Universidade do Minho
Escola de Engenharia

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Inhibition of Fungal Growth and Mycotoxin Production by Lactic Acid Bacteria

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Inhibition of Fungal Growth and Mycotoxin Production by Lactic Acid Bacteria

PhD in Chemical and Biological Engineering

Work done under the guidance of
Doctor Luís João Abrunhosa Pereira
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Inhibition of Fungal Growth and Mycotoxin Production by Lactic Acid Bacteria

ABSTRACT

Lactic acid bacteria (LAB) are frequently used in fermentation processes and have been gaining attention in biopreservation approaches against fungal contaminations. Fungal contaminations can be responsible for health issues, and economic losses since several species produce toxic compounds named mycotoxins. Mycotoxins can cause several organs diseases and cancer. In this work, cell-free supernatant (CFS) of the *Lactobacillus plantarum* UM55 strain demonstrated the ability to reduce *Aspergillus flavus* growth by 32% and aflatoxin production by 91%. Additionally, this strain CFS and the CFS from strain *Lactobacillus buchneri* UTAD104 inhibited radial growth of *Penicillium nordicum* by 17% and Ochratoxin A (OTA) production by 60%. These inhibitions resulted from organic acids produced by LAB. CFS of *L. plantarum* UM55 contained lactic acid, phenyllactic acid (PLA), hydroxyphenyllactic acid (OH-PLA) and indole lactic acid (ILA), while CFS of *L. buchneri* UTAD104 had acetic acid, lactic acid, and PLA. These compounds were individually tested against *A. flavus* and *P. nordicum*, with all showing an inhibiting effect on fungal growth and mycotoxin production. PLA and acetic acid were the most effective inhibiting *A. flavus* and *P. nordicum*, respectively. When the inhibitory activity of LAB cells incorporated into an agar culture medium was tested, *L. buchneri* UTAD104 inhibited the production of OTA entirely in all conditions tested, with acetic acid being the main responsible for this effect. *L. buchneri* UTAD104 was further incorporated into edible films and coatings to prevent fungal contamination in cheese. Films containing the bacteria, maintained their physicochemical, mechanical and barrier properties when compared with control films. These films completely inhibited the growth of *P. nordicum* on the surface of cheese samples, resulting in the absence of OTA in the samples.

Some LAB strains are definitely capable of controlling mycotoxigenic fungi. The antifungal ability of these strains is mainly due to the production of organic acids. LAB strains can be incorporated into edible films and coatings for the control of mycotoxigenic fungi in cheese products.

Keywords: antifungal; biopreservation; lactic acid bacteria; mycotoxins; organic acids

Inibição do Crescimento Fúngico e Produção de Micotoxinas por Bactérias do Ácido Láctico

RESUMO

As bactérias do ácido láctico (BAL) são extensivamente usadas em processos de fermentação e têm suscitado interesse em abordagens de biopreservação para o controlo de fungos. As contaminações fúngicas podem originar problemas de saúde e avultadas perdas económicas. Algumas espécies de fungos podem, ainda, produzir compostos altamente tóxicos denominados de micotoxinas. As micotoxinas podem originar várias doenças em órgãos internos e provocar cancro. Neste trabalho, verificou-se que os sobrenadantes da estirpe *Lactobacillus plantarum* UM55 reduziram o crescimento do fungo *Aspergillus flavus* em 32% e a produção de aflatoxinas em 91%. O sobrenadante desta estirpe e da estirpe *Lactobacillus buchneri* UTAD104 mostraram também inibir o crescimento radial de *Penicillium nordicum* em 17% e a produção de ocratoxina A (OTA) em 60%. Estas inibições resultam da produção de ácidos orgânicos por partes destas estirpes. Foi detetada a presença dos ácidos lático, fenil-lático, hidroxifenil-lático e índole lático nos sobrenadantes de *L. plantarum* UM55 e dos ácidos acético, lático e fenil-lático nos sobrenadantes de *L. buchneri* UTAD104. Estes compostos foram testados individualmente contra *A. flavus* e *P. nordicum*, e todos inibiram o crescimento fúngico e a produção de micotoxinas. O ácido fenil-lático e acético foram os mais eficazes na inibição de *A. flavus* e *P. nordicum*, respetivamente. Testes com BAL incorporadas no meio de cultura demonstraram que *L. buchneri* UTAD104 suprimiu a produção de OTA em todas as condições testadas, sendo o ácido acético o principal responsável por este efeito. Esta estirpe foi incorporada em filmes e revestimentos edíveis com o objetivo de eliminar fungos no queijo. Testes aos filmes com incorporação de BAL, mostraram que estes filmes mantiveram as suas propriedades físico-químicas, mecânicas e de barreira, quando comparados com os filmes de controlo. Estes filmes inibiram completamente o crescimento de *P. nordicum* na superfície do queijo resultando na ausência de OTA nas amostras. Este trabalho demonstrou que algumas BAL podem ser utilizadas para controlar fungos micotoxigénicos. A capacidade antifúngica destas estirpes é devida, maioritariamente, à produção de ácidos orgânicos. As BAL podem ser incorporadas em filmes e revestimentos edíveis para controlar fungos micotoxigénicos em queijos.

