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GRUPO MARISTA

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CO-TUTORIZED INTERNATIONAL DOCTORAL THESIS

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VNIVERSITAT DE VALÈNCIA

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

Pontifícia Universidade Católica do Paraná
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ANTIFUNGAL AND ANTIMYCOTOXIGENIC EFFECT OF NATURAL COMPOUNDS IN FOOD

EFECTO ANTIFÚNGICO Y ANTIMICOTOXIGÉNICO DE COMPUESTOS NATURALES EN ALIMENTOS

EFEITO ANTIFÚNGICO E ANTIMICOTOXIGÊNICO DE COMPOSTOS NATURAIS EM ALIMENTOS

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ANTIFUNGAL AND ANTITOXIGENIC EFFECT OF NATURAL COMPOUNDS IN FOOD

(Efecto antifúngico y antitoxigénico de compuestos naturales en alimentos)

(Efeito antifúngico e antitoxigênico de compostos naturais em alimentos)

Co-tutorized International Doctoral Thesis

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El **Dr. Jordi Mañes Vinuesa**, Catedrático de Universidad de la Universitat de Valencia, el **Dr. Giuseppe Meca de Caro**, Catedrático de Universidad de la Universitat de Valencia y el **Dr. Fernando Bittencourt Luciano**, Profesor Titular da Pontifícia Universidade Católica do Paraná.

CERTIFICAN QUE:

El **D. Tiago de Melo Nazareth** ha realizado bajo su dirección la Tesis Doctoral que lleva por título "**Efecto antifúngico y antitoxigénico de compuestos naturales en alimentos**", y autorizan su presentación para optar al título de Doctor por la Universitat de Valencia.

Y para que así conste, expiden y firman el siguiente certificado.

Curitiba/Burjassot, 18 de mayo de 2023

Dr. Giuseppe Meca Dr. Jordi Mañes Vinuesa Dr. Fernando Bittencourt Luciano

THESIS FORMAT AND PUBLICATIONS

This thesis consists of chapters. Chapter 1 presents an introduction of the topic. Chapter 2 is the study objectives. Chapter 3 consists of three full scientific articles formatted according to the standards of the journal to which they were submitted. Chapter 4 is a general discussion of results obtained. Chapter 5 is the conclusions. Finally, Chapter 6 includes the reference of all chapters.

This thesis has been reflected in four different articles published or submitted in the following journals:

1. Aflatoxin B1 and Ochratoxin A: Generalities, Food Occurrence, Health Impact, Mitigation Methodologies, and Biocontrol – A Review. *Foods. (Under review). Impact factor: 5.561*

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“-La vida es demasiado corta para pasarla lamentándose. Tienes que estudiar, perseguir tus sueños y hacer lo que más te guste. ¡Eres magnífico!

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Dedicado a:

À minha família

Aos meus amigos

À minha vó, Eva Maria

LIST OF CONTENT

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Name	Abbreviation
Percentage peak area	%PA
Acetonitrile	ACN
Aflatoxin B1	AFB1
Aflatoxin B2	AFB2
Aflatoxin G1	AFG1
Aflatoxin G2	AFG2
Aflatoxin M1	AFM1
Antifungal peptides	AFPs
Aflatoxins	AFs
Allyl isothiocyanate	AITC
Antimicrobial peptides	AMPs
Water activity	a_w
Balkan Endemic Nephropathy	BEN
Bacterial-free supernatant	BFS
Buffer Peptone Solution	BPS
Spanish Type Culture Collection	CECT
Colony forming unit	CFU
Deoxynivalenol	DON
Essential oils	EOs
European Union	EU
Food and Agriculture Organization	FAO
Fumonisin B1	FB1
Fumonisin B2	FB2
Food and Drug Administration	FDA
Fungal growth	FG
Flame ionization detector	FID
Fungal population	FP
Fumonisins	FUM

Gas chromatograph	GC
Gas chromatograph coupled to a mass spectrometer	GC-MS
Generally recognized as safe	GRAS
Glucosinolates	GTs
Hydroxyethyl-cellulose and oriental mustard flour	H-OMF
Hepatocellular Carcinoma	HPC
High performance liquid chromatography	HPLC
Head-space solid phase microextraction	HS-SPME
International Agency for Research on Cancer	IARC
Institute of Sciences and of Food Production	ISPA
Isothiocyanates	ITCs
Lactic acid bacteria	LAB
Limit of detection	LOD
Matrix-assisted laser desorption/ionization	MALDI
Meat broth	MB
Moisture content	MC
Minimum fungicidal concentration	MFC
Mycelial growth	MG
Minimum inhibitory concentration	MIC
Man, Rogosa Sharpe	MRS
Man, Rogosa Sharpe agar	MRSa
Man, Rogosa Sharpe broth	MRSb
Mass spectrometry	MS
Mass spectrometry detector in tandem	MS/MS
Oriental mustard flour	OMF
Ochratoxin A	OTA
Ochratoxin B	OTB
Ochratoxin C	OTC
Ochratoxins	OTs
Principal components	PC
Plate count agar	PCA

Principal Component Analysis	PCA
Potato dextrose agar	PDA
Potato dextrose broth	PDB
Phenylethyl alcohol	PEA
p-hydroxybenzylisothiocyanate	p-HBIT
Phenyllactic acid	PLA
Qualified Presumption of Safety	QPS
Secondary metabolism	SM
Solid phase microextraction	SPME
Time-of-flight mass spectrometry	TOF
Ultra-high-performance liquid chromatography	UHPLC
Volatile Organic Compounds	VOCs
Yellow mustard flour	YMF
Yellow mustard flour extract	YMF-E
Zearalenone	ZEN

ABSTRACT

Fungal contamination in food is a significant concern in the industry due to economic losses caused by reduced nutritional value, changes in sensory properties, and consumer rejection. Fungi frequently spoil cereals, nuts, cheese, and cured meat. Moreover, fungal contamination poses a public health hazard by producing mycotoxins.

Nowadays, physical, chemical, and biological methods can be used to mitigate the risk of mycotoxins. Among them, synthetic fungicides are the most widely employed, but this strategy has certain drawbacks, such as environmental contamination, resistance of microbial populations, and toxicity of their residues to humans' and animals' exposure.

In order to address the challenges above, natural alternatives have been developed to enhance the management of food contaminating fungi. Against this background, this doctoral thesis focused on investigating the antifungal and antitoxigenic properties of natural substances such as allyl isothiocyanate (AITC), oriental mustard flour, yellow mustard flour, and lactic acid bacteria through *in vitro* studies.

According to the results obtained, different methodologies for applying natural compounds in food have been explored: first, AITC was tested as a fumigant to inhibit the growth of *P. verrucosum* and the production of ochratoxin A (OTA) in barley grains. Secondly, the use of AITC, a yellow mustard flour extract, and an oriental mustard flour device (H-OMF) were studied to inhibit the growth of *A. flavus* and the production of aflatoxin B1 in almonds. Lastly, lactic acid bacteria (LAB) with antifungal potential were selected, an antifungal meat-based ingredient was developed, and the produced antifungal compounds were identified.

The results showed that AITC at 50 μL/L significantly reduced the growth of *P. verrucosum* and OTA production in barley grains to undetectable levels.

Likewise, AITC at 5.07, 10.13, and 20.26 mg/L significantly decrease the population of *A. flavus* as well as the production of AFB1 in almonds.

Moreover, when almonds were treated with different concentrations of oriental mustard flour device, the levels of *A. flavus* population and AFB1 were lowered to values below the limit of detection. On the other hand, although yellow mustard extract had shown effectiveness *in vitro*, it did not demonstrate efficacy when applied to almonds.

Regarding the antifungal activity of the isolated LAB, the *Pediococcus pentosaceus* strain C15 demonstrated the highest antifungal activity *in vitro*, which led to its selection to produce a fermented broth named MB10-C15. This fermented broth served as the basis for creating a postbiotic antifungal ingredient. Characterization of the ingredient revealed significant levels of phenolic and organic acids. In addition, analysis of the volatile organic compounds demonstrated the presence of antifungal molecules such as phenylethyl alcohol, nonanoic acid, and acetic acid.

In conclusion, the findings revealed that both antifungal device (H-OMF) and AITC could be used as fumigants with the aim of improving food safety. Furthermore, the results indicated that the incorporation of the ingredient MB10-C15 in the formulation of meat products could extend their shelf life due to the high concentration of antifungal compounds present in this ingredient.

RESUMEN

La contaminación fúngica en los alimentos representa una preocupación significativa en la industria, ya que ocasiona pérdidas económicas debido a la disminución del valor nutricional, las alteraciones en las propiedades sensoriales de los alimetos y el rechazo por parte de los consumidores. Asimismo, la contaminación fúngica representa un riesgo para la salud pública debido a la producción de micotoxinas.

Hoy en día se pueden utilizar métodos físicos, químicos y biológicos para mitigar el riesgo de micotoxinas. Entre ellos, los fungicidas sintéticos son los más utilizados, pero esta estrategia presenta ciertos inconvenientes, como la contaminación ambiental, la aparición de resistencias y la toxicidad de sus residuos para los humanos y animales.

Para hacer frente a los retos mencionados, se han desarrollado alternativas naturales con objeto de mejorar la gestión de los hongos contaminantes en alimentos. En este contexto, esta tesis doctoral se centró en la investigación de las propiedades antifúngicas y antitoxigénicas de sustancias naturales como el isotiocianato de alilo (AITC), la harina de mostaza oriental, la harina de mostaza amarilla y las bacterias ácido lácticas (LAB) mediante estudios *in vitro*.

De acuerdo con los resultados obtenidos, se exploraron diferentes metodologías de aplicación: en primer lugar, se ensayó el AITC como fumigante para inhibir el crecimiento de *P. verrucosum* y la producción de ocratoxina A (OTA) en granos de cebada. En segundo lugar, se estudió el uso de AITC, extracto de harina de mostaza amarilla y un dispositivo de harina de mostaza oriental (H-OMF) para inhibir el crecimiento de *A. flavus* y la producción de Aflatoxina B1 en almendras. Por último: se seleccionaron LAB con potencial antifúngico, se elaboró un ingrediente antifúngico a base de carne y se caracterizaron los compuestos antifúngicos producidos.

Los resultados mostraron que AITC a 50 µL/L redujo significativamente el crecimiento de *P. verrucosum* y la producción de OTA en granos de cebada hasta niveles indetectables. Asimismo, el AITC a 5,07, 10,13 y 20,26 mg/L disminuyó significativamente la población de *A. flavus*, además de la producción de AFB1 en almendras. Igualmente, cuando las almendras se trataron con diferentes concentraciones del dispositivo de harina de mostaza oriental, los niveles de población de *A. flavus* y de AFB1 se redujeron a valores por debajo del límite de detección. Por otra parte, aunque el extracto de mostaza amarilla haya demostrado eficacia *in vitro*, no la demostró en las almendras.

En cuanto a la actividad antifúngica de las LAB aisladas, la cepa de *Pediococcus pentosaceus* C15 evidenció la mayor actividad antifúngica *in vitro*, lo que condujo a su selección para producir un caldo fermentado que sirvió de base para crear un ingrediente postbiótico antifúngico. La caracterización del ingrediente reveló niveles relativamente altos de ácidos fenólicos y alifáticos. Además, el análisis de los compuestos orgánicos volátiles demostró la presencia de moléculas antifúngicas como el alcohol feniletílico, el ácido nonanoico y el ácido acético.

En conclusión, los hallazgos revelaron que tanto el dispositivo antifúngico (H-OMF) como el AITC podrían emplearse como fumigantes con el propósito de mejorar la seguridad alimentaria. Además, los resultados indican que la incorporación del ingrediente MB10-C15 en la formulación de productos cárnicos podría extender su vida útil gracias a la concentración de compuestos antifúngicos presentes en dicho ingrediente.

RESUMO

A contaminação fúngica dos alimentos representa uma preocupação importante para a indústria alimentaria, porque causa perdas econômicas devido à diminuição do valor nutricional dos alimentos, alterações nas propriedades sensoriais e a rejeição por parte dos consumidores. Além disso, a contaminação fúngica representa um risco para a saúde pública devido à produção de micotoxinas.

Atualmente, podem ser utilizados métodos físicos, químicos e biológicos para mitigar o risco de micotoxinas. Entre eles, os fungicidas sintéticos são os mais utilizados, mas essa estratégia apresenta certas desvantagens, como a contaminação ambiental, resistência microbiana e toxicidade dos resíduos para humanos e animais.

Para enfrentar os desafios mencionados, foram desenvolvidas alternativas naturais com o objetivo de melhorar a gestão de fungos contaminantes em alimentos. Nesse contexto, esta tese de doutorado centrou-se na avalição das propriedades antifúngicas e antitoxigênicas de substâncias naturais, como o isotiocianato de alila (AITC), a farinha de mostarda oriental, a farinha de mostarda amarela e bactérias ácido lácticas (LAB), por meio de estudos *in vitro*.

De acordo com os resultados obtidos, diferentes metodologias de aplicação dos compostos naturais foram exploradas: primeiro, o AITC foi testado como fumigante para inibir o crescimento de *P. verrucosum* e a produção de ocratoxina A (OTA) em grãos de cevada. Em segundo lugar, foi estudado o uso de AITC, um extrato de farinha de mostarda amarela e um dispositivo de farinha de mostarda oriental (H-OMF) para inibir o crescimento de *A. flavus* e a produção de aflatoxina B1 em amêndoas. Por fim, LAB com potencial antifúngico foram selecionadas, um ingrediente antifúngico à base de carne foi elaborado e os compostos antifúngicos produzidos foram identificados.

Os resultados mostraram que o AITC a 50 μL/L reduziu significativamente o crescimento de *P. verrucosum* e a produção de OTA em grãos de cevada a níveis indetectáveis. Além disso, o AITC a 5,07, 10,13 e 20,26 mg/L reduziu

significativamente a população de *A. flavus*, assim como a produção de AFB1 em amêndoas. Da mesma forma, quando as amêndoas foram tratadas com diferentes concentrações do dispositivo de farinha de mostarda oriental, os níveis da população de *A. flavus* e AFB1 foram reduzidos a valores abaixo do limite de detecção. Por outro lado, embora o extrato de mostarda amarela tenha demonstrado eficácia *in vitro*, não teve efeito fungicida quando aplicado nas amêndoas.

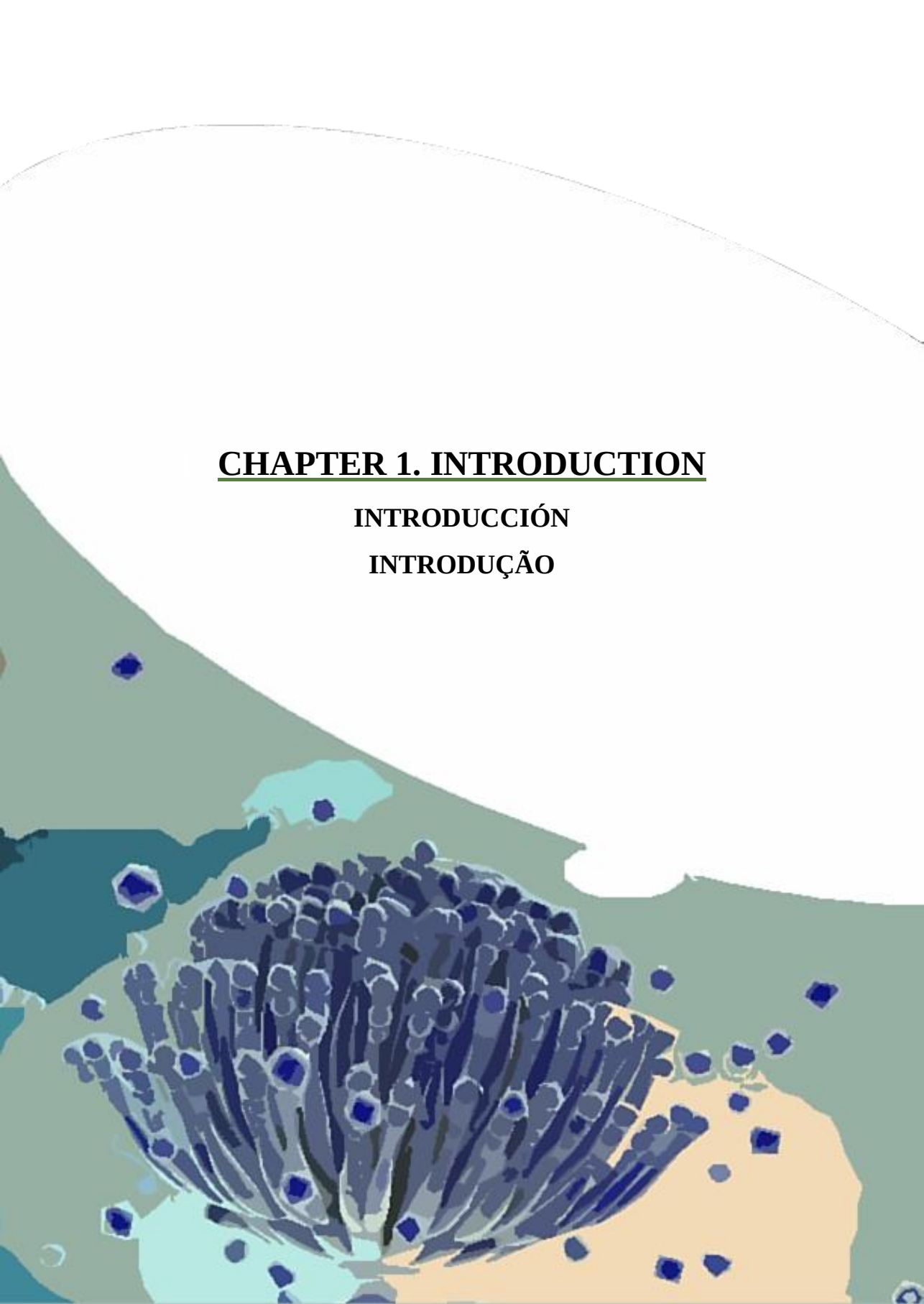
Em relação à atividade antifúngica das LAB isoladas, a cepa de *Pediococcus pentosaceus* C15 demonstrou a maior atividade antifúngica *in vitro*, o que levou à sua seleção para produzir um caldo fermentado denominado MB10-C15. Esse caldo fermentado serviu de base para criar um ingrediente pós-biótico antifúngico. A caracterização do ingrediente revelou níveis significativos de ácidos fenólicos e alifáticos. Além disso, a análise dos compostos orgânicos voláteis demonstrou a presença de moléculas antifúngicas, como álcool feniletílico, ácido nonanoico e ácido acético.

Em conclusão, os achados revelaram que tanto o dispositivo antifúngico (H-OMF) quanto o AITC poderiam ser utilizados como fumigantes com o objetivo de melhorar a inocuidade alimentaria. Além disso, os resultados indicaram que a incorporação do ingrediente MB10-C15 na formulação de produtos cárneos poderia prolongar sua vida útil devido à elevada concentração de compostos antifúngicos presentes nesse ingrediente.

CHAPTER 1. INTRODUCTION

INTRODUCCIÓN

INTRODUÇÃO



1.1 Introduction

Filamentous fungi are a diverse group of unique eukaryotic organisms ubiquitous in nature. They are remarkable for their ability to grow on a wide variety of relatively simple substrates, which they can utilize as nutrients (Lima & Santos, 2017). Additionally, they produce secondary metabolites during their development. The secondary metabolism in fungi is exceptionally complex and produces a large number of compounds with a wide variety of chemical properties. These molecules are synthesized in response to a variety of environmental abiotic and biotic signals and can confer competitive advantages on their producers (Uka et al., 2020). Fungi are biological micro-factories capable of producing substances of pharmacological interest like antibiotics, tumors suppressors, immunosuppressants, and industrial interest such as antioxidants, pigments, and organic acids (Egbuta et al., 2017). They can, however, produce a variety of toxic metabolites known as mycotoxins (Niessen, 2018).

Mycotoxins are highly toxic secondary metabolites generated by fungi that naturally affect food and animal feed (Zeidan et al., 2019). Among the mycotoxin-producing fungus, *Alternaria*, *Aspergillus*, *Penicillium*, and *Fusarium* spp. are particularly noteworthy (S. Luo et al., 2021). The number of mycotoxins discovered that are harmful to people and animals is continuously increasing, and in order to prevent their negative consequences, regulation has become more stringent (Udomkun et al., 2017). Therefore, their presence in foods is being regulated, mainly because mycotoxins result in economic losses owing to batch downgrading (Ji et al., 2016).

Human exposure to mycotoxins can happen directly or indirectly. Direct exposure occurs by ingesting contaminated grains or food products, and indirect exposure occurs when individuals ingest food such as milk and meat from contaminated animals (Peles et al., 2019). Currently, more than 400 mycotoxins have been identified, and most of them are heat stable. In other words, they can remain in

food even after thermal processing, becoming a concern for human health (Romera et al., 2018).

Mycotoxins exhibit a wide range of chemical and biological features, as well as adverse effects. Consequently, excessive exposure of animals and humans to mycotoxins may result in acute and chronic harmful effects, depending on the mycotoxin and the animal's sensitivity (Wambacq et al., 2016). These deleterious consequences are often attributed to these compounds' nephrotoxic, hepatotoxic, teratogenic, carcinogenic, estrogenic, and immunosuppressive properties (Pickova et al., 2020). Despite such deleterious effects, in the majority of the world countries, only the most common mycotoxins: some aflatoxins (AFs) (AFB1, AFB2, AFG1, AFG2 and AFM1), deoxynivalenol (DON), fumonisins (FUM) (FB1 and FB2), ochratoxin A (OTA), patulin and Zearalenone (ZEN) are legislated with *Maximum Tolerated Limits* set in foodstuffs (Alshannaq & Yu, 2017). In contrast, the legislation of feedstuffs is less strict, and few countries have established limits for mycotoxins like AFs, including European countries and the United States (US) (Changwa et al., 2018; Pinotti et al., 2016).

Among the globally regulated mycotoxins, this review will focus exclusively on AFs and OTA to provide a background to understand better the work performed. Additionally, it is essential to highlight that these mycotoxins may probably be the most toxic ever discovered, and hence, both classes of mycotoxins are highly prevalent and have a significant impact on human and animal health (Thanushree et al., 2019).

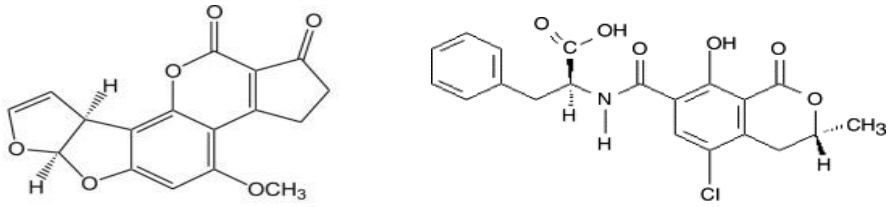
Mycotoxins were frequently discovered as a result of acute toxic incidents, most commonly in animals. In England, for example, the Turkey X disease, which claimed tens of thousands of chickens in the early 1960s, resulted in the discovery of AFs in animal feed (Richard, 2008).

Since then, AFs have risen to prominence as one of the most extensively investigated and carefully controlled mycotoxins (Tarazona et al., 2020). This

significance derives mainly from their health and economic consequences. Indeed, AFB1 (**Figure 1.1**) is the most potent natural carcinogen currently known and is directly associated with the development of liver cancer in humans and other liver pathologies such as jaundice, necrosis, cirrhosis, and hepatitis (Benkerroum, 2016). Besides, non-hepatic pathologies such as immunosuppression and growth disorders. AFB1 is designated as a Group 1 carcinogen by the International Agency for Research on Cancer (IARC). It is important to note that the group 1 compounds whose effects are harmful to humans and animals (Benkerroum, 2020).

OTA is another toxigenic fungus secondary metabolite (**Figure 1.1**). This toxin is nephrotoxic, immunosuppressive, and carcinogenic, having been recognized as a probable human carcinogen by the IARC (group 2B) (Singh & Mehta, 2020). Simplifying, the IARC classifies mycotoxins as confirmed (Group 1), probable (Group 2A), and possibly human carcinogens (Group 2B). The **Table 1.1** summarize the most prevalent and toxigenic mycotoxins classified by IARC and the degree of evidence of carcinogenicity.

Due to the adverse health effects of these toxic metabolites, consumer protection measures have been implemented by defining maximum acceptable levels in specific food categories. On the one hand, the legislation helps enhance the safety of agricultural goods and decrease mycotoxins daily intake (Logrieco et al., 2018). On the other hand, these standards have significant economic consequences since they restrict commerce from places prone to contamination and significantly reduce the economic worth of particular items in the case of contamination. Apart from regulation and legislation, socio-economic factors such as insufficient governmental policy may all contribute to mycotoxin contamination-prone conditions (Udomkun et al., 2017).



(a) (b)

Figure 1.1. Chemical structure of AFB1 (a) and OTA (b) (Gutierrez R et al., 2015; Lobeau et al., 2005).

Table 1.1. Degree of carcinogenicity of mycotoxins according to IARC.

Mycotoxin	Degree of evidence of carcinogenicity		Overall evaluation of carcinogenicity to humansa
	In humans	In animals	
Aflatoxin B1	Sufficient	Sufficient	Group 1
Aflatoxin B2		Limited	Group 1
Aflatoxin G1		Sufficient	Group 1
Aflatoxin G2		Inadequate	Group 1
Aflatoxin M1	Inadequate	Sufficient	Group 2B
Fumonisin B1	Inadequate	Sufficient	Group 2B
Fumonisin B2		Inadequate	Group 2B
Fusarin C		Limited	Group 2B
Ochratoxin A	Inadequate	Sufficient	Group 2B
Deoxynivalenol		Inadequate	Group 3
Nivalenol		Inadequate	Group 3
Zearalenone		Limited	Group 3
Citrinin	Inadequate	Limited	Group 3
Patulin	Inadequate	Inadequate	Group 3
T-2 toxin		Limited	Group 3

Group 1, carcinogenic to humans; Group 2A, probably carcinogenic to humans; Group 2B, possibly carcinogenic to humans; Group 3, not classifiable as toxic by its carcinogenicity to humans; Group 4, probably not carcinogenic to humans.

In countries with a warm climate, the inadequate storage of grains associated with the hot and humid climate favors mycotoxins to have a high incidence (Astoreca

et al., 2012). However, mycotoxins are a global problem, and according to the Food and Agriculture Organization (FAO/WHO), approximately 25% of all grains produced in the world are contaminated with mycotoxins at levels above the permitted (Eskola et al., 2020). Consequently, this contamination leads to an annual loss of 1 billion tons of grains and flour intended for human consumption. Data showed by Monbaliu et al. (2010) also evidenced that 82% of grains sampled in Europe might be contaminated with at least one type of mycotoxin. Furthermore, in the US, the Food and Drug Administration (FDA) has estimated that losses caused by mycotoxins in the animal industry can cost hundreds of millions of dollars a year (Loi et al., 2017).

Numerous authors have documented elevated AFs and ochratoxins (OTs) levels in grains, grain-based foods, and dry fruits (Alkadri et al., 2014; Duarte et al., 2010; Ghali et al., 2008; Beatriz T. Iamanaka et al., 2005). As a consequence of their high prevalence worldwide, it seems critical to develop control methods to prevent these hazardous chemicals from contaminated food or to mitigate their detrimental effects.

To inhibit the growth of toxigenic fungal agents, fungicides and pesticides have been extensively employed. However, due to their intrinsic toxicity, their usage is currently restricted (da Luz et al., 2017). As an alternative for these chemical biocides, biological control has demonstrated another potential method (Nguyen et al., 2017). For instance, it has been shown that specific atoxigenic microorganisms like lactic acid bacteria (LAB) and some fungal strains are efficient at preventing the growth of toxigenic fungi (Belkacem-Hanfi et al., 2014; Gerez et al., 2009; Rouse et al., 2008). Similarly, detoxification procedures may be used to attempt to mitigate the harmful effects of these toxins once they are synthesized (Peng et al., 2018). However, none of these strategies appears to be sufficient to address the problem of mycotoxin contamination of raw materials, as evidenced by numerous studies that reveal occasionally high levels of mycotoxin contamination, particularly in regions

with a climate conducive to the development of the fungal species that produce these compounds (Misihairabgwi et al., 2019; Oliveira et al., 2017; X. D. Sun et al., 2017; Taniwaki et al., 2019).

Over the last decade, there has been an increase in the attention to finding natural substances that may restrict mycotoxin development and synthesis. Research has shown that essential oils (EOs) and extracts from plants or spices may inhibit the growth of harmful pathogens in foods, including fungi and bacteria (Hashemi Moosavi et al., 2021; Maurya et al., 2021; Mirza Alizadeh et al., 2021). Thus, a natural way to minimize contamination of products is via EOs like the essential mustard oil.

Currently, we are facing a continuous increase in the global demand for cereals such as barley, maize, soybean, wheat, and related products, due to the constant expansion and improvement of the animal husbandry industry and population growth (Meyfroidt, 2018). Consequently, the safety of food and animal feed has become more critical, with mycotoxins being one of the most severe risks to be avoided. Among mycotoxins, AFs, FUM, OTs, trichothecenes, and ZEN are the most prominent in food and animal feed (Wan et al., 2020). However, this review will discuss various limitations imposed by AFs and OTA, including their presence in raw materials and their byproducts, health risk and adverse effects to humans and animals, current outbreaks, and current legislation to restrict their levels in food and feed. In addition, traditional methods of mycotoxin prevention and recent alternatives to biocides such as biopreservation will be reviewed. Finally, an overview of the antifungal efficacity of EOs and their mechanism of action will be presented, highlighting the potential of essential mustard oil and its substances.

1.2 AFs and fungal producers

Aflatoxin synthesis and crop contamination are lengthy biochemical process that begins with fungal invasion and continues with toxin production in contaminated crops (Abrar et al., 2013). Invasion, growth, and aflatoxin generation by fungal

pathogens are primarily influenced by an ecosystem's environmental variables, crop type, and other ecological characteristics (Demissie, 2018). Not all *Aspergillus* species generate aflatoxin, and not all *Aspergillus* species infest all kinds of crops. Thus, the degree to which agricultural products are contaminated with aflatoxin is dictated, in part, by the fungal strain predominant in the producing area (Cotty & Mellon, 2006).

Numerous studies have been conducted to determine which fungus species are capable of synthesize aflatoxin. Certain species have been incorrectly classified and recognized as AFs producers throughout time. While current molecular techniques have significantly improved this situation, there is still a great deal of information to study and understand about particular members of the *Aspergillus* genus synthesize AFs. As described in **Table 1.2**, AFs could be secreted by at least 24 species of the genus *Aspergillus*, classified into three sections: *Flavi*, *Nidulants, and Ochraceorosei* (János Varga et al., 2015).

Three *Aspergillus* species mainly generate AFs: *A. flavus*, *A. nomius*, and *A. parasiticus*, as well as other *Aspergillus* species such as *A. astellatus* (Payne & Brown, 1998; Reiter et al., 2017). AFs are the result of an extensive biosynthetic process that involves at least 27 enzyme activities (Yabe & Nakajima, 2011). Analysis of the genes sequences has demonstrated similarity among fungal species that synthesize AFs (János Varga et al., 2015). Additionally, about 30 genes are implicated in fungal AFs synthesis (J. Yu, 2012). Also, as a secondary metabolite, AFs' biosynthesis depends on other complicated mechanisms activated in response to environmental factors such as light, availability of nutrients, pH, and oxidative stress, which could activate various cell signaling pathways modulating gene expression.

Typically, these fungi develop in warm, humid tropical, and subtropical climates. Fungi of the genus *Aspergillus* may thrive at temperatures ranging from 6 to 48 °C, with the optimum temperature being 25 °C. Additionally, they may grow

at pH levels ranging from 2 to 11.2 with a water activity near 0.85. In particular, cereals, fruit, nuts, and seeds are most vulnerable to fungal infection by *Aspergillus*, primarily during the storage stage (Pitt & Hocking, 2009).

AFB1, AFB2, AFG1, and AFG2 are the main contaminants of this group, differentiated according to their fluorescence under ultraviolet (UV) light and their relative chromatographic mobility (**Figure 1.2**). Additionally, there are other AFs; there are more than a dozen in all. They are polycyclic compounds that belong to the furanocoumarin group (Ben Miri et al., 2020). The majority of them are byproducts of the hepatic metabolism of four mold-derived compounds found in food. For example, after consumption of contaminated food, AFB1 is converted in the mammalian liver by cytochrome P450 into various metabolites, the most important of which is aflatoxin M1 (AFM1) which is generated by hydroxylation of the difuranocoumarin ring's tertiary carbon (Towner et al., 2000).

Table 1.2. Fungal species that were previously identified as aflatoxin producers.

Species	Aflatoxin	Provenance	Reference
Aspergillus flavus	B1, B2, G1, G1	Nuts, cereals, and several other commodities	Frisvad et al. (2005); Giorni et al. (2007)
A. parasiticus	B1, B2, G1, G2	Peanut, maize	Frisvad et al. (2019)
A. bombycis	B1, B2		Frisvad et al. (2005)
A. pseudotamarii	B1, B2	Cereals and soil	Frisvad et al. (2019); Varga et al. (2011)
A. nomius	B1, B2, G1, G2	Wheat, Brazil nuts and other substrates	Baquião et al. (2013)
A. toxicarius	B1, B2, G1, G2	Chestnuts	Prencipe et al. (2018)
A. parvisclerotigenus	B1, B2, G1, G2	Maize	Perrone et al. (2014)
A. columnaris	B1, B2, G1, G2	Maize	Frisvad et al. (2005); Moubasher et al. (2013)
A. zhaoqingensis	B1, B2	Cereals and soil	Bao-Sheng (2011); Varga et al. (2011)
A. novoparasiticus	B1, B2, G1, G1	Maize	Iamanaka et al. (2019); Viaro et al. (2017)
A. mottae	B1, B2, G1, G2	Cereals, Brazil nuts, almond, figs, pistachio nuts	Perrone and Gallo (2017)
A. sergii	B1, B2, G1, G2	Cereals, oilseeds	Benkerroum (2020)
A. pseudocaelatus	B1, B2, G1, G2	Maize	Viaro et al. (2017)
A. transmontanensis	B1, B2, G1, G2	Cereals	Benkerroum (2020)
A. luteovirescens	B1, B2, G1, G2	Cereals	Frisvad et al. (2019)
A. minisclerotigenes	B1, B2, G1, G2	Peanut	Moral et al. (2020)
A. arachidicola	B1, B2, G1, G2	Maize, Arachis glabrata	Viaro et al. (2017)
A. austwickii	B1, B2, G1, G2,	Cereals	Moral et al. (2020)
A. aflatoxiformans	B1, B2, G1, G2	Cereals	Frisvad et al. (2019); Moral et al. (2020)
A. pipericola	B1, B2, G1, G2	Cereals	Benkerroum (2020)
A. cerealis	B1, B2, G1, G2	Cereals	Benkerroum (2020)
A. Togoensis	B1, B2	cereals	Benkerroum (2020)
A. venezuelensis	B1, B2	Cereals	Frisvad et al. (2005) Benkerroum (2020)
A. astellatus	B1, B2	Cereals and other substrates	Benkerroum (2020); Frisvad et al. (2005)
A. miraensis	B1	Cereals	Benkerroum (2020)
A. olivicola	B1	Cereals	Benkerroum (2020)
A. ochraceoroseus	B1, B2	Cereals	Frisvad et al. (2005); Varga et al. (2009);
A. rambellii.	B1	Cereals	Varga et al. (2009)

AFs generated by *Aspergillus* species are very harmful, carcinogenic and contaminate food supplies severely worldwide, resulting in significant health concerns (Mahato et al., 2019). In particular, AFB1 is highly toxic and carcinogenic and has been classified as a human carcinogen by the IARC in the 90s decade (IARC, 1993). The primary mechanism of genotoxicity is related to the production of reactive AFB1 epoxide through the activity of cytochrome P450 enzymes, which is discussed posteriorly (Fernandes De Oliveira & Leal Germano, 1997).

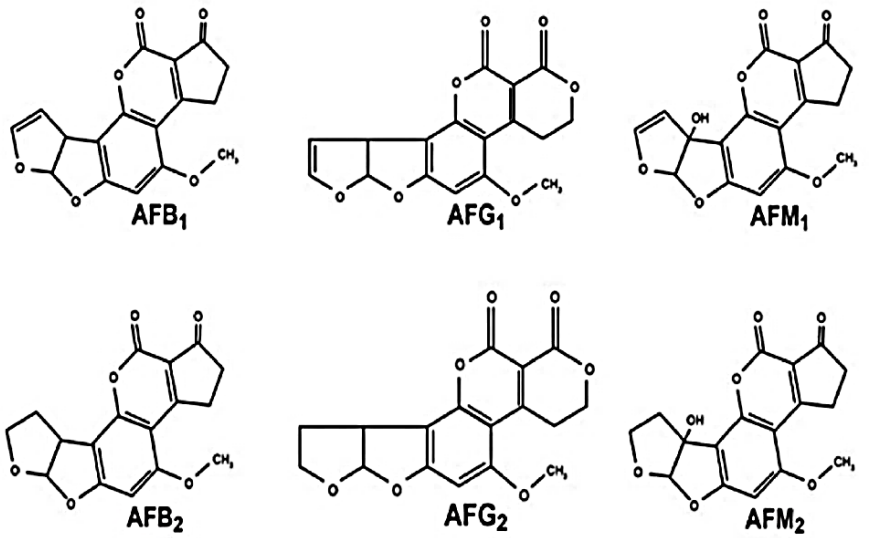


Figure 1.2. Chemical structure of the five most toxic aflatoxins (AFB1, AFB2, AFG1, AFG2, AFM1, and AFM2).

1.3 Ochratoxins and fungal producers

OTA was described for the first time in South Africa as a result of a culture of *A. ochraceus* (section Circumdati) in maize meal (Malir et al., 2016). Subsequently, it was found that *A. niger* and *A. carbonarius*, species commonly found in grapes and other fruits that mature in sunlight and high temperatures, could also produce OTA (Paterson et al., 2018). In addition to these species, several others including *Aspergillus tubingensis, A. westerdijkiae, A. steynii, A. lacticoffeatus, and*

A. sclerotioniger have been identified over time. Among *Penicillium* species, *P. verrucosum* and *P. nordicum* are known to produce OTA. *P. verrucosum* is commonly found in cereals growing in cool temperate zones, while *P. nordicum* is found in meat or cheese (Abarca et al., 2004; Cabañes et al., 2010; Ostry et al., 2013; Y. Wang et al., 2016). **Table 1.3** shows the *Aspergillus* and *Penicillium* species reported as OTA producer. shows the current identity of *Aspergillus* and *Penicillium* species that are known to produce OTA in foodstuffs.

OTA is the most abundant and most toxic ochratoxin (Mondani et al., 2020). Several metabolites related to OTA have been identified and further research has shown that more than 20 OTs metabolites are produced by molds belonging to the genera *Aspergillus* and *Penicillium*, including ochratoxin B (OTB) and ochratoxin C (OTC). These mycotoxins are a family of toxins derived from pentaketide that contain a sometimes-chlorinated isocoumarin coupled by an amide bond to an L-phenylalanine molecule. Although structurally similar, the three ochratoxins differ slightly from each other; for instance, OTB is a non-chlorinated form of OTA and OTC shows a Phenylalanyl and ethyl ester of OTA as radical (El Khoury & Atoui, 2010). However, these differences have significant effects on their respective toxic potential, with OTA being both the most prevalent and most toxic form (Mondani et al., 2020) **(Figure 1.3)**.

Similarly, to AFs producers, OTA-producing fungi are found worldwide in foodstuffs and climate variables like as temperature and humidity have a significant impact on OTA production (Pitt, 1987; Lasram et al., 2010; 2016). Thus, in cold and temperate climates, OTA is mostly generated by *P. verrucosum* or *P. nordicum* (Cabañes et al., 2010). In tropical and subtropical climates, *A. ochraceus* is mostly responsible for OTA synthesis (de Almeida et al., 2019). In addition, these fungi often produce multiple toxins simultaneously, and due to the simultaneous occurrence of OTA with other mycotoxins, such as OTB and OTC, there is a potential for synergistic toxic effects (Heussner & Bingle, 2015). The production of

OTA is greater at 0.98 a_w, irrespective of the temperature, although it tends to increase at the optimal temperature range of 25-30 °C (Taiki et al., 2011).

OTA is an organic acid with a pKa value of 7.1 and a molar mass of 403.8 g/mol. Its crystalline structure ranges from colorless to white and exhibits green fluorescence under UV light in acidic conditions and blue fluorescence in alkaline conditions. OTA is soluble in polar organic solvents such as alcohols, ketones, and chloroform in acid and neutral pH. In alkaline conditions, OTA is soluble in aqueous sodium bicarbonate solution and all alkaline solutions (Massoud et al., 2018). Its high stability is a distinguishing feature, exhibiting resistance to acidity and high temperatures. This makes it difficult to remove once foodstuffs are contaminated (Taghdisi et al., 2021). Moreover, cooking at normal conditions and even heating of OTA under various moisture conditions to 200 °C could not cause any significant change or decomposition (Trivedi et al., 1992).

Table 1.3. Species of fungi reported as OTA producers in Genus *Aspergillus* and *Penicillium.*

Species	Provenance	Reference
A. affinis	Decomposing leaves	G. Wang et al. (2023)
A. cretensis	Soil	G. Wang et al. (2023)
A. elegans	Bread, sponge	G. Wang et al. (2023)
A. flocculosus	Grapes	G. Wang et al. (2023)
A. melleus	Cereal	G. Wang et al. (2023)
A. muricatus	Peanuts	G. Wang et al. (2023)
A. ochraceopetaliformis	Sponge	G. Wang et al. (2023)
A. ostianus	Pulses	G. Wang et al. (2023)
A. ochraceus	Soya bean, nuts, red pepper, cereals, green coffee beans	Ostry et al. (2013)
A. pseudoelegans	Soil	G. Wang et al. (2023)
A. pulvericola	Indoor house dust	G. Wang et al. (2023)
A. roseoglobulosus	Decaying leaves	G. Wang et al. (2023)
A. sclerotiorum	Apple	Y. Wang et al. (2016)
A. steynii	Barley, coffee, grapes	Ostry et al. (2013)
A. subramanianii	Shelled nuts	G. Wang et al. (2023)
A. sulphureus	Soil	Y. Wang et al. (2016)
A. westerdijkiae	Decomposing leaves	Ostry et al. (2013)
A. alliaceus		Y. Wang et al. (2016)
A. albertensis		Y. Wang et al. (2016)
P. verrucosum	Cereals	Cabañes et al. (2010)
P. nordicum	Cereals and meat products	Cabañes et al. (2010)
P. thymicola	Canadian cheddar cheese	G. Wang et al. (2023)
P. radicicola	Carrots and potatoes	Cabañes et al. (2010)
P. viridicatum		Cabañes et al. (2010)
A. carbonarius	Grapes, red pepper, coffee beans	Abarca et al. (2004)
A. awamori		Abarca et al. (2004)
A. awamori var. fumeus		Abarca et al. (2004)
A. foetidus	Grapes	Abarca et al. (2004)
A. lacticoffeatus	Coffee beans	Y. Wang et al. (2016)
A. niger group		Abarca et al. (2004)
A. niger	Grapes, raisins, maize, coffee, beer	Abarca et al. (2004)
A. japonicus		Abarca et al. (2004)
A. sclerotioniger	Coffee	Ostry et al. (2013)
A. tubingensis	Grapes	Ostry et al. (2013)
A. welwitschiae	Grapes, raisins, pistachio, walnuts	G. Wang et al. (2023)
P. expansum	Pomaceous fruits and nuts	Cabañes et al. (2010)

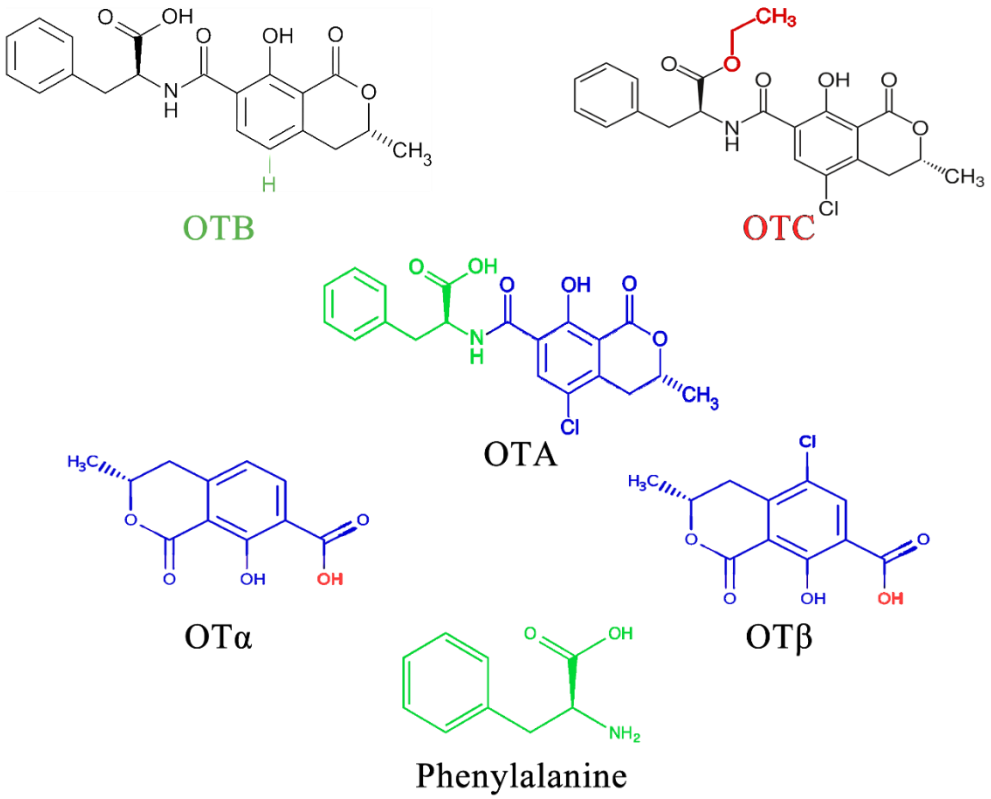


Figure 1.3. Types of ochratoxins and OTA composition.

The biosynthesis of OTA is complex and involves several precursors. L-Phenylalanine has been identified as the precursor of the phenylalanine moiety in OTA through radiolabeling experiments using 1-^{14}C-phenylalanine in the culture of *A. ochraceus*. On the other hand, the isocoumarin moiety of OTA was mainly derived through acetate condensation, with the addition of 2-^{14}C-Na-acetate. Precursor feeding experiments using 2-^{14}C-Na-acetate and 2-^{14}C-malonic acid in the liquid culture of *P. verrucosum* sp. have confirmed that both precursors are incorporated into OTA biosynthesis. The whole molecular structure of OTA has been labeled with 2-^{14}C-Na-acetate, but only the isocoumarin moiety was exclusively labeled by 2-^{14}C-malonic acid, indicating that both acetate and malonic acid are the precursors of OTA biosynthesis. Malonic acid appears to be involved in

the isocoumarin moiety biosynthesis, while acetate is transformed into both the phenylalanine moiety and the isocoumarin portion (Y. Wang et al., 2016).

1.4 Prevalence of mycotoxins in food and feed

Food is susceptible to fungal deterioration due to various factors, such as the nature of the food matrix, biological, physical, and chemical parameters, handling during harvesting and post-harvest storage, technological processes applied during processing, and storage conditions after processing. To ensure food quality and safety, it is essential to understand the factors associated with microbiological development. The factors can be divided in intrinsic and extrinsic.

Intrinsic factors are directly related to the chemical composition of the food, including its structure, composition, water activity (a_w), and pH. The microflora of the food can change depending on whether it is solid or liquid, its centesimal composition (proteins, lipids, and carbohydrates), and the presence of inhibitory substances (Baron & Gautier, 2016). Bacteria require a high a_w to carry out their metabolic activities, while molds can develop at a_w values between 0.70-0.80 (Fuchs et al., 2015). The pH value of the food also affects the microflora, with bacteria requiring a pH close to neutrality (6.5-7.5) to grow, while yeasts and molds can develop at more acidic pH levels (4.0-6.8) (Owusu-Apenten & Vieira, 2023). Extrinsic factors are associated with environmental conditions, such as temperature, gas composition, and interactions with other microorganisms, which affect the storage of the food. Molds grows optimally at room temperature, but they can also grow at refrigeration temperatures. The composition of the atmosphere can limit the presence of molds in food since they are strict aerobes and require oxygen for development (Rolfe & Daryaei, 2020).

The interactions between different microorganisms that colonize a food can also influence the presence of spoilage in the final product. The visible growth of microorganisms such as filamentous fungi, yeasts, and bacteria can lead to the rejection of the entire product at an industrial level or by the consumer. The impact

of fungal contamination on food quality can alter the nutritional and organoleptic characteristics of the food, leading to other organoleptic defects such as gas production, unpleasant flavors, and texture changes. Therefore, it is essential to consider both intrinsic and extrinsic factors in the production, processing, and storage of food to ensure food quality and safety.

Mycotoxin in agricultural commodities occurs at many stages along the food chain: pre-harvest, harvest, drying, and storage. Inadequate farming and harvesting methods, as well as insufficient drying, handling, packing, storage, and transportation conditions, encourage fungal development, increasing the risk of mycotoxin (S. Marin et al., 2013).

