007). Furthermore, a detoxification over 95% was achieved by extrusion cooking (Cazzaniga et al., 2001). TCs are also stable at neutral and acidic pH and consequently, they are not hydrolyzed in the stomach after ingestion (Yazar & Omurtag 2008). Since DON is water soluble, its level is reduced in cooked pasta (Sobrova et al., 2010). Processing cereals will substantially reduce T-2 and HT-2 contamination in most food products because T-2 and HT-2 toxins are mostly attached to the outer hull of the grain so that cleaning, sorting, sieving and, de-hulling of grains leads to marked increases in T-2 and HT-2 toxins in cereal by-products such as bran. Malting leads to substantially lower levels of T-2 and HT-2 toxins in malt,

compared to the original barley, although the ratio varies considerably (EFSA, 2011b).

ZEA is mainly produced by *F. graminearum* and also by *F. culmorum*, *F. cerealis*, *F. equiseti*, *F. verticillioides* and *F. incarnatum*. These fungal species contaminate pre and postharvest cereal crops such as corn, barley, wheat, rice and oats mainly from temperate and warm regions (Zinedine et al., 2007). ZEA and its derivatives α-zearalenol (α-ZOL) and β-zearalenol (β-ZOL) elicit estrogenic response upon binding to the estrogen receptor (Drzymala et al., 2015), although the estrogenic potential of α-ZOL is three to four times higher than that of ZEA and β-ZOL (Yang et al., 2007). Several studies have demonstrated hepatotoxic, haematotoxic, immunotoxic, genotoxic and teratogenic effects of these mycotoxins to a number of mammalian species (Zinedine et al., 2007). IARC carcinogenic evaluation of ZEA concluded that it is not classifiable regarding its carcinogenicity to humans (group 3) (IARC, 1993). Cooking does not degrade ZEA but under alkaline conditions or during extrusion cooking can be reduced more than 40%. As for T-2 and HT-2, ZEA is redistributed between milling fractions. In particular, its concentration is 3- to 30- fold higher in the by-products obtained from cleaning the raw cereals grains than the cleaned cereal grains and bran contains up to 2-fold higher concentrations (EFSA, 2011a).

Data available on emerging mycotoxins are limited due to their late recognition and understanding of their role as toxic compounds.

FUS is a sestertepene produced mostly by *F. proliferatum* and *F. subglutinans*. It is toxic to *Artemia salina*, human B lymphocytes IARC/LCL 171 and SF-9 insect cells and has teratogenic and pathogenic effects on chicken embryos (Jestoi, 2008).

MON is a very strong acid produced by *F. avenaceum*, *F. tricinctum*, *F. proliferatum*, *F. subglutinans*, and *F. verticillioides*. MON can interfere with the

tricarboxylic acid cycle and with carbohydrate mechanisms inhibiting enzymes such as pyruvate dehydrogenase (Pirrung & Nauhaus, 1996). It also inhibits glutathione peroxidase and glutathione reductase inducing oxidative damage in myoblasts (Chen et al., 1990; Reams et al., 1996). It is not a genotoxic carcinogen according to the Ames test (Knassmuller et al., 1997). MON could be stable in food processes such as baking or wet milling whereas alkaline cooking conditions may cause a partial or a complete reduction of MON depending on the temperature and time of processing. Freeze-drying did not affect the stability of MON (Abramson et al., 2002).

ENs (A, A_1, B and B_1) and BEA are a group of structurally related cyclic hexadepsipeptides consisting of three d-2 hydroxycarboxylic acid and N-methylamino acid residues linked alternately. ENs producers include *F. trincinctum*, *F. poae*, *F. sporotrichioides*, and *F. langsethiae* (Thrane, 2001). BEA is produced by *F. sambucinum*, *F. poae*, *F. langsethiae*, *F. verticillioides*, *F. sporotrichioides*, *F. proliferatum*, and *F. subglutinans* (Logrieco et al., 1998; Thrane, 2001). Finally, *F. avenaceum* can produce both ENs and small amounts of BEA (Logrieco et al., 1998). These mycotoxins are cytotoxic (Uhlig et al., 2006), and their apolar nature enables them to insert into cell membranes creating cation selective channels (Ovchinnikov et al., 1974), thereby disturbing the intracellular ionic homeostasis (Kamyar et al., 2004). Toxicological data on these mycotoxins is insufficient. It was demonstrated that thermal treatment (pasta cooking simulation) can reduce ENs in a time dependent manner whereas the variation of the pH does not produce any effect in the ENs stability (Serrano et al., 2013). A treatment of 200°C during 20 min of incubation in a model solution can completely degrade BEA. Furthermore, BEA degradation results variable from 20 to 90% in experiments carried out using crispy breads as a system used to

simulate food preparation at three different temperatures of 160, 180 and 200°C and at 3, 6, 10, 15 and 20 min incubation (Meca et al., 2012).

1.2. Bread

1.2.1. Nutritional importance and consumption

Bread in its many forms is one of the most consumed foods by humanity (Cauvain, 2015).

The basic ingredients in bread includes: flour to give strength and structure of products by starch and protein contained in the flour; water as a solvent to act with flour to form dough; yeast to act on natural sugars in the flour to generate carbon dioxide, and to make the dough rise through fermentation. Yeast is necessary for dough fermentation; salt is important not only to contribute to flavour but also to strengthen the gluten and control the action of yeast for loaf volume. Moreover, small amounts of extra ingredients are added to improve dough performance during processing or to improve the quality of finished bread. For example, sugar is added to provide nutrients to yeast in the early stages of fermentation. Fat and/or milk derivatives are used to enhance the quality of the product by contributing softness, moistness and improving flavour and texture. In addition, other functional components including additives and preservatives might be used in flour or dough to better baking quality of bread and yeast-raised fine bakers' wares (Williams & Pullen, 2007).

A healthy diet is the foundation of good health and bread is part of a healthy lifestyle. It provides most of the essential nutrients for life: carbohydrates (mainly starch), proteins, vitamins (A, B1, B2, niacin, folic acid, etc.) and minerals (calcium, magnesium, phosphorus, iodine, iron and zinc). Nutritional contributions are greater in wholemeal breads that are characterized by a bigger concentration of proteins and dietary fibers. Furthermore, many other types of breads exist

today that are enriched with different ingredients such as non-wheat grains, seeds or pulses sometimes trying to improve nutritional quality, especially with respect to fiber intake (Cauvain, 2015). The consumption of bread in Europe is of 63 kg per capita per year. It differs widely within the European countries but most of them have an average consumption of 50 kg of bread per person per year.

Moreover, bread represents a strong economic sector in Europe. The European Bakery sector is composed by more than 190,000 small and medium-sized enterprises (SMEs) and includes 2.200 large companies employing more than 2 million people in the European Union (EU) (Bread-Initiative.eu, 2016).

1.2.2. Bread spoilage

Bread is a perishable product. Microbial spoilage causes significant economic losses for the bakery industry and also for the consumers (Melikoglu & Webb, 2013). In 2011, Novozymes enterprise surveyed over 4000 bread consumers throughout Europe and found evidence that the principal cause of throwing bread away was because it had become mouldy (Van Sint Fiet, 2015).

The pH of yeast-raised bread is about 5.4-6.0 whereas in sourdough fermented bread is about 3.5-4.8. This low pH and the high water activity (a_w 0.94-0.97) which characterized bread are the main reasons why it is mostly spoiled by molds as opposed to yeast and bacteria, that are rare due to low a_w during baking (Guynot et al., 2005; Smith et al., 2004).

In the absence of preservatives, visible mould growth is observed after 3-4 days of storage at room temperature although the characteristic odour composed of volatile metabolites can be noted after 2 days (Nielsen & Rios, 2000).

Penicillium and *Aspergillus* are the most common molds causing bread spoilage (Dal Bello et al., 2007). The *Penicillium* colonies on bread produce a blue fuzzy texture or “bloom” and can be blue or green. Pigments produced by

Aspergillus can be yellow, reddish yellow or reddish brown in the ascosporic stage, or green in the conidial stage. Some species of *Aspergillus* which contaminates bread can produce AFs that are carcinogens (Nielsen & Rios, 2000). Both *Penicillium* and *Aspergillus* growth is promoted upon bread which presents values of a_w around 0.6-0.85 and temperature ranging between 22 and 30°C, because the conidia are usually abundant in the atmosphere (Smith, 2004). *Fusarium* mycotoxins can be detected in bread because this genus contaminates the raw material used to produce bread. The SCOOP project (Collection of occurrence data of *Fusarium* toxins in food and assessment of dietary intake by the population of EU member states: http://europa.eu.int/comm/food/fs/scoop/task3210.pdf) underlines that wheat and wheat products like pasta and bread represent the major source of intake of DON (Terzi el al., 2007).

The variety of the breads, the recipe used, the bakery processing, and the localization of products can affect the type and number of spoilage fungi present on bread (Pateras, 2007; Seiler, 1992; Smith et al., 2004). *Aspergillus* and *Penicillium* together with *Eurotium* are the predominant spoilages in Spanish bakery products (Guynot et al., 2005). In Northern Ireland *Penicillium* spp. is the most common bread spoilage fungi, whereas *Aspergillus* spp. is more predominant in India (Pateras, 2007). Increased acidity will have an effect such as slowing mould growth (Nielsen & Rios, 2000). Bulk-fermented bread will have a slightly longer shelf life compared with the no-time dough bread, which is due to highly alcohol content in fermented breads (Pateras, 2007).

Fungi spoilage is also influenced by relative humidity (RH) and storage temperature. Research showed that when breads were stored at 25˚C and 70% Rh, 85% of bread spoilage was caused by *Penicillium* spp. and less than 7% due to yeast spoilage (Smith et al., 2004). *Penicillium* spp. was predominant in 90 to

100% and *Aspergillus* spp. in nearly 50% of bread loaves collected from 46 bakeries over a one year period, packaged in plastic bags and stored at 22˚C for 5-6 days.

Generally, bread contains few microorganisms after baking due to thermal inactivation of viable vegetative molds and molds spores. However, bread contamination mainly results from post-bakery processes such as cooling, slicing and packaging operations either indirectly by airborne spores or directly by contact with contaminated equipment. Sliced breads have a higher probability of mold growth since the internal surfaces are exposed, condition indispensable because molds are aerobic and grow only on the surface of bread. Packaging warm bread also can promote fungal growth due to localized areas of condensate within the package. Moreover, the presence of spores in flour and their ability to spread throughout the production environment with air movement facilitate their widespread in bakeries (Pateras, 2007; Erkmen & Bozoglu, 2016). Finally, it has to be considered that reduction of mycotoxins, already present in the dough, during baking can be relatively low and it varies with the toxins (Kaushik et al., 2015).

An EC regulation (No. 1881/2006) and an EC recommendations (No. 165/2013) define maximum levels for some mycotoxins (Table 1). However, for some *Fusarium* mycotoxins like NIV, ENs, and MON no maximum level has been set yet.

Table 1. Maximum levels for mycotoxins in bread.

Mycotoxins	Foodstuffs	Maximum levels (μg/kg)
AFB_1	All cereals and products derived from cereals	2
Sum of AFB_1, AFB_2, AFG_1 and AFG_2	All cereals and products derived from cereals	4
OTA	All cereals and products derived from cereals	3
DON	Bread (including small bakery wares), pastries, biscuits, cereal snacks and breakfast cereals	500
ZEA	Bread (including small bakery wares), pastries, biscuits, cereal snacks and breakfast cereals, excluding maize-snacks and maize-based breakfast cereals	50
T-2+HT-2	Bread (including small bakery wares), pastries, biscuits, cereal snacks and pasta	25
FB_1+FB_2	Maize intended for direct human consumption, maize-based foods for direct human consumption	1000

1.3. Bread preservation techniques

1.3.1. Conventional preservatives

Conventional chemical preservatives used to inhibit the growth of undesired microorganisms and to prolong the shelf life of bakery products are weak organic acids such as propionic and sorbic acid. In particular, the forms commonly used are potassium, sodium or calcium salts of propionic and sorbic acid because they are characterized by higher water solubility and easier handling (Magan et al., 2003). These preservatives are allowed only in prepacked bread whereas they are not permitted for unpacked wheat bread made only from wheat flour, water, yeast or sourdough and salt. For prepacked unsliced bread propionate is allowed only up to 0.1% (*w/w*) of flour weight and for prepacked sliced bread and rye bread up to 0.2% of sorbate or up to 0.3% of propionate (EC, 2008).

The mechanism of action of these acids consists of decreasing phosphofructokinase activity which is a key enzyme of glycolysis and, hence, reducing the ATP yield. In fact, these acids are in their undissociated form at lower pH so that they can penetrate the plasma membrane and dissociate inside the cell acidifying cell cytoplasm. This drop of pH interferes with the activity of the aforementioned enzyme (Krebs et al., 1983). Sorbic acid can also inhibit the plasma membrane H^+-ATPase proton pump working as an antimicrobial compound (Stratford et al., 2013a, 2009).

Some disadvantages regarding the use of these preservatives have been evidenced by different authors. Sub-optimal concentrations lower than 0.03 % can result in an enhancement of fungal growth (Marin et al., 2002) and higher mycotoxin production (Arroyo et al., 2005). On the other side, the use of high concentrations of sorbate and propionate are necessary for antifungal activity but they can alter the sensory properties of the product. Furthermore, a prolonged

usage of these preservatives can lead to the development of fungal resistance to those chemicals (Levinskaite, 2012; Stratford et al., 2013b; Suhr & Nielsen, 2004). Suhr & Nielsen (2004) demonstrated that propionate has only slight effect in mold inhibition when it is included in bread at pH 6. The germination of conidia of 12 fungal species tested was only partially prevented using 0.3% of calcium propionate. Sorbate is rarely used in bread because it negatively affects bread fermentation, especially causing volume reduction, even if it seems to be more efficient in inhibiting spoilage than propionate (Lavermicocca et al., 2000).

Ethanol also showed strong inhibition of mould in bread at concentration ranging between 0.5 and 3.5% of loaf weight in both surface spraying of bread loaves and active packaging. However, an unacceptable odour affected the sensory quality of the product (Legan, 1993; Seiler, 1984). Finally, the addition of benzoic acid to bakery products has been tested but it is not authorized (Guynot et al., 2005; Suhr & Nielsen, 2004).

1.3.2. Alternative preservatives

As discussed above, the use of conventional preservatives presents several drawbacks. Additionally, health-conscious consumers and, thus, the demand for “natural” foods without chemical preservatives and additives have increased. For this reason more studies are centered on replacing traditionally preservatives by natural ones. This thesis is focused on two natural alternatives: lactic acid bacteria (LABs) and isothiocyanates (ITCs) (Axel et al., 2016). LABs encompass a heterogeneous group of Gram-positive, non-sporeforming, non-motile, aerotolerant, rod and coccus-shaped organisms, which produce lactic acid as a major end product during carbohydrate fermentation (Crowley et al., 2013). ITCs are originated by glucosinolates (GLSs) that are plant derived compounds (Axel et al., 2016).

1.3.2.1. Lactic acid bacteria

LABs represent a promising low-cost and biological solution to alleviate fungal decay of foods and also of feeds, especially with the increased pressure of consumers towards more natural food preservatives. For centuries, they have been exploited as biopreservative microorganisms which could control food spoilage and the occurrence of their mycotoxins (Crowley et al., 2013). LABs are the microorganisms most widely used as starter cultures in bakery products because they produce and release the preservative compounds *in situ* so that they can be applied, for example, in sourdough production. Biological preservation refers to the food's shelf-life extension and improvement of their microbial safety by inoculating protective cultures in the food matrix (*in situ* production of antimicrobial compounds), or incorporation of purified microbial metabolites (Gaggia et al., 2011; Pawlowska et al., 2012). Additionally, LABs play an important role in enhancing texture, sensory characteristics, nutritional value, and overall quality of the fermented products offering beneficial outcomes to consumers (Di Cagno et al., 2012; Peres et al., 2012; Ravyts et al., 2012; Vignolo et al., 2012). LABs are mainly divided into four genera: *Lactococcus*, *Lactobacillus*, *Leuconostoc* and *Pediococcus*. These microorganisms are widely used for the production of fermented foods and are also part of intestinal microflora. They are microaerophilic and their growth is strictly dependent on the available sugars. They ferment carbohydrates to produce various end-products (Dalié et al., 2010). LABs have been included in the QPS (Qualified Presumption of Safety) list for authorized use in the food and feed chain within the European Union (EFSA, 2012; EC, 2008). Furthermore, in the US, LABs are generally recognized as safe (GRAS) by the U.S. Food and Drug Administration.

LABs can act inhibiting mould growth and some of them can also potentially interact with mycotoxins. Their antifungal activity depends on

different factors such as antifungal substance together with their production levels and mode of action, temperature, incubation period and the effects of growth media, nutritional factors and pH (Dalié et al., 2010).

LABs antimicrobial activity is primarily attributed to a wide variety of metabolites that include: organic acids (lactic, acetic, formic, propionic, butyric, hydroxyl-phenyllactic acid, and phenyllactic acid (PLA)), or antagonistic compounds (carbon dioxide, ethanol, hydrogen peroxide, fatty acids, acetoin, diacetyl, antifungal compounds (propionate, phenyl-lactate, hydroxyphenyl-lactate, cyclic dipeptides and 3-hydroxy fatty acids, PLA), bacteriocins (nisin, reuterin, reutericyclin, pediocin, lacticin, enterocin, etc.), or bacteriocin-like inhibitory substances (Muhialdin et al., 2011; Reis et al., 2012; Schnürer & Magnusson, 2005). The production of the acids results in an acidic environment which restricts the growth of spoilage organisms (Schnürer & Magnusson, 2005). Additionally, organic acids increase the plasma membrane permeability and neutralize the electrochemical proton gradient, thus killing microorganisms. The production of organic acids alone does not explain the antifungal activity (Ström et al., 2002). Other mechanisms that can explain the inhibition are synergistic effects with the others antimicrobial compounds, competition of LABs for nutrients, space and exclusion of the pathogen from entry sites in the matrix, and finally alteration of spore membrane, viscosity and permeability (Pawlowska et al., 2012). Lactic and acetic acids are the most produced whereas PLA is one of the most extensively studied antifungal organic acids from LABs, which possesses broad spectrum antibacterial and antifungal action (Crowley et al., 2013). Other compounds that have been subject of many studies and have showed the highest antifungal activity were proteinaceous compounds with low molecular weight, hydrogen peroxide, phenolic compounds, hydroxyl fatty acids and reuterin (Dalié et al., 2010).

If on the one hand LABs can reduce microbial growth, on the other hand they can reduce mycotoxins. In particular, two specific processes such as binding and inhibition of biosynthesis may be involved in the interaction between LABs and the accumulation of some mycotoxins. Most data about these mechanisms focused on AFs. The inhibition of mycotoxin biosynthesis could be due to molecules releases during cell lysis and also to "anti-mycotoxigenic" metabolites produced during LAB growth.

Several studies investigated the ability of LABs to bind AFs, TCs, ZEA, OTA and FBs demonstrating that these toxins can be trapped by bacterial pellet or can be bound on the surface of the microorganisms (Dalié et al., 2010). Viability of LAB strains was not essential, suggesting that binding probably took place on the cell wall involving carbohydrates and/or protein components. Peptidoglycans and polysaccharides have been suggested to be the two most important elements responsible for the binding by LABs (Niderkorn et al. 2006, 2009). The absence of detection of degradation products of these toxins may confirm that binding, no metabolism, was the mechanisms by which toxins were removed in these studies. The efficiency of LABs to reduce mycotoxins varied depending on the strains and on the compounds tested (Kabak et al., 2009; Gratz et al., 2004; El-Nezami et al., 2002a, 2002b; Del Prete et al., 2007). The capacity of these bacteria to bind mycotoxins can be applied also to reduce their absorption in the intestinal tract, increasing the excretion of mycotoxins bound to bacterial cells. Certain LABs that show considerable adhesion to intestinal cells lose this property when they bind to mycotoxins such as AFB_1. Consequently, in the gastrointestinal tract, the bacteria–mycotoxin complex is rapidly excreted (Gratz et al., 2004; Kankaanpää et al., 2000).

1.3.2.2. Isothicyanates

ITCs are degradation products from GLSs, secondary metabolites which constitute a group of more than 140 different compounds, found in all plants belonging to the *Cruciferae* family (Fahey et al., 2001). GLSs are stored within cell vacuoles and are released when the plant suffers mechanical damage. Once in the cytoplasm they come into contact with the enzyme myrosinase. GLSs are then hydrolyzed to a number of products, ITCs being the quantitatively dominant compound. It is known that GLSs degradation products possess biological activities including beneficial effect on human health, fungicidal, herbicidal, and nematocidal properties (Dufour et al., 2012). Among them, ITCs exhibit strong antimicrobial activity and they have been added to various food products (Obaidat & Frank, 2009; Deng et al., 2014).

More information about ITCs is provided in the Book Chapter (1.5.).

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1.5. Book Chapter

Antimicrobial Activity of the Glucosinolates

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Contents

Abstract

The use of natural antimicrobial compounds is receiving much attention and is becoming very frequent by the importance that nowadays is given to natural resources. Natural components have been applied in several sectors such as agriculture, biomedicine and food preservation. The development of resistance to conventional antibiotic by pathogenic bacteria makes necessary to find alternative antimicrobials to eradicate these microorganisms. Many food products are perishable and require protection from spoilage to improve quality and shelf life. Numerous efforts are conducted to find safe natural alternatives to prevent microorganism growth in plants and food products, because of the consumer concern regarding synthetic pesticides and preservatives. Natural antimicrobials can be obtained from different sources including plants, animals, bacteria, algae, and fungi. Among them, glucosinolates and their derived products have been recognized for their benefits to human nutrition, plant defense, and as potent antimicrobial agents. This chapter describes the antimicrobial activity of glucosinolates and their hydrolysis products against different bacterial and fungal species, as well as the mechanism of action of these active compounds.

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Keywords
Glucosinolates • Isothiocyanates • Antifungal activity • Antibacterial activity • Bioactive compounds

Abbreviations

AAM	Allylamine
AC	allyl cyanide
AITC	allyl isothiocyanate
ASC	Ascorbigen
ATC	ally thiocyanate
BAM	Benzylamine
BC	benzyl cyanide
BITC	benzyl isothiocyanate
CEPT	1-cyano-2,3-epithiopropane
DIM	3,3'-di-indolylmethane
EITC	ethyl isothiocyante
GLS	Glucosinolate
I3C	Indole-3-carbinol
IAN	Indole-3-acetonitrile
ITC	Isothiocyanate
MAP	modified atmosphere packaging
MCT	medium chains triglyceride
MITC	methyl isothiocyanate
PAM	2-Phenylethylamine
PEC	2-Phenylethyl cyanide
PEITC	phenylethyl isothiocyanate
PITC	phenyl isothiocyanate
SBO	soybean oil
SFN	Sulforaphane
TC	Thiocyanate

1. Introduction

In agreement with the current trend to value the natural and renewable resources, the interest in the use of natural antimicrobial compounds is increasing for biomedical, agricultural, and especially food applications [1, 2].

Food products are perishable by nature and can be subjected to contamination by bacteria and fungi. Many of these microorganisms can cause undesirable reactions that deteriorate flavor, odor, color, sensory, and textural properties of foods. Some of them can also potentially cause food-borne illness. For all these reasons, food products require protection from spoilage during their preparation, storage, and distribution to give them desired shelf life. Furthermore, the dramatic rise of antibiotic-resistant microorganisms is of concern and includes food-borne pathogens that are also more tolerant to several food processing and preservation methods. The consumer concern regarding synthetic products, such as food additives and pesticides, and the necessity to overcome the emergence of antibiotic-resistant pathogens led to the research of alternative compounds with potent antimicrobial activity which can reduce the impact of synthetic products on human and animal health [1].

Natural antimicrobials can be obtained from different sources including plants, animals, bacteria, algae, and fungi. To select the appropriate biocidal product, the microorganism strain must be identified and the spectrum of antimicrobial activity of the compound considered [2–4]. Several reports have demonstrated the efficacy of plant-derived compounds, most of all in food applications. Antimicrobials derived from plants are mostly secondary metabolites that possess various benefits including antimicrobial properties against pathogenic and spoilage microbes. The structural diversity of plant-derived compounds is immense, and the impact of antimicrobial action they produce against microorganisms depends on their structural configuration [5].

Among the potent natural antimicrobials, glucosinolates (GLS) are an important class of secondary plant products found in seeds, roots, stems, and leaves of cruciferous plants including 16 families of dicotyledonous angiosperms, mainly *Brassicaceae* [6]. There are about 120 different GLS identified, derived from amino acids (alanine, leucine, isoleucine, valine, phenylalanine, tyrosine, and tryptophan) and a number of chain-elongated homologues [7]. They are classified as aliphatic, aromatic, methylthioalkyl, and heterocyclic, which have a thioglucoside component in common structure and differ at their side chains [8].

Located within vacuoles, GLS are physically separated but accompanied by β-thioglucosidase enzymes known as myrosinases [9]. Following plant tissue disruption, the enzyme and GLS come into contact, which, in the presence of water, generates a hydrolysis forming an aglycone moiety, glucose, and sulfate.

The aglycone moiety is unstable and rearranges to form three main groups of substances: nitriles, thiocyanates (TCs), and isothiocyanates (ITCs) (Fig. 1) [3, 10, 11].

GLS and their enzymatic hydrolysis products are responsible for a characteristic pungent flavor [12, 13]. These compounds have shown several biological activities including plant defense (against insects and microbial infections) and benefits to human health (anticarcinogenic, antioxidant, and antimicrobial properties) and might be potential natural agents for food preservation [14]. Their response to microbial population varies according to their structural characteristics. The biocidal effect of cruciferous tissues on other microorganisms has been attributed mainly to volatile degradation products of GLS, released from their plants. Among derived products, ITCs are the major inhibitors of microbial activity, and they have been studied mainly for food preservation and plant pathogen control [5, 12]. ITCs are volatile substances that

display an inhibitory effect on several microorganism species at low concentrations [15].

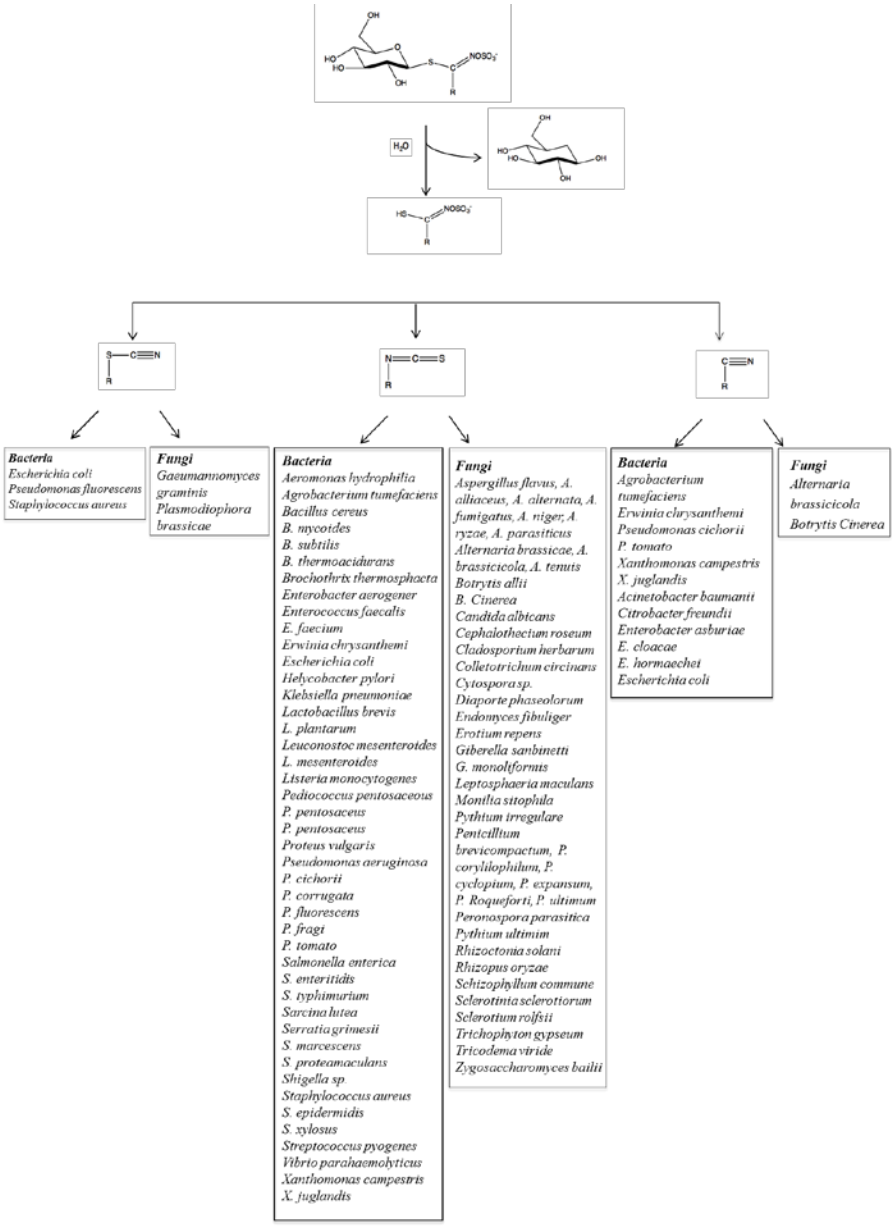

Figure 1. Enzymatic degradation of glucosinolates and the antifungal spectrum activity of their derived products.

Therefore, the objective of this chapter was to present results of studies on antimicrobial activity of GLS and their enzymatically degradation products and highlight important aspects on the application.

2. Antibacterial Activity

GLS and, above all, their hydrolysis products elicit a wide spectrum of antimicrobial activity against a variety of bacteria. The concentrations of these compounds required to inhibit microorganisms are difficult to compare given differences in methodologies, materials, and test strains employed. There are considerably more data for the ITCs, and in particular for AITC, than others GLS products. Furthermore, the mode of delivery to target microorganisms has a large impact on the antibacterial effect. Dissolution of these compounds in liquid media can result in a weak antimicrobial activity, while lower concentrations in the vapor phase are sufficient to inhibit microorganisms [10]. On Table 1 are summarized some studies which report the antibacterial potential of these compounds.

Horseradish vapors, containing GLS hydrolysis products, showed stronger antibacterial activities against several bacterial strains [16, 17]. Later, also studied and compared were the bacteriostatic and bactericidal effects of AITC, EITC, and MITC against 10 strains. MITC was the most effective, in both solution and vapor phases, followed by AITC and EITC. *Escherichia coli* and *Staphylococcus aureus* appeared to be the more resistant strains, while the least resistant were *Bacillus subtilis*, *Bacillus mycoides*, and *Serratia marcescens*.

Virtanen [18] reported the antimicrobial activity of BITC and β-phenylethyl, m-methoxybenzyl, and methoxybenzyl ITCs against *S. aureus*. The activity of these ITCs was higher than the antimicrobial activity of a series of aliphatic ITCs. Zsolnai [19] demonstrated that the same concentration of AITC and PITC, used to severely inhibit the growth of yeasts and fungi, was not effective

against *Streptococcus pyogenes*, *S. aureus*, and other Gram-negative bacteria. BITC was effective against *Staphylococci* but not on other bacteria.

Kanemaru and Miyamoto [20] studied the antibacterial activity of brown mustard and its major pungent compound, AITC, on the growth of *E. coli* 3301, *S. aureus* IFO 3761, *Proteus vulgaris* IFO 3851, *Pseudomonas fragi* IFO 3458, and *Pseudomonas aeruginosa* IFO 3755. To prepare the extract of black mustard extract was prepared as 20 % mustard in ethanol (70 %) after myrosinase treatment. AITC was also dissolved in 70 % ethanol to form an equivalent concentration. The nutrient broth in which the bacteria were cultured contained the mustard extract or AITC and was stored at 30 °C on a shaker. Turbidimetry was used to determine bacterial growth. The results obtained evidenced that the antibacterial effect of mustard was mainly due to AITC. The concentrations of mustard in the medium that inhibited bacterial growth for 24 h were 0.138 %, 0.104 %, 0.064 %, 0.043 %, and 0.089 % and those of AITC were 14.5, 12.3, 6.5, 3.6, and 7.2 ppm for *S. aureus*, *E. coli*, *P. vulgaris*, *P. fragi*, and *P. aeruginosa*, respectively. A bacteriostatic effect was shown by mustard on *S. aureus* and *E. coli* (0.8 %), while the effect was bactericidal on *P. aeruginosa* at 0.2 %.

Shofran et al. [21] tested the antimicrobial activity of sinigrin and four sinigrin hydrolysis products, in broth culture, against different species of bacteria. Sinigrin is a GLS that, upon injury or mechanical disruption of plant tissue, is hydrolyzed by myrosinase producing up to four distinct compounds: AITC, allyl TC (ATC), allyl cyanide (AC), and 1-cyano-2,3-epithiopropane (CEPT). Sinigrin had little effect upon the growth of microorganisms [22], but its hydrolysis products were effective in inhibition of growth. The species of bacteria studied in the experiment were *E. coli* 33625, *E. coli* NC101, *Pseudomonas fluorescens* MD13, *Aeromonas hydrophilia* 7966, *S. aureus* 4220, *B. subtilis* IS75, *Pediococcus pentosaceus* FFL48,

Leuconostoc mesenteroides FFL44, *Lactobacillus brevis* MD42, and *Lactobacillus plantarum* MOP3.

Table 1. Antibacterial potential of glucosinolate-derived products against several species on food products.

Glucosinolate derivative	**Bacterial strain**	**Food product**	**Reference**
Allyl isothiocyanate	*Bacillus thermoacidurans*	Fresh apple juice	[44]
Allyl isothiocyanate	*Bacillus subtilis* IFO-13722 *Bacillus cereus* IFO-13494 *Staphylococcus aureus* IFO-12732 *Staphylococcus epidermidis* IFO-12993 *Escherichia coli* JCM-1649 *Salmonella typhimurium* A TCC-14028 *Salmonella enteritidis* JCM-189 *Vibrio parahaemolyticus* IFO-12711 P. *Pseudomonas aeruginosa* IFO-13275	Fresh beef Cured pork Sliced raw tuna Cheese Egg sandwich Noodles Pasta	[45]
Allyl isothiocyanate Phenethyl isothiocyanate Allyl thiocyanate	*Staphylococcus aureus* *Escherichia coli* O157:H7 *Staphyloccus typhimurium* *Listeria monocytogenes* *Serratia grimesii* *Lactobacillus sake*	Cooked roast beef	[46]
Allyl isothiocyanate Phenethyl isothiocyanate Allyl thiocyanate 1-butane isothiocyanate	*Pseudomonas* spp. *Enterobacteriaceae* Lactic acid bacteria	Precooked roast beef slices	[47]

(continued)

Table 1. *(continued)*

Glucosinolate derivative	Bacterial strain	Food product	Reference
Methyl isothiocyanate Allyl isothiocyanate	Rifampicin-resistant strain of *Salmonella* Montevideo streptomycin-resistant strains of *Escherichia coli* O157:H7 and *Listeria monocytogenes* Scott A	Apples Tomatoes Iceberg lettuce	[48]
Allyl isothiocyanate	*Escherichia coli* O157:H7	Fresh ground beef	[49]
Allyl isothiocyanate	*Pediococcus pentosaceus* *Staphylococcus carnosus* *Escherichia coli* O157:H7	Dry fermented sausage	[50]
Allyl isothiocyanate	*Lactobacillus algidus* *Leuconostoc mesenteroides* *Leuconostoc carnosum* *Carnobacterium maltaromaticum* *Carnobacterium divergens* *Brochothrix thermosphacta* *Serratia proteamaculans*	Marinated pork	[51]
Allyl isothiocyanate	*Salmonella*	Fresh cantaloupe	[52]
Allyl isothiocyanate	*Leuconostoc mesenteroides* *Lactobacillus plantarum*	Kimchi	[53]
Allyl isothiocyanate	*Escherichia coli* *Listeria monocytogenes*	Fresh cut onions	[54]
Allyl isothiocyanate	*Listeria monocytogenes* *Salmonella typhimurium*	Chicken breast	[56]
4-hydroxybenzyl isothiocyanate	*Salmonella*	Sauce with particulates	[55]

Sinigrin, AC, and CETP at 1000 ppm did not show inhibitory effects against any of the bacteria tested. ATC was inhibitory to the growth of 3 strains of Gram-

negative (*E. coli* 33625, *E. coli* NC101, *P. fluorescens* MD13) and 1 strain of Gram-positive bacteria (*S. aureus* 4220) with minimum inhibitory concentration (MIC) values ranged between 200 and 400 ppm. The antimicrobial activity of ATC was due to its conversion to AITC, sinigrin hydrolysis products with the highest antibacterial activity. AITC was effective against all the bacteria tested, except *L. plantarum* MOP3. The MIC of AITC against Gram-negative and Gram-positive non lactic acid bacteria ranged between 100 and 200 ppm, while lactic acid bacteria were more resistant with MIC between 500 and 1000 ppm. It should be highlighted that the antimicrobial activity of AITC can be different if it is used in gaseous form or dissolved in broth culture. Furthermore, a lot of factors can influence the generation of AITC from sinigrin.

Kyung and Fleming [23] tested sinigrin and its derivate products against 15 species of bacteria: *Pediococcus pentosaceus* LA3, *P. pentosaceus* LA76, *L. mesenteroides* LA10, *L. mesenteroides* LA113, *L. plantarum* LA97, *L. plantarum* LA70, *L. brevis* LA25, *L. brevis* LA200, *Listeria monocytogenes* B67, *L. monocytogenes* B70, *S. aureus* B31, *E. coli* B34, *Enterobacter aerogenes* B146, *B. subtilis* B96, and *Salmonella typhimurium* B38. Sinigrin itself was not antimicrobial because it did not inhibit growth up to 1000 ppm and microorganisms did not degrade it to its antimicrobial aglycones. AITC is known to be antimicrobial, and the MICs found ranged from 50 to 500 ppm for bacteria, including Grampositive, Gram-negative, pathogenic, and lactic acid bacteria.

Delaquis and Sholberg [24] evaluate the microbistatic and microbicidal properties of gaseous AITC against bacterial cells of *S. Typhimurium* (ATCC 14028), *L. monocytogenes* (strain 81–861), *E. coli* O157:H7 (ATCC 43895), and *Pseudomonas corrugata* (isolated from lettuce). *S. typhimurium*, *L. monocytogenes*, and *E. coli* O157:H7 were inhibited when exposed to 1000 μg L^{-1} AITC. *P. corrugate* failed to grow in the presence of 500 μg L^{-1}. Variations at

different incubation temperatures were observed. Bactericidal activities varied with strain and increased with time of exposure. The most resistant bacterium was *E. coli*.

The antibacterial properties of the GLS and their hydrolysis products became of big interest and importance also in the eradication of pathogenic microorganisms that is complicated by the development of resistance to conventional antimicrobial agents. *Helicobacter pylori* is one of the most prevalent human pathogens in the world. Gastric infections with *H. pylori* are known to cause gastritis and peptic ulcers and dramatically enhance the risk of gastric cancer. Antibiotic therapy is recommended for infected patients with gastric or duodenal ulcers or gastric mucosa-associated lymphoid tissue lymphoma, but this treatment is not universally successful. Even with the combination of two or more antibiotics, *H. pylori* is difficult to eradicate due to the development of resistance of this bacteria to these antibiotics and the persistence of organisms within gastric epithelial cells and, furthermore, due to logistic, sociologic, and economic reasons. The ITC sulforaphane (SFN) appears to overcome all of these problems. SFN is abundant in certain varieties of broccoli and broccoli sprouts in the form of its GLS precursor called glucoraphanin. It has been demonstrated that SFN is a potent bacteriostatic agent against 3 reference strains and 45 clinical isolates of *H. pylori*. The MIC for 90 % of the strains is <4 μg mL^{-1}, irrespective of their resistance to conventional antibiotics. It is a potent bactericidal agent against both extra- and intracellular *H. pylori* in vitro. Further, brief exposure to SFN eliminated intracellular *H. pylori* from a human epithelial cell line (HEp-2). Although higher concentrations are required to achieve bactericidal activity for the intracellular forms, SFN accumulates intracellularly to high levels, as its glutathione conjugate. It can be safely administered to humans

because it is present in high concentrations in edible cruciferous vegetables and can be directly delivered to the stomach [25].

Haristoy et al. [26] evaluated the effect of SFN in vivo against *H. pylori* by using human gastric xenografts in nude mice. *H. pylori* was completely eradicated in 8 of the 11 SFN-treated grafts, after short-term administration of SFN at a dose that can be achieved in the human diet.

Haristoy et al. [27] analyzed the activities of 12 ITCs including sulforaphane on 25 strains of *H. pylori* using an agar dilution assay. The ITCs tested were iberin, cheirolin, erucin, D,L-SFN, D-SFN, L-SFN, L-sulforaphane, erysolin, berteroin, alyssin, hirsutin, PEITC, BITC, and 4-(α-L-rhamnopyranosyloxy)benzyl ITC. Furthermore, the bactericidal activities of the six ITCs (cheirolin, L-sulforaphane, erysolin, berteroin, hirsutin, and 4-(α-L-rhamnopyranosyloxy)benzyl ITC) that showed the lowest MICs were determined both directly and against intracellular bacteria in cultured human epithelial cells. The MIC90 values for these ITCs ranged between 4 and 32 μg mL^{-1}. It has been demonstrated that, in addition to SFN, four (cheirolin, berteroin, hirsutin, and 4-(α-L-rhamnopyranosyloxy)benzyl ITC) of the most active compounds exhibited high bactericidal activity against both extra- and intracellular bacteria.

Ono et al. [28] screened, isolated, and identified antibacterial compounds occurring in some common foods for bactericidal use, against *E. coli* and *S. aureus*. Among the different foodstuffs studied, wasabi stems, banana fruits, coriander leaves, and mustard seeds showed antibacterial activity. In particular, the lower minimal bactericidal concentration was obtained for wasabi stems, so their activity was highest. The compound with the antibacterial activity was identified as the 6-methyl-sulfinylhexyl ITC. The ethyl, butyl, hexyl, and octyl homologues of this ITC were determined in some *Cruciferae* plants. The main component contained in wasabi was the hexyl homologue, whereas horseradish contained

the ethyl and hexyl homologues. Broccoli, Chinese cabbage, radish, and turnip almost exclusively contained the butyl homologue, and cabbage contained only the hexyl homologue. These homologues were also active against *E. coli* and *S. aureus* with minimal bactericidal concentration ranged between 0.1 and 2.0 mg mL^{-1}.

Liu and Yang [29] studied the stability and the antimicrobial activity of AITC in two medium-chain triglyceride (MCT) and soybean oil (SBO), dispersed in an oilin-water system during long-term storage. It has been shown that the stability and antimicrobial activity were affected by the content, type, and oxidative stability of the oil. In particular, high oil content is favorable for AITC stability in the emulsion. AITC with MCT were more effective than AITC with SBO in inhibiting Gram-negative bacteria *E. coli* O157:H7, *Salmonella enterica*, and *Vibrio parahaemolyticus* and Gram-positive bacteria *S. aureus* and *L. monocytogenes*.

Luciano and Holley [30] evaluated the antibacterial activity of AITC against *E. coli* O157:H7 at different pH values and examined the inhibitory action of this compound against two enzymes important in the metabolism of this food-borne pathogen (thioredoxin reductase and acetate kinase). AITC showed greater antimicrobial activity at low pH values (4.5 and 5.5). Decomposition products of this ITC were also studied, and they did not show antibacterial activity toward *E. coli* O157:H7. Only AITC is antimicrobial in its original form. Furthermore, it has been demonstrated that only 1 µL L^{-1} of AITC could decrease the activity of thioredoxin reductase and AITC at 10–100 µL L^{-1} was able to significantly inhibit both thioredoxin reductase and acetate kinase.

The antimicrobial properties of different GLS autolysis products of *Hornungia petraea* were investigated against two isolates of *S. aureus*, *Salmonella enteritidis*, *Klebsiella pneumoniae*, *Sarcina lutea*, *E. coli*, *Shigella* sp., and *Bacillus*

cereus. The tested compounds were showed to be active against all tested microorganisms, with the activity ranging from 1 to 1250 mg mL^{-1} for inhibitory and 1 to 5000 mg mL^{-1} for microbicidal activity. In particular, the assays showed a very high antibacterial activity of the tested ITCs against *S. lutea* [31].

Olaimat and Holley [32] determined the minimum inhibitory and minimum bactericidal concentrations of AITC from mustard against five strains each of *Salmonella* and *L. monocytogenes* individually and combined. The MIC and MBC values of AITC ranged from 60 to 100 ppm and 120 to 180 ppm, respectively, at 37 °C and ranged from 10 to 40 ppm and 200 to 600 ppm, respectively, at 21 °C against both pathogens. AITC had no antimicrobial activity at low temperatures (4 °C or 10 °C) and alkaline pH over 10, but at neutral pH, *L. monocytogenes* is reduced. At acidic pH, AITC was more effective against *Salmonella*. However, AITC was more effective at combinations of 21 °C and neutral pH against *L. monocytogenes* and at combinations of higher temperature and acidic pH against *Salmonella*.

A lot of data are available about the antimicrobial activity of ITCs, but the results are difficult to compare. Accordingly, Wilson et al. [33] studied the antibacterial activity of a large number of ITCs on a wide range of microorganisms, using for all the same experimental conditions. Ten ITCs were tested, and, among them, six were investigated for the first time: SFN, iberin, AITC, BITC, MITC, PITC, PEITC-, propyl-, 3-methylthiophenyl-, and 3-methylthiopropyl-ITC. The bacteria tested were fourteen and included 8 Gram-positive species (*B. cereus* CIP 78.3, *B. subtilis* ATCC 6633, *Enterococcus faecalis* G9h, *Enterococcus faecium* ATCC 19434, *L. plantarum* DSM 9843 [299v], *L. monocytogenes* LC 10, *S. aureus* ATCC 6538, and *Staphylococcus xylosus* LC 57) and 6 Gram-negative species (*K. pneumoniae* DSM 681, *E. coli* ATCC 25922, *P. aeruginosa* DSM 1128, *S. enteritidis* LC 216, *S. typhimurium* LC 443, and *S. marcescens* LC 448). A turbidimeter was used to

monitor the growth of bacteria, and the antimicrobial activity was expressed as antimicrobial efficacy index that is a function of the growth delay, the reduction in the maximum population, and the reduction in maximum specific growth rate. All the ITCs tested displayed antimicrobial activity, depending on the target bacteria and the structural features of the molecule considered. BITC showed the highest value of antimicrobial efficacy index, followed by PEITC. Different from other studies, AITC was the least active ITC, and not necessarily aromatic ITCs were more active than aliphatic compound. For example, 3-methylthiopropyl-ITC was much more active than PITC. Gram-negative bacteria were overall more sensitive to ITCs than Gram-positive bacteria, and considerable variations in sensitivity were evidenced between species even within the same Gram type.