Palavras chave: ácidos orgânicos; antifúngico; bactéria do ácido láctico; biopreservação; micotoxinas

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LIST OF ABBREVIATIONS

AFB1	Aflatoxin B1
AFB2	Aflatoxin B2
AFG1	Aflatoxin G1
AFG2	Aflatoxin G2
AF	Aflatoxins
AFM1	Aflatoxin M1
ASTM	American Society for Testing and Materials
aw	Water activity
CFS	Cell-free supernatant
CPA	Cyclopiazonic acid
DMSO	Dimethyl sulfoxide
DON	Deoxynivalenol
EB	Elongation at Break
EC	European Commission
EFSA	European Food Safety Authority
EU	European Union
FAO	Food Agriculture Organization
FB1	Fumonisin B1
FB2	Fumonisin B2
GRAS	Generally Regarded as Safe
GYEP	Glucose-yeast extract-peptone
HPC	Hydroxypropyl Cellulose
IARC	The International Agency for Research on Cancer
IC_{50}	Inhibitory Concentration to reach 50% inhibition
IC_{90}	Inhibitory Concentration to reach 90% inhibition

ILA	Indole lactic acid
LAB	Lactic Acid Bacteria
MCE	Methylcellulose ester
MEA	Malt Extract Agar
MIC	Minimum Inhibitory Concentration
MRS	Man, Rogosa and Sharpe
MSA	Malt Soft Agar
MUM	Micoteca da Universidade do Minho
OD	Optical Density
OH-PLA	Hydroxyphenyllactic acid
OTA	Ochratoxin A
PBS	Phosphate Buffered Saline
PLA	Phenyllactic acid
PMTDI	Provisional Maximum Tolerable Daily Intake
PVOH	Poly(vinyl alcohol)
QPS	Qualified Presumption of Safety
RASFF	Rapid Alert System for Food and Feed
RH	Relative Humidity
SD	Standard Deviation
SEM	Scanning Electron Microscopy
TDI	Tolerable Daily Intake
TE	Tensile Strength
UV	Ultra violet
WHO	World Health Organization
WPC	Whey Protein Concentrate
WVP	Water Vapour Permeability
WVTR	Water Vapour Transmission Rate
YES	Yeast Extract Sucrose
ZEN	Zearalenone

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CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1 RESEARCH MOTIVATION

Moulds play an important role in spoilage of food products. Food contamination by moulds causes great economic losses and several health risks due to the toxicity and pathogenicity of some species (Gerez et al. 2010). Fungi are a major problem at any stage of the food processing chain (i.e, in the field, during post-harvest, processing, storage and handling steps) since they are able to grow in diverse environmental conditions (Schnurer and Magnusson 2005; Leyva-Salas et al. 2017). Additionally, fungi are able to produce toxic compounds that cause serious health hazards designated as mycotoxins (Murphy et al. 2006). Mycotoxins are secondary metabolites produced by many filamentous fungi that occur naturally in agriculture commodities and processed food products. When ingested by humans and animals, these compounds have toxic properties which include carcinogenic, neurotoxic, nephrotoxic, immunosuppressive and estrogenic effects even at low concentrations (Rocha et al. 2014). Two of the most relevant mycotoxins, from a health hazard point of view, are aflatoxins and ochratoxin A (OTA).

Mycotoxins can enter the food chain directly via plant products, or indirectly from animal derived products (such as milk and meat), when animals are exposed to diets contaminated with mycotoxins. These toxins are small and very stable molecules which are extremely difficult to remove, and can enter the food chain keeping their toxic properties (Jard et al. 2011). Mycotoxins are natural food contaminants, thus its complete elimination is extremely difficult, still their levels should be reduced to the minimum that is technologically possible. Consequently, preventive measures should be applied to reduce fungal contamination and resulting mycotoxins production and accumulation.

In this work, in order to minimize the problem of fungal contamination and mycotoxin production a biopreservation strategy is proposed. Biopreservation is briefly defined as the control of one organism by another and has received much attention to extend the food product shelf life and improved its microbial safety (Dalié et al. 2010). This approach meets consumer demand for naturally produced foods, with minimal processing, and without the addition of chemical preservatives (Lucera et al. 2012a).

The most studied microorganisms for biopreservation strategies are lactic acid bacteria (LAB). These bacteria occur naturally in many food systems, and have been extensively used for their fermentation and preservation properties, and thus are considered a safe organism to be consumed (Leroy and De Vuyst 2004; Oliveira et al. 2014).

Lactic acid bacteria have been reported to produce several antimicrobial and antifungal compounds. These include organic acids (Lavermicocca et al. 2003; Peláez et al. 2012; Cizeikiene et al. 2013), reuterin (Magnusson 2003), fatty acids (Sjögren et al. 2003; Broberg et al. 2007), hydrogen peroxide (Ponts et al. 2006), proteinaceous compounds and cyclic dipeptides (Gourama and Bullerman 1997; Magnusson and Schnurer 2001; Ström et al. 2002; Atanassova et al. 2003; Dal Bello et al. 2007). Several studies have already reported the use of LAB as an effective method to inhibit the growth of pathogenic bacteria, yeasts and moulds (Trias et al. 2008; Yang et al. 2012; Cizeikiene et al. 2013; Coelho et al. 2014; Siroli et al. 2015). However, despite being extensively studied as a biopreservative, a limited number of studies address the effect of LAB as an inhibitory agent of mycotoxin production (Gourama and Bullerman 1997; Florianowicz 2001; Dalié et al. 2012; Gerbaldo et al. 2012; Kapetanakou et al. 2012; Belkacem-Hanfi et al. 2014; Gerez et al. 2014; Oliveira et al. 2014; Sangmanee and Hongpattarakere 2014; Deepthi et al. 2016; Sellamani et al. 2016; Marie et al. 2018).