Once generated, mycotoxins are present throughout the fungal colony: hyphae, mycelium, spores, and the substrate on which the colony developed (Bhat et al., 2010). Mycotoxins are mostly acquired orally via infected foods, although they may also be acquired by inhalation or even contact (Zain, 2011).

It should also be noted that human and animal consumers are exposed to several mycotoxins in the majority of cases due to three main reasons: (I) numerous fungus species may generate the same mycotoxin, (II) conversely, the same species is sometimes capable of generating several mycotoxins concurrently, and (III) meals or rations, in case of animals, are usually made up of several foods or foods prepared from several raw materials, each of which can be a source of a different toxins (Alassane-Kpembi et al., 2017).

Mycotoxins can contaminate many food products such as cereals (maize, wheat, rice, barley), oilseeds (peanuts, cotton), dry and dried fruits (pistachios, nuts, dried figs), spices and Animal-derived foodstuffs (Chinaza Godswill Awuchi et al., 2021; Pankaj et al., 2018). According to global occurrence data reported over the last decade, AFs had a prevalence of 55% and a maximum level of 1,642 μg/kg in raw cereal grains, whereas OTA had a prevalence of 29% and a maximum level of 1,164 μg/kg. Other mycotoxins such as FUM, DON, and ZEN had a prevalence of 61, 58,

and 46%, and maximum levels of 71,12 μg/kg, 41,20 μg/kg, and 3,049 μg/kg, respectively (Lee & Ryu, 2017).

Recently, large surveys on mycotoxin occurrence across the world have suggested that the prevalence of mycotoxins may be higher than the 25% reported by FAO. For example, Streit et al. (2013) found that approximately 72% of the 17,300 feed samples collected from different parts of the world over an eight-year period contained mycotoxins. Similarly, Kovalsky et al. (2016) reported that mycotoxin contamination in feeds could be as high as 79% or more in approximately 2,000 samples from 52 countries. The authors noted that these frequencies are significantly higher than FAO's estimate and suggested that further investigation is needed to determine mycotoxin contamination rates at the global level.

Likewise, a ten-year global survey was conducted to assess mycotoxin contamination in feed and determine regional differences and year-to-year variation. The survey analyzed concentrations of several mycotoxins in nearly 75,000 samples of feed and feed raw materials collected from 100 countries between 2008 and 2017. The results showed that 88% of the samples were contaminated with at least one mycotoxin, and mycotoxin occurrence varied regionally, with climate playing a significant role (Gruber-Dorninger et al., 2019). In another survey conducted between 2004-2011 on agricultural commodities, 72% of the 19,000 samples analyzed showed detectable AFs, FUM, DON, ZEN, or OTA levels. This survey also showed an increase in aflatoxin contamination by 11% (2005) to 52% (2009) and 40% (2011) (Schatzmayr & Streit, 2013). Additionally, survey results on food grains indicate that the incidence of mycotoxins may range between 60 and 80%, depending on the factors such as mycotoxin of interest, kind of food, the analytical technique employed, and the detection limit of the equipment (Eskola et al., 2020; H. J. Lee & Ryu, 2017).

Because of the increase in incidence affecting all regions, the occurrence of mycotoxin contamination does not seem to be restricted to one-off climatic events. It could possibly be related to global climate change (Leggieri et al., 2021).

Mycotoxins may even be found in foods that have been industrially processed to remove fungi. Indeed, their structural stability makes them resistant to the high temperatures encountered during manufacturing and food processing (Kabak, 2009). To make matters worse, toxicological investigations using in vivo and *in vitro* models have revealed potential interactions between many distinct mycotoxins. These interactions may be categorized as antagonistic, additive, or synergistic and vary in intensity and ratio depending on the mycotoxins involved (Alassane-Kpembi et al., 2017; Smith et al., 2016).

1.4.1 Aflatoxin occurrence

AF contamination has been observed in food and feed, including groundnuts, maize, wheat, rice, barley, spices, and cocoa, mainly associated with fungal infection under pre and postharvest circumstances. Apart from food, commercial goods such as peanut butter, cooking oil, and cosmetics could also be contaminated with AFs. Even a trace amount of AFs is toxic to humans and dangerous to mammals (Mahato et al., 2019). **Table 1.4** provides a summary of recent studies that have identified AFB1 contamination in various commodities, highlighting the prevalence of this issue in the past decade.

As previously mentioned, the susceptibility of crops to fungal spoilage and subsequent toxin generation is determined by several environmental and intrinsic crop characteristics, including moisture content, nutritional content, and pH (Fountain et al., 2014; Magan et al., 2011). Genetical and environmental interactions may also influence postharvest susceptibility through their effects on grain integrity and density. Therefore, the extended cultivation of adapted crops to the environmental factor in which they are inserted has been associated with resistance to fungal spoilage (Mutiga et al., 2019).

Although the effect of environmental conditions on food susceptibility is well known, it is worth emphasizing that these factors do not work in isolation; instead, they combine with intrinsic plant characteristics to aid fungal invasion and toxin production (Tai et al., 2020).

Crop's nutritional composition has a significant effect on its susceptibility. For instance, a study carried out with maize, maize germ, maize endosperm, and wheat revealed that small amounts of soluble sugars had no impact on the synthesis of AFB1 by *A. flavus*. In contrast, when these concentrations were raised to at least 6%, AFB1 production was substantially increased, which suggests a positive relationship between the two variables. The authors also discovered that AFs levels significantly increased when maize oil was added to substrates (J. Liu et al., 2016).

Several studies on the impact of a_w on aflatoxin concentrations in agricultural products have been published (Battilani et al., 2011; Medina et al., 2017b; Sahar et al., 2015). In general, low water activity is required to minimize aflatoxin accumulation in grains after harvest. AFB1 contamination may be prevented if the grain water activity is maintained at or below 0.9 (Dorner, 2008; Lahouar et al., 2016). As discussed before, nutritional content, water activity, and temperature are interdependent, but they interact to induce aflatoxin synthesis (Ribeiro et al., 2006).

It is primordial to identify and reduce AFs in foods because the level of contamination determines the level of human exposure. In other words, regions with high contamination levels will probably evidence a high level of human dietary intake. Therefore, identifying, and quantifying mycotoxins in food and feed is a significant issue for ensuring food safety because food and feed contamination are chronic global problems.

Table 1.4. Reports of AFs occurrence in food commodities over the last ten years.

Food	Country	Percentage of contaminated samples	Mycotoxin	Concentration (µg/kg)	Method of detection	Reference
Maize flour	Iran	80.0% of 10	AFB1	<LOQ–1060	UHPLC–MS/MS	Amirahmadi et al.(2018)
Maize	Brazil	25.7 and 7.4% of 148	AFB1 and AFG1	0.5 to 49.9	HPLC–MS/MS	Oliveira et al. (2017)
Maize	Korea	13.6% of 66	AFB1	0.02 to 0.48	HPLC	D. M. Kim et al. (2013)
Maize flour	Italy	26.0% of 50	AFB1	0.17 to 3.75	HPLC-FLD	Armorini et al.(2015)
Maize	Haiti	55.0% of 20	Sum of AFs	185.9 ± 303.9	HPLC-DAD	Aristil et al. (2020)
Maize	Zimbabwe	23.7% of 338	AFB1	0.57 to 26.6	ELISA	Murashiki et al. (2017)
Maize	Ethiopa	8% of 100	AFB1	26.6	HPLC–MS/MS	Getachew et al. (2018)
Maize	Serbia	57.2% of 360	AFB1	1.3 to 88.8	HPLC-FLD	Janić Hajnal et al. (2017)
Maize	Uganda	25.8 (256)	AFTotal	0 to 3,760	TLC	Sserumaga et al. (2020)
Maize	Kenya	100%	Sum of AFs	2.14 to 411	UHPLC-FLD	Nabwire et al. (2020)
Dried Fruits	Iran	56.8% of 88	AFB1	0.3 to 8.4	HPLC-FLD	Heshmati et al. (2017)
Fermented meat products	Croatia	8.3 and 58.3% of 180	AFB1and OTA	<0.05-7.83	HPLC-FLD	Markov et al. (2013)
Infant formulae	Mexico	20% of 55	AFM1	0.040 to 0.450	HPLC	Quevedo-Garza et al. (2020)
Milk	China	80%	AFM1	0.005 to 0.10	ELISA and HPLC-MS/MS	Xiong et al. (2020)
Milk	Lebanon	58%	AFM1	0.011 to 7.350	HPLC	Daou et al. (2020)
Wheat	China	6.2% of 178	AFB1	0.03 to 0.12	HPLC–MS/MS	Y. Zhao et al. (2018)
Nuts	Zimbabwe	12.5% of 208	AFB1	0.7 to 175.9	HPLC-FLD	Maringe et al (2017)
Rice	Ecuador	7.0% of 230	AFB1	4.9 to 47.4	HPLC-FLD	Ortiz et al. (2013)
Rice	Pakistan	18.4% of 1027	AFB1	1.1 to 32.9	HPLC-DAD	Firdous et al. (2014)
Rice	Brazil	11.2% of 187	AFB1	63.32	HPLC-FLD	Katsurayama et al. (2018)
Sorghum	Ethiopia	94.4% of 90	AFB1	33.1	ELISA	Taye et al. (2016)
Vegeteble oil	Sri Lanka	34%	Sum of AFs	4.0	HPLC-FLD	Karunarathna et al. (2019)

1.4.2 OTA occurrence

Similarly, the consumption of food is a common route of exposure for both humans and animals to OTs. These mycotoxins are primarily found in cereal grains, such as barley, rye, wheat, maize, sorghum, and oats, as well as in coffee beans and grapes, including wine and raisins (Chinaza Godswill Awuchi et al., 2021). Other commodities, including cottonseed, nuts, and dried beans, may also contain ochratoxins. In addition, meat products, particularly pork and kidney, such as sausages, bacon, and ham, are also sources of human exposure (Pizzolato Montanha et al., 2018). In particular, barley and dry-cured sausage are two food items with high prevalence of OTA. The barley contamination can occur during crop development or in storage, while dry-cured sausage can become contaminated during the production process. Both barley and dry-cured sausage are popular ingredients in many food products, and therefore, the presence of OTA in these items can have a significant impact on food safety (Andrade et al., 2019; Luz et al., 2021a). **Table 1.5** summarize recent studies that have identified OTA contamination in various commodities in last ten years.

Ochratoxin contamination is more prevalent in temperate regions, with northern European countries, the Balkans, and Canada having the highest levels due to optimal production conditions of 24 °C temperature and 19-22% moisture content (Gurikar et al., 2023).

Table 1.5. Reports of ochratoxin A occurrence in food commodities over the last ten years.

Food	Country	Percentage of contaminated samples	Concentration (µg/kg)	Method of detection	Reference
Baby foods	Turkey	34.7% of 150	<0.5	HPLC	Hampikyan et al. (2015)
Barley	Egypt	20% of 15	1.13-2.15	HPLC-FLD	Ben Hassouna et al. (2022)
Barley	United States	6% of 60	0.16–185.24	HPLC-FLD	Kuruc et al. (2015)
Beers	Czech Republic	81% of 132	0.001-0.195	UPLC-FLD	Belakova et al. (2015)
Beer	Portugal	10.6% of 84	<0.43-11.25	HPLC-FLD	Silva et al. (2020)
Beer	Spain	20% of 40	0.24 to 54.76	HPLC-MS/MS	Carballo et al. (2021)
Breakfast cereals	Serbia	33.7% of 136	0.07-3.00	HPLC-FLD	Torović et al. (2017)
Cereals	Uganda	8.3 to 100% of 105	0.1-16.4	ELISA	Echodu et al. (2019)
Cocoa bean	Brazil	22.8% of 123	0.25–7.2	HPLC-FLD	Pires et al. (2019)
Coffee	Portugal	25% of 6	1.45-1,031	HPLC-FLD	Benites et al. (2017)
Maize	Pakistan	71.0% of 46	2.14-214	HPLC	Wajih ul Hassan et al. (2020)
Dried grapes	Iran	57.5% of 23	0.16-8.40	HPLC-FLD	Heshmati & Mozaffar i Nejad (2015)
Dry-Cured Meat	Croatia	19.2% of 250	0.24-4.81	HPLC-MS/MS	Lešić et al. (2022)
Dry wine	Serbia	52.2% of 113	0.026	HPLC-FLD	Torović et al. (2020)
Fermented coffees	Brazil	21.4% of 14	<0.64-0.87	HPLC-FLD	Costa da Silva et al. (2021)
Pasteurized Milk	China	25.8% of 120	>0.049-18.8	HPLC-MS/MS	Z. Zhang et al. (2022)
Milk	Italy	36.4% of 33	<0.3-3	HPLC/FLD	Lippolis et al. (2020)
Raisin	USA	93% of 40	0·06-11·4	HPLC	Palumbo et al. (2011)
Salamis	Italy	12.8% of 172	0.07–5.66	HPLC-FLD	Altafini et al. (2019)
Sorghum	Tunisia	24 of 064	1.04-27.8	HPLC-FLD	Lahouar et al. (2018)
Wheat	United States	13% of 58	0.17–14.94	HPLC-FLD	Kuruc et al. (2015)
Wine	Croatia	92.8% of 110	0.003–0.163	HPLC-FLD	Žurga et al. (2019)
Wine	Italy	71.9% of 57	Mean of 0.13	HPLC-FLD	Di Stefano et al. (2015)

1.5 Effect of AFs in human and animal health

Mycotoxins have a detrimental impact on human and animal health. These effects, hence, may vary significantly depending on the toxin, its structure, dosage, and duration of exposure (Zain, 2011). Thus, a difference may be established between acute mycotoxicosis, which occurs after a brief exposure to high levels of toxins, and subacute poisoning, which occurs following a protracted exposure to low levels of toxins (many weeks to several months). Mycotoxins' primary adverse effects include carcinogenic, mutagenic, immunosuppressive, hepatotoxic, neurotoxic, and irritating potential (Janik et al., 2020).**Table 1.6** summarize the most frequent producer of AFs and OTA, the most frequently contaminated foodstuffs, and their proven effects on health.

Table 1.6. Main associated fungi producers of AFs and OTA, the frequently contaminated foods, and primordial toxic effects.

Mycotoxins	Main producers	Food	Toxicity	Reference
Aflatoxins B1, B2, G1, and G2	*Aspergillus flavus* *A. parasiticus* *A. nomius*	Maize, peanuts, wheat, cottonseed, nuts, rice, dry fruits, and spices.	Carcinogenicity Genotoxicity Hepatotoxicity Immunotoxicity Teratogenicity	Y. Luo et al. (2018)
Ochratoxin A	*Penicillium verrucosum* *A. ochraceus* *A. carbonarius*	Cereals, cocoa, coffee beans, wine, grape juice, beer, spices, cured meat products.	Hepatotoxicity Immunotoxicity Nephrotoxicity Teratogenicity	Tao et al. (2018)

Aflatoxin is an acronym composed of letters A for *Aspergillus*, FLA for *flavus*, and TOXIN. Since the most well-known and well-publicized episode of aflatoxicosis occurred in England around the turn of the twentieth century (1960), many young turkeys contracted the previously unknown Turkey-X disease. It was subsequently discovered that these bird fatalities were caused by severe liver damage

sustained when the farm animals consumed ground-nut meals contaminated with *A. flavus* molds. Furthermore, even after this episode, AFs have been detected at measurable amounts in many popular commercial brands of peanut butter (Richard, 2008).

The toxicity of AFs, particularly AFB1, is dose and time-dependent and depends on other factors such as age, sex, the species and its tolerance, and nutritional condition. While the effects of these toxins are well established in animals, they have been recorded in humans after acute hazardous accidents or due to regular exposure of particular populations to geographical regions with high rates of food contamination. AFB1 is the most toxic, with hepatotoxic, hepatocarcinogenic, mutagenic, and teratogenic effects in a wide variety of animals, most notably young chicken (Bintvihok & Kositcharoenkul, 2006; Nayak & Sashidhar, 2010). Additionally, AFs and their metabolites may be found in the flesh or other products of animals fed aflatoxin-contaminated diets, posing a risk to human health (Bintvihok & Kositcharoenkul, 2006).

Human cases of acute aflatoxicosis are very uncommon nowadays (Fung & Clark, 2004). These hazardous incidents lead to the occurrence of eating high amounts of AFs and seem to occur only under specific unique circumstances of poverty that induce the populace to consume raw materials that are usually prohibited. For instance, the biggest aflatoxin poisoning event occurred in Kenya in 2004, when more than 300 instances of acute aflatoxicosis were recorded, culminating in the death of around 100 individuals as a consequence of consuming maize infected with high levels of AFB1 (Lewis et al., 2005).

The liver is the target organ of AFs. Indeed, the production of a very unstable molecule derivative during hepatic metabolism results in its contact with macromolecules in liver cells and, when these interactions are abundant, the cell death (Deng et al., 2018). Thus, acute aflatoxicosis symptoms include jaundice associated with hemorrhagic necrosis of the liver and hepatic encephalopathy; these

symptoms are fatal in more than 25% of patients (Lewis et al., 2005). Adults are usually more tolerant to AFs than children because cells renew faster, reducing the deleterious effect (J. H. Williams et al., 2004).

Aflatoxicosis in poultry is characterized by mortality, reduced growth rates, negative feed conversions, decreased egg production, listlessness, anorexia, fatty liver, and poor pigmentation. Birds that consume contaminated feed are immunosuppressed and more susceptible to stress and diseases. Aflatoxicosis causes significant changes such as increased liver size, paleness of liver, and enlargement of the bile duct, and can also cause lesions in kidney and spleen tissue. Histopathologically, vacuolar degeneration, fatty changes, and hepatocyte degeneration are observed in affected animals, along with infiltration of different lymphocytic cells and bile duct hyperplasia. In addition to the above effects, aflatoxin also has a carcinogenic effect on poultry.

The toxin is metabolized in the liver and converted into an active metabolite called aflatoxin B1-8,9-epoxide, which can bind to DNA and form adducts that can lead to genetic mutations. These mutations can eventually result in the development of liver cancer in birds. The risk of developing liver cancer due to aflatoxin exposure is higher in birds that are exposed to the toxin during early stages of life (Abidin et al., 2011). For this reason, Gholami-Ahangaran & Zia-Jahromi (2011) evaluated incorporating a commercial nano compound to the feed to reduce the effect of aflatoxin in broiler chickens induced to experimental aflatoxicosis. Although adding Nanocid to a regular diet had no impact on performance, adding Nanocid to a diet containing 3 ppm aflatoxin increased cumulative body weight gain, cumulative feed intake, and reduced feed conversion rate substantially over the last two weeks of the experimental period.

Although the liver is AFB1's primary target organ, it may also affect other physiological processes, as shown in **Figure 1.4**. Nonetheless, the primary toxicity of AFB1 is still associated with its hepatotoxicity and the interaction of the epoxide

derivative with hepatocyte DNA. Indeed, this interaction is responsible for developing liver tumors after chronic exposure to low AFB1 concentrations.

As previously mentioned, AFB1 is the most dangerous naturally occurring carcinogen known to humans. The IARC categorized it as a Group 1 human and animal carcinogen in 1993. This categorization is based on epidemiological evidence demonstrating a significant association between AFB1 exposure and the development of liver cancer or Hepatocellular Carcinoma (HPC) (Marchese et al., 2018). HPC is the most prevalent kind of cancer globally and the third most cause of cancer mortality (Mikhail & He, 2011).

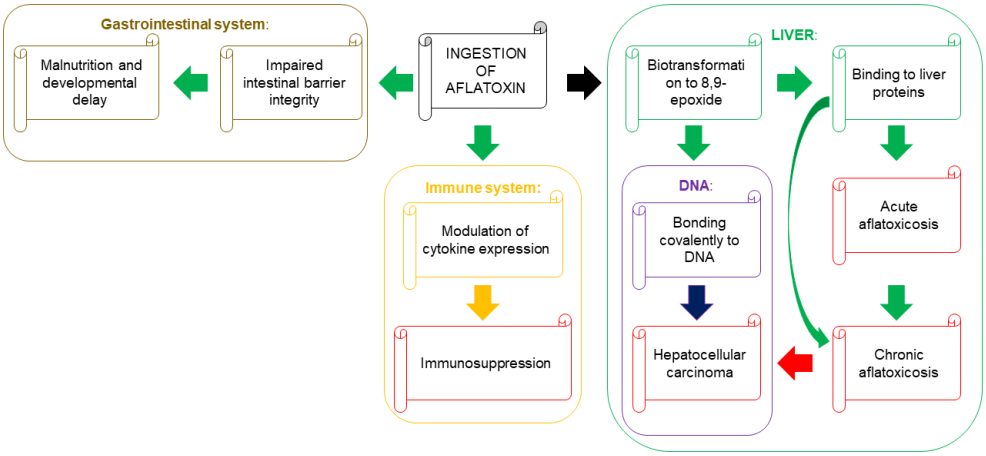


Figure 1.4. Different physiological levels of AFB1 toxicity.

AFB1 is metabolized in the liver by two cytochromes P 450 after intake (CYP1A2 and CYP3A4). The biotransformation of AFB1 into the two epoxides AFB1-8,9-endo-epoxide and AFB1-8,9-exo-epoxide is catalyzed by these two enzymes (Marchese et al., 2018). However, the latter is more prevalent, more reactive, and capable of intercalating between DNA bases to produce 8,9-dihydro-8-(N7-guanyl)-9-hydroxy-AFB1 (AFB1-N7-Gua) adducts endowing AFB1 with mutagenic characteristics. In humans, mutations in codon 249 of the p53 gene,

activation of mitotic recombination, and minisatellite rearrangements that promote genomic instability all contribute to liver tumor development and, therefore, the establishment of HPC (Ayob et al., 2021).

Apart from its carcinogenic properties, AFB1 acts as an immunosuppressive agent, impairing resistance to microorganisms. Indeed, AFB1 acts as a modulator of the cellular response by inhibiting the activity of B and T lymphocytes as well as inflammatory cytokines (S. Marin et al., 2013). It leads to the development of persistent infections and presents a significant danger to immunocompromised people. For instance, aflatoxins' adducts like AFB1 plus albumin exacerbate immune system damage in HIV-positive individuals (Jiang et al., 2008).

To summarize, aflatoxin exposure may also cause changes in the gastrointestinal tract. For instance, epidemiological studies have shown a link between AFB1 exposure and developmental delay in infants (Felicia Wu et al., 2014). Additionally, the frequent occurrences of malnutrition in emerging nations may exacerbate aflatoxins' effects. Since protein deficiency disrupts liver oxidases, promoting aflatoxin deposition in the body. Aflatoxin exposure is known to impair animal development and cause enterocyte damage, contributing to leaky gut (McMillan et al., 2018).

1.6 Effects of ochratoxin in human and animal health

The similarity in toxicities between ochratoxin A and ochratoxin C is notable, despite ochratoxin A being more frequently encountered. On the other hand, ochratoxin B's potency is lower by several times (Haschek & Voss, 2013). The human and animal exposure to OTs is particularly high and have been detected in serum, plasma, and milk. While the concentrations of OTs in grains may vary, they can occasionally reach levels that can cause diseases (Arroyo-Manzanares et al., 2021).

The mechanism of OTA toxicity involves its binding to plasma proteins, particularly albumin, which facilitates its transport to the kidneys, liver, and other

tissues (Ropejko & Twarużek, 2021). OTA has also been shown to inhibit protein synthesis and mitochondrial respiration, induce DNA damage, and alter gene expression in various cell types, including renal cells, hepatocytes, and lymphocytes (Kőszegi & Poór, 2016).

One of the most well-known toxic effects of OTA is their nephrotoxicity. The primary target of OTA is the epithelial cells of proximal tubules of the kidneys. The severity of nephrotoxicity can vary depending on the dose and duration of exposure, as well as other factors such as age, sex, and genetic susceptibility. Animal studies have shown that long-term exposure to OTA results in its accumulation in the renal cortex, where it causes oxidative stress, inflammation, apoptosis, and necrosis of renal cells, leading to renal dysfunction and fibrosis, while short-term exposure can cause acute renal failure (Imaoka et al., 2020).

In humans, the exposure has been associated with a specific form of chronic kidney disease known as Balkan Endemic Nephropathy (BEN). BEN is a progressive disease that affects people living in certain regions of the Balkans, and it is believed to be caused by long-term exposure through contaminated food and water sources (Samuel et al., 2021).

In animals, OTA is a highly toxic on broilers, porcine dogs and rats; in these species, OTA is primarily a nephrotoxin and causes severe nephrotoxicity. Among then, porcine are the most sensitive to nephrotoxic effects. Other toxicities include myelotoxicities in mice, and hepatic and cardiac lesions in rats (Chinaza G Awuchi et al., 2022). Animal exposed to OTA may experience reduced growth, poor body weight gain, and depression in behavior (Furian et al., 2022).

OTA also causes severe immunosuppression, resulting in teratogenic, mutagenic, and immunotoxic effects that increase mortality (Furian et al., 2022). In animal studies, OTA have been shown to reduce the production of antibodies and the activity of immune cells such as lymphocytes and macrophages (Xu et al., 2017). In humans, this mycotoxin has been described as modulator of humoral and cellular

immunity, inflammation, nitrosative stress and gut immunity, which can increase the risk of infections and cancer (D. E. Marin & Taranu, 2015).

Regard the OTA carcinogenicity, animal studies have shown that long-term exposure can increase the incidence of tumors, including kidney and liver tumors (Stoev, 2021). In humans, this mycotoxin has been associated with an increased risk of urinary tract tumors, as well as other types of cancer such as breast cancer (Hope & Hope, 2012).

Microscopic alterations within organs due to OTA exposure include degeneration of liver tissue and proliferation of epithelium of the biliary channel, resulting in individual cell necrosis and DNA adduct formation. In kidneys, OTA causes necrosis of tubular epithelial cells, glomerular infiltration, and distended glomerular spaces. OTA also causes atrophy of follicles and reduction in the lymphocytic mass present in the medulla within the bursa. Within the thymus, OTA exposure results in the reduction of lymphocytic mass in the parenchyma and localized congested areas (Abidin et al., 2011).

To summarize, the nephrotoxicity of OTs is well-established, and chronic exposure to these toxins has been linked to the development of chronic kidney disease. In addition, OTs can suppress the immune system, increase the risk of cancer, and cause other toxic effects such as reproductive and neurological toxicity. The mechanism of toxicity of OTs is not fully understood, but it is thought to involve the inhibition of protein synthesis in cells. Further research is needed to better understand the mechanisms of toxicity of OTs in general.

1.7 Recent outbreaks

Populations at highest risk hail from regions where there is minimal to no implementation of regulations or implementation of primary prevention strategies to mitigate the risk of contamination.

Nowadays, outbreaks are more common in tropical and subtropical climates, with a few occurring in colder zones. Additionally, the Mediterranean zones have shown more susceptibility to AFs and OTs contamination due to climate change, which causes related changes in mycotoxins' typical occurrence regions, in other words, changes in average temperatures, CO_2 levels, and rainfall patterns (Medina et al., 2017a). Climate change has resulted in a rise in agricultural contamination with fungus and mycotoxins on a global scale and this broad dispersion, along with the very high toxicity, have provoked significant chemical and biological interest and attention, which is well justified. Therefore, establishing practical, sensitive, and robust analytical techniques is critical for identifying and quantifying mycotoxins present, sometimes, in trace quantities in food and feed. Mycotoxins are detected using a variety of chromatographic and sensor-based techniques, which permitted the detection and prevention of outbreaks (Prietto et al., 2015).

In April 2004, rural Kenya had one of the country's biggest aflatoxicosis epidemics, with 317 cases and 125 fatalities. The epidemic was caused by aflatoxin-contaminated domestic maize, but the degree of regional contamination and the condition of maize in commercial markets were unclear. This epidemic occurred during severe regional and national food scarcity caused by a prolonged drought and lousy harvest (Lewis et al., 2005).

Wouters et al. (2013) described an outbreak of a major aflatoxicosis epidemic in dogs in southern Brazil in 2011. After being given diets including cooked maize meal as a standard component, 65 dogs from nine separate farms developed aflatoxicosis. Among them, 60 died. The farmers obtained the infected maize products from the same source, either whole maize grain or cornmeal. For the animals, a combination of leftover meat and maize provided by local sources was used to make maize polenta, which was given to the dogs. Two of the dogs died after many days of rejecting food and exhibiting anorexia, polydipsia, icteric mucous membranes, hematemesis, hematochezia, or melena, as well as skin, eye, ear, and

mouth hemorrhage. The main necropsy findings were jaundice, hemorrhages in many organs, and a yellowish enlarged liver with an increased lobular pattern.

In June 2016, an epidemic of an unknown illness was described in various areas of central Tanzania, affecting groups of individuals. A quick epidemiological survey was performed in the impacted villages, followed by a thorough house-to-house interview of chosen families. Between 14 May and 14 November 2016, 68 cases were reported, 20 of which resulted in death, culminating in a mortality rate of 30%. Over half of the cases were children under 15 years old, and the suspect food seemed to be locally produced maize (Kamala et al., 2018).

1.8 Legislation

Over 100 nations have aflatoxin laws, which aim to safeguard human and animal health but also impose economic costs on countries attempting to export grains and other aflatoxin-contaminated products (F. Wu, 2015). These economic consequences must be weighed against the rule's regulatory safeguards (Dimitri et al., 1998). It is critical to recognize that, even in countries with antimycotoxin laws, many people eat crops that have not been inspected, particularly in countries with extensive subsistence farming. As a result, contamination, exposure, and a lack of control may negatively impact global commerce and health (F. Wu, 2015).

People and animals have developed severe health problems because of the frequent consumption of AFs and OTA and this worldwide concern motivated various nations to establish stringent restrictions for these mycotoxins to improve the quality of commercialized products and to protect the own population health (Alshannaq & Yu, 2017).

In general, the AFs limit is between 2 and 30 μg/kg for food destinated to human ingestion around the world. On the one hand, the European Union (EU) sets stricter standards regarding AFB1 and total AFs in products intended for direct consumption, allowing up to 2 μg/kg and 4 μg/kg, respectively. On the other hand, the US sets the maximum level of AFB1 and total AFs in 10 μg/kg in every finished

product. In contrast, although OTA seems to be less toxic, the limit of maximum tolerable range from 2 to 10 μg/kg in these regions.

In Brazil, the maximum tolerated limit of mycotoxins in food is determined by the ANVISA's RDC nº 722, July 1, 2022. Similarly, the FDA's legislation (2019) in the US and the European Commission's (EC No. 165/2010 and 2022/1370) determine the maximum tolerable values of mycotoxins in maize and its derivative products. Although the maximum tolerable levels in Brazil are generally higher, with each new regulation, the legislation becomes increasingly stringent. **Table 1.7** provides a summary of the tolerable limit of AFs and OTA in foods in Brazil, the EU, and the US. Currently, the FDA has not set regulatory guidelines for OTA in food or feed in US.

Table 1.7. Limit maximum tolerable of AFs and OTA in food commercialized in Brazil, Europe Union (EU), and United States (US).

Region	Food category	AFB1 (µg/kg)	Sum of AFs (µg/kg)	AFM1 (µg/kg)	OTA (µg/kg)
Brazil	Cereal-based for infant	–	1.0	–	2.0
	Cocoa beans	–	10.0	–	10.0
	Peanuts, Brazil nuts	–	20.0	–	
	Coffee	–	–	–	10.0
	Nuts, walnuts, pistachios, hazelnuts and almonds	–	10.0	–	–
	Cereals and cereal products	–	5.0	–	10.0
	Maize and maize products	–	20.0	–	20.0
	Dehydrated and dried fruits	–	10.0	–	10.0
	Cocoa and chocolate	–	–	5.0	5.0
	Powdered milk	–	–	5.0	–
	milk	–	–	0.5	–
	Cheese	–	–	2.5	–
	Grape juice, grape, wine and its derivatives	–	–	–	2.0
EU	Cereal-based for infant	0.10	–	–	0.5
	Peanuts and tree nuts	2.0	4.0	–	–
	Brazil nuts, hazelnuts	5.0	10.0	–	–
	Coffee	–	–	–	3.0 to 5.0
	Almonds, pistachios and apricot kernel	8.0	10.0	–	–
	Cereals and cereal products	2.0	4.0	–	3.0 to 5.0
	Maize and maize products	5.0	10.0	–	–
	Dehydrated and dried fruits	2.0	4.0	–	2.0 to 8.0
	Cocoa powder	–	–	–	3.0
	Powdered milk	–	–	–	–
	Milk	–	–	0.05	–
	Infant milk	–	–	0.025	–
	Grape juice, grape, wine and its derivatives	–	–	–	2.0
US	Foods	–	20.0	–	–
	Brazil nuts	–	20.0	–	–
	Peanuts and Peanut products	–	20.0	–	–
	Pistachio nuts	–	20.0	–	–
	Milk	–	–	0.5	–
	Animal Feeds	–	20.0-300.0	–	–

1.9 methods for avoiding or mitigating the presence of mycotoxins in foods

Preventing the growth of fungi in food and inhibiting toxin production is the most effective approach to reduce mycotoxins in the food chain. Various physical, chemical, and biological methods have been utilized at both industrial and laboratory levels to achieve this goal (Y. Liu et al., 2020).

Numerous measures may be taken to mitigate the danger of mycotoxins exposure and the associated health and socio-economic issues. These may either avoid contamination by restricting fungal growth (FG) or act later FG to remove or decrease the toxin's availability. In the first instance, treatments are mostly carried out in the field, before harvest, to prevent the fungal infection from spreading. In the case of post-harvest treatments, the methods are mostly directed at decontaminating the substrates after toxin production or decreasing the toxin's availability by restricting its absorption by exposed organisms (Awuchi et al., 2021).

Contamination of cereal grains and feed is unavoidable, and cost-effective detoxification methods are unavailable. Thus, frequent quality monitoring of animal-derived foods, raw materials, and feed must be considered essential for the welfare of food animals, economic viability, and consumer food safety (Peles et al., 2019).

1.9.1 Good agricultural practice

The first critical point in limiting the incidence of *Aspergillus* or *Penicillium* isolates, and their mycotoxins contamination is to adopt agricultural practices that can create an unfavorable environment for the proliferation of fungal spores present in the soil. These practices include ploughing the soil before sowing, weeding, respecting the specific sowing time for each type of crop and the optimal harvest time, manuring, soil amendment and fertilization, irrigation management, and crop rotation with crops that are less susceptible to the growth of *Aspergillus* spp. (Torres et al., 2014).

Mycotoxin contamination may be minimized using certain cultural practices, curing, drying methods, and storage procedures. Nonetheless, these

techniques may be incompatible with small-scale agriculture in emerging nations, particularly tropical regions (Liang et al., 2006). Thus, developing mycotoxin-resistant varieties is a multi-step process that may include direct selection for resistance to FG and aflatoxin formation, indirect selection for resistance or tolerance to biotic factors or environmental stresses, or selection for morphological characteristics that inhibit or delay fungal introduction or growth (Torres et al., 2014). Due to a scarcity of resistance genes, the creation of cultivars that are resistant to preharvest mycotoxin contamination has been restricted. Numerous efforts have been made to produce mycotoxin-resistant cultivars, resulting in the creation of select resistant types that were gradually released as improved germplasm in several regions of the world. However, total resistance to mycotoxin contamination has not been reached, and genetic attempts continue (Upadhyaya et al., 2002).

Similarly, mechanical damage should be avoided since it increases the grain's susceptibility to fungal infection and insect infestation. For instance, it is preferred to harvest maize cobs with their leaves intact and avoid damaging the leaves since they are critical in protecting the cobs from insects, particularly weevils like *Sitophilus zeamais,* which are the most frequent pest of maize crops. Not only can these insects increase the surface area of the ear that is vulnerable to fungal infection, but they may also wet the grain via their metabolic activity, promoting fungus growth(Manu et al., 2019). Mechanical damage caused by grinding should also be minimized since it facilitates insect penetration. Even if the climatic circumstances are not optimal, cereal lesions caused by insect infestations may result in mycotoxin contamination of grains (Omotayo et al., 2019). The fungus may then grow inside, where they are isolated from environmental conditions and in direct contact with nutrients, generating a micro atmosphere (Ndemera et al., 2020).

Harvesting at the optimal time is also critical in avoiding the growth of fungus. Harvesting should occur shortly after physiological maturity to minimize mycotoxin contamination. Crops harvested at immaturity, on the other hand, must

be dried promptly and effectively to achieve moisture levels that are no longer conducive to mold growth (10-13% for cereals), thus avoiding any mold development throughout the storage period (Neme & Mohammed, 2017).

Climate conditions in many developing nations, which often combine inadequate early drying with excessive air humidity, have a significant role in crop contamination and the frequently high mycotoxin levels observed in agricultural raw materials. Additionally, although a longer drying time in the field exposes the grain might reduce the moisture, this technique increases the time exposure to insect attack, resulting in increased losses during storage (D. Kumar & Kalita, 2017). Therefore, unfortunately, implementing these storage and drying practices is often difficult for farmers with small parcels of land, especially when general climatic conditions are unfavorable (tropical and subtropical regions). To make matters worse, in general, the effect of crop rotation and most agricultural practices on toxicity is more limited than the impact of environmental factors (temperature and humidity).

1.9.2 Chemical approaches

Synthetic antifungal substances are chemical compounds that are designed to prevent the growth and spread of fungi. They are commonly used in the treatment of food products to prevent spoilage and improve shelf life (Leyva Salas et al., 2017). While synthetic antifungal substances have several advantages, they also have some disadvantages that must be considered (Leyva Salas et al., 2017).

One of the main advantages of fungicides is their effectiveness. They are designed to be highly potent and can provide long-lasting protection against a wide range of fungi. Synthetic fungicides are not only effective in combating food contamination, but they are also relatively inexpensive and easy to use, making them a popular choice among food producers. Consequently, their use has become a widespread practice (Ons et al., 2020). Europe is the continent that makes the most

use of fungicides for this purpose between 1990-2020, as demonstrated in **Figure 1.5.**

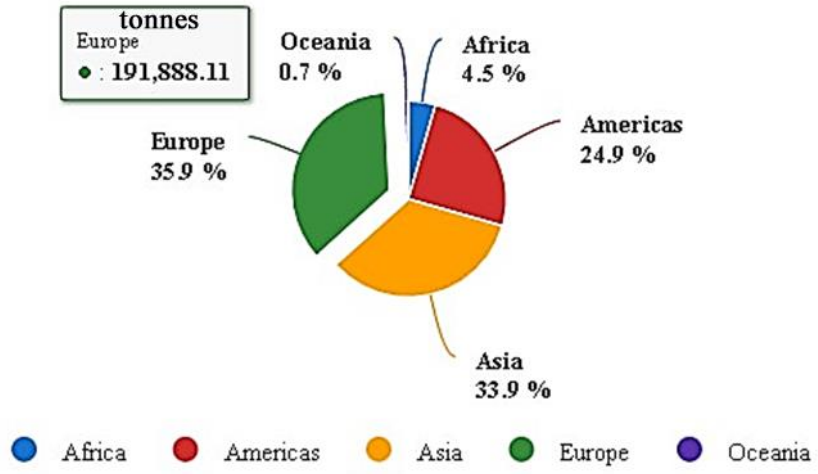


Figure 1.5. Total use of fungicides and bactericides in continents between 1990-2020 (FAOSTAT 2022).

In addition, fungicides are often more convenient than natural alternatives. They can be manufactured in large quantities and can be easily transported and stored without the risk of spoilage. This makes them an ideal choice for food producers who need to treat large quantities of food quickly and efficiently since biopesticides depend on availability of plant sources (Lahlali et al., 2022).

Despite their many advantages, synthetic antifungal substances also have some disadvantages. This strategy appears to be reaching its limits for several factors: environmental contamination and negative impacts on animal and plant biodiversity; loss of effectiveness due to the emergence of resistance in microbial population; and lastly, the inevitable toxicity of these substances in animals after chronic exposure. One of the main concerns is their potential impact on human health and the environment. Some synthetic antifungal substances have been shown toxic

effects and there is a risk of residues remaining on food products after treatment (Hossain et al., 2022).

The world's population of 7 billion people is predicted to expand by 70 million people every year until it reaches 9.2 billion in 2050, a 30% increase. This increased population density is predicted to raise food production demand by 70%, mainly owing to shifts in dietary choices in emerging nations toward higher-quality foods, such as greater intake of meat and dairy products and increased use of grains for animal feed. Additional agricultural land is scarce. Any development will mainly damage forests and natural ecosystems, which are natural enemies of wild animals, crops, and crop pests. Even more than that, rather than food, more agricultural land will be used to create bio-based raw resources such as biofuels or fibers (Carvalho, 2017; Popp et al., 2013).

Consequently, we will need to increase food production efficiency by using less land, water, energy, fertilizers, and pesticides. This is particularly important considering the limitations we face. To ensure sustainable production, it is crucial that we address the challenge of minimizing output losses caused by pests in agricultural production (Popp et al., 2013).

When fungicides are used on plants or products, they can either harm the cell membrane of the fungal pathogen or impede vital cellular processes. In some cases, fungicides can act as preventive measures by creating a protective barrier that immobilizes toxigenic fungi and reduces or prevents their colonization of the plant (Zubrod et al., 2019). However, it is important to exercise caution when using fungicides because their potential impact on human health and the environment, as well as the risk of developing resistant strains of fungi, must be carefully considered (Matumba et al., 2021). Ultimately, it is important to strike a balance between the benefits and risks of using synthetic antifungal substances in food production.

In relation to mycotoxin detoxification, various chemical agents, such as acids, bases, reducing agents, and oxidizing agents, have been employed to convert

mycotoxins into less toxic products through structural modification. Among them, ozone and ammonia are the most studied chemical detoxification treatments (Conte et al., 2020).

Ozone can be applied to disinfect vegetables, fruits, and cereals as well as for mycotoxin detoxification (Botondi et al., 2021). The mechanism of the antifungal property of ozone gas is explained through the damage it does to the fungal membrane, increase in mitochondrial degradation, cytoplasmic disintegration and plasmolysis (Ong & Ali, 2015). Moreover, ozone has been shown to degrade AFs and OTA (Agriopoulou et al., 2016; Torlak, 2019). The oxidizing agents react with the functional groups within the mycotoxin molecules, resulting in a change in their molecular structures that allows for the formation of products that have fewer double bonds, molecular weight, and less toxicity (L. Wang et al., 2016).

In addition to ozone, other bases such as ammonia have also been used to reduce several mycotoxins, including FUM, AFs, and OTs, to non-detectable levels (Y. Luo et al., 2018). However, the application of certain bases such as potassium hydroxide and sodium hydroxide may result in unwanted and toxic reactions. Seed treatment using ammonia has also been found to suppress the growth of mycotoxigenic fungi (Jalili et al., 2011).

1.9.3 Detoxifying by physical methods

Physical methods have been used traditionally for decontamination of mycotoxins in food and feed if prevention measures were not successful. These methods involve several procedures such as dehulling, heating, plasma, sorting and separation, radiation, immersing and washing, and adsorption. The effectiveness of these techniques depends on the degree of contamination and the distribution of mycotoxins within the product. Nevertheless, these methods can result in uncertain outcomes and may lead to significant product losses (Y. Luo et al., 2018).

Sorting, dehulling or washing is usually applied before processing method. They are commonly used method to remove low-quality particles from food and

preserve its quality. Cereal grains, for example, can be sorted based on various physical characteristics like density, color, shape, and size, along with identifying broken grains with FG. Since mycotoxin contamination is unevenly distributed among grains, sorting, washing or separating damaged food can significantly decrease the level of contamination (Matumba et al., 2015). Immersing and washing contaminated grains in water and discarding the floating fractions can generally remove some amount of AFs and FUM. Additionally, as reported by Milani and Heidari (2017), cleaning and scouring procedures can significantly reduce ochratoxin contamination grains.

Studies have revealed that ionizing radiation, such as gamma radiation, electron beams, or X-rays, can be a safe and effective alternative to chemical treatments for eliminating microorganisms from food and feed or reducing mycotoxin levels (Sebaei et al., 2022). This technology is known as food irradiation, which involves a physical-cold process used in the food industry in many countries. In particular, Khalil et al. (2021) have also indicated that gamma radiation can effectively reduce the growth of *A. flavus* and *A. ochraceus*, and can reasonably decrease AFs and OTA levels by 33.3-61.1% depending on the mycotoxin.

UV radiation effectiveness depends on different conditions such as time and wavelength exposure. In some cases, UV can stimulate sporulation and FG, but a shorter wavelength incidence has the opposite effect in biological organisms. García-Cela et al. (2015) demonstrated that UV-B and UV-A were effective against *A. carbonarius* and *A. parasiticus*, and consequently, reduced the OTA and AFs production in a time-dependent manner.

The use of chemical methods for mycotoxin reduction can have negative consequences, such as altering the nutritional value and palatability or leaving toxic residues. Biological methods, on the other hand, can be limited by factors such as long degradation time or incomplete degradation. As a result, adsorption has emerged as a promising option for the treatment of mycotoxin (Yan Li et al., 2018).

Adsorption involves both chemical and physical forces and is thus the most widely applied method to protect animals against mycotoxins. By using a variety of adsorbents, including clay, activated charcoal, and other modified polymers, mycotoxins can be effectively bound and immobilized, reducing their toxic impact by avoiding their absorption from gastrointestinal tract (Čolović et al., 2019). However, it can be challenging to choose efficient adsorbents since various mycotoxins may occur simultaneously in foods, and their toxic effects may be amplified due to synergistic interactions (Yan Li et al., 2018). Some chemicals form weak interactions with mycotoxins due to their polarity, solubility, molecular size, shape, and surface area, causing adsorption between adsorbents and mycotoxins (Daković et al., 2005).

Despite its effectiveness, the safety of adsorbent materials, removal from feed, and disposal of adsorption chemicals and adsorbent-mycotoxin complexes remain a concern, and some chemical adsorbents have been forbidden as detoxification materials in the food industry by the EU (Azam et al., 2021)

1.9.4 Biocontrol of toxigenic fungi and biodegradation of mycotoxins

In the literature, there have been several reports of conventional physical, chemical and adsorption-related technologies for the elimination or inactivation of mycotoxins (Stoev, 2013). Unfortunately, these approaches have certain drawbacks in terms of safety concerns, nutritional value losses, and palatability losses, in addition to their limited effectiveness and cost implications. Using mycotoxin-adsorbing compounds to bind mycotoxins in the gastrointestinal tract of animals and subsequently reduce their bioavailability and toxicities has shown significant promise in recent years, particularly in feed industry applications, according to current research. However, there are many different types of adsorption agents, and their effectiveness in avoiding mycotoxicosis differs from one another (Zain, 2011). For instance, adsorption agents are pretty beneficial in preventing aflatoxicosis, but they are not as successful in preventing other mycotoxins (Stoev, 2013). As a result,

there is a high need for decontamination technologies that are efficient, practical, and ecologically friendly.

To overcome the challenges previously mentioned, biological control techniques have been developed with the aim of managing foodborne pathogens more efficiently and quickly. Biocontrol is characterized by the control of pathogenic microorganisms or their derivatives through natural sources such as microorganisms, plant-derived fungicides, and detoxifying enzymes. Due to its ease of application and cost-effectiveness, biocontrol has emerged as a promising method for mitigating fungal spoilage at various stages of food production, leading to the recognition of biological management as an environmentally friendly alternative to synthetic compounds (Tian et al., 2016).

Currently, bio-protective crops, ferments, and purified molecules with antifungal activity are being developed. In recent years, numerous microbial strains with potential antifungal properties have been isolated from various sources such as cereals, fruits, vegetables, and meat. Research has also shown an increase in food shelf life and a decrease in fungal contamination, particularly from *Aspergillus* and *Penicillium*, as reported by Salas et al. (2017). LAB are the microorganisms most commonly employed for the bio-preservation of food, especially in dairy, bakery, meat, and fermented vegetable products, as well as grains, seeds, fruits, and alcoholic beverages like beer and wine. Furthermore, *Trichoderma* spp. is the preeminent fungal genus employed in plant biocontrol, whereby it provides beneficial effects such as growth promotion and induction of defenses against biotic and abiotic stresses (Adnan et al., 2019). Additionally, microorganisms such as *Debaryomyces hansenii* yeast and *Penicillium* spp. fungi are used to combat the decay of meat products fermented by fungi (Delgado et al., 2019; Núñez et al., 2015).

Alternatively, biodetoxification techniques offer a viable solution for managing mycotoxins. This method involves using microorganisms or enzymes to break down mycotoxins into non- or less harmful compounds (Taylor & Draughon,

2001). To achieve this, live or dead microorganisms may be used to bind the toxins to their cell wall components or decompose them into less harmful substances (S. Kim et al., 2017). However, if mycotoxins are only adsorbed and not completely degraded, there is a risk of their delayed release in gastrointestinal tract (Schaarschmidt & Fauhl-Hassek, 2021).

Nowadays, numerous studies have identified fungal and bacterial strains that can effectively break down mycotoxins. Moreover, these microorganisms are readily available from diverse types of samples, such as food, soil, and feces. Nonetheless, the addition of living microorganisms to food may raise worries about food quality, and consumers may be reluctant to eat meals enhanced with microorganisms. Hence, it is imperative that the microorganisms employed in food and feed as additives fulfill certain criteria such as being safe for application, nonpathogenic, capable of producing stable and non-toxic metabolites, proficient in mycotoxin degradation, capable of forming irreversible complexes, active during storage, not generating unpleasant odors or tastes, retaining nutritional value, and requiring minimal cultivation and production efforts. Consequently, various microorganisms have been suggested as potential detoxifiers for food and feed purposes. However, only a few have undergone thorough testing to determine their actual efficacy in food (J Varga & Tóth, 2005). Perhaps, the use of mycotoxin-degrading enzymes produced by bacteria and fungi might overcome these constraints (Loi et al., 2017).