AITC, BITC, and PEITC purified from cruciferous plants were evaluated against 15 isolates of methicillin-resistant *S. aureus* (MRSA) isolated from diabetic foot ulcer patients. In general, the AITC always presented the higher MIC values and thus lower antimicrobial activity, while BITC and PEITC presented the lowest MIC. Therefore, these ITCs showed the highest antimicrobial activity. The AITC and PEITC were essentially bacteriostatic, whereas BITC was bactericidal in 11 isolates of MRSA. Based on this, BITC is more effective in suppressing MRSA strains than PEITC. The antibacterial effectiveness of these compounds depends on the dose tested and on the chemical structure [34].

GLS and their derivate products are useful also in inhibiting the growth of pathogenic bacteria that can contaminate vegetable seeds. This contamination can occur at any point, from the field to the sprouting process and during subsequent handling of sprouts until they are consumed. Populations of *E. coli* O157:H7 have been reported to reach 10^6–10^7 cfu g^{-1} of sprouts produced from contaminated seeds. *E. coli* O157:H7 causes life-threatening hemorrhagic colitis, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura in the

young, old, and immunocompromised. The efficacy of AITC in killing *E. coli* O157:H7 on dry and wet alfalfa seeds was investigated. AITC was lethal to *E. coli* inoculated onto agar disks, but, unfortunately, the enhanced effectiveness of AITC in killing the pathogen onto alfalfa seeds is offset by a dramatic reduction in seed viability. Nevertheless, the use of AITC for the purpose of killing *E. coli* O157:H7 in other fields and, perhaps, other pathogens on alfalfa seed holds promising [35].

GLS hydrolysis products also displayed antimicrobial activity against plant pathogenic microorganisms, and this feature reinforces the potential for using them as alternatives to the traditional chemical control of phytopathogenic bacteria. Aires et al. [36] evaluated the antibacterial effects of GLS hydrolysis products against six relevant plant pathogenic Gram-negative bacteria, using a disc diffusion assay: *Agrobacterium tumefaciens*, *Erwinia chrysanthemi*, *Pseudomonas cichorii*, *Pseudomonas tomato*, *Xanthomonas campestris*, and *Xanthomonas juglandis*. The GLS hydrolysis products used in the in vitro assay were AITC, AC, SFN, BITC, benzyl cyanide (BC), PEITC, 2-phenylethyl cyanide (PEC), indole-3-acetonitrile (IAN), indole-3-carbinol (I3C), and ascorbigen (ASC). A mix of AITC, BITC, and PEITC also was tested. The strongest inhibitory effect was showed by PEITC and SFN. Among the different GLS hydrolysis products studied, the ITCs were more efficient than the other products, and the antimicrobial effects were dose-dependent.

A transgenic *Arabidopsis thaliana* that overexpressed p-hydroxybenzyl GLS was used to evaluate the capacity of GLS and their breakdown products to influence and modify the natural rhizosphere community. It was showed that the proteobacteria and also the fungal community in the rhizosphere of the transgenic plant were significantly affected. Modification of the GLS content of the plant could be an alternative to the use of pesticides [37].

Aires et al. [38] evaluated the antimicrobial activity of intact GLS and their hydrolysis products and microbial catabolites, against human pathogenic or gastrointestinal tract bacteria: the Gram-positive *E. faecalis*, *S. aureus*, and *Staphylococcus saprophyticus* and the Gram-negative *Acinetobacter baumannii*, *Citrobacter freundii*, *Enterobacter asburiae*, *Enterobacter cloacae*, *Enterobacter hormaechei*, *E. coli* (two strains), *Hafnia alvei*, *Klebsiella oxytoca*, *K. pneumoniae*, *Morganella morganii*, *Proteus mirabilis*, *P. aeruginosa*, *S. typhi*, and *Stenotrophomonas maltophilia*. The intact GLS examined were sinigrin, glucoraphanin, glucotropaeolin, gluconasturtiin, and indole glucobrassicin, while the enzymatic hydrolysis products were AITC, SFN, BITC, PEITC, I3C, AC, BC, PEC, and 3,3'-di-indolylmethane (DIM). Allylamine (AAM), benzylamine (BAM), and 2-phenylethylamine (PAM), which are microbial metabolites of GLS, were also tested. Among the compounds tested, only ITCs were effective, but GLS, nitriles, and amines were ineffective at all the doses used. The highest activity was shown by SFN and BITC. IAN had some inhibitory activity against the Gram-negative bacteria. I3C had some inhibitory effects against the Gram-positive bacteria but had no effect, even at the highest dose, against the Gram-negative bacteria. The compound, the concentration used, and the microorganism tested influence the antimicrobial activity of the GLS hydrolysis products. Some of these were more effective than conventional antibiotics in inhibiting the growth of pathogenic microorganisms, such as ITCs. The data reported in this study demonstrate the potential for using these natural antimicrobials as an alternative or in combination with antibiotic-based therapies for treating infectious diseases.

Some ITCs display a synergy with conventional antibiotics. Tajima et al. [39] examined different hydroxy ITCs for antimicrobial synergism with various antibiotics against *E. coli* and *S. aureus*. It was demonstrated that 2-(4-hydroxyphenyl) ethyl ITC displayed antimicrobial synergism with aminoglycosides,

such as streptomycin, against *E. coli* and *S. aureus* grown in glucose-containing medium. However, small changes in the concentrations of both ITC and streptomycin affect their combined action from synergism to suppression of antimicrobial activity. The mechanism of synergism and suppression remains unclear [40].

Palaniappan et al. [41] examined the synergistic interaction between natural antimicrobials and antibiotics to which the target bacteria were resistant. Among the agents studied, AITC was effective in reducing the MIC of erythromycin when tested against *S. pyogenes*.

The antibacterial effect in vitro of PEITC and its synergistic effect with antibiotics against different *E. coli* from human and animal were demonstrated by Freitas et al. [42].

Many of the older references about the antimicrobial properties of ITCs were often related to the use of these compounds as preservatives in foods. Tressler and Joslyn [43] suggested that the Romans added large quantities of mustard seed to crushed grape for preservative purposes. The use of mustard oils to fruit juices and wines has apparently been practiced for generations in some parts of the world. Kosker et al. [44] showed the possibility of using AITC as preservative in fresh apple cider at a concentration of 20 ppm. Furthermore, it was shown that the thermal resistance of *Bacillus thermoacidurans* can be greatly reduced using AITC 10 ppm in buffer and fruit juices. Kostova et al. [9] studied the use of AITC in the disinfection of eggs. It was reported that AITC could control the growth of microorganisms on the surface of goose and hen eggs by application of the solution or as vapor. This method was not pursued because the AITC was absorbed through the shell.

The major pungent component of black mustard (*Brassica nigra*) and brown mustard (*B. juncea*), which is the same as that of wasabi (*Eutrema wasabi*

Maxim.), is AITC. The antimicrobial activity of brown mustard AITC vapor and the possibility of its use as modified atmosphere packing were studied by Isshiki et al. [45]. The bacteria used were *B. subtilis* IFO-13722, *B. cereus* IFO-13494, *S. aureus* IFO-12732, *Staphylococcus epidermidis* IFO-12993, *E. coli* JCM-1649, *S. typhimurium* A TCC-14028, *S. enteritidis* JCM-1891, *V. parahaemolyticus* IFO-12711, and *P. aeruginosa* IFO-13275. First, the antibacterial activity of AITC vapor, against each microorganism, was evaluated in Petri dishes, and then application experiments were carried out with different foods. AITC vapor inhibited the growth of all microorganisms examined in the experiments. In the application experiments, none of the tested samples were spoiled after 7 days, while the controls grew sufficiently after 2 days [45].

Ward et al. [46] evaluated the effectiveness of different concentrations of a volatile distillate extracted from fresh horseradish root against the growth of spoilage and pathogenic bacteria inoculated on agar and roast beef slices at 12 °C. The distillate was composed by about 90 % AITC and 9 % 2-phenethyl ITC, and the bacteria tested were *S. aureus*, *E. coli* O157:H7, *S. typhimurium*, *L. monocytogenes*, *Serratia grimesii*, and *Lactobacillus sake*. *L. sake* was the most resistant: 20000 nL distillate L^{-1} air were required to completely inhibit growth on agar. On the other side 4000 nL distillate L^{-1} air completely inhibited the growth of *S. aureus*, *E. coli* O157:H7, *S. typhimurium*, *L. monocytogenes*, and *S. grimesii* on agar for 7 days in aerobic storage at 12 °C. These bacteria were more resistant when inoculated on roast beef: 20,000 nL distillate L^{-1} were required to completely inhibit the growth, and *L. sake* was weakly inhibited at this concentration.

Delaquis et al. [47] determined the effect of vaporized horseradish essential oil (HEO) on microbial growth in precooked roast beef slices contaminated with *Pseudomonas* spp. and *Enterobacteriaceae* and lactic acid

bacteria. The slices were stored 28 days at 4 ± 2 °C in air or 100 % N_2 with and without HEO. The results showed that 20 µL L^{-1} of HEO inhibited the growth of most spoilage bacteria and *Pseudomonas* spp. And *Enterobacteriaceae* were strongly inhibited than lactic acid bacteria that were more resistant. The chemical changes and sensory properties of precooked roast beef treated with HEO were also evaluated and revealed that the development of off-flavors and odors derived from fat oxidation products was delayed by HEO.

The bactericidal activity of AITC and MITC was tested on iceberg lettuce inoculated with a rifampicin-resistant strain of *Salmonella Montevideo* and streptomycin-resistant strains of *E. coli* O157:H7 and *L. monocytogenes* Scott A in sealed containers at 4 °C for 4 days. MITC was more active against *L. monocytogenes* than the other bacteria, while AITC showed stronger activity against *E. coli* O157:H7 and *S. Montevideo*. Furthermore in this study, the AITC was tested also on tomato stem scars and skin contaminated with *S. Montevideo* and on apple stem scars contaminated with *E. coli* O157:H7. *S. Montevideo* inoculated on tomato skin was more sensitive to AITC than that on stem scars. Treatment with vapor generated from 500 mL of AITC caused an 8-log reduction in bacteria on tomato skin but only a 5-log reduction on tomato stem scars. The bactericidal activity of AITC was weaker for *E. coli* O157:H7 on apple stem scars; only a 3-log reduction in bacteria occurred when 600 mL of AITC was used [48].

The incorporation of mustard flour (non-deheated) as an ingredient in packaged ground beef to inactivate *E. coli* O157:H7 was tested by Nadarajah et al. [49]. The results showed that it is possible to use mustard flour at levels of between 5 and 10 % to eliminate *E. coli* O157:H7 from fresh ground beef. The sensory evaluation of cooked ground beef was carried out and showed that there were no significant differences between the overall sensory acceptability of ground beef formulated with 5 % and 10 % mustard [49].

Four sausage batters (17.59 % beef, 60.67 % pork, and 17.59 % pork fat) were inoculated with *P. pentosaceus* and *Staphylococcus carnosus* and a five-strain cocktail of nonpathogenic variants of *E. coli* O157:H7. Microencapsulated AITC was added to three batters at 500, 750, or 1000 ppm to determine its antimicrobial effects. *E. coli* O157:H7 was reduced by 6.5 log10 CFU g^{-1} in sausages containing 750 and 1000 ppm AITC after 21 and 16 days of processing, respectively. *E. coli* O157:H7 numbers were reduced by 4.75 log10 CFU g^{-1} after 28 days of processing in treatments with 500 ppm AITC, and the organism was not recovered from this treatment beyond 40 days [50].

The antimicrobial activity of AITC against growth of typical spoilage bacteria (*Lactobacillus algidus*, *L. mesenteroides*, *Leuconostoc carnosum*, *Carnobacterium maltaromaticum*, *Carnobacterium divergens*, *Brochothrix thermosphacta*, *Serratia proteamaculans*) from marinated pork was also investigated in vacuum-packed pork meat. MICs for AITC were difficult to determine because of the absence of gastight barrier between the wells of a single plate used in the experiment. As AITC exerts antimicrobial activity in both liquid and gas phases, the addition of AITC to one well affected bacterial growth in adjacent wells. In fact, the addition of AITC completely inhibited the growth of *S. proteamaculans* and *B. thermosphacta* even in control wells containing no AITC. To determine the MIC for AITC in liquid phase, experiments with sealed wells would have to be carried out. The ability of AITC to exert antimicrobial effects in its gas phase even at low concentrations may make it more useful for applications in modified atmosphere-packaged foods [51].

AITC was also incorporated into chitosan coatings to develop an antimicrobial application against *Salmonella* that would improve the safety and extend shelf life of whole fresh cantaloupe. It has been demonstrated that with AITC concentrations increasing from 10 to 60 µL mL^{-1}, the antibacterial effects of

coating treatments against *Salmonella* increased, and no visual changes in overall appearance and color of cantaloupe rind and flesh due to coating treatments were observed [52].

AITC was encapsulated using gum Arabic and chitosan to overcome the problem of its high volatility to investigate the effect of microencapsulated AITC as a natural additive on the shelf life and quality of Kimchi, a traditional Korean fermented vegetable food. Encapsulated AITC addition to Kimchi resulted in positive changes in pH, titratable acidity, and microbial analysis compared to that of control. The number of *Leuconostoc* and *Lactobacillus* species in Kimchi decreased with an increase in the concentration of AITC. However, with regard to sensory analysis, AITC concentrations of 0.10 % or lower are recommended for manufacturing Kimchi [53].

The antimicrobial effect of AITC entrapped in alpha and beta cyclodextrin inclusion complexes (IC) against different target organisms, among them *Escherichia coli* and *Listeria monocytogenes*, was determined. AITC entrapped in beta IC exhibited a significantly better antimicrobial effect compared to unentrapped AITC. The antimicrobial effect of beta IC was determined during aerobic storage of packaged fresh-cut onions. This application of beta IC (200 mL L^{-1}) to packaged fresh-cut onions effectively decreased numbers of *L. monocytogenes* [54]. ITCs are used in food active packaging to reduce, inhibit, or retard the growth of microorganisms on food products. White mustard essential oil (WMEO) showed antimicrobial activity against *Salmonella* recovered from inoculated frozen vegetables and chicken particulates. The antibacterial property was due to the production of 4-hydroxybenzyl ITC obtained by the hydrolysis of the GLS sinalbin, present in white mustard essential oil derived from white mustard seeds (*Sinapis alba L.*) [55].

AITC in combination with modified atmosphere packaging (MAP) was tested to control the growth of L. monocytogenes and *S. typhimurium* on fresh chicken breasts during refrigerated storage for 21 days. On day 21, the microbial counts in the products packaged with AITC and MAP were lower than ambient air and MAP, even if AITC was less effective against *L. monocytogenes* than *S. typhimurium*. Furthermore, vapor AITC has been found to be more effective than liquid AITC [56], but its strong odor can limit its use in food systems. The use of AITC as a flavoring substance has been evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and by the EFSA (European Food Safety Authority) Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (AFC). This report concluded that there were no safety concerns from AITC consumption at the estimated levels of intake [57].

3. Antifungal Activity

One of the first studies that demonstrated antifungal activity of cruciferous plant was carried out in the 1930s, when these authors demonstrated in vitro toxicity of volatile compounds (AITC, PITC, MITC, EITC, ethyl TC, allyl sulfide, ethyl sulfide, and sinigrin) toward certain fungi (*Colletotrichum circinans*, *Botrytis allii*, *Aspergillus niger*, *A. alliaceus*, and *Gibberella saubinetti*) [58]. The antifungal property was corroborated by Hooker et al. [59], and after this, many others investigations were followed. In general, volatile sulfur compounds demonstrate more potent inhibitory effects toward fungi than bacteria [60].

Studies have shown that GLS did not present antimicrobial activity in their intact form, only after enzymatic hydrolysis. Therefore, sinigrin which is one of the most important GLS present in oriental mustard presented no effect against *Paecilomyces fumosoroseus* [61]. Sinigrin also did not affect *Alternaria brassicae* (causative agent of black spot) in Czapek-Dox agar medium, as well as sclerotium

formation of *Sclerotinia sclerotiorum* (causative agent of stem rot) [62]. Native GLS had no fungitoxic activity, whereas their hydrolytic products, in particular glucoiberin, glucoerucin, glucoheirolin, and glucotropaeolin, inhibited growth of *Rhizoctonia solani*, *S. sclerotiorum*, *Diaporthe phaseolorum*, and *Pythium irregulare* with different inhibitory responses depending upon the chemical nature of the hydrolytic products [3, 63].

The composition of hydrolysis products from GLS varies according to substrate, pH conditions, presence of ferrous ions, and specific protein factors. The chemical nature of the breakdown products depends mainly on the structure of the GLS, plant species, and reaction conditions [64]. They are classified as nitriles, TCs, epithionitriles, oxazolidine-2-thiones, ITCs, and epithioalkanes with different antimicrobial activity [65].

From these groups, ITC is the major inhibitor of microbial populations, and differences in the potential are related to the nature of their side chain [66]. Assays with ITCs have been conducted directly as a component of a growth medium and a model food system and (generally more antimicrobial effective) in the gaseous form [49]. Volatiles released from GLS, predominantly 2-propenyl GLS, showed toxic effects to the blackleg fungus, *Leptosphaeria maculans*, in vitro [67]. AITC gaseous at 0.1 mg L^{-1} for 4 h showed a fungistatic effect against *Botrytis cinerea* (gray mold) reducing by over 45 % the incidence of the gray mold on strawberries [68]. AITC (2 ppm) inhibited the growth of *Penicillium roqueforti*, *P. corylophilum*, *Eurotium repens*, *A. flavus*, and *Endomyces fibuliger* on rye bread slices in airtight environment [69].

Sellam et al. [70] demonstrate that ITCs were effective in vitro in different development stages of *A. brassicicola* and *A. brassicae*. Moreover, antifungal activity of 57 substituted derivatives of PEITC was determined on *A. niger*, *Penicillium cyclopium*, *Rhizopus oryzae*, *A. flavus*, *A. oryzae*, *A. fumigatus*, *P.*

brevicompactum, *Cladosporium herbarum*, *Trichoderma viride*, *Alternaria tenuis*, *Monilia sitophila*, *Cytospora* sp., *Schizophyllum commune*, *Fusarium* sp., *Cephalothecium roseum*, and *Trichophyton gypseum* in culture medium. The authors describe that several PEITC derivatives, as well as the most active natural ITC analogues, represent remarkable antifungal compounds; however, there are some differences in their antifungal potential [13].

Several studies have been conducted using glucosinolate-derived products against molds and yeast (Table 2). These examples show the efficiency of ITCs against saprophytic and parasitic fungal species, usually applied at low levels in culture medium, food products, and plant defense. In agricultural sciences, ITCs such as AITC have been effective fumigants on the control of insects and fungal species [71]. Among ITCs, allyl isothiocyanate (AITC) is one of the most studied.

Beneficial biological effects have been reported including antibacterial, antifungal, anti-nematode, and anti-insect activities [72]. Its uses as natural preservative have been growing because of its food origin and low toxicity [30]. The antimicrobial activity of AITC, as well as other ITCs, is related with the concentration of the compound applied, time of exposure, strains, microbial loading, temperature, food composition, pH conditions, water activity, and on diffusion of the vapor in food packaging systems [48, 71, 73]. However, its use on food products usually is limited by the interference of organoleptic characteristics, its poor aqueous solubility, instability at high temperature, and intrinsic food compounds.

GLS-derived products have also been presenting antibiocidal potential against yeast. Kyung and Fleming [23] reported that AITC showed antifungal effects against fermentative yeasts on culture media with an MIC $\leq$ 4 ppm. *Candida albicans*, a fungus potentially pathogenic to human, was inhibited by fresh cauliflower juice (*Brassica oleracea* var. *botrytis*) [74].

Table 2. Antifungal potential of glucosinolate-derived products against several species on food products.

Glucosinolate derivative	**Fungal strain**	**Food product**	**Reference**
4-Hydroxybenzyl Isothiocyanate	*Zygosaccharomyces bailii*	Acidified fruit drink	[75]
Yellow and oriental mustard (based on allyl isothiocyanate and p-hydroxybenzyl isothiocyanate)	*Aspergillus parasiticus* CECT 2681	Peanut, cashew, almonds, walnut, pistachio, hazelnut	[81]
Allyl isothiocyanate	*Penicillium roqueforti, P. corylophilum, Eurotium repens, A. flavus, Endomyces fibuliger*	Rye bread slices	[69]
Allyl benzyl phenyl Isothiocyanates	*Gibberella moniliformis strains* 2983, 5847, 5850	Bread	[78]
Allyl isothiocyanate	*Botrytis cinérea*	Strawberries	[68]
Allyl isothiocyanate	*Aspergillus parasiticus*	Fresh pizza crust	[77]
Allyl isothiocyanate	*Aspergillus parasiticus, Fusarium poae*	Wheat flour	[80]
Benzyl isothiocyanate	*Alternaria alternata*	Tomato	[79]
Ethyl isothiocyanate	*Penicillium expansum*	Apple	[71]

In the use of BITC and 3- and 4-methoxybenzyl ITCs, antifungal effects against *Aspergillus fumigatus* and *C. albicans* were revealed with MIC of 1 μg mL^{-1} (Radulovic et al., 2012). An essential oil obtained from white mustard seeds containing 25 mg L^{-1} of 4-hydroxybenzyl isothiocyanate (4HBITC)/L was able to stabilize an acidified fruit drink against acid-tolerant bacteria (*Gluconobacter* species) and preservativeresistant yeast (*Zygosaccharomyces bailii*) for 28 days at ambient temperature [75].

Thus, the ability of ITCs to reduce mycotoxigenic molds and mycotoxins was also investigated. *P. expansum* (patulin producer) was inhibited with > 50 mg of AITC, whereas *A. parasiticus* (aflatoxin producer) in culture medium was sensible to doses > 5 [76]. *Aspergillus parasiticus* was inactivated in fresh pizza crust after 30 days of AITC exposition and suppressed aflatoxin formation [77]. AITC, BITC, and PITC inhibited the growth of *Gibberella moniliformis* strains 2983, 5847, and 5850 and reduced 2.1–89.7 % of the mycelium size. ITCs also reacted with FB_2 in bread reducing the levels by 73–100 % [78]. Benzyl-ITC showed antifungal activity against *Alternaria alternata* on tomato [79] and ethyl-ITC against *P. expansum* on apple [71], both patulin producers. AITC gaseous at 0.1 μL L^{-1} was investigated to reduce aflatoxin produced by *A. parasiticus* and beauvericin and enniatins produced by *Fusarium*. The authors observed reduction of 6.9 % to 23 % mycotoxin levels while at 10 μL L^{-1}; AITC completely inhibited the production of mycotoxins for 30 days [80]. In a commercial packaging simulation, GLS present in yellow and oriental mustard flours reduced aflatoxin B_1, B_2, G_1, and G_2 in nuts (peanut, cashew, almonds, walnut, pistachio, and hazelnut). This reduction ranged from 83.1 to 87.2 % in the oriental mustard flour, whereas it was 27.0–32.5 % in the yellow flour [81]. AITC reacted with beauvericin in solution, reducing from 20 % to 100 %, and in a food system, beauvericin was reduced from 10 % to 65 %, in a dose-dependent manner [82]. AITC, BITC, and

PITC diminished fumonisin B_1 (FB_1) and B_2 (FB_2) levels in solution from 42 % to 100 %, and on fumigation treatment (50, 100, and 500 μL L^{-1}), ITCs were able to reduce 53–96 % of FB_1 and 29–91 % of FB_2 contained in corn products, with four reaction products identified through the reaction [83].

3.1. Plant Protection

GLS-derived products have been recognized as antimicrobial agents, and several studies demonstrated the ability to control soil-borne plant pathogens [84–87]. The GLS content in plant reaches about 1 % (highly variable) of dry weight in some tissues of *Brassica vegetables* [88]. Plant species and age are the major determinants of GLS composition [89], but also other factors such as nutritional status of the plant, fungal infection, and insect damage have significant effect on the content in growing plants [64].

Qualitative and quantitative differences of GLS composition vary also among plant organs [89]. GLS are found mainly in seeds, siliques, and young leaves, while intermediate contents are detected in leaves, stems, and roots [90]. Indole GLS and their hydrolysis products found in large amounts in roots may be related to their higher stability in the soil than air [91]. These compounds play a role in the development of root disease, caused by *Plasmodiophora brassicae* [92]. Volatile compounds from macerated *Brassicae* root tissue inhibited the fungal pathogen of wheat, *Gaeumannomyces graminis* [87]. Nevertheless, roots of a transgenic *Arabidopsis thaliana* had altered the profile of GLS compared with non-transgenic, with influence in the microbial community on roots and active populations in the rhizosphere [37]. The rhizospheric strains of *Fusarium* showed a protective effect on *Lepidium sativum* against *Pythium ultimum*. Accumulation of ITCs in roots not only increases resistance of the plant but also gives a competitive advantage to *Fusarium* strains [93].

Degradation products of GLS showed an inhibition of *L. maculans* at concentrations greater than 40 μg mL^{-1} [94]. Cauliflower plants (*Brassica oleracea* var. *botrytis*) infected by *Peronospora parasitica* resistant to downy mildew presented higher sinigrin content than the susceptible variety. The susceptible seedlings exhibited a 12 % decrease in glucobrassicin and a 25 % increase in methoxyglucobrassicin when compared with healthy ones six days after treatment whereas no difference in glucobrassicin and a 10 % increase in methoxyglucobrassicin were observed in healthy and inoculated resistant seedlings [95].

The disease resistance may be dependent on fungal pathogen species and the composition of GLS-derived products present in the plant [91]. *Arabidopsis thaliana* mutant extracts were investigated on *B. cinerea* and *Alternaria brassicicola* isolates. *A. brassicicola* was more affected by aliphatic GLS and ITCs, while *B. cinérea* isolates showed variable composition-dependent sensitivity to GLS and their hydrolysis products [96]. Propenyl ITC and EITC demonstrated fungistatic potential at 0.3 μL, which inhibited mycelial growth and completely suppressed conidial and chlamydospore germination of four *Fusarium oxysporum* isolates. EITC, BITC, and PEITC were fungitoxic to *F. oxysporum* conidia and chlamydospores [97]. ITCs released from cabbage tissues were effective toward *P. parasitica*, *P. ultimum*, and *Sclerotium rolfsii* [98]. PEITC inhibited the growth of a range of fungi, oomycetes, and bacteria [99]. Pedras and Sorensen [100] observed that 5-(methylsulfamyl)-pentyl-1-ITC, 6-(methylsulfamyl)-hexyl-1-ITC, and 6-(methylsulfinyl)-hexyl-1-ITC inhibited spore germination of *Phoma lingam* virulent isolate BJ 125 at a concentration of 5 X 10^{-4} M. *Alternaria* infection was positively correlated with GLS content in 33 oilseed rape lines (*Brassica napus L.* ssp. *oleifera*) [101].

4. Structure Activity Relationships

The mechanism of ITC antimicrobial action is unclear, but some hypotheses have been proposed. The central electrophilic carbon of ITCs (R-N = C = S) undergoes rapid reaction with hydroxyls, amines, and thiols, generating products such as carbamates, thiourea, and thiocarbamates, respectively [102, 103]. Thereby, AITC reacted with glutathione, amino acids, proteins, water, alcohol, and sulfites [104, 105], and it was able to disintegrate the cysteine disulfide bond through an oxidative process [104, 106].

Zsolnai [19] reported that thioglycolate and cysteine could diminish the antibacterial action of ITCs. The study also describes that the antimicrobial action of ITCs may be linked to the inhibition of sulfhydryl enzymes. This finding is consistent with those observations of Luciano et al. [106], who reported that AITC was able to react with glutathione and cysteine naturally present in meat, which interfered on their antimicrobial activity. In addition, the presence of proteic substances reduced genotoxic activity of AITC, PEITC [107], and MITC [108], on which the compounds were able to cause DNA damage in *Salmonella*, *E. coli*, and human cells (Hep G2) [109].

Kojima and Ogawa [110] suggested that ITCs act by inhibiting the oxygen uptake by yeast through the uncoupler action of oxidative phosphorylation in the mitocondria of yeast, inhibiting the coupling between the electron transport and phosphorylation reactions, thus hindering the ATP synthesis. However, the levels to achieve both enzymatic and oxygen uptake inhibitions used in the study were 200 times greater than the actual MIC of the ITCs for those organisms [30].

It is not clear if AITC crosses membranes and enters the cytoplasm of prokaryotic and eukaryotic cells or if it has an effect on cell membranes. Inside a cell, AITC can react with glutathione, sulfites, amino acids, oligopeptides, proteins, and water [111]. Delaquis and Mazza [10] suggest that AITC might cause

inactivation of essential intracellular enzymes through oxidative cleavage of disulfide bonds. Lin et al. [112] observed damages on the bacteria cell by exposition to AITC, creating pores on cell membranes and inducing leaking of cellular substances. AITC was able to modify the internal structure of *L. monocytogenes* when compared to non-treated cells when analyzed by transmission electron microscopy [104]. On the other hand, Ahn et al. [113] observed no damage in cell wall or leakage of ATP when AITC was tested against *L. monocytogenes*. The reduction of ATP could be the result of inhibition of enzymes related to ATP formation or depletion of proton motive force.

The mechanism of fungal death by ITCs was investigated by Calmes et al. [114]. Exposure of AITC, PEITC, and BITC in *A. brassicicola* decreased oxygen consumption rate, intracellular accumulation of reactive oxygen species (ROS), and mitochondrial membrane depolarization. The two major regulators of the response to oxidative stress, MAP kinase AbHog1 and the transcription factor AbAP1, were activated in the presence of ITCs. Once activated by ITC-derived ROS, AbAP1 may promote the expression of different oxidative-response genes. Besides, fungal strains deficient in AbHog1 or AbAP1 were hypersensitive to ITCs, and it might be useful to understand the mechanism of fungal resistance. In other studies, the authors [13] suggest some differences on the mode of action of 57 ITCs and related compounds investigated against *A. niger*, *P. cyclopium*, *Rhizopus oryzae*, and other species. These variations occurred in compounds in which -NCS group is directly bound on the aromatic moiety compared with the bounds on aliphatic radical. Normally, aromatic ITCs are more toxic than aliphatic, and the fungal toxicity of aliphatic ITCs decreased with the increasing length of the side chain [64].

Furthermore, it may be considered that AITC degraded in aqueous solution at 37 °C, generating allyl dithiocarbamate, diallyl tetra- and pentasulfide,

sulfur, and N, N'-diallylthiourea, dependent on temperature and pH conditions [104] However, there is no information relating this degradation to ITC's antimicrobial potential [30].

5. Conclusions

With the current trend, natural compounds are preferred and widely studied. Considering the data from several studies carried out, it may be observed that glucosinolates demonstrate a biocidal effect after their enzymatic hydrolysis. These breakdown products show a huge antibacterial and antifungal capacity, and they may be used on food preservation as well as plant defense. Several studies have demonstrated that the structure of glucosinolates and the microbial strain are responsable for their antimicrobial potential. Among the GLS hydrolysis products, ITCs are the main group that demonstrated an efficiency to reduce microbial growth. Allyl isothiocyanate is the most investigated ITCs against microorganisms, and its use as a fumigant agent on food preservation has been investigated.

There is not enough information regarding the mechanism behind the antimicrobial activity of GLS. Studies indicated that the central electrophilic carbon of ITCs may react with hydroxyls, amines, and thiols. However, it is not clear if ITC crosses the membrane and enters the cytoplasm or if they have an effect on cell membranes. Thus, further studies are necessary to clarify the mechanism of these active compounds on microorganisms and evaluate the feasibility application of GLS products as food preservative through fumigation treatment.

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2. OBJECTIVES

2. OBJECTIVES

The **general objective** of the present work is the application of chemical and biological methods to reduce the presence of fungi and mycotoxins in bread.

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

1. To evaluate the risk exposure to mycotoxins present in bread loaves purchased from different supermarkets of Valencia.
2. To study the inhibition of the *P. expansum* growth and PAT production in wheat tortillas packed with oriental mustard flour, yellow mustard flour and AITC.
3. To study the permanence of the AITC into the packaging using different systems of volatilization.
4. To study the use of ITCs to extend the shelf life of bread loaf and piadina contaminated with *A. parasiticus* by inhibiting the growth of the fungus and the production of AFs.
5. To evaluate AITC ability to react with α-ZOL and ZEA, to identify reaction products and to determine *in vitro* bioaccessibility and bioavailability of the studied mycotoxins and the reaction products.
6. To study shelf life extention and reduction of AFs employing diverse LABs in bread loaf contaminated with *A. parasiticus*.
7. To evaluate the ability of LABs to reduce the bioaccessibility of AFB_1 and AFB_2 using an *in vitro* dynamic digestion model.

2. OBJETIVOS

El **objetivo general** del presente trabajo es la aplicación de métodos químicos y biológicos para reducir la presencia de hongos y micotoxinas en pan.

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

1. Evaluar el riesgo de exposición a micotoxinas presentes en muestras de pan de molde procedentes de los comercios de Valencia.
2. Estudiar la inhibición del crecimiento de *P. expansum* y la reducción de PAT en tortillas de trigo envasadas con harina de mostaza oriental, harina de mostaza amarilla y AITC.
3. Estudiar la permanencia del AITC en el envase utilizando diferentes sistemas de volatilización.
4. Estudiar la utilización de ITCs para alargar la vida útil del pan de molde y de piadina contaminados con *A. parasiticus* inhibiendo el crecimiento del hongo y la producción de AFs.
5. Evaluar el potencial del AITC para reaccionar con α-ZOL y ZEA, identificar los productos de reacción y determinar la bioaccesibilidad y biodisponibilidad *in vitro* de las micotoxinas estudiadas y de los productos de reacción.
6. Estudiar la extensión de la vida útil y la reducción de AFs debido a la acción de distintas bacterias ácido lácticas (BALs) en pan de molde contaminado con *A. parasiticus*.
7. Evaluar la capacidad de las BALs para reducir la bioaccesibilidad de AFB_1 y AFB_2 mediante el uso de un modelo de digestión dinámico *in vitro*.

3. RESULTS

3.1. Dietary exposure to mycotoxins through the consumption of comercial bread loaf in Valencia, Spain

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3.1. Dietary exposure to mycotoxins through the consumption of commercial bread loaf in Valencia, Spain

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Abstract

In this study, 80 commercial samples of bread loaves were purchased from different supermarkets located in Valencia (Spain). These samples were investigated for the presence of legislated and non-legislated mycotoxins. Results showed that samples were contaminated with Aflatoxins (AFs), Zearalenone (ZEA) and Enniatins (ENs) with a frequency of 20, 96, and 65% respectively. Aflatoxin B_1 (AFB_1), Aflatoxin B_2 (AFB_2) and Aflatoxin G_1 (AFG_1) were detected with concentrations ranged from 0.5 to 7.1 µg/kg. The samples contaminated with AFB_1 showed values exceeding the maximum limit allowed in the EU. The sum of AFs also exceeded the maximum limit allowed in 6 samples. ENs contamination data ranged from 0.2 to 54 µg/kg and ENB was the most prevalent one. ZEA values ranged from 27 to 905 µg/kg and 30% of the contaminated samples were above the limits enforced by the EU. Finally, dietary exposure of the population living in Valencia to AFs, ENs and ZEA was estimated using the deterministic approach, through the evaluation of the consumption of commercial loaf bread and relating this data with the contamination of the loaf bread, for the calculation of the estimated daily intake (EDI) for each mycotoxin detected.

Keywords: Mycotoxins, LC-MS/MS, loaf bread, risk assessment, estimated daily intake.

1. Introduction

Mycotoxins are secondary metabolites produced by a wide variety of filamentous fungi, including species from the genera *Aspergillus*, *Fusarium*, *Penicillium, Alternaria* and *Claviceps* that grow under different climatic conditions on agricultural commodities. Approximately 25% of all cereals produced in the world are contaminated with mycotoxins and they are also found in other foods such as spices, coffee, nuts and fruits (Marin, Ramos, Cano-Sancho, & Sanchis, 2013; Zöllner & Mayer-Hel, 2006). Mycotoxins comprise a variety of chemical structures with biological properties (Varga, Glauner, Berthiller, Krska, Schuhmacher, & Sulyok, 2013). The most important mycotoxins in foods and animal feed are: aflatoxins (AFs), produced by *Aspergillus* species; ochratoxin A (OTA) produced by both *Aspergillus* and *Penicillium*; trichothecenes (TCs) [type A: HT-2 and T-2 toxin, and type B: deoxynivalenol (DON)], zearalenone (ZEA), fumonisin B_1 (FB_1) and B_2 (FB_2). There are also emerging mycotoxins such as fusaproliferin (FUS), moniliformin (MON), beauvericin (BEA), and enniatins (ENs) produced mainly by *Fusarium* species that are commonly found in grains and grain-derived products (Marin, Ramos, Cano-Sancho, & Sanchis, 2013; Krska et al., 2008). These mycotoxins can be harmful to both human and animal health, even after the food or feed product has been processed. Most mycotoxins are stable compounds to many processing operations such as heating, sorting, trimming, cleaning, milling, brewing, cooking, baking, frying, roasting, canning, flaking, alkaline cooking, nixtamalization and extrusion (Bullerman & Bianchini, 2007). These toxins may produce acute toxicity (ex. oestrogenic effect) as well as long-term effects, namely carcinogenicity, mutagenicity, teratogenicity or immunotoxicity in animals and humans (Bennett & Klich, 2003). Humans are mainly exposed to mycotoxins by cereals and cereal-derived products. Bread is a staple food worldwide and, like other perishable products, is susceptible to fungal

contamination. Spoilage of bakery products represents a significant source of economic losses to the industry and a potential safety risk due to the production of mycotoxins by different molds (ex. *Aspergillus* and *Penicillium*) (Cauvain 2012a; Saranraj & Geetha, 2012; Smith, Daifas, El-Khoury, Koukoutsis, & El-Khoury, 2004). Bread posses a relatively high water activity (a_w = 0.94-0.97) with a pH of approximately 6 (Legan, 1993). These properties are favorable for to the germination and growth of a wide range of molds. Bread loaves have a higher probability of mold growth since they are commonly sliced, which increases the surface area for microbial spoilage (Cauvain, 2012b). The Commission Regulation (EC) No 1881/2006 establishes maximum levels for mycotoxin contamination in foods. Indicative maximum levels for the sum of T-2 and HT-2 toxins have been recently issued (Commission recommendation 2013/165/EU) while ENs, BEA, FUS, NIV and MON, that belong to the group of emerging mycotoxins, do not present any specific legislation yet.

The occurrence of mycotoxins has been highly investigated in several foods as breakfast cereals (Iqbal, Rabbani, Asi, & Jinap, 2014), Italian cereal products (Juan, Mañes, Raiola, & Ritieni, 2013), pasta (Serrano, Font, Mañes, & Ferrer, 2013) and cereals (Meca, Zinedine, Blesa, Font, & Mañes, 2010). Monitoring studies for mycotoxin presence in foodstuffs have to be conducted continuously in order to obtain reliable information about the exposure of human population to these toxic compounds (Rodríguez-Carrasco, Ruiz, Font, & Berrada, 2013).

The goals of this study were: a) to determine the presence of AFB_1, AFB_2, AFG_1, AFG_2, OTA, HT-2 and T-2, DON, ZEA, FB1 and B2, FUS, BEA, ENB, ENB_1, ENA and ENA_1 in 80 bread loaf samples; b) to evaluate the risk exposure of the population in Valencia to the these mycotoxins through the deterministic risk assessment focused on the evaluation of the Estimated Daily Intake (EDI) and its

comparison with the Tolerable Daily Intake for each compound studied.

2. Materials and methods

2.1. Chemicals and reagents

AFB_1, AFB_2, AFG_1, AFG_2, OTA, HT-2 and T-2, DON, ZEA, FB1 and B2, FUS, BEA, ENB, ENB_1, ENA, ENA_1 (purity of all mycotoxins > 99%), formic acid (analytical grade, purity > 98%) and ammonium formate (analytical grade, purity ≥ 99.0%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Methanol (LC-MS grade, purity ≥ 99.9%) was purchased from Fisher Scientific (Hudson, NH, USA). Deionized water (<18MΩ cm resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Chromatographic solvents and water were filtered through a 0.45 μm cellulose filter from Scharlau (Barcelona, Spain). All stock solutions were prepared by dissolving 1 mg of the mycotoxin in 1 mL of pure methanol, obtaining a 1mg/mL solution. These stock solutions were then diluted with pure methanol in order to obtain the appropriated work solutions. All solutions were stored in darkness at -20°C before use.

2.2. Bread samples

A total of 80 commercial packages of bread loaves were purchased from different supermarkets located in Valencia (Spain) from January to July 2015. These samples were studied and divided into six categories according to the Spanish Ministry of Agriculture, Food and Environment (MAGRAMA - Ministerio de Agricultura, Alimentación y Medio Ambiente, 2014). MAGRAMA is responsible for proposing and implementing government policies against change climate, and for the protection of natural heritage, biodiversity, sea, water, rural development, agricultural, livestock and fisheries resources, and food. The six categories studied were: 16 white, 16 whole wheat, 16 crustless white, 16 crustless whole wheat, 16

special bread loaves. Special bread loaves include multi-grain, oatmeal, corn, kamut, rye, lactose and gluten free. All products were within their shelf lives. Bread slices of each sample were finely ground, added to a plastic bag and kept at -20 °C until analysis.

2.3. Mycotoxin extraction

Extraction of mycotoxins was performed using the method described by Serrano et al. (2013) with some modifications. Each bread sample was ground using a food grinder (Oster Classic grinder 220-240V, 50/60 Hz, 600W; Oster, Valencia, Spain). The bread powder obtained was vigorously shaken and three 5 g-aliquots of each sample were weighed in 50 mL plastic tubes. Then, 25 mL of methanol were added to each tube and samples were extracted using an Ultra Ika T18 basic ultraturrax (Staufen, Germany) for 3 min. The organic extract was centrifuged at 4000 rpm for 5 min at 5°C and the supernatant was transferred to a flask and evaporated to dryness with a Büchi Rotavapor R-200 (Postfach, Switzerland) at 35°C. The residue was dissolved in 5 mL of methanol, transferred to a 15 mL plastic tube and evaporated to dryness with gaseous nitrogen at 35°C using a multi-sample Turbovap LV Evaporator (Zymark, Hoptikinton, USA). Then, the extract was reconstituted in 1 mL of methanol, filtered through a 13mm/0.22 µm filter and transferred to a 1 mL glass vial.

2.4. LC-MS/MS analysis

The liquid-chromatography (LC) system (Agilent 1200 Chromatograph, Agilent Technologies, Palo Alto, CA, USA) was consisted of a binary LC-20AD pump and a SIL-20AC homoeothermic auto sampler. The LC was coupled to a 3200QTRAP mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with an ESI interface in positive mode for detection in multiple reactions

monitoring (MRM). A CMB-20A controller Analyst Software 1.5.2 was used for data acquisition and processing. The separation of mycotoxins was performed on a Gemini NX C18 column (150×2.0 mm I.D, 3.0 µm, Phenomenex, Palo Alto, CA) at room temperature (20 °C). The mobile phase was composed of solvents A (5 mM ammonium formate and 0.1% formic acid in water) and B (5 mM ammonium formate and 0.1% formic acid in methanol) at a flow rate of 0.25 mL/min. The elution gradient was established initially with 10% B, increased to 80% in 1.5 min, then kept constant from 1.5 to 4 min, increased to 90% from 4 to 10 min, increased again to 100% from 10 to 14 min and finally returned to the initial conditions for 10 min. The injection volume was 20µL. The main MS parameters were optimized and finally set as follows: nebulizer gas (GS1), 55 psi; auxiliary gas (GS2), 50 psi; curtain gas (CUR) 15 psi; capillary temperature 550 °C; ion spray voltage (IS) 5500 V. Nitrogen was used as the nebulizer, heater, curtain and collision gas. The precursor-to-product ion transitions were *m/z* 313.1/241.3–284.9 for AFB_1, *m/z* 315.1/259.0–286.9 for AFB_2, *m/z* 329.0/243.1–311.1 for AFG_1, *m/z* 331.1/313.1–245.1 for AFG_2, *m/z* 404.3/102.1-358.1 for OTA, *m/z* 484.3/185.1-215.1 for T2, *m/z* 442.2/215.4-262.8 for HT-2*, m/z* 297.1/161.0-249.2 for DON*, m/z* 319.0/282.9-301.0 for ZEA, *m/z* 722.4/334.3-352.3 for FB_1, *m/z* 706.4/336.2-318.3 for FB_2, *m/z* 355.0/175.0-246.7 for FUS, *m/z* 801.2/784.1-244.1 for BEA, *m/z* 657.3/196.1-214.0 for ENB, m/z 671.2/214.2-228.1 for ENB_1, *m/z* 699.4/210.2-228.2 for ENA, *m/z* 685.4/214.2-210.2 for ENA_1.

2.5. Dietary exposure

One of the most important aspects assessing the risk of mycotoxins is to determine the degree of human exposure to these compounds. Therefore, the dietary exposure of Valencia's population to mycotoxins present in bread loaves was calculated through Estimated Daily Intakes (EDIs) as described below:

EDI (ng/kg bw/day) = mean conc. (ng/kg) * bread consumption (kg/kg bw/day)

Bread consumption data were available in the statistical database of the Spanish Ministry of Agriculture, Food and Environment (MAGRAMA). Consumption of bread loaves was 0.12, 0.03, 0.07 and 0.03 g/kg bw/day respectively for white, whole wheat, special and crustless breads loaves (MAGRAMA, 2014). Assuming 70 kg as the average body weight (bw) for the population in Valencia, the daily consumption per kg of bw was calculated.

The health risk characterization of each mycotoxin was performed by dividing the EDI previously calculated with the tolerable daily intake (TDI) (ng/kg bw/day) of the respective mycotoxins (when available) as indicated in the equation:

$$\%TDI = (EDI/TDI)*100$$

International expert groups have not specified the TDI for AFs because no completely safe level can be established for chemicals that cause cancer. Exposure through food should be reduced to As Low As Reasonably Achievable (ALARA). Therefore, the calculated EDI cannot be directly compared with tolerable level for AFs. Since no TDI is available for ENs, the approximation to the risk assessment was carried out according to the safety guidelines established for other *Fusarium* mycotoxins. A hypothetic value of 1000 ng/kg bw/day was used (JECFA, 2001; SCF, 2002). The TDI for ZEA was set at 250 ng/kg bw/day (EFSA, 2014).