To aid the antifungal functions of LAB, it is proposed the incorporation of these bacteria into edible films and coatings. These edible packaging can improve food quality and safety, while providing a semi permeable barrier to water, vapour and gases between the food and the surrounding atmosphere (Silva-Weiss et al. 2013). For the successful application of edible films and coatings containing living cells in food products, a series of factors have to be taken in account: the combination of film forming materials, method cell incorporation in the film matrix, parameters of film production, type of LAB strain, storage conditions, method of application on food products and type of food product. The possibility of using an edible matrix as carrier for LAB can lead to better

survival rates of cells during storage, eventually improve some sensorial characteristics, give a better control of dosage and allow applications in a vast range of food products. So far, the few works combining viable bacteria and edible films described their application in a limited range of food products such as fruit, bread, fish and beef (Tapia et al. 2007; Gialamas et al. 2010; Altamirano-Fortoul et al. 2012; De Lacey et al. 2012; Soukoulis et al. 2014c). Despite the fact that in most of the studies found in literature LAB were used for their probiotic properties, some studies focused on LAB antimicrobial properties (Gialamas et al. 2010; Concha-Meyer et al. 2011; Sánchez-González et al. 2013; Sánchez-González et al. 2014; Pereira et al. 2018; Ye et al. 2018; Bambace et al. 2019; Settier-Ramírez et al. 2019). However, none of them focused on the inhibition of filamentous fungi.

In this work, LAB isolated from various sources (mainly milk and silage) were tested for their ability to inhibit fungal growth, as well as mycotoxin production. The compounds responsible for this inhibition were identified by chromatography. Following, LAB strains with good results for the inhibition of fungal growth and consequent mycotoxin production were incorporated in edible films and applied in food products. The search for LAB with antifungal properties and the study of their metabolites is worth being investigated since it can be an efficient way to improve shelf life and inhibit the growth of pathogenic organisms in food products, without altering nutritional value of those products (Ananou et al. 2007).

1.2 MAIN OBJECTIVES

The main objective of this work is the selection of LAB with antifungal activity against mycotoxigenic fungi and its inhibitory effects on mycotoxins production. It is also pretended to determine the antifungal metabolites responsible for that inhibition. The final goal is to apply these antifungal LAB in food products by incorporation into edible films and coatings. This work will be mainly focused in the inhibition of fungal strains that are producers of aflatoxins and OTA.

So, the specific objectives to be accomplished in this work are:

1. Identification of lactic acid bacteria capable of inhibiting fungal growth and mycotoxin production and further testing of the most active strains and their cell free supernatants;

2. Determination of lactic acid bacteria metabolites that are responsible for the inhibition of fungal growth and mycotoxin production and calculation of their inhibitory concentrations;

3. Application of an active lactic acid bacteria strain in edible films and coatings to prevent fungal development in cheese products.

1.3 THESIS OUTLINE

This dissertation is divided into five chapters, where two of them describe experimental work (CHAPTER 3 and CHAPTER 4), one of them refers to bibliographic revision of literature related to the scope of this thesis (CHAPTER 2), the last chapter states the most relevant conclusions and future perspectives of this work (CHAPTER 5) and the current one provides a short introduction (CHAPTER 1).

CHAPTER 1 provides a brief introduction on the theme of this dissertation, motivation and main objectives.

CHAPTER 2 consists in a literature review of works regarding the use of lactic acid bacteria as biocontrol/biopreservation strategy to inhibit fungal growth and mycotoxin production and the metabolites that are responsible for that inhibition. It also presents the main issues of fungal contamination, highlighting the mycotoxin problem and its toxicity and effects on human and animal health. Moreover, it refers how the use of edible films and coatings could aid the use of lactic acid bacteria, with antifungal activity, in food products.

In CHAPTER 3, 98 strains of lactic acid bacteria are tested for their antifungal ability against mycotoxigenic fungal strains. Most active strains, *L. plantarum* UM55 and *L. buchneri* UTAD104, are further tested for their effect on fungal growth, against *Aspergillus flavus* and *Penicillium nordicum*, respectively. Inhibition of the mycotoxin production of these strains is also evaluated. Cell free supernatants of these LAB strains are characterized to determine the compounds responsible for the

antifungal activity Inhibitory concentrations for fungal growth and mycotoxin production are calculated for the detected antifungal compounds.

In CHAPTER 4, *L. buchneri* UTAD104 is incorporated into edible films and coatings. Characterization of these films against control films (without bacteria) is presented as well as the film's capacity to inhibit *P. nordicum* and ability of cells to survive in the films. Antifungal edible films and coatings are used to cover cheese samples and antifungal efficiency of the films and coatings is evaluated.

Finally, CHAPTER 5 presents the main conclusions from this thesis, and gives some suggestions of further research in this field.

It is also included two appendix sections, APPENDIX A and APPENDIX B, with the original data that are presented along this thesis in Chapter 3 and Chapter 4, respectively.

CHAPTER 2

BIBLIOGRAPHIC REVISION

2. BIBLIOGRAPHIC REVISION

2.1 THE PROBLEM OF FUNGAL CONTAMINATION

Fungi are eukaryotic, single or multi-celled, multinucleated organisms that are heterotrophic in nature, possess a chitinous cell wall and, in general, present a filamentous growth in multicellular colonies. These organisms are ubiquitous, being able to occupy a vast range of environments, such as soil, water, air and several other materials (Magan and Aldred 2007; Dix 2012). It is estimated that nearly 1.5 to 5.1 millions of different fungi exist, but only nearly 120 000 species have been identified and described (O'Brien et al. 2005; Hibbett et al. 2011; Hawksworth and Lücking 2017).

Moulds play a significant role in human life by acting as natural environmental decomposers, in the preparation of certain foods (such as cheese) and in the production of antibiotics and other chemical molecules (Bhat et al. 2010), while others have been used in fermentation processes, for products such as wine, beer, soy sauce and sake (Dung et al. 2005; Chang et al. 2007). Biotechnological processes also use fungal metabolism to produce valuable compounds for industrial applications (e.g., organic acids and enzymes) (Schuster et al. 2002; Sauer et al. 2008).