1.9.5 Lactic acid bacteria as a potential biocontrol agent

LAB is a group of bacteria that are Gram-positive and can tolerate oxygen, but do not form spores or respire. They play a crucial role in fermenting a variety of foods and drinks. These bacteria are distinguished by their capability to utilize carbohydrates during fermentation to produce lactic acid, which is one of the primary products of fermentation (Papadimitriou et al., 2016). In addition to lactic acid, LAB also produce a range of organic compounds that contribute to the sensory qualities

of the fermented product, such as flavor, texture, and aroma, which are unique to each product.

The core group of the LAB consists of four genera: *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus*. However, recent changes to the taxonomy of this group have added several new genera, including *Aerococcus*, *Alloiococcus*, *Carnobacterium*, *Dolosigranulum*, *Enterococcus*, *Globicatella*, *Lactococcus*, *Oenococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella* (Khalisanni Khalid, 2011).

LAB can also be classified into different genera/species based on their acid production characteristics by fermenting sugars and their growth at specific temperatures. They can also be classified as homofermentative or heterofermentative organisms based on their ability to ferment carbohydrates. The homofermentative lactic acid bacteria, such as *Lactococcus* and *Streptococcus*, yield two molecules of lactates from one glucose molecule, whereas heterofermentative lactic acid bacteria, such as *Leuconostoc*, *Wiessella*, and some lactobacilli, generate lactate, ethanol, and carbon dioxide from one molecule of glucose.

In recent years, the classification of lactic acid bacteria has undergone a major revision due to the diversity of the original genus *Lactobacillus*, which made it challenging to classify, name, and distinguish between different lactobacilli. As a result, scientists have reclassified the genus into 25 genera, including the original *Lactobacillus*, *Paralactobacillus*, and 23 novel genera. These novel genera include *Amylolactobacillus, Acetilactobacillus, Agrilactobacillus, Apilactobacillus, Bombilactobacillus, Companilactobacillus, Dellaglioa, Fructilactobacillus, Furfurilactobacillus, Holzapfelia, Lacticaseibacillus, Lactiplantibacillus, Lapidilactobacillus, Latilactobacillus, Lentilactobacillus, Levilactobacillus, Ligilactobacillus, Limosilactobacillus, Liquorilactobacillus, Loigolactobacilus, Paucilactobacillus, Schleiferilactobacillus,* and *Secundilactobacillus* (Zheng et al., 2020).

According to the old taxonomic classification, the most extensively studied LAB species for their antifungal action belong to the genera *Lactobacillus*, *Pediococcus*, and *Leuconostoc* (Shi & Maktabdar, 2021).

The use of LAB as a biopreservation strategy was preferred over other organisms proposed since the EU has included these microorganisms in the QPS (Qualified Presumption of Safety) list. Additionally, in the US, LAB are generally recognized as safe (GRAS) by the Food and Drug Administration (FDA) (Russo et al., 2017b).

Generally, the inhibitory activity of LAB is associated with the metabolites synthesized during the fermentation process, although competition for nutrients and space are also considered a mechanism of action of these microorganisms in foods (Siedler et al., 2019). During fermentation, LAB produce a wide range of antifungal metabolites that prevent the mycotoxin production, including organic acids, phenolic acids, volatile acids, CO2, hydrogen peroxide, antimicrobial peptides (AMPs), fatty acids, ethanol, and diacetyl (**Figure 1.6**). These metabolites can act synergistically or additively, making it difficult to determine the exact mechanism of LAB's antifungal action (Nasrollahzadeh et al., 2022).

Organic acids such as lactic, acetic, and propionic acids are known to have antifungal properties, primarily due to their ability to disrupt the fungal cell's proton gradient, which is necessary for many cellular processes. Lactic acid, for example, can diffuse across the cell membrane in a hydrophobic form and decompose in the cell, releasing H+ ions and causing the cytoplasm to become acidic. Acetic and propionic acids also have antifungal properties, but their effectiveness depends on the low pH induced by lactic acid. Additionally, these acids can inhibit the absorption of amino acids by fungal cells (H. Chen et al., 2021).

Antifungal peptides (AFPs) are small, cationic peptides produced by LAB that can disrupt the fungal membrane or interfere with the proton gradient on the cell membrane. Lactoferricin B, for example, has been shown to interact with the fungal surface and disrupt the fungal membrane, making it an effective antifungal (Struyfs et al., 2021).

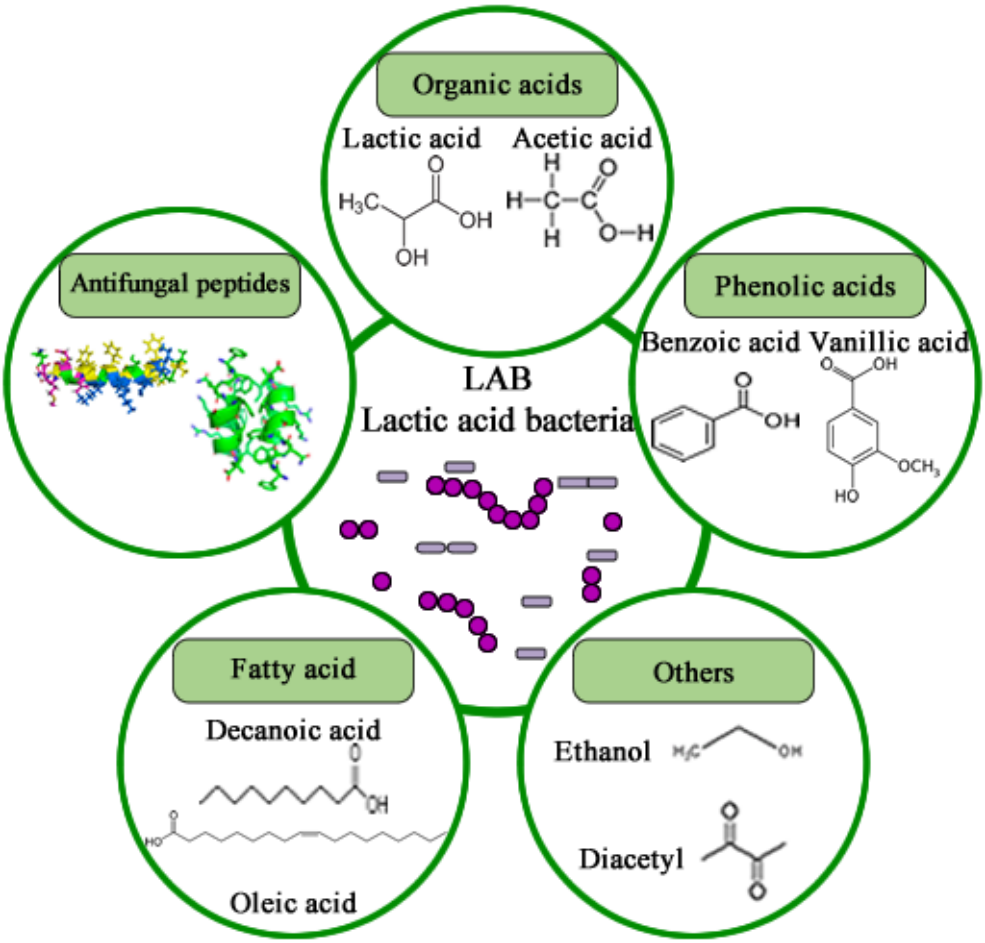


Figure 1.6. Chemical structure of antifungal compounds produced by LAB.

AFPs are a type of AMPs that exhibit broad antifungal activity and are a current research focus for natural biological control agents. AFPs can be obtained from plant, animal, and microbial sources and possess amino acids linked by peptide bond (Bahar & Ren, 2013).

Previous studies have reported the identification of various phenolic compounds in foods or media that have been fermented with LAB. These studies have demonstrated that such compounds exhibit various properties, including antioxidant, antifungal, and antitoxigenic activities (Antognoni et al., 2019; Guimarães et al., 2018). The primary phenolic acids produced by the LAB family are Phenyllactic acid (PLA) and its derivative, 4-hydroxyphenylactic acid. PLA has been identified as a key metabolite that contributes to the antifungal activity of fermented media by LAB, thereby extending the shelf life of food (Guimarães et al., 2018; Mishra et al., 2022). Other phenolic acids produced by LAB during the fermentation include succinic acid, 4-hydroxybenzoic acid, vanillic acid, caffeic acid, p-coumaric acid, salicylic acid, ferulic acid, and benzoic acid (Salman et al., 2022).

1.9.5.1 Detoxification of mycotoxin by LAB

The process of mycotoxin detoxification in foods by LAB involves three potential mechanisms. These include LAB enzyme degradation, adsorption by LAB cells, and interaction between mycotoxins and LAB metabolites (**Figure 1.7**). The crucial role in this detoxification process is played by the proteolytic enzymes of LAB (Sheikh-Zeinoddin & Khalesi, 2019). Additionally, the cell wall of certain LAB strains has been suggested to adsorb mycotoxins in specific foods, which is linked to the presence of polysaccharides, protein, and peptidoglycans in the cell wall (Muhialdin et al., 2020). The exact mechanisms of mycotoxin removal and degradation by LAB cells and metabolites remain unclear, with several mechanisms being proposed, including degradation via proteolytic enzymes and the binding of specific metabolites to mycotoxins (Muhialdin et al., 2020).

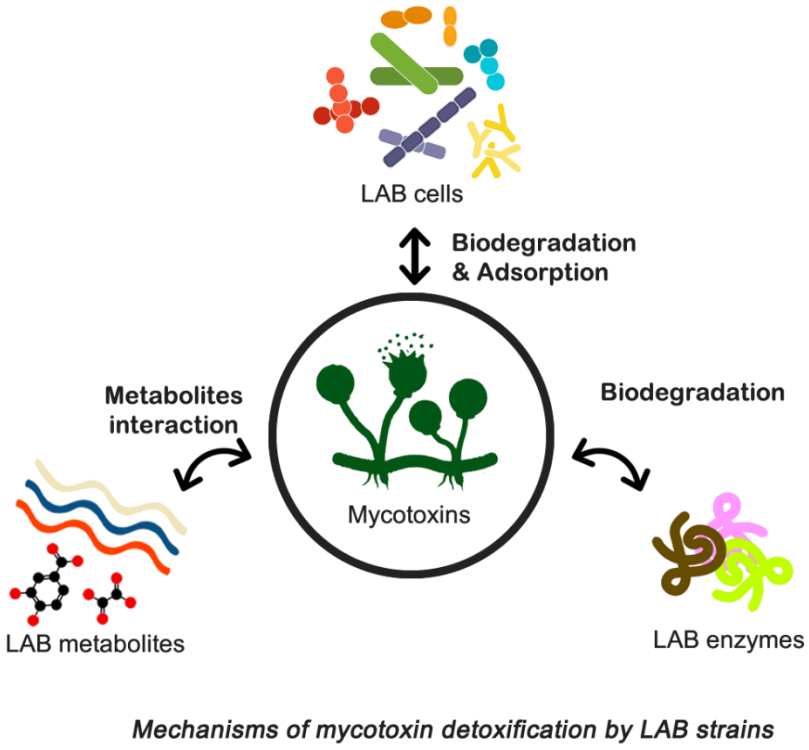


Figure 1.7. Three potential mechanisms of mycotoxin detoxification in foods by lactic acid bacteria (LAB).

1.9.6 Essential oils

Currently, there is a trend towards the use of natural compounds to ensure food safety, which has led to research on natural fungicides for the control of postharvest fungal disorders in agricultural products. These natural phytosanitares can be obtained from different sources, including microorganisms (Ribes et al., 2018).

Zubrod et al. (2019) have described that fungicides derived from plant-based products exhibit reduced environmental impact and lower risk to human health compared to agrochemicals, as they degrade more rapidly. Consequently, such

fungicides ensure the safety of the environment and consumers alike. One strategy involving plant-origin compounds involves the utilization of bioactive secondary metabolites, including phenols, terpenes, aliphatic alcohols, aldehydes, ketones, and EOs, which have been extensively investigated. EOs, comprising complex mixtures of volatile and lipophilic substances primarily obtained from plants via steam distillation (Santos et al., 2013), consist of diverse molecules such as terpenes and hydrocarbons, and exhibit considerable *in vitro* antimicrobial potential. However, due to the substantial doses required to observe antimicrobial effects and the potential flavor impact on food, the use of EOs is somewhat limited (Sharma et al., 2021).

Over the past decade, oregano, cinnamon, thyme, rosemary, fennel, clove, and eucalyptus have been the most used EOs to combat mycotoxigenic fungi and their mycotoxins. The inhibitory effects of EOs on FG and mycotoxin synthesis have been demonstrated through various pathways, such as the modification of FG rate and extension of the lag phase, disruption of cell permeability, and manipulation of gene expression patterns and metabolic processes associated with the electron transport chain (Salman et al., 2022).

1.9.6.1 Mustards and Allyl Isothiocyanate as biocontrol agent

Mustard is an herbaceous plant belonging to the *Cruciferae* or *Brassicaceae* family. The varieties that exist are white or yellow mustard (*Brassica hirta* or *Sinapis alba*), black or royal mustard (*Brassica nigra*) and oriental or brown mustard (*Brassica juncea*), which have a high potential against FG (Lietzow, 2021; Torrijos et al., 2019).

These plants are characterized by the presence of secondary metabolites known as glucosinolates (GTs), the hydrolysis of which gives off a very characteristic pungent odor (Bell et al., 2021). GTs play an important role in plant defense, so that when plants suffer physical damage they are exposed to fungal alterations, among other possible attacks, so these substances are hydrolyzed by the

enzyme myrosinase, producing isothiocyanates (ITCs) in the presence of H_2O, among other products (Plaszkó et al., 2021).

ITCs possess multiple properties, including biocidal activity (fungicidal, bactericidal, and insecticidal) (Ş. Kurt et al., 2011), and their potential against FG is widely described (Ekanayake et al., 2012). In addition, they exhibit herbicidal, antioxidant and anticarcinogenic activity (Higdon et al., 2007; Sánchez-Pujante et al., 2017).

In the case of oriental mustard, hydrolysis of glucosinolate sinigrin by myrosinase gives rise to allyl isothiocyanate (AITC). In yellow mustard, hydrolysis of glucosinolate sinalbin by myrosinase generates p-hydroxybenzylisothiocyanate (p-HBIT). Both ITCs were evaluated, demonstrating their fungal inhibitory capacity, both in the case of AITC (Yingbin Li et al., 2020) and p-HBIT (Meca et al., 2012).

Mustard also contains other bioactive compounds, such as phenolic acids, which are characterized by their high antioxidant activity and are used by plants for their growth and resistance to pests and pathogens, among other functions (Cartea et al., 2010).

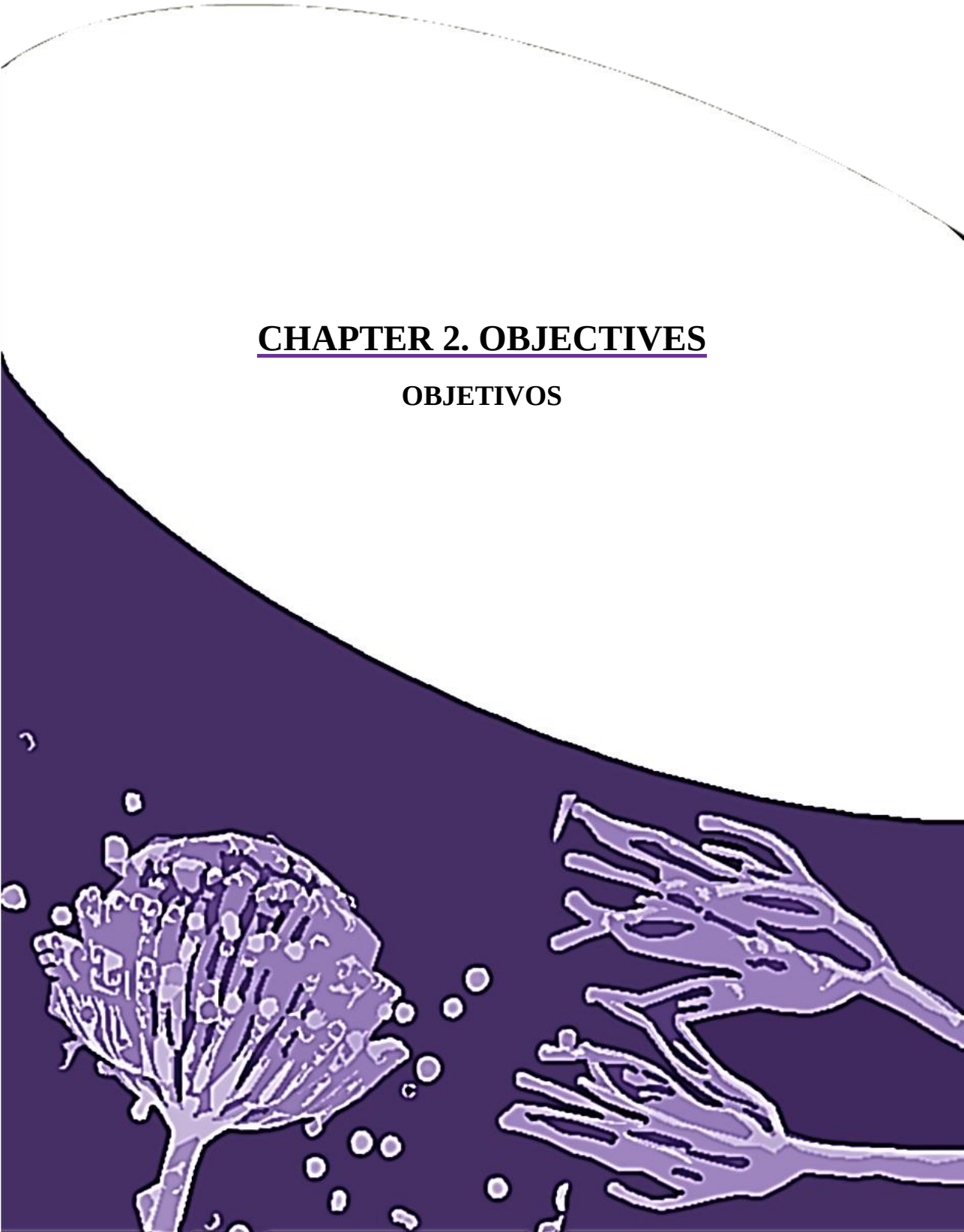
AITC is a volatile compound that is related to several beneficial effects on human health including antiangiogenic, anti-inflammatory, neuroprotective, and anticarcinogenic potential (Bhattacharya et al., 2013). AITC is the most studied and potent antimicrobial among ITCs, due to its antimicrobial action at lower doses (**Table 1.8**) (Saladino et al., 2017a). In this context, some authors have demonstrated the ability of AITC to volatilize and inhibit the growth of mycotoxigenic entities, such as *F. graminearum*, *A. parasiticus*, *P. expansum*, and *F. poae*, demonstrating a dose dependent effect and with mitigating effects on mycotoxin production starting at 10 μL/L in gas phase (Azaiez et al., 2013; Manyes et al., 2015; Nazareth et al., 2016). In addition, AITC can react directly with mycotoxins forming new compounds and reducing their presence in food solutions and matrices (Bordin et al., 2017).

Table 1.8. Recent reports about the use of AITC Antifungal to prevent the fungal grwoth and extend the shelf life of foods.

Food or primary commodity	Application mode	Dosage (µL/L)	Fungi evaluated	Reference
Barley	Hydroxyethyl-cellulose gel disk	50	*Penicillium verrucosum*	Quiles et al. (2019)
Wheat	Hydroxyethyl-cellulose gel disk	50	*P. verrucosum*	Quiles et al. (2019)
Corn	Hydroxyethyl-cellulose gel disk	50	*Aspergillus flavus*	Quiles et al. (2019)
Blackberry	12h exposition to the compound in paper towel	0.5, 1, 2, 5, and 10	Natural fungal growth.	Park et al.(2023)
Maize	Paper filter containing AITC during storage (30d)	0.125, 0.25, 0.5, 1, and 5	*A. flavus*	Nazareth et al. (2020)
Chicken breast	Multilayer carrageenan/chitosan coatingn containg AITC, applied by immersion. Storage during 21d	20 and 200.	Natural fungal growth	Moller et al. (2023)
Maize	Paper filter containing AITC during storage (30d).	30 and 300.	*A. parasiticus*	Evangelista et al. (2021)
Maize	Paper filter containing AITC during storage (30d)	30 and 300	*Fusarium verticillioides*	Evangelista et al. (2021)
Maize	Paper filter containing AITC during storage (30d)	30 and 300	*F. graminearum*	Evangelista et al. (2021)
Grape	Injection of gaseous phase AITC in the firstday (14d of total storage)	25 µg/mL	*A. niger*	Yang et al. (2021)
Grape	Injection of gaseous phase AITC in the first day of storage (14d of total storage).	25 µg/mL	*A. carbonarius*	Yang et al. (2021)
Grape	Injection of gaseous phase AITC in the first day of storage (14d of total storage).	25 µg/mL	*A. ochraceus*	Yang et al. (2021)
Maize	Paper filter containing AITC during storage (15d).	25 µg/mL	*A. niger*	Yang et al. (2021)
Maize	Paper filter containing AITC during storage (15d).	25 µg/mL.	*A. carbonarius*	Yang et al. (2021)
Maize	Paper filter containing AITC during storage (15d).	25 µg/mL	*A. ochraceus*	Yang et al., 2021
Barley	Paper filter containing AITC during storage (90d)	50	*P. verrucosum*	De Melo Nazareth et al. (2019)○
Almonds	Hydroxyethyl-cellulose antifungal device and a paper filter containing AITC during storage (15d).	5.07, 10.13, and 20.26 mg/L	*A. flavus*	Nazareth (2022)
Pita bread	Active packaging system containing AITCduring storage (7d)	8, 16, 33 or 50 mg	*P. verrucosum*	Torrijos et al. (2019)
Bread	Filter disk in Petri dishes with bread slices (10d of storage)	1 µL	*Rhizopus stolonifer*	Clemente et al. (2019)

CHAPTER 2. OBJECTIVES

OBJETIVOS



2.1 OBJECTIVES

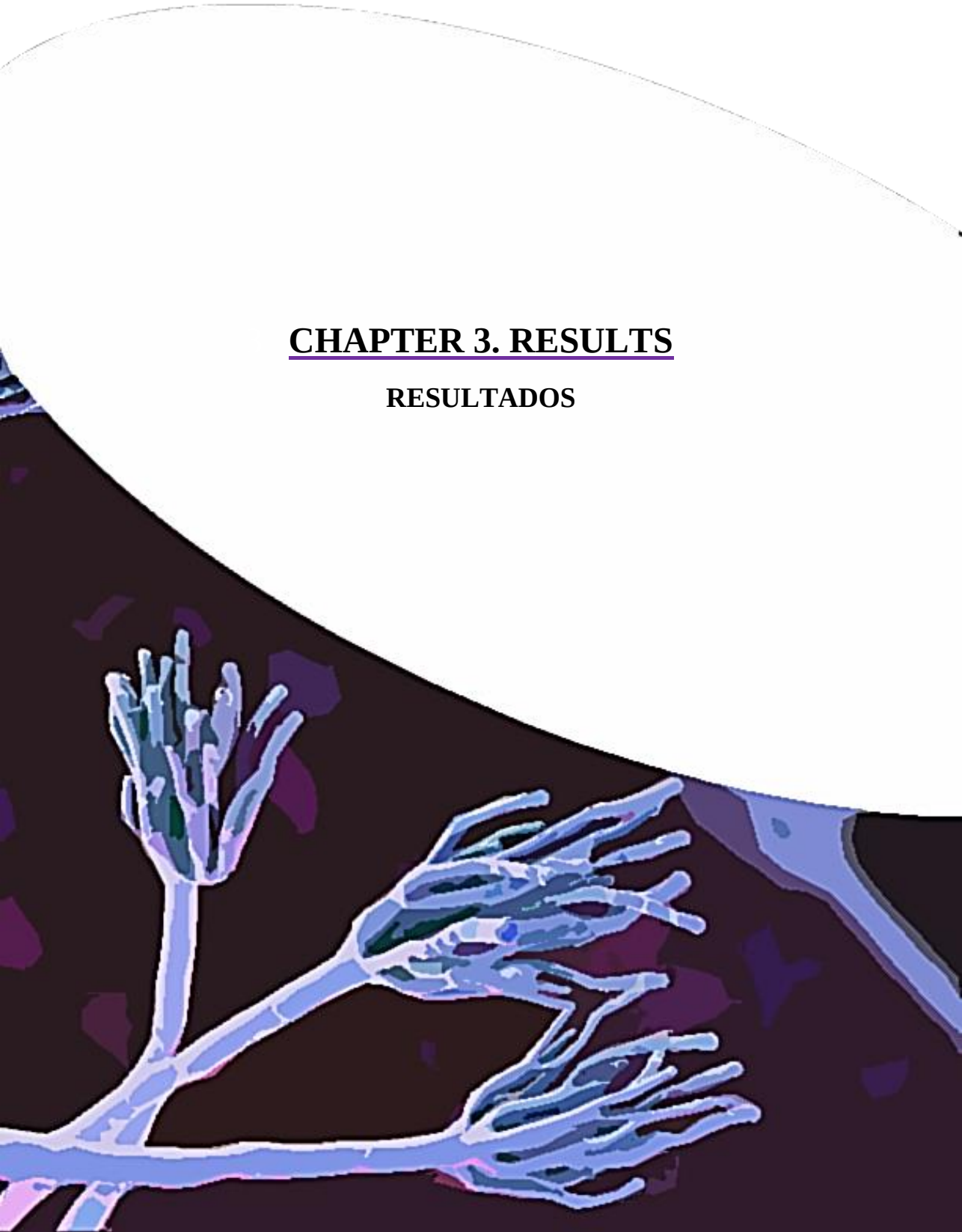
The **general objective** of this study was to investigate the antifungal and antitoxigenic activity of natural compounds, in particular AITC, oriental mustard flour, yellow mustard flour and lactic acid bacteria and evaluate their practical application in foods.

In order to achieve this goal, the following **specific objectives** have been established:

1. Assess the effectiveness of AITC in preventing the growth of *P. verrucosum* and production of OTA on barley stored under different moisture content (MC) over a period of 90 days. Additionally, determine the concentration of AITC remaining in the grains after treatment.
2. Evaluate the *in vitro* antifungal potential of the AITC, and a freeze-dried yellow mustard flour extract (YMF-E). Then, develop an antifungal device based on hydroxyethyl-cellulose and oriental mustard flour (H-OMF) and determine its antifungal effect. Finally, assess the efficacy of all treatments in avoiding the growth of *A. flavus* and preventing the production of AFB1 in almonds.
3. Isolate lactic acid bacteria with antifungal properties from traditional dry-cured sausages and develop an antifungal ingredient with potential application in the manufacture of meat products. Moreover, characterize and identify the metabolites responsible for the antifungal ingredient capacity.

CHAPTER 3. RESULTS

RESULTADOS



3.1 Antifungal and antimycotoxigenic activity of allyl isothiocyanate on barley under different storage conditions

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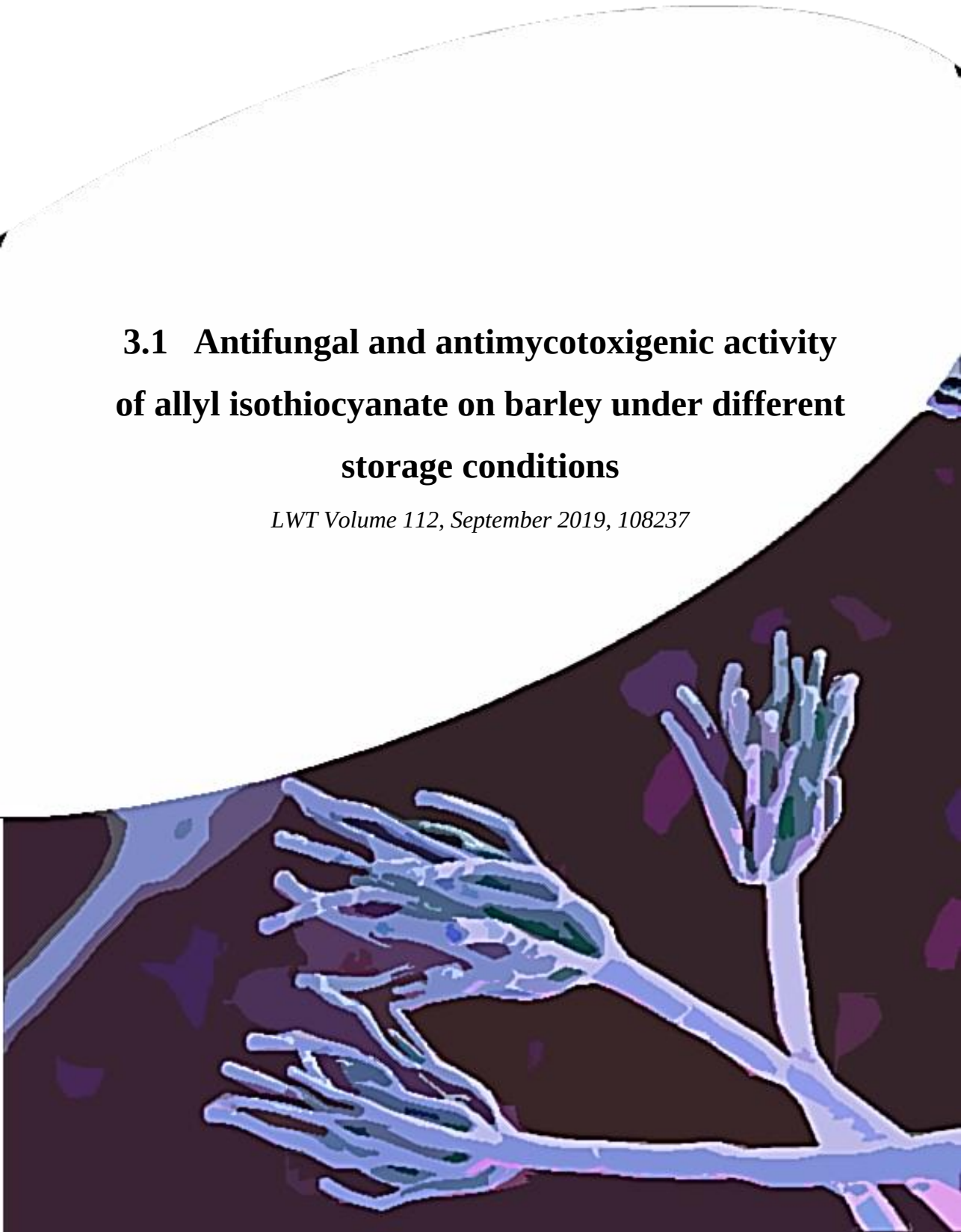


3.1.1 Introduccion

Barley, hops, yeast and water represent the main ingredients in beer production, and their quality is essential to confer the specific organoleptic characteristics of beer and its acceptance by consumers. Actually, beer is the most consumed alcoholic drink in the America and Europe, representing around 34.8% of all alcohol beverages consumed in the world (Hammer et al., 2018).

Barley grains are commonly colonized by toxigenic fungi of genre *Penicillium, Aspergillus and Fusarium.* Some species of fungi, such as *P. verrucosum* is known to produce OTA during the plant growth in the field or grain storage. The beer contamination by mycotoxins is an industrial and public health concern, because the consumption of mycotoxin and its long time exposure can lead to carcinogenic, teratogenic and mutagenic effects in animals as well as humans (Binder, 2007; Zain, 2011). Specifically, OTA is a nephrotoxic, mutagenic and carcinogenic mycotoxin characterized by IARC as a compound belonging to group 2B, a group for possibly carcinogenic compound (Kujawa, 1994).

Numerous studies have been investigated the incidence of mycotoxins in beer and several countries have stipulated a specific regulation for OTA in foodstuffs (Bellver Soto et al., 2014; Bertuzzi et al., 2018; Pascari et al., 2018; Peters et al., 2017). The Commission of the European Community 1881/2006 fixed the OTA limit in unprocessed grains at 3 µg/kg whereas the limit for grains intended for animal feed is 250 µg/kg. On the other hand, the Joint FAO/WHO Expert Committee on Food Additives (ECFA) established a tolerable weekly intake of OTA for humans in 112 ng/kg body weight/day, on the basis of experiments that evaluated the deterioration of renal function in pigs (FAO/WHO, 2008).

Beer production has diverse operations which can impact on final levels of mycotoxins, but these operations cannot totally reduce OTA due its capacity to withstand after thermic treatment (Kabak, 2009). For this reason, it is necessary

identifying the operations able to decrease mycotoxin level and associating with mitigation strategies.

In order to minimize economical losses promoted by OTA, some authors have been proposing different strategies, such as fungicide application in the field or silos, addition of microorganisms during the fermentation process to bind or degrade mycotoxin and the use of hot water treatment (Luz et al., 2018; Mateo et al., 2007; H. Zhang et al., 2016). However, avoiding the fungal contamination is the better way to prevent high mycotoxin levels in final products and their byproducts. As these byproducts may be used in animal feeding, the mitigation process can also reduce the OTA impact in animal production. In addition, specific studies are needed to better understand the risk of OTA for animals and especially, the risk for the general population through the ingestion of products of animal origin containing OTA and/or its metabolites.

Currently, the application of natural antimicrobials in foodstuffs has gained importance due the safe status of these compounds to consumers (Luciano & Holley, 2009). Isothiocyanates (ITCs) are aliphatic and aromatic compounds found in cruciferous vegetables belonging to the families *Brassicaceae*, *Capparaceae* and *Caricaceae.* These compounds are responsible for defense system in plants and they arise from the hydrolysis of GTs by the enzyme myrosinase after tissue damage promoted by phytopathogens (Alvarez et al., 2015; Troncoso-Rojas & Tiznado-Hernández, 2014).

Among the ITC, it has been reported that AITC is a volatile compound that is related to several beneficial effects to human health including antiangiogenic, anti-inflammatory, neuroprotective and anticarcinogenic potential (Bhattacharya et al., 2013). But this compound is mainly known by its inhibitory activity against toxigenic and food spoilage microorganisms including species from the genera *Escherichia*, *Salmonella*, *Clostridium*, *Listeria*, *Aspergillus, Penicillium and Fusarium* (Alanazi et al., 2018; Azaiez et al., 2013; Cardiet et al., 2012; Lopes et al.,

2018b; Meira et al., 2017; Olaimat et al., 2018; Quiles et al., 2015a). Moreover, many studies demonstrate that AITC can be used through fumigation to avoid postharvest fungal spoilage in fruits, cereals and bread (Meca et al., 2012; Nielsen & Rios, 2000; Wang & Chen, 2010). However, currently, there is no data about the influence of AITC treatment in the conservation of barley designated to beer production.

The aims of this work were evaluating the efficacy of AITC treatment (50 µL/L) to avoid the *P. verrucosum* growth and OTA production on barley with different MC levels during 90d. Moreover, the residual concentration of AITC in grains was determined.

3.1.2 Material and methods

3.1.2.1 Chemicals

Methanol for extraction process was purchased from Fisher Scientific (New Hempshire, USA). Deionized water (18 MΩ) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA). Methanol, formic acid (HCOOH), ammonium formate and OTA (98% purity) were purchased from Sigma Aldrich (St. Louis, MO, USA). Potato dextrose agar (PDA) and buffered peptone water were purchased from Oxoid (Basingstoke, UK).

3.1.2.2 Inoculum preparation

The strain of *Pencillium verrucosum* VTT D-01847 was obtained from culture collection of VTT (Research Centre of Finland). The strain was cultured in PDA medium at 25 ° C for 7 d. Then 5 mL of peptone water were added on top of the culture and conidia were scraped to form a suspension. The suspensions were transferred to a falcon tube and adjusted to a concentration of 10^5 conidia/L using a Neubauer chamber. These solutions were used to contaminate the grains.

3.1.2.3 AITC fumigation and antifungal activity in barley grains

The fumigation process was carried out according to Nazareth et al. (2016) with some modifications. Barley grains (10 g) were separated into 50 mm Petri dishes, placed on a rigid carton base and transferred to 1 L jars (**Figure 3.1.1**). Then, samples were contaminated with *P. verrucosum* suspension to obtain a final concentration of 3 x 10^3 spores/g. The jars were stored for 3 d at room temperature to allow the fungi stabilization. In addition, 50 mm Petri dishes containing 10 mL of NaCl, KCl, KNO_3 or K_2SO_4 saline solution were used to generate MC in grain of 13, 16, 19 and 21%, respectively. So, a paper filter containing AITC at 50 µL/L (in relation to jar volume) were added to the jar lids. Jars were hermetically closed for 24 h to allow total volatilization and contact of AITC with the grains contaminated. Finally, jars were opened up by small holes in the lids to allow ventilation. The experiment was realized in duplicates during 90 d and sampling occurred every 30 d.

3.1.2.4 Microbiological analysis

After incubation time, barley samples (10 g) were placed into a stomacher bag containing 90 mL of 0.1 % peptone water and homogenized with a stomacher (IUL, Barcelona, Spain) during 1 min. So, the mixture was serially diluted with 0.1% peptone water. Then, aliquots of 100 µL were plated on acidified PDA (pH 3.5) and plates were incubated for 7 d at 25 ºC prior to counting. Results are expressed in Log CFU/g.

3.1.2.5 OTA extraction

Extraction of OTA was performed with the method described by Hontanaya et al. (2015) with some modifications. First, 5 g of samples were weighed in a falcon tube (50 mL) and 25 mL of methanol were added. Then, the mixture was extracted using the Ultraturrax basic T18 (IKA Works) for 3 min. The extract was centrifuged at 4000 x g for 5 min at 4 °C. Next, the supernatant was evaporated with a rotary evaporator (Büchi Rotavapor R-200) at 40 °C. The dry extract was reconstituted in

5 mL of methanol, transferred to a 15 mL plastic tube, and evaporated using a Turbovap Nitrogen Evaporator (Zymark) at 40 °C under 5 psi pressure. After evaporation, samples were reconstituted in 1 mL of methanol, filtered through a 0.22 μm filter and injected in liquid chromatography coupled to the mass spectrometry detector in tandem (HPLC-MS/MS).

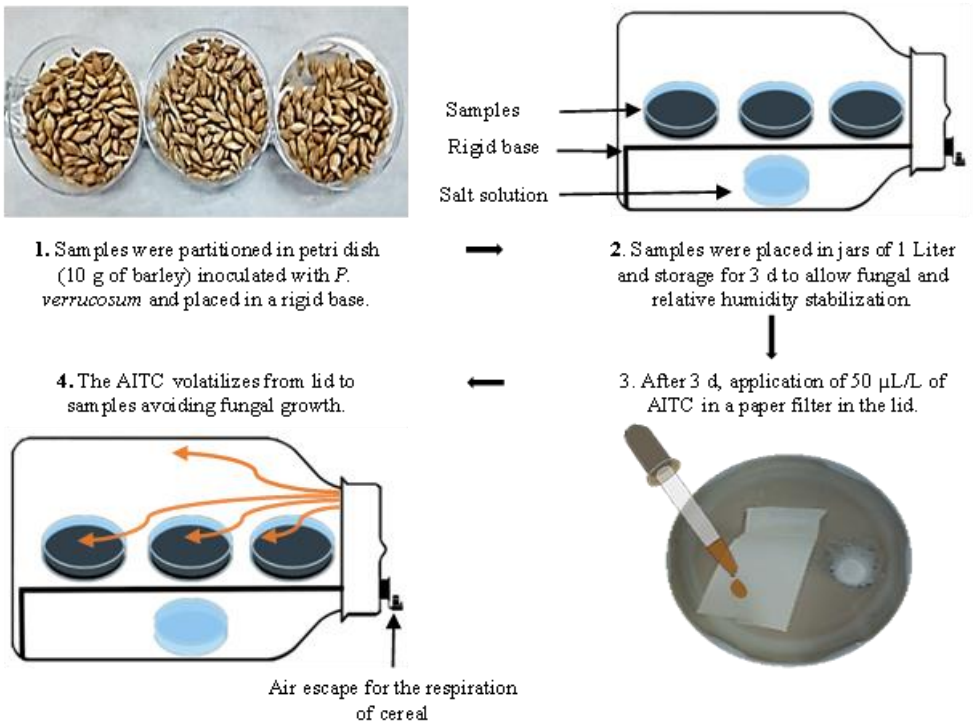


Figure 3.1.1. Assay fumigation of barley grains by Allyl Isothiocyanate (AITC).

3.1.2.6 HPLC-MS/MS analysis

The analytical instrument was an Agilent 1200 liquid-chromatograph (Agilent Technologies, Palo Alto, CA, USA) with a binary system LC-20AD pump and coupled to an A3200QTRAP mass spectrometer (Applied Biosystems, Foster City, CA, USA) containing an ESI interface used in positive mode for detection. The Auto sampler was a SIL-20AC homoeothermic and the Analyst Software 1.5.2 was used for data analysis. The stationary phase was an analytical column Gemini NX

C18 column (150×2.0 mm I.D, 3.0 μm, Phenomenex, Palo Alto, CA) at room temperature (20 °C) and the mobile phase was 5 mM ammonium formate and 0.1% formic acid in water (A) and 5 mM ammonium formate and 0.1% formic acid in methanol (B) at a flow rate of 0.25 μL/min. The gradient mobile phase was 10% of B increasing to 80% (B) in 1.5 min, hold for 2.5 min, then increased to 90% (B) in 10 min, 100% (B) in 14 min and returned to the initial condition. The injection volume was 20 μL with 10 min of equilibration mode. The main MS conditions were ion spray voltage 5500V; capillary temperature 550 ºC; nebulizer gas (GS1) at 55 psi; auxiliary gas (GS2) at 50 psi; curtain gas (CUR) at 15 psi; and nitrogen was used as the nebulizer, heater, curtain and collision gas. Finally, the precursor for ion transitions were m/z 404.3/102.1–404.3/239.0 and 404.3/358.1 for OTA. The validation parameters for detection and quantification are shown in **Table 3.1.1**.

Table 3.1.1. Results of validation method parameters for quantification of Ochratoxin A (OTA) and Allyl Isothiocyanate (AITC).

Analytical instrument	Analyte	LOD*	LOQ*	Concentration*	Recovery (%)	RSD_r (%)
LC-MS/MS	OTA	0.25	0.4	0.4	68.1	4.9
				0.8	79.1	3.6
				1.2	76.1	9.1
GC-FID	AITC	200	700	700	73.0	3.3
				1.4	71.3	12.2
				2.1	71.7	4.3

LOD, limit of detection; LOQ, limit of quantification; RSD_r, Relative standard deviation for recovery; LC-MS/MS, liquid chromatography with tandem mass spectrometry; GC-FID, gas chromatography with flame ionization detector; *μg/Kg. (n=9)

3.1.2.7 Residual AITC extraction and quantification

The extraction was performed as described by Nazareth et al. (2018). Samples of 5g was added to Falcon tubes (15 mL) containing 10 mL of methanol. The mixture was extracted for 30 min in a water bath at 40 °C. Subsequently, the

tubes were placed in an ultrasonic bath for 10 minutes, followed by centrifugation at 4000 x g for 5 minutes at 20 °C. The supernatant was recovered and filtered through a nylon membrane filter (0.22 μm). Finally, an aliquot of 10 μL was injected in a gas chromatograph (GC) coupled with flame ionization detector (FID) (GC 6890, Agilent Technologies Inc., Santa Clara, Calif., U.S.A.).

The stationary phase was a 30 mm× 0.25 mm CP-SIL 88 fused capillary column (Varian, Middelburg, Netherlands). The initial temperature was 200 °C, whereas the detector temperature was 250 °C. The H_2 at 5 mL/min was used as carrier gas. Ionization gasses was carried out with H_2 40 mL/min and purified air at 450 mL/min. The gradient of temperature was set as follow: 60 °C was kept for 1 min, increasing in 8 °C per min up to 100 °C and kept for 5 min, then increasing in 15 °C per min up to 200 °C. The total time of chromatography was 13.2 min per sample. In addition, the quantification of AITC was performed comparing the samples areas with the standards areas from the points standards curve (0.1-10 mg/L).

3.1.2.8 Evaluation of barley moisture

MC quantification was performed submitting the barley grains samples to the same condition described previously. However, the samples were not contaminated with *P. verrucosum*.

Grains (10 g) was weighed in mortars and placed into incubator at 105 °C for 24 h. Then, samples were cooled with a desiccator to room temperature. Samples were weighed and the MC on barley grains was determined considering the weight loss. The MC test was carried out after 0, 10, 30, 60 and 90 d in triplicates.

3.1.2.9 Statistical analysis

The software prism version 3.0 (GraphPad, La Jolla, CA, USA) for Windows was used for the statistical analysis of data. The experiments were realized in triplicate and the differences among groups were analyzed by one-way-ANOVA

followed by Tukey post-hoc test for multiple comparisons. The level of significance considered was $p \leq 0.05$.

3.1.3 Results and Discussion

3.1.3.1 Antifungal activity of AITC in barley grains

Recently, the search for new substances from natural sources has gained importance worldwide as an alternative to synthetic fungicides. Among these natural compounds AITC has been evaluated for its antifungal potential. Besides, this compound is recognized as GRAS being permitted as a food preservative in US.

For the first time, the antifungal activity of AITC was evaluated against *P. verrucosum* employing a silo simulator assay with barley grain storage under different MC conditions.

The influence of the MC as well as the antifungal activity of AITC on *P. verrucosum* population are shown in **Table 3.1.2**. The MC showed a proportional effect on fungal population (FP), when higher was the MC on grain higher was the FP. In the control group, the MC of 16, 19 and 21% increased the population in 2.1, 4.1 and 4.8 Log CFU/g, respectively. However, 13% of MC could not provide an ideal condition of growth and the population increased only by 0.2 Log CFU/g after 90 d of storage. These results demonstrate that the MC must be monitored to avoid the fungal development, since the uncontrolled MC can lead to ideal conditions for FG.

After 24 h of experiment, the control samples showed a population of 3.4, 3.4, 3.2 and 3.3 Log CFU/g for 13, 16, 19 and 21% MC, respectively. However, the fumigation with AITC at 50 µL/L could significantly reduce the FP by 1.5 Log CFU/g for 13% and 21% MC and reduce the FP below the limit of quantification (1.2 Log CFU/g) for 16 and 19 % MC at day 1.

Table 3.1.2. Efficacy of gaseous Allyl Isothiocyanate against *Penicillium verrucosum* growth in barley stored for 90 d.

Population of *Penicillium verrucosum* in log CFU/g (Mean ± SD)				
	Days			
	1	30	60	90
Control				
NaCl (13%)	3.4 ± 0.0^{A}	$<1.2 \pm 0.0^{A}$	3.4 ± 0.0^{A}	3.6 ± 0.2^{A}
KCl (16%)	3.4 ± 0.1^{A}	2.5 ± 0.2^{B}	5.1 ± 0.1^{B}	5.5 ± 0.1^{B}
KNO_3 (19%)	3.2 ± 0.1^{A}	5.3 ± 0.1^{C}	7.8 ± 0.0^{C}	7.3 ± 0.3^{C}
K_2SO_4 (21%)	3.3 ± 0.1^{A}	6.2 ± 0.1^{D}	8.3 ± 0.1^{D}	8.1 ± 0.1^{D}
50 µL/L				
NaCl (13%)	1.9 ± 0.2^{B}	$<1.2 \pm 0.0^{A}$	$<1.2 \pm 0.0^{E}$	$<1.2 \pm 0.0^{E}$
KCl (16%)	$<1.2 \pm 0.0^{C}$	$<1.2 \pm 0.0^{A}$	$<1.2 \pm 0.0^{E}$	$<1.2 \pm 0.0^{E}$
KNO_3 (19%)	$<1.2 \pm 0.0^{C}$	$<1.2 \pm 0.0^{A}$	$<1.2 \pm 0.0^{E}$	$<1.2 \pm 0.0^{E}$
K_2SO_4 (21%)	1.8 ± 0.1^{B}	$<1.2 \pm 0.0^{A}$	$<1.2 \pm 0.0^{E}$	$<1.2 \pm 0.0^{E}$

Barley grains were contaminated with *P. verrucosum* at 10^3 conidias/g and stored by 3 d, then, the grains were treated with Allyl isothiocyanate for 24 h and the fungal population was evaluated. Different letters represents statistical differences among treatments ($p \leq 0.05$). (n=6).

After 90 d of storage, a significant difference could be observed in the control group among the different levels of MC tested. The populations in this group reached 3.6, 5.5, 7.3 and 8.1 Log CFU/g for 13, 16, 19 and 21% MC, respectively. On the other hand, the AITC could reduce the FP to levels below the limit of quantification after 30 d and this effect remained for up to 90 d independently of MC condition. These results reinforce the importance of implementing several hurdles to improve food safety, such as the use of AITC fumigation and MC control to avoid FG.

Saladino (2017b) investigated the growth inhibition by AITC in bread loaf contaminated with *A. parasiticus*. The loaf bread slices were treated with AITC at

concentrations of 0.5, 1 or 5 µL/L. The treatment with 5 µL/L increased the shelf life of bread in 4 days, including these treatments led to mycotoxin reduction above 60%. Manyes et al. (2015) demonstrated the antifungal activity of the AITC against *P. expansum*. After 20 d of incubation the *P. expansum* was completely inhibited by AITC at 50 µL/L. Our results corroborate with these previous studies, since AITC have been shown a strong antifungal effect after 5 µL/L of treatment.