In addition, two exposure scenarios were considered to calculate mean values in the EDI study: the lower bound scenario (LB) and the upper bound scenario (UB). The first one underestimates contamination and exposure levels, while the second one generally overestimates them. The LB was obtained by assigning a zero value to those samples in which the analyte was non-detected or non-quantified and using these values to estimate dietary exposure. UB dietary

exposure was estimated by assigning the Limit of Detection (LOD) to all samples with non-detected results and the Limit of Quantification (LOQ) to all samples with less than the LOQ but more than the LOD (EFSA, 2010).

3. Results and discussion

3.1. Method validation

The method validation consisted in studies of linearity, recovery, repeatability, reproducibility, LODs and matrix effect for each mycotoxin. For the estimation of linearity and matrix effects, the standard calibration curves were carried out for each mycotoxin by plotting the signal intensity versus the mycotoxin concentration. All mycotoxins exhibited good linearity over the working range in the standard solution, a matrix-matched calibration assay and a fortified sample assay. The resulting coefficients of determination (R^2) were always higher than 0.9923. Linearity was evaluated using matrix-matched calibrations in triplicate at concentrations between 5 and 500 μg/Kg for mycotoxins with high sensitivity and between 50 and 2000 μg/Kg for mycotoxins with lewer sensitivity. The matrix effect (Table 1) for each mycotoxin was calculated according to the formula defined as the percentage of the matrix-matched calibration slope divided by the slope of the standard calibration and multiplied by 100. Recovery analyses were performed in triplicate during 3 consecutive days by spiking blank samples at three levels: LOQ, 2 times LOQ and 10 times LOQ. Spiked samples were left overnight at RT to allow solvent evaporation and stabilization of the mycotoxins on the matrix. Results were between 72% and 97% and relative standard deviation (RSD) was lower than 17%. The values for intra-day repeatability (n = 3), expressed as repeatability relative standard deviation (RSDr), ranged from 7.4% to 11.7%; and inter-day reproducibility (n = 5), expressed as reproducibility relative standard deviation (RSDR), ranged from 8.1% to 17.2% for the same linearity

addition values. LODs and LOQs were calculated by analyzing blank samples spiked with the standard mycotoxins (Table 1); they were determined as the lowest concentration of the selected compounds that produce a chromatographic peak at a signal-to-noise ratio (S/N) of 3 and 10 for LOD and LOQ, respectively.

Table 1. LODs, LOQs and matrix effects (ME) (%) for different mycotoxins in bread loaf.

Mycotoxin	LOD (µg/Kg)	LOQ (µg/Kg)	ME (%)
AFB_1	0.08	0.27	37
AFB_2	0.08	0.27	29
AFG_1	0.16	0.53	27
AFG_2	0.30	1.00	34
OTA	0.05	0.17	102
FB1	50.00	166.67	132
FB2	30.00	100.00	139
ZEA	7.80	26.00	106
T-2	1.76	5.87	72
HT-2	4.95	16.50	77
ENA	2.50	8.33	14
ENA_1	0.50	1.67	21
ENB	0.03	0.10	49
ENB_1	0.06	0.20	49
BEA	7.00	23.33	32
DON	20.50	68.33	60
FUS	0.65	2.17	35

3.2. Occurrence of mycotoxins in bread loaf

The occurrence of the aforementioned mycotoxins was determined in 80 units of bread loaf. The mycotoxins detected were: AFB_1, AFB_2, AFG_1, ZEA, ENA_1, ENB and ENB_1. Results of the natural occurrence of AFs are summarized in Table 2.

Table 2. Presence of Aflatoxins in analyzed samples.

Samples	N. of samples	Aflatoxins (µg/Kg)											
		Positive samples and frequency (%)	AFB_1			Positive samples and frequency (%)	AFB_2			Positive samples and frequency (%)	AFG_1		
			Mean	Max. level	Min. level		Mean	Max. level	Min. level		Mean	Max. level	Min. level
White	16	2 (12.5)	5.6	7.1	4.2	2 (12.5)	3.6	4.2	3.1	1 (6.2)	2.9	2.9	ND
Whole wheat bread	16	1 (6.2)	6.1	6.1	ND	3 (18.7)	2.2	3.2	0.5	0	0	ND	ND
Special	16	1 (6.2)	5.2	5.2	ND	0	ND	ND	ND	1 (6.2)	2.5	2.5	ND
Crustless white	16	0	ND	ND	ND	3 (18.7)	4.1	5.3	1.0	0	ND	ND	ND
Crustless whole wheat	16	0	ND	ND	ND	5 (31.3)	1.8	3.5	0.8	0	ND	ND	ND

The frequency of contamination of total samples with AFs (AFB_1, AFB_2 and/or AFG_1) was 20% with concentrations ranging from 0.5 to 7.1 μg/kg. AFs are often detected in cereals cereal-based products due to their stability to environmental conditions and through harsh processes, such as baking (Marin, S., Ramos, A.J., Cano-Sancho, G., & Sanchis, V., 2013).

Frequencies of sample contamination with AFB_1 were 12.5, 6.2 and 6.2% for white, whole wheat and special bread loaves, respectively. The average concentration found was of 5.6 μg/kg and the highest level (7.1 μg/kg) was detected in a white bread sample. Two samples of white (4.2 and 7.1 μg/kg), one of special (5.2 μg/kg) and one of whole wheat bread (6.1 μg/kg) were contaminated with AFB_1, whereas this mycotoxin was not found in crustless bread. All samples contaminated with AFB_1 showed values exceeding the maximum limit (2.0 μg/kg) set by the European legislation for this mycotoxin in bread (European Commission, 2010).

AFB_2 was detected in a total of 13 samples, namely, two white, three whole wheat, three crustless white and five crustless whole wheat bread. The mean concentration of AFB_2 in the contaminated samples was 2.7 μg/kg, and the highest and the lowest were 5.3 and 0.5 μg/kg, respectively. AFG_1 was only detected in 2 samples at 2.9 μg/kg in a white bread unit and 2.5 μg/kg in a special bread unit. These units were also contaminated with AFB_1. Overall, six bread samples (7.5%) presented a sum of AFs that exceeded the maximum allowed (4.0 μg/kg) by the EC for the presence of total AFs in bread (European Commission, 2010).

Iqbal et al. (2014) analyzed aflatoxins, ochratoxin A and zearalenone in breakfast cereals. Their results have shown that 41% of the samples were positive for the presence of AFs, twofold higher than the frequency obtained in our study. The authors have also shown that 16% and 8% of the samples presented levels of

AFB_1 and total AFs, respectively, above the limits enforced by the European legislation. Moreover, the co-occurrence and risk assessment of different mycotoxins in cereals and cereal-based products from Mediterranean area was also evaluated (Serrano et al., 2012), where 10.2% of the samples were contaminated with AFs at much higher concentrations (4.2-66.7 μg/kg) than the contamination found in our study.

Among all emerging mycotoxins tested, only ENs (Table 3) were found in the bread loaves sampled. The frequency of contamination of ENs (ENA_1, ENB, ENB_1) was 96.2%. Only ENA was not detected in the samples analyzed. The co-occurrence of ENs was found in 61 samples (76.2%). ENA_1 was found in 14 samples (17.5%), with higher frequencies in whole wheat (31.3%), special (25%) and white (18,3%) breads. ENB was the mycotoxin most frequently detected (96.2% of total samples) at an average concentration of 13.7 μg/kg (0.4 - 54 μg/kg). ENB_1 was detected in 63 of the 80 samples (78.7%) with frequencies of 81.3, 81.3, 75.0, 87.5 and 68.8% for white, whole wheat, special, crustless white an crustless whole wheat bread, respectively. The average concentration of this mycotoxin in the contaminated samples was 5.1 μg/kg, with values ranging from 0.2 to 14.8 μg/kg. ENB was also found as the most prevalent enniatin in Italian cereal products (Juan et al., 2013). However, the levels of enniatins in these products were ten-fold higher (ENA_1 8.3 μg/kg, ENB 133.60 μg/kg and ENB_1 8.1 μg/kg) than the concentrations found in the present study.

Meca et al. (2010) also investigated the presence ENs, BEA and FUS in cereals available in the Spanish market. The authors showed a frequency of 73.4% for ENA_1 (ranging from 33.38 to 814.42 mg/kg), 7.8% for ENB (ranging from 2.23 to 21.37 mg/kg) and 4.6% of ENB_1 (ranging from 4.34 to 45.94 mg/kg). Our study detected lower values of ENA_1, ENB_1, ENB in bread.

Table 3. Presence of Enniatins in analyzed samples.

Samples	N. of samples	Enniatins (μg/Kg)											
		Positive samples and frequency (%)	ENA_1			Positive samples and frequency (%)	ENB			Positive samples and frequency (%)	ENB_1		
			Mean	Max. level	Min. level		Mean	Max. level	Min. level		Mean	Max. level	Min. level
White	16	3 (18.7)	<LOQ	<LOQ	<LOQ	15 (93.8)	9.8	18.7	2.0	13 (81.3)	2.9	6.0	0.2
Whole wheat bread	16	5 (31.3)	2.4	2.6	2.2	16 (100)	16.5	41.1	1.3	13 (81.3)	6.5	14.8	1.5
Special	16	4 (25)	2.6	2.6	<LOQ	16 (100)	16.9	54.0	0.4	12 (75.0)	6.3	14.0	0.2
Crustless white	16	1 (6.2)	<LOQ	<LOQ	<LOQ	16 (100)	14.8	8.7	1.4	14 (87.5)	4.6	13.0	0.4
Crustless whole wheat	16	1 (6.2)	<LOQ	<LOQ	<LOQ	14 (87.5)	10.6	31.0	1.0	11 (68.8)	5.1	13.0	2.4

Results of ZEA contamination in the bread samples analyzed are presented on Table 4. This mycotoxin was detected in 52 out of the 80 samples studied (65.0%). The frequency for white, whole wheat, special, crustless white and crustless whole wheat bread loaves was 50.0, 56.2, 56.2, 87.5 and 75.0%, respectively. The average concentration of ZEA in the contaminated samples was 89.6 µg/kg, while the highest and lowest values were 905 and 27 µg/kg, respectively. Thirty percent of the samples were above the limits enforced by the European legilsation (European Commission, 2006) for ZEA in bread (50 µg/kg).

Table 4. Presence of Zearalenone in analyzed samples.

Samples	N. of samples	**Zearalenone (µg/Kg)**			
		Positive samples and frequency (%)	**ZEA**		
			Mean	Max. level	Min. level
White	16	8 (50)	56.8	80.0	36.0
Whole wheat bread	16	9 (56.2)	48.8	100.0	29.0
Special	16	9 (56.2)	178.6	905.0	27.0
Crustless White	16	14 (87.5)	96.8	214.0	40.0
Crustless whole wheat	16	12 (75.0)	67.0	135.0	30.0

3.3. Estimation of the daily intake

The EDIs and health risk characterization of the mycotoxins (% of relevant TDI) detected in the samples analyzed are presented on table 5. EDIs for ENs ranged from 0.005 (ENA_1 LB) to 0.808 ng/kg bw/day (ENB UB) and for the sum of ENs the EDIs were 1.037 and 1.079 ng/Kg bw/day, respectively for the LB and UB scenarios. Considering that there is no TDI available for this class of mycotoxin, an estimated risk assessment was carried out according to the guidelines for other *Fusarium* mycotoxins, using a TDI of 1000 ng/kg bw/day. In this case, all EDI values

were lower than the TDI. The health risk characterization expressed as %TDI for ENs ranged from 0.001 to 0.081%, while it was found as 0.104% (LB) and 0.108% (UB) for the sum of ENs.

Table 5. Mycotoxins' exposure estimation and risk assessment of the population in Valencia through the consumption of bread.

Mycotoxin	EDI (ng/kg bw/day)		% TDI	
	LB	UB	LB	UB
AFB_1	0.030	0.035	-	-
AFB_2	0.022	0.026	-	-
AFG_1	0.008	0.018	-	-
Sum of AFs	0.021	0.078	-	-
ZEA	2.380	2.923	0.952	1.169
ENA_1	0.005	0.047	0.001	0.005
ENB	0.808	0.808	0.081	0.081
ENB_1	0.223	0.224	0.022	0.022
Sum of ENs	1.037	1.079	0.104	0.108

The EDIs calculated for ZEA were 2.380 and 2.923 ng/kg bw/day considering the LB and UB scenarios, respectively. The %TDI was 0.952% for LB and 1.169% for UB. EDI values calculated for ZEA through the consumption of different commodities commonly consumed in Catalonia ranged between 0.3-0.5 ng/kg bw/day (Cano-Sancho et al., 2012), which is 5-6 times lower than the results found in the present study. Moreover, Aldana et al. (2014) found EDIs of 0.049 and 0.090 μg/kg bw/day in Portugal and the Netherlands, respectively, through the consumption of contaminated wheat flour, which is the main ingredient of bread.

EDIs of AFs ranged from 0.008 (AFG_1 LB) to 0.035 ng/kg bw/day (AFB_1 UB)

and 0.021 (LB) and 0.078 (UB) ng/kg bw/day for the sum of AFs. EDIs of AFB_1, AFB_2, AFG_1 and AFG_2 obtained by García-Moraleja et al. (2015) for the adult Spanish population through the consumption of coffee were of 0.003, 0.001, 0.006 and 0.014, respectively. However, there is a lack of studies to evaluate the EDI of AFs in the total diet of the Spanish population. In addition, the EDI of AFs could not be compared with the TDI because carcinogenic compounds do not have a specific TDI (Azaiez, Font, Mañes, & Fernández-Franzón, 2015). Exposure of carcinogenic substances through food consumption should be reduced to As Low As Reasonably Achievable (ALARA).

4. Conclusion

Bread loaves purchased in the community of Valencia were tested positive to aflatoxins (AFs), zearalenone (ZEA) and enniatins (ENs) with a frequency of 20, 96 and 65%, respectively. More importantly, some samples presented levels of AFB1 (5% of total samples), ZEA (30% of total samples) and sum of AFs (7.5% of total samples) that exceeded the maximum content established by the European legislation. Although the contamination levels for some mycotoxins are above the maximum limits allowed, all calculated EDIs were lower than the correspondent TDIs. However, further studies are necessary to analyze the levels of these mycotoxins in several other foods to have a complete risk assessment about the intake of these mycotoxins in the whole diet of the population in Valencia.

Conflict of interest

The authors declare that are no conflicts of interest.

Acknowledgements

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3.2. Bioactive compounds from mustard flours for the control of patulin production in wheat tortillas

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3.2. Bioactive compounds from mustard flours for the control of patulin production in wheat tortillas

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Abstract

Patulin (PAT) is a toxic fungal metabolite produced by *Penicillium*, *Aspergillus* and *Byssochlamys* growing especially in fruit and cereals. PAT exhibits a number of toxic effects in animals and its presence in food is undesirable. In this study the reduction of the mycotoxin PAT produced by a strain of *Penicillium expansum,* on wheat tortillas was studied using volatile bioactive compounds present in the oriental and yellow mustard flour and also using the standard solution of the antifungal compound allyl isothiocyanate (AIT), developing an active packaging with two different systems of release of those bioactive compounds. Also the kinetic of volatilization of the compounds used in the bioactive packaging was evaluated using the technique of the gas chromatography (GC) coupled to the flame ionization detector (FID). The PAT was extracted from the samples using the QUECHERS methodology and was determined using the technique of the liquid chromatography (LC) coupled to the mass spectrometry detector in tandem (MS/MS). The maximum of volatilization of the AIT in the bioactive packaging is produced between 1 and 24h depending on the volatilization technique and is stable during two months, whereas the reduction of PAT evidenced in the samples treated ranged from 80 to 100%.

Keywords: Glucosinolates, isothiocyanates, *Penicillium expansum*, chemical control, PAT.

1. Introduction

Patulin (Fig. 1) (PAT) is a toxic secondary metabolite produced by a wide range of fungal species of the genera *Penicillium*, *Aspergillus* and *Byssochlamys*. Among the different genera, the most important PAT producer is *P. expansum* (Moake, Padilla-Zakour, & Worobo, 2005). PAT has been found as a contaminant in many mouldy fruits, vegetables, cereals and other foods. However, the major sources of contamination are apples and apple products, which are also the most important source of PAT in the human diet (Baert et al., 2007; Murillo-Arbizu, Amézqueta, González-Peñas, & de Cerain, 2009; Reddy et al., 2010).

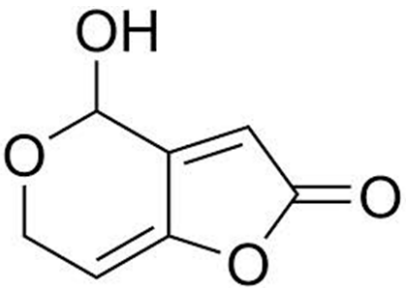

Figure 1. Patulin chemical structure.

PAT has been classified in Group 3 by IARC that means not classifiable as to its carcinogenicity to humans, although it has been shown to cause neurotoxic and mutagenic effects in animals (IARC, 2002). In 1995, the Joint Food and Agriculture Organization/ World Health Organization Expert Committee on Food Additives (JECFA, 1995) recommended a provisional tolerable daily intake (pTDI) of 0.4 μg PAT/kg body weight/day based on long-term exposure (JECFA, 1995). As a result, the levels of PAT in fruits are subjected to legislative control. The Codex Alimentarius recommends levels of PAT in fruits and fruit juices to be lower than

0.05 mg/kg.

PAT causes gastrointestinal effects as distension, ulceration and hemorrhage in acute and short-term *in vivo* studies. Recent studies have also demonstrated that PAT alters the intestinal barrier function. PAT has electrophilic properties and high reactivity to cellular nucleophiles. At cellular level it can cause enzyme inhibition and chromosomal damage. PAT causes cytotoxic and chromosome-damaging effects mainly by forming covalent adducts with essential cellular thiols (Fliege, & Metzler, 2000; Glaser, & Stopper, 2012).

Vegetables like broccoli, cauliflower, cabbage, Brussels sprouts, belong to the Brassica genus and are widely consumed. A healthy diet should include Brassica vegetables because these vegetables are rich in health-promoting compounds like ascorbic acid, soluble fiber, selenium, glucosinolates (GLS), etc. Among these compounds, GLS have been extensively studied in the past decades. GLS are secondary metabolites that can be classified as aliphatic, aromatic or indolic depending on their side chain (Fahey, Zalcmann, & Talalay, 2001). GLS are hydrolysed by a group of endogenous β-glucosidases termed myrosinase (Fig. 2). Myrosinase (MYR) is stored separately from GLS in the plants, but will mix with GLS upon tissue damage (Kissen, Rossiter, & Bones, 2009). Hydrolysis of the thioglucosidic bond by myrosinases releases an aglycone that can either rearrange into an isothiocyanate or be converted to other products such as nitriles, epithionitriles or organic thiocyanates depending on the presence of specific proteins and certain structural prerequisites.

Fungi growth inhibition by isothiocyanates has been reported since the late 1930's (Luciano & Holley, 2009). These compounds are very unique in comparison to other essential oils, since they are only formed when the plant cell suffers some kind of injury such as insect bite, grinding, milling or fungi contamination in the presence of water (Luciano & Holley, 2009). Then, the isothiocyanate precursors,

called GLS, are transformed by the enzyme myrosinase. Therefore, isothiocyanates are not present in dry mustard flour, unlesswater is added to it.

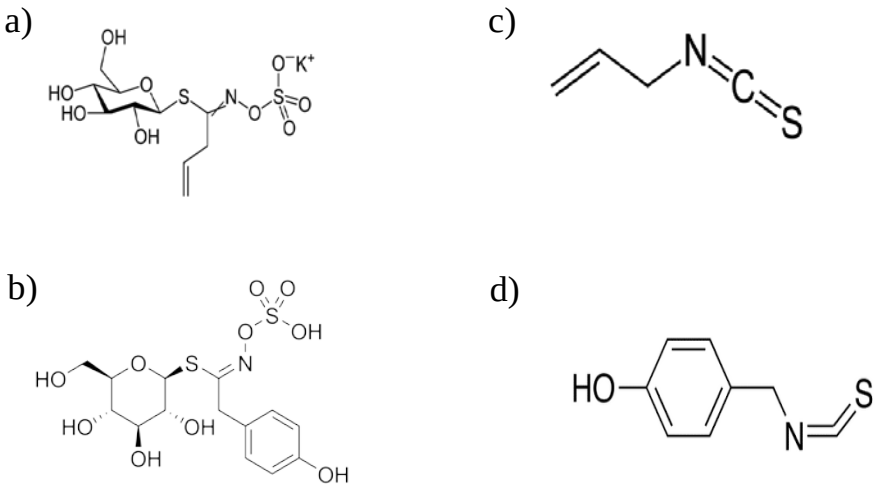

Figure 2. Chemical structure of the bioactive GLCs a) sinigrin and b) sinalbin c) and of the ITCs c) AIT and d) PHBITC.

ITCs exhibit biocidal activity against microorganisms including fungi (Nielsen & Rios 2000) and bacteria (Luciano & Holley 2011), as well as insects (Tsao, Yu, Potter, & Chiba, 2002b) and nematodes (Flemming, Turner, & Hunt, 2006). In particular, it has been demonstrated that AIT effectively inhibits the growth of a variety of pathogenic microorganisms at low concentrations (Lin, Preston, & Wei, 2000; Luciano & Holley 2009). The potential of AIT as a natural antimicrobial in different food matrices, including chicken breast (Shin, Harte, Ryser, & Selke, 2010), ground beef (Nadarajah, Han, & Holley, 2005), dry-cured ham (Graumann & Holley 2007), fermented dry sausages (Chacon, Muthukumarasamy, & Holley, 2006), and tuna meet (Hasegawa, Matsumoto, Hoshino, & Iwashita, 1999) has been studied.

The aims of this study were to study a) the quantity of the GLS present in yellow and oriental mustard flours b) the kinetic of volatilization of the antimicrobial AIT present in two active packaging and c) the inhibition of the *Penicillium expansum* growth and PAT production in wheat tortillas treated with AIT.

2. Materials and methods

2.1. Materials and chemicals

PAT, sinalbin and sinigrin (98% purity), formic acid (HCOOH), AIT (94% purity), para-hydroxybenzylisothiocyanate (PHBITC), tetrabutylammonium hydrogen sulfate (TBA), ammonium formate, and sodium chloride (NaCl) were obtained from Sigma-Aldrich (St. Louis, USA). Oriental (*Brassica juncea*) and yellow mustard (*Brassica alba*) flours were provided by G.S. Dumm dry mustard millers (Hamilton, Ontario, Ca). Methanol was purchased from Fisher Scientific (New Hempshire, USA). 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. The strain of *Penicillium expansum* CECT 2278, was obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain). The plastic trays used for the experiments were composed by multilayer polyethylene (13" × 9.6" clear, rectangular, with an oxigen trasmission of 6509 $cm^3/mil/m^2/24h$) and were provided by Saplex (Barcelona, Spain).

2.2. GLS extraction and determination

GLS from oriental and yellow mustard flours were extracted using the method of Prestera et al. (1996) with modifications. Twenty grams of each flour were placed in a 50 mL glass tube and autoclaved at 115 °C during 15 minutes to

inactivate the enzyme myrosinase. Then, the samples were added with 200 mL of boiling distilled water in a 500 mL Erlenmeyer flask and the mixture was stirred for 10 min at 350 rpm. The mixtures were cooled at room temperature, centrifuged at 2500 rpm for 5 min at 4 °C and filtered through Whatman no. 4 filter paper into 50 mL screw-capped tubes. The extracts were filtered again through a 0.22 µM filter. Separation and quantification of GLS were performed using a Shimadzu LC system (Shimadzu, Japan), equipped with a Gemini C18 column (4.6 × 150 mm i.d. 5 µm; Phenomenex, Palo alto, CA). Elution was carried out isocratically for 20 min at a flow rate of 1 mL/min, using a solvent system containing 20% (v/v) acetonitrile and 80% water+0.02 M tetrabutylammonium hydrogen sulfate (pH 5.5). The injection volume used was 20 µL. A UV detector was used to measure the absorbance at 227 nm in order to verify and quantify the presence of GLS sinigrin (SN) and sinalbin (SA) with reference retention time of 2.23 and 1.83 min. respectively.

2.3. Headspace analysis of AIT

The study of the release of the AIT used to preserve the wheat tortillas (stored in plastic trays) against *P. expansum* growth and PAT production was carried out performing two different sets of experiments and in particular:

1) It was evaluated the AIT released in the plastic tray by the conversion of the GLS contained in the oriental mustard flour into AIT through myrosinase conversion in presence of water. The flour (4g) was weighted and placed in a small plastic bag containing 3 mL of water, and after the homogenization of the mustard flours matrices were pasted on the cover tray.

2) It was evaluated the AIT released by the evaporation of a 50 µL of the standard solution of the AIT placed on a 2.5 × 2.5 cm paper-filter plastic and pasted on the cover tray.

The AIT evaporated in the headspace of the plastic trays described above was determined using a gas chromatograph (GC) equipped with flame ionization detector (FID) (GC 6890, Agilent Technologies Inc., Santa Clara, Calif., U.S.A.), equipped with a 30m × 0.25 mm CP-SIL 88 fused capillary column (Varian, Middelburg, Netherlands). The inlet temperature was set at 200 °C, whereas the detector temperature was 250 °C. H_2 served as carrier gas (5 ml/min), and the FID gasses were H_2 (40mL/min), and purified air (450 mL/min). The temperature program was as follow: initial temperature of 60 °C was held for 1 min, raised at 8 °C/min until 100 °C and held for 5 min, then raised at 15 °C/min until 200 °C, totalizing 16.6 min per sample analysis. Identification and quantification of AIT was carried out comparing the samples areas with points standards curve (1-100 mg/Kg).

Test trays were stored at room temperature (0-2 months) and then 100 µL of the head space was injected in the chromatograph through a septum applied in the tray cover. Three replicates were carried out for each test condition.

2.4. Wheat tortillas food system experiments

In a multilayer plastic tray of 1 L (Saplex, Barcelona, Spain) was inserted a sample of wheat tortilla (10 g). The samples were treated with:

a) Three quantities of oriental and yellow mustard flours (0.5, 1 and 2 g) placed in a small plastic bag with the same quantity of water to promote the reaction of conversion of the GLS into ITCs and pasted on the tray plastic cover.

b) Three different quantities of the standard solution of the AIT (50-100-200 µL/L) placed on a 2.5 × 2.5 cm paper-filter pasted on the plastic tray cover.

The wheat tortillas (50 g) were contaminated with one mL of *P. expansum* CECT 2681 grown in Potato Dextrose Broth (PDB) (Oxoid, UK) medium containing 10^6 conidia/mL. Conidial concentration was measured by optical density at 600

nm in sterile water and adjusted to 10^6 conidia/mL in PDB as reported Kelly, Grimm, Bendig, Hempel, & Krull, (2006). The control group did not receive any mustard flours or AIT treatments. The plastic trays were closed and incubated at 23 °C during 30 days.

2.5. PAT extraction

For wheat tortilla samples 10 ± 0.01 g test portion was weighed into a 50 mL Falcon polypropylene tube (Becton Dickinson, Le Pont de Claix, France). Ten milliliters of acetonitrile were added in all samples, and the resulting slurry was vigorously hand-mixed and placed onto an automated shaker for 5 min.

Partitioning Step: A magnesium sulfate ($MgSO_4$)/sodium chloride (NaCl) salt mixture (4:1, w/w) (5.0 ± 0.2) was added to the slurry, which was immediately and vigorously hand shaken for a few seconds before centrifugation at room temperature at 4000 g for 15 min.

Cleanup by Dispersive Solid-Phase Extraction (dSPE): The resulting acetonitrile-based supernatant (6 mL) was transferred to a 15 mL Falcon polypropylene tube already filled with 400 mg of Primary Secondary Amine (PSA), 400 mg of C_{18}, and 1200 mg of $MgSO_4$, and the tubes were vigorously hand-shaken for about 30 s. After centrifugation (4000 g at room temperature for 10 min), the supernatant (1 mL) was transferred into a new 15 mL Falcon polypropylene tube filled beforehand with 10 µL of a 5% formic acid solution in water (v/v). Sample was mixed and evaporated to dryness at 40 °C under a stream of nitrogen.

Final Treatment of the Extract: The residue was reconstituted in H_2O (200 µL) before being filtered over a Polytetrafluoroethylene (PTFE) syringe filter, 0.2 µm, 13 mm, and collected into a amber glass vial for further liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis (Desmarchelier, Mujahid, Racault, Perring, & Lancova, 2011).

2.6. LC-MS/MS PAT determination

LC analysis was performed on a 150 mm × 4.6 mm, 2.1 mm i.d., 2.5 µm, Gemini C18 column (Phenomenex, Palo Alto, CA) using an Agilent 1100 binary pump system. The mobile phase was constituted by H_2O (A), and acetonitrile (B). A linear gradient program was set up with 0.2 min, 5% B; 2.5 min, 95% B; a hold at 95% B for 2 min; and a return to 5% B in 0.5 min (the LC column was reconditioned at 5% B for an additional 10 min). The flow rate was 0.3 mL/min, and 20 µL of the extract were injected. MS detection was performed using an 4000 QTrap (Applied Biosystems, Toronto, CA) equipped with a Turbo Ion Spray ionization source. MS tuning was performed in positive electrospray ionization (ESI) by syringe-infusing separately a 10 µg/mL solution of PAT. The block source temperature was maintained at 500 °C, and the gas set values were as follows: curtain gas, 40 psi; nebulizer gas, 30 psi; turbo gas, 30 psi; collision gas, 1.2×10^4 psi. The entrance potential and the collision exit potential were settled at 10 and 15 V, respectively, and the electrospray capillary voltage was set at +4 kV. Quantitative analysis was performed using tandem MS in selected reaction monitoring (SRM) mode using as quantification transition the ion with a *m/z* of 153, whereas as analyte confirmation the ions with a *m/z* of 109 and 81. Data processing was carried out using Analyst software 1.5. PAT was quantitated by means of an external calibration curve (Desmarchelier et al., 2011).

3. Results and discussion

3.1. GLS evaluation in yellow and oriental mustard flours

The GLS presents in the yellow and oriental mustard flours were analytically characterized to know the total amount of the GLS that can be converted in ITCs through the action of the enzyme myrosinase. Both flour matrices were extracted with an aqueous extraction and analyzed using the LC-DAD technique (Fig. 3).

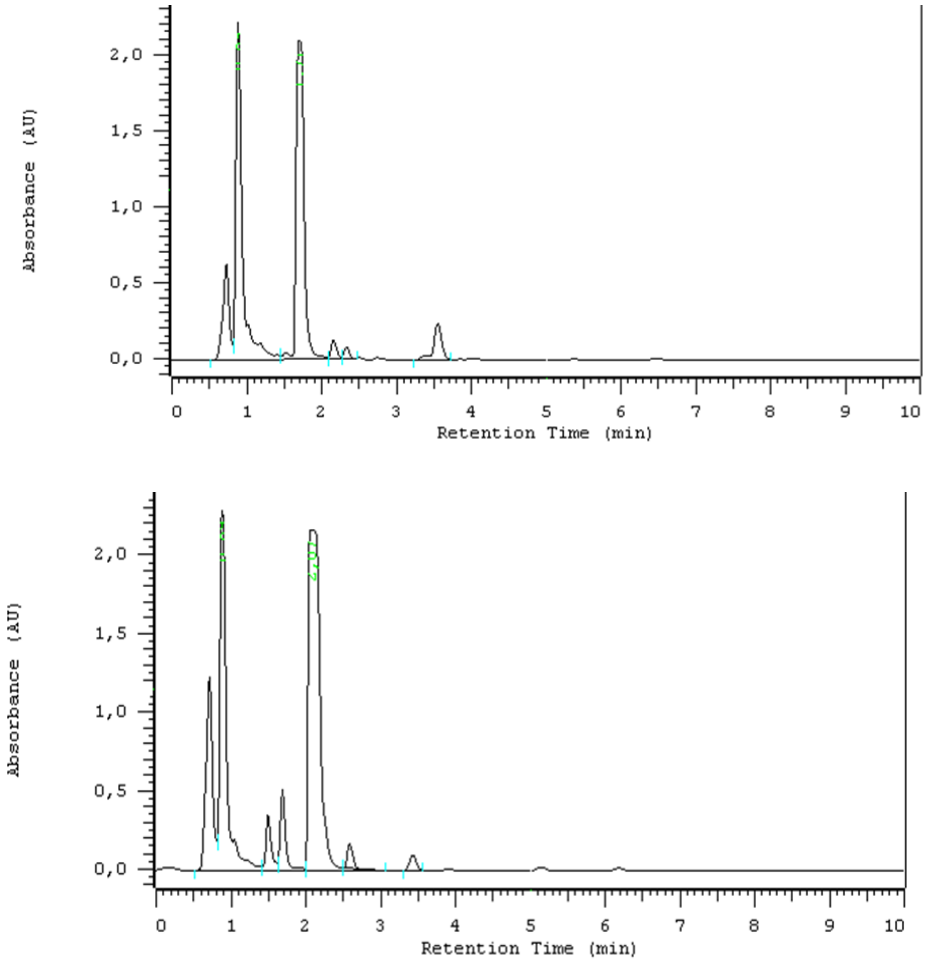

Figure 3. LC-DAD chromatograms of the a) sinalbin and b) sinigrin presents in the yellow and oriental mustard flours respectively.

The principal GL present in the oriental mustard flour was the sinigrin (SN), with a retentij detected in the quantity of 46 g/Kg, whereas the yellow mustard flour was characterized for the presence of the GL sinalbin (SA) in the concentration of 42 g/Kg. These two GLS, SN and SA, are the precursor of the antimicrobial compounds AIT and parahydroxybenzyl isothiocyanate (PHITC)

respectively that are the compounds that through the reaction with the enzyme myrosinase were used to reduce the growth of the strain of *P. expansum* inoculated on wheat tortillas.

To improve the safety of bakery products, the addition of other agents during manufacture to control the presence of the mycotoxigenic fungi is of great interest. One alternative to the synthetically antimicrobial compounds with promise is the mustard flour, which has natural antimicrobial properties. All plants in the Brassicaceae family contain GLS as secondary metabolites, and yellow (*B. juncea*) and oriental (*B. alba*) mustards contains the GLS SN and SA. Upon physical damage of the plant tissue, hydrolysis of GLS is catalyzed by the endogenous enzyme myrosinase in the presence of moisture to produce the antimicrobials compounds AIT and PHBITC (Delaquis, & Mazza, 1995; Ekanayake et al., 2006). The mechanism of action of these antimicrobial compounds is uncertain, but it may inhibit essential enzymes and cause membrane damage (Lin et al., 2000).

3.2. Gas chromatography evaluation of the AIT volatilization

The volatilization of the bioactive compound AIT employed to reduce the growth of the strain of *P. expansum* CECT 2278 in wheat tortillas samples was studied using three different methodologies. The first methodology was based on the employment of the oriental mustard flour inserted in a small plastic bag with water that catalyze the reaction conversion of the GL SN in the AIT mediated by the enzyme myrosinase, whereas the second methodology was developed absorbing 50 µL of the AIT standard solution on a paper filter pasted under the plastic tray cover used for the wheat tortillas storage. The volatilization of the bioactive compound AIT was studied with and without the presence of the food matrix employed in this study at room temperature during two months storage.

In particular as shown in Fig. 4a, using the oriental mustard flour, the maximum level of AIT detected in the headspace with and without the sample were of 34.3 and 50.7 mg/L respectively. This difference on the AIT volatilization could be related to several factors:

a) During the incubation time studied the concentration of the AIT decrease until 15 mg/L evidenced at 24 h incubation, and then continue to decrease probably due to the absorption of the AIT in the plastic matrix that compose the tray and also for the absorption of the bioactive compound in the food matrix tested.

b) Another important aspect that has to be considered is that the AIT during the volatilization can be converted in other compounds with less antimicrobial activity as thiocyanates and nitrils (Meca, Luciano, Zhou, Tsao, & Mañes, 2012).

Employing the second AIT volatilization system (50 µL of AIT standard solution absorbed on a paper filter) the maximum AIT dispersion in the headspace was detected during the first incubation hour and was of 130 and 115 mg/L considering the presence and the absence of the food matrix present in the plastic tray (Fig. 4b). The concentration of the bioactive compound in the head space decrease between the second and the fifth incubation hour arriving at 20 mg/L at 48h incubation and remain constant to 5 mg/L until the end of the experiments. Employing this AIT volatilization system the concentration of the AIT present in the headspace in the first 48h was 2.0 fold highest than the data evidenced using the oriental flour as AIT generation system. This application of the AIT has the advantage to promote a more rapid AIT volatilization in the first incubation hours reducing the possibility of growth/germination of the mycotoxigenic fungi in food matrices. Considering the data evidenced in ours study the presence of the food matrix does not influence significatively the vaporization of the AIT.

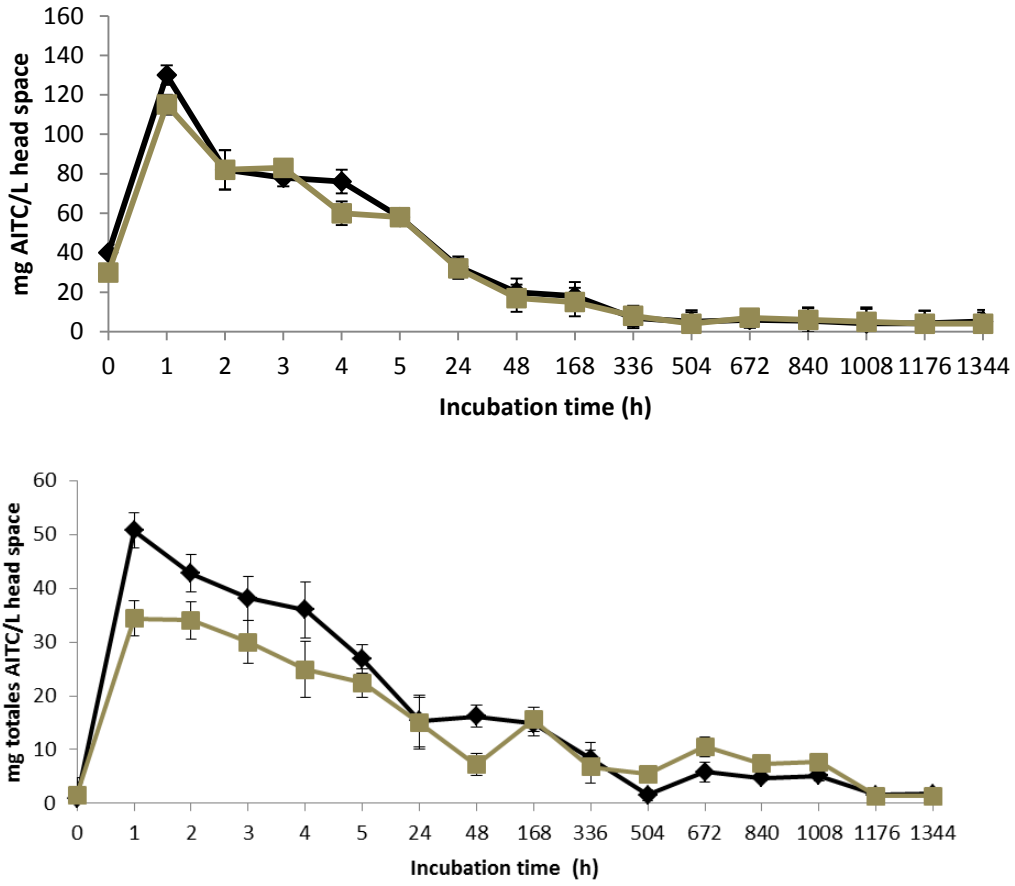

Figure 4. Volatilization kinetics of the AIT contained in the plastic trays a) employing 4 g of oriental mustard placed in a small plastic bag containing 3mL of water to promote the sinigrin conversion in AIT, b) employing the AIT released by the evaporation of a 100 µL of the standard solution of the bioactive compound placed on a 2.5 x 2.5 cm paper-filter and pasted on the cover tray (Black=with food, grey= without food).

3.3. PAT reduction in wheat tortillas

The sample preparation for PAT analysis in wheat tortillas was based on the

QuEChERS procedures described in AOAC International official method 2007.01 and CEN standard method (2008). All the parameters optimized for LC-MS/MS analysis are described below:

The limit of detection (LOD) was defined as the lowest concentration producing a chromatographic peak with a signal-to-noise ratio (S/N)≥3.

Despite closely depending on the cleanliness of the MS source, and thus submitted to small variations over the time, the LOD was broadly estimated within a 0.2 μg/kg for the food matrix studied. The limit of quantitation (LOQ) was arbitrarily defined as the lowest fortification level, that was, 5 μg/kg.

Internal standard corrected recoveries were within a 94-104% range, whereas precision data, that is, RSDr and RSD_{IR}, were of 1.3 and 3.3 %, respectively.

All of these results were compliant with the analytical requirements of the European Committee for Standardization (2010).

The bioactive compound AIT used in this study was tested for the reduction of the *P. expansum* growth in wheat tortillas samples and also the production by the microorganism of the mycotoxin PAT was evaluated to understand if the fungal growth reduction can influence the mycotoxin reduction. The wheat tortillas were treated with two different AIT applications as explained in the material and method section.

The PAT present in the food products treated with those two methodologies was analyzed using the technique of the LC-MS/MS (Fig. 5).

As evidenced in the Fig. 6, the mean reduction of the PAT observed using the two different AIT applications was of 92.58%. The highest PAT reduction was evidenced in the experiments carried out using 2.0 g of yellow mustard flour with a 99.1%, whereas the lowest was observed using the 0.5 g of oriental mustard flour with 85.5%. Comparing the results obtained using the two mustard flours no

significative differences were detected using 0.5 and 1.0 g of both flours, whereas using 2.0 g, the PAT reduction observed using the yellow and oriental mustard flour were of 99.1 and 92.9% respectively. The results obtained using the standard solutions of the AIT were comparable with the data obtained with the two flours matrices employed.

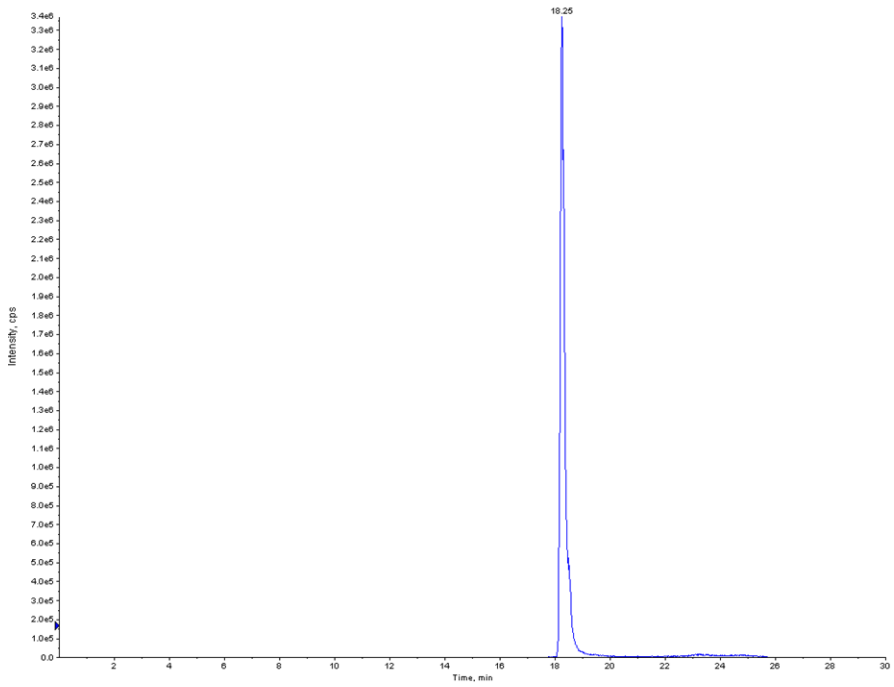

Figure 5. LC-MS/MS chromatogram of the patulin present in the control wheat tortilla treated without AIT treatment.

This article can be considered the first where a chemical approach based on the use of the bioactive compounds ITCs was employed to reduce the PAT produced by *P. expansum* in food matrices, whereas the use of other strategies to

reduce the PAT present in food products was evaluated by many authors.

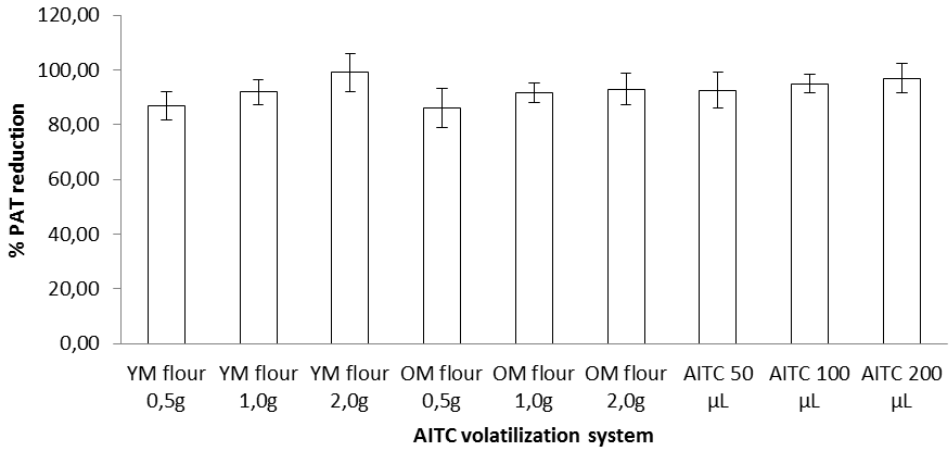

Figure 6. Patulin reduction in wheat tortillas contaminated with the strain of *Penicillium expansum* CECT 2278 (patulin producer) and treated with yellow (YM) and oriental (OM) mustard flours and also with three different quantities of the standard solution of the AIT.

In particular Drusch, Kopka, & Keading, (2007) studied the stability of PAT in an aqueous juice-like model system. At acidic pH, the presence of ascorbic acid reduced the stability of PAT. After 34 days, PAT was reduced to 30% of its initial concentration in the presence of ascorbic acid compared to 68–71% in samples without ascorbic acid. Conditions during storage (presence of light, oxygen and/or metal ions) influenced the stability of PAT. Furthermore, it was possible to induce degradation of PAT by either generating hydroxyl radicals or by adding the rather stable radical diphenyl-1-picrylhydrazyl (DPPH). The data evidenced by the authors indicate that PAT is decomposed by free radicals generated by oxidation of ascorbic acid to dehydroascorbic acid. The percentages of PAT reduction evidenced in this study are 0.5 fold lower than the data evidenced in our study.