However, contamination by fungi represents one of the main problems in food industry, with some species being responsible for several diseases in plants and animals. Fungi can develop in food under favourable conditions of temperature, relative humidity, pH, nutrient availability, and oxygen and, in general, these organisms have a great ability to adapt to different environmental conditions. They are resistant to osmotic pressures, and can tolerate acidic media and a wide range of temperatures (Magan and Aldred 2007). The aptitude of filamentous fungi to

contaminate food causes high economic losses, and several health risks, due to toxicity and pathogenicity of some species (Gerez et al. 2010).

Aspergillus is one of the most studied fungal genera due to its environmental, health and economic impact. This genus was defined in 1729 and includes 339 species, and it is present in numerous different environments (Bennett 2010; Samson et al. 2014). Most species have been characterized based on its morphological and physiological features, and more recently by molecular techniques. Aspergilli are found in soil, where they contribute to the decomposition of organic matter, and are a known pathogen of plants, animals and insects. *Aspergillus* species have a high metabolic versatility and their airborne conidia can be dispersed easily by air movements or insects, and are also able to grown in a wide range of substrates and climatic conditions (Gourama and Bullerman 1995a). Some species of *Aspergillus* are also common contaminants of human food and animal feed. Major agriculture commodities affected by Aspergilli include corn, rice and other cereal grains, peanuts, cottonseed, tree nuts and fruits (Hill et al. 1983; Cotty 1994; Leong et al. 2004; Giorni et al. 2007; Reddy et al. 2009a; Rodrigues et al. 2009). Additionally, *Aspergillus* spp. can be allergenic, toxigenic and pathogenic to humans and other animals. They can cause several diseases such as avian aspergillosis and bovine mycotic abortion (Ainsworth and Austwick 1973). Other major factor of concern for *Aspergillus* contamination is that several species, including, *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus nomius*, *Aspergillus ochraceus*, *Aspergillus parasiticus* and *Aspergillus versicolor* have the ability of producing toxic compounds named mycotoxins (Sweeney and Dobson 1998). The main mycotoxins produced by *Aspergillus* are: aflatoxins (AF), ochratoxin A, sterigmatocystin, fumonisins, cyclopiazonic acid (CPA), patulin, aspergillic acid and citrinin (Smith and Ross 1991; Keller et al. 1997; Vaamonde et al. 2003; Sabater-Vilar et al. 2004; Frisvad et al. 2007).

However, *Aspergillus* genus also include species that are important for fermentation processes, such as *Aspergillus sojae*, *Aspergillus oryzae* and *Aspergillus tamarii* that are used in the production of oriental fermented foods, and as hosts for heterologous gene expression (Campbell-Platt and Cook 1989). Also, *A. niger* is a species of great biotechnological interest since it is used for the production of organic acids (e.g. citric acid) and extracellular enzymes (Schuster et al. 2002; Baker 2006).

One of the most referred species is *A. flavus*, which occurs in soil and contaminate a vast number of agricultural commodities in the field and during storage. *A. flavus* grows as a saprophyte in the soil where it acts as a nutrient recycler, supported by plant and animal waste and its ability to survive adverse environments allows it to win the competition with other microorganisms for substrates in the soil (Hedayati et al. 2007). They can cause ear rot in corn, rot in mature peanuts, and affect the quality of cotton; it is a pathogen of honeybees, among other species; and, in humans, it can be considered an allergen and cause infections in immunosuppressed people (Klich 2007). *A. flavus* (microscopic image, cultured in PDA medium and as maize contaminant) is shown in Figure 2.1.

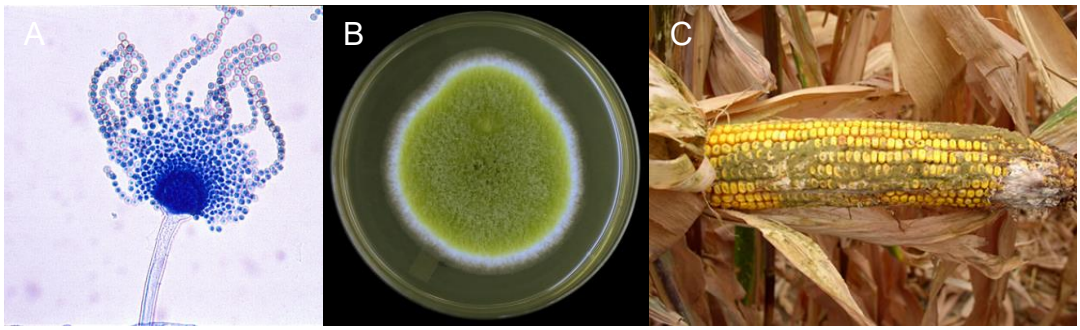


Figure 2.1. *A. flavus* microscopic morphology (A); cultured in PDA medium (B) and in maize (C). Adapted from Hedayati et al. (2007) and Schmale and Munkvold (2009).

Penicillium is another common fungal genus that occurs in a vast range of habitats, from vegetation to soil, to air, household and several food products. It is well distributed worldwide, and it is also responsible for food waste and health problems. In nature, its main function is to decompose organic materials, thus causing damage in many food crops at the pre- and post-harvest stages, and compromising the quality and safety of commodities (Frisvad and Samson 2004; Pitt and Hocking 2009). Several *Penicillium* species are also able to produce mycotoxins. OTA, patulin and citrinin are the most significant ones, with legal limits been imposed, for certain food products, in some countries (Samson et al. 2004), but *Penicillium* spp. can also produce other less known mycotoxins, such as: cyclopiazonic acid, cyclopiamine, cyclopiamide, cyclopaldic acid, cyclopolic acid, xanthomegnin, viomellein, vioxanthin, pencicillic acid, verrucosidin, roquefortine C, penitrem A, rugulovasin, fumigaclavine A and B, isofumigaclavine A

and B, botryodiploidin, chaetoglobosin, communesin B, viridic acid, cardiotoxin terrestric acid, tryptoquialanins and territrems (Frisvad and Samson 2004).