3.1.3.2 OTA reduction by AITC

Several authors have demonstrated the ability of AITC to avoid the production of mycotoxins in different food stuffs such as nuts (Hontanaya et al., 2015; Lopes et al., 2018a), bread (Saladino et al., 2016a), pizza (Quiles et al., 2015a) and wheat flour (Nazareth et al., 2016). However, during our research no studies were found demonstrating the AITC's effectiveness in avoiding the OTA production, as well as the application of this compound in barley destined for animal feed.

The production of OTA in barley grains inoculated with *P. verrucosum* and treated with AITC are shown in **Table 3.1.3**. The grains were stored under different MC conditions for 90 d.

As results, OTA was only quantified in the control group, so the treatment was able to inhibit the mycotoxin production at undetectable levels. OTA was found in the control group after 30 d for MC of 16, 19 and 21% and at day 90 for the control group with 13% of MC. Although the control group with 13% MC grew only 0.2 log during the experiment, this MC did not inhibit mycotoxin production, reaching values of 0.5 µg/Kg. Therefore, this value is below the limit stipulated by the European Community (EC 1881/2006), and these grains could be used as human unprocessed food and animal feed.

Table 3.1.3. Effect of allyl isothiocyanate treatment to avoid ochratoxin A production by *Penicillium verrucosum* on barley stored under different conditions of humidity.

Ocratoxin A production by *Penicillium verrucosum* (MEAN of µg/Kg)				
	Days			
	1	30	60	90
Control				
NaCl (13%)	ND	ND	<LOQ^{A}	0.5^{A}
KCl (16%)	ND	<LOD^{A}	0.5^{B}	0.7^{A}
KNO_3 (19%)	ND	<LOD^{A}	137.2^{C}	246.2^{B}
K_2SO_4 (21%)	ND	82.3^{B}	742.1^{D}	1515.9^{C}
50 µL/L				
NaCl (13%)	ND	ND	ND	ND
KCl (16%)	ND	ND	ND	ND
KNO_3 (19%)	ND	ND	ND	ND
K_2SO_4 (21%)	ND	ND	ND	ND

Different letters represents statistical differences among treatments ($p \leq 0.05$). n=6. ND, not detected; LOD, limit of detection; LOQ, limit of quantification (µg/kg).

The MC exercised direct influence on OTA's concentration. The greater the MC, the higher the OTA concentration found during the analysis. Consequently, after 90 d grains containing MC of 16, 19 and 21% presented OTA concentrations of 0.7, 246.2 and 1515.9 µg/kg, respectively. These MC have provided OTA production to levels above the limits set by European Community 1881/2006 for feed and food. Therefore, MC above 13% must be avoided in barley storage to prevent mycotoxin production, increase the shelf life of grain, and increase its safety.

3.1.3.3 Residual AITC

Table 3.1.4 shows the residual concentration of AITC. The grains were fumigated for 24 h and storage time was 90 d.

Table 3.1.4. Residual concentration of Allyl Isothiocyanate (AITC) in barley after 24 h of treatment and 90 d of stored.

	Concentration of AITC in barley (mg/Kg) (Mean ± SE)			
	Days			
Moisture	1	30	60	90
NaCl (13%)	22.0 ± 1.5^{A}	20.30 ± 0.4^{A}	11.2 ± 0.1^{A}	9.1 ± 2.7^{AB}
KCl (16%)	34.7 ± 8.1^{A}	21.9 ± 0.5^{A}	15.0 ± 1.3^{B}	13.4 ± 1.4^{A}
KNO3 (19%)	25.5 ± 2.7^{A}	11.6 ± 0.3^{B}	7.1 ± 1.1^{C}	5.9 ± 1.7^{B}
K2SO4 (21%)	75.2 ± 1.6^{B}	29.1 ± 0.4^{C}	13.1 ± 0.2^{AB}	4.4 ± 1.4^{B}

Different letters represents statistical differences among moistures ($p \leq 0.05$). (n=6).

At day 1, grains with MC of 13, 16, 19 and 21% presented 22.0, 34.7, 25.5 and 75.2 mg/kg of AITC, respectively. In addition, statistical analysis demonstrated that there was no significate difference among the MC of 13, 16 and 19%. However, when the MC is above 19% the capacity of AITC absorption by grain increased probably because of their softer hull. Regardless of the MC, AITC was able to penetrate the grain and was slowly released, reaching values of 9.1, 13.4, 5.9 and 4.4 mg/kg for grains with MC of 13, 16, 19 and 21% at day 90, respectively. These results suggested that residual effect of AITC extended the shelf-life of barley grain for 90 d, but its effect in the organoleptic characteristic of beer and barley germination should be studied due a strong mustard flavors and its phytotoxicity (Shin et al., 2010; Q. Yu et al., 2016). However, its residual effect would be not a concern since the germination and kilning step can accelerate the AITC volatilization.

Nazareth et al. (2018) and Tracz et al. (2017) evaluated the residual concentrations of gaseous AITC in the corn kernels. These authors demonstrated that AITC was gradually released during storage. However, dose of 50 µL/L was not detected after 60 d of experiment. Whereas the AITC was detected after 90 d in our work. The difference among our results for these previous studies could be explained by the different capacity of absorption of different matrices. Therefore, barley grains had better absorb AITC compared to maize grains, allowing longer protection time.

3.1.3.4 Moisture content on barley grains

The **Figure 3.1.2** shows the average of MC in barley grains during 90 d of storage. The use of saturated salt solution of NaCl, KCl, KNO_3 and K_2SO_4 produced a mean of 13.4; 15.8; 19.0 and 20.6% of MC, respectively. Simulating the ideal condition of storage (13%) and unfavorable storage conditions (above 15%). Our results demonstrated that the higher MC (21%) led to higher FP and increased OTA production. Moreover, the higher MC reduced the shelf life of product, once the FG was visible after 14 d of storage (data not shown). Therefore, drying the grains can prevent fungal spoilage during storage.

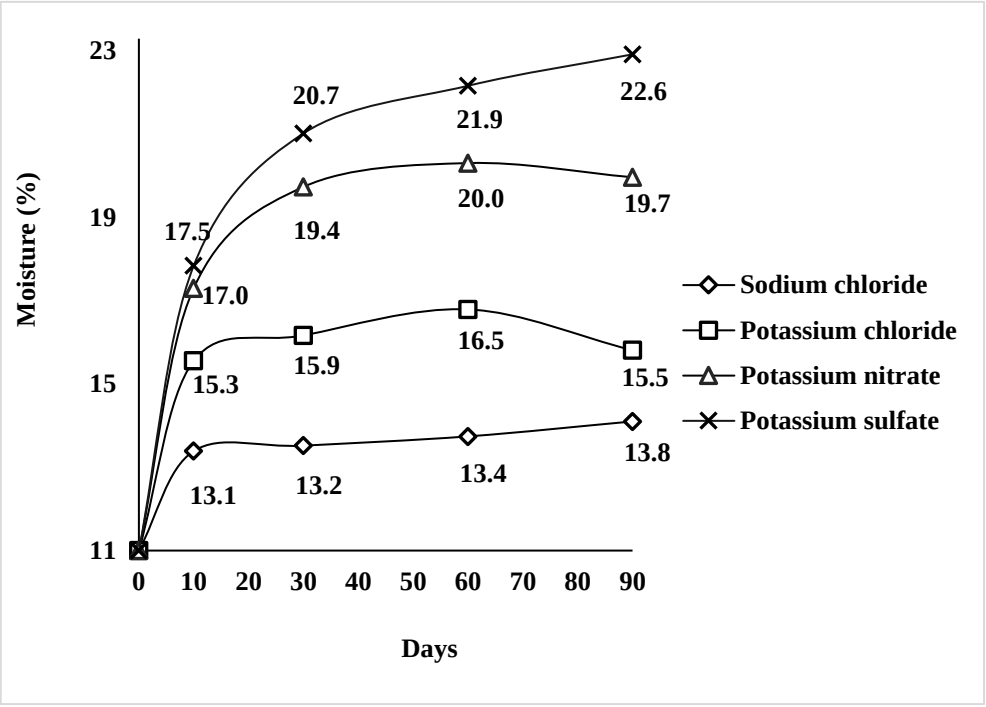
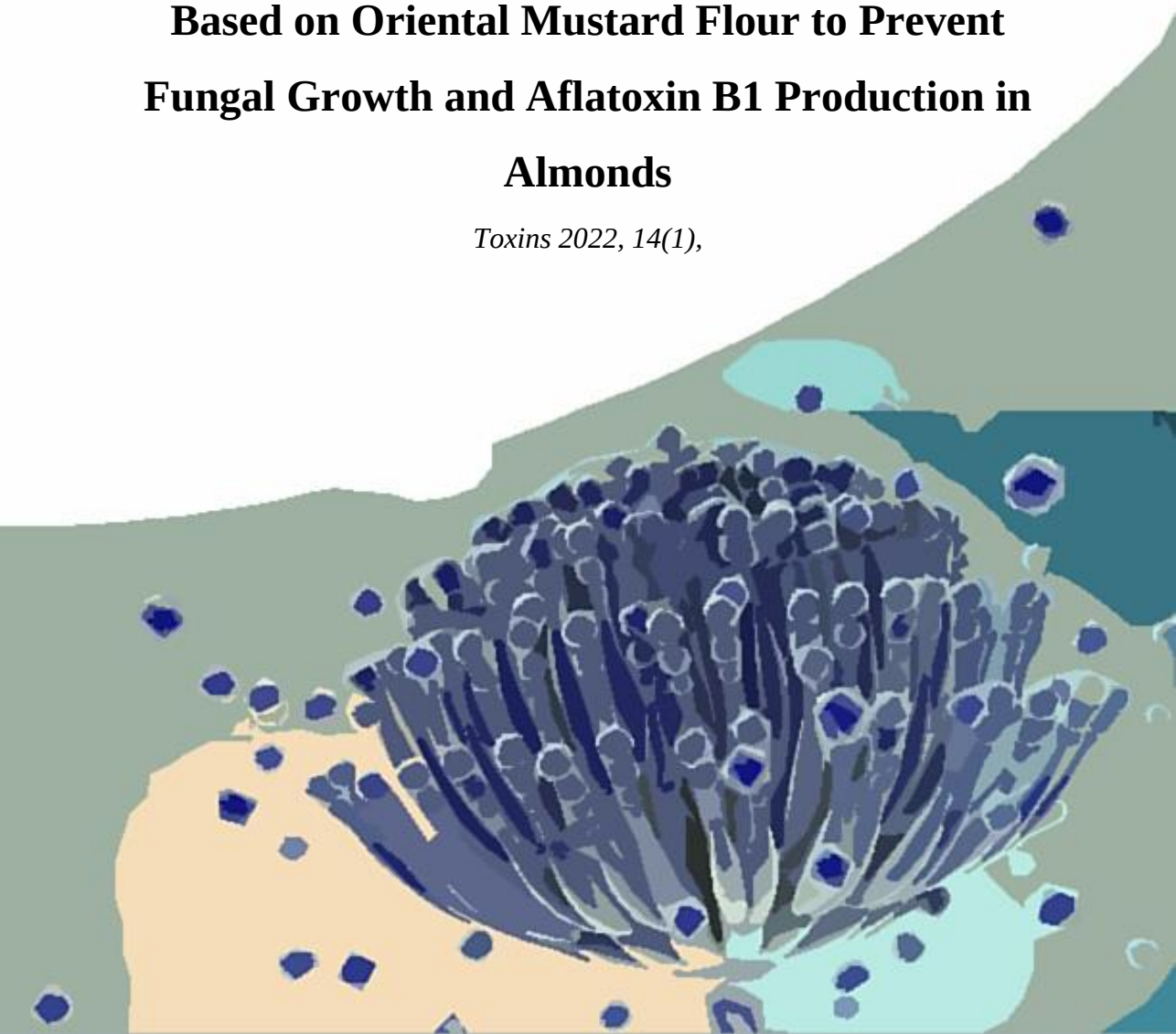


Figure 3.1.2. Moisture content in barley grains kept in controlled conditions by salt solutions

3.2 Development of an Antifungal Device Based on Oriental Mustard Flour to Prevent Fungal Growth and Aflatoxin B1 Production in Almonds

Toxins 2022, 14(1),



3.2.1 Introduction

Mycotoxins are secondary fungal metabolites produced mainly by the genera *Aspergillus*, *Penicillium*, and *Fusarium* (Anfossi et al., 2016; Njobeh et al., 2010; Terzi et al., 2014). Among the metabolites produced, AFs are produced by fungi of the genus *Aspergillus*, predominantly by two species, *A. flavus* and *A. parasiticus*, which can produce four main AFs, B_1, B_2, G_1, and G_2. According to IARC, AFs are classified into group 1: carcinogens to humans. The AFB_1 is the most toxic and can cause chronic liver damage, affect the immune system, growth, and malnutrition (Rushing & Selim, 2019). AFs are present in cereals, spices, and dry fruits such as almonds, peanuts, and oilseeds, contaminating directly pre-harvest and postharvest food (P. Kumar et al., 2017).

Dry fruits such as almonds and nuts have beneficial effects, decreasing risk factors related to diabetes and cardiovascular diseases, with anti-inflammatory and antioxidant properties, and increasing bone health. Moreover, they are also rich in sugar and fat, mono- and polyunsaturated fats, and sterols (Alasalvar et al., 2020; Aune et al., 2016; Becerra-Tomás et al., 2019). Nevertheless, on the other hand, almonds are very susceptible to FG and mycotoxin production. This fact occurs because of inadequate storage conditions, environmental issues (humidity and temperature), and high sugar content (Saladino et al., 2016b).

Several methods have been used to prevent FG and mycotoxin production in foods. Among the strategies used are pre- and postharvest actions, which reduce the amount of contamination. Examples of pre-harvest actions are developing genetically modified cultivar lines, crop rotation, and changes in planting time, while post-harvesting methods include food drying, storage, and preservatives used (Rushing & Selim, 2019).

Increasing interest in alternate preservation approaches for inactivating microbes and enzymes in foods has emerged from the need to ensure food safety while addressing such demands for nutrition and qualitative qualities (Le Lay et al.,

2016). Flavour, odour, colour, texture, and nutritional value are all critical quality characteristics. This growing demand for natural preservatives originating from plants, animals, or microorganisms has expanded the possibilities for their application (Torrijos et al., 2021). Natural antimicrobials may be used alone or in combination with other innovative preservation technologies to enable the eradication of more conventional methods (Tiwari et al., 2009).

The use of EOs has been investigated with great interest to the food industry due to their broad spectrum of activity, low tendency to induce resistance, and safety for consumption (Jamil et al., 2016). Plants belonging to the *Brassicaceae* family are rich in bioactive compounds, highlighting the GTs, which in an enzymatic reaction give ITCs (Vig et al., 2009). *Sinapis alba* (yellow or white mustard) and *Brassica juncea* (brown or oriental mustard) are rich in GTs and, in the presence of water, form ITCs' aromatic compounds. In oriental mustard, myrosinase forms AITC from the main glucosinolate, sinigrin (Nadarajah et al., 2005). Concerning yellow mustard, the ITC formed is p-HBIT from the main glucosinolate sinalbin (Ekanayake et al., 2012).

AITC is a volatile compound that is reported to exhibit numerous beneficial effects, including antimicrobial, anticarcinogenic, cardioprotective, and neuroprotective properties (J. R. Williams et al., 2015) and shows antifungal activity towards mycotoxigenic *Aspergillus* (Hontanaya et al., 2015), *Penicillium* (Tunc et al., 2007), and *Fusarium* species (Nazareth et al., 2016). Moreover, AITC is considered a GRAS compound by the FDA, and the IARC has classified it as non-carcinogenic (class 3) (Tracz et al., 2017).

Likewise, YMF has shown significant antimicrobial activity against food pathogens, such as bacteria and fungi (Andini et al., 2021; Drakopoulos et al., 2020).

In order to minimize the economic loss caused by fungi, the objectives of this work were, first, to evaluate the *in vitro* antifungal potential of the AITC and a freeze-dried yellow mustard flour extract (YMF-E). Then, it was to develop an

antifungal device that contains hydroxyethyl-cellulose and oriental mustard flour (H-OMF) and determine its antifungal effect. Finally, it was to evaluate the efficacy of all treatments in avoiding the growth of *A. flavus* and preventing the production of AFB_1 in almonds. These data may support a new strategy to preserve almonds and mitigate AFB_1 contamination.

3.2.2 Results and Discussion

3.2.2.1 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

Using broth microdilution, *A. flavus* demonstrated sensitivity to AITC and YMF-E, as shown in **Table 3.2.1**. The MIC for AITC and YMF-E were 7.90 mg/L and 390 mg/L, respectively. The MFC of these compounds was previously compared with the results of MIC. AITC required a four-fold greater amount to have fungicidal effects. Regarding YMF, the quantity required was eight-fold greater than MIC. Thus, AITC was the compound that demonstrated more effectiveness against this toxigenic strain.

Table 3.2.1. Minimum Inhibitory Concentration and minimum fungicidal concentration obtained using allyl isothiocyanate and freeze-dried yellow mustard flour extract against *Aspergillus flavus* ISPA 8111.

Minimum Inhibitory Concentration/Minimum Fungicidal Concentration				
	Compounds			
	AITC (mg/L)		YMF-E (mg/L)	
Fungi	MIC	MFC	MIC	MFC
A. flavus	7.90	31.61	390	3,130

MFC: minimum fungicidal concentration; MIC: Minimum inhibitory concentration; AITC: allyl isothiocyanate; YMF-E: freeze-dried yellow mustard flour extract (n = 8).

Many authors have studied the inhibition of FG using natural compounds. Nielsen and Rios (2000) tested the volatilization of EOs such as AITC against several

fungi. Regarding *A. flavus*, the MIC was 3.5 mg/L in the gas phase. Other authors, such as Clemente et al. (2019), also described the antifungal activity of AITC in a liquid medium. These authors identified that the MIC values for *A. flavus*, *A. ochraceus*, and *A. niger* were 6.25 mg/L, 6.25 mg/L, and 3.13 mg/L, respectively. However, MFC results were higher, ranging from 6.25 mg/L for *A. niger* and *A. flavus* strains and 25 mg/L for *A. ochraceus*.

Quiles et al. (2018) reported MIC for several fungi strains using a concentrated extract of YMF non-autoclaved. The results ranged from 238.2 for *P. camemberti* to 15,000 mg/L for *A. flavus*, *A. parasiticus*, and *A. carbonarius*. In addition, this extract showed a fungicidal effect with MFC values ranging from 1875 mg/L against *P. nordicum*, *P. commune*, and *P. brevicompactum* to 15,000 mg/L towards *A. flavus*, *A. parasiticus*, and *A. carbonarius*.

YMF-E does not seem to have a substantial impact on *Aspergillus* spp., as does *Penicillium* spp., based on the findings reported by the other authors (Quiles et al., 2018; Torrijos et al., 2021). Conversely, we noticed that YMF-E had a lower MIC and MFC value in this study. Our findings, nonetheless, support previous studies since large doses are required to suppress *A. flavus* growth.

3.2.2.2 Oriental Mustard Flour (OMF) In Vitro Activity Against A. flavus

Since the H-OMF device could not be diluted in a Potato Dextrose Broth (PDB), its antifungal activity was determined by measuring MG. **Table 3.2.2** shows the *in vitro* antifungal effect of the H-OMF device against *A. flavus* during an incubation time of 7 days at 25 °C. Control samples reached a mean MG of 50 mm in diameter on the fifth day. Regardless of the incubation time, a dosage of 30 mg/L of H-OMF exhibited a fungicidal effect and inhibited visual MG. On day 3, a dosage of 25 mg/L of H-OMF reduced MG by 80%. On the fifth day, the same treatment showed a reduction by 50%. On the seventh day, this concentration provided a mycelial reduction by 26%. The dose of 12.5 mg/L of H-OMF significantly reduced MG on days 3 and 5; however, this dosage did not avoid the FG. On the other hand,

the concentration of 6.2 mg/L of H-OMF did not show an antifungal effect in the MG after 5 days of storage.

Table 3.2.2. *In vitro* antifungal activity of oriental mustard flour antifungal device based on hydroxyethyl-cellulose (H-OMF) against *Aspergillus flavus* ISPA 8111.

Mycelial Growth (mm of Diameter) after Fumigation by H-OMF			
Treatment	Days		
	3	5	7
Control	25 ± 1.2^{A}	50 ± 2.5^{A}	50 ± 5.5^{A}
30 mg/L	ND	ND	ND
25 mg/L	5 ± 1.3^{B}	25 ± 3.4^{B}	37 ± 5.1^{B}
12.5 mg/L	9 ± 0.8^{C}	34 ± 2.2^{C}	38 ± 4.2^{B}
6.2 mg/L	9 ± 1.1^{C}	48 ± 1.6^{A}	50 ± 6.1^{A}

OMF: oriental mustard flour; ND: the mycelial growth was not detected; different capital letters represent statistical differences ($p \leq 0.05$) (n = 9).

In general, only the concentration of 30 mg/L of H-OMF demonstrated a fungicidal effect; the other doses, such as 12.5 and 25 mg/L, only presented a fungistatic effect, which allowed the FG during storage. Nevertheless, it should be highlighted that these concentrations also exhibited a significant difference regarding the control group. Therefore, these results suggest that H-OMF may inhibit the FG in a dose-dependent manner, and concentrations higher than 30 mg/L of H-OMF might be necessary to achieve a fungicidal effect in complex matrices.

Manyes et al. (2015) evaluated the capacity of AITC deposited inside a disc of sterile paper to avoid the micellar growth of *A. parasiticus* and *P. expansum*. The authors demonstrated that the MG was not observed when AITC amounts greater than 50 mg were deposited in the Petri dishes' centre inoculated with *P. expansum*. Concerning *A. parasiticus*, 25 mg was able to inhibit the MG completely. Saladino et al. (2016a) reported that OMF decreased the mycelial diameter growth of *A. parasiticus* by 48.2–60.4% when using 0.1 to 1 g of OMF incubated for 24 h. In our

study, better results were found using a smaller amount of OMF, such as 0.030 g. This fact may be explained by the difference in the strains and the applied method by Saladino and colleagues.

3.2.2.3 Determination of the FP in Natural Almonds

Natural chemicals are increasingly being used to mitigate fungus development in foodstuffs. As shown in **Table 3.2.3,** on day 0, all treatments had significantly shown less FP than on days 7 and 15. All treatments of YMF-E tested did not inhibit the FG when compared with the control group. However, 2000 and 4000 mg/L of H-OMF decreased the FP to levels below the limit of detection (LOD) (1.22 log CFU/g) on the 15th day. The same result was observed for the treatments with 5.07, 10.13, and 20.26 mg/L of AITC. Likewise, these concentrations decreased the FP to levels below the LOD on days 7 and 15. This fact may be explained because OMF in the presence of water was converted in AITC. However, this reaction did not occur with YMF-E. Concerning the residual FP, on day 0, there were no statistical differences among the control group and the treatments tested, which could suggest that antifungal compounds were slowly released over time.

Ground yellow mustard seeds and yellow mustard seeds have been paired with meats, especially as condiments for fermented sausages (Luciano et al., 2011). White or yellow mustard (*Sinapis alba*) and oriental or brown mustard (*Brassica juncea*) are known to contain a high concentration of GTs, and these substances are cleaved in the presence of moisture by myrosinase to produce ITCs and a few other minor molecules such as thiocyanates and nitriles (Quiles et al., 2018). On the one hand, the predominant glucosinolate in yellow mustard is called sinalbin, which is cleaved by myrosinase to form ρ-HBIT. Although the antifungal effect of yellow mustard is not well understood, authors have attributed it to the synthesis of ITC compounds such as p-HBIT (Luciano et al., 2011). On the other hand, sinigrin is the major glucosinolate molecule found in oriental mustard seeds; these are nitrogen- and sulphur-containing metabolites that serve as AITC precursors. Similarly, AITC

is enzymatically synthesized when sinigrin is hydrolysed by myrosinase in a humid environment (Bahmid et al., 2020). Thus, OMF may be used as a natural supply of AITC that is gradually released into the headspace of silo systems (Dai & Lim, 2014).

Table 3.2.3. The residual population of *Aspergillus flavus* ISPA 8111 in almonds treated with allyl isothiocyanate, the antifungal device of hydroxyethyl-cellulose and oriental mustard flour, and freeze-dried mustard flour extract.

Treatment	Concentration	Fungal Population in log CFU/g (Mean ± SD) Days		
		0	7	15
Control	-	5.11 ± 0.26^{A}	9.24 ± 0.34^{A}	10.52 ± 0.08^{A}
AITC	5.07 mg/L	5.10 ± 0.16^{A}	$\leq 1.22 \pm 0.00^{B}$	$\leq 1.22 \pm 0.00^{B}$
	10.13 mg/L	5.10 ± 0.11^{A}	$\leq 1.22 \pm 0.00^{B}$	$\leq 1.22 \pm 0.00^{B}$
	20.26 mg/L	5.70 ± 0.51^{A}	$\leq 1.22 \pm 0.00^{B}$	$\leq 1.22 \pm 0.00^{B}$
H-OMF	2000 mg/L	5.90 ± 0.20^{A}	$\leq 1.22 \pm 0.00^{B}$	$\leq 1.22 \pm 0.00^{B}$
	4000 mg/L	5.80 ± 0.34^{A}	$\leq 1.22 \pm 0.00^{B}$	$\leq 1.22 \pm 0.00^{B}$
YMF-E	100 g/L	5.60 ± 0.27^{A}	8.91 ± 0.29^{A}	$10,31 \pm 0.04^{A}$
	160 g/L	5.40 ± 0.53^{A}	9.96 ± 0.10^{A}	$10,68 \pm 0.16^{A}$
	200 g/L	5.70 ± 0.21^{A}	8.96 ± 0.17^{A}	$10,15 \pm 0.12^{A}$

AITC: allyl isothiocyanate; H-OMF: hydroxyethyl-cellulose device with oriental mustard flour; YMF-E: yellow mustard flour extract. Different capital letters represent statistical differences among treatments ($p \leq 0.05$). (n = 18).

Different mechanisms of action for ITCs' antimicrobial activity have been suggested, including regulation of sulfhydryl enzymes, inhibition of RNA production, partial suppression of DNA synthesis, and inhibition of protein synthesis through the ITCs' molecule (Turgis et al., 2009). Although the antimicrobial mechanisms of ITCs are not fully understood, it is believed that their antimicrobial action is linked to their reactivity with proteins, which may disrupt in vivo biochemical processes. In other words, the carbon of the ITC molecule (R-N=C=S)

is extremely electrophilic and interacts quickly with amines, thiols, and hydroxyls. Consequently, they may readily target the thiols and amines of the amino acid structure found in proteins. However, they primarily prefer to attack the sulfhydryl groups (Dufour et al., 2015).

Authors also described that ITCs' antibacterial action is a result of their amphiphilic nature. Although most authors agree that thiocyanate moiety is a critical component of ITCs' antibacterial action, some authors have nevertheless described that ITCs exert their influence primarily through generating oxidative stress (Nielsen & Rios, 2000; Troncoso et al., 2005; Tunc et al., 2007).

Our research group has demonstrated the antifungal capacity of AITC against a variety of fungi, such as *Aspergillus* and *Penicillium* (Luciano et al., 2011; Manyes et al., 2015).

Lopes et al. (2018a) and Tracz et al. (2017) showed the inhibition of FG at levels of 2.5 and 500 µL/L of gaseous AITC (2.53 and 506.50 mg/L considering AITC density of 1.013 kg/m^3). Nazareth et al. (2018) reported a reduction of *A. parasiticus* CECT 2681 and *F. verticillioides* CECT 2983 at levels below the detection limit using a single treatment of 50 µL/L (50.65 mg/L) of AITC in corn kernels stored for 150 days. Our data show that a lower concentration of AITC was necessary to achieve comparable results. This difference can be justified because food matrices and fungal strains are not the same as reported previously (Nazareth et al., 2018). Quiles et al. (2019) demonstrated the growth inhibition of *P. verrucosum* using AITC; at the concentration of 500 µL/L (506.50 mg/L), the dispositive also reduced the FP to undetectable levels. Our study reported concentrations lower than those required in that study, which the differences in the applied methods could justify.

The antifungal activity of AITC was also evidenced by Suhr and Nielsen (2003)using essential mustard oil with 99% of AITC on pieces of bread inoculated with 10^6 spores/mL of *P. roqueforti*, *P. corylophilum*, and *A. flavus*. In that study,

the inhibition of the fungi was observed at concentrations of 1 μL/L of AITC (99%). Quiles et al. (2015a) investigated the ability of AITC and OMF plus water to prevent the development of *A. parasiticus* in fresh pizza crust using active packaging devices for 30 days of storage. The growth of *A. parasiticus* was inhibited with AITC at 5 and 10 μL/L (5.07 and 10.13 mg/L, respectively) and OMF at 850 mg after 30 days. The treatment with 10.13 mg/L of AITC reduced the FP by more than 5 log CFU/g, and OMF at 850 mg reduced by 1.5 log CFU/g compared to the control group (8.99 log CFU/g).

Despite the differences between the studies mentioned above, this study proved that AITCs have intense antifungal activity against *A. flavus*, and natural compounds associated with novel devices, such as H-OMF, might be effective against mycotoxigenic strains in different types of food. Indeed, we propose that further research might be conducted to determine the fumigant potential of H-OMF in other dry fruits.

3.2.2.4 Aflatoxin Determination

To reproduce natural contamination of the almonds, *A. flavus* ISPA 8111 was used as an Afs' producer. The results are shown in **Table 3.2.4,** and the samples were analysed from day 0 to day 15. As shown in **Table 3.2.4**, the treatments with H-OMF (2000 mg/L and 4000 mg/L) and AITC (5.07, 10.13, and 20.26 mg/L) reduced the production of AFB_1, which could have resulted from the FG inhibition. On the other hand, YMF-E (100, 160, and 200 g/L) had no effect inhibiting AFB_1 production compared to the control group. Conversely, treatment with YMF-E significantly increased AFB_1 production compared to the control group and over time. These results suggest that YMF-E should be avoided to prevent AFB_1 production, or higher doses must be assayed.

This study may be considered the first in which a device based on hydroxyethyl-cellulose and OMF was applied to avoid the content of AFB_1 in almonds. Nonetheless, the use of AITC to reduce the growth of the fungi and

mycotoxin production has been studied previously by other authors. Nazareth et al. (2016) evaluated the capacity of AITC to reduce the production of the mycotoxin by *A. parasiticus* and *F. poae* in wheat flour. The results showed that the application of AITC in a concentration of 10 µL/L (10.13 mg/L) reduced the biosynthesis of mycotoxins entirely for 30 days. Tracz et al. (2017) showed the capacity of AITC to reduce the production of mycotoxins in corn kernels in a concentration of 50, 100, and 500 µL/L (50.65, 101.30, and 506.50 mg/L, respectively), and all the treatments were capable of avoiding the production of mycotoxins. The data obtained in our study corroborate with these studies using AITC since the antitoxigenic effect was achieved applying concentrations lower than 20.26 mg/L.

Table 3.2.4. Effect of allyl isothiocyanate, the antifungal device of hydroxyethyl-cellulose and oriental mustard flour, and yellow mustard flour extract treatment on aflatoxin B_1 (AFB_1) production by *Aspergillus flavus* in almonds.

Treatment	**Concentration**	**The Concentration of AFB_1 in µg/kg (Mean ± SD)**	
		Days	
		7	15
Control	-	36.22 ± 4.21^{A}	71.67 ± 2.84^{A}
AITC	5.07 mg/L	$\leq LOD^{C}$	≤LOD
	10.13 mg/L	$\leq LOD^{C}$	≤LOD
	20.26 mg/L	$\leq LOD^{C}$	≤LOD
H-OMF	2000 mg/L	$\leq LOD^{C}$	≤LOD
	4000 mg/L	$\leq LOD^{C}$	≤LOD
YMF-E	100 g/L	267.38 ± 43.09^{B}	276.43 ± 186.83^{B}
	160 g/L	314.13 ± 131.49^{B}	364.41 ± 77.78^{B}
	200 g/L	395.21 ± 143.14^{B}	575.90 ± 169.61^{B}

AITC: allyl isothiocyanate; H-OMF: antifungal device of hydroxyethyl-cellulose with oriental mustard flour; YMF-E: yellow mustard flour extract; LOD: limit of detection was 0.30 µg/kg; A, B, and C: capital letters represent statistical differences among treatments at the same time point ($p \leq 0.05$). The samples were previously analysed for the presence of AFB_1 ($n = 9$).

Considering OMF's effectiveness in reducing mycotoxin production, Quiles et al. (2015a) reported that pizza crust contaminated with *A. parasiticus*, when treated with 10 μL/L (10.13 mg/L) of AITC in filter paper and 850 mg of OMF, reduced the AFs' (B_1, B_2, G_1, and G_2) production below the limits of quantification (≤LOQ). Hontanaya et al. (2015) evaluated the effectiveness of OMF in reducing the FG of *A. parasiticus* and aflatoxin production in dry fruits (peanut, cashew, walnut, almond, hazelnut, and pistachio). The use of OMF reduced the aflatoxin production by 83.1–87.2%. Saladino et al. (2016a) used OMF (0.1, 0.5, and 1 g of flour) to derivate isothiocyanates and avoid aflatoxin production in Italian *piadina* contaminated with *A. parasiticus*. The reduction of aflatoxin production ranged from 60.5 to 89.3%. The same authors evaluated the use of isothiocyanates' derivative from OMF and YMF by the water addition to reduce the formation of mycotoxins produced by *P. expansum*. The mycotoxin reduction ranged from 80 to 100%.

Recently, several authors have carried out intensive studies on the role of genes' expression in the synthesis of AFs. Nevertheless, the regulation mechanism remains poorly recognized. Among the genes evaluated, aflR and aflS are strictly linked to the aflatoxin pathway, and their down-regulation, caused by environmental or nutritional factors, may lead the fungus to a suppressed aflatoxin production (Peromingo et al., 2017). Within this frame of reference, Nazareth et al. (2020) evaluated the AFB_1 production and the transcriptional profile of *A. flavus*, applying sublethal dosages of AITC. All treatments increased the expression of genes involved in the synthesis of AFB_1, although this mycotoxin has been substantially decreased because of the antifungal action. Alternatively, the findings indicated a widespread overexpression of the AFB_1 gene cluster, which seems to be associated with the stressful condition induced by AITC activity. AITC also resulted in aberrant regulation of the examined genes, including those encoding critical transcription factors such as veA and laeA, which are also associated with the AFB_1 production.

Overall, the device H-OMF reduced *A. flavus* growth and AFB_1 production in the lab-scale system, demonstrating an important candidate for silo fumigation. Furthermore, this antifungal device could be scaled up to be tested in a large-scale experiment or a real silo trial due to its manufacturability. Therefore, it is also essential to note that further studies will be conducted to evaluate this antifungal device as a fumigator in 100-L scale and full-scale silos.

In a real-life situation, in order to maintain the overall quality during a longer storage time (more than 9 months), different physical barriers such as modified atmosphere packaging and refrigeration could be applied (García-Pascual et al., 2003). The use of low barrier packaging material combined with refrigeration on the storage conditions can maintain the quality of almonds for up to 12 months when stored at refrigeration temperature (2 °C) (García-Pascual et al., 2003). Therefore, due to the manufacturability of the antifungal device, it could be used in packaging based on permeable plastics or simply deposited on the bottom of storage containers.

3.2.3 Materials and Methods

3.2.3.1 Chemicals

AFB_1 standard solution (purity > 99%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The hydroxyethyl-cellulose was provided from Sigma-Aldrich (St. Louis, MO, USA). Methanol (99%) and formic acid (99%) used for liquid chromatography were HPLC grade and obtained from VWR Chemicals (Radnor, PA, USA). Ammonium formate was obtained from Sigma-Aldrich (St. Louis, MO, USA). Microbiological media such as PDB, potato dextrose agar (PDA), and Peptone Water were obtained from Liofilchem Products (Roseto Degli Abruzzi, Italy). YMF and oriental mustard flour (OMF) were provided by G.S. Dunn dry mustard millers (Hamilton, ON, Canada). AITC (95.1% purity) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

3.2.3.2 Microorganism and Culture Conditions

The mycotoxigenic strain of *A. flavus* ISPA 8111 used in this study was obtained from the Institute of Food Production Science (ISPA, Bari, Italy). This microorganism was stored in sterile glycerol at −80 °C before use. Then the microorganism was grown in PDB at 25 °C, and, after growth, the PDA Petri dishes were inoculated in the dark.

3.2.3.3 Preparation of the Freeze-Dried Yellow Mustard Extract (YMF-E)

The method previously described by Quiles et al. (2018) was used to extract water-soluble components with minor modifications. YMF (2 g) was homogenised in 25 mL of distilled water for 5 min at 7000 rpm using an Ultra Ika T18 basic Ultra-Turrax (Staufen, Germany). After centrifuging the extracts for 15 min at 5000× *g*, the supernatant was collected and deposited on polypropylene trays. Afterwards, the supernatant was freeze-dried for 72 h in a FreeZone 2.5 L Labconco (Kansas, MO, USA). The powder produced was kept at a temperature of 4 °C until its use in the antifungal activity test.

3.2.3.4 Manufacture of H-OMF Antifungal Device

In a Petri dish, 1.5 g of hydroxyethyl-cellulose (gelling agent), 10 mL of water, and 2 or 4 g of OMF were combined to form the gel device. As illustrated in **Figure 3.2.**1, the lid of the Petri dish was previously punctured to allow the volatilisation of the AITC. The antifungal device was then inserted into the jars to study its antifungal effect.

3.2.3.5 Determination of the Minimum Inhibitory Concentration and the Minimum Fungicidal Concentration of AITC and YMF-E

The MIC was determined by quadruplicate, using broth microdilution in a 96-well plate according to the protocol M38-A2 of the Clinical Laboratory Standard Institute, with adaptations (2008). The fungus was first grown on PDA and incubated

for 7 days at 25 °C. The fungal suspension was prepared by harvesting the spores from the surface of the plates with peptone water 0.1%. After counting the number of spores using a Neubauer chamber, the inoculum was adjusted to 2×10^4 spores/mL in a PDB medium (Liofilchem, Italy). Then, 100-µL aliquots of fungal suspension were added to each well. Different concentrations of AITC (ranging from 0.98 mg/L to 506.50 mg/L) and YMF-E (ranging from 90 to 100,000 mg/L) were added by microdilution. The microplate was filled to a final volume of 200 µL/well. The plate was incubated for 48 h at 25 °C before a visual reading was performed. Control groups were prepared with PDB and fungal suspension (positive control) and only PDB (negative control) containing 200 µL as the final volume.

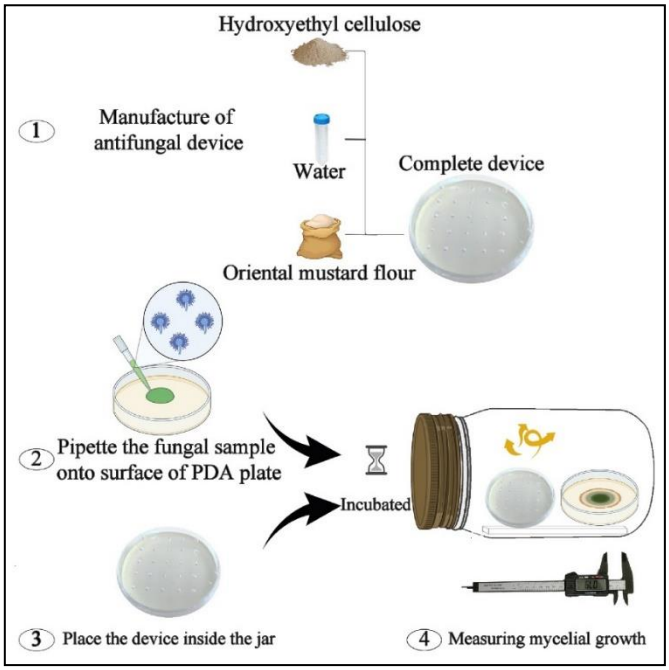


Figure 3.2.1. The small silo system used to determine the *in vitro* antifungal activity of mustard flour device based on hydroxyethyl-cellulose (H-OMF) against *Aspergillus flavus* ISPA 8111.

The MFC was determined after MIC determination according to the protocol described by Espinel-Ingroff et al. (2012). First, 10-µL aliquots of each well showing

complete inhibition of FG were withdrawn and cultured in PDA plates for 72 h at 25 °C. The negative control group was also inoculated in PDA plates and incubated for 72 h at 25 °C. The MFC was then established as the lowest dilution that yielded fewer than three colonies.

3.2.3.6 In Vitro Antifungal Activity of H-OMF against A. flavus ISPA 8111

Petri dishes (50-mm diameter) containing 6.2, 12.5, 25, and 30 mg of OMF were prepared to mix the flour with 10 mL of sterile distilled water (to improve the conversion of the glucosinolate sinigrin into AITC) and 1.5 g of hydroxyethyl-cellulose (a gelling agent). Simultaneously, Petri dishes (50-mm diameter) containing PDA were inoculated with 10 µL of *A. flavus* ISPA 8111 at 10^4 spores/mL. After, the dishes were inserted into the jars without the lids, and then the jars were hermetically closed (**Figure 3.2.1**). The control group did not receive any treatment. Jars were kept at 25 °C for 7 days. Finally, on days 3, 5, and 7, the inhibitory effect of H-OMF was monitored by measuring the mycelia growth diameter on a scale of mm.

3.2.3.7 Microbiological Assay with Natural Almonds

The natural almonds were autoclaved, dried at 28 °C for 24 h, contaminated with *A. flavus* ISPA 8111 in a concentration of 10^6 spores/g, and then portioned in samples of 20 g. After 24 h of incubation, three different treatments were carried out:

- Filter papers (2.5 × 2.5 cm) containing AITC at 5.07, 10.13, and 20.26 mg/L (related to jar volume) were prepared and adhered to the lids of the jars (**Figure 3.2.2a**).
- H-OMF antifungal device was manufactured by mixing 2 and 4 g of OMF with 10 mL of sterile distilled water and 1.5 g of hydroxyethyl-cellulose. The device was then placed into the jars, reaching a final concentration of 2000 and 4000 mg/L (related to jar volume) (**Figure 3.2.2b**). These values

represented 80- and 160-folds of the minimal dose to avoid the MG of *A. flavus* significantly.

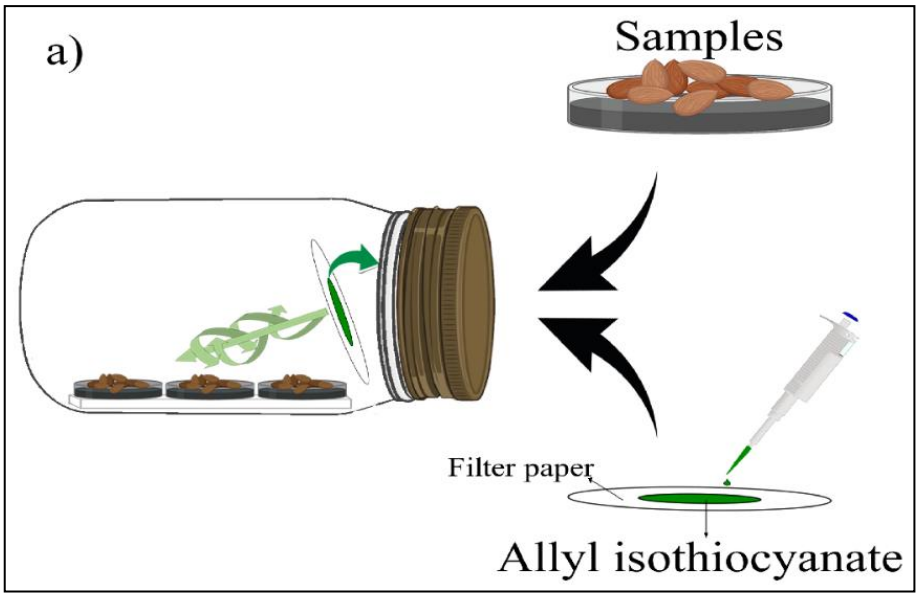
- A spray of YMF was prepared with 0.5 (32 folds MFC), 0.8 (51.2 folds MFC), and 1 g (64 folds MFC) of YMF-E with 5 mL of sterile distilled water. The treatment was carried out by spraying the extract on the surface of almonds to reach final concentrations of 100, 160, and 200 g/L (**Figure 3.2.2c**).

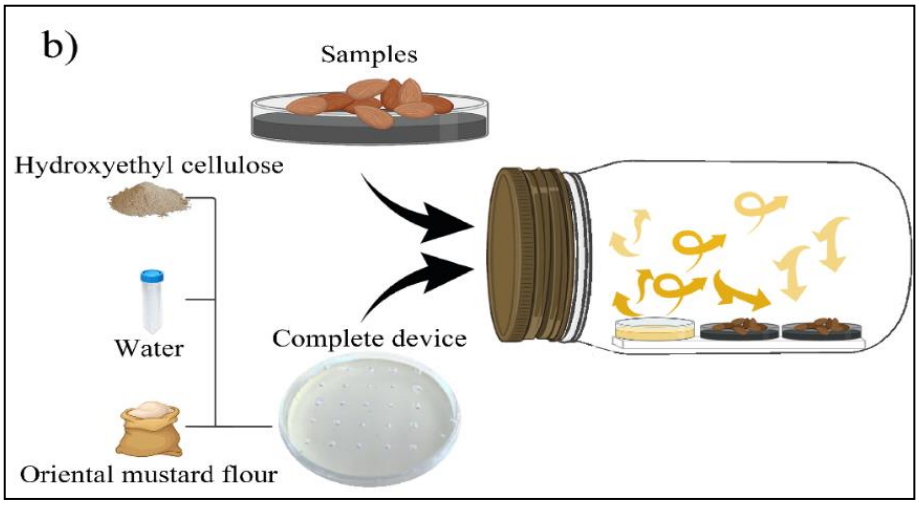
The samples were placed in 50-mm Petri dishes and transferred to 1-L glass jars. Then, the jars were hermetically closed (**Figure 3.2.2**). Control samples did not receive treatments. Experiments were performed in triplicate for 15 days and kept at 25 °C. Finally, the almonds were microbiologically analysed on days 0, 7, and 15.

3.2.3.8 Determination of the FP in Natural Almonds

The numbers of moulds in almonds' kernels during storage were analysed according to Hashemi and Raeisi (2018).

After incubation, 20 g of each sample were transferred to a sterile plastic bag containing 180 mL of sterile peptone water 0.1% (Liofilchem, Italy) and homogenised with a stomacher (IUL, Barcelona, Spain) for 60 s. Serial dilutions of the suspensions were performed in sterile plastic tubes with 0.1% peptone water. After that, aliquots of 0.1 mL were plated on Petri dishes containing PDA (Liofilchem, Italy), and the plates were incubated at 25 ◦C for 7 days before microbial counting. The results were expressed in a log of colony-forming unit/g of almond (log CFU/g). All analyses were conducted in triplicate.


a)
Samples
Filter paper
Allyl isothiocyanate


b)
Samples
Hydroxyethyl cellulose
Water
Oriental mustard flour
Complete device

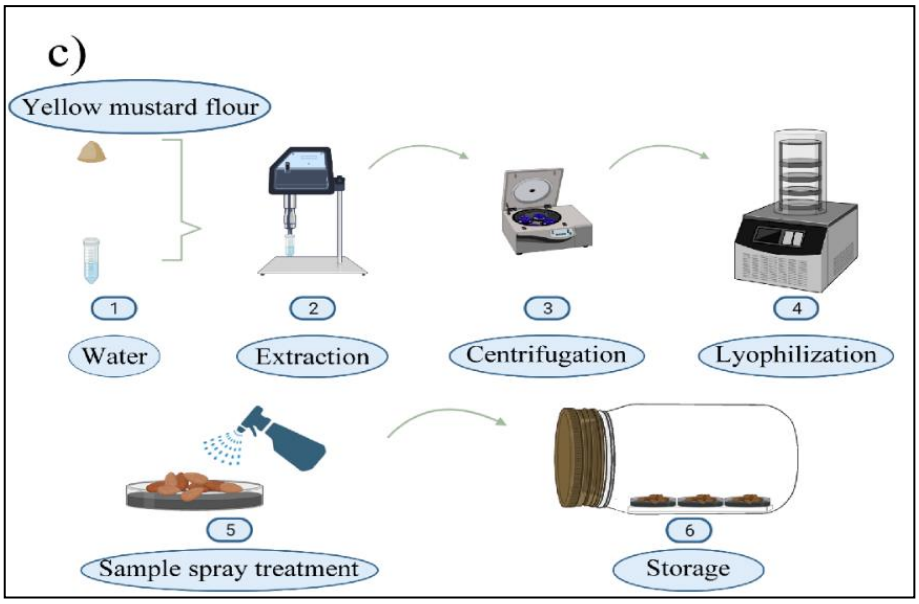


Figure 3.2.2. Silo system used to determine the volatile antifungal activity of: (a) allyl isothiocyanate (AITC); (b) the antifungal device based on hydroxyethyl-cellulose and oriental mustard flour (H-OMF); and (c) the spray of freeze-dried yellow mustard flour extract (YMF-E). Natural almonds were contaminated with *Aspergillus flavus* ISPA 8111 and stored for 15 days.