Yun et al. (2008) studied the effects of organic acids, amino acids, and ethanol on the radio-degradation of PAT by gamma irradiation in an aqueous

model system. The PAT, dissolved in distilled water at a concentration of 50 ppm, was practically degraded by the gamma irradiation at the dose of 1.0 kGy, while 33% of the PAT remained in apple juice. In the aqueous model system, the radio-degradation of PAT was partially inhibited by the addition of organic acids, amino acids, and ethanol. The proportions of remaining PAT after irradiation with the dose of 1.0 kGy in the 1% solution of malic acid, citric acid, lactic acid, acetic acid, ascorbic acid, and ethanol were 31.4%, 2.3%, 31.2%, 6.1%, 50.8%, and 12.5%, respectively. During 30 days of storage, the remaining PAT was reduced gradually in the solution of ascorbic acid and malic acid compared to being stable in other samples. The amino acids, serine, threonine, and histidine, inhibited the radio-degradation of PAT. It was suggested that 1 kGy of gamma irradiation (recommended radiation doses for radicidation and/or quarantine in fruits) is effective for the reduction of PAT, but the nutritional elements should be considered because the radio-degradation effects are environment dependent.

Gao, Yue, Yuan, Fu, & Peng, (2009), evaluated the ultrasonic degradation technology of PAT to reduce the content of this bioactive compound in apple juice and improve the security of the food product. Based on the single-factor test, the optimum condition of ultrasonic was developed through orthogonal design. The results showed that the best process parameters of PAT ultrasonic degradation in apple juice is power 420 W, time 90 min, frequency 28 kHz, temperature 30 °C, with the PAT degradation rate of 69.43%. There is minor impact on the key quality parameters of apple juice. The data evidenced in this study are 30% lowest than the data evidenced in our study.

Zhu, Koutchma, Warriner, Shao, & Zhou, (2013) evaluated the feasibility of monochromatic ultraviolet (UV) radiation at 253.7 nm as a possible commercial application for the reduction of PAT in fresh apple cider and juice. It was shown that 56.5%, 87.5%, 94.8% and 98.6% reduction of PAT can be achieved,

respectively, in the model solution, apple cider, apple juice without ascorbic acid addition and apple juice with ascorbic acid addition in 2-mm thickness sample initially spiked by 1 mg/L of PAT after UV exposure for 40 min at UV irradiance of 3.00mWcm^2.

Funes, Gómez, Resnik, & Alzamora, (2013) investigated the effect of pulsed light (PL) dose on PAT degradation in McIlvaine buffer, apple juice and apple purée. The exposure of all samples to PL doses between 2.4 and 35.8 J/cm^2 resulted in a significant decrease in PAT levels. PAT reduction in McIlvaine buffer did not depend markedly on the initial concentration of the mycotoxin. At the maximum dose tested, the remaining average PAT level dissolved in McIlvaine buffer ranged from 5 to 15%, while in apple juice the values declined up to 22%. In apple purée naturally contaminated with 29 mg/kg of PAT, exposure to a PL dose of 12 J/cm2 provoked a 51% reduction in PAT concentration, while no residual contamination was detected for higher irradiation times. These results suggested that PL treatment would be a potential alternative method to reduce PAT contamination in apple products. However, further investigations need to be conducted to evaluate toxicological safety of PAT degradation product(s). The results evidenced in this study are 1.0 fold lower than the data produced in our study.

4. Conclusion

The results evidenced in this study demonstrate that the bioactive compounds present in yellow and oriental mustard flours can reduce the growth of the strain of *Penicillium expansum*, PAT producer in wheat tortillas samples. No significative differences were observed on the reduction of PAT produced by the strain tested using the two different methodologies of the release of the ITCs. Also the results obtained by the kinetic of the ITCs volatilization demonstrate that

this isothiocyanates are stable in the bioactive packaging maintaining antifungal concentration during two months. The results obtained in this study could be considered of particular interest considering the potential application that those bioactive compounds could have in the industries to improve the shelf life of many food products.

Acknowledgements

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3.3. Reduction of the aflatoxins B_1, B_2, G_1 and G_2 in Italian piadina by isothiocyanates

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3.3. Reduction of the aflatoxins B_1, B_2, G_1 and G_2 in Italian piadina by isothiocyanates

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Abstract

Aflatoxins (AFs) are mycotoxins produced mainly by the molds *Aspergillus flavus*, *A. parasiticus* and *A. nomius*. These mycotoxins are contaminants of cereals. AFB_1, the most abundant and toxic metabolite, is known to cause several toxic responses, such as hepatotoxicity, teratogenicity and mutagenicity. Isothiocyanates (ITCs) are natural compounds produced by the enzymatic hydrolysis of glucosinolates (GLs), which have shown potent antimicrobial activity in food applications. In this study, ITCs derived from oriental and yellow mustard (0.1, 0.5 and 1 g of flour) were used to avoid the production of AFs in piadina (a typical Italian flatbread) contaminated with *A. parasiticus* CECT 2981. In addition, the antifungal activity of the ITCs toward *A. parasiticus* was also evaluated. The mustard flours employed in this study inhibited the growth of *A. parsiticus*, reducing the mycelium size by 12.2 to 80.6%, noticing that the oriental mustard flour was more active. The ITCs produced *in situ* also reduced the AFs biosynthesis in Italian piadina. In particular, the use of oriental mustard flour reduced the AFs content by 60.5 to 89.3%, whereas the reduction caused by yellow mustard flour ranged from 41.0 to 69.2%. Therefore, yellow and oriental mustard flour could be used as sources of ITCs in intelligent packaging systems to increase the shelf life and safety of piadina.

Keywords: Aflatoxins, oriental and yellow mustard flour, isothiocyanates, mycotoxin reduction, *Aspergillus* spp.

1. Introduction

Aflatoxins (AFs) are a group of mycotoxins produced mainly by *Aspergillus flavus, Aspergillus parasiticus* and *A. nomius* (Bayman & Cotty, 1993). These species are ubiquitous in nature and as saprophytes they grow on a wide variety of substrates, including decaying plant and animal debris. Aflatoxins are carcinogenic contaminants found in foods and animal feed that are frequently responsible for health and economic concerns in many countries. Aflatoxin B_1 is the most toxic metabolite among aflatoxins and presents hepatotoxic, teratogenic and mutagenic properties. It has been classified as a Class 1 human carcinogen by the International Agency for Research on Cancer (IARC, 2002).

Generally, agricultural commodities are naturally contaminated with *Aspergillus spp*. in the field and it may be difficult to completely prevent aflatoxin formation in these products (Kumar, Shukla, Singh, Prasad, & Dubey, 2008; Reddy, Reddy, & Muralidharan, 2009). In addition, aflatoxins are resistant to food processing and it is very difficult to mitigate them once they are present in foods and food ingredients (IARC, 2002). Presence of AFs is one of the main food-safety concerns in field crops, particularly in commodities that are produced in tropical and subtropical weather, where the high temperature and humidity promote the growth and proliferation of *Aspergillus spp*. Examples of crops that are frequently contaminated with AFs include rice (Bansal, Tam, Cavlovic, & Kwong, 2011; Makun, Dutton, Njobeh, Mwanza, & Kabiru, 2011), peanuts (Shank, Wogan, Gibson, & Nondasuta, 1972), beans (Pitt et al., 1994), herbs and spices (Adzahan, Jalili, & Jinap, 2009; Candlish et al., 2001; Colak, Bingol, Hampikyan, & Nazli, 2006), chillies (Paterson, 2007), processed spices (Cho et al., 2008), dried fruits (Trucksee & Scott, 2008), wheat (Riba, Bouras, Mokrane, Mathieu, Lebrihi, & Sabaou, 2010) and corn (Kim et al., 2013).

Glucosinolates (GLs) are bioactive metabolites (Manson et al., 1997)

present in plants of the Brassicaceae family, and their hydrolysis products possess antimicrobial properties against foodborne microorganisms (Luciano & Holley, 2009). More than 300 GLs have been described and they are cultivar-dependent (Tsao, Peterson, & Coats, 2002b). Sinigrin is the major GL found in oriental (*Brassica juncea*) and black (*Brassica nigra*) mustard, whereas sinalbin (SNB) is the major glucosinolate in yellow mustard (*Sinapis alba*) (Kushad et al., 1999). After hydrolysis, these GLs can generate allyl isothiocyanate (AITC) and ρ-hydroxybenzyl isothiocyanate (ρ-HBITC), respectively (Fig. 1).

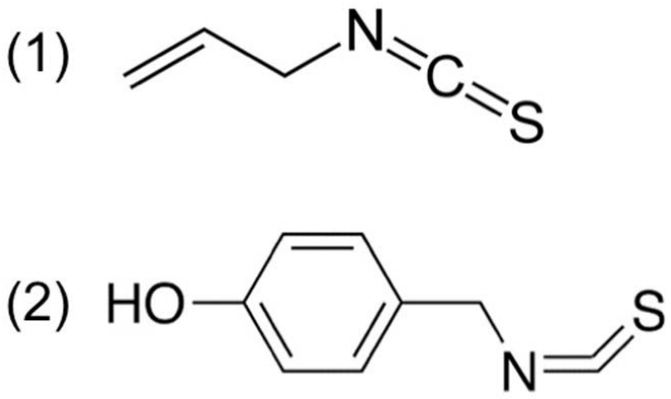

Figure 1. Molecular structure of the principal isothiocyanates formed in *Brassica juncea* and *Sinapis alba*, which are (1) allyl isothiocyanate and (2) ρ-hydroxybenzyl isothiocyanate, respectively.

ITCs exhibit biocidal activity against microorganisms including fungi (Nielsen & Rios, 2000) and bacteria (Luciano & Holley, 2010), as well as insects (Tsao, Yu, Potter, & Chiba, 2002a) and nematodes (Flemming, Turner, & Hunt, 2006). In particular, it has been demonstrated that AITC effectively inhibits the growth of a variety of pathogenic microorganisms at low concentrations (Lin, Preston, & Wei, 2000; Luciano & Holley, 2009). The potential of AITC as a natural

antimicrobial in different food matrices has been studied, including chicken breast (Shin, Harte, Ryser, & Selke, 2010), ground beef (Nadarajah, Han, & Holley, 2005), dry-cured ham (Graumann & Holley, 2007), fermented dry fermented sausage (Chacon, Muthukumarasamy, & Holley, 2006) and tuna meat (Hasegawa, Matsumoto, Hoshino, & Iwashita, 1999).

Italian piadina is a traditional flatbread that contains high-moisture (>15%) and is very susceptible to microbial, especially fungal, spoilage (Pittia & Antonello, 2016). In addition, mycotoxigenic fungi can potentially grow in this product, which represents a food safety concern.

The objectives of the present study were to observe the effect of volatile ITCs produced by the addition of water to oriental or yellow mustard flour against 1) the growth of *Aspergillus parasiticus* CECT 2681 *in vitro* and 2) the production of aflatoxins by this same microorganism inoculated on Italian piadina.

2. Materials and methods

2.1. Chemicals

AFs B_1, B_2, G_1, G_2, and sinigrin (98% purity), phosphate buffer saline (PBS) at pH 7, formic acid (HCOOH), AITC, tetrabutylammonium hydrogen sulfate (TBA), ammonium formate, and sodium chloride (NaCl) were obtained from Sigma–Aldrich (St. Louis, USA). Sinalbin and ρ-HBITC were gently provided by Prof. Alberto Ritieni of the University of Naples “Federico II”. Oriental and yellow mustard flours were provided by G.S. Dunn Dry Mustard Millers (Hamilton, Ontario, Canada). Methanol was purchased from Fisher Scientific (New Hempshire, USA). Deionized water (<18 MX 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. *Aspergillus parasiticus* CECT 2681 was obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain).

2.2. Glucosinolates extraction and determination from oriental and yellow mustard flours

GLs from oriental and yellow mustard flours were extracted using the method of Presteraet al. (1996) with modifications. Twenty grams of flour were introduced in a 50 mL glass tube and autoclaved at 115 °C during 15 minutes to inactivate the enzyme mirosinase (Luciano, Belland, & Holley, 2011). Then, samples were added with 200 mL of boiling distilled water in a 500 mL Erlenmeyer flask and the mixture was stirred for 10 min at 350 rpm. The mixtures were cooled at room temperature, centrifuged at 2500 rpm for 5 min at 4 °C and filtered through Whatman no. 4 filter paper into 50 mL screw-capped tubes. The extracts were filtered again through a 0.22 µm filter. Separation and quantification of GLs was performed using a Shimadzu LC system (Shimadzu, Japan), equipped with a Gemini C18 column (4.6 × 150 mm i.d. 5 µm; Phenomenex, Palo alto, CA). Elution was carried out isocratically for 20 min at a flow rate of 1 mL/min, using a solvent system containing 20% (v/v) acetonitrile and 80% water + 0.02 M tetrabutylammonium hydrogen sulfate (pH 5.5). The injection volume used was 20 µL. GLs were detected at 227 nm. All samples were filtered through a 0.22 mm syringe filter (Phenomenex) prior to injection (20 µl) into the column. Quantification of GLs was carried out by comparing peak areas of investigated samples to the calibration curve of sinigrin and sinalbin standards (1-100 mg/L).

2.3. Small-scale piadina experiments

Samples of Italian piadinas (10 g approximately) were individually introduced in multilayer polyethylene plastic bags for food packaging (Saplex, Barcelona,

Spain). The samples were treated with three different quantities of oriental and yellow mustard flour (0.1, 0.5 and 1 g) that were put in Petri dish bottoms and added with 2 mL of water to promote the activation of mirosinase, and formation of ITCs vapors. The Petri dish bottoms were placed inside the plastic bag together with the piadina samples. Then, samples were contaminated with 1 mL of *Aspergillus parasiticus* CECT 2681 grown in potato dextrose broth (PDB, Oxoid, UK) containing 10^6 conidia/mL. Conidial concentration was measured by optical density at 600 nm in sterile water and adjusted to 10^6 conidia/mL in PDB as reported Kelly, Grimm, Bendig, Hempel, & Krull (2006). The control group did not receive any treatment with mustard flour. The plastic bags were sealed and incubated at 23 °C for 15 days. Then, bags were opened and the samples were extracted for AFs quantification using liquid chromatography coupled to the mass spectrometry in tandem (LC-MS/MS).

2.4. Aflatoxin extraction

Extraction of aflatoxins was carried out using the method described by Liu et al. (2013) with a few modifications. Briefly, 5 g of finely ground piadina samples (Oster Classic grinder, Oster, Valencia, Spain) were weighed in a 50 mL plastic tube. Samples were added with 0.5 g of sodium chloride (NaCl) and 25 mL of a methanol/water mixture (80:20, V/V). Then, samples were extracted using an Ultra Ika T18 basic Ultraturrax (Staufen, Germany) for 3 min. The mixture was centrifuged at 4500 *g* for 5 min and the supernatant was evaporated to dryness with a Büchi Rotavapor R-200 (Postfach, Switzerland). The residue was re-dissolved in 1 mL of extraction solvent, filtered through a 0.22 µM filter and injected into the LC-MS/MS system.

2.5. LC-MS/MS aflatoxins identification and quantification

The liquid-chromatography analysis system consisted of a binary LC-20AD pump, a SIL-20AC homoeothermic auto-sampler, a CTO-20A column oven and a CMB-20A controller and an Analyst Software 1.5.2 was used for data acquisition and processing. The separation of AFs was performed on a Gemini NX C18 column (150x2.0 mm I.D, 3.0 µm; Phenomenex, CA, USA) at room temperature (20 °C). The mobile phase was composed of solvents A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile) at a flow rate of 0.2 mL/min. After 0.6 min of holding time, 10% of B reached 95% at 1.6 min and was kept constant for 0.3 min. Afterwards, the column was re-equilibrated with 10% solvent A until the end of the run at 4.0 min. An API-4000 triple-quadruple MS/MS system (Applied Biosystems, Foster City, CA, USA) equipped with ESI interface in positive mode was used for detection in multiple reactions monitoring (MRM) mode. The main MS parameters were optimized and finally set as follows: nebulizer gas (GS1), 55 psi; auxiliary gas (GS2), 50 psi; curtain gas (CUR) 15 psi; capillary temperature 550 °C; ion spray voltage (IS) 5500 V. Nitrogen was used as the nebulizer, heater, curtain and collision gas. The precursor-to-product ion transitions were m/z 313.3/241.3-228.5, m/z 315.3/259.0-288.4, m/z 329.7/243.3-200.5, m/z 331.9/189.3-217.1 for AFB_1, AFB_2, AFG_1 and AFG_2 respectively.

2.6. Effect of ITCs on Aspergillus parasiticus growth

Aspergillus parasiticus CECT 2681 employed in this assay was firstly inoculated on potato dextrose agar (PDA) medium for 7 days. Agar plugs (5 mm diameter) containing the fungal mycelium were removed from the margins of actively growing cultures and were transferred to commercially available Petri dishes (100 mm x 15 mm), which are physically divided in 2 halves (Insulab, Valencia, Spain). The inoculum was placed in the middle of the half-plate

containing PDA, while oriental or yellow mustard flour (0.1, 0.5 and 1 g) was added on the other side of the plate (Fig. 2).

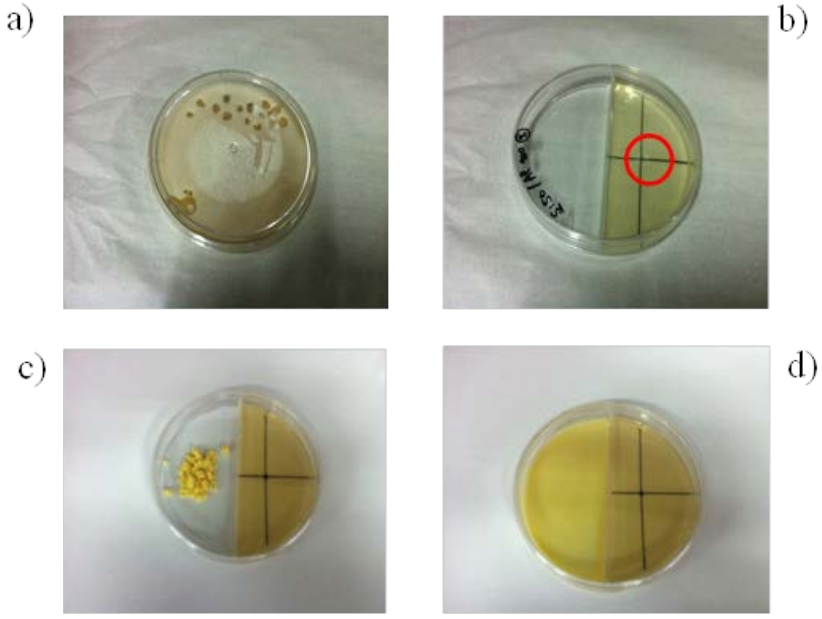

Figure 2. Schematization of the antifungal assay performed to test the antimicrobial activity of ITCs produced by the hydrolysis GLs present in yellow and oriental mustard flours toward the *Aspergillus parasiticus* CECT 2681. a) An agar plug was removed from a grown culture of *A. parasiticus*; b) the plug was introduced on top of the PDA located on the right side of the petri dish; c) the left side of the petri dish received different amounts of mustard flour; d) and it was added with water to promote myrosinase activation and, therefore, formation of gaseous ITCs.

The flours were mixed with 2 mL of water to promote the activation of myrosinase, and therefore, the conversion of GLs into ITCs. Immediately after the addition of water, plates were sealed with parafilm and incubated at 25 °C during 148h in the dark. The diameter of radial mycelial growth was determined after 24, 48, 100, 124 and 148 h. When mycelial growth was asymmetrical, four diameter

measurements were taken and averaged. The inhibitory effect on mycelia growth was calculated as the percentage of mycelium size inhibition comparing the treated and control fungi:

IG(%) = Dc- Dt/Dc*100;

where IG means growth inhibition, Dc is the mycelium diameter (mm) in control dishes and Dt is the mycelium diameter (mm) in treated dishes (Kurt, Gunes, & Msoylu, 2011).

3. Results and discussion

3.1. GLs determination in oriental and yellow mustard flours

Fig. 3 shows the LC-DAD chromatograms of the glucosinolates determined in the two flour matrices employed in this study. The oriental mustard flour is characterized for the presence of sinigrin with a retention time (RT) of 1.6 min, whereas the yellow mustard flour is contains sinalbin with a RT of 2.3 min. Concentrations of the GLs detected in the two matrices analyzed were 46.04 and 78.25 g/Kg, respectively. Sinigrin and sinalbin are the precursors of the antimicrobial compounds AITC and ρ-HBITC, respectively. Studies have shown that ITCs exhibit biocidal activity against microorganisms including fungi and bacteria, as well as insects and nematodes. In particular, it has been demonstrated that AITC effectively inhibits a variety of pathogenic fungi at low concentrations (Isshiki, Tokuoka, Mori & Chiba, 1992; Lin et al., 2000; Luciano & Holley, 2009; Mari, Leoni, Iori & Cembali, 2002; Nielsen & Rios, 2000).

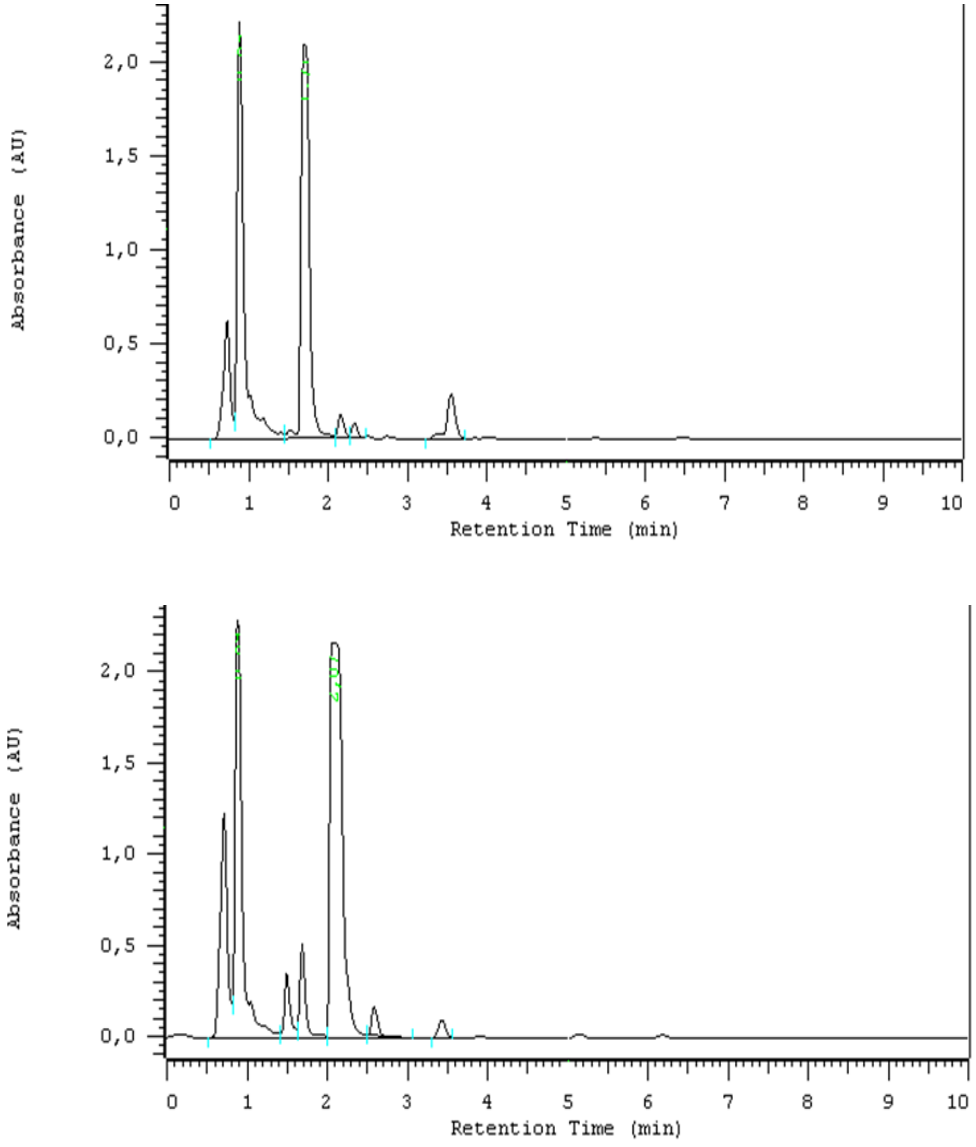

Figure 3. LC-DAD chromatograms of a) sinigrin (SNG) and b) sinalbin (SNB) present in the oriental and yellow mustard flour, respectively.

3.2. Inhibition of AFs production in Italian piadina

Inhibition of AFs B_1, B_2, G_1, and G_2 production in Italian piadina spiked with *Aspergillus parasiticus* was tested. Piadina is elaborated with wheat flour, water and olive oil in a pizza-like shape. Typically, this product suffers the contamination by *Aspergillus* and *Penicillium* strains during storage (Belz et al., 2012). In this study, *Aspergillus parasiticus* CECT 2981 was inoculated on piadina to naturally produce AFs. After inoculation, the product was introduced in a storage plastic bag and treated with three concentrations of yellow and oriental mustard flours as explained on Section 2.3. As shown on Fig. 4, inhibition of AFs production by ITCs produced by the mustard flours was dose-dependent and oriental mustard was more effective in inhibiting the production of AFB_1, with an average reduction of 78.6%. The highest reduction was achieved with the use of 1 g of mustard flour, which lowered AFB_1 production by 89.3% in comparison to the control group. Average reduction of AFB_2 and AFG_1 was 74.5%. Again, the use of 1 g of oriental mustard flour was the most efficient treatment, resulting in 82.7 and 87.3% reduction of AFB_2 and AFG_1, respectively. The results found for AFG_2 were similar to those found for AFG_1.

p-HBITC derived from yellow mustard flour also inhibited the production of AFs by *Aspergillus parasiticus* CECT 2981. The mean reductions of AFs belonging to the group B in piadina were 55.7 and 59.6% for AFB_1 and AFB_2, respectively. In addition, the yellow flour also presented a dose-dependent response with the highest reductions in AFs production when used at 1 g/package (Fig. 4). The average production of AFs belonging to the group G was reduced by 58.5%. The highest reductions were also observed when using 1 g of yellow flour, where AFG_1 and AFG_2 concentrations were lowered by 67.2 and 69.2%, respectively.

Comparing the AFs reduction data produced by the application of yellow mustard and oriental mustard, it is clear that the latter has much higher potential

to be applied as an anti-AFs agent. This phenomenon may be partially related to the stability of the isothiocyanates generated by both flours used. ρ-HBITC, which is formed in yellow mustard flour, is less stable than AITC (Luciano & Holley, 2009). Moreover, ρ-HBITC is much less volatile than AITC, which may set these antimicrobials apart when they are used as gaseous antimicrobial agents.

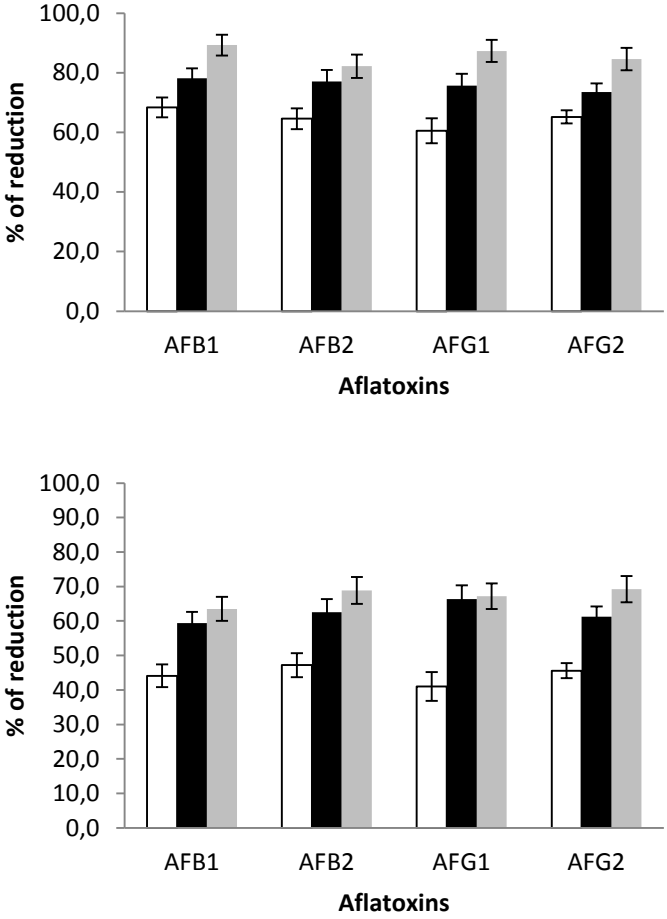

Figure 4. Reduction rates of AFs B_1, B_2, G_1, G_2 in Italian piadina contaminated with the strain of *Aspergillus parasiticus* CECT 2681 promoted by the presence of a) oriental and b) yellow mustard flour (white = 0.1 g, black = 0.5 g, grey = 1 g).

Other authors have used essential oils to inhibit the growth and, consequently, the production of aflatoxins in culture media and food products. Soliman and Badea (2002) studied the inhibitory activity of 12 essential oils derived from medicinal plants against *A. flavus*, *A. parasiticus*, *A. ochraceus* and *Fusarium moniliforme*. Anise, thyme and cinnamon essential oils were the most efficient in inhibiting the growth of all fugal species and when applied to wheat, 2% of these oils were necessary to completely inhibit fugal growth. In addition, it is important to highlight that anise and thyme essential oils were able to completely inhibit aflatoxins, ochratoxin A and fumonisins production when applied at 0.1% to wheat grains. Moreover, Kumar et al. (2008) also evaluated 14 odoriferous angiospermic essential oils against several fungal species, including a toxigenic strain of *Aspergillus flavus*. Thyme essential oil (*Thymus vulgaris* L.) showed highest antifungal efficacy and totally inhibited the mycelial growth of *A. flavus* at 700 µl/l. It also exhibited a broad fungitoxic spectrum against *Fusarium oxysporum*, *Cladosporium herbarum*, *Curvularia lunata*, *Aspergillus terreus*, *Aspergillus niger*, *Aspergillus fumigatus*, *Alternaria alternata* and *Botryodiploidia theobromae*, which are species implicated with food spoilage. The oil also showed significant antiaflatoxigenic efficacy and totally inhibited AFB_1 production when used at 600 µl/l.

Another study used the essential oil of *Zataria multiflora* to inhibit the growth and aflatoxin formation by *A. flavus* in culture media and white cheese (Gandomi et al., 2009). Although *Z. multiflora* essential oil completely inhibited the fungal growth in potato dextrose agar at levels ≥ 400 ppm, it was not able to inhibit the production of aflatoxin in cheese even at 1000 ppm. The main constituents of this essential oil were phenolic compounds such as carvacrol, thymol and eugenol.

Razzaghi-Abyaneh et al. (2008) also tested the essential oil of summer savory leaves (*Satureja hortensis* L.) against the production of AFB_1 and AFG_1 by *Aspergillus parasiticus* NRRL 2999 in potato dextrose broth. This essential oil not only inhibited the production of mycotoxins, but also reduced the fungal growth in a dose-dependent manner. Carvacrol and thymol were identified as the major components of the essential oil, and these could also reduce the levels of AFB_1 and AFG_1 when used individually. The calculated IC_{50} of carvacrol against AFB_1 and AFG_1 were 0.50 and 0.06 mM, respectively, whereas these levels were found as 0.69 and 0.55 mM for thymol. Similarly to the mustard essential oils (specially that derived from oriental mustard), these compounds could inhibit the production of AFs at very low concentrations. More recently, the aqueous extract of *Ocimum tenuiflorum* (holy basil) was used to reduce the levels of AFB_1 in rice (Panda & Mehta, 2013). The combination of the extract with high temperature (85 °C/4 h) was able to decrease the concentration of this mycotoxin by 74.7% *in vitro* and by 70.2% in rice. This was a direct detoxification and it was not dependent on fungi growth inhibition. Direct detoxification was also found when allyl isothiocyanate from oriental mustard was added to fumonisin and beauvericin-spiked foods (Azaiez, Meca, Manyes, & Fernández-Franzón, 2013; Meca, Luciano, Zhou, Tsao, & Mañes, 2012). Isothiocyantes are very reactive compounds (Zhang, 2004), and their electrophilic nature enables their reaction with thiol, amino and hydroxyl groups, forming conjugates, dithiocarbamate and thiourea structures (Cejpek, Valusek, & Velisek, 2000). Therefore, the direct reaction of isothiocyanates and AFs should also be tested as this may lead to direct detoxification of aflatoxin-contaminated food products.

3.3 Antifungal activity of oriental and yellow mustard flours in vitro

Yellow or oriental mustard flours were added with water to activate myrosinase and placed in the same environment as PDA inoculated with *Aspergillus parasiticus* (Fig. 2). The data related to the inhibition of the mycelial growth are presented on Table 1. Reduction of mycelial growth was directly proportional to the incubation time and quantity of mustard flour used. Yellow mustard flour inhibited the mycelial growth by 12.10 to 21.36% at 24 h, whereas the oriental mustard decreased the mycelial diameter by 48.2 to 60.4% at this same time point. Fungal growth inhibition was more pronounced at longer incubation times with the highest reductions observed at 148h, where the fungal diameter was inhibited by 30.15% with 1 g of yellow mustard and by 80.6% with 1 g of oriental mustard flour. Similarly to the results observed for the effect of these flours in the production of AFs in piadina, the gas generated from the oriental mustard flour was much stronger in affecting *Aspergillus* growth than the volatile products derived from the yellow flour. Again, this may be explained by higher volatility of allyl isothiocyanate in comparison to ρ-hydroxybenzyl isothiocyanate (Luciano & Holley, 2010).

This article can be considered the first where the antifungal activity of gaseous ITCs formed from food matrices *in situ* was tested on an *Aspergillis parasiticus* strain. This could be used as a strategy to avoid the growth of this and other mycotoxinogenic species in several food products. Other authors have used pure isothiocyanates and extracts of *brassica* plants to inhibit the growth of several fungi. In special, Dhingra, Jham, Rodrigues, Silva, and Costa (2009) have used synthetic mustard oil to inhibit the growth of *Aspergillus glaucus* and *A. parasiticus* in groundnuts. The products were fumigated with the oil at 100 ppm and stored at controlled room temperature (25 °C) for 90 days. The oil was able to keep the population of both *Aspergillus glaucus* and *A. parasiticus* below the

inoculation level during the entire period of storage when the moisture content was kept below 8.2%, whereas non-fumigated groundnuts presented fungal growth after 15 days. Moreover, mustard oil treatment was also able to significantly reduce the rate of fungal growth at higher moisture content (up to 10.5%). Tunc, Chollet, Chalier, Preziosi-Belloy, and Gontard (2007) also evaluated the effect of AITC against *Penicillium notatum* growth at 30 °C. The oil presented a minimum inhibitory concentration (MIC) of 3.8 mmol/L of air. Since AITC is a very pungent compound, the authors also tested the possible synergistic effect of AITC and other antimicrobial gases in order to reduce the sensorial impact that this oil may have in food products. Synergism was found for the combination of AITC with sulfur dioxide and cinnamaldehyde. The present study did not evaluate the influence of the gases formed from oriental and yellow mustard flours on the taste and aroma from piadina, but it is predicted to be very low. As presented on Section 3.1, the oriental mustard flour contained 46.04 g of sinigrin/Kg, while the yellow mustard flour had 78.25 g of sinalbin/Kg. Considering an ideal situation where the whole GLs content would be converted in ITCs, these flours would form 11.48 ng of AITC/g and 30.39 ng of ρ-HBITC/g, respectively. In this study, these quantities would result in a maximum of 0.146 and 0.387 ppm of AITC and ρ-HBITC, respectively, after total volatilization. Nielsen and Rios (2000) have shown that the taste and aroma of AITC were only recognized at concentrations higher than 2.4 ppm (gaseous phase) in rye bread and 1.8-3.5 ppm in hot dog bread. However, further studies are necessary to evaluate the maximum AITC and ρ-HBITC gaseous concentrations that can be used to treat piadina without negatively impacting its sensory profile.

Table 1. *Aspergillus parasiticus* CECT 2681 mycelia growth inhibition (%) produced by ITCs generated by glucosinolates present in oriental and yellow mustard flours.

		Incubation time (h)				
Strain	**Mustard flour (g)**	**24**	**48**	**100**	**124**	**148**
Aspergillus parasiticus CECT 2681	Yellow 0.1	12.10±0.5	17.25±0.8	20.16±0.7	22.47±0.4	25.47±1.3
	Yellow 0.5	15.36±0.3	21.36±2.1	24.55±0.8	26.47±0.5	29.31±1.2
	Yellow 1.0	21.36±1.1	24.56±2.2	26.74±1.1	28.51±0.8	30.14±1.1
	Oriental 0.1	48.25±2.2	53.88±2.6	58.69±3.3	64.25±3.7	68.46±3.2
	Oriental 0.5	52.26±3.4	55.36±2.5	60.14±3.5	65.69±3.1	71.04±2.9
	Oriental 1.0	60.47±3.9	64.01±3.3	70.36±3.1	74.25±3.3	80.69±3.3

4. Conclusion

The present study showed the capacity of the AITC and ρ-HBITC to reduce the concentration of AFs naturally produced in wheat piadina by *Aspergillus parasiticus*. In addition, ITCs were able to reduce the mycelial growth of the mycotoxigenic fungus *in vitro*. This study shows that these compounds may be formed *in situ* through the addition of water to inexpensive products such as mustard flour, and be used as natural preservatives for bakery products and other foods that are commonly contaminated by *Aspergillus* species. Further studies will investigate the possible development o a bioactive sachet that could be included inside the packed product that promotes the controlled vaporization of the AITC through GLCs myrosinase conversion, increasing the shelf life of the treated product. Also the reaction between the ITCs and AFs, and the impact of ITCs on the sensory properties of piadina will be studied.

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3.4. Shelf life improvement of the loaf bread using allyl, phenyl and benzyl isothiocyanates against Aspergillus parasiticus

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3.4. Shelf life improvement of the loaf bread using allyl, phenyl and benzyl isothiocyanates against *Aspergillus parasiticus*

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Abstract

Fungal growth inhibition and aflatoxins (AFs) reduction using allyl (AITC), benzyl (BITC) and phenyl (PITC) isothiocyanates were studied in loaf bread contaminated with *Aspergillus parasiticus*. Two inoculated loaf bread slices were introduced into a plastic tray together with paper filters or small plastic bags paper filters soaked with AITC, BITC or PITC, the final concentration inside the package was of 0.5, 1 or 5 µL/L. The plastic trays, incubated at room temperature, were visual examined for the shelf life evaluation during 8 days. The quantification of the AFs was carried out using liquid chromatography coupled to mass spectrometry (LC-MS/MS). Shelf life increase of three and four days was obtained with small plastic bag paper filter and paper filter soaked with AITC 5 µL/L, respectively. These treatments also showed the highest reductions of AFs. All treatments with small plastic bag paper filter significantly reduced the content of AFs at percentages above 60% except the reduction of AFB_1 in the samples treated with BITC 0.5 µL/L and PITC 1 µL/L. The AFs reduction observed in the packaging with paper filter were above 60% only using AITC at the concentrations of 1 and 5 µL/L.

Keywords: A*spergillus spp*., Loaf bread, Isothiocyanates, Shelf life improvements, aflatoxin reduction.

1. Introduction

Aflatoxins (AFs) are a group of natural food toxins which are recognized as toxic and carcinogenic secondary metabolites mainly produced by certain strains of *Aspergillus flavus, Aspergillus paraciticus* and *Aspergillus nomius* (Iqbal, Mustafa, Asi, & Jinap, 2013). AFs are found as contaminants in various agricultural commodities including bread, corn, peanut, cottonseed, Brazil nut, pistachio nut, fig, spices and copra (El-tawila, Neamatallah, & Serdar, 2013). The International Agency for Research on Cancer (IARC) has classified AFB_1 as a group I carcinogen which primarily affects the liver (IARC, 2002; Iqbal, Asi, & Jinap, 2014).

Acute exposure to AFs can cause aflatoxicosis, and in severe hepatotoxicity cases the mortality rate is approximately 25%. Chronic exposure to AFs is associated with hepatocellular carcinoma, especially in the presence of hepatitis B infection. Other probable health impacts are immunological suppression, impaired growth and nutritional interference (Strosnider et al., 2006). These impacts have been demonstrated in various species of livestock and fish, and while they may have similar effects on humans, causal evidence is still lacking.

In bakery products, preservatives (salts of propionic and sorbic acids) are added to prevent growth of spoilage fungi. However, in recent years, there has been consumer pressure to reduce the use of such preservatives. Previous studies have suggested that the use of suboptimal concentrations of these preservatives may stimulate the growth and AFs production of some spoilage fungi of bread (Arroyo, Aldred, & Magan, 2005). Thus, suboptimal doses could pose a problem and allow mould spoilage to occur. In the last decade, however it has increased the interest for biological methods to prevent the fungal growth using lactic acid bacteria (LAB) or natural antimicrobial compounds (Gerez, Torino, Obregozo, & Font de Valdez, 2010; Ryan, Dal Bello, & Arendt, 2008; Ryan et al., 2011).

Bread is known as a high moisture product with a_w values between 0.96 and 0.98 (Smith, Daifas, El-Khoury, Koukoutsis, & El-Khoury, 2004). As demonstrated by Doerry (1990), microbial spoilage is the main cause for shelf-life in intermediate and high moisture food products. Nowadays, mould growth is still a cause of high losses to the bread-producing industry (Pateras, 2007; Smith et al., 2004).

Glucosinolates are metabolites found in plants belonging to the family *Brassicaceae* (Nielsen & Rios, 2000). These compounds are located within vacuoles and are released when the plant suffers mechanical damage (e.g. wounding, cutting). Once in the cytoplasm, they are hydrolyzed by the enzyme myrosinase (EC 3.2.1.147), resulting in the formation of three main groups of substances: nitriles, thiocyanates and isothiocyanates (ITCs) (Delaquis & Mazza, 1995; Luciano & Holley, 2009). The last group contains diverse compounds with strong antimicrobial activity and they have been added to various food products (Lin, Preston & Wei, 2000; Obaidat & Frank, 2009). Food processing may influence the production of ITCs in different way bringing glucosinolates into contact with myrosinase, degrading them, leaching them or preserving them by thermal inactivation of the enzyme responsible of the conversation (Deng, Zinoviadou, Galanakis, Orlien, Grimi et al., 2014). Allyl, benzyl and phenyl isothiocyanates (AITC, BITC and PITC) have been previously studied due to their antibacterial (Wilson et al., 2013) and antifungal properties (Smolinska, Morra, Knudsen & James, 2003). These compounds are strong electrophilic reagents and can react easily with nucleophiles such as amines, amino acids, alcohols, water, and sulfites during food treatment and under physiological conditions (Cejpek, Valusek, & Valisek, 2000). Recently, it has been found that AITC was able to react with the aminic groups of BEA in aqueous solutions and foods (Azaiez, Meca, Manyes, & Fernandez-Franzón, 2013a; Meca, Luciano, Zhou, Tsao, & Mañes, 2012).

The aims of this study were: a) to study the antifungal activity of AITC, BITC and PITC against *Aspergillus parasiticus* in an *in vitro* model, b) to evaluate the potential industrial application of these compounds as preservative of loaf bread against *A. parasiticus* spoilage, evaluating the shelf life and AFs production using two different bioactive compounds devices as the sticker and the filter.

2. Materials and methods

2.1. Chemicals

AFB_1, AFB_2, AFG_1, AFG_2, formic acid (HCOOH), AITC, BITC and PITC, ammonium formate, and sodium chloride (NaCl) were obtained from Sigma-Aldrich (St. Louis, USA). HPLC-MS grade methanol was purchased from Fisher Scientific (New Hempshire, USA). Deionized water (<18 MX 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. The strain of *A. parasiticus* CECT 2681 was obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain). Potato dextrose agar (PDA) and potato dextrose broth (PDB) were provided by Oxoid (Madrid, Spain).

2.2. Antifungal activity of AITC, PITC, and BITC in liquid medium

The fungal strain of *A. parasitcus* was cultured in PDB and the antifungal activity was assessed with the method of Bolivar et al. (2011) with some modifications (Mikiciuk, Mikiciuk & Szterk, 2016a; Mikiciuk, Mikiciuk, Wronska & Szterk, 2016b). AITC, BITC and PITC oils were dissolved in water with 2% of DMSO to facilitate compounds solution and to obtain concentrations ranging from 10 to 15000 mg/L.

For the antifungal tests, 9.8 mL of PDB were added to screw-capped

tubes, followed by 0.1 mL *A. parasiticus* (OD~0.6). After the inoculum was added, the tubes were treated with 0.1 mL of the ITCs solution obtaining final concentrations that ranged from 0.1 to 150 mg/L. Control groups contained 9.9 mL of PDB with water at 2% dimethyl sulfoxide and 100 µL of inoculum. The tubes were incubated for 48h and 72h at 30 °C under orbital shaking to calculate Minimum Inhibotory Concentration (MIC) and Minimum Fungicidal Concentration (MFC), respectively. MIC and MFC are defined as the lowest concentration of an antimicrobial compound that will inhibit the visible growth of a microorganism after 48 and 72h of incubation, respectively. After that, 100 µL of each tube were inoculated in PDA Petri dishes and incubated at 30 °C during 48h. The cells colonies were also counted to perform the viability curve of the microorganism exposed at the different concentration of the ITCs. All the experiments were performed in triplicate.

2.3. Samples preparation and antifungal treatment

The recipe for loaf bread preparation: 600 g of wheat flour, 20 g of sucrose, 10 g of NaCl, 40 g of yeast for bakery products (Levital, Spain) and 350 mL of water. The ingredients were kneaded manually for 5 min and the dough produced was left rising for 1 h at room temperature. Baking was performed at 200°C for 40 min in a deck oven (MIWE, Arnstein, Germany). The oven was presteamed (300 mL of water) before loading. The breads were kept for 30 min on cooling racks at room temperature. Breads were cut in slices of 30 g each.

Each slice was inoculated in 9 spots with 100 µL of a suspension containing $1x10^7$ conidia/mL *A. parasiticus* CECT 2681. Conidial concentration was measured by optical density at 600 nm and adjusted to 10^7 conidia/mL in PDB as reported Kelly, Grimm, Bendig, Hempel, and Krull (2006). Inoculated bread slices

received two different antimicrobial treatments to test respectively a rapid and a controlled release of the volatile active compounds:

1. Two inoculated bread slices were introduced in the plastic tray together with paper filters (2.5 x 2.5 cm) soaked with AITC, BITC and PITC giving a final concentration of 0.5, 1 or 5 µL/L of the essential oils inside the package.