Still, some *Penicillium* species are important to the food industry with some of them involved in the production of speciality cheeses, such as Camembert and Roquefort (Nelson 1970; Karahadian et al. 1985) and fermented dry-cured meats (such as traditional southern European cured sausages, salami and dried cured hams) (Bruna et al. 2001; López-Díaz et al. 2001; Ludemann et al. 2010; Meftah et al. 2018). Also, their degradative ability has been used in the recycling of organic matter, and in the industrial production of organic acids and enzymes (Paterson et al. 2004; Adsul et al. 2007; Li et al. 2007; Terrasan et al. 2010). Still, its most acknowledgeable impact is the production of penicillin, which is of extreme importance in the treatment of bacterial infections (Chain et al. 1940; Abraham et al. 1941).

In terms of health hazard, among the *Penicillium* species, *Penicillium nordicum and Penicillium verrucosum* are of special importance since they are the only known OTA producers in this genus. These species have common morphological characteristics; however, they colonize different environments. *P. verrucosum* is found mainly in plant-derived material, being the principal source of OTA contamination in cereals in temperate climates, while *P. nordicum* contaminates dry-cured meats and cheeses and other fat and protein rich substrates. This species is very salt tolerant, thus can thrive and produce OTA in these environments (Cabañes et al. 2010).

P. nordicum is normally found in air and surfaces of hams and dry-cured sausages manufacturing plants, and are responsible for the extensive presence of OTA (45%) in the surface of these products, which constitute and health risk for consumers (Battilani et al. 2007; Iacumin et al. 2009). For this reason, limits of OTA allowed in meat have been introduced in some countries (e.g. in Italy 1 μg/kg) (Iacumin et al. 2017). Since *P. nordicum* contaminates finished food products, its control is of crucial importance. *P. nordicum* structure, cultivated in YES agar medium and as a sausage contaminant is shown in Figure 2.2.

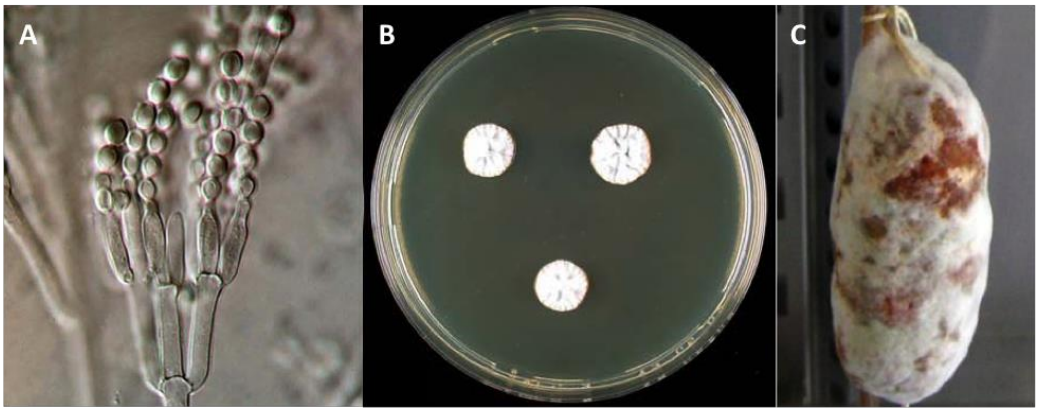


Figure 2.2. *P. nordicum* microscopic morphology (A); cultured in YES medium (B) and in dry sausages (C). Adapted from Frisvad et al. (2004) and Meftah et al. (2018).

2.2 MYCOTOXINS

Mycotoxins are low molecular weight secondary metabolites produced by some fungal species that are highly toxic, and that are not required for the normal fungal development (Richard 2007). These toxins are small and quite stable molecules which are extremely difficult to remove or eradicate, and can enter the food chain maintaining their toxic properties (Sweeney and Dobson 1998; Placinta et al. 1999). Even when food is subjected to processing, such as cooking and freezing, mycotoxins are not inactivated. It has been determined that mycotoxin production can be altered under different fungal growth conditions such as substrate composition, temperature, water activity (a_w), pH, oxygen concentration and microbial interactions (Hymery et al. 2014).

Although their functions are not completely understood, mycotoxins are thought to give a selective advantage to the producing fungi within their ecosystems. The production of mycotoxins by many fungal species and the preservation of their multigenic biosynthesis pathways indicate that these secondary metabolites are probably used for survival functions. Thus, it is considered that mycotoxins may act as chemical signals for communication or to inhibit the growth of competitors in the same ecologic niche (Magan and Aldred 2007).

Some species of filamentous fungi of the genera *Aspergillus, Penicillium, Fusarium*, *Alternaria* and *Claviceps* are the main producers of mycotoxins (Panigrahi 1997; Sweeney and Dobson 1998; Placinta et al. 1999). These fungal strains invade crops and are able to grow in

commodities under favourable conditions of temperature and a_w (Bhat et al. 2010). Factors influencing mycotoxin occurrence in the food chain are represented in Figure 2.3. The contamination of food and feed with mycotoxin is a global problem, with studies showing extensive mycotoxin contamination in both developing and developed countries. It is difficult to accurately assess the global economic impact of mycotoxins; still, some reviews estimated that 25% of the world's agricultural commodities are contaminated with these toxins (Fink-Grernmels 1999).