3.2.3.9 AFB_1 Extraction

The AFB_1 extraction was performed using the method previously described by Huang et al. (2010) with some adaptations. First, samples were ground and homogenized, and 5 g were taken in Falcon tubes of 50 mL with 25 mL of methanol. Then the extract was homogenised for 3 min by Ultra Ika T18 basic Ultraturrax (Staufen, Germany) at 13,500 RPM. Next, the extracts were centrifuged at 4800× *g* for 5 min at 4 °C, and the supernatant was transferred and evaporated using a Büchi Rotavapor R-200 (Postfach, Switzerland). Finally, the obtained residue was resuspended in 2 mL of methanol, filtered through a 0.22-µm syringe filter, transferred to a glass vial, and injected into an LC-MS/MS system.

3.2.3.10 LC-MS/MS Analysis

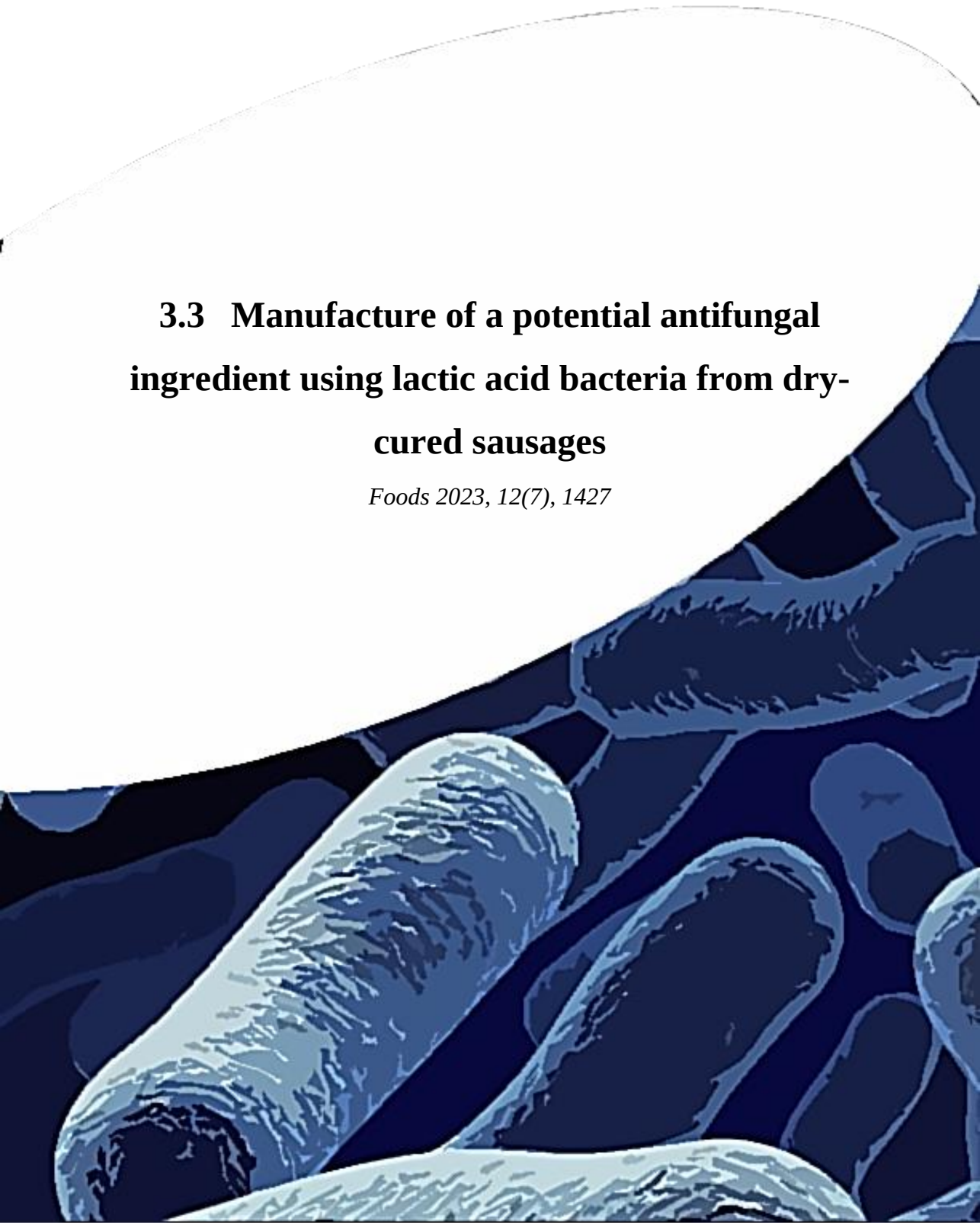
The liquid chromatography system was composed of an LC-20AD pump connected to a 3200QTRAP mass spectrometer (Applied Biosystems, Foster City, CA, USA) through an ESI interface operating in positive ion mode. The established stationary phase was a Gemini NX C18 column (150 × 2.0 mm I.D, 3.0 mm) obtained from Phenomenex (Palo Alto, CA, USA). The mobile phases were solvent A (5 mM ammonium formate and 0.1% formic acid in water) and solvent B (5 mM ammonium formate and 0.1% formic acid in methanol) at a flow rate of 0.25 mL/min. The elution was carried out employing a gradient starting with 10% of B, increasing to 80% up to 1.5 min, and the proportion was kept constant until the fourth min. The ratio was again increased to 90% up to the 10th min. Phase B was then increased to 100% until the 14th min. The time interval between injections was 10 min to return to initial conditions. The injection volume of the samples was 20 µL. The nebulizer, the makeup gas, and the curtain gas were set at 55, 50, and 15 psi, respectively. Furthermore, the capillary temperature was set at 550 °C, and the ion spray voltage was set at 5500 V. Finally, the precursor-to-product ion transitions were m/z 313.3/241.3–228.5 for AFB_1 (Quiles et al., 2016).

3.2.3.11 Statistical Analyses

The assays were performed in triplicate (n = 9). Analysis of variance (ANOVA) was followed by Tukey's test using the software GraphPad Prism 5. The results obtained are expressed as mean ± standard deviation. Statistical differences were considered significant if $p \leq 0.05$.

3.3 Manufacture of a potential antifungal ingredient using lactic acid bacteria from dry-cured sausages

Foods 2023, 12(7), 1427



3.3.1 Introduction

Due to their high nutritional content, meat and its derivatives are an important food category in many people's diets. Consuming processed meats like sausages, hot dogs, and luncheon meats has grown mainstream (Manassi et al., 2022).

Dry-cured meat products are a type of traditional food that is manufactured and eaten across the globe. Their market dominance is well-known, owing to customers' stringent expectations for high-quality and safe foodstuffs. Consumption of these fungus-infected foods increases the risk of exposure to mycotoxins, a worldwide public health concern (Lippolis et al., 2016).

Dry-cured meats are mostly comprised of muscle tissue, and their surface physical-chemical characteristics, such as low a_w, neutral to low pH, high salt content, and protein content, influence the microbial flora that grows on their exterior layers (Simoncini et al., 2015). Although dry-fermented sausages have low a_w and high salt content, alterations of these properties influence the metabolism by facilitating mycotoxin biosynthesis (Rodríguez et al., 2012).

Several species are recognized for producing mycotoxins, including *Aspergillus*, *Alternaria*, *Claviceps*, *Fusarium*, and *Penicillium* (De Ruyck et al., 2015). In certain environmental and substrate conditions over meat products, *A. flavus*, *A. parasiticus*, *A. ochraceus*, *P. nordicum*, *P. polonicum*, and *P. verrucosum* produce mycotoxins, posing a risk to consumers through their growth in salami, dry-cured hams, and other meat products (Perrone et al., 2015; Sánchez-Montero et al., 2019).

P. nordicum is the most important OTA producing species frequently detected in cold-chain protein foods such as dry-cured ham, salami, and salted fish. In particular, this fungus grows well at temperatures of about 15 °C and salt concentrations of more than 5% NaCl (Ferrara et al., 2015).

Mycotoxin contamination of these products may occur at any stage along the manufacturing chain, from animals contaminated in feed through the end product's manufacture or storage. To make matters worse, despite having been detected in dry-cured meats and cheese worldwide, most nations do not regulate mycotoxins in this kind of food. Although the EU has published a new amending regulation and concluded that additional monitoring for OTA occurrence is required before setting maximum levels, this mycotoxin will soon be regulated (Commission Regulation (EC) No 2022/1370).

The quest to make healthier meat products has prompted studies to minimize saturated fat, salt, and cholesterol. Better composition of unsaturated fatty acids and integration of postbiotics, probiotics, and prebiotics were also encouraged. These functional additives may benefit human health while improving meat products' nutrition (Thøgersen & Bertram, 2021). Thus, contemporary customers desire high-quality, safe, minimally processed, and chemical-free foodstuffs.

Lactic Acid Bacteria (LAB) are either naturally present in meals or introduced as pure cultures to a variety of dietary items. LAB have a GRAS classification, and it is estimated that fermented foods make up 25% of the European diet and 60% of the diet in many developing nations (Raman et al., 2022). LAB are often used as starting cultures in the production of acidophilus milk, yogurt, buttermilk, cottage cheeses, hard cheeses, and soft cheeses, among other dairy products (H. Li et al., 2013). The cohabitation of LAB and yeast is also crucial for the success of other biotechnological applications, such as the production of sourdough bread (Olaniyan & Adetunji, 2021). Ancient traditions of employing LAB in food and animal feed, together with a new understanding of the favorable health benefits of probiotic LAB use, imply that they might serve as viable alternatives to chemical preservatives.

Regarding the antifungal activity of bioprotective cultures, it is often the consequence of the synergistic impact of many compounds since organic acids are

not the only known active molecules. Antifungal action may require other molecules, such as fatty acids (Garnier et al., 2020), reuterin (Schmidt et al., 2018), cyclic dipeptides, and proteinaceous substances (Crowley et al., 2013), among others.

Phenolic acids are the most representative subgroup of phenolic compounds; they are important for fermented products because of their relationship with the sensory characteristics of foods. LAB have the ability to metabolize and release bound phenolic acids in their free form, and some of them have demonstrated a broad spectrum of antimicrobial activity, making them an important preservative to consider for foods (Omedi et al., 2019). For instance, salicylic and gallic acid have shown antifungal potential against postharvest fungi such as *P. expansum* and *F. graminearum* (da Rocha Neto et al., 2015; Pagnussatt et al., 2014).

The use of probiotics in meat products is regarded as an attractive strategy for enhancing their healthfulness, as the fermentation carried out by probiotics can generate health-improving compounds, typically through the hydrolysis of polysaccharides, proteins, and fats, as well as biologically active compounds such as peptides, organic acids, and conjugated linoleic acid (Karwowska et al., 2022). In addition, fermented sausages are significant matrices for probiotic delivery since they may be taken without heat treatment, which increases the bacteria' and fungi' survival rate.

Therefore, a continuous survey of OTA-producing strains, especially during ripening, could support safety assurance in dry-cured meat production. Against this background, the objective of this study was to isolate LAB with antifungal properties from traditional dry-cured sausages and to develop an antifungal ingredient with potential application in the manufacture of dry-cured meat products. Moreover, characterization and identification of the responsible metabolites of the antifungal ingredient properties were given.

3.3.2 Materials and Methods

3.3.2.1 Chemicals

Each analyte had at least 95% of purity. Sigma-Aldrich (St. Louis, MO, USA) provided the phenolic acids 1,2–dihydroxybenzene, 3–(4–hydroxy–3–methoxyphenyl) propionic, benzoic acid, caffeic acid, gallic acid, p–Coumaric acid, sinapic acid, syringic acid, vanillic acid, and vanillin. Bachem (Bubendorf, Switzerland) provided the PLA. MP Biomedicals (Santa Ana, CA, USA) provided the ferulic acid. The lactic acid and acetic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Solvents suitable for liquid chromatography (≥ 99.9% purity), including acetonitrile (ACN), ethyl acetate, formic acid, and methanol, were provided by VWR Chemicals (Radnor, Pennsylvania, USA). Sigma-Aldrich supplied ammonium formate (≥ 99.5%), C18, magnesium sulfate ($MgSO_4$), sodium chloride (NaCl), and PDB (P6685). Liofilchem (Roseto degli Abruzzi, Teramo, Italy) provided de Man, Rogosa Sharpe broth (MRSb) (Oxoid CM359), and MRS agar (MRSa) (Oxoid CM361), plate count agar (PCA) (Oxoid CM0463) and potato dextrose agar (PDA) (Oxoid CM0139). Water was deionized with <18 MΩ/cm using a Milli-Q purification system (Millipore Corp., Bedford, MA, USA).

3.3.2.2 Sampling isolation, cultivation, and preliminary characterization of LAB isolates

Sampling, isolation, cultivation, and preliminary characterization LABs were collected from various Spanish homemade-fermented products obtained from local shops in the Valencia region: “longaniza de payés,” “fuet,” “longaniza de Pascua,” and “longaniza classic.” Samples (20 g) were added to 180 mL of sterile physiological saline solution and were homogenized for 5 min. Simultaneously, bacteria were isolated by scraping a swab dipped in 0.1% peptone water (Liofilchem, Roseto degli Abruzzi, Teramo, Italy; Oxoid LP 0037) over the food surface. The swabs were then submerged in tubes containing MRSb to recover the bacteria, and the tubes were kept anaerobic at 37 ºC for LAB culture. Appropriate dilutions were

plated onto MRSa and cultures anaerobically for 48 h at 37 ºC. Colonies were purified on MRSa, after being randomly chosen from MRSa plates containing 15 to 300 colonies (Ben Belgacem et al., 2009). Microorganisms were first evaluated microscopically for Gram reaction and morphology as well as catalase production. Only Gram-positive and catalase-negative isolates were investigated, and the strains were maintained at -80 ºC in MRSb containing 20% glycerol.

Biochemical and physiological tests were used to describe the microorganisms. The bacteria were evaluated for their capacity to grow at different temperatures (15, 30, and 45 ºC), at different pH values (3, 5, and 6), and MRSb supplemented with NaCl (3, 6.5, and 10%).

3.3.2.3 Fungal culture and inoculum preparation

Fungal strains purchased from the Spanish Type Culture Collection (Valencia, Spain) were *A. parasiticus* CECT 2681, *P. commune* CECT 20767, *P. griseofulvum* CECT 2605–T, and *P. nordicum* CECT 2320. *A. flavus* ITEM 8111 was acquired from the Institute of Sciences for Food Production's microbial culture collection (Bari, Italy). The VTT Culture Collection provided *P. verrucosum* VTT D–01847 (Espoo, Finlandia). The fungal strains were stored at -80 ºC in sterile glycerol (30% v/v).

The fungal strains were recovered by adding 1 mL of the glycerinated solution to 9 mL PDB media. After incubating the infected PDB for 72 h at 25 °C, aliquots were plated on PDA Petri plates to harvest spores. The experiments were performed with these spores.

3.3.2.4 Screening of antifungal properties by overlay method

The first screening of antifungal activity was performed using the bacterial isolates grown overnight in 10 mL of MRSb at 37 ºC. Then, the overlay assay was used to determine the antifungal activity of LAB isolates (Jesper & Johan, 2001). Bacterial suspensions (10 μL) were inoculated on MRSa plated and incubated at 37

ºC for 48 h. Afterward, the agar plates were filled with 10 mL of soft (7% agar) PDA containing 10^6 conidia/mL and were incubated at 30 ºC. The inhibition growth zone was determined 48 h later and measured on a cm scale. The experiments were conducted in triplicate.

3.3.2.5 Identification of isolate by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) fingerprinting

LAB was identified by extracting the isolated cultures according to Maier et al. (2006). The MALDI-TOF MS was a Microflex L20 (Bruker Daltonics, Billerica, MA, USA) mass spectrophotometer equipped with an N_2 laser. The sample spectra were acquired in positive linear ion mode, voltage acceleration of 20 kV, and the mass ranged from 2,000 to 20,000 Da. Moreover, the spectra were obtained in triplicate as suggested by MALDI Biotyper Realtime Classification. The identification of the samples was the spectra of the largest log score organism. Finally, the results were compared with the MBT 7854 y MBT 7311_RUO database (Bruker Daltonics).

3.3.2.6 Preparation of the bacteria-free supernatant (BFS) and development of a postbiotic antifungal ingredient

Meat broth (MB) was formulated by modifying the composition of the MRSb, as plotted in supplementary material **Table S1**. Meat extract and yeast extract were substituted with freeze-dried pork loin at different concentrations: 2.00 (MB2), 4.00 (MB4), 8.00 (MB8), and 10.00 (MB10) g/L. The ingredients were mixed and dissolved in distilled water for 10 min, and the pH was adjusted to 6. Then, the media were autoclaved at 121 ºC for 21 min. Afterward, the isolated LAB were defrosted and incubated in MRSb at 37 ºC for 12 h to reach the exponential growth phase. Subsequently, the LAB were placed at 10^7 CFU/mL in 50 mL of the MB and incubated at 37 ºC for 48 h. After fermentation, the LAB were mechanically separated by centrifugation at 4,000× *g* for 10 min to separate the BFS. Finally,

BFS were kept at -80 ºC for 24 h and freeze-dried to obtain the postbiotic antifungal ingredient (de Melo Nazareth et al., 2019). Freeze-dried BFS of fermented MRSb was carried out as a control group.

3.3.2.7 Determination of sensitivity of food-borne toxigenic fungi to bacteria isolates

3.3.2.7.1 In vitro antifungal activity of samples by agar diffusion method

The agar diffusion test was used initially to determine the sensitivity of food spoilage and toxin-producing fungi to the BFS. The method was previously described by Fredua-Agyeman et al. (2019) with some modifications.

Petri dishes containing PDA were infected with collected fungal conidial grown on PDA plates. The conidia of the fungal strains described in section 2.3 were scraped off the agar and distributed on another PDA plate using sterile cotton swabs. Then, using sterile pipette tips, 10 mm diameter wells were created, and each well was filled with 100 µL of freeze-dried BFS resuspended in sterile water at a concentration of 250 g/L. A sterile water solution containing freeze-dried MRSb was elaborated as a control. After that, the PDA plates were incubated at 25 ºC for 72 hours to enable either FG or BFS diffusion through the agar. Finally, the antifungal activity was determined by measuring the inhibition zone around the well on a cm scale.

3.3.2.7.2 Microdilution Susceptibility Test to Freeze-Dried BFS

To establish the MIC and the MFC, the microdilution test was carried out in 96-well microplates following the CLSI M38-A2 (2008) criteria, although with a few changes. Firstly, the microplates were filled with 100 µL of PDB. Next, plates were filled with PDB containing freeze-dried BFS at a concentration of 340 g/L and then serially diluted 2-fold to reach the final concentration of 0.33 g/L. A volume of 100 µL of PDB containing $5x10^3$ conidia/mL of the toxigenic fungi described by

section 2.3 was added to each well. The positive control consisted of contaminated PDB with fungal spores, whereas the negative control used PDB that had not been infected. Then, the microplates were incubated for 72 h at 25 ºC. Each dose was tested in four replicates, and the experiment was carried out two times (n=8).

Three parameters were determined: MIC50, MIC100, and MFC. After the incubation period, the MIC50 was established as the minimum concentration at which the BFS inhibited 50% of the fungal colonies, whereas the MIC100 was defined as the minimum concentration of BFS at which no growth was seen in the microplate. The MFC was then determined by reculturing 10 µL of the concentration matching the MIC100 and higher concentrations assessed on PDA plates. After 72 hours of incubation at 25 ºC, the MFC concentration that prevented FG was determined. The plates were rechecked after seven days of incubation.

3.3.2.8 Extraction and quantification of organic acids

The organic acids were extracted from the fermented MRSb and MB10, according to Özcelik et al. (2016). First, 5 mL of the BFS was aliquoted into a separated tube and treated with 1 mL of metaphosphoric acid. After 2 minutes of homogenization, the tubes were centrifuged at 4,000× g for 10 min. The BFS was filtered using a membrane filter (0.45 µm). Finally, the BFS was diluted in water: formic acid (0.1%) and injected into an HPLC system.

The system applied for the chromatographic determination was an Agilent 1100 (Santa Clara, CA) HPLC equipped with an autosampler, binary pump, and vacuum degasser. Results were expressed in g/L. The column used as the stationary phase was a Rezex™ ROA-Organic Acid H+ (8%) (150 x 7.8 mm, Ea Phenomenex®, Torrance, California, USA). The mobile phases consisted of water as solvent A and ACN as solvent B, both acidified with 0.1% formic acid and eluted using the gradient (5% B at 0 min, 95% B at 30 min, and 5% at 35 min). Each analysis began with a 3-minute equilibration of the column. The flow rate was set at 0.3 mL/min, and the

sample volume was set to 20 µL. The photo diode array detector (Agilent G1315B) was applied with at 210 nm to monitor the chromatogram.

Individual stock solutions of each examined organic acid were produced by dissolving the compounds in the mobile phase. At least six-point calibration curves were created for acetic and lactic acid. The concentrations of organic acids were determined by comparing the peak area to the calibration curve standard area. Results were expressed in g/L.

3.3.2.9 Extraction and quantification of phenolic acids

The phenolic acids of the fermented MRSb and the MB10 were purified using the QuEChERS extraction described by Brosnan et al. (2014). In 50-mL tubes, a solution of 4 g $MgSO_4$, 1 g NaCl, and 10 mL ethyl acetate with 1% formic acid (v/v) were added, followed by the addition of 10 mL of the BFS. For 1 min, the tubes were vortexed and cooled on ice. Next, the solution was centrifuged at 4 °C and 4,000× g for 10 min. The supernatant was combined with 900 mg of $MgSO_4$ and 150 mg of C18 and vortexed for 1 min before centrifuging as previously described. Finally, the samples were filtered with a 0.22 µm pore filter and evaporated under nitrogen flow.

The phenolic acids analysis was performed in an Agilent 6450 Ultra High-Definition Accurate Mass QTOF-MS equipment, coupled to an Agilent Dual Jet Stream Electrospray Ionization (ESI) interface in negative ionization mode under the conditions described by Denardi-Souza et al. (2018). MassHunter Qualitative Analysis software version B.08.00 was used to handle data integration and elaboration. The results were expressed in µg/L.

3.3.2.10 Volatile Organic Compounds (VOCs) determination

VOCs emitted by the bacteria after fermentation in MRSb and MB10 media were determined by head-space solid phase microextraction (HS-SPME) technique

and subsequent analysis in a gas chromatograph coupled to a mass spectrometer (GC-MS) following the methodology of Luz et al. (2021b) with minor modifications.

For this purpose, 10 mL of the BFS, obtained as described in section 2.6, was placed in a 20 mL glass vial. The VOCs were extracted for 45 min at 50 °C with a divinylbenzene/carbon wide range/polydimethylsiloxane (DVB/C-WR/PDMS) coated fiber (80 µm x 10 mm) (Agilent Technologies, Santa Clara, CA, USA). Then, the fiber was desorbed for 10 min at 250 °C in splitless mode on an Agilent 7890A gas chromatograph coupled to an Agilent 7000A triple quadrupole mass spectrometer equipped with an electron impact (EI) source. The column used for chromatographic separation was an HP-5MS (30 m x 0.25 mm, 0.25 µm) (Agilent Technologies). The temperature ramp was programmed as follows: 40 °C held for 2 min and raised to 160 °C at 6 °C/min; then ramped up to 260 °C at 10 °C/min and held for 4 min. The carrier gas was Helium (99.99%) at a flow rate of 2.5 mL/min. Compound detection was performed in Full Scan mode in an *m/z* range of 40-450 Da.

The compounds were identified by comparison of their mass spectra with those recorded in the NIST 09 library. In addition, linear retention indices (LRI) were calculated based on the retention time of a solution of alkanes (C8-C20) tested under the same conditions as the samples and compared with the existing literature.

3.3.2.11 Statistical analysis

For statistical analysis, GraphPad Prism version 3.0 software was employed. The differences ($p \leq 0.05$) between de groups of the organic acids and phenolic acids composition were analyzed by a one-way ANOVA followed by the Tukey post hoc test for multiple comparisons.

3.3.3 Results and discussion

3.3.3.1 Isolation, screening, and identification of antifungal bacteria strains

A total of 102 bacteria were isolated from different handmade dry Spanish sausages. Among these, 42 isolates were classified as LAB since they were gram-positive, could grow in micro-aerobic conditions, and lacked catalase activity (**Table 3.3.1**). Further characterization of bacteria strains highlighted that these isolates have grown in the following conditions: temperature ranging from 15 to 45 ºC; pH ranging from 3.5 to 6; and growth in MRS-NaCl at 3, 6.5, and 10%. Therefore, these bacteria were selected, and after the characterization, a first screening was performed using the overlay approach to assess the antifungal properties of the 42 bacteria isolated; they were assayed against six spoilage fungal strains *in vitro* that commonly contaminate cured sausage products. As presented in **Table 3.3.2**, only 14 bacteria isolates (C11, C12, C13, C15, C20, C28, C56, C58, C60, C66, C69, C71, C72, and C79) demonstrated inhibitory activity against all fungi examined, with varying degrees of inhibition depending on the fungal strain tested. In particular, *P. griseofulvum* CECT 2605–T and *P. commune* CECT 20767 were the most susceptible strains, with inhibition zones ranging from 1.5-3.0 cm and 1.0-3.0 cm, respectively. In contrast, *Aspergillus* strains (*A. parasiticus* CECT 2681 and *A. flavus* ITEM 8111) were the most resistant to the LAB, with inhibition zones ranging from 0.3-1.7 cm. An example of overlay methodology is given in **Figure 3.3.1**. These findings agree with those obtained by previous authors (Russo et al., 2017a), which noticed that *Aspergillus* strains are more resistant to LAB than other fungal genera.

The 14 gram-positive bacteria with antifungal activity against all fungal strains were identified by MALDI-TOF-MS and classified regarding the MTB 7854 and MBT 7311_RUO databases (Bruker Daltonics). The classification to species level was the following: *Pediococcus pentosaceus* C11, *P. pentosaceus* C12, *P. pentosaceus* C13, *P. pentosaceus* C15, *Lactiplantibacillus plantarum* C20, *P. pentosaceus* C28, *P. pentosaceus* C56, *P. pentosaceus* C58, *L. plantarum* C60, *P. pentosaceus* C66, *P. pentosaceus* C69, *P. pentosaceus* C71, *P. pentosaceus* C72, and *P. pentosaceus* C79. Thus, the bacteria identified with potential inhibitory properties

were studied to develop an antifungal ingredient against dry-cured meat spoilage agents.

Table 3.3.1. Characterization of LAB isolated from dry-cured sausages.

Preliminary characterization				Growth at temperature	Growth in pH	Growth in MRS-NaCl (%)	
Bacteria	Morphology	Gram*	Catalase	15 to 45 °C	3.5 to 6	3 to 6.5	10
C1	Coccoid	P	-	+	+	+	-
C4	Coccoid	P	-	+	+	+	-
C5	Coccoid	P	-	+	+	+	-
C6	Coccoid rod	P	-	+	+	+	-
C11	Coccoid	P	-	+	+	+	-
C12	Coccoid	P	-	+	+	+	-
C13	Coccoid	P	-	+	+	+	-
C14	Coccoid rod	P	-	+	+	+	+
C15	Coccoid	P	-	+	+	+	+
C16	Rod-shaped	P	-	+	+	+	+
C17	Rod-shaped	P	-	+	+	+	+
C19	Coccoid	P	-	+	+	+	+
C20	Rod-shaped	P	-	+	+	+	+
C27	Rod-shaped	P	-	+	+	+	+
C28	Coccoid	P	-	+	+	+	+
C29	Rod-shaped	P	-	+	+	+	+
C30	Rod-shaped	P	-	+	+	+	+
C31	Coccoid	P	-	+	+	+	+
C35	Coccoid	P	-	+	+	+	-
C36	Coccoid	P	-	+	+	+	+

*P means positive; + signifies visual growth, and – means no visual growth.

Table 3.3.1. *Continuation.*

Preliminary characterization				Growth at temperature	Growth in pH	Growth in MRS-NaCl (%)	
Bacteria	Morphology	Gram*	Catalase	15 to 45 °C	3.5 to 6	3 to 6.5	10
C37	Coccoid	P	-	+	+	+	+
C38	Coccoid	P	-	+	+	+	-
C39	Coccoid	P	-	+	+	+	+
C44	Coccoid	P	-	+	+	+	+
C45	Coccoid	P	-	+	+	+	+
C46	Coccoid rod	P	-	+	+	+	-
C47	Coccoid	P	-	+	+	+	+
C48	Coccoid	P	-	+	+	+	+
C50	Coccoid	P	-	+	+	+	+
C56	Coccoid	P	-	+	+	+	+
C57	Coccoid	P	-	+	+	+	+
C58	Coccoid	P	-	+	+	+	+
C59	Coccoid	P	-	+	+	+	-
C60	Rod-shaped	P	-	+	+	+	+
C66	Coccoid	P	-	+	+	+	+
C68	Coccoid	P	-	+	+	+	+
C69	Coccoid	P	-	+	+	+	+
C70	Coccoid	P	-	+	+	+	-
C71	Coccoid	P	-	+	+	+	+
C72	Coccoid	P	-	+	+	+	+
C79	Coccoid	P	-	+	+	+	+
C80	Coccoid	P	-	+	+	+	+

*P means positive; + signifies visual growth, and – means no visual growth.

Table 3.3.2. Antifungal activity of LAB by overlay technique.

Selected Bacteria	*A. parasiticus*	*A. flavus*	*P. commune*	*P. verrucosum*	*P. griseofulvum*	*P. nordicum*
	Inhibition zone (cm)					
C1	0.0	0.0	2.4	2.0	3.0	1.0
C4	0.0	0.1	1.5	1.7	3.0	0.9
C5	0.3	0.0	2.5	1.4	3.0	1.0
C6	0.3	0.0	2.7	1.7	3.0	1.1
C11	1.0	0.4	3.0	2.0	3.0	1.4
C12	0.5	0.3	3.0	1.7	2.3	1.3
C13	0.7	0.6	3.0	1.8	2.1	1.0
C14	0.5	0.0	2.0	1.4	3.0	1.1
C15	0.4	0.6	3.0	1.6	3.0	1.2
C16	0.5	0.0	3.0	1.5	0.6	0.7
C17	0.3	0.0	0.6	2.0	3.0	0.8
C19	0.3	0.0	1.6	1.7	3.0	1.0
C20	0.8	0.9	3.0	1.7	1.9	1.3
C27	0.5	0.0	0.9	1.7	1.3	0.5
C28	1.0	0.8	3.0	1.7	1.8	1.3
C29	0.4	0.0	2.9	1.7	1.2	0.9
C30	0.6	0.0	3.0	1.4	1.0	0.9
C31	0.5	0.0	3.0	1.7	1.8	0.9
C35	0.4	0.0	3.0	1.7	1.6	1.1
C36	0.0	0.0	2.7	1.6	3.0	1.0
C37	0.4	0.0	3.0	2.5	1.0	0.8
C38	0.2	0.0	2.4	1.5	3.0	1.1
C39	0.4	0.0	0.0	1.8	1.6	0.4

Table 3.3.2. *Continuation.*

Selected Bacteria	*A. parasiticus*	*A. flavus*	*P. commune*	*P. verrucosum*	*P. griseofulvum*	*P. nordicum*
	Inhibition zone (cm)					
C44	2.0	0.0	1.4	1.7	3.0	1.0
C45	0.4	0.0	1.1	1.6	3.0	0.8
C46	0.2	0.0	0.5	2.0	3.0	0.7
C47	0.3	0.0	3.0	1.6	2.5	1.1
C48	0.5	0.0	3.0	1.7	1.5	1.1
C50	0.9	0.0	2.5	1.5	1.8	0.8
C56	1.5	1.2	3.0	2.1	1.6	1.1
C57	1.0	0.0	2.8	1.8	0.2	0.6
C58	1.5	1.4	3.0	1.5	3.0	1.5
C59	0.5	0.0	0.6	2.0	1.5	0.5
C60	1.2	1.7	1.6	1.5	2.5	1.2
C66	1.0	1.1	1.0	2.1	0.8	0.6
C68	1.0	0.0	3.0	2.2	2.5	1.1
C69	0.4	0.5	3.0	2.5	3.0	1.1
C70	0.0	0.0	3.0	1.3	3.0	1.2
C71	1.5	1.2	2.6	1.5	3.0	1.3
C72	1.0	1.1	3.0	1.5	1.5	0.9
C79	0.4	0.3	2.7	1.7	3.0	1.1
C80	0.0	0.0	2.7	1.7	3.0	1.0

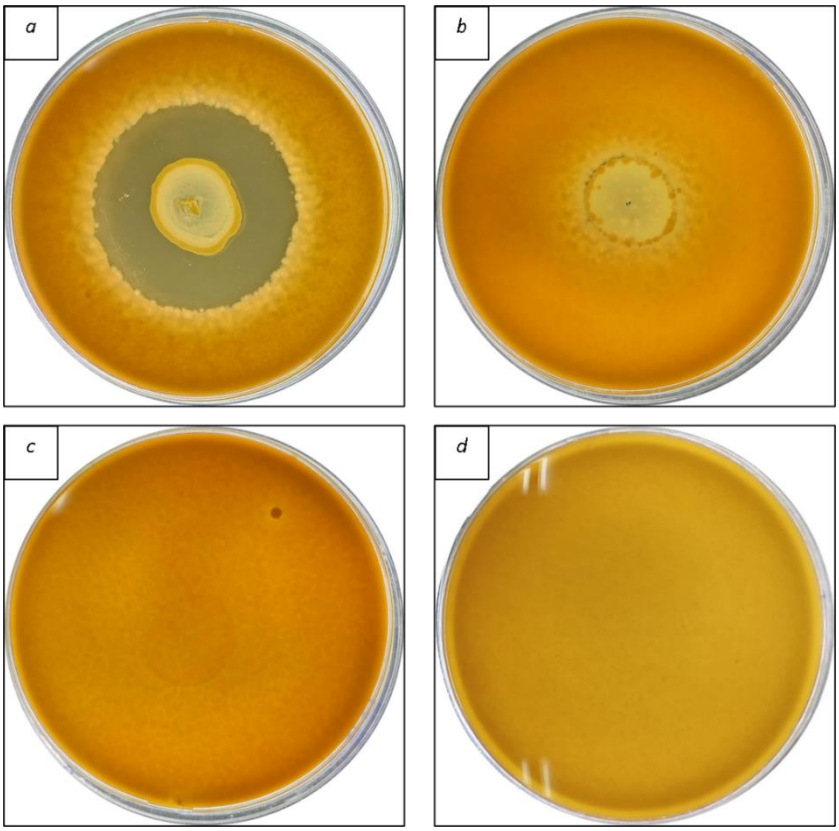


Figure 3.3.1. Screening of lactic acid bacteria (LAB) through overlay method. Strong inhibitory action (a); slightly inhibitory effect (b); no inhibitory action (c); control (d).

The LAB with antifungal activity are poorly documented, while more attention has been exploited its antibacterial activity. The LAB that produces bacteriocins are isolated from a severe category of food. For example, Delcarlo et al. (2019) isolated 22 LAB with antimicrobial action from mussels on the Argentina coast. Parlindungan et al. (2021) identified novel probiotic candidates from fermented meats and characterized them by bacteriocin production. Furthermore, Ivanovic et al. (2021) isolated, identified, and characterized antibacterial LAB from traditional cheese.

Only a few studies from scientific literature cite a broad spectrum of antifungal activity for LAB. Most of them showed higher strain-specific antifungal activity against one or two mold species (Hernández et al., 2022). For this reason, one of the goals of this study is to isolate a large-spectrum strain. In this work, we observed that antifungal activity was strain-dependent, as well as fungal species and methodologies analyzed. Moreover, it seems that dry-cured sausages can be used as a potential source of antifungal LAB because they have significant antagonistic properties against the microorganisms tested.

3.3.3.2 In vitro antifungal study of the MB formulated

Two methods were performed to determine the fungal sensitivity to bacterial isolates: agar diffusion test and microdilution of BFS in 96-well plates.

The MB was formulated by changing the composition of the MRSb by subtracting the yeast extract and meat extract and adding freeze-dried pork loin as a protein source at 2.00, 4.00, 8.00, and 10.00 g/L (MB2, MB4, MB8, and MB10, respectively). These new formulations were fermented with the 14 bacteria strains identified in **section 3.3.3.1**, and a first qualitative evaluation was performed using the agar-diffusion test to assess the antifungal properties. Moreover, the fermented MRSb was also prepared with the same bacteria strains to compare the effectiveness of the newly formulated ingredient.

After fermentation, only the MB10 exhibited antifungal activity similar to the fermented MRSb used as a control reference. As presented in **Table 3.3.3**, the inhibition halos obtained varied according to the fungal strain tested and the bacteria used as inoculum. In particular, the most sensitive strains to the MB10 were *P. commune* CECT 20767, *P. nordicum* CECT 2320, and *P. verrucosum* VTT–D01847. These strains were susceptible to all the formulated MB, with inhibition halos observed on the PDA plates ranging from 0.2 cm (+) to inhibition halos greater than 0.4 cm (+++). The fungal strains with the most significant resistance were those of the *Aspergillus* genera, in which only *Pediococcus pentosaceus* C12, *Pediococcus*

pentosaceus C15, and *Lactiplantibacillus plantarum* C60 showed inhibitory activity. This finding agrees with the previous characterization of the LAB isolates by overlay method since *Aspergillus* strains showed higher resistance to the LAB than *Penicillium* strains. For this reason, the formulation MB10 and *P. pentosaceus* C12, *P. pentosaceus* C15, and *L. plantarum* C60 isolates were selected, and the preliminary results of the antifungal activity were confirmed through the MIC50, MIC100, and MFC determination *in vitro*. The MB10 and MRSb fermented for 48 h obtained the highest inhibition halo zone and the antifungal effect of MB10 fermented for 24, 48 and 72 h are plotted in **Table S2**.

Table 3.3.3. Antifungal activity of Bacterial free supernatant (BFS) extract in agar diffusion method. LAB fermented MRS broth (MRSb) and a Meat broth (MB10) for 48 h at 37 ºC. Then, the supernatant was freeze-dried and resuspended at a concentration of 250 g/L. These bacteria were previously selected due to their antifungal effect in the overlay method.

Antifungal activity of the BFS of the fermented MRS broth and the Meat Broth (MB10)

LAB	Fungal strain											
	A. flavus		*A. parasiticus*		*P. commune*		*P. griseofulvum*		*P. nordicum*		*P. verrucosum*	
	MRSb	MB10	MRSb	MB10	MRSb	MB10	MRSb	MB10	MRSb	MB10	MRSb	MB10
C11	+	-	-	-	+	+	+	+	+	+	+	+
C12	+	+	++	+	++	++	++	++	+	+	++	++
C13	-	-	+	-	+	+	+	+	+++	+++	+++	+++
C15	++	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++
C20	-	-	+	-	+	+	+	+	++	++	+	+
C28	+	-	-	-	+	+	+	+	+++	+++	+++	+++
C56	-	-	+	-	+	+	+	+	+	+	+	+
C58	-	-	-	-	+	+	++	++	+++	+++	+++	+++
C60	+	+	++	++	++	++	++	++	+++	+++	++	++
C66	+	-	-	-	++	++	+++	+++	++	++	+++	+++
C69	-	-	+	-	+	+	-	-	++	++	+	+
C71	-	-	+	-	+	+	-	-	+	+	+	+
C72	+	-	-	-	++	+	++	++	++	++	+++	+++
C79	+	-	-	-	++	+	++	++	++	++	+++	+++

(+) Represents a growth inhibition halo of 0.2 cm; (++) represents a growth inhibition halo of between 0.2 to 0.4 cm; (+++) represents a growth inhibition halo greater than 0.4 cm.

Similar findings In literature indicated that a range of theories might explain LAB's antifungal activities; Schnürer & Magnusson (2005) found that the suppression of mold growth on an agar plate is the consequence of a complex interaction of multiple chemicals and metabolites, all of which contribute to the overall antifungal activity.

The microdilution assay demonstrated that the BFS of the fermented MRSb was the most active extract against all fungi tested, regardless of the bacteria isolated (**Table 3.3.4**). The MIC50 obtained ranged from 5-21 g/L, the MIC100 ranged from 21-85 g/L, and the MFC ranged from 21-170 g/L. However, for MB10 formulation, the MIC50, MIC100, and MFC parameters ranged from 11-43 g/L, 21-85 g/L, and 43-170 g/L, respectively. It is important to underline that the BFS of the MRSb and the MB10 fermented by *P. pentosaceus* C15 showed the lower concentrations required to achieve FG against all the toxigenic strains tested compared to *P. pentosaceus* C12 and *L. plantarum* C60 fermented BFS. Although the BFS of the MRSb evidenced a higher antifungal effect, a poor difference was evidenced when compared to MB10. In some cases, there were no significant differences ($p \leq 0.05$) between the treatments, e.g., MB10 fermented by *P. pentosaceus* C15 had no statistical difference to MRSb comparing the average values of MIC100 and MFC (**Table 3.3.4**). In other words, *P. pentosaceus* C15 was able to ferment MB10 and produce antifungal compounds, which inhibited the FG at similar conditions to MRSb. Besides, MRSb fermented by *P. pentosaceus* C15 obtained MIC50, MIC100, and MFC values lower than *L. plantarum* C60 and *P. pentosaceus* C12, regardless of the fungal strain essayed.

Table 3.3.4. Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of Bacterial-free supernatant (BFS). MRS broth (MRSb) and Meat Broth (MB10) were fermented by LAB for 48 h at 37 °C and centrifuged to obtain the BFS.

	BFS concentration (g/L) of the fermented MRSb								
	Lactiplantibacillus plantarum C60			*Pediococcus pentosaceus* C12			*Pediococcus pentosaceus* C15		
Fungi	MIC[1]	MIC[2]	MFC	MIC[1]	MIC[2]	MFC	MIC[1]	MIC[2]	MFC
A. flavus	11	85	170	21	85	170	11	21	85
A. parasiticus	11	43	170	11	43	170	11	21	85
P. commune	21	43	85	21	43	85	11	21	85
P. griseofulvum	21	43	170	21	43	170	5	21	170
P. nordicum	11	43	85	11	43	85	5	21	21
P. verrucosum	21	43	85	21	43	85	11	43	43
Mean	16	50	128	18	50	128	9	25	82
	BFS concentration (g/L) of the MB10								
Fungi	MIC[1]	MIC[2]	MFC	MIC[1]	MIC[2]	MFC	MIC[1]	MIC[2]	MFC
A. flavus	21	85	170	43	85	170	21	43	85
A. parasiticus	21	43	170	21	43	170	21	43	85
P. commune	43	85	85	43	85	85	21	21	85
P. griseofulvum	43	85	170	43	85	170	11	21	170
P. nordicum	21	43	85	43	85	85	11	21	43
P. verrucosum	43	85	85	43	85	85	11	43	43
Mean	32*	71	128	39*	78*	128	16*	35	85

(1): 50% of visual inhibition growth; (2): 100% of visual inhibition growth. (*): results were statistically analyzed by the t-test, comparing the Bacterial free supernatant (BFS) of MRSb with the BFS of MB10. Means are significantly different if $p \leq 0.05$

The MRSb was applied in both experiments as a control group since previous studies have demonstrated its antifungal activity while fermented by LAB and producing antimicrobial substances. For instance, Taroub et al. (2019) identified *P. pentosaceus* and *L. plantarum* strains that showed good antifungal activity against *A. niger* and *A. carbonarius*. In addition, the strains showed a high capacity for the degradation of OTA, one of the main toxins produced by these fungi. However, the authors could not identify the compounds responsible for the antifungal and

antitoxigenic activity of the strains. In other studies, the application of MRS fermented by *L. plantarum* inhibited the *A. flavus and F. verticillioides* growth and reduced the production of aflatoxin and fumonisin in cereals such as maize (de Melo Nazareth et al., 2019).

Therefore, MRSb has proven to be an interesting medium for fermenting bacteria as antimicrobial metabolites are produced. However, the use of MRSb has some disadvantages, such as its high cost and the complexity of some of their ingredients that convert MRSb might not be allowed as an ingredient in meat products. Therefore, one of the objectives of the work was to develop a meat broth with similar antifungal characteristics but, at the same time, that could be incorporated as an antifungal ingredient.

Overall, the *P. pentosaceus* C15 showed higher antifungal potential in the agar diffusion test and microdilution of BFS; hence, these results led us to select this strain for the fermentation of MB10 and elaborate a postbiotic antifungal ingredient.

3.3.3.3 Chemical characterization of the postbiotic antifungal ingredient

3.3.3.3.1 Organic and phenolic acid production

The metabolites generated after fermentation of the antifungal strains selected were investigated in the BFS of the MRSb and MB10. As plotted in **Figure 3.3.2**, two organic acids, lactic acid and acetic acid, were identified in both formulations. For lactic acid, the mean values of this metabolite ranged from 2.49-3.00 g/L, whereas for acetic acid, the concentration detected was lower, with mean values ranging from 0.24-0.38 g/L. The highest amount of lactic acid was detected in the fermented MRSb (2.973 g/L) and MB10 (3.00 g/L) fermented by the *P. pentosaceus* C15 strain. There were no statistical differences in lactic acid production between these two formulations ($p \leq 0.05$). Regarding acetic acid, the higher concentration ($p \leq 0.05$) was also quantified in the fermented MB10 with *P.*

pentosaceus C15 (0.38 g/L), followed by the MRSb of *P. pentosaceus* C15 (0.32 g/L) and the fermented MRSb with *L. plantarum* C60 (0.31 g/L).

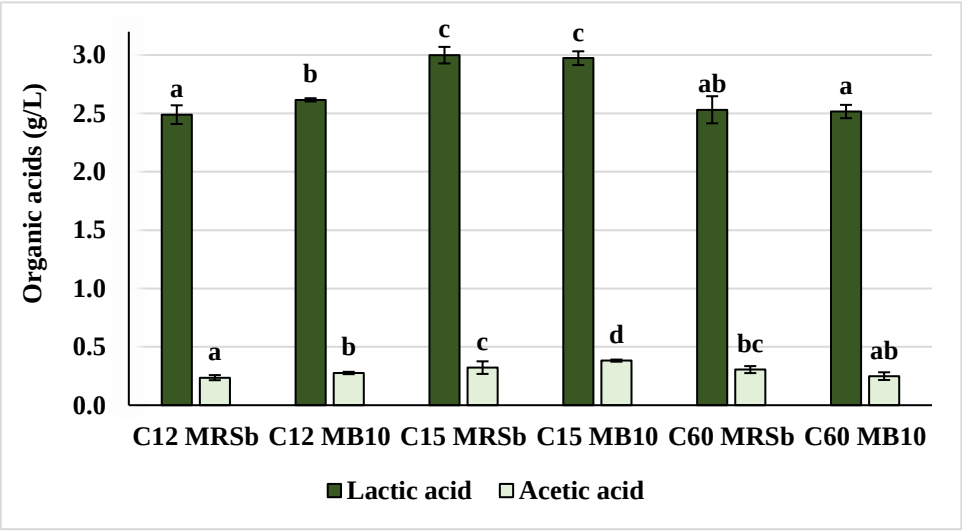


Figure 3.3.2. The concentration of organic acids produced by LAB in meat broth (MB10) and MRS broth (MRSb) after incubation for 48 h at 37 ºC. The meat broth was prepared with 10% freeze-dried loin pork and fermented by *P. pentosaceus* C12, *P. pentosaceus* C15, and *L. plantarum* C60.

Concerning the phenolic acids, a total of 12 different metabolites, described in the literature as antifungals, were identified and quantified through the UHPLC Q-TOF MS technique. Among them, it is noteworthy that the concentration of benzoic acid, PLA, syringic acid, and vanillic acid stood out regardless of the fermented broth and bacteria tested (**Table 3.3.5**). In general, it could be noted that MRSb produced higher concentrations of phenolic acids compared to its analog MB10. However, in most cases the difference was not significant ($p \leq 0.05$). In view of these results, we suggest further studies to evaluate the synergistic effect of these four compounds in order to evaluate their antifungal activity and to increase the understanding about the mechanism of antimicrobial action of LAB.

The selected microorganisms grew on the MB10 and produced a broad variety of metabolites previously described in the literature as antimicrobial compounds. The phenolic acid most produced by *P. pentosaceus* C12 in MB10 was syringic acid, showing values of 19.47 µg/L what was significantly higher than bacteria C60. Aziz et al. (1998) demonstrated that syringic acid at 300 mg/L inhibited the *A. flavus and A. parasiticus* growth and the aflatoxin production. In addition, Ren et al. (2009) demonstrated that lower doses of syringic acid (100 mg/L) avoided the growth of *A. niger*. Therefore, it seems that higher concentration of phenolic acids is demanded to achieve an antifungal effect when they are applied in isolation, so that these findings reinforce the hypothesis that LAB antifungal effect is obtained via a synergistic effect.

Table 3.3.5. The concentration of phenolic compounds in Man, Rogosa and Sharpe broth (MRSb) and Meat broth (MB10) fermented by antifungal LAB. The broths were fermented for 48 h at 37 °C.