2. Two inoculated bread slices were introduced in the plastic tray together with small plastic bags paper filters (2.5 x 2.5 cm) soaked with AITC, BITC and PITC, giving a final concentration of 0.5, 1 and 5 µL/L of the essential oils inside the package.

A control group without antimicrobial treatment was used for each set of assays. All the experiments were performed in triplicate. A total of 60 plastic trays, correspondent to 120 slices, were used for this experiment. All plastic trays were closed hermetically and incubated at room temperature during 10 days. During that time each day the bread slices were examined to control the visible fungal growth and to establish the effect of the treatment on the shelf life. Then, all packages were opened and samples analyzed for AFs using liquid chromatography coupled to mass spectrometry in tandem (LC-MS/MS).

2.4. Aflatoxin extraction

AFs extraction was performed using the method described by Hontanaya, Meca, Luciano, Mañes, and Font (2015). Briefly, the two bread slices were finely grounded with a blender (Oster Classic grinder, Oster, Valencia, Spain) and 5 g samples were placed in a 50mL plastic tube. Then, 0.5 g of sodium chloride (NaCl) and 25 mL of a methanol/water (80:20, V/V) mixture were added. Samples were homogenized using Ultra Ika T18 basic Ultraturrax (Staufen, Germany) for 3 min. The mixture was centrifuged at 4500 x *g* for 5 min and the supernatant was evaporated to dryness with a Büchi Rotavapor R-200 (Postfach, Switzerland). The

residue was re-dissolved in 1 mL of extraction solvent, filtered through a 0.22 µM syringe filter and injected to the LC-MS/MS system.

2.5. AFs identification and quantification by LC-MS/MS

LC-MS/MS analyses were performed with a system consisting of an Agilent 1200 chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled to a 3200QTRAP mass spectrometer (Applied Bio-systems, AB Sciex, Foster City, CA, USA) equipped with a turbo ionspray electrospray ionisation (ESI) interface. The instrument data were collected and processed using the Analyst version 1.5.2 software. Separation of analytes was performed using a reversed-phase analytical column (Gemini C18 column, 150 X 2 mm, I.D. 3 µm particle size), equipped with a security guard cartridge C18 (4 X 2 mm, I.D.; 5 µm) all from Phenomenex, Madrid, Spain. The mobile phases were composed of two eluents, both containing 5 mM ammonium formate (Sigma-Aldrich, St. Louis, USA), the eluent A was water + 0.1% formic acid and the eluent B methanol + 0.1% formic acid. The elution gradient was established initially with 10% eluent B, increased to 80% in 1.5 min and kept constant during 2.5 min. The eluent B was increased to 90% in 6 min and then 100% in 4 min. Afterwards, the initial conditions were maintained for 5 min. The flow rate was 0.25 mL/min. MS/MS analysis was achieved in the selected reaction monitoring (SRM) mode using ESI in positive mode. For LC-MS/MS analysis, scheduled SRM was used with a 120 s SRM detection window and 1 s of target scan time. The applied parameters were: ion spray voltage, 5500 V; source temperature, 450 °C; curtain gas, 20; ion source gas 1 (sheath gas), 50 psi; ionsource gas 2 (drying gas), 55 psi. Nitrogen served as nebulizer and collision gas. The ionization and fragmentation parameters used for the detection and quantification of the AFs were set according to Liu et al. (2013).

3. Results and discussion

3.1. Antifungal activity of AITC, BITC and PITC

The data related to the growth inhibition induced by AITC, BITC and PITC on *A. parasiticus* in liquid medium are shown in Fig. 1. All the three ITCs resulted active against the *A. parasiticus* and reduction of fungal growth and AFs were proportional to the concentrations of the bioactive compounds: % of viability decreased with increasing concentration of the antimicrobial. Fungal growth is higher after 72 h than 48 h because the fungi have more time to develop. The viability is lower than 50% using AITC, BITC or PITC with concentrations ≥ 0.5 mg/L. MIC and MFC values, defined respectively as the lowest concentration of an antimicrobial that inhibits the growth or kills the microorganism, were calculated for each compound (Table 1). AITC and BITC showed the lowest MIC of 20 mg/L, while the MIC obtained for PITC was 50 mg/L. On the other side, BITC was the active compound with the lowest MFC (20 mg/L), while AITC and PITC showed a MFC value of 50 mg/L.

Applications of ITCs as antimicrobial substances have been tested by several authors. Azaiez et al. (2013a) studied the efficacy of AITC, BITC and PITC as antimicrobials against three mycotoxigenic strains of *Gibberella moniliformis* on solid medium. The ITCs employed inhibited the growth of the three strains, reducing 2.1-89.7% of the mycelium size. The reduction resulted proportional to the incubation time employed but no to the quantities of the bioactive compounds tested. BITC was the compound with the lowest inhibitory activity on the three mycotoxigenic strains.

Mejía-Garibay, Palou, and López-Malo (2015) quantified the antimicrobial activity of the essential oil (EO) of black mustard (*Brassica nigra*) when applied by direct contact into the liquid medium or by exposure in the vapor phase against *Aspergillus niger, Aspergillus ochraceus,* or *Penicillium citrinum* (in laboratory

media or in a bread-type product).

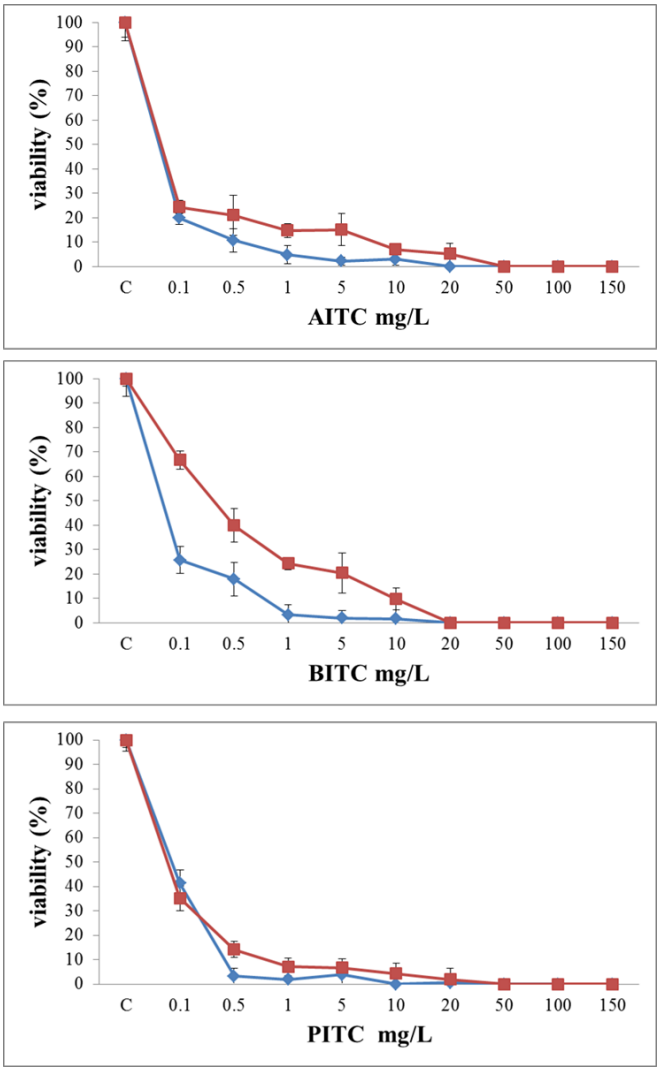

Figure 1. Viability curves of *Aspergillus parasiticus* exposed at different concentrations of AITC, BITC and PITC at 48h (blue) and 72h (red) of incubation.

Table 1. Minimum inhibitory concentration and minimum fungicidal concentration evidenced by AITC, BITC and PITC on *Aspergillus parasiticus*.

Compound	*A. parasiticus*	
	MIC (mg/L)	MFC (mg/L)
AITC	20	50
BITC	20	20
PITC	50	50

AITC is the most abundant compound of *B. nigra* EO (more than 98% of the identified components). Black mustard EO applied by direct contact into the liquid medium inhibited the growth of *A. ochraceus* and *P. citrinum* using 2 µL/mL (MIC), while for *A. niger,* the MIC of *B. nigra* EO was 4 µL/mL. Mold exposure to 41.1 µL/L of *B. nigra* EO in vapor phase showed that *P. citrinum* and *A. niger* growth was delayed 10 days, while *A. ochraceus* growth was delayed 20 days. Exposure to concentrations ≥47 µL of *B. nigra* EO per liter of air (MIC) inhibited the growth of the tested molds to 30 days, and they were not able to recover after further incubation time into an environment free of EO due to the fungicidal effect. Mustard EO was also effective against the previous molds inhibiting their growth for 30 days in a bread-type product by vapor contact.

Manyes, Luciano, Mañes, and Meca (2015) tested the antifungal activity of AITC against *Aspergillus parasiticus* and *Penicillium expansum* employing solid medium. The *P. expansum* strain was inhibited with >50 mg of AITC after 20 days incubation, whereas the strain of *A. parasiticus* was sensible to AITC doses >5 mg.

Janatova et al. (2015) tested the antifungal activity of seven volatile EO components from plants against *Aspergillus niger*: AITC, carvacrol, cinnamaldehyde, diallyl disulfide, eugenol, thymol, and thymoquinone. The evaporation rate of these compounds pure and encapsulated was also determined. In this experiment AITC appeared to be almost ineffective and only

slightly effective when encapsulated. The evaporation rate assay confirmed that AITC had completely evaporated during the first day. The difference from previous studies, in which the AITC resulted highly effective, could be explained by the lack of restriction of air circulation in petri dishes. AITC probably escaped before antifungal activity was produced.

3.2. Shelf life improvement of loaf bread treated with AITC, PITC and BITC and mycotoxin reduction

After evaluation of AITC, BITC and PITC antimicrobial activity in the *in vitro* model, their capability to inhibit fungal growth and reduce AFs production was tested in a food system. It is important to underline that AITC have been granted GRAS status according to the U.S. Food and Drug Administration (FDA, 2005) for its use as a shelf-life extension agent or antispoilage agent in food. In Japan, AITC is permitted for use as a preservative and AITC-containing antimicrobial films are commercially available (Lee, 2005). In Europe, The Panel on Food Additives and Nutrient Sources added to Food (ANS) provides a scientific opinion on the safety of AITC when used as a food preservative that deals with the safety of AITC for the proposed uses as a food additive (preservative) (EFSA, 2010). The Panel derived an ADI of 0.018 mg/kg bw/day which was rounded up to 0.02 mg/kg bw/day based on a LOAEL of 9 mg/kg bw/day (EFSA; 2010). No scientific opinions and no restrictions about the use of BITC and PITC have already been emitted.

The shelf life improvement of the samples treated with AITC, BITC and PITC is showed in Table 2. In the control experiment (loaf bread without antimicrobial treatment) the growth of *A. parasiticus* resulted visible after four days of incubation, while in the treatment with paper filter and the small plastic bag paper filter soaked with AITC 5 µL/L the growth started after eight and seven days of incubation, showing an improvement of the shelf life of four and three days,

respectively (Fig. 2). All the other treatments did not extend the shelf life of the samples.

Figure 2. Visual observation of the loaf breads contaminated with *Aspergillus parasiticus* at six days of incubation, in the a) control experiment where is clearly visible the presence of the fungal contamination, b) and c) the loaf breads packaged with paper filter and small plastic bag paper filter soaked with AITC 5µL/L, respectively, where is possible to observe the absence of the mycotoxigenic fungi.

Considering that the strain of the *A. parasiticus* used in this study was AFs producer, the studied loaf breads were extracted for AFs detection and quantification using the LC-MS/MS. As evidenced in Table 3, the two treatments that showed an improvement of the shelf life produced the highest reductions of the AFs production. In particular, the reduction levels ranged from 91.1 to 94.4% in the breads treated with AITC 5 µL/L on paper filter and from 89.9 to 100.0% for AITC 5 µL/L small plastic bag paper filter considering all the four aflatoxins. Using the paper filter treatments, the % of reduction ranged from 5.3 to 91.8% for AFB_1, from 7.2 to 94.0 % for AFB_2, from 0.6 to 91.1 % for AFG_1 and from 7.8 to 94.4% for AFG_2. All treatments tested employing small plastic bag paper filter significantly reduced the content of AFs with percentages ranging from 78.5 to 100.0% using AITC, from 59.0 to 89.7 using BITC and from 57.9 to 82.7% using PITC. The percentage of reduction ranged between 57.9 and 89.9% for AFB_1, between 66.3 to 100% for AFB_2, between 60.4 and 91.1 for AFG_1 and from 71.1 to 98.1 for AFG_2.

Table 2. Shelf life, expressed in days, of the loaf breads contaminated with *Aspergillus parasiticus* and packaged together with a) paper filters soaked with AITC, BITC and PITC and b) small plastic bag paper filters soaked with AITC, BITC and PITC (+ = Loaf breads contaminated with visible colonies of *Aspergillus parasiticus* and, - = Loaf breads without any visible sign of *Aspergillus parasiticus* growth).

a)

Treatment	Incubation time (days)							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Control	-	-	-	+	+	+	+	+
AITC 0.5 µL/L	-	-	-	+	+	+	+	+
AITC 1 µL/L	-	-	-	+	+	+	+	+
AITC 5 µL/L	-	-	-	-	-	-	-	+
BITC 0.5 µL/L	-	-	-	+	+	+	+	+
BITC 1 µL/L	-	-	-	+	+	+	+	+
BITC 5 µL/L	-	-	-	+	+	+	+	+
PITC 0.5 µL/L	-	-	-	+	+	+	+	+
PITC 1 µL/L	-	-	-	+	+	+	+	+
PITC 5 µL/L	-	-	-	+	+	+	+	+

b)

Treatment	Incubation time (days)							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Control	-	-	-	+	+	+	+	+
AITC 0.5 µL/L	-	-	-	+	+	+	+	+
AITC 1 µL/L	-	-	-	+	+	+	+	+
AITC 5 µL/L	-	-	-	-	-	-	+	+
BITC 0.5 µL/L	-	-	-	+	+	+	+	+
BITC 1 µL/L	-	-	-	+	+	+	+	+
BITC 5 µL/L	-	-	-	+	+	+	+	+
PITC 0.5 µL/L	-	-	-	+	+	+	+	+
PITC 1 µL/L	-	-	-	+	+	+	+	+
PITC 5 µL/L	-	-	-	+	+	+	+	+

Table 3. AFs reduction evidenced in the loaf breads contaminated with *Aspergillus parasiticus* and treated with AITC, BITC and PITC on a) soaked paper filters and b) small plastic bag paper filters.

a)

Treatment		% of Reduction			
		AFB_1	**AFB_2**	**AFG_1**	**AFG_2**
AITC	0.5 µL/L	38.9 ± 4.4	41.0 ± 3.6	45.6 ± 4.2	34.5 ± 3.0
AITC	1 µL/L	77.3 ± 2.3	79.5 ± 4.3	80.8 ± 1.7	87.6 ± 0.9
AITC	5 µL/L	91.8 ± 1.7	94.0 ± 2.7	91.1 ± 4.1	94.4 ± 2.7
BITC	0.5 µL/L	7.9 ± 0.5	7.2 ± 3.7	0.6 ± 1.2	7.8 ± 5.9
BITC	1 µL/L	34.6 ± 4.2	32.5 ± 4.6	24.8 ± 5.6	29.3 ± 2.6
BITC	5 µL/L	48.6 ± 3.8	47.0 ± 1.0	44.0 ± 4.6	48.9 ± 1.7
PITC	0.5 µL/L	5.7 ± 3.4	8.4 ± 5.2	26.2 ± 2.2	17.0 ± 3.9
PITC	1 µL/L	5.3 ± 3.3	27.7 ± 2.3	28.5 ± 2.2	33.9 ± 3.8
PITC	5 µL/L	8.7 ± 1.6	21.7 ± 0.6	32.0 ± 0.6	26.1 ± 0.5

b)

Treatment		% of Reduction			
		AFB_1	**AFB_2**	**AFG_1**	**AFG_2**
AITC	0.5 µL/L	78.5 ± 0.5	86.7 ± 4.7	85.8 ± 0.5	88.3 ± 4.0
AITC	1 µL/L	84.7 ± 0.6	88.0 ± 6.3	87.0 ± 0.6	90.7 ± 1.8
AITC	5 µL/L	89.9 ± 1.9	100.0 ± 0.9	91.1 ± 2.1	98.1 ± 2.8
BITC	0.5 µL/L	59.0 ± 8.9	66.3 ± 6.1	60.4 ± 2.5	71.1 ± 1.2
BITC	1 µL/L	73.9 ± 4.7	75.9 ± 6.9	77.7 ± 5.7	84.1 ± 3.4
BITC	5 µL/L	86.1 ± 1.4	89.2 ± 3.1	86.1 ± 2.0	89.7 ± 2.9
PITC	0.5 µL/L	66.6 ± 1.4	72.3 ± 4.5	74.2 ± 2.1	72.3 ± 4.3
PITC	1 µL/L	57.9 ± 3.1	71.1 ± 5.1	68.3 ± 4.8	72.8 ± 3.0
PITC	5 µL/L	69.9 ± 2.7	79.5 ± 4.4	79.3 ± 5.0	82.7 ± 3.1

All these reduction levels were above 60% except the reduction of AFB1 in the samples treated with BITC 0.5 µL/L and PITC 1 µL/L. On the other hand, the AFs reduction observed in the packaging with paper filter was above 60% only using AITC at the concentrations of 1 and 5 µL/L. Active packaging with ITCs has been studied by other authors. Quiles et al. (2015) tested active packaging devices containing AITC or oriental mustard flour (OMF) to inhibit the growth of *A. parasiticus* and AFs production in fresh pizza crust after 30 days, obtaining similar results to our study. The antimicrobial and anti-aflatoxin activities were compared to a control group (no antimicrobial treatment) and to a group added with commercial preservatives (sorbic acid + sodium propionate). *A. parasiticus* growth was only inhibited after 30 days by AITC in filter paper at 5 µL/L and 10 µL/L, AITC sachet at 5 µL/L and 10 µL/L and OMF sachet at 850 mg + 850 mL of water. AFs production was inhibited by all antimicrobial treatments in a dose-dependent manner. More importantly, AITC in a filter paper at 10 µL/L, AITC sachet at 10 µL/L, OMF sachet at 850 mg + 850 mL of water and sorbic acid + sodium propionate at 0.5 and 2.0 g/Kg completely inhibited AFs formation.

Nazareth, Bordin, Manyes, Meca, Manes, and Luciano (2016) evaluated the capacity of gaseous AITC in inhibiting the production of AFs by *A. parasiticus* in wheat flour. Petri dish lids filled with 2 g of wheat flour were inoculated with 10^4 conidia/g of *A.parasiticus* placed in a 1 L mason jar. AITC was added at 0.1, 1 or 10 µL/L in the gaseous phase. Ten µL/L AITC totally inhibited the production of AFs while 0.1 and 1 µL/L were able to inhibit AFs production in up to 23.0% and 52.3%, respectively.

Otoni, Soares, Silva, Medeiros, and Baffa Junior (2014) evaluate the antifungal effect of AITC against another strain of *Aspergillus* AFs producer, *A. flavus*, developing an AITC-containing sachet (similar to small plastic bag paper filters employed in our study) to control *A. flavus* sporulation in peanuts during a

90 day period. The differences between then survival of A. flavus in the control and AITC-treated peanuts were remarkable. AITC-containing sachets were able to reduce the *A. flavus* cell count by four log cycles after a month of storage at 25°C, a temperature that simulates the commercial distribution and storage processes of peanuts.

AITC, BITC and PITC have been also employed in paper filter inserted into jar of 1 L at final concentrations of 50, 100, 500 µL/L to reduce FB_2 present in loaf bread contaminated with *Gibberella moniliformis* (Azaiez et al., 2013a). All ITCs showed similar reduction profile and the mean FB_2 reduction after 24h incubation was 84.9%. AITC at 500 µL/L presented the highest reduction (95.77%) whereas the lowest occurred with the 50 µL/L PITC treatment.

Moreover the same concentrations of AITC (50, 100, 500 µL/L) were tested by Azaiez, Meca, Manyes, Luciano, and Fernández-Franzón (2013b) to reduce FBs in corn kernels and corn flour contaminated with *Gibberella moniliformis*. ITC fumigation treatment was able to reduce 53 to 96% of FB_1, 29 to 91% of FB_2 and 29 to 96% of FB_3. The higher degradation rates of FBs were found with higher doses of ITCs.

Nielsen & Rios (2000) tested volatile EO and oleoresins (OL) from spices and herbs against a range of fungi commonly found on bread: *Penicillium commune*, *P. roqueforti*, *Aspergillus flavus* and *Endomyces fibuliger* were assayed on hot-dog bread and rye bread. *A. flavus* was more resistant than the other microorganisms while *P. roqueforti* was the most sensitive. Results of sensory evaluation showed, that hot-dog bread was more sensitive to AITC than rye bread. The minimal concentration of AITC that had a fungicidal effect was 2.4 mg/mL in gas phase for rye bread and between 1.8 and 3.5 mg/mL in gas phase for hot-dog bread. These findings showed that an improvement of on shelf-life of rye bread could be achieved by active packaging with AITC. However, hot-dog bread, may

nevertheless require the additional effect of other preserving factors to avoid off-flavor formation.

The effects of AITC (18 and 36 μg/L) in vapor phase on *Pseduomonas aeruginosa* in fresh catfish fillet using modified atmosphere (MA; 49% CO_2, 0.5% O_2, and 50.5% N_2), at different temperatures (8, 15, and 20 ◦ C) were evaluated by Pang et al. (2013). Lag phase, maximum growth rate, and shelf life parameters were studied to detect the antimicrobial effects. Both gaseous AITC and MA alone inhibited the growth potential of *P. aeruginosa* effectively, prolonging the shelf life by 1.5 to 3.4 times compared to the control at temperatures between 8 and 20 ˚C. Synergistic effect was observed at 8 °C, extending the shelf life of fresh catfish by more than 6.5 times (≥ 550 h), so that this combination may be used as an effective antimicrobial system to improve the shelf life of fresh catfish fillet.

Hontanaya et al. (2015) evaluated the reduction of the AFs present in dried fruits (peanut, cashew, walnut, almond, hazelnut and pistachio) by ITCs generated by the enzymatic hydrolysis of the glucosinolates (GLCs) present in oriental and yellow mustard flours. An in vitro model and a food system were used. The ITCs produced through GLCs hydrolysis reduced the *A. parasiticus* growth in both systems. The AFs reduction ranged meanly from 83.1 to 87.2% using the oriental mustard flour, whereas employing the yellow flour the mean reduction observed ranged from 27.0 to 32.5%. In the food system experiments carried out employing only the oriental mustard flour the mean AFs reduction observed ranged from 88 to 89%.

4. Conclusion

In this study it has been demonstrated that AITC, BITC and PITC possess antimicrobial activity against *A. parasiticus* in liquid medium. In the loaf bread, AITC showed the highest activity, being the only one of the three compounds that

improved the shelf life of the loaf bread contaminated with the fungus and reduced significantly the production of AFs in the packaging using paper filter. However, BITC and PITC, such as AITC, showed a significantly reduction of the AFs in almost all the concentrations used in the packaging with a controlled release of the active compounds.

The use of ITCs in active packaging devices could be a natural alternative to avoid the growth of mycotoxinogenic fungi and inhibit the production of AFs in different food products in substitution of common commercial preservatives.

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3.5. Reaction of zearalenone and α-zearalenol with allyl isothiocyanate, characterization of reaction products, their bioaccessibility and bioavailability in vitro

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3.5. Reaction of zearalenone and α-zearalenol with allyl isothiocyanate, characterization of reaction products, their bioaccessibility and bioavailability *in vitro*

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Abstract

This study investigates the reduction of zearalenone (ZEA) and α-zearalenol (α-ZOL) on a solution model using allyl isothiocyanate (AITC) and also determines the bioaccessibility and bioavailability of the reaction products isolated and identified by MS-LIT. Mycotoxin reductions were dose-dependent, and ZEA levels decreased more than α-ZOL, ranging from 0.2 to 96.9% and 0 to 89.5% respectively, with no difference ($p \leq 0.05$) between pH 4 and 7. Overall, simulated gastric bioaccessibility was higher than duodenal bioaccessibility for both mycotoxins and mycotoxin-AITC conjugates, with duodenal fractions representing ≥63.5% of the original concentration. Simulated bioavailability of reaction products (α-ZOL/ZEA-AITC) was lower than 42.13%, but significantly higher than the original mycotoxins. The cytotoxicity of α-ZOL and ZEA in Caco-2/TC7 cells was also evaluated, with toxic effects observed at higher levels than 75 µM. Further studies should be performed to evaluate the toxicity and estrogenic effect of α-ZOL/ZEA-AITC.

Keywords: Zearalenone, α-Zearalenol, allyl isothiocyanate, reaction products, chemical reduction.

1. Introduction

Zearalenone (ZEA) and its derivative α-zearalenol (α-ZOL) are non-steroidal estrogenic mycotoxins produced by fungi belonging to the genus *Fusarium* and *Gibberella* (Yang, Wang, Liu, Fan, & Cui, 2007). These fungal species contaminate pre and postharvest cereal crops such as corn, barley, wheat, rice and oats mainly from temperate and warm regions (Zinedine, Soriano, Molto, & Mañes, 2007). Several studies have demonstrated hepatotoxic, haematotoxic, immunotoxic, genotoxic and teratogenic effects of these mycotoxins to a number of mammalian species (Zinedine et al., 2007). ZEA elicit estrogenic response upon binding to the estrogen receptor (Drzymala et al., 2015). Moreover, it can be metabolized in the human body to α-ZOL, which possesses three to four times higher estrogenic activity (Wang et al., 2014).

There are some strategies proposed for the detoxification and biodegradation of ZEA in foods through chemical/enzymatic methods using ozone (McKenzie et al., 1997), H_2O_2 (Abd Alla, 1997) and lactonohydrolase (Takahashi-Ando, Kimura, Kakeya, Osada, & Yamaguchi, 2002); biological methods using lactic acid bacteria (Mokoena, Chelule, & Gqaleni, 2005), *Aspergillus niger* strain FS10 (Sun et al., 2014) and *Lactobacillus plantarum* Lp22, Lp39 and Lp4 (Zhao et al., 2015); and physical methods such as the use of adsorbent materials (Avantaggiato, Havenaar, & Visconti, 2003; Ramos, Hernández, Plá-Delfina, & Merino, 1996) or extrusión (Cetin & Bullerman, 2005). However, there is little information concerning the metabolites produced through these processes and their potential toxicity.

Glucosinolates (GLs) are a group of phytochemicals found in vegetables of the *Brassicaceae* (*Syn. Cruciferae*) family, which includes broccoli, cauliflower, mustard and horseradish (Meca, Luciano, Zhou, Tsao, & Mañes, 2012). Damage to the plant tissue leads to the hydrolysis of GLs by endogenous myrosinase,

producing numerous biologically active compounds, including isothiocyanates (ITCs), thiocyanates and nitriles (Borges, Simões, Saavedra, & Simões, 2014). ITCs have several biological activities including plant defense (against insects and microbial infections) (Luciano & Holley, 2009; Mansour et al., 2012; Santos, Faroni, Sousa, & Guedes, 2011), benefits to human health (chemopreventive and anti-angiogenic properties) (Cavell, Sharifah, Donlevy, & Packham, 2011; Fimognari, Turrini, Feruzzi, Lenzi, & Hrelia, 2012; Zhang, 2004) and might be used as natural food preservatives (Borges et al., 2014; Saavedra et al., 2010). Allyl isothiocyanate (AITC) is one of the most common ITC, which has been reported as potent antimicrobial (Luciano & Holley, 2009). Previous studies have also demonstrated its capacity to react with mycotoxins such as beauvericin (BEA) (Meca, Luciano, et al., 2012) and fumonisins (FBs) (Azaiez, Meca, Manyes, Luciano, & Fernández-Frazón, 2013) in buffered solutions and in food matrices. AITC was able to react with both mycotoxins forming adducts, which may reduce their toxicity.

Toxins ingested through food products can be degraded or modified by metabolic processes of the human body, and only a fraction of the initial content may be accessible for absorption (Angelis, Monaci, Mackie, Salt, & Visconti, 2014). In this sense, bioavailability is defined as the portion of ingested contaminant that reaches the bloodstream (Kabak & Ozbey, 2012). These studies in combination with cell models can provide important information concerning the impact of these compounds on human health (Meca, Mañes, Font, & Ruiz, 2012). Bioavailability and toxicity evaluated through cellular systems has been widely used by rapid and cost-effective assays of easy standardization, which reduce the use of experimental animals and enables the investigation of specific mechanisms using different cultured cells (Fernández-Garcia, Carvajal-Lérida, & Pérez-Gálvez, 2009).

The objective of the present study was to assess the potential of AITC to react with α-ZOL and ZEA in buffered solutions and to determine the bioaccessibility and bioavailability in vitro of the reaction products.

2. Materials and methods

2.1. Materials and apparatus

ZEA (MW = 318.36 g/mol; P98% purity) and a-ZOL (MW = 320.38 g/mol; 97% purity) standards, AITC (MW = 99.15 g/mol; 95% purity), formic acid (HCOOH), potassium chloride (KCl), potassium thiocyanate (KSCN), monosodium phosphate (NaH_2PO_4), sodium sulfate ($NaSO_4$), sodium chloride (NaCl), sodium bicarbonate ($NaHCO_3$), urea, a-amylase, hydrochloric acid (HCl), pepsin, pancreatin and bile salts were obtained from Sigma–Aldrich (St. Louis, MO, USA). The stock solutions were prepared in methanol and kept at -20 °C. Acetonitrile, methanol and ethyl acetate of LC–MS grade were purchased from Fisher Scientific (New Hampshire, USA). Deionized water (<18MX cm resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Cell culture materials including Dulbecco's modified Eagle's medium (DMEM), penicillin, amphotericin B, HEPES, no essential aminoacids (NEAA), streptomycin, phosphate buffer saline (PBS), Hank's balanced salt solution (HBSS) and dimethyl sulfoxide (DMSO) were also provided by Sigma-Aldrich. Fetal calf serum (FCS) was purchased from Cambrex Co. (Belgium).

2.2. Reduction of α-ZOL and ZEA with AITC in vitro

ZEA and α-ZOL standards were diluted to 78 µM in phosphate buffer at pH 4 or 7. AITC at 2, 20, 100 or 200 mM was added to the reaction vials (final volume of 1 ml), which were tightly closed, shaken with the use of a vortex for 1 min and kept at room temperature. Aliquots were draw after 0, 4, 8, 24 and 48 h of

reaction for further analyses. Assays were carried out in triplicate and compared with a standard curve ranging from 0.3 to 300 µM. The results were expressed in percentage (%) of reduction of mycotoxins based on a control sample prepared with the mycotoxin standard.

2.2.1. HPLC analysis

ZEA and α-ZOL were determined using Merk HPLC with a diode array detector (LC-DAD) L-7455 (Merk, Darmstadt, Germany) at 236 nm and Hitachi Software Model D-7000 version 4.0 was used for data analysis. A Gemini C18 column (Phenomenex, Torrance, USA) 4.6 X 150 mm, 3 µm particle size was used as the stationary phase. The isocratic mobile phase was consisted of water/acetonitrile (55:45, v/v) with a flow rate of 0.7 ml/min. The samples were filtered through 0.22 µm nylon membrane and 20 µl was injected into HPLC system. There was a new peak identified on LC-DAD chromatograms corresponding to the reaction product of AITC and either α-ZOL or ZEA. The structures of these compounds were confirmed by a linear ion trap spectrometer (MS-LIT). Assuming that 1 mol of AITC and 1 mol of α-ZOL or ZEA produces 1 mol of adducts, the molecular weight (MW) was considered 418.7 and 418.4 g/mol of ZEA-AITC and α-ZOL-AITC respectively. This ratio was used to calculate its theoretical concentration.

2.2.2. MS-LIT characterization of α-ZOL and ZEA-AITC

A 3200 QTRAP^{TM} linear ion trap mass spectrometer (AB SCIEX Concord, Ontario, Canada) coupled to a Turbo Ion Spray source was used. This instrument is based on a triple-quadrupole path (QqQ) in which the third quadrupole also operates as a linear ion trap (QqLIT) with improved performance. In the QqLIT configuration, Q TRAP^{TM} operates in enhanced resolution (ER) and enhanced

product ion (EPI) scan modes. Applied Biosystem/MDS SCIEX Analyst software version 1.3.2 was used for data acquisition and processing.

The electrospray ionization mass spectrometry (ESI-MS) analyses were performed in positive ion mode. The equipment was set as follows: ESI needle voltage at 5500 V, curtain gas at 35 (arbitrary units), GS1 and GS2 were set to 35 and 40 °C, respectively, and probe temperature at 350 °C. Nitrogen served both as turbo-gas and collision gas. The method was optimized based on mycotoxin reduction, and α-ZOL/ZEA-AITC reaction products were obtained from the combination of 200 mM of AITC and 78 µM of each mycotoxin (α-ZOL or ZEA). Products were extracted from the buffer solution with 3 ml of ethyl acetate (99.9%). The extraction was repeated with another 3 ml of ethyl acetate and the solvent was evaporated using nitrogen flow. The isolate was diluted in 1 ml of methanol and infused into the ion source at a flow rate of 20 µl/min introduced via a model 11 Harvard infusion pump. Full-scan spectra were analyzed for the identification of products formed through the reaction between ZEA or α-ZOL and AITC. Spectra were preliminarily recorded by connecting the Harvard infusion pump to the interface. The characterization of isolated compounds were performed using the modality of ER scan, the mass range from 200 to 500 Da to obtain the general spectra of the molecule. The utilization of the mass spectrometry associated at the detection with the linear ion trap allowed the total characterization of the isolated compounds.

2.3. In vitro digestion model

The static *in vitro* digestion model used was performed according to Gil-Izquierdo, Zafrilla, and Tomás-Barberá (2002) with some modifications. All digestive solutions were warmed to 37 ± 3 °C before the experiment. Methanol solutions (1 ml) of α-ZOL, ZEA or adducts (α-ZOL/ZEA-AITC) were added to

polyethylene tubes at 15, 30 or 60 µM. Then, digestion started by adding 0.6 ml of artificial saliva [10 ml KCl (89.6 g/l), 10 ml KSCN (20 g/l), 10 ml NaH_2PO_4 (88.8 g/l), 10 ml Na_2SO_4 (57 g/l), 1.7 ml NaCl (175.3 g/l), 20 ml $NaHCO_3$ (84.7 g/l), 8 ml urea (25 g/l) and 290 mg of α-amylase completed to 0.5 L and adjusted the pH to 6.8 to the tubes]. The solution was homogenized, added to 10 ml of water and pH was adjusted to 2 with HCl 1 N. Immediately after, 0.05 ml of pepsin solution (0.04 g/ml in HCl 0.1 N) was added and the samples were incubated at 37 °C for 2 h in a shaker water bath (100 rpm) (Stuart, SBS30, Staffordshire, UK). To simulate the duodenal compartment, 20 ml of water was added and the pH was increased to 6.5 with $NaHCO_3$ 1 N, followed by addition of 0.125 ml of a solution of pancreatin (4 g/l) and bile salts (25 g/l) (1:1; v/v). The mixture was homogenized and incubated at 37 °C for 2 h in a water bath with orbital shaker at 100 rpm. Samples of 5 ml were drawn to evaluate the concentrations of the compounds after the gastric and duodenal digestion (bioaccessibility). These aliquots were centrifuged at 4000 rpm and 4 °C for 5 min. The supernatant obtained was filtered and injected into the LC to quantify the mycotoxins and reaction products.

2.4. Cell culture

Caco-2/TC7 cell were routinely maintained and grown with DMEM supplemented with 25 mM HEPES, 1% NEAA, 100 U/ml penicillin, 100 mg/ml streptomycin, 2.5 lg/ml amphotericin B, and 10% heat inactivated FBS. Incubation conditions were pH 7.4, 37 °C and 5% CO_2 in a 95% relative humidity atmosphere. Absence of mycoplasma was checked routinely using the Mycoplasma Stain Kit (Sigma-Aldrich, St. Louis-MO, USA).

2.5. In vitro cytotoxicity assays

The cytotoxicity of ZEA and a-ZOL in Caco-2/TC7 cells was determined by MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte trazolium bromide and protein content (PC) assays. MTT assay measures the ability of live cells, but not dead cells, to reduce a colourless tetrazolium salt to purple formazan salt by the mitochondria; whereas PC assay indicates the relative determination of cell number by measuring the collective protein content on a cell culture dish. The MTT assay was performed as described by Ruiz, Festila, and Fernández (2006) with some modifications and PC assay was analyzed according to the procedure described by Pichardo et al. (2007). Caco-2/TC7 cells were plated in 96-well tissue culture plates at a density of $3x10^4$ cells per well and grown to 90% confluence. The growth medium was removed and ZEA and α-ZOL, at concentrations from 6.25 to 100 µM, were added to the medium, allowing uptake to proceed for 24 h. Then, the medium was removed and each well received 200 µl of fresh medium containing 50 µl of MTT for 4 h (37 °C in darkness). The resulting formazan was solubilized with DMSO. The cell viability or protein content was determined using an automatic ELISA plate reader MultiscanEx (Thermo Scientific, MA, USA) wit wavelength of 570 nm.

PC assay was also conducted spectrophotometrically at 24 and 48 h in the same 96-well culture plates where the MTT assay was performed. The medium with formazan was then removed and the cells were washed with PBS and homogenized in NaOH for 2 h at 37 °C. Then, the NaOH was removed and an acidic solution of Coomassie Brilliant Blue was added at room temperature.

Protein content was measured after 30 min at 620 nm using an automatic ELISA plate reader MultiscanEx (Thermo Scientific, MA, USA). Results were expressed as percentage relative to control cells (1% DMSO). Mean inhibition concentration (IC_{50}) values were calculated from full dose-response curve.

2.6. In vitro bioavailability

Differentiated Caco-2/TC7 were exposed to α-ZOL/ZEA-AITC adducts produced through α-ZOL/ZEA and AITC reaction to analyze the *in vitro* bioavailability of these compounds according to Meca, Mañes, et al. (2012). Briefly, Caco-2/TC7 cells were seeded at 1.35 X 10^6 cells/cm^2 on a 6-well Transwell Permeable Supports, 12 mm diameter (Corning, NY, USA) and 0.4 mm of pore size, and grown for 21 days until morphological differentiation. The medium was renewed every 2–3 days. After this period, apical (upper compartment) and basolateral (lower compartment) medium were removed, and cells were washed twice with PBS, followed by a final wash with HBSS (transport medium). The apical solution composed of 1.5 ml of HBSS and α-ZOL/ZEA-AITC adducts (at initial levels of 15, 30 and 60 µM) was subjected to simulated duodenal digestion (Fig. 1) and digested sample obtained was diluted in HBSS (1:1, v/v). Bioavailability was assessed by transepithelial passage of this solution to the basolateral side, which contained 0.5 ml of HBSS. Control samples composed by transport medium with methanol 1%, were also evaluated. Aliquots (150 µl) were drawn from the basolateral compartment after 1, 2, 3 and 4 h of incubation and analyzed by LC-DAD.

2.7. Statistical analysis

Graphpad Prism version 6.0 (Graphpad Software Inc., La Jolla, CA, USA) was used for the statistical analysis of data. All experiments were performed in triplicate and differences between groups analyzed with one-way ANOVA followed by the Tukey HSD post-hoc test for multiple comparisons. The level of significance considered was $p \leq 0.05$.

3. Results and discussion

3.1. ZEA and α-ZOL in vitro reduction

The reaction between α-ZOL/ZEA (78 µM) and AITC (2, 20, 100 or 200 mM) were monitored by LC-DAD in PBS at different pH levels (4 and 7) and incubation times (0, 4, 8, 24 and 48 h). As shown on Table 1, the reduction ranged from 0 to 89.5 ± 1.2% for α-ZOL and 0.2 ± 0.3% to 96.9 ± 2.4% for ZEA. There was no difference ($p \leq 0.05$) between the pH levels investigated. The effect of AITC was dose-dependent towards both mycotoxins. However, the reaction occurred rapidly with most doses reaching a plateau already at 0 h, with a few exceptions that presented this same reaction plateau after 4 h.

AITC seems to be more reactive with ZEA than α-ZOL. At 20 mM (pH 4), AITC was able to reduce more than half of ZEA's initial concentration, whereas α-ZOL was reduced by a maximum of 28.9 ± 8.9% with this same dose. ZEA was also reduced by 74.5 ± 10.3% (pH 4) and 77.2 ± 3.6% (pH 7) promptly after addition of 100 mM of AITC, while α-ZOL was reduced by 54.1 ± 3.8% and 67.7 ± 6.4% at pH 4 and 7, respectively. ZEA and α-ZOL are important mycotoxins in animal production, and the use of allyl isothiocyante in animal feed could be an alternative to mitigate this problem, since 20 mM AITC can reduce levels up to 68.0% *in vitro*.

Previous studies have reported the use of gaseous allyl, benzyl and phenyl isothiocyanates (ITCs) to reduce mycotoxins levels. Meca, Luciano, et al. (2012), evaluated the reduction of beauvericin (BEA) by AITC in buffered solutions and in wheat flour. AITC at 1 mM was able to reduce this mycotoxin (25 mg/l) in buffered solutions by 100% after 48 h. Similar to the results obtained in this study, no significant difference was found for the extent of reaction between AITC and BEA at pH 4 and 7.

Table 1. Reduction of α-ZOL and ZEA through *in vitro* reaction with AITC at different pH and incubation time.

Time (h)	pH 4				pH 7			
	AITC concentration (mM)[a]							
	2	20	100	200	2	20	100	200
	α-ZOL reduction ± SD (%)[b]							
0	NE	$27.0^{aA} \pm 7.1$	$54.1^{aB} \pm 3.8$	$83.3^{aC} \pm 4.7$	NE	$17.3^{aA} \pm 3.2$	$67.7^{aBC} \pm 6.4$	$80.3^{aC} \pm 2.0$
4	NE	$28.9^{aA} \pm 8.9$	$74.2^{bBC} \pm 3.5$	$89.5^{bBC} \pm 1.2$	NE	$17.8^{aA} \pm 4.2$	$67.9^{aB} \pm 5.1$	$81.5^{aC} \pm 2.8$
8	NE	$24.4^{aA} \pm 6.6$	$73.3^{bC} \pm 2.8$	$89.5^{bC} \pm 0.5$	NE	$16.4^{aA} \pm 6.0$	$65.4^{aB} \pm 2.7$	$81.6^{aC} \pm 3.4$
24	NE	$26.4^{aA} \pm 5.2$	$74.5^{bB} \pm 3.1$	$88.5^{bB} \pm 1.1$	NE	$19.6^{aA} \pm 9.8$	$70.6^{aB} \pm 2.3$	$78.9^{aBC} \pm 7.7$
48	NE	$27.7^{aA} \pm 6.5$	$73.0^{bB} \pm 2.7$	$88.6^{bB} \pm 2.1$	NE	$23.2^{aA} \pm 6.9$	$69.3^{aB} \pm 4.9$	$77.6^{aBC} \pm 8.2$
	ZEA reduction ± SD (%)[b]							
0	$0.4^{aA} \pm 0.5$	$51.6^{aBC} \pm 6.5$	$74.5^{aCD} \pm 10.3$	$83.2^{aD} \pm 11.9$	$1.5^{aA} \pm 1.5$	$44.5^{aB} \pm 2.2$	$77.2^{aD} \pm 3.6$	$82.1^{aD} \pm 5.3$
4	$0.2^{aA} \pm 0.3$	$54.9^{aB} \pm 10.5$	$92.2^{aC} \pm 3.4$	$96.9^{aC} \pm 2.4$	$1.6^{aA} \pm 1.4$	$41.5^{aB} \pm 3.4$	$88.4^{abC} \pm 7.6$	$95.6^{bC} \pm 2.6$
8	$0.4^{aA} \pm 0.6$	$52.2^{aB} \pm 9.2$	$90.6^{aC} \pm 3.7$	$96.8^{aC} \pm 2.7$	$1.1^{aA} \pm 0.5$	$31.9^{aB} \pm 11.0$	$90.4^{bC} \pm 3.3$	$95.2^{bC} \pm 2.1$
24	$0.9^{aA} \pm 0.6$	$60.2^{aB} \pm 9.7$	$90.8^{aC} \pm 8.0$	$92.3^{aC} \pm 3.0$	$0.7^{aA} \pm 1.0$	$47.0^{aB} \pm 15.9$	$92.2^{abC} \pm 6.9$	$95.4^{bC} \pm 2.3$
48	$0.4^{aA} \pm 0.6$	$68.0^{aBC} \pm 5.5$	$87.1^{aCDE} \pm 10.5$	$95.4^{aE} \pm 1.4$	$0.5^{aA} \pm 0.5$	$47.8^{aB} \pm 15.5$	$85.4^{abD} \pm 3.3$	$94.1^{abDE} \pm 3.5$

NE: no effect observed in mycotoxins levels.

Different lower case letters show significant difference ($p \leq 0.05$) in the same column, whereas different capital letters denote significant difference ($p < 0.05$) within the same row.

[a] Concentrations of AITC (allyl isothiocyanate) evaluated (2, 20, 100, 200 mM) to reduce 78 µM of a-ZOL (α-Zearalenol)/ZEA (zearalenone) in different incubation time.

[b] Percentage of reduction was calculated based on a blank prepared for each assay.

In addition, wheat flour treated with gaseous AITC (50, 100 and 500 µL/l) was able to reduce the initial BEA concentration (25 mg/Kg) by 10-65% in a dosedependent fashion.

Reaction of isothiocyanates (allyl, benzyl and phenyl – 1 mg/l) and fumonisins (FB_1, FB_2 and FB_3 – 1 mg/l) at pH 4, 7 and 9 was also surveyed (Azaiez, et al., 2013). The reduction of FB_1 and FB_2 in solution ranged from 42% to 100% in a time-dependent manner and was greatly influenced by pH. In general, lower pH levels facilitated the reaction between ITCs and FBs, where four reaction products were identified. Moreover, gaseous ITCs were used to fumigate corn kernels and corn flour contaminated with FBs. ITC fumigation (50, 100 and 500 µL/l) was able to reduce 53%-96% of FB_1, 29%-91% of FB_2 and 29%-96% of FB_3. Reduction of these FBs could be due to the free amino group contained in these mycotoxin structures, which act as an electron donor and react with the electrophile carbon present within the isothiocyanate (ITC) group.