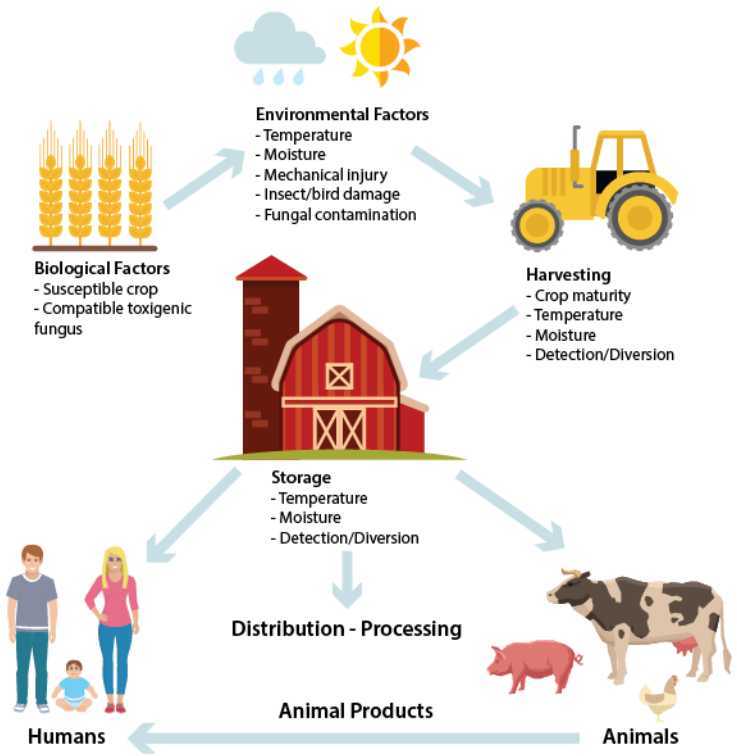


Figure 2.3. Factors influencing mycotoxin occurrence in the food chain (adapted from Paterson and Lima (2010)).

Mycotoxins can get into the food chain directly via plant products, such as cereal grains, coffee, oil seeds, spices, fruit juices, and beverages (wine and beer) among others, or indirectly from animal fed with contaminated feed, due to their residues or modified forms accumulating in meat, milk and other animal products.

The term mycotoxin was defined in 1962 after an unidentified disease killed 100 000 turkeys in England (Blount 1961). This disease was then linked to a peanut meal contaminated

with toxic metabolites produced by *A. flavus*. The compound was isolated and designated as aflatoxin. This alerted to the possibility that moulds could produce deadly metabolites and soon, a vast number of new secondary fungal metabolites was found to be toxic (Bennett and Klich 2003).

Up until now, approximately 600 compounds, produced by more than 100 fungal species, are recognized as mycotoxins (Kabak et al. 2006; Castillo et al. 2016). These are generally classified according to structural similarities and major toxic effects . The most relevant mycotoxins, from a toxigenic point of view are: aflatoxins, OTA, patulin, fumonisins, zearalenone (ZEN), trichothecenes and ergot alkaloids (Bennett and Klich 2003).

The toxic effect of mycotoxins can be dependent of several factors including the amount ingested, mycotoxin type, period of exposure, metabolism and mode of action. However, the consumption of these compounds can lead to carcinogenic, mutagenic, teratogenic, neurotoxic, nephrotoxic, immunosuppressive, and estrogenic effects at low concentrations in both humans and animals, with the main health hazards of mycotoxin consumption being related to chronic exposure (e.g. cancer, kidney damage and immune suppression) (Bennett and Klich 2003). Additionally, the effects of food contamination with mycotoxins are particularly relevant when the nutritional base of a population is very dependent on one single type of food. If this source is contaminated, it can lead to prolonged exposure to mycotoxins and end in chronic mycotoxicoses.

In addition to the impact in human health, mycotoxins cause significant crop damage (FAO/IAEA 2001). Crops contaminated with large amounts of mycotoxins have to be destroyed and other less contaminated crops are redirected to animal feed. This may lead to illness, reduced growth and, eventually, death of the animals. Additionally, products derived from contaminated animals, namely meat and milk, can contain toxin residues and derived mycotoxin products resulting from their metabolization (Van Egmond 1989). All these factors have an important impact on commercial trade.

According to the data of the EU Rapid Alert System for Food and Feed (RASFF), mycotoxins were the cause for a large number of rejections at the border; with nuts, seeds, fruits, vegetables, herbs and spices, cereals, and bakery products being the most affected (Marin et al. 2013).

The mycotoxins more frequently found in food and feed are: aflatoxins; OTA; trichothecenes (HT-2 toxin, T-2 toxin and deoxynivalenol (DON)), zealeralenone, fumonisins, patulin and ergot

alkaloids. The European Commission (EC) regulates the maximum levels for all these toxins in foodstuff (EC 2015, 2018). The EC and the European Food Safety Authority (EFSA) also recommends the monitoring of sterigmatocystin, phomopsins, citrinin and Alternaria toxins (EFSA 2011, 2012b, 2012c, 2013). Other mycotoxins can also be found in foodstuff such as: emerging mycotoxins (fusaproliferin, moniliformin, beauvericin, and enniatins); altenuene; alternariol methyl ether; altertoxin and tenuazonic acid among other toxins (Marin et al. 2013). The most relevant fungal species producing mycotoxins are listed in Table 2.1.