Phenolic compound	**The concentration of phenolic compounds Mean ±SD (µg/L)**					
	Pediococcus pentosaceus C12		*Pediococcus pentosaceus* C15		*Lactiplantibacillus plantarum* C60	
	MRSb	MB10	MRSb	MB10	MRSb	MB10
1,2–Dihydroxybenzene	9.39 ± 2.02^{A}	2.51 ± 0.41^{B}	5.86 ± 2.74^{CD}	5.64 ± 2.22^{CD}	7.12 ± 1.3^{D}	4.64 ± 1.39^{C}
Propionic acid	7.14 ± 1.79^{A}	7.08 ± 1.34^{A}	9.66 ± 2.64^{A}	9.36 ± 0.62^{A}	6.19 ± 2.85^{A}	13.44 ± 5.58^{B}
Benzoic acid	31.65 ± 4.42^{A}	18.09 ± 5.35^{B}	24.69 ± 0.72^{C}	22.12 ± 7.32^{BC}	19.38 ± 5.91^{BC}	23.18 ± 0.35^{BC}
Caffeic acid	4.07 ± 1.46^{A}	3.99 ± 2.46^{A}	8.37 ± 1.26^{B}	7.72 ± 1.87^{BC}	3.40 ± 0.97^{A}	6.27 ± 1.55^{C}
DL–3–Phenyllactic acid	18.17 ± 3.52^{A}	18.30 ± 1.54^{AB}	31.90 ± 7.49^{C}	22.68 ± 3.25^{B}	16.98 ± 3.66^{A}	15.85 ± 0.61^{A}
Ferulic acid	N.D	N.D	4.79 ± 1.81^{AB}	5.85 ± 4.02^{AB}	3.70 ± 0.72^{A}	6.18 ± 1.26^{B}
Gallic acid	N.D	N.D	8.04 ± 2.83^{A}	6.06 ± 1.88^{A}	6.58 ± 2.05^{A}	7.44 ± 1.25^{A}
p–Coumaric acid	22.59 ± 9.91^{A}	9.37 ± 1.72^{B}	6.28 ± 0.07^{B}	7.56 ± 0.44^{B}	5.29 ± 1.47^{B}	4.99 ± 2.03^{B}
Sinapic acid	4.87 ± 1.04^{AB}	6.43 ± 0.44^{AC}	5.85 ± 2.38^{AD}	7.86 ± 3.66^{CD}	3.54 ± 0.86^{B}	7.22 ± 1.32^{CD}
Syringic acid	26.78 ± 3.52^{A}	19.47 ± 5.28^{B}	17.73 ± 4.12^{B}	21.12 ± 0.39^{B}	17.42 ± 1.40^{B}	12.83 ± 3.40^{C}
Vanillic acid	20.84 ± 4.56^{AC}	18.81 ± 2.82^{A}	30.83 ± 6.19^{B}	25.18 ± 1.13^{D}	27.60 ± 2.94^{BD}	24.00 ± 2.12^{CD}
Vanillin	11.68 ± 1.26^{A}	13.76 ± 1.08^{A}	16.85 ± 5.47^{A}	13.73 ± 1.72^{A}	7.57 ± 1.65^{B}	8.46 ± 1.51^{B}

Different letters mean a significant statistical difference in the concentration of phenolic compounds among fermented broths ($p \leq 0.05$)

Regarding *P. pentosaceus* C15 and *L. plantarum* C60, vanillic acid was the most produced phenolic acid, reaching values of 25.18 and 24.00 µg/L in MB10 and 30.83 and 27.60 µg/L in MRSb, respectively. An oxidized derivative of vanillin, vanillic acid is a monohydroxybenzoic acid composed of a 4–hydroxybenzoic acid with a methoxy group at position 3 (Ren et al., 2009). It has been noted for its antioxidant properties, but its antifungal activity is poorly reported, and more research is required. In contrast, its precursor, vanillin, has been the subject of extensive research, and its antifungal activity varies depending on the microorganism tested (Fitzgerald et al., 2005; Pei et al., 2021).

These findings also suggest that the antifungal ingredient obtained from MB10 could be incorporated into meat food products to provide a significant source of phenolic and organic acids.

LAB can synthesize a wide variety of antifungal compounds such as organic acids, phenolic acids, AMPs, diacetyl and reuterin (Amiri et al., 2021). During fermentation, carbon metabolism produces organic acids such as lactic acid, acetic acid, and propionic acid (Punia Bangar et al., 2022). Among these metabolites, the most studied are organic acids. It is important to mention that these metabolites can constitute synergistic activity. However, this synergistic activity's exact mechanism is unknown (Chen et al., 2021).

The results also suggest that the combination of organic acids and phenolic acids produced by LAB could be responsible for the inhibitory activity of the spoilage fungi *in vitro*. However, it is essential to underline that the antifungal properties of the MB10 are not probably produced exclusively by organic and phenolic acids; for instance, several volatile substance can act synergistically and potentialize the antifungal properties (Garnier et al., 2020). Moreover, the decrease in pH collaborates to a more efficient antifungal activity (Cortés-Zavaleta et al., 2014). In this context, Peyer et al. (2016) found that organic acids and phenolic acids generated by some LAB strains as antifungal metabolites have synergistic actions

against *F. culmorum*. Likewise, in bakery products, organic acids and AFPs produced by LAB showed a significant synergistic effect which allowed the biopreservation and enhanced the shelf life of quinoa and rice bread (Axel et al., 2016).

In addition to synergistic compound effect, the combination of different microorganisms can also promote a positive effect. For instance, Ruggirello et al. (2019) associated yeast with LAB and obtained a great antifungal effect against strains of *Aspergillus* and *Penicillium* genera, fermenting cocoa beans. The authors described that the antifungal potential could be a result of association between organic acids of LAB with proteinaceous substances of yeasts. According to Christ-Ribeiro et al. (2019) phenolic chemicals avoid fungal development by inhibiting the production of cell wall components including glucan, chitin, and mannoproteins, as well as cell membrane components like ergosterol. This process happens by damaging the cell wall and cell membrane, affecting nutrient influx regulation. As a result, phenolic chemicals impede fungal cell production of proteins, amino acids, and sphingolipids. Moreover, they obstruct electron transit and the preservation of cellular integrity.

3.3.3.3.2 VOC analysis in MB10 formulation

The fermented Meat Broth VOCs (MB10) were analyzed through HS-SPME coupled to the GC-MS technique. A total of 24 compounds were identified through MS comparison with the NIST library and calculation of the LRI and belonged to several chemical classes: acid (2), alcohol (8), aldehyde (6), ketone (5), and pyrazine (2) (**Table S3**). Moreover, the percentage peak area (%PA) of each identified compound was calculated, and the results obtained are reported in **Table 3.3.6**.

Table 3.3.6. Percentage area (%) of the volatile organic compounds (VOCs) identified in the formulated Meat Broth (MB10) fermented with different lactic acid bacteria strains (*P. pentosaceus* C12, *P. pentosaceus* C15, and *L. plantarum* C60). Results are expressed as mean ± standard deviation.

Nº	Rt	Compound	Control	C15	C60	C12
Acid						
1	3.11	Acetic acid	n.d	13.74 ± 1.12^{A}	11.11 ± 0.62^{AB}	8.95 ± 2.63^{B}
2	15.49	Nonanoic acid	n.d	5.45 ± 1.69^{A}	6.94 ± 1.00^{A}	3.70 ± 1.13^{A}
Alcohol						
3	8.97	2–Octanol	8.12 ± 2.16^{A}	1.77 ± 0.16^{B}	0.39 ± 0.05^{B}	0.87 ± 0.28^{B}
4	11.19	1–Octanol	2.16 ± 0.07^{A}	2.70 ± 0.10^{AB}	3.03 ± 0.25^{AB}	4.16 ± 1.11^{B}
5	11.62	2–Nonanol	n.d	0.58 ± 0.08^{A}	0.53 ± 0.14^{A}	0.66 ± 0.06^{A}
6	13.43	Phenylethyl alcohol	1.24 ± 0.13^{A}	6.57 ± 0.45^{B}	1.60 ± 0.20^{A}	2.44 ± 0.88^{A}
7	13.6	1–Nonanol	1.26 ± 0.17^{A}	3.65 ± 0.05^{B}	1.95 ± 0.18^{AB}	3.80 ± 1.49^{B}
8	15.88	1–Decanol	n.d	1.22 ± 0.12^{A}	2.18 ± 0.70^{A}	1.86 ± 0.35^{A}
9	16.27	2–Undecanol	0.86 ± 0.03^{A}	1.15 ± 0.16^{A}	1.09 ± 0.03^{A}	1.76 ± 0.42^{B}
Aldehyde						
10	6.25	Heptanal	3.51 ± 0.43^{AB}	1.04 ± 0.21^{B}	2.13 ± 0.35^{B}	5.71 ± 2.66^{A}
11	8.89	Octanal	13.45 ± 2.80^{A}	3.81 ± 0.33^{B}	3.75 ± 0.63^{B}	4.98 ± 0.06^{B}
12	11.02	Benzeneacetaldehyde	20.31 ± 2.76^{A}	19.27 ± 2.39^{A}	19.82 ± 3.34^{A}	18.36 ± 3.74^{A}
13	11.42	Nonanal	1.24 ± 0.13^{A}	2.91 ± 0.53^{A}	2.43 ± 0.16^{A}	2.94 ± 1.44^{A}
14	15.82	2–Decenal	0.97 ± 0.18^{A}	0.53 ± 0.07^{A}	1.44 ± 0.13^{A}	1.47 ± 0.72^{A}
15	18.5	Dodecanal	1.28 ± 0.07^{A}	0.27 ± 0.05^{B}	0.81 ± 0.19^{AB}	0.94 ± 0.56^{AB}
Alkane						
16	10.77	Decane, 2–methyl	n.d	0.85 ± 0.05^{A}	2.42 ± 0.04^{B}	1.81 ± 0.63^{B}
17	11.69	Undecane	1.66 ± 0.41^{AB}	1..47 ± 0.31^{AB}	0.78 ± 0.22^{A}	2.21 ± 0.66^{B}
Ketone						
18	5.78	2–Heptanone	n.d	1.73 ± 0.40^{A}	2.80 ± 0.53^{A}	2.82 ± 0.72^{A}
19	8.71	2–Octanone	2.52 ± 0.45^{A}	2.89 ± 0.19^{A}	1.92 ± 0.15^{A}	2.32 ± 0.95^{A}
20	11.28	2–Nonanone	1.59 ± 0.03^{AB}	2.82 ± 0.55^{B}	0.88 ± 0.05^{A}	2.13 ± 1.28^{AB}
21	16.11	2–Undecanone	n.d	0.63 ± 0.10^{A}	1.29 ± 0.38^{B}	0.82 ± 0.12^{AB}
22	20.45	2–Tridecanone	n.d	0.60 ± 0.08^{A}	2.13 ± 0.35^{B}	1.13 ± 0.20^{A}
Pyrazine						
23	4.55	Pyrazine, methyl–	10.24 ± 1.00^{A}	5.30 ± 0.09^{B}	6.91 ± 1.73^{B}	4.34 ± 1.11^{B}
24	6.37	Pyrazine, 2,5–dimethyl	29.58 ± 2.79^{A}	19.05 ± 1.87^{B}	21.68 ± 0.80^{B}	19.79 ± 0.07^{B}

n.d= no-detected.

Aldehydes were the most abundant compounds in the samples analyzed and represented a proportion between 27.8-40.8% of the total VOCs detected (**Figure 3.3.3**). In particular, five linear aldehydes (heptanal, octanal, nonanal, 2–decenal, and dodecanal) were identified in all MB10; nonetheless, the greatest observed concentration was of an aromatic aldehyde, benzeneacetaldehyde, which %PA ranged between 18.4 and 20.3% depending on the formulation analyzed. Saturated aldehydes are lipid-derived volatiles produced mainly by the oxidation of oleic acid, a characteristic fatty acid of raw pork meat (Domínguez et al., 2019; Estévez et al., 2003; Wang et al., 2018). Regarding benzeneacetaldehyde, this compound could be synthesized using phenylalanine as a precursor through the Maillard reaction during the sterilization step of the MB10 since it is mainly detected in cooked meat (Tamura et al., 2022; Watanabe et al., 2015). It was noted that aldehyde content in the fermented MB10 was statistically lower ($p \leq 0.05$) in comparison with the control group, which corroborated the findings of Kwaw et al. (2018), which described a decline of aldehydes in mulberry juice when LAB fermentation was applied.

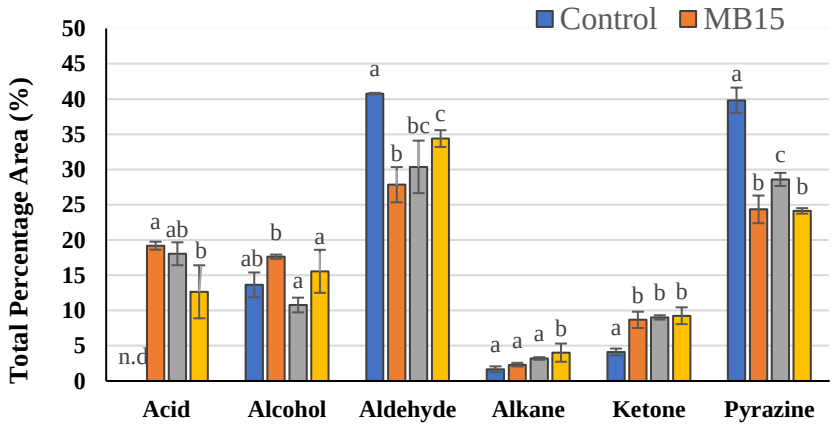


Figure 3.3.3. Total percentage area (%) of the chemical classes identified in the volatile fraction of the Meat Broth formulated with 10% of lyophilized pork loin and fermented by *P. pentosaceus* C12 (MB12), *P. pentosaceus* C15 (MB15), and *L. plantarum* C60 (MB60).

Pyrazines were the second abundant group detected in MB10 formulations (%PA ranging from 24.1 to 39.8). It was observed that the %PA statistically decreased in the fermented MB10 formulations ($p \leq 0.05$) compared to control formulations. This phenomenon was also described by Kurt et al. (2023), which evidenced that the pyrazine content in spirulina water solutions (4% w/v) was reduced after LAB fermentation.

The greatest variety of chemical compounds found in the MB10 were alcohols, representing a mean %PA value ranging from 10.8 to 17.6%. Among the alcohols identified, it was detected phenylethyl alcohol (PEA) in a higher proportion (6.6 ± 0.5 %PA) on the MB10 fermented with C15 (MB10-C15) in comparison with other formulations ($p \leq 0.05$). This active compound has been studied for its antifungal potential and could explain the lower MIC and MFC values detected in the MB10-C15, as reported previously in section 3.2. For instance, Gong et al. (2019) determined that the antifungal properties of *Enterobacter absuriae* Vt–7 were mainly due to the volatile antifungal PEA, and it effectively controlled the development of

the toxigenic fungi *A. flavus* in peanuts. Similar results were obtained by Wonglom et al. (2019), which associated the antifungal potential of *Trichoderma* sp. T76–12/2 against *Sclerotium* fruit rot due to the synthesis of PEA and other VOCs.

Regarding ketones, LAB not only significantly increased ($p \leq 0.05$) the ketone levels in the formulated MB10 but also introduced three new ketones that were not present in the control group, such as 2–heptanone, 2–undecanone, and 2–tridecanone (**Table 3.3.6**). Some ketones may be synthesized through microbial oxidation of fatty acids, and this could explain its higher proportion in the MB10 formulations when compared to the control formulation (MB10 without fermentation) (Chen et al., 2019)**.**

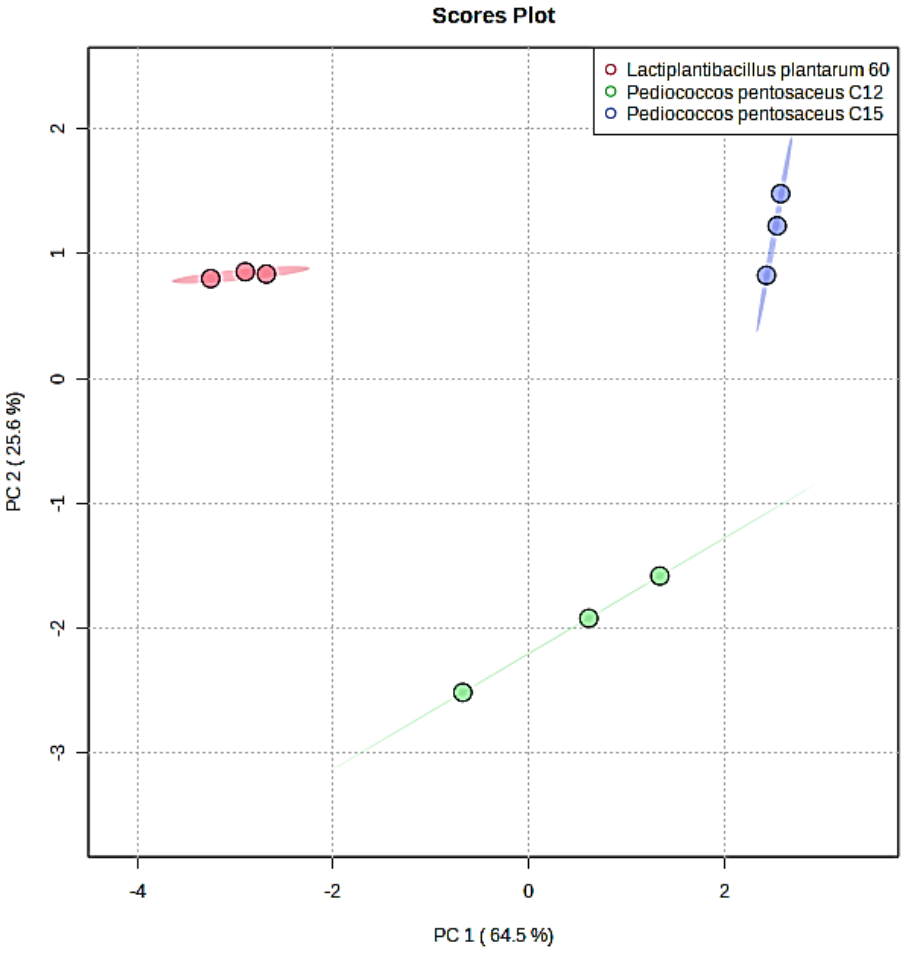
Only two acids, acetic acid and nonanoic acid were identified in the fermented MB10. Nonanoic acid is produced mainly from the degradation of unsaturated fatty acids such as oleic acid, whereas acetic acid is produced because of the heterofermentative metabolism of LAB (Pang et al., 2021). Previous studies conducted have observed an increase in these chemical compounds when LAB are employed in different food matrices such as pumpkin and watermelon juices (Mandha et al., 2021; Sun et al., 2022). Regarding its biological properties, it is important to emphasize that nonanoic acid has evidenced antimicrobial properties against several pathogens such as *Alternaria alternata*, *Botrytis cinerea* (Chen et al., 2020), *Candida albicans* (Lee et al., 2021), *Salmonella enterica* (White et al., 2021), and *Escherichia coli* O157:H7 (Cimowsky et al., 2022). Thus, volatile acids combined with the other biological cmpounds found in the MB10 (such as PEA, organic acids, and phenolic acids) could contribute to the antifungal properties of the formulated ingredient since LAB antifungal potential is related to the synergistic action of the different metabolites synthesized (Schnürer & Magnusson, 2005).

3.3.3.3.3 Principal Component Analysis (PCA) of the MB10 with LAB

In order to understand the differences between bioactive metabolite production (organic acids, phenolic acids and VOCs) in the fermented MB

formulations, a PCA was realized, and the results obtained are outlined in **Figure 3.3.4**. The sum of the first two principal components (PC) achieved 90.1% of the total explained variance, in which PC1 explained up to 64.5% of the total variance, while PC2 explained 25.6% (**Figure 3.3.4a**). The PC1 distributed on the positive axis both *P. pentosaceus* strains (C12 and C15), whereas *L. plantarum* C60 strain was positioned on the negative axis. The PC2 allowed the distinction of the C12 and C15 strains according to their bioactive metabolite profile since C12 was positioned on the negative axis and C15 on the positive.

(a)


Scores Plot
Lactiplantibacillus plantarum 60
Pediococcos pentosaceus C12
Pediococcos pentosaceus C15
PC 2 (25.6 %)
PC 1 (64.5 %)
2
1
0
-1
-2
-3
-4
-2
0
2

(b)

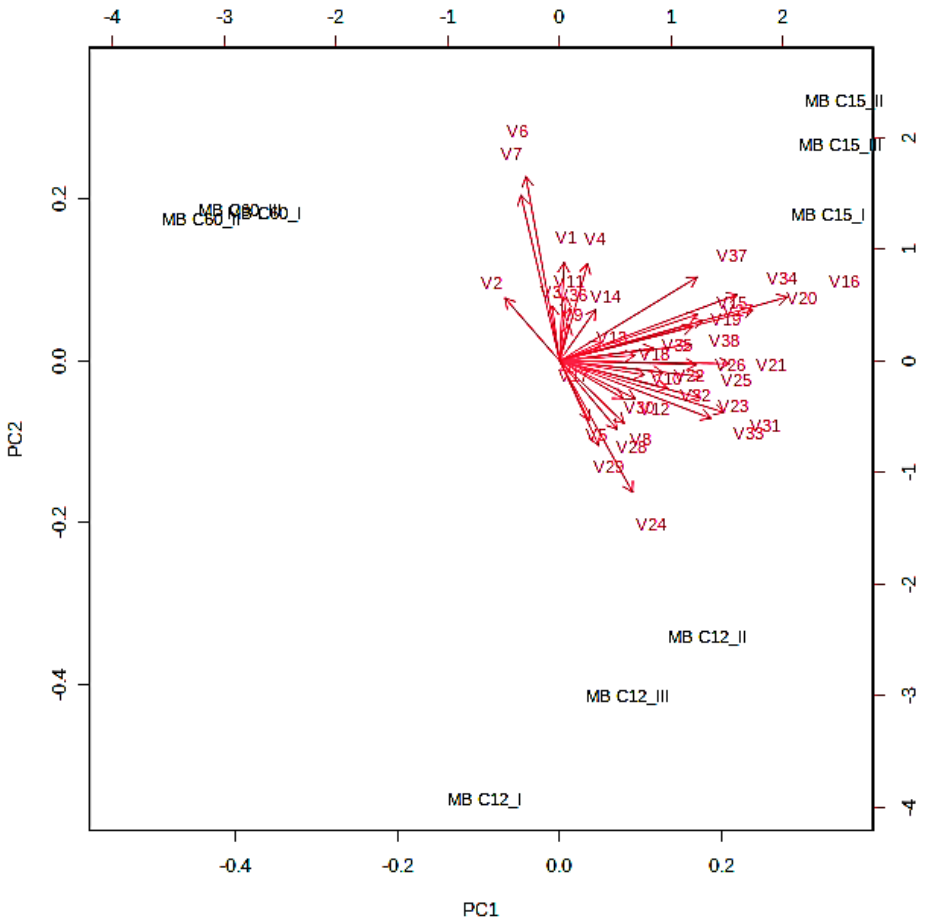


Figure 3.3.4. Principal component analysis (PCA) scores plot of the bioactive compounds (organic acids, phenolic acids, and volatile organic compounds) found in the Meat Broth fermented by *P. pentosaceus* C12 (MB C12), *P. pentosaceus* C15 (MB C15), and *L. plantarum* C60 (MB C60) (a) and relative loadings of the variables employed (b).

According to the loading plot, that represents the relative importance of the variables analyzed (**Figure 3.3.4b**), the MB10-C12 was distinguished from the

MB10-C15 for its higher volatile aldehyde production, and specifically, in some specific compounds such as heptanal (V24), 2–decenal (V28) and dodecanal (V29). The MB10 formulated with C15 was characteristic from the other formulations mainly for the higher production of different antifungal compounds such as nonanoic acid (V16) and phenyl ethyl alcohol (V20).

Regarding the MB10 formulated with *L. plantarum* strain (MB10-C60), the main variable that differences this formulation from those prepared with *P. pentosaceus* strains was the higher production of the phenolic compound 3–(4–hydrodoxy–3–methoxyphenyl) propionic (V2). Furthermore, the ferulic acid (V6) and gallic acid content (V7) positioned on the positive axis according to the second component the MB10-C60 formulation and permitted the distinction between the MBC12 formulation. The variables that positioned on the negative axis according to the first component of the MB C60 was the lower content in volatile alcohols in comparison with C12 and C15, such as 1–nonanol (V21) and 2–undecanol (V23).

Table S1. Elaboration of meat broths for fermentation for lactic acid bacteria.

Ingredient	***Concentration (g/L)***				
	MB2	**MB4**	**MB8**	**MB10**	**MRSb**
Dextrose	20.00	20.00	20.00	20.00	20.00
Meat extract	-	-	-	-	8.00
Yeast extract	-	-	-	-	4.00
Peptone	10.00	10.00	10.00	10.00	10.00
Sodium acetate	5.00	5.00	5.00	5.00	5.00
Dipotassium phosphate	2.00	2.00	2.00	2.00	2.00
Ammonium citrate	2.00	2.00	2.00	2.00	2.00
Magnesium sulfate	0.20	0.20	0.20	0.20	0.20
Manganese sulfate	0.05	0.05	0.05	0.05	0.05
Tween (mL/L)	1.00	1.00	1.00	1.00	1.00
Lyophilized pork loin	2.00	4.00	8.00	10.00	-

Table S2. Antifungal activity of formulated meat broths (MB) and MRS broth fermented by *Pediococcus pentosaceus* C15 during 24, 48, and 72 h at 37 °C. The bacterial-free supernatant (BFS) was freeze-dried, resuspended at a concentration of 250 g/L, and tested against six toxigenic fungi**.**

Fungal strain	**BFS of *Pediococcus pentosaceus* C15 (250 g/L)**					
	24 h		48 h		72 h	
	MRSb	MB10	MRSb	MB10	MRSb	MB10
Aspergillus flavus	+	-	++	++	+	+
Aspergillus parasiticus	+	-	++	+	+	+
Penicillium commune	++	+	+++	+++	+++	++
Penicillium griseofulvum	++		+++	+++	++	++
Penicillium nordicum	++	+	+++	+++	++	++
Penicillium verrucosum	++	+	+++	+++	+++	++

(+) Represents a growth inhibition halo of 0.2 cm; (++) represents a growth inhibition halo of between 0.2 to 0.4 cm; (+++) represents a growth inhibition halo greater than 0.4 cm**.**

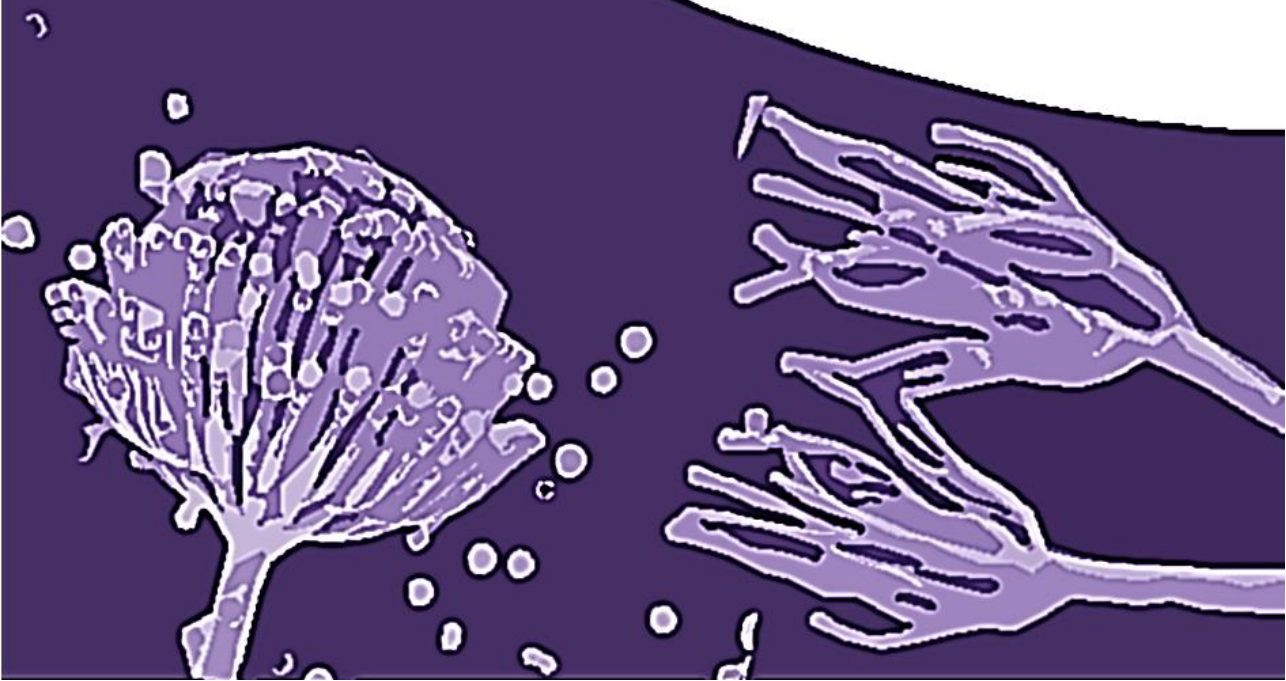
Table S3. Identification of Volatile Organic Compounds (VOCs) of the fermented Meat Broth 10, with retention time, chemical class, and calculated LRI (LRI exp.).

Nº	Rt	Compound	Class	Identif.	LRI exp.	LRI lit.
1	3.11	Acetic acid	Acid	MS		
2	4.55	Pyrazine, methyl-	Pyrazine	MS + LRI	805	807
3	5.78	2-Heptanone	Ketone	MS + LRI	859	859
4	6.25	Heptanal	Aldehyde	MS + LRI	880	882
5	6.37	Pyrazine, 2,5-dimethyl	Pyrazine	MS + LRI	885	883
6	8.71	2-Octanone	Ketone	MS + LRI	980	984
7	8.89	Octanal	Aldehyde	MS + LRI	988	991
8	8.97	2-Octanol	Alcohol	MS + LRI	991	990
9	10.77	Decane, 2-methyl	Alkane	MS + LRI	1063	1061
10	11.02	Benzeneacetaldehyde	Aldehyde	MS + LRI	1073	1071
11	11.19	1-Octanol	Alcohol	MS + LRI	1080	1079
12	11.28	2-Nonanone	Ketone	MS + LRI	1083	1083
13	11.42	Nonanal	Aldehyde	MS + LRI	1089	1089
14	11.62	2-Nonanol	Alcohol	MS + LRI	1097	1098
15	11.69	Undecane	Alkane	MS + Std	1100	
16	13.43	Phenylethyl alcohol	Alcohol	MS	1172	
17	13.60	1-Nonanol	Alcohol	MS + LRI	1180	1180
18	15.49	Nonanoic acid	Acid	MS + LRI	1261	1263
19	15.82	2-Decenal	Aldehyde	MS + LRI	1275	1270
20	15.88	1-Decanol	Alcohol	MS + LRI	1278	1279
21	16.11	2-Undecanone	Ketone	MS + LRI	1289	1273
22	16.27	2-Undecanol	Alcohol	MS + LRI	1296	1294
23	18.50	Dodecanal	Aldehyde	MS + LRI	1398	1405
24	20.45	2-Tridecanone	Ketone	MS + LRI	1493	1497

CHAPTER 4. GENERAL DISCUSSION

DISCUSIÓN GENERAL

DISCUSSÃO GERAL



4.1 GENERAL DISCUSSION

Mycotoxins are harmful secondary metabolites produced by fungi found in food, with the most common being *Alternaria*, *Aspergillus*, *Penicillium* and *Fusarium* species. Mycotoxins can cause toxic effects in humans and animals, such as cancer, liver and kidney disease, damage to the nervous system and the immune system. Exposure to mycotoxins can be through ingestion of contaminated food, as well as through inhalation of airborne fungal spores. Control of toxigenic fungi is crucial to ensure food safety. Although conventional methods such as the use of pesticides and chemical preservatives are effective, their prolonged use can lead to the development of resistance and the accumulation of toxic residues. Therefore, the use of natural compounds as growth inhibitors of toxigenic fungi in food is a promising and sustainable alternative.

The research work carried out in this doctoral thesis has focused on the study of the antifungal and antitoxigenic activity of AITC, mustard flours, and LAB *in vitro*, as well as the application of these natural strategies to reduce the growth of toxigenic fungi and mycotoxins in barley and almonds. On the other hand, a natural antifungal device (based on OMF) and a postbiotic antifungal ingredient based on meat broth fermented by LAB was elaborated.

Initially, an assay with AITC has been performed to inhibit the *P. verrucosum* growth and OTA production on barley, simulating different storage conditions by changing the MC of grains. Moreover, the residual concentration of AITC was also evaluate overtime in order to determine the possible exposure of humans and animals to the AITC since this compound has a pungent odor.

Previous results, obtained by our group, have demonstrated that OMF and YMF possess an antifungal activity, avoiding the FG in several matrices. Against this background and supported by the literature, the objective of second work was elaborate an antifungal strategy to prevent the growth of *A. flavus* and AFB1 production in almonds using AITC, OMF, and YMF. According to the structure of

the compound, different application strategies were considered, either by direct spraying, or through the development of a fumigation device. On the one hand, OMF was studied for incorporation into a hydroxyethyl-cellulose solution together with water to evaporate the AITC *in situ*. On the other hand, the hydrolysis of the glucosinolate sinalbin present in the YMF manly generate p-HBIT; this compound is not volatile, therefore, the direct application of YFM lyophilized in almonds was the most plausible option. The most important contribution of this work was the design of an antifungal device containing hydroxyethyl-cellulose and OMF.

Finally, due to the increased interesting about use of LAB as biocontrol of foods, the mainly goal of the third work was identify and characterize a LAB with antifungal potential in order to create an antifungal ingredient that could be used in the manufacture of meat products such as dry-cured sausages. The majority of studies, associated with LAB, implies the use of MRS as manner of treatment. In other words, they use the bacterial free supernatant of LAB provided of fermentation of MRS. However, MRS is not allowed as food additive and the necessity of develop a food-based broth to be fermented by LAB is well justified. Throughout this study, different meat broths were elaborated and fermented by several LAB strains. In conclusion, an antifungal ingredient was elaborated, and its application was evidenced in dry-cured sausages although these results have not been published so far.

Likewise, the evidence of inhibition of FG by the postbiotic ingredient confirmed the need to characterize the produced compounds responsible for the LAB's antifungal activity. The antifungal compounds were characterized by HPLC-DAD, LC-qTOF-MS, GC-MS, and MALDI-TOF.

Overall, the purpose of all strategies was increasing the shelf life of foods, reducing the incidence of toxigenic fungi, as well as reducing the synthesis of AFs and OTA. It is important to highlight that the results obtained in these works can be

extrapolated to other matrices and opens a wide range of possibilities for the food industry.

4.1.1 *In vitro* antifungal activity of natural compounds

4.1.1.1 Antifungal activity of YMF-E

Prior to the microdilution antifungal assay, an aqueous extract of YMF was prepared. The YMF solution was freeze-dried and resuspended in different concentrations to form a new extract (YMF-E). *A. flavus* demonstrated sensitivity to YMF-E and the MIC and MFC was respectively 390 and 3,130 mg/L.

Previous studies such as Quiles et al. (2018) reported that YMF could inhibit the FG of several fungi. The authors also demonstrated that MIC and MFC ranged from 238.2 to 15,000 mg/L and 1,875 mg/L to 15,000 mg/L, respectively. It is important to emphasize that the results depended on the fungal strains essayed. Additionally, according to the reports by Quiles et al. (2018) and Torrijos et al. (2021), it appears that YMF-E does not have a significant effect on *Aspergillus* spp. compared to *Penicillium* spp. However, in our study, we observed that YMF-E had a lower MIC and MFC. Despite this, our results are consistent with previous research indicating that high doses are needed to inhibit the growth of *A. flavus* with the same proportion.

4.1.1.2 Antifungal activity of AITC

AITC is a known for its antimicrobial activity and previous studies have demonstrated its efficacy as demonstrated in **Table 1.8.** Apart from its antifungal capacity, AITC is volatile and allow its evaporation and entire releasing through any recipient or container. Due to its characteristics AITC convert in an excellent candidate to food fumigation.

Similar to YMF-E in the microdilution assay, AITC showed an important antifungal activity against *A. flavus*. However, the concentration needed was

significantly lower. More specifically, the MIC and MFC ranged from 7.90 mg/L and 31.61 mg/L. Therefore, the concentration needed to achieve a fungicidal result was 100-fold lower than YMF-E.

Nielsen and Rios (2000) tested the volatilization of EOs such as AITC against several fungi. Other authors, such as Clemente et al. (2019), also described the antifungal activity of AITC in a liquid medium. The antifungal activity of AITC is related to its electrophilic carbon (-N-C-S) which makes it reactive towards nucleophiles, e.g., thiols (-SH), amines (-NH-, -NH_2) carboxyl (-COOH) and hydroxyl (-OH). As a result, AITC may react with structures of the microorganisms, binding to structures and destroying others essential for the maintenance of life of the microorganisms (Andini et al., 2020).

4.1.1.3 Antifungal activity of H-OMF device

Sinigrin is the major glucosinolate associated with OMF. The hydrolysis of sinigrin by enzyme mirosinase in presence of water releases AITC in a vapor phase. Thus, OMF is a natural source of AITC but, this reaction is not stable and occurs immediately which limit its application. Taking into account this background, the idea was elaborate a device that retained AITC releasing it slowly. For this purpose, a gel-forming agent (hydroxyethyl-cellulose) was applied and a dispositive was performed with different amounts of OMF. The device received the acronym H-OMF.

Since the H-OMF device could not be diluted in a PDB, its antifungal activity was determined by measuring MG. The *in vitro* antifungal effect of the H-OMF device against *A. flavus* was evaluated for 7 days at 25 °C. In general, the fungicidal effect was only observed at a concentration of 30 mg/L of H-OMF. Doses of 12.5 and 25 mg/L only showed a fungistatic effect, which allowed the FG to grow during storage. However, it is important to note that these concentrations did exhibit a significant difference from the control group. Thus, these findings suggested that the ability of H-OMF to inhibit the FG may depend on the dosage, and concentrations

higher than 30 mg/L may be required to achieve a fungicidal effect in complex matrices.

4.1.1.4 In vitro antifungal activity of the postbiotic ingredient

Before analyzing the antifungal activity of the postbiotic ingredient, it was necessary isolate, identify and elaborate a screening of possible antifungal LAB strains. From 102 bacteria isolated from different handmade dry Spanish sausages, only 42 isolates were gram-positive and catalase negative.

The 42 isolated strains were evaluated by overlay method in plates containing PDA and MRSa. In this step, the FG was evaluated in coculture with the bacteria. Of the 42 bacteria tested, merely 14 isolates exhibited inhibitory activity against all the fungi tested, and the extent of inhibition varied depending on the specific fungal strain being examined. In this experiment, *P. griseofulvum* and *P. commune* were the most susceptible strains. In contrast, *Aspergillus* strains *A. parasiticus* and *A. flavus* were the most resistant to the LAB. These findings agree with those obtained by previous authors (Russo et al., 2017a), which noticed that *Aspergillus* strains are more resistant to LAB than other fungal genera. The present findings agree with the results reported by Russo et al. (2017a), who observed that *Aspergillus* strains show increased resistance to LAB compared to other fungal genera. After that, the 14 bacteria were identified as species of *L. plantarum and P. pentosaceus* by MALDI-TOF-MS method.

After screening, several broths were prepared using different concentrations of freeze-dried pork loin meat (2.00, 4.00, 8.00, and 10.00 g/L respectively MB2, MB4, MB8, and MB10) in order to obtain an ideal broth for LAB growth that would allow the bacteria to produce the same antifungal activity as the fermented MRSb. Since the main idea was to avoid MRS in the composition of the new ingredient, which could generate concern among consumers, the use of meat as the main ingredient was planned as a natural alternative.

The MB2 to MB10 were fermented by 14 bacteria isolated, and two methods were performed to determine the fungal sensitivity to bacterial isolates: agar diffusion test and microdilution of BFS in 96-well plates.

First, the qualitative antifungal activity of MB was determined by agar diffusion assay. To perform this method, the 14 strains were incubated in MRSb (Positive control inhibition) and MB; The fermented broths were centrifuged, freeze-dried, and resuspended in a concentration of 250 g/L. The final solution was deposited in wells previously made in PDA, in which six different fungi species were sown. After fermentation, only the MB10 exhibited antifungal activity similar to the fermented MRSb. In this step, results allowed us to identify the ideal MB composition and three strains that had fungicidal activity against all strains. In consequence, we eliminated the other broths and bacteria. The strains selected were *P. pentosaceus* C12, *P. pentosaceus* C15, and *L. plantarum* C60.

Finally, the quantitative antifungal capacity of three strains was determined by microdilution antifungal assay. This method consisted of using 96-well plates to dilute the antifungal analyte in two-fold serial dilution in order to determine de MIC and MFC. Thus, the antifungal activity of MRSb and MB fermented by LAB could be quantified. This trial showed that fermentation of both MRSb and MB10 with *P. pentosaceus* C15 resulted in lower concentrations needed to inhibit FG against all tested toxigenic strains, compared to the same broths fermented by *P. pentosaceus* C12 and *L. plantarum* C60. Although the fermented MRSb had exhibited a stronger antifungal effect, the difference in efficacy was not significant when compared to MB10. In certain instances, there were no notable differences ($p \leq 0.05$) between the treatments. In other words, *P. pentosaceus* C15 was able to ferment MB10 and produce antifungal compounds, which inhibited the FG at similar conditions to MRSb.

In general, our findings indicated that *P. pentosaceus* C15 exhibited a greater antifungal potential in both the agar diffusion test and microdilution of BFS. Based

on these *in vitro* results, we proceeded to ferment MB10 with *P. pentosaceus* C15 for 48 hours at 37 °C to obtain a postbiotic antifungal ingredient. Subsequently, we centrifuged the fermented broth to remove bacteria and lyophilized it to produce a powder that would facilitate its application in food and enhance the ingredient's shelf life.

After reviewing scientific studies, it is evident that only a limited number of them have reported a broad spectrum of antifungal activity for LAB. However, most of these studies have demonstrated that LAB have higher antifungal activity against one or two specific mold species, as shown by Hernández et al. (2022). Consequently, the objective of this research was to isolate a strain that exhibits a wider range of activity against fungi. Our findings indicate that the antifungal activity of LAB varies depending on the strain, fungal species, and analytical methods used. Likewise, the findings revealed that it is hard to find a LAB with broad antifungal spectrum, so we have only identified three after several screes. Furthermore, our study suggests that dry-cured sausages could be a valuable source of antifungal LAB because they exhibit significant antagonistic properties against the microorganisms tested.

Finally, due to the antifungal activity evidenced by the postbiotic ingredient, it was necessary to identify the main compounds produced by LAB such as organic acids, phenolic acids, and VOCs to help elucidate the LAB's antifungal mechanism of action.

4.1.2 Application of natural antifungal compounds in foods

4.1.2.1 Biocontrol of P. verrucosum on barley grains

The antifungal potential of AITC, a natural compound that is recognized as GRAS and permitted as a food preservative in the US, was evaluated for the first time against *P. verrucosum* using a silo simulator assay with barley grains stored under different MC conditions.

The method of plate count agar allowed us to investigate the impact of MC and AITC treatment in the *P. verrucosum* growth. As results, the use of AITC as fumigant at 50 µL/L on barley grains stored under 13, 16, 19 and 21% of MC reduced the FP significantly, in 24 h of exposure. Moreover, AITC could reduce the FP to levels below the limit of quantification after 30 d and this effect remained for up to 90 d regardless the MC storage condition. These results highlighted the significance of implementing multiple barriers to enhance food safety, including the utilization of AITC fumigation and MC control to prevent FG.

4.1.2.2 Biocontrol of A. flavus on almonds

The *in vitro* results suggested that the application of AITC, YMF-E and, H-OMF could be efficient on almonds to control the *A. flavus* growth. In this step, three different methods of application were performed. First, AITC was socked in a paper and placed inside a silo simulator recipient containing almonds. Second, YMF-E was elaborated and directly sprayed in the surface of samples. Third, an antifungal device containing water, OMF, and hydroxyethylcellulose was elaborated and placed in the recipient to allow AITC volatilization.

The results of fungal plate count demonstrated that YMF-E did not expose any inhibition of FG. Conversely, the application of 2,000 and 4,000 mg/L of H-OMF, as well as 5.07, 10.13, and 20.26 mg/L of AITC, resulted in a significant decrease in fungal population, reaching levels that were below the LOD on both the 7th and 15th days. This phenomenon may be attributed to the conversion of OMF into AITC when water is present. Therefore, OMF could be utilized as a natural source of AITC, gradually released into the silo system's headspace. The study further established that AITCs possess potent antifungal activity against *A. flavus*. Moreover, the new device, named H-OMF, could have the potential to be effective against mycotoxigenic strains in various food types. However, it is recommended that further research is conducted to investigate the fumigant potential of H-OMF in other dry fruits.

4.1.2.3 Antitoxigenic activity of natural compounds in foods

Various studies have shown that AITC has the ability to prevent the formation of mycotoxins in different food products such as nuts, bread, pizza, and wheat flour. However, there is a lack of research on the effectiveness of AITC in preventing the production of OTA and its application in barley for human consumption and animal feed.

In the first study, the effect of different MC and AITC fumigation on OTA production in barley grains stored for 90 d was determined. The grains were contaminated with *P. verrucosum* and analyzed by high-performance liquid chromatography coupled to tandem mass detector (HPLC-MS-MS), which allowed quantification of the OTA produced.

Similar to the results evidenced by FG analysis in barley grains, the treatment with AITC inhibited OTA production, showing a significant difference on OTA content regard to the control group. Likewise, MC demonstrated an important positive correlation in the OTA production, i.e., the higher the MC, the higher levels of OTA were found in the samples. Therefore, grains with MC above 13% should be avoided to prevent mycotoxin production and ensure food and feed safety. Otherwise, the treatment with AITC could also be an alternative to prevent OTA production during 90 d.

In the second study, our objective was to investigate the effectiveness of an H-OMF device and AITC in reducing AFB1 content in almonds. To replicate the natural contamination of almonds, A. flavus was used as an aflatoxin producer, and the almonds were analyzed from day 0 to day 15 for AFB1 production using HPLC-MS/MS.

The results showed that H-OMF at concentrations of 2000 mg/L and 4000 mg/L effectively reduced AFB1 production. However, YMF-E at concentrations of 100, 160, and 200 g/L increased AFB1 production. On the other hand, AITC at concentrations of 5.07, 10.13, and 20.26 mg/L was found to be effective in reducing

mycotoxin production. The reduction in AFB1 can be attributed to the fact that these treatments inhibited the FG, preventing the fungi from activating its secondary metabolite pathway.

It is worth noting that this study is the first to utilize a device based on hydroxyethyl-cellulose and OMF to reduce AFB1 content in almonds. In contrast, the use of AITC to reduce the growth of fungi and mycotoxin production has been previously studied by other authors. Overall, our results suggest that YMF-E should be avoided as an antitoxigenic treatment, while H-OMF could be scaled up and tested in large-scale experiments or real silos.

4.1.3 Evaluation of residual concentration of AITC during storage

To establish the residual concentration of AITC in barley grains, samples were extracted using a methanol solution, and evaluated using a gas chromatograph coupled to a flame ionization detector, which allowed the evaluation of the fraction of AITC absorbed by the grains over the 90 days of fumigation. As results, the initial concentration of AITC absorbed was MC dependent, with higher moisture content resulting in higher absorption. Regardless of MC, AITC was released slowly from the matrix, leading to a prolonged shelf life of up to 90 days. However, the potential impact of residual AITC concentration on fungal recontamination should be studied.

4.1.4 Characterization of antifungal compounds in the postbiotic ingredient

4.1.4.1 Determination of phenolic compounds and organic acids content

In this part of the study, it was analyzed the main organic acids and phenolic compounds of LAB fermented broths that showed antifungal activity *in vitro*. The organic acid content was determined by HPLC-DAD and the phenolic acids were determined by UHPLC-Q-TOF/MS. Thus, we investigated the metabolites produced after fermenting MRSb and MB10 by *P. pentosaceus* C12, *P. pentosaceus* C15, and *L. plantarum* C60.

The results showed that lactic acid and acetic acid were identified in both formulations, with lactic acid having a higher concentration than acetic acid. The highest amount of lactic acid was detected in the MRSb (2.973 g/L) and MB10 (3.00 g/L) fermented by the *P. pentosaceus* C15 strain. However, there were no statistical differences in lactic acid production between these two formulations ($p \leq 0.05$). Regarding acetic acid, the higher concentration ($p \leq 0.05$) was also quantified in the fermented MB10 with *P. pentosaceus* C15 (0.38 g/L).

In relation to phenolic compounds, 12 different phenolic acids were identified, with benzoic acid, PLA, syringic acid, and vanillic acid standing out. It was observed that MRSb produced higher concentrations of phenolic acids than MB10. In particular, syringic acid was significantly higher in *P. pentosaceus* C12 in MB10, while vanillic acid was the most produced phenolic acid by *P. pentosaceus* C15 and *L. plantarum* C60 in both MRSb and MB10. The production of some phenolic acids such as PLA, vanillic and benzoic acid which had previously demonstrated antioxidant or antifungal indicate that antifungal ingredients obtained from fermentation of MB10 can be added to meat food products to provide a significant source of phenolic and organic acids.