3.2. Identification of ZEA/α-ZOL-AITC reaction products

MS-LIT total ion chromatograms of isolated α-ZOL-AITC and ZEA-AITC reaction products are shown in Fig. 1a and b. The results obtained in the present study show that ZEA and α- ZOL can react with AITC and form adducts. Despite the structural similarity, the reduction of ZEA was higher than α-ZOL (Table 1). ZEA ($C_{18}H_{22}O_5$) and its metabolite α-ZOL ($C_{18}H_{24}O_5$) contain some nucleophile groups that may react with the central carbon.

Fig. 1 shows the mass spectra obtained of ZEA-AITC and α-ZOL-AITC adduct in enhanced resolution (ER). Several diagnostic fragments were found in the spectra, confirming the structure of the compound. The molecular weight of the reaction compound ZEA-AITC is the fragment with *m/z* of 419.2 $[M + H]^+$.

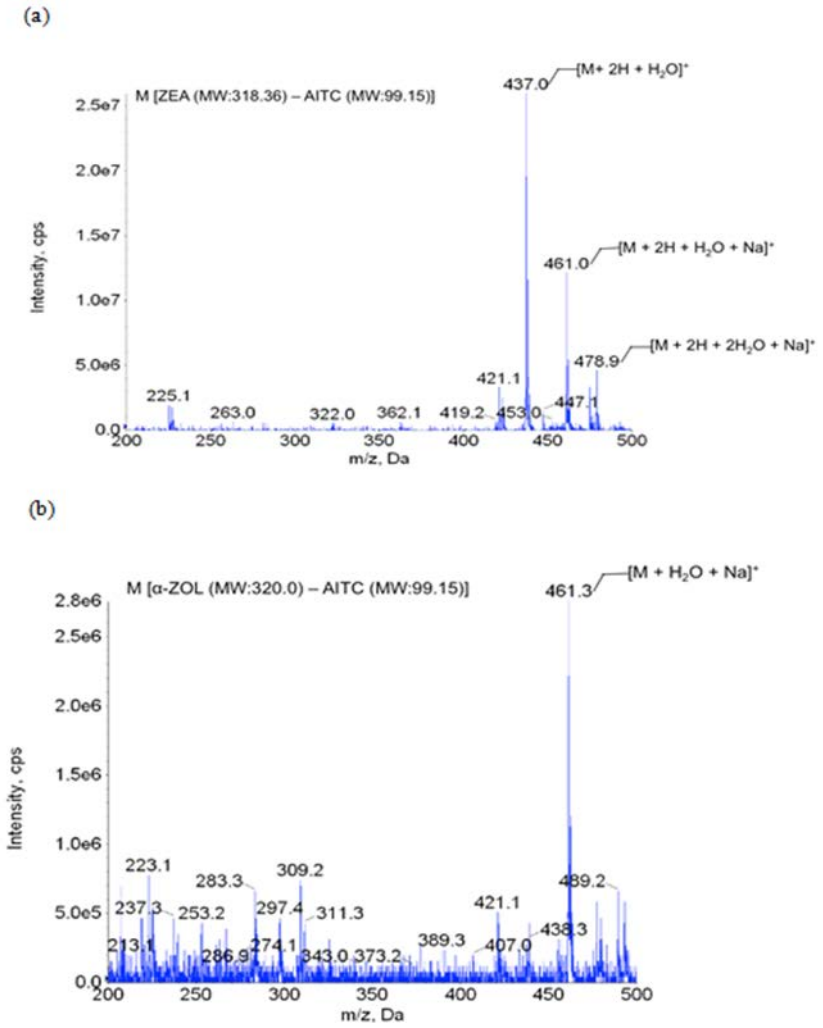

Figure 1. ER mass spectra of reaction products: (a) ZEA with AITC and (b) α-ZOL with AITC.

The fragment with *m/z* of 437.0 corresponds to $[M + 2H + H_2O]^+$, whereas the fragment with *m/z* of 461.0 represents the sodium adduct $[M +2H + H_2O + Na]^+$. Another important fragment is the ion with *m/z* of 478.9 that represents the ion corresponding to the *m/z* of 461.0 with the addition of water $[M + 2H + 2H_2O + Na]^+$. The characterization of the reaction product between α-ZOL and AITC is shown on Fig. 1b, with the ER mass spectra of the isolated compounds. The ion with *m/z* of 461.3, corresponding to the sodium adduct of α-ZOL-AITC plus a molecule of water $[M + H_2O + Na]^+$ confirm the correct identification of the α-ZOL adduct.

3.3 Bioaccessibility of α-ZOL/ZEA-AITC adducts

The bioaccessible fraction is the amount of an ingested compound that is available for absorption in the body after digestion (Versantvoort, Oomen, Van de Kamp, Rompelberg, & Sips, 2005). In this sense, the amount of α-ZOL, ZEA and α-ZOL/ZEA-AITC adducts under simulated human digestive fluids were determined and are presented on Table 2. α-ZOL showed higher gastric bioaccessibility than α-ZOL-AITC at 30 µM. Duodenal bioaccessibility was also higher for α-ZOL (80.9 ± 1.0% and 84.8 ± 5.9%) than α-ZOL-AITC (63.7 ± 7.3% and 71.4 ± 2.8%) at 30 and 60 µM, respectively. At 15 µM, α -ZOL had no difference with α -ZOL-AITC for the gastric fraction and duodenal fraction. Gastric bioaccessibility of ZEA and ZEA-AITC were also similar to all levels investigated. The results of gastric and duodenal bioaccessibility of ZEA obtained in the present study are higher than Ferrer, Manyes, Manes, and Meca (2015), which recovered 54.6 ± 3.2% and 44.3 ± 2.5% of ZEA content administered *in vitro* under gastric and duodenal model. These compounds can be structurally transformed or degraded during the digestion, but this mechanism is difficult to assess by the complex processes that comprise the digestion system (Versantovoort et al., 2005).

Table 2. *In vitro* bioaccessibility assessment of α-ZOL, ZEA and α-ZOL-AITC and ZEA-AITC adducts.

Fraction	Bioaccessibility *in vitro* (%) (Mean ± SD)					
	α-ZOL (μM)*			α-ZOL - AITC (μM)*		
	15	30	60	15	30	60
Gastric	85.0± 12.4	92.4± 3.1	91.2± 5.2	70.5± 5.4	72.8± 3.8	85.4± 2.1
Duodenal	65.0± 10.4	80.9± 1.0	84.8± 5.9	57.8± 3.4	63.7± 7.3	71.4±2.8
	ZEA (μM)*			ZEA - AITC (μM)*		
	15	30	60	15	30	60
Gastric	89.1±11.7	88.1± 2.3	94.2± 1.0	71.0± 3.9	77.4± 1.7	78.5± 3.6
Duodenal	81.5± 7.3	77.8± 4.4	81.8± 1.7	63.5± 11.3	67.3± 1.3	69.6± 4.0

ND: no detected levels.

Different capital letters show significant difference ($p \leq 0.05$) in the same column, whereas different lower case letters denote significant difference ($p < 0.05$) within the same row.

* Initial concentration of α-ZOL, ZEA, α-ZOL-AITC (allyl isothiocyanate), ZEA-AITC (15, 30 and 60 μM) evaluated bioaccessibility through *in vitro* digestion model (gastric 2 h and duodenal 2 h).

3.4. In vitro cytotoxicity and duodenal bioavailability

Caco-2/TC7 cell monolayers were used in the design of a simulated *in vitro* intestinal model in order to assess the absorption of α-ZOL/ZEA-AITC adducts. However, ZEA and α-ZOL are cytotoxic and could reduce the viability of Caco-2/TC7. This could interfere with the interpretation of the results, since the absorption should occur through viable cells. Therefore, cytotoxicity of ZEA and α-ZOL towards Caco-2/TC7 was analyzed. MTT assay shows that ZEA did not decrease cell viability at doses up to 100 μM ($p \geq 0.05$) at 24 h and ≥ 50 μM were necessary to affect ($p \leq 0.05$) Caco-2/TC7 viability at 48h. However, α-ZOL at ≥ 37.5 μM and ≥ 18.75 μM were able to reduce cell viability ($p \leq 0.05$) after 24 and 48 h, respectively (Fig. 2). The IC_{50} value of 95 μM was obtained for α-ZOL after 48 h of exposure. Results from PC assays show that ZEA did not damage the cells at doses up to 100 μM after 24 and 48 h (Fig. 3a). Moreover, α-ZOL only damaged the cells at 100 μM and ≥ 18.75 μM after 24 and 48 h of exposure, respectively (Fig. 3b).

The evaluation of the duodenal bioavailability of α-ZOL/ZEAAITC conjugates was carried out by determining the concentrations in the basolateral compartment after 1, 2, 3 and 4 h considering the initial concentrations of each compound.

The initial concentrations applied to the *in vitro* digestion model were 15, 30 and 60 μM of each compound. However, the gastric and pancreatic juices diluted the toxins. If these compounds were totally bioaccessible, their concentration in the simulated intestine would be 0.27, 0.54 and 1.08 μM in respect to their initial concentration of 15, 30 and 60 μM, respectively. As presented in Figs. 2 and 3, much higher concentrations were necessary to cause cell damage and to reduce cell viability after 24 h.

As it can be observed in Table 3, ZEA-AITC presented a higher bioavailability than α-ZOL-AITC. The former compound presented a similar profile among all

concentrations studied. The bioavailability obtained for the initial concentration of 15 μM ranged from 3.92% after 2 h to 15.84% after 4 h for α-ZOL-AITC and from 8.61 to 37.61% for ZEA-AITC. α-ZOL-AITC showed a bioavailability ranging from 0.98 (1 h) to 15.67% (4 h) for the initial concentration of 30 μM and from 0.74 to 12.72% using an initial concentration of 60 μM. Bioavailability ranging from 9.99 to 36.15% (0–4 h; 30 μM) and 10.14 to 42.13% (0–4 h; 60 μM) were obtained for ZEA-AITC. In addition, the mycotoxins themselves presented significantly lower bioavailability than the reaction products or the original mycotoxins were metabolized to a higher extent.

Videman, Mazallon, Tep and Lecouer, (2008) evaluated the metabolism and transfer of ZEA using Caco-2 cell line as a model of intestinal epithelial barrier demonstrating that ZEA easily crosses the cell barrier. After 3 h of ZEA exposure at 10 μM, about 30% crossed the cell monolayer. The metabolites produced were evaluated and the composition was as follows: 40.7 ± 3.1% α-ZOL, 31.9 ± 4.9% β-ZOL, 8.2 ± 0.9% ZEA-glucuronide and 19.1 ± 1.3% α-ZOL-glucuronide.

Another study Pfeiffer, Kommer, Dempe, Hilebrand, & Metzier, (2011) determined theabsorption of ZEA and α-zearalanol (α-ZAL) *in vitro* using monolayers of differentiated Caco-2 cells. Cells were exposed to ZEA or α-ZAL (10, 20, 30 and 40 μM) for 6 h. Unconjugated ZEA appeared to decrease with first-order kinetics at the apical side, while basolateral reached a plateau after 2 h. After 3-h incubation of 40 μM of ZEA, 57.5% were recovered unconjugated, 11.1% were metabolized to glucoronides and 26.2% were found as α- or β-ZOL. *In vivo* and *in vitro* studies of ZEA kinetics showed early appearance of dietary ZEA in the plasma demonstrating that ZEA can be efficiently absorbed in the proximal part of the small intestine (Avantaggiato et al., 2003; Kuiper-Goodman, Scott, & Watanabe, 1987; Ramos et al., 1996). Furthermore, α-ZOL, β-ZOL, ZEA-glucuronide and α-ZOL-glucuronide are rapidly produced and easily cross the cell

membranes, being detectable at both apical and basolateral sides since the first hour of exposure to ZEA. α-ZOL is the main metabolite produced by the Caco-2 cells and it shows the strongest estrogenic activity (Videmann, Mazallon, Tep, & Lecoeur, 2008). The present study suggests that the reaction products of the mycotoxins with AITC were more stable during the bioavailability study, and perhaps it could avoid the formation of more estrogenic metabolites. However, the toxic effects of these compounds still need to be assessed.

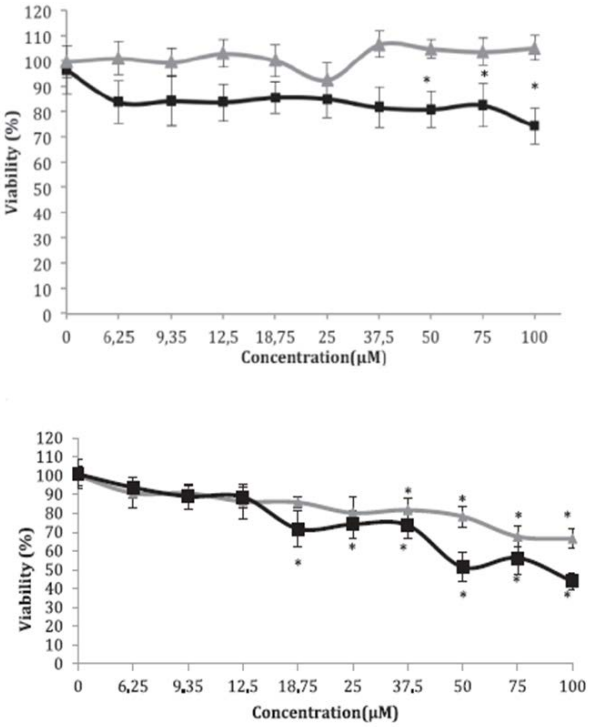

Figure 2. Dose response curve of Caco-2/TC7 cells viability in the presence of a) ZEA and b) α-ZOL measured by MTT. Cells were incubated for 24 (–▲–) and 48 h (–■–). All values are expressed as mean ± SD of 3 replicates. *Represent significant difference ($p \leq 0.05$) between the treatment and the control (100% viability).

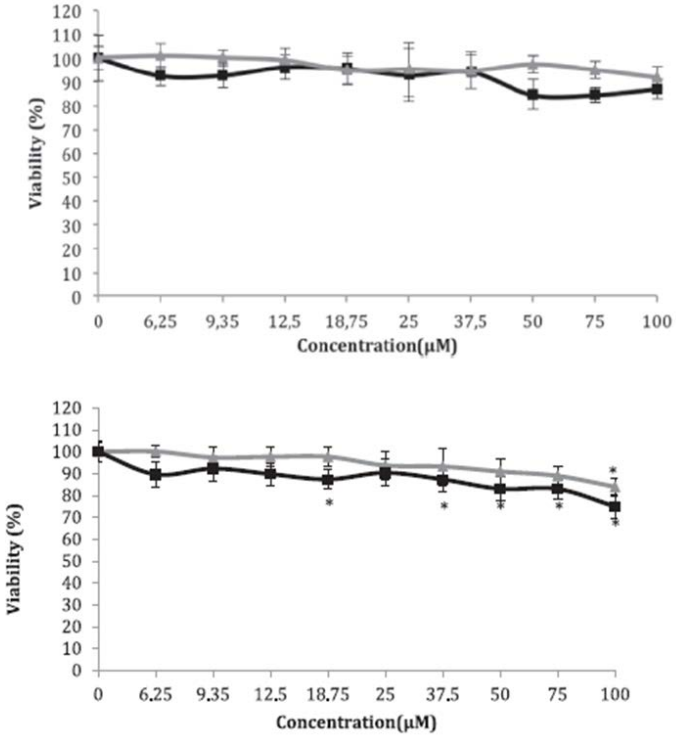

Figure 3. Dose response curve of Caco-2/TC7 cells viability in the presence of a) ZEA and b) α-ZOL measured by PC. Cells were incubated for 24 (–▲–) and 48 h (–■–). All values are expressed as mean ± SD of 3 replicates. *Represent significant difference ($p \leq 0.05$) between the treatment and the control (100% viability).

Table 3. Bioavailability of the products obtained by α-ZOL/ZEA and AITC reaction products after digestion treatment through Caco-2/TC7 cell monolayers.

Hours	Bioavailability %			
	α-ZOL (μM)	α-ZOL - AITC (μM)		
	60 (Control)	15	30	60
1	0.06 ± 0.04^{aA}	ND	0.98 ± 0.2^{bA}	0.74 ± 0.47^{abA}
2	0.28 ± 0.15^{aAB}	3.92 ± 2.6^{aA}	3.79 ± 2.8^{aB}	3.31 ± 0.40^{aA}
3	0.29 ± 0.11^{aAB}	9.52 ± 4.00^{bAB}	8.80 ± 2.8^{bBC}	4.41 ± 1.43^{abA}
4	0.48 ± 0.21^{aB}	15.84 ± 5.9^{bC}	15.67 ± 3.7^{bC}	12.72 ± 2.65^{bB}
Hours	ZEA (μM)	ZEA - AITC (μM)		
	60 (Control)	15	30	60
1	0.78 ± 0.15^{aA}	ND	9.99 ± 1.07^{bA}	10.14 ± 0.52^{bA}
2	1.06 ± 0.24^{aA}	8.61 ± 3.8^{bA}	13.82 ± 3.61^{bA}	19.34 ± 9.95^{bAB}
3	1.16 ± 0.27^{aA}	13.05 ± 4.96^{bA}	17.54 ± 6.55^{bA}	25.02 ± 4.73^{bBC}
4	1.23 ± 0.39^{aA}	37.61 ± 4.30^{bB}	36.15 ± 4.90^{bB}	42.13 ± 2.51^{bC}

ND: no detected levels.
Caco-2/TC7 cells were apically exposed to the duodenal fraction (initial concentrations of 60 μM of mycotoxin – control – and 15, 30, 60 μM of mycotoxin-AITC). The compounds evaluated were measured in the basolateral (BL) compartment after 1, 2, 3, 4 h.
Different capital letters show significant difference ($p \leq 0.05$) in the same column, whereas different lower case letters denote significant difference ($p < 0.05$) within the same row.

4. Conclusion

The results of this trial indicate that allyl isothiocyanate can react and reduce α-ZOL and ZEA *in vitro* at levels up to 96.9%, and form ZEA/α-ZOL-AITC conjugates. Reduction of mycotoxins and their reaction products were identified after gastric and duodenal treatments, probably due structural transformation during digestion. Moreover, the data obtained in this study suggested a lower bioavailability *in vitro* of ZEA, α-ZOL in comparison to their AITC conjugates. Further investigation may focus on the evaluation of the possible utilization of AITC to control ZEA and α-ZOL levels in food and animal feed. In addition, the toxicological assessment of the ZEA-AITC and α-ZOL-AITC must be performed.

Conflict of interest

The authors declare that there are no conflicts of interest.

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3.6. In vitro antifungal activity of lactic acid bacteria against mycotoxigenic fungi and their application in loaf bread shelf life improvement

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3.6. *In vitro* antifungal activity of lactic acid bacteria against mycotoxigenic fungi and their application in loaf bread shelf life improvement

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Abstract

Food spoilage caused by mycotoxigenic fungi represents an important food safety problem. Lactic acid bacteria (LAB) are used as starter cultures in a larger number of food products. In this study, 16 strains of LAB were cultivated in MRS broth under anaerobiosis. Then, cell free supernatants were obtained by centrifugation and their antifungal activity against *Aspergillus parasiticus* and *Penicillium expansum* was tested using the disc-diffusion method. Furthermore, the LABs that showed *in vitro* antifungal activity were used in bread fermentation with yeast in order to study fungal growth inhibition and aflatoxin (AF) reduction in processed bread previously inoculated with *A. parasiticus*. The compounds present in the fermented medium of six LAB strains induced inhibition of *P. expansum* growth, whereas five probiotic strains produced antifungal compounds against *A. parasiticus*. The analysis by liquid chromatography coupled to mass spectrometry in tandem showed a reduction of the AF content in bread samples fermented with yeast and LABs. The reduction of AFs ranged from 84.1 to 99.9%. Moreover, bread sample studies showed a shelf life increase of about 3-4 days.

Keywords: Antifungal compounds, lactic bacteria, mycotoxins.

1. Introduction

Moulds cause a high degree of deterioration in food, feed and other agricultural commodities and are responsible for considerable economical losses. They destroy about 30% of crop yields and damage more than 30% of perishable crops in developing countries by lowering their quality and quantity. Furthermore, moulds produce mycotoxins which are potentially toxic to consumers and cause illness and death (Matasyoh, Wagara, Nakavuma & Kiburai, 2011; Pawlowska, Zannini, Coffey & Arendt, 2012). The ubiquitous nature of moulds, their ability to colonize different substrates and the lack of effective control measures have contributed to the high incidence of mould and mycotoxin contamination in food and feed (Ahlberg, Joutsjoki, & Korhonena, 2015; Hassan, Zhou, & Bullerman, 2015).

Agricultural products are susceptible to fungal invasion such as *Aspergillus*, *Penicillium*, *Fusarium* and *Claviceps* species, which may produce mycotoxins in the field before harvest, during harvesting, or during storage and processing. Among all food contaminants, mycotoxins are of greatest concern in terms of chronic toxicity as well as economics. Their incidence depends on various factors, such as the commodity, climatic conditions, agricultural practices, storage conditions and seasonal variation (Warth, Parich, Atehnkeng, Banyopadhyay, Schuhmacher, Sulyok, et al., 2012).

Aflatoxins (AFs) are mycotoxins produced by certain species of *Aspergillus*, particularly *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius*. There are more than 20 distinct but structurally related AF compounds but the four most commonly found are known as aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2) (Hernandez-Martìnez & Navarro-Blasco, 2010; Tam et al., 2006). Aflatoxin B_1 (AFB_1) is carcinogenic and genotoxic *in*

vitro and *in vivo* (EFSA, 2007), and it has been classified in the group 1 by the International Agency for Research on Cancer (IARC, 2002). Extensive contamination of food and drinks with mycotoxins is the main problem over the world since they can also compromise the safety of food and feed supplies and adversely affect health to humans and animals (Marin, Ramos, Cano-Sancho, & Sanchis, 2013). One of the most used types of food bioconservation is fermentation, a process based on the growth of microorganisms in foods. Among bakery products, the microorganisms most widely used are lactic acid bacteria (LAB) applied as starter cultures for sourdough. LABs have been included in the QPS (Qualified Presumption of Safety) list for authorized use in the food and feed chain within the European Union (EFSA, 2012; EC, 2008); furthermore, in the US they are given the generally regarded as safe (GRAS) status by the U.S. Food and Drug Administration. Formerly, it was believed that the organic acids produced by LAB, particularly lactic and acetic acid, and also the phenolic acids produced by these bioactive strains were the main agents responsible for antifungal activity due to the lower pH. Besides organic acids, a range of other secondary metabolites produced by LABs has been identified additionally as the source of the antifungal activity (Moore, Dal Bello, & Arendt, 2008; Crowley, Mahony, & Van Sinderen, 2013; Ahlberg, Joutsjoki, & Korhonena, 2015; Hassan, Zhou, & Bullerman, 2015).

The aims of this study were: a) to evaluate the antifungal activity of LAB cell-free supernatant (CFS) against *A. parasiticus* and *Penicillium expansum*; b) to determine the inhibition of fungal growth and estimate the shelf-life of breads fermented with yeast and LAB and c) to analyze the reduction of AFs content in bread inoculated with *A. parasiticus*.

2. Materials and methods

2.1. Microorganisms and culture conditions

The strain of *A. parasiticus* CECT 2681 and *P. expansum* CECT 2268 and the lactic acid bacteria used in this study (*Bifidobacterium longum* CECT 4551*, B. bifidum* CECT 870T*, Bifidobacterium breve* CECT 4839T*, Bifidobacterium adolescentis* CECT 5781T*, Lactobacillus rhamnosus* CECT 278T*, Lactobacillus ruminis* CECT 1324*, Lactobacillus casei* CECT 4647*, L. rhamnosus* CECT 288*, Lactobacillus johnsoni* CECT 289*, L. casei* CECT 475*, Lactobacillus plantarum* CECT 749*, Lactobacillus reuteri* CECT 725*, Lactobacillus bulgaricus* CECT 4005*, Lactobacillus paracasei* CECT 4022*, Lactobacillus salivarius* CECT 4062*, L. salivarius* CECT 4305) were obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain).

All these microorganisms were maintained in sterile 18% glycerol at -80°C before use. Then, the mycotoxigenic fungi were recovered in Potato Dextrose Broth (PDB) at 25 °C. until they were inoculated to PDA Petri dishes. On the other hand, the LABs were recovered in MRS broth at 37 °C. for 48 h under anaerobic conditions before experiments (Meroth, Walter, Hertel, Brandt, & Hammes, 2003).

2.2. Preparation of cell-free supernatant

The LABs were inoculated at a concentration of 10^5 cells/mL in 2 liters of MRS broth and incubated at 37 °C. for seven days under anaerobic conditions. CFS was prepared by centrifugation at 5000 rpm for 15 min. Then, aliquots of 2, 5, 10 and 20 mL of CFS were completely dried using a flow of nitrogen through evaporator workstation Turbovap at 35 °C. (Biotage, Madrid, Spain) and each one of these were resuspended in 1 mL fresh sterile MRS broth so as to get

concentrated solutions of 1:2; 1:5; 1:10, and 1:20. The concentrated MRS solutions containing the bioactive antifungal compound produced by probiotic bacteria were filtered using a 0.22 μm filter and stored at -20 °C. until the antifungal activity test against *A. parasiticus* and *P. expansum* (Vinderola, Mocchiutti & Reinheimer, 2002).

2.3. Antifungal activity tests

The disc-diffusion method used to evaluate the antimicrobial activity of the LAB cell-free supernatant was performed according to Madhyastha, Marquardt, Masi, Borsa, and Frohllch (1994). Ten microliters of either cell-free sterile concentrated supernatant, prepared as described in the paragraph 2.2, were added on sterile discs (6-mm Whatman No. 1, Madrid Spain). The microorganisms used for the inoculums were cultured on 9 mm Petri dishes prepared with 20 mL of PDA, and incubated for 7 days at 30 °C. . After that, 1 mL of distilled water was added on the agar surface, the microorganisms were scraped with a sterile loop and 0.1 mL of the inoculums were introduced in another plate containing only 10 mL of growth medium. The treated discs were placed on the agar surface just after inoculation. After refrigeration at 4 °C. for 6 h to allow the bioactive compounds to diffuse into the agar, the plates were incubated seven days at 30 °C. . According to Castlebury, Sutherland, Tanner, Henderson, and Cerniglia (1999), 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 disc.

2.4. Baking with probiotic bacteria

The loaf bread recipe included 400 g of wheat flour, 3 g of sucrose, 6 g of NaCl, 20 g of yeast for bakery products (Levital, Spain) and 500 mL of tap water.

The probiotic bacteria were added during the baking process (Gerez, Torino, Rollan, & de Valdez, 2009). Briefly, an overnight culture of each probiotic bacteria was used to inoculate (at 1% level) 80 mL of MRS broth and was incubated for 24 h at 37 °C. in anaerobic conditions. The probiotic bacteria tested in this study were cultivated and tested in bread individually carrying out a triplicate of each experiment. Cells were harvested by centrifugation at 2000 rpm for 10 min, washed twice with sterile PBS at pH 7.4 and resuspended in 40 mL sterile water (containing ca. 5 x 10^7 CFU/mL).

The ingredients were kneaded manually for 5 min and the dough produced was left rising for 6 h at room temperature. Baking was performed at 230 °C. for 30 min in a deck oven (MIWE, Arnstein, Germany). The oven was presteamed (300 mL of water) before cooking. The loaves were kept for 30 min on cooling racks at room temperature. Loaves were cut in slices of 30 g each.

The slices were inoculated with 500 µL of a suspension containing 1 x 10^5 conidia/mL *A. parasiticus* CECT 2681. Conidial concentration was measured by optical density at 600 nm and adjusted to 10^5 conidia/mL in PDB as reported by Kelly, Grimm, Bendig, Hempel, and Krull (2006) and introduced in 1L plastic trays. The control group did not receive any treatment during baking with the probiotic bacteria.

All plastic trays were closed hermetically and incubated at room temperature during 15 days. Each day until the analysis the bread slices were examined to determine the visible fungal growth and to evaluate the shelf life extension. Then, all packages were opened and samples were used to determine the AFs content by liquid chromatography coupled to mass spectrometry in tandem (LC-MS/MS).

2.5. Aflatoxin extraction

Extraction was performed using the method described by Hontanaya, Meca, Luciano, Mañes, and Font (2015). Briefly, the two bread slices were finely grounded with a blender (Oster Classic grinder, Madrid, Spain) and 5 g samples were placed in a 50 mL plastic tube. Then, 0.5 g of sodium chloride (NaCl) and 25 mL of a methanol/water (80:20, v/v) mixture were added. Samples were homogenized using Ultra Ika T18 basic Ultraturrax (Staufen, Germany) for 3 min. The mixture was centrifuged at 4500 rpm for 5 min and the supernatant was evaporated to dryness with a Büchi Rotavapor R-200 (Postfach, Switzerland). The residue was re-dissolved in 1 mL of extraction solvent, filtered through a 0.22 µm syringe filter and injected to the LC-MS/MS system.

2.6. AF identification and quantification by LC-MS/MS

LC-MS/MS analyses were performed with a system consisting of an Agilent 1200 chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled to a 3200QTRAP mass spectrometer (Applied Bio-systems, AB Sciex, Foster City, CA, USA) equipped with a turbo ionspray electrospray ionisation (ESI) interface. The instrument data were collected and processed using the Analyst version 1.5.2 software. Separation of analytes was performed using a reversed-phase analytical column (Gemini C18 column, 150 X 2 mm, I.D. 3 µm particle size), equipped with a security guard cartridge C18 (4 X 2 mm, I.D.; 5 µm) all from Phenomenex, Madrid, Spain. The mobile phases were composed of two eluents, both containing 5 mM ammonium formate (Sigma-Aldrich, St. Louis, USA), the eluent A was water + 0.1% formic acid and the eluent B methanol + 0.1% formic acid. The elution gradient was established initially with 10% eluent B, increased to 80% in 1.5 min and kept constant during 2.5 min. The eluent B was increased to 90% in 6 min and then

100% in 4 min. Afterwards, the initial conditions were maintained for 5 min. The flow rate was 0.25 mL/min. MS/MS analysis was achieved in the selected reaction monitoring (SRM) mode using ESI in positive mode. For LC-MS/MS analysis, scheduled SRM was used with a 120 s SRM detection window and 1 s of target scan time. The applied parameters were: ion spray voltage, 5500 V; source temperature, 450 °C. ; curtain gas, 20; ion source gas 1 (sheath gas), 50 psi; ionsource gas 2 (drying gas), 55 psi. Nitrogen served as nebulizer and collision gas. The ionization and fragmentation parameters used for the detection and quantification of the AFs were set according to Liu, Qiu, Kong, Wei, Xiao, and Yang (2013).

3. Results and discussion

The CFS obtained by the fermentation of the MRS broth inoculated with the probiotic bacteria was concentrated under nitrogen flow and resuspended in fresh MRS to obtain concentrations of the dried extract of 1:2; 1:5; 1:10, and 1:20 (Section 2.3). The results related to the antifungal activity of the CFS against *A. parasiticus* (AFs producer) and *P. expansum* (PAT producer) in solid medium of PDA are shown in Table 1. The analysis of the data shows that the CFS concentrated 2:1, 5:1, and 10:1 did not possess any antifungal activity against the mycotoxigenic fungi tested. On the other side, the 20:1 concentrated CFSs, obtained by the fermentation of *B. bifidum*, *L. ruminis*, *L. rhamnosus* (CECT 288), *L. johnsoni*, *L. plantarum* and *L. bulgaricus* in MRS medium, produced the inhibition of *P. expansum* growth (Fig. 1). The same CFSs, excluding only the fermented medium obtained by the fermentation of *L. bulgaricus*, resulted effective also against *A. parasiticus*, probably due to the antimicrobial properties of fermentation products (phenolic compounds and bioactive proteins and peptides)

of LAB present in the media. The control composed of MRS broth treated in the same way did not show evidence of any antifungal property against the mycotoxigenic fungi tested (Fig. 1).

For the first time, the antifungal components present in the fermented media of the probiotic bacteria were tested on these two species of mycotoxigenic fungi, whereas the antifungal properties of bioactive components produced by LAB have been investigated by other authors. In particular, Valerio, Favilla, De Bellis, Sisto, De Candia, and Lavermicocca (2009), studied a total of 125 presumptive LAB isolates. Eight out of 17 LAB, namely *W. cibaria* (3 strains), *W. confusa* (1 strain), *Lactobacillus citreum, Lactobacillus mesenteroides, L. plantarum* and *Lactobacillus rossiae*, almost completely inhibited (more than 90%) the growth of the yeast *Endomyces fibuliger* compared to control. Fermentation products of LAB also influenced the growth of the filamentous fungus *Penicillium roqueforti*, which was inhibited by almost all strains at a percentage higher than 65.5%. In particular, *L. plantarum* was the most effective against the bread microbial contaminant, *P. roqueforti*. Finally, *L. citreum*, *W. cibaria* and *L. rossiae* completely inhibited (>98%) the growth of the other filamentous fungus, *Aspergillus niger*. The results obtained by the authors confirmed the data evidenced in our study, in particular related to the antifungal activity of the bioactive compounds produced by *L. plantarum.*

Ryan, Dal Bello, and Arendt (2008) evidenced similar results on the antifungal properties of the bioactive metabolites produced by *L. plantarum*, in fermented sourdoughs using an agar diffusion assay. *L. plantarum* in fermented sourdough showed the highest inhibitory activity against all the fungi tested. Additionally, the authors evaluated also the ability of *L. plantarum* sourdoughs to extend the shelf life of wheat bread.

Table 1. Antifungal activity on solid medium of MRS agar of the bioactive compounds presents in cell-free supernatants of the MRS broth fermented with several probiotic strains against *Aspergillus parasiticus* and *Penicillium expansum*. Calculation of antifungal activity: 8 mm diameter clearing zone (+), 10 mm diameter clearing zone (++), and more than 10 mm diameter clearing zone (+++).

	Concentration of extracelular medium							
Strains	**2:1**		**5:1**		**10:1**		**20:1**	
	P. expansum	***A. parasiticus***	***P. expansum***	***A. parasiticus***	***P. expansum***	***A. parasiticus***	***P. expansum***	***A. parasiticus***
Control	-	-	-	-	-	-	-	-
B. longum CECT 4551	-	-	-	-	-	-	-	-
B. bifidum CECT 870T	-	-	-	-	-	-	++	++
B. breve CECT 4839T	-	-	-	-	-	-	-	-
L. adolescentis CECT 5871T	-	-	-	-	-	-	-	-
L. rhamnosus CECT278T	-	-	-	-	-	-	-	-
L. ruminis CECT 1324	-	-	-	-	-	-	+	++
L casei CECT 4647	-	-	-	-	-	-	-	-
L. rhamnosus CECT 288	-	-	-	-	-	-	+	++
L. johnsoni CECT 289	-	-	-	-	-	-	+	+
L. casei CECT 475	-	-	-	-	-	-	-	-
L plantarum CECT 749	-	-	-	-	-	-	++	++
L. reuteri CECT 725	-	-	-	-	-	-	-	-
L. bulgaricus CECT 4005	-	-	-	-	-	-	+	-
L. paracasei CECT 4022	-	-	-	-	-	-	-	-
L. salivarius CECT 4062	-	-	-	-	-	-	-	-
L. salivarius CECT 4305	-	-	-	-	-	-	-	-

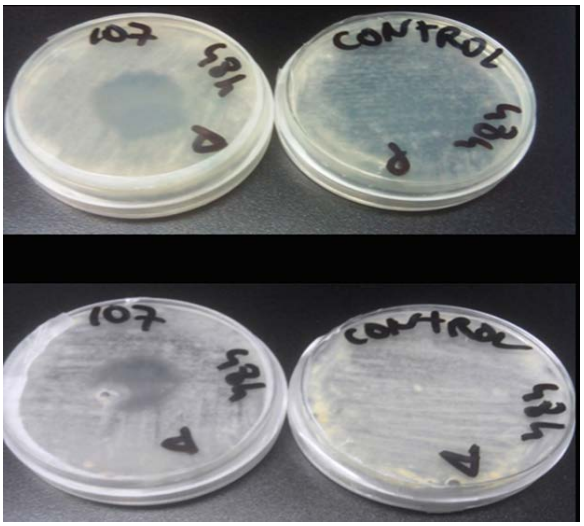

Figure 1. Inhibition zone evidenced by bioactive compounds presents in cell-free supernatant of the MRS broth fermented with the probiotic strain of *L. plantarum* concentred 20:1 on a) *Penicillium expansum* and b) *Aspergillus parasiticus*.

Comparison with the spoilage rate in the control breads revealed that *L. plantarum* sourdough breads retarded the outgrowth of *A. niger*, *F. culmorum* and *P. expansum*, but no inhibition was observed against *P. roqueforti*. All controls, *Lactobacillus sanfranciscensis* LTH2581 sourdough bread, the non-fermented control and the chemically acidified breads showed no effect against the selected fungi. The bread slices were completely spoiled after 3 days. Additionally, there was no significant difference in the moisture, pH and total titratable acidity (TTA) values for all the fermented and chemically acidified breads. The data obtained by these authors was in concurrence with the values evidenced in our study.

The strains of *L. bulgaricus, L. plantarum, L. johnsoni, L. rhamnosus* (CECT 288)*, L. ruminis* and *B. bifidum* that evidenced the highest antifungal activity against the two mycotoxigenic strains of *A. parasiticus* and *P. expansum* in the *in vitro* assay were employed for the study of the shelf life improvement of loaf

bread. In the control experiment (loaf bread fermented only with yeast, without probiotic bacteria, and stored at the same conditions of the treated loaf bread), the visual growth of the *A. parasiticus* started in the fifth day of incubation, obtaining for this sample a total shelf life of four days (Table 2). An important increment of the shelf life was observed in the loaf breads that were inoculated with probiotic bacteria (*L. bulgaricus* and *L. plantarum*) during the baking process. In the loaf breads fermented with *L. bulgaricus,* the visual fungal growth started at the eighth day of storage obtaining a total shelf life of seven days, whereas in the loaf breads fermented with *L. plantarum*, the fungal growth of *A. parasiticus* was detectable in the ninth day of incubation obtaining a total shelf life of 8 days (Fig. 2).

Table 2. Shelf life expressed in days of the loaf breads contaminated with *Aspergillus parasiticus* and fermented using several lactic acid bacteria with antifungal properties showed in the antimicrobial assay in solid medium of MRS agar.+ = Loaf breads contaminated with visible colonies of *A. parasiticus* and, - = Loaf breads without any visible sign of *A. parasiticus* growth.

	Incubation time (days)								
Strains	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Control	-	-	-	-	+	+	+	+	+
L. bulgaricus	-	-	-	-	-	-	-	+	+
L. plantarum	-	-	-	-	-	-	-	-	+
L. johnsoni	-	-	-	-	+	+	+	+	+
L. rhamnosus	-	-	-	-	+	+	+	+	+
L. ruminis	-	-	-	-	+	+	+	+	+
B. bifidum	-	-	-	-	+	+	+	+	+

The experiments carried out using the probiotic bacteria (*L. bulgaricus* and *L. plantarum*) during the fermentation of the loaf breads, evidenced an increment of the shelf life of the loaf bread compared with the control of three and four days

respectively. The loaf bread fermented with the other strains used in this part of the experiments did not show any significant improvement of the shelf life compared with the control experiment (Table 2).

Considering that the strain of the *A. parasiticus* used in this study was AFs producer, the control and treated loaf breads were extracted for AFs detection and quantification using the LC-MS/MS. In particular in the control loaf bread at the ninth day of incubation, the strain of *A. parasiticus* produced 550.16 µg/Kg of AFB_1, 166.76 µg/Kg of AFB_2, 679.34 µg/Kg of AFG_1 and 108.09 µg/Kg of AFG_2. As evidenced in Table 3, the mean reductions of AF production by *A. parasiticus* in the loaf bread fermented with the probiotic bacteria *L. bulgaricus* and *L. plantarum* were 99.9 and 99.4%, respectively.

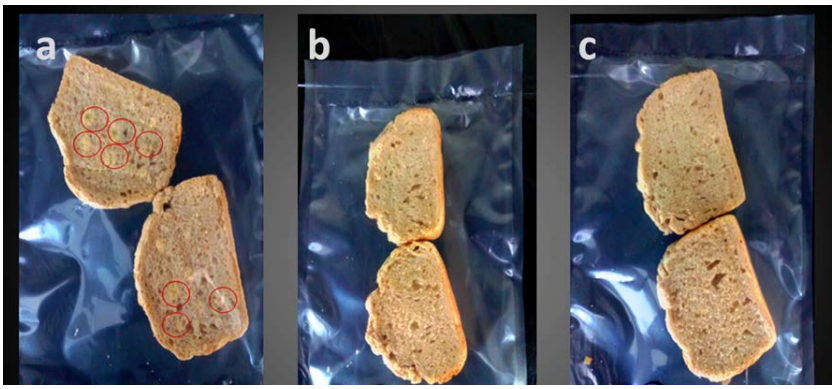

Figure 2. Visual observation of the loaf breads contaminated with *Aspergillus parasiticus* at seven day incubation, in the a) control where is clearly visible the presence of the fungal contaminant, b) and c) in the loaf bread produced with *L. bulgaricus* and *L. plantarum* respectively, where is possible to observe the absence of the mycotoxigenic fungi.

This result is very important, considered that the strains that produced the highest shelf life improvement of the loaf bread were also the ones that reduced the production of these important contaminants. The mean AFs reduction evidenced by the other probiotic strains used in the experiments

carried out in this study ranged from the 84.1 and 93.5%. Considering that the other strains do not improve the shelf life of the loaf breads compared with the control experiments, this data could have a special interest because the compounds produced by the probiotic bacteria during the fermentation of the loaf breads do not reduce the fungal growth in comparison with the control experiments but inhibited the production of the AFs by the *A. parasiticus* strains.

The present results suggest 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 bioconservation of packaged food such as loaf bread.

Table 3. AFs reduction promoted by the inhibition of the mycotoxigenic *Aspergillus parasiticus*, by fermentation of the loaf bred with antifungal lactic acid bacteria.

	% of Reduction				
Strains	**AFB_1**	**AFB_2**	**AFG_1**	**AFG_2**	**Mean**
Control	0.0	0.0	0.0	0.0	0.0
L. bulgaricus	99.9±4.4	99.9±3.3	99.9±5.5	100.0±5.7	99.9±5.2
L. plantarum	99.0±5.2	99.4±4.2	99.2±5.1	100.0±3.1	99.4±3.8
L. johnsoni	84.6±3.6	86.8±3.7	87.3±4.9	77.8±6.1	84.1±4.1
L. rhamnosus	91.4±2.6	93.6±3.9	95.6±3.5	93.3±4.1	93.5±3.9
L. ruminis	85.6±5.8	91.1±4.1	90.7±3.9	92.3±2.8	89.9±5.2
B. bifidum	76.5±2.9	93.8±2.9	84.9±4.4	88.4±3.9	85.9±4.4

4. Conclusion

In this study for the first time the components present in the fermented media of the probiotic bacteria were tested against these two mycotoxigenic fungi *A. parasiticus* and *P. expansum*. The components produced by 6 of the tested LABs showed antifungal activity against *A. parasiticus* and/or *P. expansum*. Furthermore, the use of LAB as starter cultures with yeast in manufacture bread

extended the shelf-life of contaminated bread with *A. parasiticus* and reduced AFs. The reduction of AFs production in contaminated bread is primarily due to inhibition of fungal growth by the bioactive compounds synthesized during fermentation by LAB, as well as the bond between AFs and non-viable LAB after processing. Further investigations will be focused on the isolation and identification of bioactive compounds responsible of the antifungal activity on the two mycotoxigenic strains tested.

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3.7. Influence of probiotic microorganisms on aflatoxin B_1 and B_2 bioaccessibility evaluated with a simulated gastrointestinal digestión

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3.7. Influence of probiotic microorganisms on aflatoxins B_1 and B_2 bioaccessibility evaluated with an simulated gastrointestinal

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Abstract

Aflatoxins (AFs) are produced mainly by the molds *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxin B1 (AFB1) is classified as carcinogenic to humans. The aim of this study was to evaluate the capacity of different strains of *Lactobacilli* (*Lb*.) and *Bifidobacteria* (*Bf*.) to reduce the bioaccessibility of AFB1 and aflatoxin B2 (AFB2), spiked in loaf bread, using a dynamic *in vitro* simulated gastrointestinal digestion system. Aliquots of 20 mL of gastric and duodenal fluids were sampled for the determination of the mycotoxins gastric and duodenal bioaccessibility respectively, by liquid-chromatography coupled to the mass spectrometry in tandem (LC-MS/MS). A reduction of AFs bioaccessibility compared to the control (digestion without bacterial strains) was evidenced. The strains that evidenced the highest gastric and duodenal bioaccessibility reductions of AFB1 and AFB2 were *Lb. johnsoni* CECT 289, *Lb. reuteri* CECT 725, *Lb. plantarum* CECT 220 and *Lb. casei* CECT 4180, with values ranging from 76.38 to 98.34% for AFB1 and from 77.14 to 98.66% for AFB2. These results suggest that a food enriched with specific probiotic microorganisms and consumed at the same time as food contaminated with AFs, could reduce the risk associated to the intake of these toxic compounds contained in food.

Keywords: Aflatoxins, bioaccessibility, probiotic bacteria, *Lactobacillus*, *Bifidobacterium.*

1. Introduction

Aflatoxins (AFs), a group of structurally related toxic secondary metabolites of fungi, are primarily produced by *Aspergillus flavus* and *Aspergillus parasiticus*, groups of fungi that could be found in corn, nuts, peanuts, coconut, dried fruits and beer (Frenich et al., 2009; Li et al., 2009a; Li et al., 2009b; Williams et al., 2004). There are four major AFs named as B_1, B_2, G_1 and G_2. Among them, aflatoxin B_1 (AFB_1) is classified as a carcinogenic substance of group 1 by the International Agency for Research on Cancer (IARC) as it may interfere with the inductive of specific enzymes and forbid the synthesis of RNA 5 (IARC, 2012; Merrick et al., 2013; Wild and Montesano, 2009). Owing to the highly resistance to degradation during food processing, AFB_1 could enter the food chain and provide a threat to human health (Castells et al., 2007). Therefore, the regulatory limits for AFs ($B_1 + B_2 + G_1 + G_2$), even for AFB_1, have been established in several countries. The European Commission has set strict limits for the maximum allowed levels (MAL) of AFB_1 in ground-nuts, dried fruits and their products, in which the MAL of AFB_1 could not be greater than 2 μg kg^{-1} for Retail Ready Foods (Van Egmond, 1995). It has been proved that the intake of AFB_1 over a long time may be dangerous even at a very low concentration.

Therefore, the assays with high sensitivity and specificity are required to determine AFs at trace level in foods and agricultural products.