Table 2.1. List of mycotoxins and their producing species (adapted from Marín et al. (2013))

Mycotoxin	Acronym	Producing Species
Aflatoxins B1, B2, G1, G2	AFB1 AFB2 AFG1 AFG2	*Aspergillus* section *Flavi*
Alternariol	AOH	*Alternaria alternata*
Cyclopiazonic acid	CPA	*A. flavus, A. tamarii, A. oryzae, Penicillium commune, Penicillium camembertii, Penicillium palitans, Penicillium griseofulvum*
Deoxynivalenol	DON	*Fusarium graminearum, Fusarium culmorum, Fusarium cerealis*
Ergot alkaloids	EAs	*Claviceps africana, Claviceps purpurea, Claviceps paspali, Cylindrotheca fusiformis*, *Neotyphodium* spp.
Fumonisins B1, B2	FB1 FB2	*Fusarium* section *Liseola*, *A.niger, A. alternata*
HT-2 toxin T-2 toxin	HT-2 T-2	*Fusarium acuminatum, F. poae, F. sporotrichioides, F. langsethiae*
Moniliformin	MON	*Fusarium avenaceum, Fusarium tricinctum, Fusarium* section *Liseola*
Ochratoxin A	OTA	*Aspergillus* section *Circumdati, Aspergillus* section *Nigri, Penicillium verrucosum, Penicillium nordicum*
Patulin	PAT	*Penicillim expansum, Byssochlamys nivea, Aspergillus clavatus, Paecilomyces spp.*
Penicillic acid		*A. ochraceus; Penicillium aurantiogriseum; Penicillium neoechinulatum*
Zearalenone	ZEN	*F. graminearum*, *F. culmorum*, *Fusarium equiseti*, *F. cerealis, Fusarium verticillioides, Fusarium crookwellense*

2.2.1 Aflatoxins

Aflatoxins are the most extensively characterized group of mycotoxins. Aflatoxins are difuranocoumarin derivatives produced by a polyketide pathway by several *Aspergillus* species, specially *A. flavus* and *A. parasiticus* (Gourama and Bullerman 1995a). Other *Aspergillus* species such as *Aspergillus bombycis, A. nomius*, and *Aspergillus pseudotamarii* can also produce aflatoxins, but are a less common contaminant (Klich et al. 2000; Peterson et al. 2001). The quantity and type of aflatoxins is dependent of the producing strain within each aflatoxigenic species (Bennett and Klich 2003).

Aflatoxins occur generally in tropical and subtropical regions, where high humidity and temperature favour fungal growth, with aflatoxins being produced at temperatures between 24 and 35 °C. During storage, moisture content of the substrate and the relative humidity of the environment, play an important role in the formation of aflatoxins (Milani 2013). Aflatoxins can be found in numerous important agricultural commodities including, cereals (maize, rice and millet), spices (chillies, pepper, coriander, turmeric and ginger), seeds, nuts (almond, pistachio and walnut) and milk produced by animals that consumed contaminated feed (Bhat et al. 2010).

The main aflatoxins are designated by B1, B2, G1 and G2 based on their blue or green fluorescence under UV light, and position in chromatographic elution in thin-layer chromatography (Bennett and Klich 2003). The chemical structure of these aflatoxins is represented in Figure 2.4. Aflatoxin B1 (AFB1) is the most carcinogenic of all the natural occurring mycotoxins and also the most potent natural carcinogen known, being classified as a group I carcinogen by The International Agency for Research on Cancer (IARC) (IARC 2002). Due to its cancer inducing effects and for being the major toxin produced by aflatoxigenic strains, AFB1 is the most well studied toxin. The ingestion of AFB1 contaminated diets by mammals can result in the production of aflatoxin M1 (AFM1), and its excretion into milk (chemical structure of AFM1 is shown in Figure 2.4). AFM1 carcinogenic potential is about 10 times lower than AFB1, and its carcinogenicity has only been proved in animals, still it is classified as possible carcinogenic to humans (group 2B) (IARC 2002).

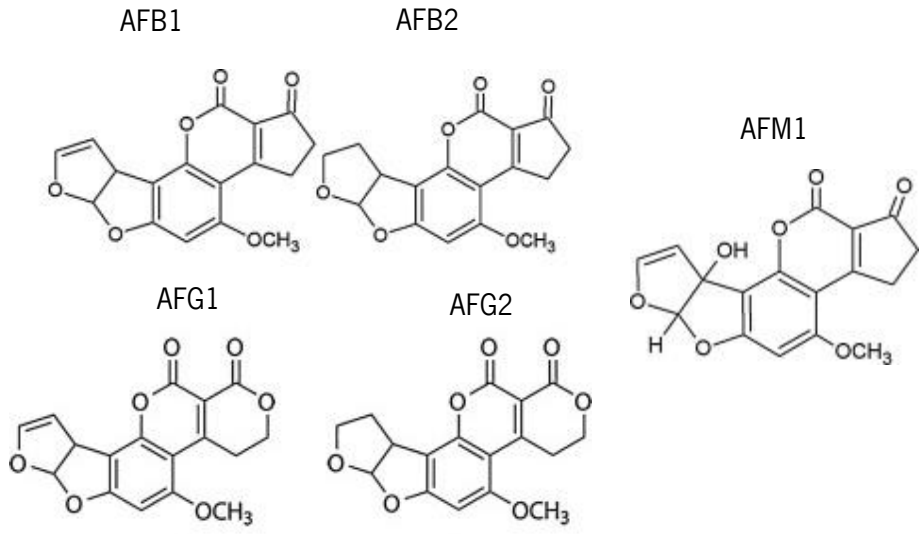


Figure 2.4. Chemical structure of aflatoxins: B1, B2, G1, G2 and M1.

Aflatoxin production results from a combination of factors that include fungal species, substrate and environment. Several physical factors affect aflatoxin production such as temperature, pH, relative humidity, a_w, moisture, light, aeration and atmospheric gases, also nutritional factors such as nutrients that favour aflatoxin biosynthesis (glucose, sucrose or fructose), glycine, glutamic acid, zinc and manganese, can influence aflatoxin production (Gourama and Bullerman 1995a).