LAB can synthesize various antifungal compounds such as organic acids, phenolic acids, AMPs, diacetyl, and reuterin. These metabolites can constitute synergistic activity, but the synergistic activity of these metabolites is not fully understood. Therefore, the results suggest that the antifungal activity of MB10 is not solely due to organic and phenolic acids. Other volatile substances and the decrease in pH also contribute to efficient antifungal activity. Christ-Ribeiro et al. (2019) evidenced that phenolic chemical compounds have a preventive effect on fungal development by exerting inhibitory effects on the biosynthesis of key cellular components, such as glucan, chitin, mannoproteins, and ergosterol, which constitute the fungal cell wall and membrane. This mode of action is attributable to the ability of phenolic compounds to inflict damage on the fungal cell wall and membrane,

leading to alterations in the regulation of nutrient influx. The consequence of these perturbations is a reduced ability of the fungal cell to synthesize key biomolecules such as proteins, amino acids, and sphingolipids. In addition, phenolic compounds are also known to impede the transit of electrons and thus compromise the structural and functional integrity of the fungal cell.

4.1.4.2 VOC composition of postbiotic ingredient

The study analyzed fermented meat broth VOCs (MB10) using HS-SPME coupled to the GC-MS technique. A total of 24 compounds were identified, including 2 alkanes, 2 acids, 7 alcohols, 6 aldehydes, 5 ketones, and 2 pyrazines.

The most abundant compounds found in the samples were aldehydes, representing 27.8-40.8% of the total VOCs detected. Among them, benzeneacetaldehyde represented between 18.4 and 20.3% depending on the formulation analyzed. It was noted that aldehyde content in the fermented MB10 was statistically lower ($p \leq 0.05$) in comparison with the control group. Similarly, pyrazines were the second most abundant group, and their content statistically decreased in the fermented MB10 formulations. Both pyrazine and aldehyde decrease content could be explained by the LAB fermentation. The alcohols identified showed a mean %PA value ranging from 10.8 to 17.6%, with PEA detected in a higher proportion on MB10 fermented with bacteria C15 in comparison with other formulations ($p \leq 0.05$). This active compound has been studied for its antifungal potential and could explain the lower MIC and MFC values detected in the MB10-C15. The LAB fermentation significantly also increased the ketone levels in the formulated MB10 and introduced three new ketones. Two acids were identified in the fermented MB10, acetic acid, and nonanoic acid. It is important to emphasize that nonanoic acid also exhibited antimicrobial properties against several pathogens as reported by Chen et al. (2020), Lee et al. (2021), White et al. (2021), and Cimowsky et al. (2022). Thus, volatile acids combined with the organic acids,

and phenolic acids found in the MB10 could contribute to the antifungal properties of the formulated ingredient.

4.1.4.3 Principal Component Analysis (PCA)

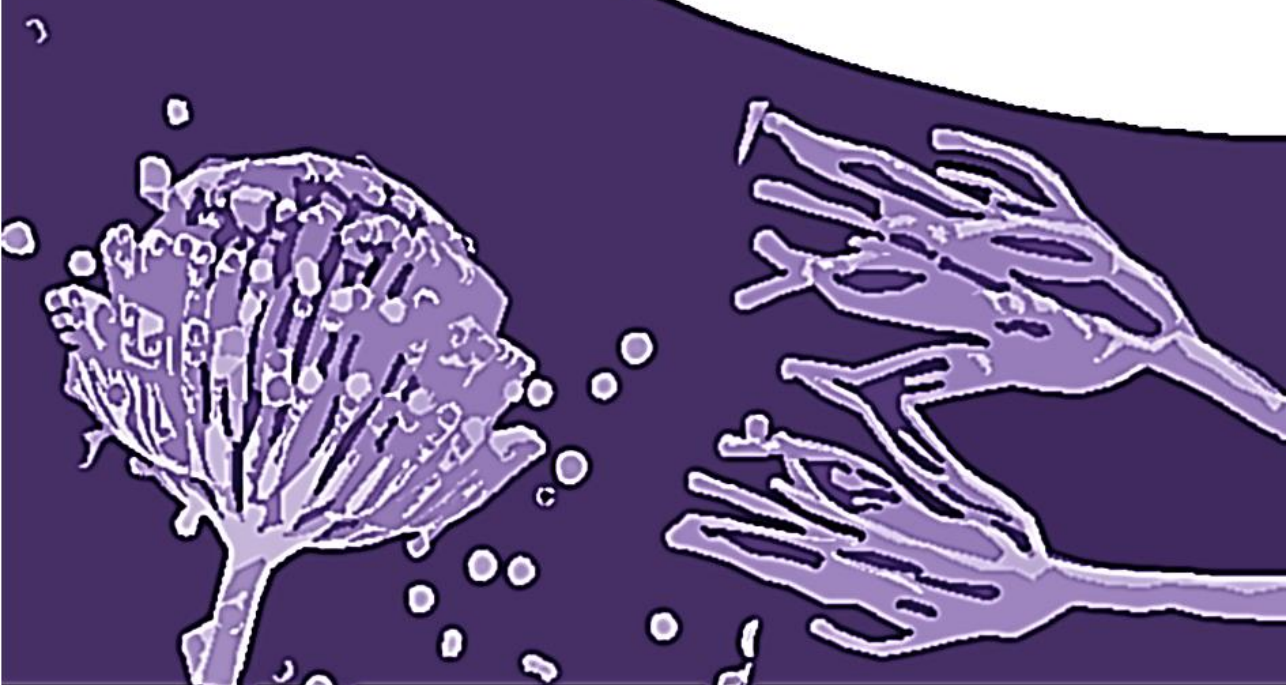
A PCA was performed to understand the differences between the organic acids, phenolic acids and VOCs produced and to try to determine which compound contributes the antifungal property or is responsible for the differentiation of the fermented MB formulations. The combined contribution of the first two principal components (PCs) accounted for 90.1% of the total variance explained. Specifically, PC1 explained 64.5% of the total variance, while PC2 explained 25.6%.

On the positive axis of PC1, both *P. pentosaceus* strains (C12 and C15) were distributed, whereas the *L. plantarum* C60 strain was positioned on the negative axis. PC2 differentiated strains C12 and C15 based on their bioactive metabolite profile, as C12 was positioned on the negative axis and C15 on the positive axis. Finally, based on the loading plot, it was observed that MB10-C12 and MB10-C15 could be distinguished by their production of volatile aldehydes, specifically compounds such as heptanal, 2-decenal, and dodecanal. MB10-C15 was characterized by the production of different antifungal compounds, such as nonanoic acid and phenylethyl alcohol, which differentiated it from other formulations. In comparison, MB10-C60, formulated with strain *L. plantarum*, was distinguished from *P. pentosaceus* formulations by the higher production of the compound 3-(4-hydroxy-3-methoxyphenyl) propionic acid.

CHAPTER 5. CONCLUSION

CONCLUSIÓN

CONCLUSÃO



5.1 CONCLUSIONS

1. The supersaturated salt solution can be used to simulate the MC conditions found in poorly managed silos. Moreover, the MC showed a proportional effect in FG and mycotoxin production. The higher the MC the higher the PF.
2. The AITC at 50 µL/L was absorbed by barley and gradually released. The gradual release of the AITC avoided the FG and OTA production for 90 days, independently of grain MC.
3. The use of AITC as natural fumigant associated with the control of MC of grains can completely avoid the fungal spoilage and mycotoxin production.
4. AITC, yellow mustard flour extract, and the H-OMF antifungal device showed antifungal effect against *A. flavus in vitro,* being AITC the most effective followed by antifungal device and YMF-E.
5. Yellow mustard flour did not show antifungal effect when sprayed in almonds.
6. AITC at 5.07, 10.13, and 20.26 mg/L avoided the growth of *A. flavus* in almonds, and consequently, inhibited the production of AFB1 to levels below the limit of detection.
7. Similarly, the antifungal device containing 2,000 and 4,000 mg/L of oriental mustard flour decreased the FP and the AFB1 concentration to levels below the limit of detection (LOD) without contact with the food.
8. A total of 42 antifungal LAB isolated from dry-cured sausages, among them 14 presented antifungal activity against six fungi of the *Aspergillus* and *Penicillium* genera. However, only three bacteria *L. plantarum* C60, *P. pentosaceus* C12, and *P. pentosaceus* C15 showed *in vitro* antifungal activity against all fungi.
9. The strain *P. pentosaceus* C15 showed higher *in vitro* antifungal activity, so this bacterium was selected for the preparation of the fermented MB10 broth in order to elaborate an antifungal postbiotic ingredient.
10. The chemical characterization highlighted that the postbiotic ingredient is rich in phenolic acids and organic acids. A total of two organic acids (lactic and acetic

acid) and 12 different phenolic acids were identified, highlighting the benzoic acid, PLA, syringic acid, and vanillic acid.

11. A total of 24 volatile organic compounds were identified in the postbiotic ingredient and the most abundant compound found was benzeneacetaldehyde. In addition, antifungal compounds were also identified, including phenylethyl alcohol, nonanoic acid, and acetic acid.

CHAPTER 6. REFERENCES

REFERENCIAS

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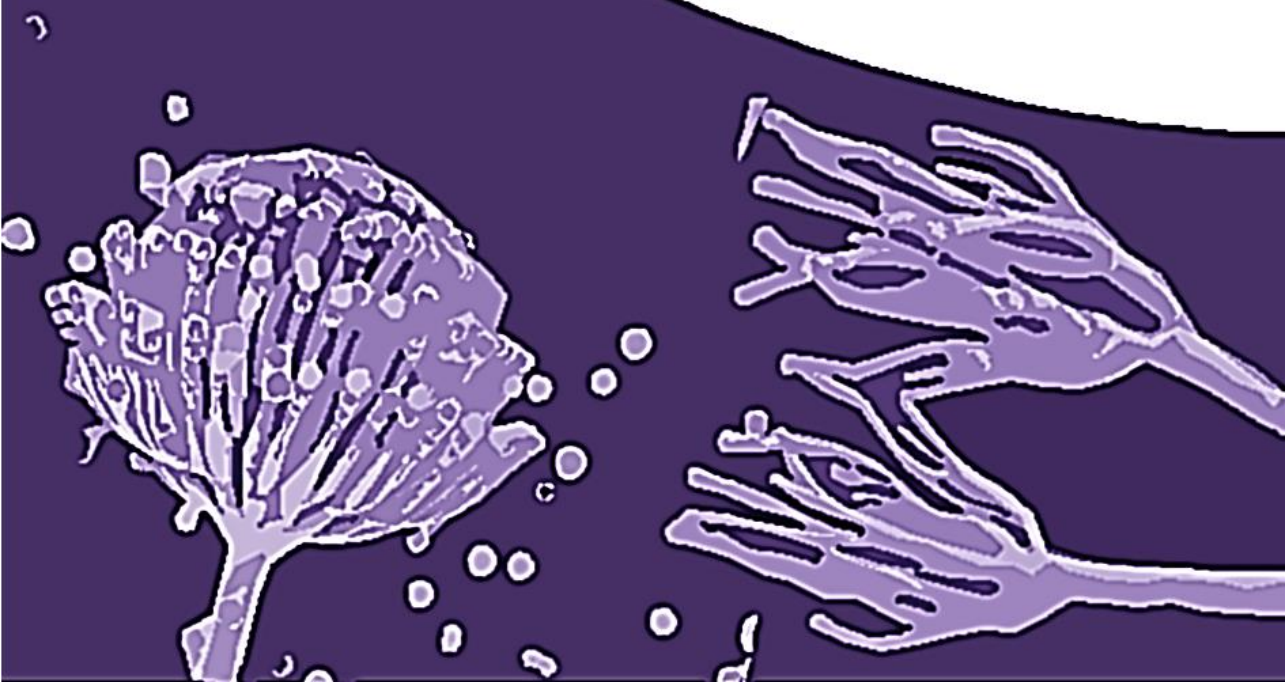

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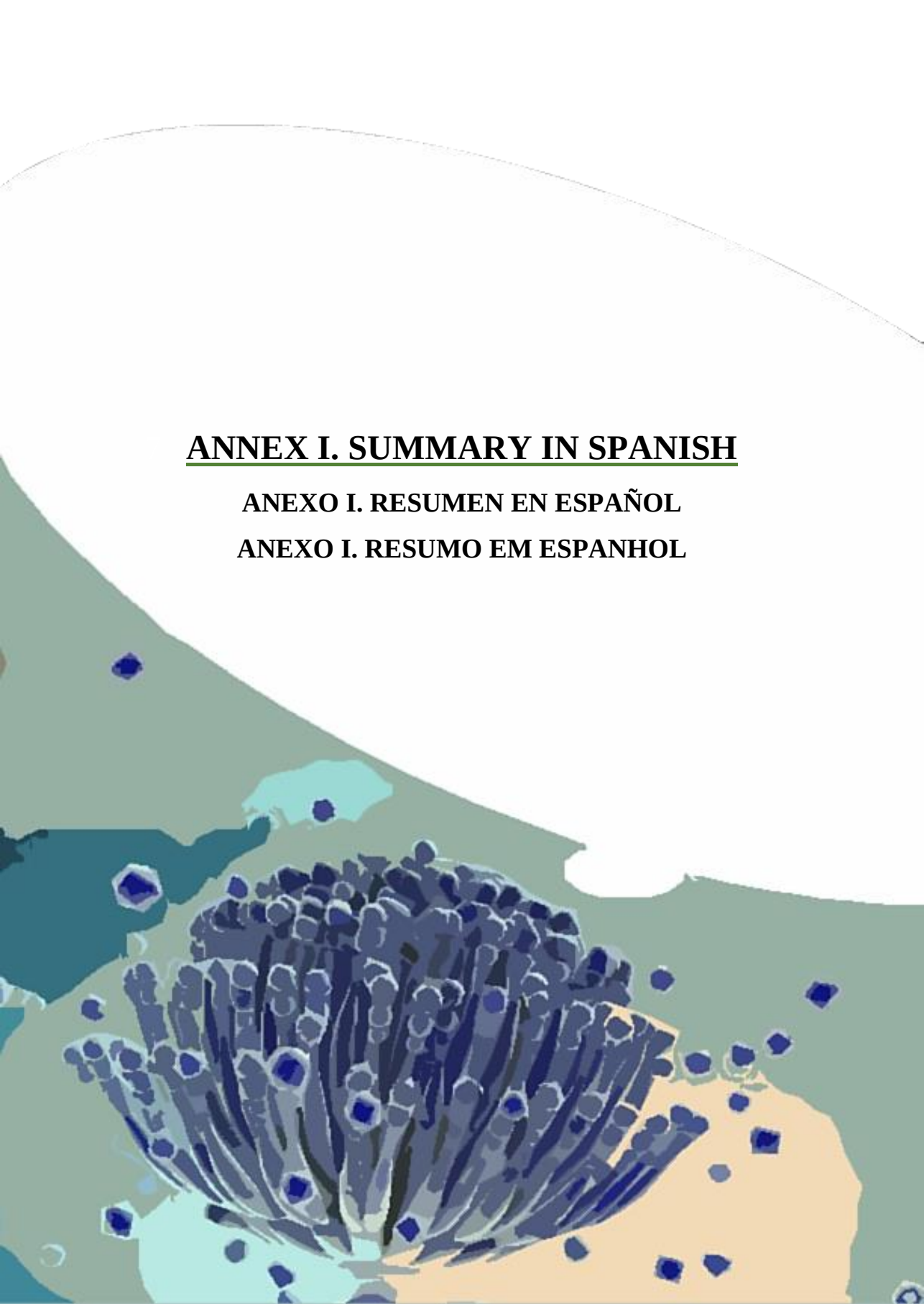
ANNEX I. SUMMARY IN SPANISH

ANEXO I. RESUMEN EN ESPAÑOL

ANEXO I. RESUMO EM ESPANHOL



7.1 CONTEXTUALIZACIÓN

Las micotoxinas son metabolitos secundarios producidos por hongos presentes en los alimentos. Entre ellos, los más comunes son *Aspergillus, Penicillium, Fusarium y Alternaria*. Las micotoxinas pueden causar efectos tóxicos en humanos y animales, como cáncer, enfermedades hepáticas y renales, daños en el sistema nervioso, en el sistema inmunitario y alteraciones reproductivas, entre otras. La exposición a las micotoxinas se produce por ingestión de alimentos contaminados, así como por inhalación de esporas fúngicas transportadas por el aire.

El control de los hongos toxigénicos en los alimentos es crucial para garantizar su seguridad. Aunque los métodos convencionales, como el uso de pesticidas y conservantes químicos, son eficaces, su uso prolongado puede provocar el desarrollo de resistencias y la acumulación de residuos tóxicos. Por ello, el uso de compuestos naturales como inhibidores del crecimiento de hongos toxigénicos en los alimentos es una alternativa prometedora y sostenible.

El trabajo de investigación realizado en esta tesis doctoral se ha centrado en el estudio de la actividad antifúngica y antitoxigénica del isotiocianato de alilo (AITC), harinas de mostazas y Bacterias ácido lácticas (LAB) *in vitro*, así como en la aplicación de estas estrategias naturales y sus productos de fermentación para reducir el crecimiento de hongos toxigénicos y micotoxinas en cebada y almendras. Por otro lado, se elaboró un dispositivo antifúngico natural, basado en harina de mostaza oriental (H-OMF) y un ingrediente antifúngico postbiótico basado en caldo de carne fermentado por LAB.

Inicialmente, se ha realizado un ensayo con AITC para inhibir el crecimiento de *P. verrucosum* y la producción de OTA en cebada, simulando diferentes condiciones de almacenamiento al cambiar el contenido de humedad (MC) de los granos. Además, también se evaluó la concentración residual de AITC a lo largo del tiempo para determinar la posible exposición de humanos y animales al AITC, ya que este compuesto tiene un olor y gusto acre.

Resultados anteriores, obtenidos por nuestro grupo, han demostrado que la harina de mostaza oriental (OMF) y la harina de mostaza amarilla (YMF) poseen una actividad antifúngica que evita el crecimiento fúngico (FG) en varias matrices. En este contexto y con el apoyo de la literatura, el objetivo del segundo trabajo fue elaborar una estrategia antifúngica para prevenir el crecimiento de *A. flavus* y la producción de AFB1 en almendras utilizando AITC, OMF e YMF. En función de la estructura del compuesto, se consideraron diferentes estrategias de aplicación, bien mediante pulverización directa, o bien mediante el desarrollo de un dispositivo de fumigación. La OMF se estudió para su incorporación en una solución de hidroxietilcelulosa junto con agua para evaporar el AITC *in situ*. Por el contrario, la hidrólisis del glucosinolato sinalbina presente en la YMF genera principalmente p-hidroxibencil-isotiocianato (p-HBIT); este compuesto no es volátil, por lo tanto, la aplicación directa de YFM liofilizado en almendras fue la opción más plausible. Además, la contribución más importante de este trabajo fue la elaboración de un dispositivo antifúngico a partir de la mezcla de hidroxietilcelulosa y OMF.

Finalmente, debido al creciente interés sobre el uso de LAB como agente de biocontrol en alimentos, el objetivo principal del tercer trabajo fue identificar y caracterizar LAB con potencial antifúngico con el fin de crear un ingrediente antifúngico que pudiera ser utilizado en la fabricación de productos cárnicos como embutidos curados en seco. La mayoría de los estudios, asociados a LAB, implican el uso de MRS como forma de tratamiento. En otras palabras, utilizan el sobrenadante libre de bacterias de LAB proporcionado por la fermentación del MRS. Sin embargo, el MRS no está permitido como aditivo alimentario y la necesidad de desarrollar un caldo de base alimentaria para ser fermentado por las LAB está bien justificada. A lo largo de este estudio, se elaboraron diferentes caldos de carne que fueron fermentados por varias cepas de *Pediococcus* y *Lactiplantibacillus* spp. En conclusión, se elaboró un ingrediente antifúngico y se evidenció su aplicación en

embutidos curados en seco, aunque estos resultados no han sido incluidos en la presente tesis.

Asimismo, la evidencia de la inhibición del FG por el ingrediente postbiótico confirmó la necesidad de caracterizar los compuestos producidos responsables de la actividad antifúngica de las LAB. Los compuestos antifúngicos se caracterizaron mediante HPLC-DAD, LC-qTOF-MS, GC-MS y MALDI-TOF.

7.2 OBJETIVOS

En general, el objetivo de todas las estrategias era aumentar la vida útil de los alimentos, reduciendo la incidencia de hongos toxigénicos, así como reducir la síntesis de AFs y OTA. Con el fin de alcanzar este objetivo, se han establecido los siguientes objetivos específicos:

1. Evaluar la efectividad del AITC en la prevención del crecimiento de *P. verrucosum* y la producción de OTA en cebada almacenada con diferentes contenidos de humedad durante un período de 90 días. Además, determinar la concentración residual de AITC en los granos después del tratamiento.
2. Evaluar el potencial antifúngico *in vitro* del AITC y del extracto de harina de mostaza amarilla liofilizada (YMF-E). Para luego, desarrollar un dispositivo antifúngico basado en hidroxietilcelulosa y harina de mostaza oriental (H-OMF) y determinar su efecto antifúngico. Además, evaluar la eficacia de todos los tratamientos para evitar el crecimiento de *A. flavus* y prevenir la producción de AFB1 en almendras.
3. Aislar bacterias ácido lácticas con propiedades antifúngicas de embutidos tradicionales curados y desarrollar un ingrediente antifúngico con potencial aplicación en la fabricación de productos cárnicos. E identificar los metabolitos responsables de la capacidad antifúngica del ingrediente.

7.3 PRINCIPALES RESULTADOS Y DISCUSIÓN GENERAL

7.3.1 Actividad antifúngica *in vitro* de los compuestos naturales

7.3.1.1 Actividad antifúngica del extracto de harina de mostaza amarilla (YMF-E)

Antes de realizar el ensayo de microdilución antifúngica, se preparó un extracto acuoso de YMF. La solución de YMF se liofilizó y se resuspendió en diferentes concentraciones para formar un nuevo extracto (YMF-E). Como resultado, *A. flavus* demostró sensibilidad a YMF-E y la concentración inhibitoria mínima (MIC) y la concentración mínima fungicida (MFC) fueron de 390 y 3.130 mg/L, respectivamente.

Estudios previos como el de Quiles et al. (2018) reportaron que YMF podría inhibir el crecimiento de varios hongos. Los autores también demostraron que la MIC y la MFC oscilaban entre 238,2 y 15.000 mg/L, y entre 1.875 mg/L y 15.000 mg/L, respectivamente. Es importante destacar que los resultados dependían de las cepas fúngicas ensayadas. Además, de acuerdo con los informes de Quiles et al. (2018) y Torrijos et al. (2021), parece ser que YMF-E no tiene un efecto significativo en *Aspergillus* spp. en comparación con *Penicillium* spp. Sin embargo, en nuestro estudio, observamos que YMF-E tenía un valor de MIC y MFC más bajo en comparación con estos estudios. A pesar de esto, nuestros resultados son consistentes con investigaciones anteriores que indican que se necesitan dosis altas para inhibir el crecimiento de *A. flavus* en la misma proporción que los hongos pertenecientes al género *Penicillium*.

7.3.1.2 Actividad antifúngica de AITC

El AITC es conocido por su actividad antimicrobiana y estudios previos han demostrado su eficacia. Además de su capacidad antifúngica, el AITC es volátil y permite su evaporación y liberación completa a través de cualquier recipiente o contenedor. Debido a sus características, el AITC se convierte en un excelente candidato para la fumigación de alimentos.

De manera similar al YMF-E, en el ensayo de microdilución, el AITC mostró una importante actividad antifúngica contra *A. flavus*. Sin embargo, la concentración necesaria fue significativamente menor. Más específicamente, la MIC y la MFC oscilaron entre 7,90 mg/L y 31,61 mg/L. Por lo tanto, la concentración necesaria para lograr un resultado fungicida fue 100 veces menor que la del YMF-E.

Nielsen y Rios (2000) probaron la volatilización de aceites esenciales como el AITC contra varios hongos. Otros autores, como Clemente et al. (2019), también describieron la actividad antifúngica del AITC en un medio líquido. La actividad antifúngica del AITC está relacionada con su carbono electrofílico (-N-C-S), que lo hace reactivo hacia nucleófilos, como tióles (-SH), aminas (-NH-, -NH2), carboxilos (-COOH) e hidroxilos (-OH). Como resultado, el AITC puede reaccionar con estructuras de los microorganismos, uniéndose a compuestos esenciales para el mantenimiento de la vida de los microorganismos (Andini et al., 2020).

7.3.1.3 Actividad antifúngica del dispositivo de hidroxietilcelulosa y harina de mostaza oriental (H-OMF)

La sinigrina es el principal glucosinolato asociado con la OMF. La hidrólisis de la sinigrina por la enzima mirosinasa en presencia de agua libera AITC en fase de vapor. Por lo tanto, la OMF es una fuente natural de AITC, sin embargo, esta reacción no es estable y ocurre de inmediato, lo que limita su aplicación. Teniendo en cuenta este antecedente, se ideó elaborar un dispositivo que retuviera el AITC, liberándolo lentamente. Para este fin, se utilizó un agente formador de gel (hidroxietilcelulosa) y se creó un dispositivo con diferentes cantidades de OMF. El dispositivo recibió la sigla H-OMF.

Dado que H-OMF no podía diluirse en un medio PDB, su actividad antifúngica se determinó mediante la medición del crecimiento micelial (MG). El efecto antifúngico *in vitro* del dispositivo H-OMF contra *A. flavus* se evaluó durante 7 días a 25 °C. En general, el efecto fungicida solo se observó a una concentración

de 30 mg/L de H-OMF. Las dosis de 12,5 y 25 mg/L solo mostraron un efecto fungistático, lo que permitió el MG durante el almacenamiento. Sin embargo, es importante destacar que estas concentraciones mostraron una diferencia significativa en comparación con el grupo control. Por lo tanto, estos hallazgos sugirieron que la capacidad de H-OMF para inhibir el FG puede depender de la dosis, y pueden ser necesarias concentraciones superiores a 30 mg/L para lograr un efecto fungicida en matrices complejas como los frutos secos.

7.3.1.4 Actividad antifúngica in vitro del ingrediente postbiótico

Antes de analizar la actividad antifúngica del ingrediente postbiótico, fue necesario aislar, identificar y elaborar un cribado de posibles cepas de LAB antifúngicas. De 102 bacterias aisladas de diferentes embutidos secos españoles caseros, solo 42 aislados fueron grampositivos y catalasa negativos.

Las 42 cepas aisladas fueron evaluadas mediante el método de superposición en placas que contenían PDA y MRSa. En esta etapa, se evaluó el crecimiento fúngico en cocultivo con las bacterias. De las 42 bacterias probadas, solo 14 aislados mostraron actividad inhibitoria contra los seis hongos probados, y el grado de inhibición varió según la cepa fúngica específica examinada. En este experimento, *P. griseofulvum* y *P. commune* fueron las cepas más susceptibles. En contraste, las cepas de *Aspergillus*, *A. parasiticus* y *A. flavus*, fueron las más resistentes al LAB. Estos hallazgos corroboran con los obtenidos por autores anteriores (Russo et al., 2017a), quienes observaron que las cepas de *Aspergillus* son más resistentes al LAB que otros géneros fúngicos. Después de eso, las 14 bacterias fueron identificadas como especies de *L. plantarum* y *P. pentosaceus* mediante el método MALDI-TOF-MS.

Después del cribado, se prepararon varios caldos utilizando diferentes concentraciones de carne de lomo de cerdo liofilizada (2, 4, 8 y 10 g/L respectivamente, MB2, MB4, MB8 y MB10) con el fin de obtener un caldo ideal para el crecimiento de LAB que permitiera a las bacterias producir la misma

actividad antifúngica que el MRSb fermentado. Dado que la idea principal era evitar el uso de MRS en la composición del nuevo ingrediente, lo cual podría generar preocupación entre los consumidores, se planificó el uso de carne como ingrediente principal como una alternativa natural.

Los medios MB2 a MB10 fueron fermentados por los 14 aislados bacterianos, y se realizaron dos métodos para determinar la sensibilidad fúngica a los aislados bacterianos: el ensayo de difusión en agar y la microdilución del BFS en placas de 96 pocillos.

Primero, se determinó la actividad antifúngica cualitativa de los MB mediante el ensayo de difusión en agar. Para realizar este método, las 14 cepas se incubaron en MRSb (control positivo de inhibición) y en los MB. Los caldos fermentados se centrifugaron, liofilizaron y resuspendieron en una concentración de 250 g/L. La solución final se depositó en pocillos previamente realizados en PDA, en el cual se sembraron seis especies diferentes de hongos. Después de la fermentación, solo el MB10 mostró actividad antifúngica similar al MRSb fermentado. En esta etapa, los resultados nos permitieron identificar la composición ideal del MB y separar tres cepas que tenían actividad fungicida contra todas las cepas fúngicas. En consecuencia, se eliminaron los otros caldos y bacterias. Las cepas seleccionadas fueron *P. pentosaceus* C12, *P. pentosaceus* C15 y *L. plantarum* C60.

Finalmente, se determinó la capacidad antifúngica cuantitativa de las tres cepas mediante el ensayo de microdilución antifúngica. Este método consistió en utilizar placas de 96 pocillos para diluir el analito antifúngico en diluciones seriadas al doble para determinar la MIC y la MFC. De esta manera, se pudo cuantificar la actividad antifúngica del MRSb y MB fermentados por LAB. Este ensayo mostró que la fermentación tanto de MRSb como de MB10 con *P. pentosaceus* C15 resultó en concentraciones más bajas necesarias para inhibir el crecimiento fúngico contra todas las cepas toxigénicas probadas, en comparación con los mismos caldos

fermentados por *P. pentosaceus* C12 y *L. plantarum* C60. Aunque el MRSb fermentado mostró un efecto antifúngico más fuerte, la diferencia en la eficacia no fue significativa en comparación con el MB10. En ciertos casos, no hubo diferencias notables ($p \leq 0,05$) entre los tratamientos. En otras palabras, *P. pentosaceus* C15 fue capaz de fermentar MB10 y producir compuestos antifúngicos, que inhibieron el crecimiento fúngico en condiciones similares al MRSb.

En general, nuestros hallazgos indicaron que *P. pentosaceus* C15 exhibió un mayor potencial antifúngico tanto en el ensayo de difusión en agar como en la microdilución de BFS. Basándonos en estos resultados *in vitro*, se fermentó el medio MB10 con *P. pentosaceus* C15 durante 48 horas a 37 °C para obtener un ingrediente postbiótico antifúngico. Posteriormente, se centrifugó el caldo fermentado para eliminar las bacterias y se liofilizó para producir un polvo que facilitara su aplicación en alimentos y mejorara la vida útil del ingrediente.

Después de revisar estudios científicos, fue evidente que solo un número limitado de ellos informó sobre un amplio espectro de actividad antifúngica de LAB. Sin embargo, la mayoría de estos estudios demostraron que las LAB tienen una mayor actividad antifúngica contra una o dos especies específicas de hongos, como lo mostró Hernández et al. (2022). En consecuencia, el objetivo de esta investigación fue aislar una cepa que exhibiera un rango más amplio de actividad contra hongos. Nuestros hallazgos indican que la actividad antifúngica de LAB varía según la cepa, la especie fúngica y los métodos analíticos utilizados. Asimismo, los hallazgos revelaron que es difícil encontrar una LAB con un amplio espectro antifúngico, por lo que se identificaron tres después de varias pruebas. Además, nuestro estudio sugirió que los embutidos curados podrían ser una fuente valiosa de LAB antifúngicas, ya que exhiben propiedades inhibitorias significativas contra los microorganismos evaluados.

Finalmente, debido a la actividad antifúngica evidenciada por el ingrediente postbiótico, fue necesario identificar los principales compuestos producidos por

LAB, como ácidos orgánicos, ácidos fenólicos y VOCs, para ayudar a dilucidar el mecanismo de acción antifúngico de las LAB.

7.3.2 Aplicación de compuestos antifúngicos naturales en alimentos

7.3.2.1 Biocontrol del P. verrucosum en granos de cebada

Se evaluó por primera vez el potencial antifúngico de AITC, un compuesto natural reconocido como GRAS y permitido como conservante de alimentos en los Estados Unidos, contra *P. verrucosum* utilizando un ensayo en un simulador de silo con granos de cebada almacenados en diferentes condiciones de contenido de humedad (MC).

El método de recuento en placa de agar nos permitió investigar el impacto del MC y el tratamiento con AITC en el crecimiento de *P. verrucosum*. Como resultado, el uso de AITC como fumigante a 50 µL/L en granos de cebada almacenados bajo 13, 16, 19 y 21% de MC redujo significativamente la FP en 24 horas de exposición. Además, AITC pudo reducir la FP a niveles por debajo del límite de cuantificación después de 30 días, y este efecto se mantuvo hasta 90 días independientemente de la condición de almacenamiento en MC. Estos resultados resaltaron la importancia de implementar múltiples barreras sanitarias para mejorar la seguridad alimentaria, incluyendo la utilización de la fumigación con AITC y el control del MC para prevenir el crecimiento fúngico (FG).

7.3.2.2 Biocontrol del A. flavus en almendras

Los resultados *in vitro* sugirieron que la aplicación de AITC, YMF-E y H-OMF podría ser eficiente en almendras para controlar el crecimiento de *A. flavus*. En esta etapa, se realizaron tres métodos de aplicación diferentes. Primero, se empapó AITC en un papel y se colocó dentro de un recipiente de simulación de silo que contenía almendras. Segundo, se elaboró YMF-E y se esparció directamente en la superficie de las muestras. Tercero, se elaboró un dispositivo antifúngico que

contenía agua, OMF e hidroxietilcelulosa, y se colocó en el recipiente para permitir la volatilización del AITC.

Los resultados del recuento de colonias fúngicas demostraron que YMF-E no mostró ninguna inhibición del FG. Por el contrario, la aplicación de 2.000 y 4.000 mg/L de H-OMF, así como 5,07, 10,13 y 20,26 mg/L de AITC, resultó en una disminución significativa de la población fúngica, alcanzando niveles que estaban por debajo del límite de detección tanto en el séptimo como en el decimoquinto día. Este fenómeno puede atribuirse a la conversión de OMF en AITC cuando hay presencia de agua. Por lo tanto, el OMF podría utilizarse como una fuente natural de AITC, que se libera gradualmente en el espacio de cabeza del sistema del silo. El estudio también estableció que los AITC poseen una potente actividad antifúngica contra *A. flavus*. Además, el nuevo dispositivo llamado H-OMF podría tener el potencial de ser efectivo contra cepas toxigénicas en varios tipos de alimentos. Sin embargo, se recomienda realizar investigaciones adicionales para dilucidar el potencial fumigante de H-OMF en otros frutos secos.

7.3.2.3 Actividad antitoxigénica de compuestos naturales en alimentos

Varios estudios han demostrado que el AITC tiene la capacidad de prevenir la formación de micotoxinas en diferentes productos alimentarios como frutos secos, pan, pizza y harina de trigo. Sin embargo, existe una falta de investigación sobre la efectividad del AITC en la prevención de la producción de OTA y su aplicación en la cebada para consumo humano y alimentación animal.

Debido a este vacío literario, en el primer estudio, se determinó el efecto de diferentes concentraciones de humedad (MC) y la fumigación con AITC en la producción de OTA en granos de cebada almacenados durante 90 días. Los granos fueron contaminados con *P. verrucosum* y se analizaron mediante cromatografía líquida de alta resolución acoplada a un detector de masas en tándem (HPLC-MS-MS), lo que permitió la cuantificación de la OTA producida.

Similar a los resultados evidenciados por el análisis de FG en granos de cebada, el tratamiento con AITC inhibió la producción de OTA, mostrando una diferencia significativa en el contenido de OTA en comparación con el grupo de control. Del mismo modo, la MC demostró una importante correlación positiva en la producción de OTA, es decir, a mayor MC, se encontraron niveles más altos de OTA en las muestras. Por lo tanto, se deben evitar los granos con una MC superior al 13% para prevenir la producción de micotoxinas y garantizar la seguridad alimentaria. De lo contrario, el tratamiento con AITC también podría ser una alternativa para prevenir la producción de OTA durante 90 días. Sin embargo, la mejor estrategia seria asociar las técnicas de reducción de humedad y la fumigación con AITC.

En el segundo estudio, nuestro objetivo fue investigar la efectividad del dispositivo H-OMF y AITC en la reducción del contenido de AFB1 en almendras. Para replicar la contaminación natural de las almendras, se utilizó *A. flavus* como productor de AFs, y las almendras se analizaron desde el día 0 hasta el día 15 para la producción de AFB1 utilizando HPLC-MS/MS.

Los resultados mostraron que H-OMF a concentraciones de 2.000 mg/L y 4.000 mg/L redujo efectivamente la producción de AFB1. Por otro lado, AITC a concentraciones de 5,07, 10,13 y 20,26 mg/L fue efectivo en la reducción de la producción de micotoxinas. La reducción en AFB1 se puede atribuir al hecho de que estos tratamientos inhibieron el FG, evitando que el hongo active su vía de metabolitos secundarios.

Vale la pena mencionar que este estudio es el primero en utilizar un dispositivo basado en hidroxietilcelulosa y OMF para reducir el contenido de AFB1 en almendras. En contraste, el uso de AITC para reducir el crecimiento de hongos y la producción de micotoxinas ha sido estudiado previamente por otros autores.

En general, nuestros resultados sugirieron que se debe evitar el uso de YMF-E como tratamiento antimicotóxico, mientras que H-OMF podría ser ampliado y probado en experimentos a gran escala o en silos reales.

7.3.3 Evaluación de la concentración residual de AITC durante el almacenamiento

Para establecer la concentración residual de AITC en granos de cebada, se extrajeron muestras utilizando una solución de metanol y se evaluaron mediante cromatografía de gases acoplada a un detector de ionización de llama, lo que permitió la evaluación de la fracción de AITC absorbida por los granos durante los 90 días de fumigación. Como resultado, la concentración inicial de AITC absorbida dependió de la MC, con un mayor contenido de humedad resultando en una mayor absorción. Independientemente de la MC, el AITC se liberó lentamente de la matriz, lo que condujo a una vida útil prolongada de hasta 90 días. Sin embargo, se debe estudiar el impacto potencial de la concentración residual de AITC en la recontaminación fúngica.

7.3.4 Caracterización de compuestos antifúngicos en el ingrediente postbiótico

7.3.4.1 Determinación del contenido de compuestos fenólicos y ácidos orgánicos

En esta parte del estudio, se analizaron los principales ácidos orgánicos y compuestos fenólicos de los caldos fermentados por LAB que mostraron actividad antifúngica *in vitro*. El contenido de ácidos orgánicos se determinó mediante HPLC-DAD y los ácidos fenólicos se determinaron mediante UHPLC-Q-TOF/MS. Así, se investigaron los metabolitos producidos después de la fermentación de MRSb y MB10 por las cepas *P. pentosaceus* C12, *P. pentosaceus* C15 y *L. plantarum* C60.

Los resultados mostraron que el ácido láctico y el ácido acético se identificaron en ambas formulaciones, siendo el ácido láctico el que presentó una

concentración más alta que el ácido acético. La mayor cantidad de ácido láctico se detectó en el MRSb (2,973 g/L) y el MB10 (3,00 g/L) fermentados por la cepa *P. pentosaceus* C15. Sin embargo, no hubo diferencias estadísticas en la producción de ácido láctico entre estas dos formulaciones ($p \leq 0,05$). En cuanto al ácido acético, también se cuantificó una mayor concentración ($p \leq 0,05$) en el MB10 fermentado con *P. pentosaceus* C15 (0,38 g/L).

Con relación a los compuestos fenólicos, se identificaron 12 ácidos fenólicos diferentes, destacando el ácido benzoico, PLA, ácido siríngico y ácido vanílico. Se observó que MRSb produjo concentraciones más altas de ácidos fenólicos que MB10. En particular, el ácido siríngico fue significativamente mayor en *P. pentosaceus* C12 en MB10, mientras que el ácido vanílico fue el ácido fenólico más producido por *P. pentosaceus* C15 y *L. plantarum* C60 tanto en MRSb como en MB10. La producción de algunos ácidos fenólicos como PLA, ácido vanílico y ácido benzoico, que previamente han demostrado tener propiedades antioxidantes o antifúngicas, indica que los ingredientes antifúngicos obtenidos a partir de la fermentación de MB10 pueden agregarse a productos alimentarios cárnicos para proporcionar una fuente significativa de ácidos fenólicos y orgánicos (además de ayudar a la inocuidad alimentaria).

Las LAB pueden sintetizar diversos compuestos antimicrobianos como ácidos orgánicos, ácidos fenólicos, AMPs, diacetilo y reuterina. Estos metabolitos pueden tener una actividad sinérgica, pero la actividad sinérgica de estos metabolitos no está totalmente establecida. Por lo tanto, los resultados sugieren que la actividad antifúngica de MB10 no se debe únicamente a los ácidos orgánicos y fenólicos. Sino a que otras sustancias volátiles junto a la disminución del pH también contribuyen a una actividad antifúngica eficiente. Christ-Ribeiro et al. (2019) evidenciaron que los compuestos fenólicos tienen un efecto preventivo sobre el desarrollo fúngico al ejercer efectos inhibitorios sobre la biosíntesis de componentes celulares clave, como glucano, quitina, manoproteínas y ergosterol, que constituyen la pared celular y la

membrana fúngica. Este modo de acción se atribuye a la capacidad de los compuestos fenólicos para causar daño en la pared celular y la membrana fúngica, lo que conduce a alteraciones en la regulación del flujo de nutrientes. Como consecuencia de estas perturbaciones, la célula fúngica tiene una capacidad reducida para sintetizar biomoléculas clave como proteínas, aminoácidos y esfingolípidos. Además, se sabe que los compuestos fenólicos obstaculizan el tránsito de electrones y, por lo tanto, comprometen la integridad estructural y funcional de la célula fúngica.

7.3.4.2 Composición del VOC del ingrediente postbiótico

En la parte del estudio relacionada con la composición de VOCs (compuestos orgánicos volátiles) del ingrediente postbiótico fermentado a partir de caldo de carne (MB10), se utilizó la técnica de HS-SPME acoplada a GC-MS. Se identificaron un total de 24 compuestos, incluyendo 2 alcanos, 2 ácidos, 7 alcoholes, 6 aldehídos, 5 cetonas y 2 pirazinas.

Los compuestos más abundantes encontrados en las muestras fueron los aldehídos, representando entre el 27,8% y el 40,8% del total de VOCs detectados. Entre ellos, el benceno acetaldehído representó entre el 18,4% y el 20,3%, dependiendo de la formulación analizada. Se observó que el contenido de aldehídos en el MB10 fermentado fue estadísticamente menor ($p \leq 0,05$) en comparación con el grupo de control. De manera similar, las pirazinas fueron el segundo grupo más abundante, y su contenido disminuyó de manera estadísticamente significativa en las formulaciones de MB10 fermentado. Tanto la disminución del contenido de pirazinas como de aldehídos podrían ser explicados por la fermentación de LAB. Los alcoholes identificados mostraron un valor medio de %PA (porcentaje del área total) que varió entre el 10,8% y el 17,6%, destacando la detección de PEA (alcohol fenetílico) en una proporción mayor en el MB10 fermentado con la bacteria C15 en comparación con otras formulaciones ($p \leq 0,05$). Este compuesto activo ha sido estudiado por su potencial antifúngico y podría explicar los valores más bajos de

MIC (concentración mínima inhibitoria) y MFC (concentración mínima fungicida) detectados en el MB10-C15. La fermentación de LAB también aumentó significativamente los niveles de cetonas en el MB10 formulado e introdujo tres nuevas cetonas. Se identificaron dos ácidos en el MB10 fermentado, ácido acético y ácido nonanoico. Es importante destacar que el ácido nonanoico también ha mostrado propiedades antimicrobianas contra varios patógenos, según lo informado por Chen et al. (2020), Lee et al. (2021), White et al. (2021) y Cimowsky et al. (2022). Por lo tanto, los ácidos volátiles combinados con los ácidos orgánicos y fenólicos encontrados en el MB10 podrían contribuir a las propiedades antifúngicas del ingrediente formulado.

7.3.4.3 Análisis de Componentes Principales (PCA)

Se realizó un análisis de componentes principales (PCA) para entender las diferencias entre los ácidos orgánicos, ácidos fenólicos y VOCs producidos, y tratar de determinar qué compuesto contribuye a la propiedad antifúngica o es responsable por la diferenciación de las formulaciones de MB fermentadas.

La contribución combinada de los dos primeros componentes principales (PCs) representó el 90,1% de la varianza total explicada. Específicamente, PC1 explicó el 64,5% de la varianza total, mientras que PC2 explicó el 25,6%.

En el eje positivo de PC1, ambas cepas de *P. pentosaceus* (C12 y C15) se distribuyeron, mientras que la cepa de *L. plantarum* C60 se posicionó en el eje negativo. PC2 diferenció las cepas C12 y C15 en función de su perfil de metabolitos bioactivos, ya que C12 se posicionó en el eje negativo y C15 en el eje positivo. Finalmente, según el *loading plot*, se observó que MB10-C12 y MB10-C15 se podían distinguir por su producción de aldehídos volátiles, específicamente compuestos como heptanal, 2-decenal y dodecanal. MB10-C15 se caracterizó por la producción de diferentes compuestos antifúngicos, como el ácido nonanoico y el alcohol fenetílico, que lo diferenciaron de otras formulaciones. En comparación,

MB10-C60, formulado con la cepa *L. plantarum*, se distinguía de las formulaciones de *P. pentosaceus* por una mayor producción del ácido propiónico.

7.4 CONCLUSIONES

1. La solución de sal supersaturada se puede utilizar para simular las condiciones de contenido de humedad (MC) encontradas en silos mal gestionados. Además, se observó que el MC tiene un efecto proporcional en el crecimiento fúngico (FG) y la producción de micotoxinas. Cuanto mayor es el MC, mayor es el FG.
2. El AITC a 50 µL/L fue absorbido por la cebada y liberado gradualmente. La liberación gradual de AITC evitó la producción de OTA y el FG durante 90 días, independientemente del MC del grano.
3. El uso de AITC como fumigante natural asociado al control del MC de los granos puede evitar completamente el deterioro fúngico y la producción de micotoxinas.
4. El AITC, el extracto de harina de mostaza amarilla y el dispositivo antifúngico H-OMF mostraron efecto antifúngico contra *A. flavus in vitro*, siendo el AITC el más efectivo, seguido del dispositivo antifúngico y el extracto de harina de mostaza amarilla.
5. La harina de mostaza amarilla no mostró efecto antifúngico cuando se pulverizó en las almendras.
6. El AITC en concentraciones de 5,07, 10,13 y 20,26 mg/L evitó el crecimiento de *A. flavus* en almendras y, en consecuencia, inhibió la producción de AFB1 a niveles por debajo del límite de detección.
7. Del mismo modo, el dispositivo antifúngico que contenía 2.000 y 4.000 mg/L de harina de mostaza oriental disminuyó la FP y la concentración de AFB1 a niveles por debajo del límite de detección sin contacto con los alimentos.
8. Se aislaron un total de 42 bacterias antifúngicas de embutidos curados, de las cuales 14 presentaron actividad antifúngica contra seis hongos de los

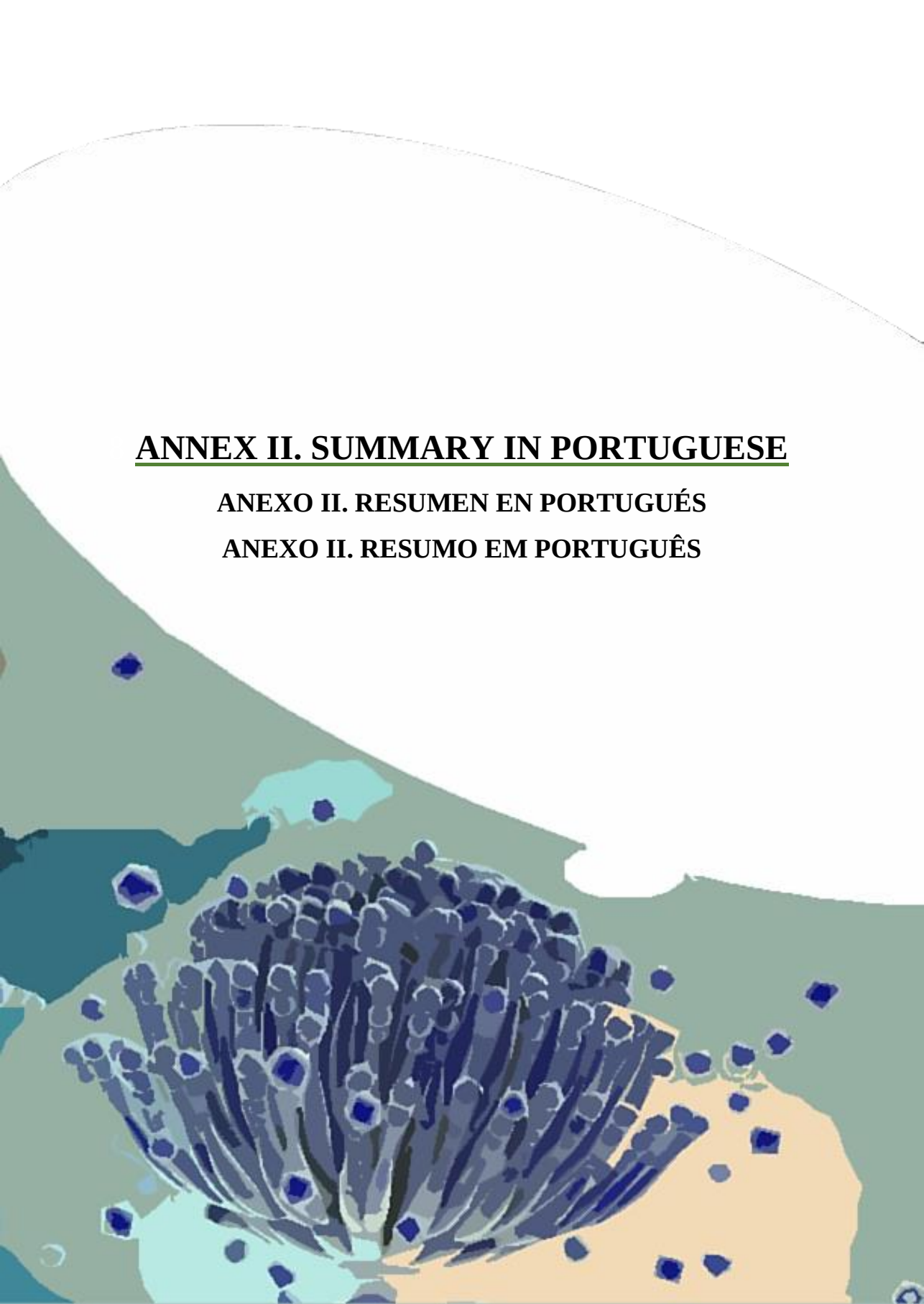
géneros *Aspergillus* y *Penicillium*. Sin embargo, solo tres bacterias, *L. plantarum* C60, *P. pentosaceus* C12 y *P. pentosaceus* C15, mostraron actividad antifúngica *in vitro* contra todos los hongos.