Even though the consumption of food contaminated with AFs should be strictly avoided due to its toxicity and carcinogenic effect, several studies show presence of AFs in different cereal products (Saladino et al., 2017; Iqbal et al., 2014; Serrano et al., 2012), sometimes above the limits enforced by the European legislation. For this reason different strategies have been developed to prevent the growth of mycotoxin producing fungi on food and feed, as well as to decontaminate and/or detoxify mycotoxin contaminated products. One of the

most used strategy to reduce the mycotoxins bioaccessibility during the gastrointestinal digestion is the employment of probiotic bacteria. *Lb*. and *Bf*. have shown AF-binding ability. This mechanism is unclear but it is suggested that is a physical phenomenon associated with bacterial cell wall structure. Peptidoglycans and polysaccharides have been suggested to be the two most important elements responsible for the binding (Kabak et al., 2009).

In human health risk assessment, ingestion of food is considered a major route for exposure to many contaminants. The total amount of an ingested contaminant (intake) does not always reflect the quantity that is available to the body, because only a smaller amount will be available for absorption. As a consequence, bioaccessibility, defined as the amount of contaminant released through the gastrointestinal tract from the food matrix and then potentially absorbable, can be considered a measure for the assessment of mycotoxin bioavailability in food (Versantvoort et al., 2005).

Recently is increasing the interest in the use of microorganisms to reduce the absorption of mycotoxins, present in food and feed, in the gastrointestinal tract. In particular, Kabak and Ozbey (2012a) studied the effectiveness of some probiotic bacteria to reduce the amount available for intestinal absorption of AFs from different contaminated food materials obtaining reductions in the bioaccessibility up to 35.6% for AFB_1, 35.5% for AFB_2, 31.9% for AFG_1 and 33.6% for AFG_2. Kabak and Ozbey (2012b) obtained a reduction between 15.5% and 31.6% in AFM_1 bioaccessibility (in milk) in the presence of probiotic bacteria and Serrano-Niño et al. (2013) showed reduction of AFM1's bioaccessibility in phosphate buffer saline (PBS) from 22.72 to 45.17% using five different probiotic strains.

This is the first report in which is evaluated the effect of the intake of a simulated food enriched with probiotic microorganisms on reducing AFs bioaccessibility if consumed at the same time as contaminated loaf bread.

The aim of this study was to evaluate the capacity of probiotic microorganisms to reduce the bioaccessibility of AFB1 and AFB_2 using a dynamic *in vitro* simulated gastrointestinal digestion system.

2. Materials and methods

2.1. Chemicals

Potassium chloride (KCl), potassium thiocyanate (KSCN), sodium dihydrogen phosphate (NaH_2PO_4), sodium sulfate (Na_2SO_4), sodium chloride (NaCl), sodium hydrogen carbonate ($NaHCO_3$), urea ($CO(NH_2)_2$), α-amylase (930 U mg^{-1} A3403), hydrochloric acid (HCl), sodium hydroxide (NaOH), formic acid (HCOOH), pepsin A (674 U mg^{-1} P7000), pancreatin (762 U mg^{-1} P1750), bile salts (B8631), phosphate buffer saline (PBS, pH 7.5) and standard solutions of AFB_1 and AFB_2 (≥98% purity), were purchased from Sigma-Aldrich (Madrid, Spain). Methanol and ethyl acetate were supplied by Fisher Scientific (Madrid, Spain). Deionized water was purchased 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.

2.2. Bacterial strains and growth conditions

Fifteen commercial probiotic strains were used in the *in vitro* system to evaluate the capacity to reduce AFs bioaccesibility during simulated gastrointestinal digestion. In particular, *Lb. rhamnosus* CECT 278T (1), *Lb. ruminis* CECT 4061T, *Lb. casei* CECT 475 (1), *Lb*. *rhamnosus* CECT 288 (2), *Lb. johnsonii*

CECT 289, *Lb. casei* CECT 4180 (2), *Lb. plantarum* CECT 220, *Lb. reuteri* CECT 725, *Lb. bulgaricus* CECT 4005, *Lb. paracasei* CECT 277, *Lb. salivarus* CECT 4062, *Bifidobacterium Longum* CECT 4551, *Bf. bifidum* CECT 870T, *Bf*. *breve* CECT 4839T, and *Bf. adolescentis* CECT 5781T were obtained from the Spanish Type Culture Collection (CECT Valencia, Spain), in sterile 18% glycerol. The bacterial strains were tested individually and were added in the simulated saliva before the gastric digestion step at ~10^5 CFU mL^{-1} to simulate the intake of a food enriched with probiotic microorganisms and consumed at the same time as spiked loaf bread produced in this study.

For longer survival and higher quantitative retrieval of the cultures, they were stored at −80 °C. When needed, the recovery of strains was undertaken by two consecutive subcultures in appropriate media prior to use.

2.3. Loaf bread preparation

The recipe for loaf bread preparation was composed by: 600 g of wheat flour, 20 g of sucrose, 10 g of NaCl, 40 g of yeast for bakery products (Levital, Spain) and 350 mL of water. The ingredients were kneaded manually for 5 min and the dough produced was left rising for 1 h at room temperature. Baking was performed at 200 ºC for 40 min in a deck oven (MIWE, Arnstein, Germany). The oven was presteamed (300 mL of water) before loading. The breads were kept for 30 min on cooling racks at room temperature. Twenty grams of loaf bread were spiked with 90 µL AFB_1 and AFB_2 at 14.79 and 6.14 mg kg^{-1}, in 9 spot of 10 µL each to cover significatively the bread loaf surface respectively, using a stock methanolic solution (1000 µM) of each AFs. After 12 h contact at room temperature to completely remove the solvent, the bread was used for the *in vitro* dynamic digestion.

2.4. In vitro dynamic digestion model

Gastrointestinal digestion in the *in vitro* dynamic model was carried out using 5L bioreactors Infors (Bottmingen, Switzerland) (Fig. 1) 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 2 g. The incubation temperature was maintained at 37 °C.

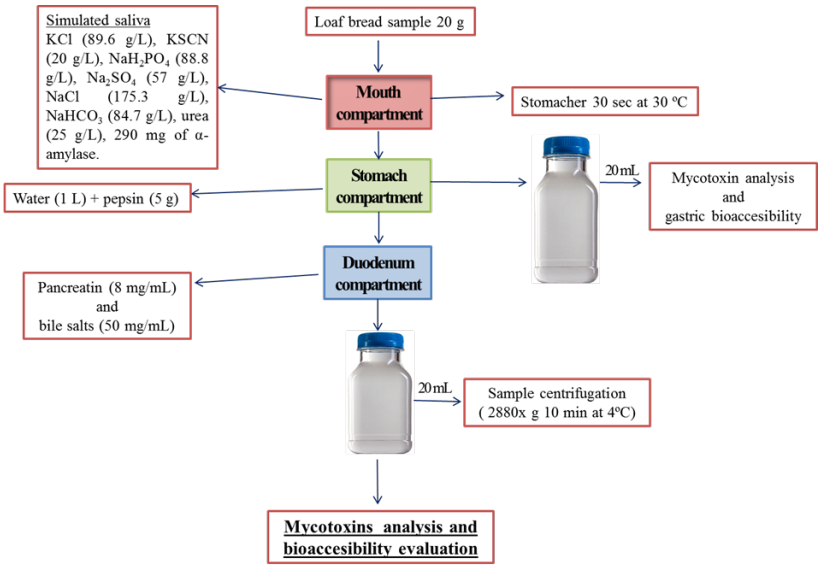

Figure 1. Schematic representation of the *in vitro* simulated gastrointestinal digestion system used for the digestion of the loaf bread spiked with AFB_1 and AFB_2.

Twenty grams of spiked loaf bread were mixed with 60 mL of artificial saliva (composed of: 10 mL of KCl (89.6 g L^{-1}), 10 mL of KSCN (20 g L^{-1}), 10 mL of NaH_2PO_4 (88.8 g L^{-1}), 10 mL of Na_2SO_4 (57 g L^{-1}), 1.7 mL of NaCl (175.3 g L^{-1}), 20 mL $NaHCO_3$ (84.7 g L^{-1}), 8 mL of urea (25 g L^{-1}), and 290 mg of α-amylase). The bacterial strains were also added individually at ~105 CFU mL^{-1} 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 (14 800 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 2 h. 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 $NaHCO_3$ (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^{-1}) and 25 mL of bile salts (50 mg mL^{-1}) 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)

2.5. Mycotoxin extraction from the simulated intestinal fluids

AFs B_1 and B_2 contained in gastric and gastric+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 liquid-chromatography coupled to the mass spectrometry in tandem (LC-MS/MS).

2.6. LC-MS/MS aflatoxin identification and quantification

The liquid-chromatography system consisted of a binary LC-20AD pump, a SIL-20 A C homoeothermic auto-sampler, a CTO-20A column oven and a CMB-20A controller (Agilent, Santa Clara, USA) an Analyst Software 1.5.2 (Applied Biosystems, Foster City, USA) was used for data acquisition and processing. The separation of AFs was performed on a Gemini NX C18 column (150 X 2.0 mm I.D, 3.0 mm; Phenomenex, CA, USA) at room temperature (20 °C).

The mobile phase was composed of solvents A (5 mM ammonium formate and 0.1% formic acid in water) and B (5 mM ammonium formate and 0.1% formic acid in methanol) at a flow rate of 0.25 mL/min. The elution gradient was established initially with 10% eluent B, increased to 80% in 1.5 min, then kept constant from 1.5 to 4 min, increased to 90% from 4 to 10 min, increased again to 100% from 10 to 14 min and finally return to the initial conditions and requilibrate during 10 min. The injection volumen was 20 mL. An API-4000 triple-quadruple MS/MS system (Applied Biosystems, Foster City, CA, USA) equipped with ESI interface in positive mode was used for detection in multiple reactions monitoring (MRM) mode. The main MS parameters were optimized and finally set as follows: nebulizer gas (GS1), 55 psi; auxiliary gas (GS2), 50 psi; curtain gas (CUR) 15 psi; capillary temperature 550 °C; ion spray voltage (IS) 5500 V. Nitrogen was used as the nebulizer, heater, curtain and collision gas. The precursor-to-product ion transitions were m/z 313.1/241.3-284.9 and m/z 315.1/259.0-286.9 for AFB_1 and AFB_2, respectively. Quantification of AFs was carried out by comparing peak areas

of investigated samples with the calibration curve performed with standards (concentrations ranging from 0.1 to 100 μg L^{-1}).

2.7. 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 intestinal fluids (free from contamination of AFs) (n=3) at levels of AFs (0.1-100 μg L^{-1}). The recoveries evidenced for AFB_1 and AFB_2 were 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 AFs were 0.04 and 0.15 μg L^{-1} for AFB_1 and 0.21 and 0.72 μg L^{-1} for AFB_2, respectively.

3.2. Evaluation of the AFs contained in the gastric and duodenal fluids (bioaccessibility)

Tables 1 and 2 show the bioaccessibility data of the AFs B_1 and B_2 present in loaf bread spiked with these two contaminants (the initial concentrations present in the food matrix were 14.79 and 6.14 mg kg^{-1} for AFB_1 and AFB_2, respectively), after being digested using an in vitro method to mimic the conditions of human

gastrointestinal digestion in presence of different probiotic strains. In particular, in the control experiment (consisting of a spiked loaf bread digested without probiotic strains), the AFB_1 bioaccessibility of the stomach and the duodenal digestion were 53.89 and 25.76%, respectively. All tests performed with the probiotic strains, evidenced a reduction of the AFB_1 bioaccessibility compared with the control experiments. The mean AFB_1 bioaccessibility (considering both stomach and duodenal compartments) ranged from 0.94% (with *Lb. johnsoni*) to 30.71% (with *Lb. bulgaricus*). The lowest AFB_1 bioaccessibility was obtained with *Lb. johnsoni*, displaying gastric and duodenal bioaccessibility reductions of 98.09 and 96.73%, respectively. In the treated samples, the mean AFB_1 bioaccessibility reduction was equal to 59.12%. The strains that evidenced the highest AFB_1 bioaccessibility reductions were *Lb. johnsoni*, *Lb.reuteri*, *Lb. plantarum* and *Lb. casei* (2), with values ranging from 76.38 to 98.34%.

Regarding AFB_2, its bioaccessibility at gastric and duodenal levels were 57% and 36.48%, respectively. In the samples treated with the probiotic strains during the gastrointestinal digestion, the mean lower and higher bioaccessibility for this contaminant were detected in samples treated with *L. reuteri* (3.09%) and *Bf. Bifidum* (39.90%). When comparing the data of the treated samples with those of the control ones, a mean AFB_2 bioaccessibility reduction of 52.65% was noted. The strains that showed the highest bioaccessibility reductions of AFB_1 (*Lb. johnsoni*, *Lb. reuteri*, *Lb. plantarum* and *Lb. casei* (2)) also showed the highest AFB_2 bioaccessibility reductions (77.14-98.66%). In particular, as can be observed in Tables 1 and 2, the relation between bioaccessibility reductions of AFB_1 and AFB_2 is rather similar among the same probiotic strain and also among all the strains.

The observed differences among probiotic strains in reducing AFs bioaccesibility is unclear, however it has been speculated that cell surface hydrophobicity can be related to AF-binding (Oatley et al., 2000). It is thought that

AF molecules are bound on the cell wall components of specific bacteria so that the different efficacy of the bacteria might be due to completely different binding sites present in different strains or minor differences in similar binding sites that varies in a strain dependent manner (Hernandez-Mendoza et al., 2009). In previous studies have been found that some strains of *Lb.* and *Bf.* have AF-binding ability. El-Nezami et al. (1998) showed that within 24 h cultures of *Lb. rhamnosus* strain GG and *Lb. rhamnosus* strain LC-705 were able to remove approximately 80% of the AFB_1. In other research by Peltonen et al. (2000) the binding of AFB_1 by *Lb. paracasei* F19, *Bf. lactis* Bb-12, *Lb. crispatus* M247 and MU5, *Lb. salivarius* LM2-118 and *Lb. johnsonii* LJ-1 was 12 found to range from 5.8 to 31.3%. *Lb johnsonii* LJ-1 and *Lb paracasei* F19 were the best binders with approximately 30% binding. Kabak and Var (2004) determined that the ability of *Lb. acidophilus* NCC12, *Lb. acidophilus* NCC36, *Lb. acidophilus* NCC68, *Bf. bifidum* Bb13, *Bf. bifidum* NCC3881 and *Lb. rhamnosus* to bind AFM_1 ranged between 25.7–32.5% and 21.2–29.3% in phosphate-buffered saline and skimmed milk, respectively.

Table 1. Gastric and duodenal bioaccessibility reduction of AFB_1 present in loaf bread, subjected to digestion with probiotic microorganisms. Significantly different from the control, $p \leq 0.05$ (*), $p \leq 0.001$ (**), $p \leq 0.0001$ (***).

Samples	**Aflatoxin B_1**			
	Concentration (ppm)	**Bioacc. (%)**	**Mean**	**Bioacc. Red. (%)**
Control[1] S[2]	7.97±0.40	53.89	39.82	
Control D[3]	3.81±0.10	25.76		
Bf. longum S	3.97±0.20	26.81	17.80	50.25***
Bf. longum D	1.30±0.030	8.79		65.88***
Bf. bifidum S	4.82±0.20	32.60	22.38	39.51***
Bf. bifidum D	1.80±0.01	12.17		52.76***
Bf. breve S	3.18±0.20	21.49	16.53	60.12***
Bf. breve D	1.70±0.08	11.56		55.11***

Table 1. *(continued)*

Samples	Aflatoxin B_1			
	Concentration (ppm)	**Bioacc. (%)**	**Mean**	**Bioacc. Red. (%)**
Bf. adolescentis S	5.45±0.40	36.88	26.05	31.57**
Bf. adolescentis D	2.25±0.20	15.21		40.94***
Lb. rhamnosus (1) *S*	3.20±0.40	40.15	25.82	25.49**
Lb. rhamnosus (1) *D*	1.70±0.03	11.49		55.38***
Lb. ruminis S	3.31±0.03	22.35	16.58	58.58***
Lb. ruminis D	1.60±0.01	10.82		58.01***
Lb. casei (1) *S*	2.44±0.02	16.47	12.29	69.43***
Lb. casei (1) *D*	1.20±0.03	8.11		68.50***
Lb. rhamnosus (2) *S*	3.45±0.40	23.30	16.31	56.73***
Lb. rhamnosus (2) *D*	1.38±0.07	9.30		63.89***
Lb. johnsoni S	0.15±0.01	1.03	0.94	98.09***
Lb. johnsoni D	0.12±0.02	0.84		96.73***
Lb. casei (2) *S*	0.46±0.03	3.12	2.72	94.21***
Lb. casei (2) *D*	0.34±0.03	2.31		91.02***
Lb. plantarum S	0.13±0.01	0.90	0.98	98.34***
Lb. plantarum D	0.16±0.01	1.06		95.90***
Lb. reuteri S	1.43±0.03	9.67	7.88	82.06***
Lb. reuteri D	0.90±0.02	6.09		76.38***
Lb. bulgaricus S	6.74±0.40	45.58	30.71	15.41*
Lb. bulgaricus D	2.34±0.03	15.84		38.49**
Lb. paracasei S	5.26±0.40	35.57	25.25	34.00**
Lb. paracasei D	2.20±0.05	14.93		42.06***
Lb. salivarus S	5.69±0.70	38.50	28.22	28.55**
Lb. salivarus D	2.64±0.30	17.93		30.39**

[1]The control consisted of spiked loaf bread digested without probiotic strains.
[2]Stomach.
[3]Duodenum.

Kabak et al. (2009) studied the release of AFB_1 and ochratoxin A (OTA) from different food products in the gastrointestinal tract in the absence and presence of probiotics, as possible adsorbents. The average bioaccessibility of AFB_1 and OTA

without probiotics was about 90% and 30%, respectively, depending on several factors such as food product, contamination level, compound and type of contamination (spiked versus naturally contaminated). The six probiotic bacteria showed a variable AFB_1 and OTA binding capacities, which depended on the bacterial strain, toxin, type of food and contamination level. A reduction of 37% and 73% was observed for the AFB_1 and OTA bioaccessibility in the presence of probiotic bacteria, respectively.

Raiola et al. (2012) analyzed 27 samples of dried pasta characterized by size, packaging and marketing intended for young children consumption, by liquid chromatography (LC) and liquid chromatography–tandem mass spectrometry (LC–MS/MS) for Deoxynivalenol (DON), OTA and AFB_1 determination. The samples that showed the highest amounts of one of the mycotoxins were cooked for 10 min, digested with an in vitro gastrointestinal protocol and bioaccessibility values were calculated. Seven of the 27 samples exceeded by 120-225% the European legal limit set for DON in processed cereal-based baby foods (Commission Regulation (EC) 1126/2007). The mean value of gastric bioaccessibility of DON was 23.1%, whereas the mean duodenal bioaccessibility was equal to 12.1%.

Kabak and Ozbey (2012) investigated the bioaccessibility of AFs from various spiked food matrices (peanut, pistachio, hazelnut, dried figs, paprika, wheat and maize) and evaluated the effectiveness of six probiotic bacteria in reducing AF bioaccessibility using an in vitro digestion model. The bioaccessibility of AFs from seven food matrices ranged from 85.1% to 98.1% for AFB_1, 83.3% to 91.8% for AFB_2, 85.3% to 95.1% for AFG_1 and 80.7% to 91.2% for AFG_2. The bioaccessibilities of all four compounds were independent of the spiking level and food matrix. The inclusion of probiotic bacteria showed a significant ($p < 0.05$) reduction in the bioaccessibility of AFs: up to 35.6% for AFB_1, 35.5% for AFB_2, 31.9% for AFG_1 and 33.6% for AFG_2. AF-binding activity of probiotic bacteria in simulated

gastrointestinal conditions was reversible, and 10.3–39.8% of bound AFs were released back into the digestion juices from the bacteria–AF complexes.

Table 2. Gastric and duodenal bioaccessibility reduction of AFB_2 present in loaf bread, subjected to digestion with probiotic microorganisms. Significantly different from the control, $p \leq 0.05$ (*), $p \leq 0.001$ (**), $p \leq 0.0001$ (***).

Samples	**Aflatoxin B_2**			
	Concentration (ppm)	**Bioac. (%)**	**Mean**	**Bioac. Red (%)**
Control[1] S[2]	3.50±0.2	57.00	46.74	
Control D[3]	2.24±0.3	36.48		
Bf. longum S	3.00±0.1	48.86	36.64	14.29*
Bf. longum D	1.50±0.3	24.43		33.04**
Bf. bifidum S	3.10±0.2	50.49	39.90	11.43*
Bf. bifidum D	1.80±0.1	29.31		19.64*
Bf. breve S	2.20±0.3	35.83	24.43	37.14**
Bf. breve D	0.80±0.1	13.03		64.29***
Bf. adolescentis S	2.80±0.4	45.60	33.39	20.00**
Bf. adolescentis D	1.30±0.2	21.17		41.96**
Lb. rhamnosus (1) *S*	2.10±0.2	34.20	24.43	40.00**
Lb. rhamnosus (1) *D*	0.90±0.1	14.66		59.82***
Lb. ruminis S	2.20±0.3	35.83	24.43	37.14***
Lb. ruminis D	0.80±0.2	13.03		64.29***
Lb. casei (1) *S*	2.00±0.3	32.57	21.99	42.86***
Lb. casei (1) *D*	0.70±0.1	11.40		68.75***
Lb. rhamnosus (2) *S*	2.40±0.2	39.09	31.76	31.43**
Lb. rhamnosus (2) *D*	1.50±0.3	24.43		33.04**
Lb. johnsoni S	0.80±0.1	13.03	8.96	77.14***
Lb. johnsoni D	0.30±0.08	4.89		86.61***
Lb. casei (2) *S*	0.50±0.2	8.14	4.89	85.71***
Lb. casei (2) *D*	0.10±0.02	1.63		95.54***
Lb. plantarum S	0.40±0.1	6.51	3.66	88.57***
Lb. plantarum D	0.05±0.01	0.81		97.77***

Table 2. *(continued)*

Lb. reuteri S	0.35±0.06	5.70	3.09	90.00***
Lb. reuteri D	0.03±0.01	0.49		98.66***
Lb. bulgaricus S	2.30±0.08	37.46	27.69	34.29***
Lb. bulgaricus D	1.10±0.3	17.91		50.89***
Lb. paracasei S	2.20±0.2	35.83	26.06	37.14**
Lb. paracasei D	1.00±0.2	16.29		55.36***
Lb. salivarus S	2.30±0.3	37.46	31.76	34.29**
Lb. salivarus D	1.60±0.2	26.06		28.57**

[1]The control consisted of spiked loaf bread digested without probiotic strains.
[2]Stomach.
[3]Duodenum.

4. Conclusions

The present study showed the capacity of probiotic bacteria to reduce the bioaccessibility of AFB_1 and AFB_2 in spiked loaf bread. In particular, the highest bioaccesibility AFs reduction was obtained when the spiked loaf bread was digested together with *Lb. johnsoni* CECT 289, *Lb. casei* CECT 4180, *Lb. plantarum* CECT 220 and *Lb*. *reuteri* CECT 725, reaching reduction up to 98.66%.

Results from this study suggest that a food enriched with specific probiotic microorganisms and consumed at the same time as food contaminated with AFs, could reduce the risk associated to the intake of these toxic compounds contained in food.

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4. GENERAL DISCUSSION

4. DISCUSIÓN GENERAL

El trabajo de investigación llevado a cabo a lo largo de esta Tesis Doctoral se ha concretado en el análisis de micotoxinas en pan de molde y en el estudio de métodos químicos y biológicos capaces de reducir la presencia de hongos y micotoxinas en pan y productos similares a base de cereales.

En el primer trabajo se ha validado un método analítico para el análisis de diecisiete micotoxinas en pan. Posteriormente se ha evaluado la exposición de la población valenciana a dichas micotoxinas a través del consumo de pan de molde y el riesgo asociado a su exposición mediante el análisis de 80 muestras de pan de molde procedentes de pequeños comercios y grandes superficies de la ciudad de Valencia.

La presencia de estas micotoxinas en las muestras analizadas planteó la necesidad de reducir estos compuestos o evitar su producción en el pan. Para ello se han estudiado métodos químicos y biológicos, como los isotiocianatos (ITCs) y algunas bacterias lácticas (BALs), y sus efectos sobre el crecimiento del hongo y la producción de micotoxinas. Los ITCs se encuentran en la mostaza oriental y mostaza amarilla por lo que se estudia el efecto de estas harinas sobre los hongos y distintos sistemas de volatilización para determinar su efectividad y posible aplicación en la industria alimentaria. Por otro lado, el efecto antifúngico de los ITCs se ha estudiado tanto *in vitro* como en productos derivados de cereales. El AITC es el ITC que presenta mayor efecto antifúngico, por lo que se ha evaluado su capacidad de reaccionar con zearalenona (ZEA) y α-zearalenol (α-ZOL) en soluciones tamponadas, además de la bioaccesibilidad y de la biodisponibilidad tanto de las micotoxinas como de los productos de reacción formados.

Las BALs engloban un grupo heterogéneo de microorganismos capaces de producir ácido láctico a partir de la fermentación de azúcares. Su utilización en la producción de numerosos alimentos hace que su empleo como bioconservante

sea una alternativa a otros métodos para prevenir el desarrollo de los hongos en los alimentos, por lo que se han utilizado las BALs con el objetivo de extender la vida útil del pan. Además, se ha estudiado la capacidad de algunas BALs para reducir la bioaccesibilidad de las aflatoxinas B_1 (AFB_1) y B_2 (AFB_2) a través de un modelo de digestión dinámico *in vitro*.

4.1. Micotoxinas en pan de molde

4.1.1. Validación del método analítico

En un primer trabajo se validó un método para el análisis de 17 micotoxinas por cromatografía liquida acoplada a espectrometría de masas en tándem (CL-EM/EM), previa extracción con metanol y utilización del homogeneizador Ultra-Turrax®. Las micotoxinas estudiadas son: AFB_1, AFB_2, aflatoxina G_1 (AFG_1), aflatoxina G_2 (AFG_2), ocratoxina A (OTA), toxina HT-2 (HT-2) y toxina T-2 (T-2), deoxinivalenol (DON), ZEA, fumonisina B_1 (FB_1) y fumonisina B_2 (B_2), fusaproliferina (FUS), beauvericina (BEA), eniatina B (ENB), eniatina B_1 (ENB_1), eniatina A (ENA) y eniatina A_1 (ENA_1).

En la validación del método se han evaluado la linealidad, la precisión mediante el estudio de la repetibilidad (intradía) y reproducibilidad (interdía), la exactitud con el estudio de la recuperación, la sensibilidad con los límites de detección (LDs) y límites de cuantificación (LQs) y por último el efecto matriz (EF). Para la estimación de la linealidad y del efecto de la matriz se han realizado rectas de calibrado para cada micotoxina. Todas las micotoxinas mostraron buena linealidad con coeficientes de correlación (R^2) siempre superiores a 0,9923. Para evaluar el efecto matriz (Tabla 1), la pendiente de la recta de calibrado obtenida al adicionar el patrón al extracto se comparó con la pendiente de la recta patrón en metanol. Se observó una supresión de la señal (14-77%) para la mayoría de las micotoxinas, mientras que para cuatro de las micotoxinas se observó un aumento

de la señal (106-139%). Los análisis de recuperación se han realizado por triplicado a tres concentraciones diferentes (LQ, 2 veces el LQ y 10 veces el LQ), utilizando la recta de calibrado de distintas concentraciones de micotoxinas adicionadas a los extractos. Los resultados obtenidos varían entre 72% y 97% y la desviación estándar relativa es inferior al 17%. La repetibilidad intradía (n = 3) oscila entre el 7% y el 12%; y la reproducibilidad interdía (n = 3) oscila entre el 8% y el 17%. El LD es la cantidad más baja de analito que puede ser detectada en una muestra. El LQ es la cantidad más baja de analito en una muestra que puede ser utilizado para la cuantificación (Tabla 1). Los LD varían desde 0,03 µg/Kg de la ENB hasta 20,5 µg/Kg del DON.

Tabla 1. LD, LQ y EM (%) de diferentes micotoxinas en pan de molde.

Micotoxinas	LD (µg/Kg)	LQ (µg/Kg)	EM (%)
AFB_1	0,08	0,27	37
AFB_2	0,08	0,27	29
AFG_1	0,16	0,53	27
AFG_2	0,3	1	34
OTA	0,05	0,17	102
FB_1	50	166,67	132
FB_2	30	100	139
ZEA	7,8	26	106
T-2	1,76	5,87	72
HT-2	4,95	16,5	77
ENA	2,5	8,33	14
ENA_1	0,5	1,67	21
ENB	0,03	0,1	49
ENB_1	0,06	0,2	49
BEA	7	23,33	32
DON	20,5	68,33	60
FUS	0,65	2,17	35

LD= límite de detección; LQ= límite de cuantificación; EM= efecto matriz.

4.1.2. Análisis de micotoxinas en muestras de pan de molde comercializadas en Valencia

Mediante esta metodología se han analizado 80 muestras de pan de molde, seleccionadas al azar, y compradas en distintos comercios de la ciudad de Valencia entre enero y julio 2015. De las diecisiete micotoxinas estudiadas se han detectado ocho: AFB_1, AFB_2, AFG_1, AFG_2, ZEA, ENA_1, ENB y ENB_1.

Las AFs (AFB_1, AFB_2 y/o AFG_1) se encontraron en el 20% de las muestras a concentraciones que oscilan entre 0,5 y 7,1 µg/kg. Todas las muestras contaminadas con AFB_1 muestran valores superiores al límite máximo (2 µg/kg) establecido por la Legislación Europea para el pan. La AFB_2 se ha encontrado en 13 muestras mientras la AFG_1 solo en 2 muestras que también estaban contaminadas por la AFB_1. En total, 6 muestras de pan superaron el límite máximo legislado (4 µg/kg) en Europa para la suma de AFs (EC, 2006). Otros autores han detectado AFs, en cereales y productos derivados procedentes de países Mediterráneos (Serrano et al., 2012) y en cereales para el desayuno procedentes de Pakistán (Iqbal et al., 2014). En este último trabajo, el 16% (38 muestras de 237) y el 8% (19 muestras de 237) de las muestras de cereales para el desayuno presentaban niveles de AFB_1 y suma de AFs, respectivamente, por encima de los límites establecidos en la legislación (Iqbal et al., 2014).

De las cuatro ENs estudiadas, la ENA es la única EN que no se ha encontrado en las muestras analizadas. La incidencia de la ENB es la más alta de todas las micotoxinas con un 96% (76 muestras de 80), seguida de la ENB_1 con un 79% (63 muestras) y la ENA_1 con un 18% (14 muestras). Las concentraciones encontradas oscilaron entre 0,2 y 54 µg/kg. La ENB fue la micotoxina que se detectó con mayor frecuencia en los cereales italianos analizados por Juan et al., 2013. Sin embargo, los niveles de ENs encontrados en otros estudios superan las

concentraciones obtenidas en el presente estudio (Meca et al., 2010; Juan et al., 2013).

La ZEA se ha detectado en el 65% (52) de las muestras de pan con un rango de concentración entre 27 and 905 μg/kg. El 30% (24) de las muestras están por encima de los límites máximo legislados en Europa (EC, 2006) para ZEA en pan (50 μg/kg).

4.1.3. Estimación de la exposición a micotoxinas a través del pan de molde y evaluación del riesgo

El grado de exposición de la población a micotoxinas a través de los alimentos depende de la composición de la dieta en la zona geográfica considerada y de la contaminación por micotoxinas de los alimentos consumidos por dicha población. La ingesta diaria estimada (IDE) se calcula multiplicando los datos de consumo de los alimentos analizados por la media de los niveles de micotoxina encontrada en los mismos y se expresa considerando el peso corporal medio de 70 kg. En el estudio realizado sobre la exposición a micotoxinas de la población valenciana se han considerado dos escenarios posibles de exposición: nivel bajo de exposición, asignando un valor de cero a aquellas muestras <LD o <LQ; nivel de exposición alto, asignando el valor del LD para muestras <LD y el valor del LQ para muestras >LD pero <LQ (EFSA, 2010). La información sobre el consumo de pan de molde se ha obtenido a partir de la bases de datos de consumo en hogares valencianos del Ministerio de Agricultura, Alimentación y Medio Ambiente (MAGRAMA, 2014).

Se ha realizado una aproximación a la evaluación del riesgo comparando los valores de IDE obtenidos con los valores de ingesta diaria tolerable (IDT) establecidos para algunas de las micotoxinas por las instituciones y comité científicos competentes (Comité Mixto FAO/OMS de Expertos en Aditivos

Alimentarios, JECFA; Comité Científico sobre la Alimentación Humana, SCF; Autoridad Europea de Seguridad Alimentaria, EFSA). Para las micotoxinas emergentes no se han establecido IDTs por lo que se ha realizado una estimación de la exposición de la población a estas micotoxinas a través de la dieta y se ha comparado con las IDTs del DON de 1000 ng/kg p.c. por día.

Las IDEs obtenidas para las ENs varían entre 0,005 y 0,808 ng/kg p.c. por día y para la suma de ENs la ingesta calculada resulta aproximadamente de 1 ng/kg p.c. por día. La exposición alimentaria a la ZEA mediante el pan de molde ha sido estimada entre 2,380 y 2,923 ng/kg p.c. por día. Comparando con otros estudios realizados en Europa, las IDEs obtenidas para ZEA a través del consumo de diferentes productos comúnmente consumidos en Cataluña (Cano-Sancho et al., 2012) y mediante el consumo de harina de trigo, principal ingrediente del pan, en Portugal y en los Países Bajos (Aldana et al., 2014), las IDEs obtenidas en nuestro estudio son mas bajas.

Las IDEs calculadas para las AFs varían entre 0,008 y 0,035 ng/kg p.c. por día y entre 0,021 y 0,078 ng/kg p.c. por día para la suma de AFs. Valores de ingesta de 0,003, 0,001, 0,006 y 0,014 ng/kg p.c. por día se han obtenido para AFB_1, AFB_2, AFG_1 y AFG_2 respectivamente a través del consumo de café (García-Moraleja at al., 2015). Sin embargo, no hay estudios publicados que evalúen las IDEs de AFs en la dieta total de la población valenciana. Por otro lado, no existen IDTs para las AFs debido a que no se puede establecer un nivel considerado como seguro para productos cancerígenos genotóxicos.

En la aproximación de la evaluación del riesgo por exposición a micotoxinas a través del consumo de pan de molde por la población valenciana, el riesgo más alto es debido a la ZEA, siendo aproximadamente del 1% de la IDT. La estimación del riesgo de las ENs varía entre lo 0,001% y lo 0,081% de la IDT considerada, alcanzando lo 0,1% para la suma de ENs.

Todas las IDEs calculadas demuestran que la contribución del pan de molde a la ingesta de micotoxinas está por debajo de la IDT establecida incluso teniendo en cuenta un escenario de exposición sobreestimado por lo que el consumo de pan de molde no supone un riesgo para la población valenciana, sin embargo habría que considerar que hay muchos otros alimentos susceptible de ser contaminados por micotoxinas y que la exposición a través del pan de molde es solo una pequeña parte de la exposición total.

4.2. Reducción de hongos y micotoxinas

4.2.1. Empleo de ITCs

Los glucosinolatos (GLSs) son metabolitos bioactivos presentes en plantas de la familia *Brassicaceae*, como col, coliflor, brócoli, coles de Bruselas y mostaza. Los productos de hidrólisis de los GLSs, mayoritariamente los ITCs, poseen propiedades antimicrobianas frente diferentes microorganismos patógenos que se pueden encontrar en los alimentos. En los vegetales, los GLSs se encuentran en vacuolas específicas distintas de donde está la mirosinasa, enzima responsable de catalizar la reacción de hidrólisis. Cuando la planta sufre un daño físico, sustrato y enzima entran en contacto produciéndose los ITCs y sus consiguientes efectos. La sinigrina es el GLS principal de la mostaza oriental (*Brassica juncea*) mientras la sinalbina de la mostaza amarilla (*Sinapis alba*). Después de la hidrólisis, la sinigrina genera AITC y la sinilbina genera ρ-hidroxibencil isotiocianato (ρ-HBITC) (Saladino et al., 2016).

4.2.1.1. Actividad antimicrobiana in vitro

La actividad antimicrobiana de las harinas de mostaza oriental y amarilla se ha estudiado a distintas concentraciones (0,1, 0,5 y 1 g) añadiendo agua (2 mL) para activar la reacción catalizada por la mirosinasa. Se han colocando las harinas

en medio sólido PDA inoculado con *A. parasiticus*. La inhibición del crecimiento del hongo ha sido expresada como porcentaje de reducción del diámetro del micelio del hongo tratado con harina con respecto al control no tratado con las harinas.

La inhibición del crecimiento del hongo es directamente proporcional al tiempo de incubación y a la cantidad de harina de mostaza utilizada. La mayor reducción se ha observado con 1 g de mostaza oriental a las 148 h con una reducción del diámetro del micelio del 81% y del 30% con 1 g de harina de mostaza amarilla.

Los compuestos generados a partir de la harina de mostaza oriental han sido mas efectivos en la inhibición del crecimiento de *A. parasiticus* que los productos volátiles derivados de la harina amarilla y eso puede ser debido a la mayor volatilidad del AITC en comparación con el ρ-HBITC (Luciano y Holley, 2010).

Es la primera vez que se ha evaluado la actividad antifúngica de los ITCs obtenidos a partir de matrices alimentarias *in situ* frente *A. parasiticus*. Otros autores han utilizado ITCs puros o extractos de plantas del genero *brassica* para estudiar la inhibición del crecimiento de diferentes hongos. El aceite sintético de mostaza se ha utilizado frente a *A. glaucus* y *A. parasiticus* en cacahuetes (Dhingra et al., 2009) logrando mantener la población de los mismos por debajo de la inoculación inicial durante todo el período de almacenamiento. También se ha estudiado el efecto del AITC frente a *P. notatum* (Nielsen & Rios, 2000).

Por otro lado, se ha evaluado la actividad antimicrobiana *in vitro* de soluciones estándar de AITC, BITC y PITC en medio líquido en concentraciones que variaban entre 0,1 y 150 mg/L frente *A. parasiticus* para calcular la mínima concentración inhibitoria (MCI) y la mínima concentración fungicida (MCF). La MCI es la concentración más baja de un antimicrobiano que inhibe el crecimiento de

un microorganismo mientras la MCF se refiere al agente que disminuye en 99,9% las colonias a partir de un inoculo inicial.

Los tres ITCs estudiados son activos frente a *A. parasiticus* y la inhibición es proporcional a las concentraciones ensayadas. AITC y BITC muestran un MCI por debajo de 20 mg/L, mientras que la MCI obtenida para el PITC es de 50 mg/L. El BITC es el compuesto activo con menor MCF (20 mg/L), mientras que el AITC y el PITC muestran un valor de MCF de 50 mg/L.

Azaiez et al. (2013a) han evaluado la actividad antimicrobiana de AITC, BITC y PITC frente tres cepas de *Gibberella moniliformis* en medio sólido alcanzando el 90% de reducción del micelio. Manyes et al. (2015) han estudiado la actividad antifúngica del AITC frente *A. parasiticus* y *P. expansum* en medio sólido resultando el primer hongo más sensible al compuesto antimicrobiano. Por otra parte, Janatova et al. (2015) han evaluado diferentes aceites esenciales volátiles de los cuales, el AITC resulta ser casi ineficaz frente *A. niger* y sólo presenta algún efecto cuando el AITC se encuentra encapsulado. La baja actividad del AITC podría ser explicada por la falta de restricción a la circulación del aire en las placas Petri utilizadas en el ensayo, por lo tanto el AITC probablemente desaparece antes de que la actividad antifúngica se produzca.

4.2.1.2. Estudio de volatilización del AITC

Para determinar la permanencia del compuesto bioactivo AITC en un envase se han estudiado dos sistemas de volatilización diferentes. El primero se basa en el empleo de harina de mostaza oriental insertada en una pequeña bolsa de plástico con agua para catalizar la reacción de conversión de la sinigrina en AITC mediante la enzima mirosinasa, mientras que para el segundo sistema se impregna una tira de papel de filtro con 50 µL de la solución estándar del AITC, y se pega a la tapa del envase. La volatilización del compuesto con ambos sistemas

se ha estudiado sin y con el alimento (tortilla de trigo) en el envase, a temperatura ambiente inyectando 100 µL del espacio de cabeza del envase a un cromatógrafo de gases con detector de ionización de llama (CG-DILL) en 16 diferentes tiempos a lo largo de dos meses.

Utilizando la mostaza oriental, el nivel máximo de AITC detectado en el espacio de cabeza con y sin el alimento ha sido de 34,3 y 50,7 mg/L, respectivamente. La diferencia es debida probablemente a la absorción del AITC por parte de la matriz alimentaria. Además, hay que considerar que el plástico también absorbe el compuesto y que durante la volatilización el AITC puede convertirse en otros compuestos como tiocianatos y nitrilos (Meca et al., 2012).

Empleando el segundo sistema de volatilización del AITC (50 µL de solución estándar de AITC) la máxima concentración se ha detectado durante la primera hora de incubación y ha sido de 130 y 115 mg/L con y sin el alimento, respectivamente. Empleando este sistema la concentración del compuesto bioactivo presente en el espacio de cabeza en las primeras 48 h ha sido 2 veces más alta que en el caso del empleo de la harina como sistema de generación del AITC. Esta aplicación tiene la ventaja de promover una volatilización del AITC más rápida en las primeras horas de incubación reduciendo la posibilidad de crecimiento/germinación de los hongos micotoxigénicos en matrices alimentarias.

Considerando los resultados obtenidos en este estudio se observa que la aplicación de AITC impregnado en tiras de papel de filtro es más efectiva que la utilización de las bolsas de plástico con agua y harina de mostaza y por otro lado la presencia del alimento no influye de forma destacable en la volatilización del AITC.

4.2.1.3. Efectos sobre el hongo y las micotoxinas producidas por éste

4.2.1.3.1. Reducción de PAT en tortillas de trigo

En el siguiente estudio se han tratado muestras de 10 g de tortillas de trigo contaminadas con *P. expansum* y envasadas en bolsas de plástico con:

- 0,5, 1 y 2 g de harina de mostaza oriental o amarilla colocados en una pequeña bolsa de plástico (pegada en el interior del envase) con la misma cantidad de agua para promover la reacción de producción de los ITCs.
- 50, 100 y 200 µL/L de AITC en tiras de papel de filtro pegadas en el interior de los envases.

Considerando todos los tratamientos estudiados se ha obtenido una reducción media de la PAT del 93%. La mayor reducción de PAT se ha evidenciado en los estudios con 2 g de harina de mostaza amarilla, obteniendo una reducción del 99%, mientras que la más baja se ha observado empleando 0,5 g de harina de mostaza oriental (86%). Tenido en cuenta sólo los envases con harina de mostaza, se han detectado reducciones del 99% y 93% utilizando 2 g de harina amarilla y oriental, respectivamente.

Para conocer la cantidad total de GLSs presentes en la harina de mostaza amarilla y oriental que pueden convertirse en ITCs se han caracterizado analíticamente los GLSs. Ambas matrices de harina se han extraído con una solución acuosa y se han analizado mediante cromatografía líquida acoplada a detector diodo array (CL-DAD). La sinigrina se ha detectado a la concentración de 46 g/kg mientras que la sinalbina a 42 g/kg.

Este estudio puede ser considerado el primero en el cual se ha empleado un enfoque químico basado en el uso de compuestos bioactivos para reducir la PAT producida por *P. expansum* en los alimentos. La PAT se encuentra en fruta y derivados, especialmente manzana y zumo de manzana, pero también puede contaminar otros alimentos como los cereales (Assunção et al., 2016) o

encontrarse en el mismo alimento cereales y manzanas contaminadas como en algunos cereales de desayuno. Diferentes estrategias de reducción han sido evaluadas por otros autores. Drusch et al. (2007) han demostrado que la PAT puede descomponerse por los radicales libres generados por oxidación del ácido ascórbico a ácido deshidroascórbico. Los porcentajes de reducción de la micotoxina evidenciados en este estudio resultan 0,5 veces más bajos que los resultados obtenidos en nuestro estudio. Yun et al. (2008) han demostrado que la adición de ácidos orgánicos, aminoácidos y etanol inhibe parcialmente la degradación de la PAT por gamma irradiación en un sistema modelo acuoso. Gao et al. (2009) han evaluado la utilización del ultrasonido para reducir el contenido de PAT en zumo de manzana obteniendo una reducción del 30% más baja con respecto a nuestro estudio. Se han logrado reducciones de PAT del 57%, 88%, 95% y 99%, respectivamente en solución (tampón citrato con pH 3,4), sidra de manzana, zumo de manzana sin ácido ascórbico y con adición de ácido ascórbico mediante el uso de radiación ultravioleta monocromática (UV) a 253,7 nm (Zhu et al., 2013). Funes et al. (2013) han evaluado el efecto de la luz pulsada sobre la degradación de la PAT en tampón McIlvaine en zumo de manzana y puré de manzana, obteniendo resultados más bajos que los datos obtenidos en nuestro estudio.

En nuestro estudio todos los envases utilizados han logrado reducciones de la PAT superiores al 80% y los resultados obtenidos utilizando las soluciones estándar de AITC son comparables con los datos obtenidos con las dos harinas de mostaza empleadas.

4.2.1.3.2. Inhibición de la producción de AFs en piadina italiana

La inhibición de la producción de AFs ha sido estudiada en muestras de piadina italiana inoculadas con *A. parasiticus* y envasadas con tres

concentraciones diferentes de harina de mostaza oriental y amarilla (0,1, 0,5 y 1 g). En todos los ensayos realizados la inhibición obtenida dependía de las dosis utilizadas siendo la harina de mostaza oriental la más eficaz. La mayor reducción se ha obtenido utilizando 1 g de harina de mostaza oriental logrando una disminución de la producción del 89% de la AFB_1, del 83% de la AFB_2 y del 87% de la AFG_1 en comparación con el grupo control. Los resultados obtenidos para AFG_2 son similares a los de la AFG_1. Las reducciones obtenidas con la harina amarilla son todas inferiores al 70%.