9. La cepa *P. pentosaceus* C15 mostró una mayor actividad antifúngica *in vitro*, por lo que esta bacteria fue seleccionada para la preparación del caldo MB10 fermentado con objeto de elaborar un ingrediente postbiótico antifúngico.
10. La caracterización química destacó que el ingrediente postbiótico es rico en ácidos fenólicos y ácidos orgánicos. Se identificaron un total de dos ácidos orgánicos (ácido láctico y ácido acético) y 12 ácidos fenólicos diferentes, destacando el ácido benzoico, PLA, ácido siríngico y ácido vanílico.
11. Se identificaron un total de 24 compuestos orgánicos volátiles en el ingrediente postbiótico y el compuesto más abundante encontrado fue el benceno acetaldehído. Además, también se identificaron compuestos antifúngicos, como el alcohol fenetílico, el ácido nonanoico y el ácido acético.

ANNEX II. SUMMARY IN PORTUGUESE

ANEXO II. RESUMEN EN PORTUGUÉS

ANEXO II. RESUMO EM PORTUGUÊS



8.1 CONTEXTUALIZAÇÃO

As micotoxinas são metabólitos secundários produzidos por fungos presentes nos alimentos. Entre eles, os mais comuns são os fungos dos gêneros *Alternaria, Aspergillus*, *Penicillium* e *Fusarium*. As micotoxinas podem causar efeitos tóxicos em seres humanos e animais, tais como câncer, doenças hepáticas e renais, danos no sistema nervoso e no sistema imunológico. A exposição às micotoxinas pode ocorrer através da ingestão de alimentos contaminados, bem como pela inalação de esporos fúngicos transportados pelo ar.

O controle de fungos toxigênicos em alimentos é crucial para garantir sua segurança. Embora métodos convencionais, como o uso de pesticidas e conservantes químicos, sejam eficazes, seu uso prolongado pode levar ao desenvolvimento de resistência fúngica e acúmulo de resíduos tóxicos. Portanto, o uso de compostos naturais como inibidores do crescimento de fungos toxigênicos em alimentos é uma alternativa promissora e sustentável.

O trabalho de pesquisa realizado nesta tese de doutorado concentrou-se no estudo da atividade antifúngica e antimicotóxica do isotiocianato de alila (AITC), farinhas de mostarda e bactérias ácido lácticas (LAB) *in vitro*, bem como na aplicação dessas estratégias naturais e seus produtos de fermentação para reduzir o crescimento de fungos toxigênicos e micotoxinas em cevada e amêndoas. Além disso, foi desenvolvido um dispositivo antifúngico natural, com base em farinha de mostarda oriental (H-OMF), e um ingrediente antifúngico pós-biótico baseado em caldo de carne fermentado por LAB.

Inicialmente, foi conduzido um ensaio com AITC para inibir o crescimento de *P. verrucosum* e a produção de OTA em cevada, simulando diferentes condições de armazenamento ao alterar o teor de umidade dos grãos (MC). Além disso, a concentração residual de AITC ao longo do tempo também foi avaliada para determinar a possível exposição de humanos e animais ao AITC, uma vez que este composto possui um odor e sabor pungente.

Resultados anteriores, obtidos pelo nosso grupo, demonstraram que a farinha de mostarda oriental (OMF) e a farinha de mostarda amarela YMF possuem atividade antifúngica que previne o crescimento fúngico (FG) em várias matrizes. Nesse contexto e com o ajuda da literatura, o objetivo do segundo trabalho foi elaborar uma estratégia antifúngica para prevenir o crescimento de *A. flavus* e a produção de AFB1 em amêndoas utilizando AITC, OMF e YMF. Com base na estrutura do composto, diferentes estratégias de aplicação foram consideradas, seja por pulverização direta ou pelo desenvolvimento de um dispositivo de fumigação. A OMF foi estudada para sua incorporação em uma solução de hidroxietil celulose juntamente com água para evaporar o AITC *in situ*. Por outro lado, a hidrólise da glucosinolato sinigrina presente na YMF gera principalmente p-hidroxibencil-isotiocianato (p-HBIT); esse composto não é volátil, portanto, a aplicação direta de YMF liofilizada em amêndoas foi a opção mais plausível. Além disso, a contribuição mais importante deste trabalho foi a elaboração de um dispositivo antifúngico a partir da mistura de hidroxietilncelulose e OMF.

Por fim, devido ao crescente interesse no uso de LAB como agentes de biocontrole em alimentos, o principal objetivo do terceiro trabalho foi identificar e caracterizar LAB com potencial antifúngico, a fim de criar um ingrediente antifúngico que pudesse ser utilizado na fabricação de produtos cárneos, como embutidos curados a seco. A maioria dos estudos relacionados às LAB envolve o uso de meio de cultura MRS como forma de tratamento. Em outras palavras, utilizam o sobrenadante livre de bactérias LAB fornecido pela fermentação do meio MRS. No entanto, o MRS não é permitido como aditivo alimentar, e a necessidade de desenvolver um caldo de base alimentar para ser fermentado pelas LAB poderia ser a solução. Ao longo deste estudo, diferentes caldos de carne foram elaborados e fermentados por várias cepas de *Pediococcus* e *Lactiplantibacillus* spp. Como conclusão, foi elaborado um ingrediente antifúngico e sua aplicação em embutidos

curados a seco foi evidenciada, embora esses resultados ainda não tenham sido publicados até o momento.

Além disso, a evidência da inibição do crescimento fúngico pelo ingrediente pós-biótico confirmou a necessidade de caracterizar os compostos produzidos responsáveis pela atividade antifúngica das LAB. Os compostos antifúngicos foram caracterizados por meio de HPLC-DAD, LC-qTOF-MS, GC-MS e MALDI-TOF.

8.2 OBJETIVOS

Em geral, o objetivo de todas as estratégias foi aumentar a vida útil dos alimentos, reduzindo a incidência de fungos toxigênicos, bem como reduzir a síntese de AFs e OTA. Para alcançar esse objetivo, os seguintes objetivos específicos foram estabelecidos:

1. Avaliar a eficácia do AITC na prevenção do crescimento de *P. verrucosum* e na produção de OTA em cevada armazenada com diferentes teores de umidade durante um período de 90 dias. Além disso, determinar a concentração residual de AITC nos grãos após o tratamento.
2. Avaliar o potencial antifúngico *in vitro* do AITC e do extrato de farinha de mostarda amarela liofilizada (YMF-E). Em seguida, desenvolver um dispositivo antifúngico baseado em hidroxietil celulose e farinha de mostarda oriental (H-OMF) e determinar seu efeito antifúngico. Ademais, avaliar a eficácia de todos os tratamentos para evitar o crescimento de *A. flavus* e prevenir a produção de AFB1 em amêndoas.
3. Isolar bactérias ácido lácticas com propriedades antifúngicas de embutidos tradicionais curados e desenvolver um ingrediente antifúngico com potencial aplicação na fabricação de produtos cárneos. Além disso, caracterizar e identificar os metabólitos responsáveis pela capacidade antifúngica do ingrediente.

8.3 RESULTADOS PRINCIPAIS E DISCUSSÃO GERAL

8.3.1 Atividade antifúngica *in vitro* dos compostos naturais

8.3.1.1 Atividade antifúngica do extrato de farinha de mostarda amarela (YMF-E)

Antes de realizar o ensaio de microdiluição de compostos antifúngicos, um extrato aquoso de YMF foi preparado. A solução de YMF foi liofilizada e ressuspendida em diferentes concentrações para formar um novo extrato (YMF-E). Como resultado, *A. flavus* demonstrou sensibilidade ao YMF-E, e a concentração inibitória mínima (MIC) e a concentração mínima fungicida (MFC) foram de 390 e 3.130 mg/L, respectivamente.

Estudos anteriores, como Quiles et al. (2018), relataram que a YMF poderia inibir o crescimento de vários fungos. Os autores também demonstraram que a MIC e a MFC variavam entre 238,2 e 15.000 mg/L, e entre 1.875 mg/L e 15.000 mg/L, respectivamente. É importante destacar que os resultados dependiam das cepas fúngicas testadas. Além disso, de acordo com os resultados de Quiles et al. (2018) e Torrijos et al. (2021), parece que o YMF-E não tem um efeito significativo em *Aspergillus* spp. em comparação com *Penicillium* spp. No entanto, em nosso estudo, observamos que o YMF-E apresentou um valor de MIC e MFC mais baixo em comparação com esses estudos. Apesar disso, nossos resultados são corroboraram com pesquisas anteriores que indicam que altas doses de YMF são necessárias para inibir o crescimento de *A. flavus* na mesma proporção dos fungos pertencentes ao gênero *Penicillium*.

8.3.1.2 Atividade antifúngica do AITC

O AITC é um composto conhecido por sua atividade antimicrobiana e estudos anteriores têm demonstrado sua eficácia. Além de sua capacidade antifúngica, o AITC é volátil e permite sua evaporação e liberação completa através de qualquer recipiente ou contêiner. Devido às suas características, o AITC se torna um excelente candidato para a fumigação de alimentos.

De forma semelhante ao YMF-E, no ensaio de microdiluição, o AITC mostrou uma atividade antifúngica significativa contra *A. flavus*. No entanto, a concentração necessária foi significativamente menor. Mais especificamente, a MIC e a MFC variaram entre 7,90 mg/L e 31,61 mg/L. Portanto, a concentração necessária para obter um resultado fungicida foi 100 vezes menor do que a da YMF-E.

Nielsen e Rios (2000) testaram a volatilização de óleos essenciais, como o AITC, contra vários fungos. Outros autores, como Clemente et al. (2019), também descreveram a atividade antifúngica do AITC em meio líquido. A atividade antifúngica do AITC está relacionada à sua carbonila eletrofílica (-N-C-S), que o torna reativo a nucleófilos, como tióis (-SH), aminas (-NH-, -NH2), carboxilas (-COOH) e hidroxilas (-OH). Como resultado, o AITC pode reagir com estruturas dos microrganismos, ligando-se a algumas estruturas e destruindo outras essenciais para a manutenção da vida dos microrganismos (Andini et al., 2020).

8.3.1.3 Atividade antifúngica do dispositivo de hidroxietil celulose e farinha de mostarda oriental (H-OMF)

A sinigrina é o principal glucosinolato associado à OMF. A hidrólise da sinigrina pela enzima mirosinase na presença de água libera AITC em fase de vapor. Portanto, a OMF é uma fonte natural de AITC, no entanto, essa reação não é estável e ocorre imediatamente, limitando sua aplicação. Levando em consideração esse antecedente, foi elaborado um dispositivo que retivesse o AITC, liberando-o lentamente. Para esse fim, utilizou-se um agente formador de gel (hidroxietil celulose) e criou-se um dispositivo com diferentes quantidades de OMF. O dispositivo recebeu a sigla H-OMF.

Uma vez que o H-OMF não pôde ser diluído em meio PDB, sua atividade antifúngica foi determinada por meio da medição do crescimento micelial (MG). O efeito antifúngico *in vitro* do dispositivo H-OMF contra *A. flavus* foi avaliado durante 7 dias a 25 °C. De modo geral, o efeito fungicida só foi observado na concentração de 30 mg/L de H-OMF. As doses de 12,5 e 25 mg/L mostraram apenas

um efeito fungistático, permitindo o MG durante o armazenamento. No entanto, é importante ressaltar que essas concentrações apresentaram diferença significativa em comparação com o grupo controle. Portanto, esses achados sugeriram que a capacidade do H-OMF de inibir o FG pode depender da dose, sendo necessárias concentrações superiores a 30 mg/L para obter um efeito fungicida em matrizes complexas, como frutas secas.

8.3.1.4 Atividade antifúngica in vitro do ingrediente pós-biótico

Antes de analisar a atividade antifúngica do ingrediente pós-biótico, foi necessário isolar, identificar e realizar um rastreamento de possíveis cepas antifúngicas de LAB. De 102 bactérias isoladas de diferentes embutidos secos caseiros espanhóis, apenas 42 isolados eram gram-positivos e catalase negativos.

Os 42 isolados foram avaliados utilizando o método de sobreposição em placas contendo PDA e MRSa (Overlay). Nesta etapa, o crescimento fúngico foi avaliado em cocultura com as bactérias. Das 42 bactérias testadas, apenas 14 isolados mostraram atividade inibitória contra os seis fungos testados, e o grau de inibição variou de acordo com a cepa fúngica específica examinada. Neste experimento, P. *griseofulvum* e *P. commune* foram as cepas mais suscetíveis. Em contraste, as cepas de *Aspergillus*, *A. parasiticus* e *A. flavus* foram as mais resistentes às LAB. Esses achados corroboram com os resultados obtidos por autores anteriores (Russo et al., 2017a), que observaram que as cepas de *Aspergillus* são mais resistentes às LAB do que outros gêneros fúngicos. Após isso, as 14 bactérias foram identificadas como espécies de *L. plantarum* e *P. pentosaceus* por meio do método MALDI-TOF-MS.

Após a seleção, vários caldos foram preparados utilizando diferentes concentrações de carne de lombo de porco liofilizada (2, 4, 8 e 10 g/L respectivamente, MB2, MB4, MB8 e MB10) com o objetivo de obter um caldo ideal para o crescimento das LAB que permitisse que as bactérias produzissem a mesma atividade antifúngica do MRSb fermentado. Uma vez que a ideia principal era evitar o uso de MRS na composição do novo ingrediente, o que poderia gerar preocupação

entre os consumidores; o uso de carne como ingrediente principal foi então planejado como uma alternativa natural.

Os meios MB2 a MB10 foram fermentados pelas 14 bactérias isoladas, e dois métodos foram utilizados para determinar a sensibilidade fúngica aos isolados bacterianos: o ensaio de difusão em ágar e a microdiluição do BFS em placas de 96 poços.

Primeiramente, foi determinada a atividade antifúngica qualitativa dos MB pelo ensaio de difusão em ágar. Para realizar esse método, as 14 cepas foram incubadas em MRSb (controle positivo de inibição) e nos MB. Os caldos fermentados foram centrifugados, liofilizados e ressuspendidos em uma concentração de 250 g/L. A solução final foi depositada em poços previamente preparados em PDA, nos quais foram semeadas seis espécies diferentes de fungos. Após a fermentação, apenas o MB10 mostrou atividade antifúngica semelhante ao MRSb fermentado. Nesta etapa, os resultados permitiram identificar a composição ideal do MB e selecionar três cepas que apresentaram atividade fungicida contra todas as cepas fúngicas. Como resultado, os outros caldos e bactérias foram eliminados. As cepas selecionadas foram *P. pentosaceus* C12, *P. pentosaceus* C15 e *L. plantarum* C60.

Finalmente, a capacidade antifúngica quantitativa das três cepas foi determinada pelo ensaio de microdiluição de compostos antifúngicos. Esse método envolveu o uso de placas de 96 poços para diluir o analito antifúngico em diluições seriadas para determinar a MIC (Concentração Inibitória Mínima) e a MFC (Concentração Fungicida Mínima). Dessa forma, foi possível quantificar a atividade antifúngica do MRSb e do MB fermentados pelas cepas LAB. Esse ensaio demonstrou que a fermentação tanto do MRSb quanto do MB10 com *P. pentosaceus* C15 resultou em concentrações mais baixas necessárias para inibir o crescimento fúngico contra todas as cepas toxigênicas testadas, em comparação com os mesmos caldos fermentados por *P. pentosaceus* C12 e *L. plantarum* C60. Embora o MRSb

fermentado tenha mostrado um efeito antifúngico mais forte, a diferença de eficácia não foi significativa em comparação com o MB10. Em certos casos, não foram observadas diferenças significativas ($p \leq 0,05$) entre os tratamentos. Em outras palavras, *P. pentosaceus* C15 foi capaz de fermentar o MB10 e produzir compostos antifúngicos, que inibiram o crescimento fúngico em condições semelhantes ao MRSb.

De maneira geral, nossas descobertas indicaram que *P. pentosaceus* C15 apresentou um maior potencial antifúngico tanto no ensaio de difusão em ágar quanto na microdiluição do BFS. Com base nesses resultados *in vitro*, o meio MB10 foi fermentado com *P. pentosaceus* C15 por 48 horas a 37 °C para obter um ingrediente pós-biótico antifúngico. Posteriormente, o caldo fermentado foi centrifugado para remover as bactérias e liofilizado para produzir um pó que facilitasse sua aplicação em alimentos e melhorasse a vida útil do ingrediente.

Após uma breve revisão dos estudos científicos, ficou evidente que apenas um número limitado deles relatou um amplo espectro de atividade antifúngica de LAB. No entanto, a maioria desses estudos demonstrou que as LAB têm maior atividade antifúngica contra uma ou duas espécies específicas de fungos, conforme demonstrado por Hernández et al. (2022). Portanto, o objetivo deste trabalho foi isolar uma cepa que apresentasse um espectro mais amplo de atividade antifúngica. Nossas descobertas indicam que a atividade antifúngica das LAB varia de acordo com a cepa, a espécie fúngica e os métodos analíticos utilizados. Além disso, os resultados revelaram que é difícil encontrar uma LAB com amplo espectro antifúngico, razão pela qual foram identificadas apenas três após várias avaliações. Além disso, nosso estudo sugere que os embutidos curados podem ser uma fonte valiosa de LAB antifúngicas, uma vez que exibem propriedades inibitórias significativas contra os microrganismos avaliados.

Por fim, devido à atividade antifúngica evidenciada pelo ingrediente pós-biótico, foi necessário identificar os principais compostos produzidos pelas LAB,

como ácidos orgânicos, ácidos fenólicos e VOCs (compostos orgânicos voláteis), para ajudar a elucidar o mecanismo de ação antifúngica das LAB.

8.3.2 Aplicação de compostos antifúngicos naturais em alimentos

8.3.2.1 Biocontrole de P. verrucosum em grãos de cevada

O potencial antifúngico de AITC, um composto natural reconhecido como GRAS (geralmente reconhecido como seguro) e permitido como conservante de alimentos nos Estados Unidos, foi avaliado pela primeira vez contra *P. verrucosum* usando um ensaio simulador de silo com grãos de cevada armazenados em diferentes condições de umidade.

O método de contagem em placa de ágar permitiu investigar o impacto do MC e do tratamento com AITC no crescimento do *P. verrucosum*. Como resultado, o uso de AITC como fumigante a uma concentração de 50 µL/L em grãos de cevada armazenados com 13%, 16%, 19% e 21% de MC reduziu significativamente a população fúngica em 24 horas de exposição. Além disso, o AITC foi capaz de reduzir a população fúngica a níveis abaixo do limite de quantificação após 30 dias, e esse efeito foi mantido até 90 dias, independentemente da condição de armazenamento em MC. Esses resultados destacaram a importância da implementação de múltiplas barreiras sanitárias para melhorar a segurança alimentar, incluindo a fumigação com AITC e o controle do teor de umidade para prevenir o crescimento fúngico.

8.3.2.2 Biocontrole de A. flavus em amêndoas

Os resultados *in vitro* sugeriram que a aplicação de AITC, YMF-E e H-OMF pode ser eficiente em amêndoas para controlar o crescimento de *A. flavus*. Nesta etapa, foram realizados três métodos de aplicação diferentes. Primeiro, o AITC foi embebido em um papel e colocado dentro de um recipiente de simulação de silo que continha amêndoas. Segundo o YMF-E foi preparado e espalhado diretamente na

superfície das amostras em forma de spray. Terceiro, um dispositivo antifúngico contendo água, OMF e hidroxietil celulose foi preparado e colocado no recipiente para permitir a volatilização do AITC.

Os resultados da contagem de colônias fúngicas demonstraram que o YMF-E não mostrou nenhuma inibição do FG. Por outro lado, a aplicação de 2.000 e 4.000 mg/L de H-OMF, bem como 5,07, 10,13 e 20,26 mg/L de AITC, resultou em uma diminuição significativa da população fúngica, alcançando níveis abaixo do limite de detecção tanto no sétimo quanto no décimo quinto dia. Esse fenômeno pode ser atribuído à conversão de OMF em AITC na presença de água. Portanto, o OMF pode ser usado como uma fonte natural de AITC, que é liberado gradualmente no espaço livre do sistema de silo. Esse estudo também estabeleceu que o AITC possui uma potente atividade antifúngica contra *A. flavus*. Além disso, o novo dispositivo chamado H-OMF pode ter o potencial de ser efetivo contra cepas toxigênicas em vários tipos de alimentos. No entanto, recomenda-se realizar pesquisas adicionais para investigar o potencial fumigante do H-OMF em outros frutos secos e alimentos.

8.3.2.3 Atividade antitoxigênica de compostos naturais em alimentos

Vários estudos têm demonstrado que o AITC tem a capacidade de prevenir a formação de micotoxinas em diferentes produtos alimentares, como frutos secos, pão, pizza e farinha de trigo. No entanto, existe uma falta de pesquisa sobre a efetividade do AITC na prevenção da produção de OTA e sua aplicação na cevada para consumo humano ou alimentação animal.

Devido a essa lacuna na literatura, no primeiro estudo, determinou-se o efeito de diferentes concentrações de umidade (MC) e a fumigação com AITC na produção de OTA em grãos de cevada armazenados por 90 dias. Os grãos foram contaminados com *P. verrucosum* e analisados por cromatografia líquida de alta resolução acoplada a um detector de massas em tandem (HPLC-MS/MS), a qual permitiu a quantificação da OTA produzida.

Similar aos resultados evidenciados pela análise de FG em grãos de cevada, o tratamento com AITC inibiu a produção de OTA, mostrando uma diferença significativa no conteúdo de OTA em comparação com o grupo controle. Da mesma forma, a MC demonstrou uma importante correlação positiva na produção de OTA, ou seja, quanto maior a MC, maiores níveis de OTA foram encontrados nas amostras. Portanto, grãos com MC superior a 13% devem ser evitados para prevenir a produção de micotoxinas e garantir a segurança alimentar. Caso contrário, o tratamento com AITC também poderia ser uma alternativa para prevenir a produção de OTA por 90 dias. No entanto, a melhor estratégia seria associar técnicas de redução de umidade e a fumigação com AITC.

No segundo estudo, nosso objetivo foi investigar a efetividade do dispositivo H-OMF e AITC na redução do conteúdo de AFB1 em amêndoas. Para replicar a contaminação natural das amêndoas, *A. flavus* foi usado como produtor de AFs, e as amêndoas foram analisadas do dia 0 ao dia 15 para a produção de AFB1 utilizando HPLC-MS/MS.

Os resultados mostraram que H-OMF em concentrações de 2.000 mg/L e 4.000 mg/L reduziu efetivamente a produção de AFB1. No entanto, YMF-E em concentrações de 100, 160 e 200 g/L aumentou a produção de AFB1. Por outro lado, constatou-se que AITC em concentrações de 5,07, 10,13 e 20,26 mg/L foi eficaz na redução da produção de micotoxinas. A redução de AFB1 pode ser atribuída ao fato de que esses tratamentos inibiram o FG, impedindo que o fungo ative sua via de metabólitos secundários.

Vale ressaltar que este estudo é o primeiro a utilizar um dispositivo baseado em hidroxietil celulose e OMF para reduzir o teor de AFB1 em amêndoas. Em contraste, o uso de AITC para reduzir o crescimento de fungos e a produção de micotoxinas foi previamente estudado por outros autores.

Em geral, nossos resultados sugerem que o uso de YMF-E como tratamento antitoxigênico deve ser evitado, enquanto H-OMF pode ser expandido e testado em experimentos em grande escala ou em silos reais.

8.3.3 Avaliação da concentração residual de AITC durante o armazenamento

Para determinar a concentração residual de AITC em grãos de cevada, foram extraídas amostras utilizando uma solução de metanol e avaliadas por cromatografia gasosa acoplada a um detector de ionização de chama, o que permitiu a avaliação da fração de AITC absorvida pelos grãos durante os 90 dias de fumigação. Como resultado, a concentração inicial de AITC absorvida dependeu da MC, com um maior teor de umidade resultando em uma maior absorção. Independentemente da MC, o AITC foi liberado lentamente da matriz, o que levou a uma vida útil prolongada de até 90 dias. No entanto, o impacto potencial da concentração residual de AITC na recontaminação fúngica deve ser estudado.

8.3.4 Caracterização dos compostos antifúngicos no ingrediente pós-biótico

8.3.4.1 Determinação do teor de compostos fenólicos e ácidos orgânicos

Nesta parte do estudo, foram analisados os principais ácidos orgânicos e compostos fenólicos presentes nos caldos fermentados por LAB que apresentaram atividade antifúngica *in vitro*. O teor de ácidos orgânicos foi determinado por HPLC-DAD e os ácidos fenólicos foram determinados por UHPLC-Q-TOF/MS. Assim, foram investigados os metabólitos produzidos após a fermentação do MRSb e do MB10 pelas cepas *P. pentosaceus* C12, *P. pentosaceus* C15 e *L. plantarum* C60.

Os resultados mostraram que o ácido láctico e o ácido acético foram identificados em ambas as formulações, sendo o ácido láctico aquele que apresentou uma concentração mais alta do que o ácido acético. A maior quantidade de ácido láctico foi detectada no MRSb (2,973 g/L) e no MB10 (3,00 g/L) fermentados pela cepa *P. pentosaceus* C15. No entanto, não houve diferenças estatísticas na produção

de ácido láctico entre essas duas formulações ($p \leq 0,05$). Em relação ao ácido acético, também foi quantificada uma concentração maior ($p \leq 0,05$) no MB10 fermentado com *P. pentosaceus* C15 (0,38 g/L).

Quanto aos compostos fenólicos, foram identificados 12 ácidos fenólicos diferentes, com destaque para o ácido benzoico, PLA, ácido siríngico e ácido vanílico. Observou-se que o MRSb permitiu concentrações mais altas de ácidos fenólicos do que o MB10. Em particular, o ácido siríngico foi significativamente maior quando MB10 foi fermentado pela cepa *P. pentosaceus* C12, enquanto o ácido vanílico foi o ácido fenólico mais produzido por *P. pentosaceus* C15 e *L. plantarum* C60 tanto em MRSb quanto em MB10. A produção de alguns ácidos fenólicos, como PLA, ácido vanílico e ácido benzóico, que previamente demonstraram ter propriedades antioxidantes ou antifúngicas, indica que os ingredientes antifúngicos obtidos a partir da fermentação de MB10 podem ser adicionados a produtos cárneos para fornecer uma fonte significativa de ácidos fenólicos e orgânicos (além de contribuir para a segurança alimentar).

As LAB são capazes de sintetizar diversos compostos antimicrobianos, como ácidos orgânicos, ácidos fenólicos, AMPs (péptidos antimicrobianos), diacetil e reuterina. Esses metabólitos podem ter uma atividade sinérgica, mas a atividade sinérgica desses metabólitos ainda não está completamente elucidada. Portanto, os resultados sugerem que a atividade antifúngica do MB10 não se deve exclusivamente aos ácidos orgânicos e fenólicos, mas outras substâncias voláteis e a redução do pH também contribuem para uma atividade antifúngica eficaz.

Christ-Ribeiro et al. (2019) evidenciaram que os compostos fenólicos têm um efeito preventivo sobre o desenvolvimento fúngico ao exercer efeitos inibitórios sobre a biossíntese de componentes celulares chave, como glicanos, quitinas, manoproteínas e o ergosterol, que compõem a parede celular e a membrana fúngica. Esse modo de ação é atribuído à capacidade dos compostos fenólicos de causar danos na parede celular e na membrana fúngica, resultando em alterações na regulação do

fluxo de nutrientes. Como consequência dessas perturbações, a célula fúngica tem sua capacidade de sintetizar biomoléculas essenciais reduzida, como proteínas, aminoácidos e esfingolipídios. Além disso, é sabido que os compostos fenólicos dificultam o fluxo de elétrons e, portanto, comprometem a integridade estrutural e funcional da célula fúngica.

8.3.4.2 Identificação dos VOC no ingrediente pós-biótico

Na parte do estudo relacionada com a composição de VOCs (compostos orgânicos voláteis) do ingrediente pós-biótico fermentado a partir de caldo de carne (MB10), foi utilizada a técnica de HS-SPME acoplada a GC-MS. Foram identificados um total de 24 compostos, incluindo 2 alcanos, 2 ácidos, 7 álcoois, 6 aldeídos, 5 cetonas e 2 pirazinas.

Os compostos mais abundantes encontrados nas amostras foram os aldeídos, representando entre 27,8% e 40,8% do total de VOCs detectados. Entre eles, o benzaldeído representou entre 18,4% e 20,3%, dependendo da formulação analisada. Observou-se que o teor de aldeídos no MB10 fermentado foi estatisticamente menor ($p \leq 0,05$) em comparação com o grupo controle. Da mesma forma, as pirazinas foram o segundo grupo mais abundante, e seu teor diminuiu de forma estatisticamente significativa nas formulações de MB10 fermentado. Tanto a diminuição do teor de pirazinas como de aldeídos podem ser explicadas pela fermentação de LAB. Os álcoois identificados apresentaram um valor médio de %PA (percentagem da área total) variando entre 10,8% e 17,6%, com destaque para a detecção de PEA (álcool fenetílico) em maior proporção no MB10 fermentado com a bactéria C15 em comparação com outras formulações ($p \leq 0,05$). Este composto ativo tem sido estudado pelo seu potencial antifúngico e pode explicar os valores mais baixos de MIC (concentração mínima inibitória) e MFC (concentração mínima fungicida) detectados no MB10-C15. A fermentação de LAB também aumentou significativamente os níveis de cetonas no MB10 formulado e introduziu três novas cetonas. Foram identificados também dois ácidos no MB10 fermentado, ácido

acético e ácido nonanoico. É importante destacar que o ácido nonanoico também tem demonstrado propriedades antimicrobianas contra vários patógenos, conforme relatado por Chen et al. (2020), Lee et al. (2021), White et al. (2021) e Cimowsky et al. (2022). Portanto, os ácidos voláteis combinados com os ácidos orgânicos e fenólicos encontrados no MB10 podem contribuir para as propriedades antifúngicas do ingrediente formulado.

8.3.4.3 Análise de Componentes Principais (PCA)

Foi realizado uma análise de componentes principais (PCA) para entender as diferenças entre os ácidos orgânicos, ácidos fenólicos e VOCs produzidos, e tentar determinar qual composto contribui para a propriedade antifúngica ou é responsável pela diferenciação das formulações de MB fermentadas.

A contribuição combinada dos dois primeiros componentes principais (PCs) representou 90,1% da variância total explicada. Especificamente, o PC1 explicou 64,5% da variância total, enquanto o PC2 explicou 25,6%.

No eixo positivo do PC1, ambas as cepas de *P. pentosaceus* (C12 e C15) se distribuíram, enquanto a cepa de *L. plantarum* C60 se posicionou no eixo negativo. O PC2 diferenciou as cepas C12 e C15 com base em seu perfil de metabólitos bioativos, já que C12 se posicionou no eixo negativo e C15 no eixo positivo. Finalmente, de acordo com o gráfico de loading, observou-se que MB10-C12 e MB10-C15 podiam ser distinguidos por sua produção de aldeídos voláteis, especificamente compostos como heptanal, 2-decenal e dodecanal. MB10-C15 foi caracterizado pela produção de diferentes compostos antifúngicos, como ácido nonanoico e álcool fenetílico, que o diferenciaram de outras formulações. Em comparação, MB10-C60, formulado com a cepa *L. plantarum*, diferenciou-se das formulações de *P. pentosaceus* por uma maior produção do composto ácido propiônico.

8.4 CONCLUSÕES

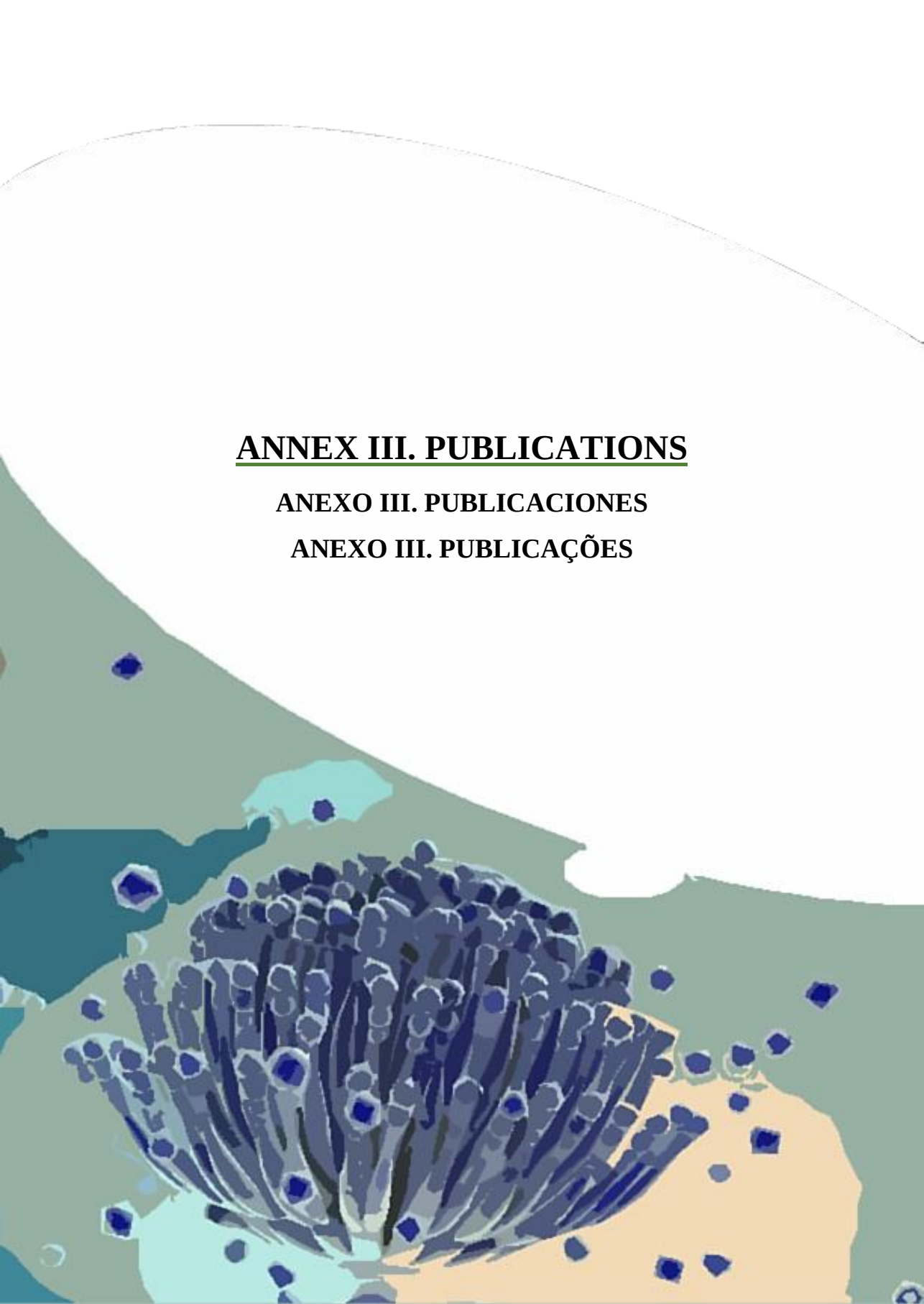
1. A solução salina supersaturada pode ser utilizada para simular as condições de teor de umidade (MC) encontradas em silos mal gerenciados. Além disso, observou-se que o MC tem um efeito proporcional sobre o crescimento fúngico e a produção de micotoxinas. Quanto maior o MC, maior o FG.
2. O AITC a 50 µL/L foi absorvido pela cevada e liberado gradualmente. A liberação gradual de AITC evitou a produção de OTA e o FG por 90 dias, independentemente da MC do grão.
3. O uso de AITC como fumigante natural associado ao controle do MC dos grãos pode evitar completamente o deterioro fúngico e a produção de micotoxinas.
4. O AITC, o extrato de farinha de mostarda amarela e o dispositivo antifúngico H-OMF mostraram efeito antifúngico contra *A. flavus in vitro*, sendo o AITC o mais efetivo, seguido pelo dispositivo antifúngico e pelo extrato de farinha de mostarda amarela.
5. A farinha de mostarda amarela não mostrou efeito antifúngico quando pulverizada nas amêndoas.
6. O AITC em concentrações de 5,07, 10,13 e 20,26 mg/L evitou o crescimento de *A. flavus* em amêndoas e, consequentemente, inibiu a produção de AFB1 a níveis abaixo do limite de detecção.
7. Da mesma forma, o dispositivo antifúngico contendo 2.000 e 4.000 mg/L de farinha de mostarda oriental reduziu o crescimento fúngico e a concentração de AFB1 a níveis abaixo do limite de detecção sem entrar em contato com os alimentos.
8. Um total de 42 bactérias ácido lácticas antifúngicas foram isoladas de embutidos curados, dos quais 14 apresentaram atividade antifúngica contra seis fungos dos gêneros *Aspergillus* e *Penicillium*. No entanto, apenas três bactérias, *L. plantarum* C60, *P. pentosaceus* C12 e *P. pentosaceus* C15, mostraram atividade antifúngica *in vitro* contra todos os fungos.

9. A cepa *P. pentosaceus* C15 mostrou uma maior atividade antifúngica in vitro, pelo que esta bactéria foi selecionada para a preparação do caldo fermentado MB10 para produzir um ingrediente pós-biótico antifúngico.
10. A caracterização química destacou que o ingrediente pós-biótico é rico em ácidos fenólicos e ácidos orgânicos. Um total de dois ácidos orgânicos (ácido lático e ácido acético) e 12 ácidos fenólicos diferentes foram identificados, com destaque para o ácido benzoico, PLA, ácido siríngico e ácido vanílico.
11. Um total de 24 compostos orgânicos voláteis foram identificados no ingrediente pós-biótico, e o composto mais abundante encontrado foi o benzaldeído. Além disso, também foram identificados compostos antifúngicos, como álcool fenetílico, ácido nonanoico e ácido acético.

ANNEX III. PUBLICATIONS

ANEXO III. PUBLICACIONES

ANEXO III. PUBLICAÇÕES



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Antifungal and antimycotoxigenic activity of allyl isothiocyanate on barley under different storage conditions

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ABSTRACT

The present study evaluated the efficacy of allyl isothiocyanate (AITC) in avoiding the growth of *Penicillium verrucosum* and consequent ochratoxin A (OTA) production in barley during storage for 90 d. Environmental humidity was controlled using saturated salt solutions and moisture content (MC) of the grain was analyzed. Moreover, the residual concentration of AITC on stored barley was also examined. Samples with 20.6% of MC presented the highest absorption of AITC with levels ranging from 75 to 4 mg/kg at day 1 and 90, respectively. The population of *P. verrucosum* was significantly reduced after 24 h of AITC exposure. After 90 d, the non-treated control group reached a fungal population of 8.3 log CFU/g and 1.5 mg/kg of OTA. On the other hand, AITC at 50 μL/L was able to reduce the fungal population as well as the production of OTA to levels that were below the limit of detection, independently of the MC and the time of exposure. In conclusion, the AITC was absorbed by the barley and released gradually, inhibiting the growth of *P. verrucosum* and the production of mycotoxin. Therefore, AITC could be used as a fungicide to prolong the shelf life of the barley improving its safety.

1. Introduction

Barley, hops, yeast, and water are the main or sole ingredients in beer production, and their quality is essential to confer its acceptance by consumers. Beer is the most consumed alcoholic drink in America and Europe, representing around 34.8% of all alcoholic beverages consumed in the world (WHO, 2014).

Barley is commonly colonized by the toxigenic fungi from the genera *Penicillium, Aspergillus,* and *Fusarium.* Some species of fungi, such as *P. verrucosum* is known to produce ochratoxin A during the plant growth in the field or throughout grain storage. Beer contamination by mycotoxins is a public health concern since longterm exposure to these substances can lead to carcinogenic, teratogenic and mutagenic effects in humans (Binder, 2007; Zain, 2011). Specifically, OTA is nephrotoxic, mutagenic and carcinogenic classified by the International Agency for Research on Cancer as a compound belonging to group 2B – possibly carcinogenic compounds (IARC, 1993).

Numerous studies have investigated the incidence of mycotoxins in beer and several countries have stipulated specific regulations for OTA in foodstuffs (Bertuzzi, Rastelli, Mulazzi, Donadini, & Pietri, 2018; Pascari, Ramos, Marín, & Sanchís, 2018; Peters et al., 2017, Bellver Soto, Fernández-Franzón, Ruíz, & Juan-García, 2014). The Commission of the European Community 1881/2006 fixed the OTA limit in unprocessed grains at 3 μg/kg whereas the limit for grains intended for animal feed is 250 μg/kg. On the other hand, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) established a tolerable weekly intake of OTA for humans in 112 ng/kg body weight/day, on the basis of experiments that evaluated the deterioration of renal function in pigs (FAO/WHO, 2008).

Beer production has diverse operations which can impact on final levels of mycotoxins, but these operations cannot totally reduce OTA due to its capacity to withstand thermic treatments (Kabak, 2009).

In order to minimize economic losses promoted by OTA, some authors have proposed different strategies, such as fungicide application in the field or during storage, addition of microorganisms during the fermentation process to bind or degrade OTA, and the use of hot water treatment (Luz, Ferrer, Mañsirenes, & Meca, 2018; Mateo, Medina, Mateo, Mateo, & Jiménez, 2007; Zhang, Apaliya, Mahunu, Chen, & Li, 2016). Ideally, avoidance of fungal contamination is a better path to prevent high mycotoxin levels in beer and its byproducts. As these

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toxins

MDPI

Article

Development of an Antifungal Device Based on Oriental Mustard Flour to Prevent Fungal Growth and Aflatoxin B1 Production in Almonds

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Abstract: The present study describes the manufacture of an antifungal device composed of oriental mustard flour and hydroxyethyl-cellulose (H-OMF) and evaluates its efficacity in inhibiting *Aspergillus flavus* growth and aflatoxin B1 (AFB_1) production in almonds. Additionally, it compares the H-OMF with allyl isothiocyanate (AITC) and a freeze-dried extract of yellow mustard flour (YMF-E); such substances were previously described as antifungal. Minimum inhibitory concentration (MIC), Minimum fungicidal concentration (MFC), the H-OMF in vitro antifungal activity, and the residual fungal population, as well as the production of AFB_1 in almonds were determined. AITC and YMF-E showed significant antifungal activity in vitro. Additionally, the in vitro activity of H-OMF avoided mycelial growth by applying 30 mg/L. Almonds treated with AITC (5.07, 10.13, and 20.26 mg/L) and H-OMF (2000 and 4000 mg/L) showed a reduction in the population of *A. flavus* and the production of AFB_1 to values below the limit of detection. YMF-E showed effectiveness by in vitro methodologies (MIC and MFC) but did not show efficacy when applied in almonds. Our findings indicated that the hydroxyethyl-cellulose-based device containing oriental mustard flour might be utilised as a fumigant to increase the safety of almonds and could be extended to other cereals or dry fruits.

Keywords: natural antimicrobials; fungi; mycotoxins; food safety; AITC; *Aspergillus flavus*; hydroxyethyl-cellulose-based device

Key Contribution: This work focused on the study of the antifungal activity of bioactive compounds, especially allyl isothiocyanate (AITC), freeze-dried extract of yellow mustard flour (YMF-E), and the mixture of oriental mustard flour and hydroxyethyl-cellulose (H-OMF). AITC, YMF-E, and H-OMF showed in vitro antifungal activity against *A. flavus*, but only H-OMF and AITC inhibited the growth of *A. flavus* and prevented the production of aflatoxin B_1 in almonds. The results demonstrated that H-OMF could be used as a fumigant to prevent fungal growth on nuts. Therefore, this manuscript is of interest, important from a food safety point of view, as it addresses the possibility of limiting the biosynthesis of carcinogenic aflatoxins and finding new strategies to prevent fungal growth and mycotoxin production.

Citation: Nazareth, T.M.; Torrijos, R.; Bocate, K.P.; Mañes, J.; Luciano, F.B.; Meca, G.; Vila-Donat, P. Development of an Antifungal Device Based on Oriental Mustard Flour to Prevent Fungal Growth and Aflatoxin B1 Production in Almonds. *Toxins* **2022**, *14*, 5. https://doi.org/10.3390/toxins14010005

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1. Introduction

Mycotoxins are secondary fungal metabolites produced mainly by the genera *Aspergillus*, *Penicillium*, and *Fusarium* [1–3]. Among the metabolites produced, aflatoxins (AFs) are produced by fungi of the genus *Aspergillus*, predominantly by two species, *Aspergillus flavus* and *Aspergillus parasiticus*, which can produce four main AFs, B_1, B_2, G_1, and G_2. According to International Cancer Research Agency (IARC), AFs are classified into group 1:

foods

MDPI

Article

Manufacture of a Potential Antifungal Ingredient Using Lactic Acid Bacteria from Dry-Cured Sausages

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Abstract: The growing interest in functional foods has fueled the hunt for novel lactic acid bacteria (LAB) found in natural sources such as fermented foods. Thus, the aims of this study were to isolate, identify, characterize, and quantify LAB's antifungal activity and formulate an ingredient for meat product applications. The overlay method performed a logical initial screening by assessing isolated bacteria's antifungal activity in vitro. Next, the antifungal activity of the fermented bacteria-free supernatants (BFS) was evaluated by agar diffusion assay against six toxigenic fungi. Subsequently, the antifungal activity of the most antifungal BFS was quantified using the microdilution method in 96-well microplates. The meat broth that showed higher antifungal activity was selected to elaborate on an ingredient to be applied to meat products. Finally, antifungal compounds such as organic acids, phenolic acids, and volatile organic compounds were identified in the chosen-fermented meat broth. The most promising biological candidates belonged to the *Lactiplantibacillus plantarum* and *Pediococcus pentosaceus*. *P. pentosaceus* C15 distinguished from other bacteria by the production of antifungal compounds such as nonanoic acid and phenyl ethyl alcohol, as well as the higher production of lactic and acetic acid.

Keywords: *Pediococcus pentosaceus*; *Lactiplantibacillus plantarum*; antifungal activity; organic acids; phenolic acids; volatile organic compounds

Citation: Nazareth, T.d.M.; Calpe, J.; Luz, C.; Mañes, J.; Meca, G. Manufacture of a Potential Antifungal Ingredient Using Lactic Acid Bacteria from Dry-Cured Sausages. *Foods* **2023**, *12*, 1427. https://doi.org/10.3390/foods12071427

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1. Introduction

Due to their high nutritional content, meat and its derivatives are an important food category in many people's diets. Consuming processed meats such as sausages, hot dogs, and luncheon meats has grown mainstream [1]. Between 1998 and 2018, global meat consumption rose by 58%, reaching a total of 360 million tonnes [2].

Dry-cured meat products are a type of traditional food that are manufactured and eaten across the globe. Their market dominance is well-known, owing to customers' stringent expectations for high-quality and safe foodstuffs. Consumption of these fungus-infected foods increases the risk of exposure to mycotoxins; this is a worldwide public health concern [3].

Dry-cured meats mostly comprise muscle tissue, and their surface physical-chemical characteristics, such as low water activity (A_w), neutrality to low pH, high salt content, and protein content, influence the microbial flora that grows on their exterior layers [4]. Although dry-fermented sausages have low A_w and high salt content, alterations of these properties influence the metabolism by facilitating mycotoxin biosynthesis [5].

Several species are recognized for producing mycotoxins, including *Aspergillus*, *Alternaria*, *Claviceps*, *Fusarium*, and *Penicillium* [6]. In certain environmental and substrate conditions over meat products, *Aspergillus flavus*, *A. parasiticus*, *A. ochraceus*, *P. nordicum*, *P. polonicum*, and *P. verrucosum* produce mycotoxins, posing a risk to consumers through their growth in salami, dry-cured hams, and other meat products [7,8].

Penicillium nordicum is the most important ochratoxin A (OTA) producing species frequently detected in cold-chain protein foods such as dry-cured ham, salami, and salted fish.

(Under review)

Aflatoxin B1 and Ochratoxin A: Generalities, Food Occurrence, Health Impact, Mitigation Methodologies, and Biocontrol – A Review.

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