Otros autores han estudiado diferentes aceites esenciales y su capacidad para inhibir el crecimiento del hongo y reducir la producción de AFs en distintos medios de cultivo y alimentos. Soliman y Badeaa (2002) han evaluado 12 aceites esenciales procedentes de plantas medicinales frente *A. Flavus*, *A. parasiticus*, *A. ochraceus* y *F. moniliforme* demostrando que los aceites de anís, tomillo y canela son los más efectivos en inhibir el crecimiento de dichas especies. El crecimiento fúngico se ha inhibido completamente al aplicar el 2% de estos aceites al trigo, por otro lado con el 0,1% se ha conseguido inhibir totalmente la producción de AFs, OTA y FBs. Kumar et al. (2008) han evaluado 14 aceites esenciales frente a distintos hongos incluyendo una cepa tóxica de *A. flavus*. El tomillo resulta ser el más efectivo y ha inhibido totalmente la producción de AFB_1 a la concentración de 600 µL/L. En otro estudio se ha observado que el aceite esencial de *Zataria multiflora* compuesto principalmente por carvacrol, timol y eugenol inhibe completamente el crecimiento y la formación de AFs por *A. flavus* en medio de cultivo pero no en el queso, incluso utilizando 1000 ppm (Gandomi et al., 2009). Razzaghi-Abyaneh et al. (2008) han demostrado la capacidad del aceite esencial de *Satureja hortensis L.*, compuesto principalmente por carvacrol y timol, de inhibir el crecimiento de *A. parasiticus* y la producción de AFB_1 y AFG_1.

En nuestro estudio se observa que el AITC y el ρ–HBITC pueden reducir la concentración de AFs producidos por *A. parasiticus* en piadina de trigo, siendo el AITC el más eficaz. Además, se ha demostrado que el AITC y el ρ–HBITC pueden formarse *in situ* a través de la adición de agua a las harinas de mostaza por lo que podrían ser utilizados como conservantes naturales en alimentos contaminados por especies de *Aspergillus*.

4.2.1.3.3. Aumento de la vida útil del pan de molde empleando AITC, BITC y PITC

Los estudios de vida útil aportan datos del tiempo que puede un producto conservar inalteradas sus propiedades organolépticas y físicoquímicas. Mediante la utilización de los ITCs se pretende prevenir el crecimiento del hongo y la presencia de micotoxinas. Se ha estudiado la capacidad de los ITCs para aumentar la vida útil del pan de molde envasado con respecto al control no tratado con los ITCs. En las muestras de pan de molde utilizadas como control se ha observado un crecimiento visible del hongo *A. parasiticus* al cuarto dia, sin embargo 5 µL/L de AITC impregnado en una tira de papel de filtro (liberación rápida) pegada en el interior del envase ha conseguido un alargamiento de 4 días. Por otro lado se ha observado una extensión de tres días utilizando la misma tira de papel de filtro pero insertada en una pequeña bolsa de plástico para que el compuesto se liberara en el interior del envase en un tiempo más prologado (liberación lenta). Considerando que el *A. parasiticus* es un hongo productor de AFs, se ha estudiado también la reducción de estas micotoxinas. Los dos tratamientos estudiados y que han logrado alargar la vida útil son los mismos que han mostrado los mayores porcentajes (entre 90 y 100%) de reducción de las cuatro AFs. En los estudios en los que se han utilizado los envases de liberación rápida todas las reducciones son proporcionales a la cantidades de compuestos utilizadas pero solo se han obtenido reducciones superiores al 60% empleando AITC a

concentraciones de 1 o 5 µL/L mientras con los envases de liberación lenta las reducciones en casi todos los casos son superiores al 60%.

Otros autores han utilizado los ITCs en envases para inhibir el crecimiento de diferentes hongos y reducir la producción de micotoxinas. Quiles et al. (2015) han obtenido resultados similares a nuestros estudios utilizando envases con AITC o harina de mostaza para inhibir el crecimiento de *A. parasiticus* y la producción de AFs en masas de pizza fresca. Después de treinta días no se observaba crecimiento del hongo en los envases de liberación rápida y lenta de AITC a concentraciones de 5 y 10 µL/L y en los envase con 850 mg de harina de mostaza oriental y 850 ml de agua. Todos los tratamientos han inhibido la producción de AFs proporcionalmente a las concentraciones utilizadas tanto con los dos tipos de envases con AITC de liberación rápida y lenta como con la harina de mostaza oriental. Nazareth et al. (2016) han logrado una inhibición total de la producción de AFs utilizando 10 µL/L de AITC en harina de trigo contaminada con *A. parasiticus* mientras reducciones del 23 y 52% se han obtenido empleando respectivamente 0,1 y 1 µL/L de AITC. La actividad antifúngica del AITC se ha estudiado también frente el hongo *A. flavus* en cacahuetes utilizando envases parecidos a nuestros envases de liberación lenta obteniendo una reducción significativa de 4 unidades logarítmicas con respecto al control (Otoni et al., 2014). El AITC a 50, 100 y 500 µL/L se ha utilizado para inhibir la producción de FB_2 en pan de molde (Azaiez et al., 2013a) y de FBs en grano y harina de maíz (Azaiez et al., 2013b). Las reducciones más altas se han obtenido con la mayor concentración de AITC (500 µL/L): 96% de FB_2 en pan de molde, FB_1 y FB_3 en maíz y 91% de FB_2 en maíz. Nielsen y Rios (2000) han evaluado diferentes aceites esenciales, entre ellos el AITC, en pan de centeno y pan para perritos calientes frente diferentes hongos que se encuentran en el pan. La concentración mínima de AITC con efecto fungicida ha sido de 2,4 mg/mL en fase gaseosa para el pan de

centeno y entre 1,8 y 3,5 mg/mL en fase gaseosa para pan de perritos caliente, sin embargo este último tipo de pan resulta más sensible al AITC tras la prueba sensorial. La reducción de AFs se ha estudiado también en frutos secos (maní, anacardo, nuez, almendra, avellana y pistacho) mediante el empleo de los ITCs derivados de la harina de mostaza oriental y amarilla. Se han obtenidos reducciones entre 88 y 89% utilizando la harina de mostaza oriental (Hontanaya et al., 2015). Por último, se ha observado un efecto sinérgico entre el AITC y el uso del atmósfera modificada (49% CO_2, 0,5% O_2, and 50,5% N_2) en la extensión de la vida útil de pescado fresco contaminado con *Pseudomonas aeruginosa* (Pang et al., 2013).

En nuestro estudio el AITC tiene la mayor actividad, siendo el único de los tres compuestos que ha logrado alargar la vida útil del pan contaminado con el hongo y, al mismo tiempo, reducir significativamente la producción de AFs. Sin embargo, BITC y PITC, como AITC, muestran una reducción significativa de las AFs en casi todas las concentraciones utilizadas en el envase a liberación lenta de los compuestos activos. El empleo de envases activos con ITCs podría ser una válida alternativa al uso de conservantes comerciales para controlar el crecimiento de hongos y la producción de AFs.

4.2.1.4. Acción directa del AITC sobre las micotoxinas y productos de reacción producidos

4.2.1.4.1. Reacción AITC – ZEA/α-ZOL, identificación de los productos de reacción, estudio de bioaccesibilidad y biodisponibilidad

Además de estudiar la capacidad de los ITCs de inhibir el crecimiento de hongos micotoxigénicos y las micotoxinas producidas por dichos hongos, se ha evaluado la acción directa del AITC sobre patrones de micotoxinas mediante reacciones directas entre el AITC y la ZEA o el α-ZOL. Para dicho estudio se ha

utilizado el AITC a diferentes concentraciones (2, 20, 100 y 200 mM) y a dos pH (4 y 7). Tras poner en contacto el AITC con las micotoxinas se ha agitado la solución a diferentes tiempos (0, 4, 8, 24 y 48 h) y se ha analizado mediante CL-DAD. Los resultados se han expresado en porcentaje de reducción respecto a un control que contenía solo las micotoxinas. El efecto del AITC fue dosis-dependiente para ambas micotoxinas logrando una reducción máxima del 89% para el α-ZOL y del 97% para la ZEA. No se observa diferencia significativa entre los pH estudiados. Casi todas las reacciones alcanzaron el valor máximo de reducción al tiempo cero. El AITC resulta ser más reactivo con ZEA que con α-ZOL ya que se observa siempre una mayor reducción de dicha micotoxina.

En otros estudios, se han utilizado el AITC y también el BITC y el PITC para reducir otras micotoxinas. El AITC se ha ensayado con la BEA en soluciones tamponadas y en la harina de trigo (Meca et al., 2012). La BEA se ha reducido completamente después de 48 h en solución tamponada con 1 mM de AITC. Como en nuestro estudio no se ha observado diferencia entre los dos pH ensayados y los resultados fueron proporcionales a la concentración de AITC utilizada (50, 100 y 500 µL/L) obteniendo en la harina de trigo reducciones entre el 10 y el 65%. Azaiez et al. (2013a) han evaluado 1 mg/L de AITC, BITC y PITC para reducir las FB_1 y FB_2 a pH 4, 7 y 9 en soluciones tamponadas obteniendo porcentajes de reducción entre 42 y 100%. En este caso los diferentes pH tienen un efecto distinto según el compuesto estudiado. Las mayores reducciones se han obtenido con el AITC y el PITC. Estos mismos ITCs se han utilizado en maíz y harina de maíz a concentraciones de 50, 100 y 500 µL/L. El PITC ha sido más eficiente contra la FB_3 en el maíz y la FB_2 en la harina de maíz, mientras que el AITC ha presentado mayor actividad contra FB_1 y FB_3 en ambos productos. BITC reacciona con todas las FBs de una manera similar, con la excepción de la FB_3 en la harina, ya que el compuesto bioactivo es menos reactivo. La reducción media de las FBs

en el maíz oscila entre 62 y 87%, mientras que en la harina de maíz la reducción de las tres toxinas varía entre 66 y 68%.

Además de estudiar la reducción de ZEA y α-ZOL, en este trabajo se han identificado los productos de reacción. En los cromatogramas obtenidos por CL-DAD, se observa un pico correspondiente al producto de reacción del AITC con la ZEA o el α-ZOL. Se ha tenido en cuenta que 1 mol de AITC y 1 mol de α-ZOL o ZEA producen 1 mol de aductos y que los pesos moleculares considerados para los aductos son 418,7 y 418,4 g/mol para ZEA-AITC y α-ZOL-AITC, respectivamente. Esta proporción se ha utilizado para calcular las concentraciones teóricas. La estructura de estos productos ha sido confirmada mediante espectrometría de masas de trampa iónica lineal (EM-LIT) a través de los diferentes fragmentos que se han encontrado en los espectros.

Por último, se ha estudiado la bioaccesibilidad y la biodisponibilidad de las dos micotoxinas y de los productos de reacción formados con el compuesto antimicrobiano.

La bioaccesibilidad se define como la solubilización de un determinado compuesto de la matriz alimentaria durante el proceso de digestión gastrointestinal. La fracción solubilizada, denominada fracción bioaccesible, está disponible para su absorción a través de la pared gástrica e intestinal y su posterior paso a la circulación sistémica. La biodispinibilidad es la cantidad de un nutriente o contaminante alimentario que tras su solubilización durante la digestión gastrointestinal es absorbida a través del epitelio gastrointestinal y llega a la circulación sistémica.

La bioaccesibilidad se ha estudiado a través de un modelo de digestión simulada *in vitro* a tres concentraciones diferentes (15, 30 y 60 μM) de las micotoxinas y de los productos de reacción. Se han determinado las cantidades de α-ZOL, ZEA y de los aductos α-ZOL/ZEA-AITC después de las fases gástrica y

duodenal de la digestión. La bioaccesibilidad es similar para la ZEA y su producto de reacción con el AITC a todas las concentraciones estudiadas y en los dos compartimentos. El mismo efecto se ha observado para el α-ZOL y su producto de reacción a 15 µM. Por otro lado, el aducto del α-ZOL ha mostrado una bioaccesibilidad gástrica menor que el α-ZOL a 30 µM y una bioaccesibilidad duodenal menor que la micotoxina a 30 y 60 µM. Posteriormente, se ha calculado la biodisponibilidad celular utilizando el modelo de células Caco-2 y analizando los niveles de las micotoxinas en el compartimento basolateral después de 1, 2, 3 y 4 h. La biodisponibilidad no presenta diferencias significativas entre las concentraciones estudiadas obteniendo después de 4 h un valor de 0,5 µM para α-ZOL, 1 µM para ZEA, 16 µM para 15 y 30 µM de α-ZOL-AITC, 13 µM para 60 µM de α-ZOL-AITC, 38 µM para 15 µM de ZEA-AITC, 36 µM para 30 µM de ZEA-AITC y 42 µM para 60 µM de ZEA-AITC.

Videmann et al. (2008) han demostrado que los metabolitos de la ZEA como α-ZOL, β-ZOL, ZEA-glucurónido y α-ZOL-glucurónido se producen rápidamente y cruzan fácilmente las membranas celulares. El α-ZOL es el principal metabolito producido por las células Caco-2 y también posee la mayor actividad estrogénica.

En nuestro estudio la biodisponibilidad de los productos de reacción ha resultado mayor que la de las micotoxinas de forma aislada, lo que puede ser debido a la formación de productos de degradación de las micotoxinas. Videmann et al. (2008) observaron que estos productos, que son más tóxicos, se forman rápidamente, por lo tanto la producción de los aductos con el AITC podría evitar la formación de metabolitos con mayor efecto estrogénico. Sin embargo, es necesario realizar estudios de los efectos tóxicos de los compuestos para conocer realmente si se está consiguiendo una detoxificación de estas micotoxinas.

4.2.2. Empleo de BALs

4.2.2.1. Empleo de BALs para alargar la vida útil del pan

En primer lugar se ha realizado un ensayo previo para evaluar la actividad antimicrobiana de los componentes antifúngicos presentes en el medio fermentado de 16 BALs frente *A. parasiticus* y *P. expansum*. De las BALs estudiadas, *Bifidobacterium bifidum*, *Lactobacillus ruminis*, *L. rhamnosus* (CECT 288), *L. johnsoni*, *L. plantarum* y *L. bulgaricus* han inhibido el crecimiento de *P. expansum* y estas mismas BALs, excepto *L. bulgaricus*, han resultado activas también frente *A. parasiticus*. Las 6 cepas con actividad antifúngica *in vitro* han sido empleadas en la fermentación de pan contaminado con *A. parasiticus* y de esta forma se ha estudiado la posibilidad de alargar la vida útil de este producto inhibiendo el crecimiento fúngico y la producción de AFs.

Tras este estudio, se ha observado en los panes fermentados con *L. bulgaricus* y *L. plantarum* un importante incremento de la vida útil de tres y cuatro días, respectivamente, si se comparan con los controles preparados sin BALs. Posteriormente, se han analizado las AFs de las muestras de pan fermentado con BALs, observándose una reducción total de las micotoxinas, respecto al control (pan fermentado únicamente con levadura). La reducción media de las AFs oscila entre 84 y 100%. Los porcentajes más altos se han obtenido con *L. bulgaricus* (99%) y *L. plantarum* (100%), que son también las cepas que más han alargado la vida útil del pan. Las otras 5 cepas utilizadas no han aumentado la vida útil del pan pero han reducido las micotoxinas, este efecto podría ser debido a las uniones que se establecen entre las AFs y las BALs una vez que hayan perdido viabilidad después del procesado.

En este estudio se ha evaluado la actividad antifúngica del medio fermentado por diferentes LABs, mientras que otros autores estudian la actividad antimicrobiana de compuestos bioactivos producidos por las mismas. Valerio et

al. (2009) han estudiado diferentes cepas de BALs observando que el *L. plantarum* es el más efectivo frente *P. roqueforti*. Ryan et al. (2008) han evidenciado resultados similares a los de nuestro estudio. El *L. plantarum* presenta la mayor actividad antimicrobiana frente todos los hongos ensayados en medio sólido. Además, los autores han observado una inhibición del crecimiento de *A. niger*, *F. culmorum* y *P. expansum* en muestras de pan de trigo fermentado con *L. plantarum*, en comparación con muestras de pan control.

4.2.2.2. Influencia de BALs en la bioaccesibilidad de las AFs B_1 y B_2

Tras llevar a cabo una digestión dinámica simulada en presencia de 15 BALs, se ha estudiado la capacidad de reducir la bioaccesibilidad de las AFs B_1 y B_2 contenidas en pan de molde con respecto al control (digestión sin bacterias). Para calcular la bioaccesibilidad se han tomado alícuotas de los fluidos de la digestión y se han analizado por CL-EM/EM.

En primer lugar, se ha validado un método para la detección y cuantificación de las AFs en los fluidos de la digestión. Se han obtenido recuperaciones del 88 y 84% para ambas AFs, LDs de 0,04 y 0,21 µg/L y LQs de 0,15 y 0,72 µg/L para la B_1 y la B_2, respectivamente. Las precisiones intradía e interdía varían entre 2,6 y 4,2%.

En segundo lugar se ha calculado la bioaccesibilidad y la reducción de las micotoxinas respecto al control, empleando diferentes BALs. La bioaccesibilidad duodenal del control es del 26 y del 36% para las AFB_1 y AFB_2, respectivamente. Las mayores reducciones de bioaccesibilidad se han obtenido con las siguientes BALs para ambas las AF_S: el *L. johnsoni* (97% AFB_1 – 87% AFB_2), el *L. plantarum* (96% AFB_1 – 98% AFB_2), el *L. casei* (2) (91% AFB_1 – 96% AFB_2) y el *L. reuteri* (76% AFB_1 – 99% AFB_2). El mecanismo de acción de las bacterias no está claro aunque se piensa que las diferencias observadas entre las cepas en la reducción de la

bioaccesibilidad de las AFs puedan ser debido a la habilidad de los probióticos para unirse a las AFs. La capacidad de unión entre los probióticos y las AFs se ha observado que es especie y cepa específica y que se establece a través de los componentes de la pared celular (Oatley et al., 2000; Hernández-Mendoza et al., 2009).

Otros autores han demostrado la habilidad de diferentes cepas de *Lactobacillus* y *Bifidobacterium* para unirse a la AFB_1 (El-Nezami et al., 1998; Peltonen et al., 2000) y la AFM_1 (Kabak & Var, 2004). Kabak et al. (2009) han estudiado el efecto sobre la AFB_1 presente en diferentes productos alimenticios en el tracto gastrointestinal y en ausencia y presencia de BALs, como posibles adsorbentes. Las seis bacterias estudiadas han mostrado diferente capacidad de unirse a la AFB_1, lo que dependía de diversos factores (cepa bacteriana, toxina, tipo de alimento y nivel de contaminación) y además, se ha observado una reducción máxima de la bioaccesibilidad de la AFB_1 del 37% en presencia de las bacterias probióticas.

Kabak & Ozbey (2012) han investigado la eficacia de seis bacterias probióticas en la reducción de la bioaccesibilidad de las AFs presentes en diferentes matrices alimenticias (cacahuete, pistacho, avellana, higos secos, paprika, trigo y maíz) utilizando un modelo de digestión *in vitro*. La inclusión de bacterias probióticas han reducido significativamente la bioaccesibilidad de las AFs: hasta el 36% para la AFB_1 y la AFB_2, hasta el 32% para la AFG_1 y hasta el 34% para AFG_2.

Tanto los ITCs como las BALs consiguen una buena extensión de la vida útil del pan hasta 4 días y una reducción de las micotoxinas hasta el 100%. La única diferencia se refiere a la forma en que se aplican: los ITCs en envases y las BALs como cultivos starters. Las BALs pueden ser fácilmente aceptadas por el consumidor ya que desde hace mucho tiempo se utilizan también en otros

alimentos. Además, como se ha observado en los resultados del último estudio, un alimento enriquecido con determinadas BALs podría reducir el riesgo asociado a la ingesta de las AFs, disminuyendo sus bioaccesibilidad. Por otro lado, el hecho que los ITCs se apliquen en envases puede tener interés para el consumidor ya que se trata de compuestos naturales presentes en los alimentos. Además, estos compuestos reaccionan con las micotoxinas y originar productos de reacción que pueden ser menos tóxicos y pueden reducir la bioaccesibilidad de estas sustancias tóxicas.

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5. CONCLUSION

5. CONCLUSIONES

1. Se detectaron AFs, ZEA y ENs en el 20, 65 y 96% de las muestras de pan commercial analizadas, respectivamente. En algunas de ellas, AFs y ZEA superaron los límites máximos establecidos por la Unión Europea.

2. Aunque todos los valores de IDE fueron más bajos de los valores de IDT establecidos, se necesitan estudios adicionales para tener una evaluación mas completa del riesgo de estas micotoxinas en la población de Valencia.

3. Tanto los envases con AITC como los envases con harina de mostaza han reducido la PAT en tortillas de trigo contaminadas con *P. expansum*. Considerando todos los tratamientos estudiados se ha obtenido una reducción media de la PAT del 93%.

4. Las tiras de papel de filtro impregnadas de AITC promueven una volatilización más rápida del AITC que el envase con las bolsas de plástico con agua y harina de mostaza. Además, la presencia del alimento no influye de forma destacable en la volatilización del AITC.

5. De las harinas de mostaza empleadas para reducir la producción de AFs en piadina italiana, la harina de mostaza oriental resultó más efectiva con una reducción maxima del 89,3% mientras que la harina de mostaza amarilla del 69,2%. De los ITCs, empleados como compuestos aislados, únicamente el AITC ha alargado la vida útil y, al mismo tiempo, reducido las AFs en pan de molde contaminado con *A. parasiticus* alcanzado el 100% de reducción.

6. El AITC puede reaccionar y reducir α-ZOL y ZEA *in vitro* a niveles de hasta el 97%, formando aductos con el AITC. Se observó una reducción de α-ZOL y ZEA y de sus productos de reacción tras el tratamiento de la digestión. Por otro

lado la biodisponibilidad de las micotoxinas fue inferior al la de los productos de reacción, lo que puede ser debido a la formación de otros productos de degradación durante el proceso. Es necesario completar estos estudios con la evaluación de la toxicidad de los productos de reacción.

7. Entre las BALs empleadas, únicamente *L. bulgaricus* y *L. plantarum* han logrado extender la vida útil. Todas las BALs estudiadas han reducido la producción de AFs entre el 76,5% y el 100%.

8. Algunas de las BALs estudiadas lograron reducir la bioaccesibilidad de las AFB_1 y AFB_2 contenidas en el pan contaminado hasta un 99%. Estos resultados sugieren que un alimento enriquecido con BALs puede reducir el riesgo asociado a la ingesta de estos compuestos tóxicos contenidos en los alimentos.

9. Tanto los ITCs en envases como las BALs empleadas como ingredientes consiguen reducir las micotoxinas hasta el 100% y extender la vida útil del pan hasta 4 días. Además pueden reducir la bioaccesibilidad de las micotoxinas. Los consumidores demandan alimentos seguros por lo que la utilización de los ITCs y de las BALs puede ser una alternativa a los conservantes clásicos.

ANNEX

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Dietary exposure to mycotoxins through the consumption of commercial bread loaf in Valencia, Spain

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ABSTRACT

In this study, 80 commercial samples of bread loaves were purchased from different supermarkets located in Valencia (Spain). These samples were investigated for the presence of legislated and non-legislated mycotoxins. Results showed that samples were contaminated with Aflatoxins (AFs), Zearalenone (ZEA) and Enniatins (ENs) with a frequency of 20, 96, and 65% respectively. Aflatoxin B_1 (AFB_1), Aflatoxin B_2 (AFB_2) and Aflatoxin G_1 (AFG_1) were detected with concentrations ranged from 0.5 to 7.1 μg/kg. The samples contaminated with AFB_1 showed values exceeding the maximum limit allowed in the EU. The sum of AFs also exceeded the maximum limit allowed in 6 samples. ENs contamination data ranged from 0.2 to 54 μg/kg and ENB was the most prevalent one. ZEA values ranged from 27 to 905 μg/kg and 30% of the contaminated samples were above the limits enforced by the EU. Finally, dietary exposure of the population living in Valencia to AFs, ENs and ZEA was estimated using the deterministic approach, through the evaluation of the consumption of commercial loaf bread and relating this data with the contamination of the loaf bread, for the calculation of the estimated daily intake (EDI) for each mycotoxin detected.

1. Introduction

Mycotoxins are secondary metabolites produced by a wide variety of filamentous fungi, including species from the genera *Aspergillus*, *Fusarium*, *Penicillium*, *Alternaria* and *Claviceps* that grow under different climatic conditions on agricultural commodities. Approximately 25% of all cereals produced in the world are contaminated with mycotoxins and they are also found in other foods such as spices, coffee, nuts and fruits (Marin, Ramos, Cano-Sancho, & Sanchis, 2013; Zöllner & Mayer-Hel, 2006). Mycotoxins comprise a variety of chemical structures with biological properties (Varga et al., 2013). The most important mycotoxins in foods and animal feed are: aflatoxins (AFs), produced by *Aspergillus* species; ochratoxin A (OTA) produced by both *Aspergillus* and *Penicillium*; trichothecenes (TCs) [type A: HT-2 and T-2 toxin, and type B: deoxynivalenol (DON)], zearalenone (ZEA), fumonisin B_1 (FB_1) and B_2 (FB_2). There are also emerging mycotoxins such as fusaproliferin (FUS), moniliformin (MON), beauvericin (BEA), and enniatins (ENs) produced mainly by *Fusarium* species that are commonly found in grains and grain-derived products (Krska et al., 2008; Marin et al., 2013). These mycotoxins can be harmful to both human and animal health, even after the food or feed product has been processed. Most mycotoxins are stable compounds to many processing operations such as heating, sorting, trimming, cleaning, milling, brewing, cooking, baking, frying, roasting, canning, flaking, alkaline cooking, nixtamalization and extrusion (Bullerman & Bianchini, 2007). These toxins may produce acute toxicity (ex. Oestrogenic effect) as well as long-term effects, namely carcinogenicity, mutagenicity, teratogenicity or immunotoxicity in animals and humans (Bennett & Klich, 2003). Humans are mainly exposed to mycotoxins by cereals and cereal-derived products. Bread is a staple food worldwide and, like other perishable products, is susceptible to fungal contamination. Spoilage of bakery products represents a significant source of economic losses to the industry and a potential safety risk due to the production of mycotoxins by different molds (ex. *Aspergillus* and *Penicillium*) (Cauvain, 2012a, pp. 614–658; Saranraj & Geetha, 2012; Smith, Daifas, El-Khoury, Koukoutsis, & El-Khoury, 2004). Bread posses a relatively high water activity ($a_w = 0.94–0.97$) with a pH of approximately 6 (Legan, 1993). These properties are favorable for to the germination and growth of a wide range of molds. Bread loaves have a higher probability of mold growth since they are commonly sliced, which increases the surface

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Bioactive compounds from mustard flours for the control of patulin production in wheat tortillas

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ABSTRACT

Patulin (PAT) is a toxic fungal metabolite produced by *Penicillium*, *Aspergillus* and *Byssochlamys* growing especially in fruit and cereals. PAT exhibits a number of toxic effects in animals and its presence in food is undesirable. In this study the reduction of the mycotoxin PAT produced by a strain of *Penicillium expansum*, on wheat tortillas was studied using volatile bioactive compounds present in the oriental and yellow mustard flour and also using the standard solution of the antifungal compound allyl isothiocyanate (AIT), developing an active packaging with two different systems of release of those bioactive compounds. Also the kinetic of volatilization of the compounds used in the bioactive packaging was evaluated using the technique of the gas chromatography (GC) coupled to the flame ionization detector (FID). The PAT was extracted from the samples using the QUECHERS methodology and was determined using the technique of the liquid chromatography (LC) coupled to the mass spectrometry detector in tandem (MS/MS). The maximum of volatilization of the AIT in the bioactive packaging is produced between 1 and 24 h depending on the volatilization technique and is stable during two months, whereas the reduction of PAT evidenced in the samples treated ranged from 80 to 100%.

1. Introduction

Patulin (Fig. 1) (PAT) is a toxic secondary metabolite produced by a wide range of fungal species of the genera *Penicillium*, *Aspergillius* and *Byssochlamys*. Among the different genera, the most important PAT producer is *Penicillium expansum* (Moake, Padilla-Zakour, & Worobo, 2005). PAT has been found as a contaminant in many moldy fruits, vegetables, cereals and other foods. However, the major sources of contamination are apples and apple products, which are also the most important source of PAT in the human diet (Baert et al., 2007; Murillo-Arbizu, Amézqueta, González-Peñas, & de Cerain, 2009; Reddy et al., 2010).

PAT has been classified in Group 3 by IARC that means not classifiable as to its carcinogenicity to humans, although it has been shown to cause neurotoxic and mutagenic effects in animals (IARC, 2002). In 1995, the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives (JECFA, 1995) recommended a provisional tolerable daily intake (pTDI) of 0.4 μg PAT/kg body weight/day based on long-term exposure (JECFA, 1995). As a result, the levels of PAT in fruits are subjected to legislative control. The Codex Alimentarius recommends levels of PAT in fruits and fruit juices to be lower than 0.05 mg/kg.

PAT causes gastrointestinal effects as distension, ulceration and hemorrhage in acute and short-term *in vivo* studies. Recent studies have also demonstrated that PAT alters the intestinal barrier function. PAT has electrophilic properties and high reactivity to cellular nucleophiles. At cellular level it can cause enzyme inhibition and chromosomal damage. PAT causes cytotoxic and chromosome-damaging effects mainly by forming covalent adducts with essential cellular thiols (Fliege & Metzler, 2000; Glaser & Stopper, 2012).

Vegetables like broccoli, cauliflower, cabbage, Brussels sprouts, belong to the Brassica genus and are widely consumed. A healthy diet should include Brassica vegetables because these vegetables are rich in health-promoting compounds like ascorbic acid, soluble fiber, selenium, glucosinolates (GLS), etc. Among these compounds,

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Reduction of the aflatoxins B_1, B_2, G_1 and G_2 in Italian piadina by isothiocyanates

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ABSTRACT

Aflatoxins (AFs) are mycotoxins produced mainly by the molds *Aspergillus flavus*, *Aspergillus parasiticus* and *A. nomius*. These mycotoxins are contaminants of cereals. AFB_1, the most abundant and toxic metabolite, is known to cause several toxic responses, such as hepatotoxicity, teratogenicity and mutagenicity. Isothiocyanates (ITCs) are natural compounds produced by the enzymatic hydrolysis of glucosinolates (GLs), which have shown potent antimicrobial activity in food applications. In this study, ITCs derived from oriental and yellow mustard (0.1, 0.5 and 1 g of flour) were used to avoid the production of AFs in piadina (a typical Italian flatbread) contaminated with *A. parasiticus* CECT 2981. In addition, the antifungal activity of the ITCs toward *A. parasiticus* was also evaluated. The mustard flours employed in this study inhibited the growth of *A. parasiticus*, reducing the mycelium size by 12.2–80.6%, noticing that the oriental mustard flour was more active. The ITCs produced *in situ* also reduced the AFs biosynthesis in Italian piadina. In particular, the use of oriental mustard flour reduced the AFs content by 60.5–89.3%, whereas the reduction caused by yellow mustard flour ranged from 41.0 to 69.2%. Therefore, yellow and oriental mustard flour could be used as sources of ITCs in intelligent packaging systems to increase the shelf life and safety of piadina.

1. Introduction

Aflatoxins (AFs) are a group of mycotoxins produced mainly by *Aspergillus flavus*, *Aspergillus parasiticus* and *A. nomius* (Bayman & Cotty, 1993). These species are ubiquitous in nature and as saprophytes they grow on a wide variety of substrates, including decaying plant and animal debris. Aflatoxins are carcinogenic contaminants found in foods and animal feed that are frequently responsible for health and economic concerns in many countries. Aflatoxin B_1 is the most toxic metabolite among aflatoxins and presents hepatotoxic, teratogenic and mutagenic properties. It has been classified as a Class 1 human carcinogen by the International Agency for Research on Cancer (IARC, 2002).

Generally, agricultural commodities are naturally contaminated with *Aspergillus* spp. in the field and it may be difficult to completely prevent aflatoxin formation in these products (Kumar, Shukla, Singh, Prasad, & Dubey, 2008; Reddy, Reddy, & Muralidharan, 2009). In addition, aflatoxins are resistant to food processing and it is very difficult to mitigate them once they are present in foods and food ingredients (IARC, 2002). Presence of AFs is one of the main food-safety concerns in field crops, particularly in commodities that are produced in tropical and subtropical weather, where the high temperature and humidity promote the growth and proliferation of *A.* spp. Examples of crops that are frequently contaminated with AFs include rice (Bansal, Tam, Cavlovic, & Kwong, 2011; Makun, Dutton, Njobeh, Mwanza, & Kabiru, 2011), peanuts (Shank, Wogan, Gibson, & Nondasuta, 1972), beans (Pitt et al., 1994), herbs and spices (Adzahan, Jalili, & Jinap, 2009; Candlish et al., 2001; Colak, Bingol, Hampikyan, & Nazli, 2006), chillies (Paterson, 2007), processed spices (Cho et al., 2008), dried fruits (Trucksee & Scott, 2008), wheat (Riba, Bouras, Mokrane, Mathieu, Lebrihi, & Sabaou, 2010) and corn (Kim et al., 2013).

Glucosinolates (GLs) are bioactive metabolites (Manson et al., 1997) present in plants of the Brassicaceae family, and their hydrolysis products possess antimicrobial properties against

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Shelf life improvement of the loaf bread using allyl, phenyl and benzyl isothiocyanates against *Aspergillus parasiticus*

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ABSTRACT

Fungal growth inhibition and aflatoxins (AFs) reduction using allyl (AITC), benzyl (BITC) and phenyl (PITC) isothiocyanates were studied in loaf bread contaminated with *Aspergillus parasiticus*. Two inoculated loaf bread slices were introduced into a plastic tray together with paper filters or small plastic bags paper filters soaked with AITC, BITC or PITC, the final concentration inside the package was of 0.5, 1 or 5 μL/L. The plastic trays, incubated at room temperature, were visual examined for the shelf life evaluation during 8 days. The quantification of the AFs was carried out using liquid chromatography coupled to mass spectrometry (LC-MS/MS). Shelf life increase of three and four days was obtained with small plastic bag paper filter and paper filter soaked with AITC 5 μL/L, respectively. These treatments also showed the highest reductions of AFs. All treatments with small plastic bag paper filter significantly reduced the content of AFs at percentages above 60% except the reduction of AFB_1 in the samples treated with BITC 0.5 μL/L and PITC 1 μL/L. The AFs reduction observed in the packaging with paper filter were above 60% only using AITC at the concentrations of 1 and 5 μL/L.

1. Introduction

Aflatoxins (AFs) are a group of natural food toxins which are recognized as toxic and carcinogenic secondary metabolites mainly produced by certain strains of *Aspergillus flavus, Aspergillus paraciticus* and *Aspergillus nomius* (Iqbal, Asi, Zuber, Akram, & Batool, 2013). AFs are found as contaminants in various agricultural commodities including bread, corn, peanut, cottonseed, Brazil nut, pistachio nut, fig, spices and copra (El-tawila, Neamatallah, & Serdar, 2013). The International Agency for Research on Cancer (IARC) has classified AFB_1 as a group I carcinogen which primarily affects the liver (IARC, 2002; Iqbal, Asi, & Jinap, 2014).

Acute exposure to AFs can cause aflatoxicosis, and in severe hepatotoxicity cases the mortality rate is approximately 25%. Chronic exposure to AFs is associated with hepatocellular carcinoma, especially in the presence of hepatitis B infection. Other probable health impacts are immunological suppression, impaired growth and nutritional interference (Strosnider et al., 2006). These impacts have been demonstrated in various species of livestock and fish, and while they may have similar effects on humans, causal evidence is still lacking.

In bakery products, preservatives (salts of propionic and sorbic acids) are added to prevent growth of spoilage fungi. However, in recent years, there has been consumer pressure to reduce the use of such preservatives. Previous studies have suggested that the use of suboptimal concentrations of these preservatives may stimulate the growth and AFs production of some spoilage fungi of bread (Arroyo, Aldred, & Magan, 2005). Thus, suboptimal doses could pose a problem and allow mould spoilage to occur. In the last decade, however it has increased the interest for biological methods to prevent the fungal growth using lactic acid bacteria (LAB) or natural antimicrobial compounds (Gerez, Torino, Obregozo, & Font de Valdez, 2010; Ryan, Dal Bello, & Arendt, 2008; Ryan et al., 2011).

Bread is known as a high moisture product with a_w values between 0.96 and 0.98 (Smith, Daifas, El-Khoury, Koukoutsis, & El-Khoury, 2004). As demonstrated by Doerry (1990), microbial spoilage is the main cause for shelf-life in intermediate and high moisture food products. Nowadays, mould growth is still a cause of high losses to the bread-producing industry (Pateras, 2007; Smith

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Reaction of zearalenone and α-zearalenol with allyl isothiocyanate, characterization of reaction products, their bioaccessibility and bioavailability *in vitro*

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ABSTRACT

This study investigates the reduction of zearalenone (ZEA) and α-zearalenol (α-ZOL) on a solution model using allyl isothiocyanate (AITC) and also determines the bioaccessibility and bioavailability of the reaction products isolated and identified by MS-LIT. Mycotoxin reductions were dose-dependent, and ZEA levels decreased more than α-ZOL, ranging from 0.2 to 96.9% and 0 to 89.5% respectively, with no difference ($p \leqslant 0.05$) between pH 4 and 7. Overall, simulated gastric bioaccessibility was higher than duodenal bioaccessibility for both mycotoxins and mycotoxin-AITC conjugates, with duodenal fractions representing $\geqslant$63.5% of the original concentration. Simulated bioavailability of reaction products (α-ZOL/ZEA-AITC) were lower than 42.13%, but significantly higher than the original mycotoxins. The cytotoxicity of α-ZOL and ZEA in Caco-2/TC7 cells was also evaluated, with toxic effects observed at higher levels than 75 μM. Further studies should be performed to evaluate the toxicity and estrogenic effect of α-ZOL/ZEA-AITC.

1. Introduction

Zearalenone (ZEA) and its derivative α-zearalenol (α-ZOL) are non-steroidal estrogenic mycotoxins produced by fungi belonging to the genus *Fusarium* and *Gibberella* (Yang, Wang, Liu, Fan, & Cui, 2007). These fungal species contaminate pre and post-harvest cereal crops such as corn, barley, wheat, rice and oats mainly from temperate and warm regions (Zinedine, Soriano, Molto, & Mañes, 2007). Several studies have demonstrated hepatotoxic, haematotoxic, immunotoxic, genotoxic and teratogenic effects of these mycotoxins to a number of mammalian species (Zinedine et al., 2007). ZEA elicit estrogenic response upon binding to the estrogen receptor (Drzymala et al., 2015). Moreover, it can be metabolized in the human body to α-ZOL, which possesses three to four times higher estrogenic activity (Wang et al., 2014).

There are some strategies proposed for the detoxification and biodegradation of ZEA in foods through chemical/enzymatic methods using ozone (McKenzie et al., 1997), H_2O_2 (Abd Alla, 1997) and lactonohydrolase (Takahashi-Ando, Kimura, Kakeya, Osada, & Yamaguchi, 2002); biological methods using lactic acid bacteria (Mokoena, Chelule, & Gqaleni, 2005), *Aspergillus niger* strain FS10 (Sun et al., 2014) and *Lactobacillus plantarum* Lp22, Lp39 and Lp4 (Zhao et al., 2015); and physical methods such as the use of adsorbent materials (Avantaggiato, Havenaar, & Visconti, 2003; Ramos, Hernández, Plá-Delfina, & Merino, 1996) or extrusion (Cetin & Bullerman, 2005). However, there is little information concerning the metabolites produced through these processes and their potential toxicity.

Glucosinolates (GLs) are a group of phytochemicals found in vegetables of the *Brassicaceae* (*Syn. Cruciferae*) family, which includes broccoli, cauliflower, mustard and horseradish (Meca, Luciano, Zhou, Tsao, & Mañes, 2012). Damage to the plant tissue leads to the hydrolysis of GLs by endogenous myrosinase, producing numerous biologically active compounds, including isothiocyanates (ITCs), thiocyanates and nitriles (Borges, Simões, Saavedra, & Simões, 2014). ITCs have several biological activities including plant defense (against insects and microbial infections) (Luciano & Holley, 2009; Mansour et al., 2012; Santos, Faroni,

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In vitro antifungal activity of lactic acid bacteria against mycotoxigenic fungi and their application in loaf bread shelf life improvement

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ABSTRACT

Food spoilage caused by mycotoxigenic fungi represents an important food safety problem. Lactic acid bacteria (LAB) are used as starter cultures in a larger number of food products. In this study, 16 strains of LAB were cultivated in MRS broth under anaerobiosis. Then, cell free supernatants were obtained by centrifugation and their antifungal activity against *Aspergillus parasiticus* and *Penicillium expansum* was tested using the disc-diffusion method. Furthermore, the LABs that showed *in vitro* antifungal activity were used in bread fermentation with yeast in order to study fungal growth inhibition and aflatoxin (AF) reduction in processed bread previously inoculated with *A. parasiticus*. The compounds present in the fermented medium of six LAB strains induced inhibition of *P. expansum* growth, whereas five probiotic strains produced antifungal compounds against *A. parasiticus*. The analysis by liquid chromatography coupled to mass spectrometry in tandem showed a reduction of the AF content in bread samples fermented with yeast and LABs. The reduction of AFs ranged from 84.1 to 99.9%. Moreover, bread sample studies showed a shelf life increase of about 3–4 days.

1. Introduction

Moulds cause a high degree of deterioration in food, feed and other agricultural commodities and are responsible for considerable economical losses. They destroy about 30% of crop yields and damage more than 30% of perishable crops in developing countries by lowering their quality and quantity. Furthermore, moulds produce mycotoxins which are potentially toxic to consumers and cause illness and death (Matasyoh, Wagara, Nakavuma, & Kiburai, 2011; Pawlowska, Zannini, Coffey, & Arendt, 2012). The ubiquitous nature of moulds, their ability to colonize different substrates and the lack of effective control measures have contributed to the high incidence of mould and mycotoxin contamination in food and feed (Ahlberg, Joutsjoki, & Korhonena, 2015; Hassan, Zhou, & Bullerman, 2015).

Agricultural products are susceptible to fungal invasion such as *Aspergillus*, *Penicillium*, *Fusarium* and *Claviceps* species, which may produce mycotoxins in the field before harvest, during harvesting, or during storage and processing. Among all food contaminants, mycotoxins are of greatest concern in terms of chronic toxicity as well as economics. Their incidence depends on various factors, such as the commodity, climatic conditions, agricultural practises, storage conditions and seasonal variation (Warth, Parich, Atehnkeng, Banyopadhyay, Schuhmacher, Sulyok, et al., 2012).

Aflatoxins (AFs) are mycotoxins produced by certain species of *Aspergillus*, particularly *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius*. There are more than 20 distinct but structurally related AF compounds but the four most commonly found are known as aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2) (Hernandez-Martínez & Navarro-Blasco, 2010; Tam et al., 2006). Aflatoxin B_1 (AFB_1) is carcinogenic and genotoxic *in vitro* and *in vivo* (EFSA, 2007), and it has been classified in the group 1 by the International Agency for Research on Cancer (IARC, 2002). Extensive contamination of food and drinks with mycotoxins is the main problem over the world since they can also compromise the safety of food and feed supplies and adversely affect health to humans and animals (Marin, Ramos, Cano-Sancho, & Sanchis, 2013). One of the most used types of food bioconservation is fermentation, a process based on the growth of microorganisms in foods. Among bakery products, the microorganisms most widely used are lactic acid bacteria (LAB) applied as starter cultures for sourdough. LABs have been included in the QPS (Qualified Presumption of Safety) list for authorized use in the food and feed chain within the European Union (EFSA, 2012; EC, 2008);

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Influence of probiotic microorganisms on aflatoxins B_1 and B_2 bioaccessibility evaluated with a simulated gastrointestinal digestion

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ABSTRACT

Aflatoxins (AFs) are produced mainly by the molds *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxin B_1 (AFB_1) is classified as carcinogenic to humans. The aim of this study was to evaluate the capacity of different strains of *Lactobacilli* (*Lb.*) and *Bifidobacteria* (*Bf.*) to reduce the bioaccessibility of AFB_1 and aflatoxin B_2 (AFB_2), spiked in loaf bread, using a dynamic *in vitro* simulated gastrointestinal digestion system. Aliquots of 20 mL of gastric and duodenal fluids were sampled for the determination of the mycotoxins gastric and duodenal bioaccessibility respectively, by liquid-chromatography coupled to the mass spectrometry in tandem (LC–MS/MS). A reduction of AFs bioaccessibility compared to the control (digestion without bacterial strains) was evidenced. The strains that evidenced the highest gastric and duodenal bioaccessibility reductions of AFB_1 and AFB_2 were *Lb. johnsoni* CECT 289, *Lb. reuteri* CECT 725, *Lb. plantarum* CECT 220 and *Lb. casei* CECT 4180, with values ranging from 76.38 to 98.34% for AFB_1 and from 77.14 to 98.66% for AFB_2. These results suggest that a food enriched with specific probiotic microorganisms and consumed at the same time as food contaminated with AFs, could reduce the risk associated to the intake of these toxic compounds contained in food.

1. Introduction

Aflatoxins (AFs), a group of structurally related toxic secondary metabolites of fungi, are primarily produced by *Aspergillus flavus* and *Aspergillus parasiticus*, groups of fungi that could be found in corn, nuts, peanuts, coconut, dried fruits and beer (Frenich et al., 2009; Li et al., 2009a,b; Williams et al., 2004). There are four major AFs named as B_1, B_2, G_1 and G_2. Among them, aflatoxin B_1 (AFB_1) is classified as a carcinogenic substance of group 1 by the International Agency for Research on Cancer (IARC) as it may interfere with the inductive of specific enzymes and forbid the synthesis of RNA 5 (IARC, 2012; Merrick et al., 2013; Wild and Montesano, 2009). Owing to the highly resistance to degradation during food processing, AFB_1 could enter the food chain and provide a threat to human health (Castells et al., 2007). Therefore, the regulatory limits for AFs ($B_1 + B_2 + G_1 + G_2$), even for AFB_1, have been established in several countries. The European Commission has set strict limits for the maximum allowed levels (MAL) of AFB_1 in ground-nuts, dried fruits and their products, in which the MAL of AFB_1 could not be greater than 2 $\mu g\,kg^{-1}$ for Retail Ready Foods (Van Egmond, 1995). It has been proved that the intake of AFB_1 over a long time may be dangerous even at a very low concentration. Therefore, the assays with high sensitivity and specificity are required to determine AFs at trace level in foods and agricultural products.

Even though the consumption of food contaminated with AFs should be strictly avoided due to its toxicity and carcinogenic effect, several studies show presence of AFs in different cereal products (Saladino et al., 2017; Iqbal et al., 2014; Serrano et al., 2012), sometimes above the limits enforced by the European legislation. For this reason different strategies have been developed to prevent the growth of mycotoxin producing fungi on food and feed, as well as to decontaminate and/or detoxify mycotoxin-contaminated products. One of the most used strategy to reduce the mycotoxins bioaccessibility during the gastrointestinal digestion is the employment of probiotic bacteria. *Lb.* and *Bf.* have shown AF-binding ability. This mechanism is unclear but it is suggested that is a physical phenomenon associated with bacterial cell wall

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