Aflatoxins can cause acute or chronic toxicity in both human and animal populations (Newberne and Butler 1969; Eaton and Gallagher 1994; Bhat et al. 2010). Acute aflatoxicosis (diseases caused by aflatoxins) can ultimately result in death, while chronic aflatoxicosis can result in cancer, immune suppression and other pathologic conditions (Zain 2011). In humans, the main concern of aflatoxins consumption is liver cancer, although the handling of contaminated grains may also cause lung cancer (Kelly et al. 1997). Liver damage is caused by consumption of contaminated feed being reported in poultry, fish, rodents and primates. Still, different animal species show different susceptibilities to aflatoxins, with age, sex, diet, weight, and consumption of other mycotoxins or pharmacologically active agents influencing the magnitude of the susceptibility (Cullen and Newberne 1994). Due to these differences, it has been difficult to extrapolate the effects of aflatoxins in humans. Particularly in the case of acute toxicity, since very few cases in humans have been reported, and are usually originated from developing countries.

In developed countries the regulation of aflatoxin levels in food protects humans from significant aflatoxin ingestion. However, in underdeveloped countries, absence or insufficient food regulations results in higher exposure to aflatoxins (Cotty 1994). In 1974 an outbreak of hepatitis, in Western India, resulted in the death of 100 persons. It is hypostasized that the consumption of aflatoxin-contaminated maize was the cause of the disease. It was estimated that each adult consumed 2 to 6 mg of aflatoxin daily over a period of a month (Krishnamachari et al. 1975). In 1988, 13 children died of acute encephalopathy in Malaysia during a Chinese festival. Investigations determined that the cause of death was related to the consumption of a Chinese noodle contaminated with aflatoxins and boric acid (Lye et al. 1995). One of the most severe episodes of human aflatoxin poisoning took place in Kenya in 2004, as a result of the ingestion of contaminated maize. A total of 317 cases of poisoning were reported with a case fatality rate of 39%, resulting in 125 deaths. Public health officials analysed the maize from the affected area and found aflatoxin B1 concentration over 4400 μg/kg, when the limit imposed by Kenyan authorities for aflatoxins in food is 20 μg/kg. The fungi species responsible for the contamination was not identified. (Azziz-Baumgartner et al. 2005). Other outbreak in the same area of Kenya, in 1981, resulted in 20 cases of aflatoxin poisoning and 12 deaths. Again, consumption of contaminated maize was found to be the source of aflatoxin exposure. In this case, levels of aflatoxins in maize grains reached 12000 μg/kg. After the 2004 outbreak, the Kenyan government implemented measures of clinical disease surveillance for acute aflatoxicosis and together with FAO initiated a public education campaign for aflatoxin prevention. Additionally, house grown maize were surveyed during high-risk months (April to June) for aflatoxin contamination (Johnni et al. 2011).

More recently, in 2016, in Tanzania, an outbreak of an unknown disease caused health problems in 68 persons, which resulted in 20 deaths, half of the patients were below the age of 15 years. It was determined that the cause of the disease was the consumption of home grown maize contaminated with high levels of aflatoxins (10 to 51100 μg/kg). Apart from aflatoxins, maize samples were also contaminated with high levels of fumonisins (945 to 12 630 μg/kg) (Kamala et al. 2018).

Hepatotoxicity from aflatoxicosis is caused by the absorption of aflatoxins after consumption of contaminated food that is then transported to the liver through the circulatory system. Approximately 1 to 3% of aflatoxins bind irreversibly to proteins and DNA bases to form adducts

(a segment of DNA bound to a chemical that can originate cancer). The disruption of proteins and DNA bases in hepatocytes results in liver toxicity. The hepatotoxicity of aflatoxins manifests itself in the early stages of the disease as anorexia, malaise and low-grade fever; and then progresses to potentially lethal acute hepatitis with vomiting, abdominal pain and eventually death. Even with supportive care, fatality rates for acute poisoning range from 25 to 40% (Azziz-Baumgartner et al. 2005). Hepatocellular carcinoma caused by aflatoxins affects particularly individuals with hepatitis B. This association complicates many of the epidemiological studies of the consequences of chronic exposure to aflatoxins (Peers and Linsell 1973; Li et al. 2001).

Consequently, it was defined that the lethal dose for aflatoxin consumption in adults is between 10 and 20 mg. However, reports of a woman that ingested over 40 mg of aflatoxin in a suicide attempt and survived may indicate that the lethal dose for aflatoxin consumption can be higher than the previously defined values (Willis et al. 1980).

The lower limits for aflatoxins consumption are the ones imposed by the European Union (EU) that define a maximum of 2 μg/kg of AFB1 and 4 μg/kg for total consumption of aflatoxins in cereals and products derived from cereals (0.1 μg/kg for infant cereals), except for maize and rice, which have higher limits (5 μg/kg for AFB1 and 10 μg/kg for the four aflatoxins). In raw milk aflatoxin M1 is limited to 0.05 μg/kg and in infant milk to 0.025 μg/kg (EC 2018).

2.2.2 Ochratoxin A

Ochratoxin A was discovered in 1965 as a metabolite of *A. ochraceus* (currently classified as *Aspergillus westerdijkiae*), during a research to identify new mycotoxins (Van Der Merwe et al. 1965). Since then, ochratoxins have been found in several species of the genera *Aspergillus*, such as *Aspergillus carbonarius*, *Aspergillus cretensis*, *Aspergillus flocculosus*, *Aspergillus fresenii*, *Aspergillus muricatus*, *Aspergillus pseudoelegans*, *Aspergillus pulvericola*, *Aspergillus melleus, Aspergillus roseoglobulosus, Aspergillus steynii, Aspergillus sclerotiorum, Aspergillus westerdijkiae, Aspergillus occultus* and *A. niger* among others, and also in *Penicillium* species, namely *P. nordicum* and *P. verrucosum* (Abarca et al. 1994; Frisvad et al. 2004; Visagie et al. 2014). Its chemical structure consists of a para-chlorophenolic group containing a

dihydroisocoumarin moiety that is amide-linked to L-phenylalanine (Figure 2.5). It is considered to be one of the most relevant mycotoxins due to its toxic effects.

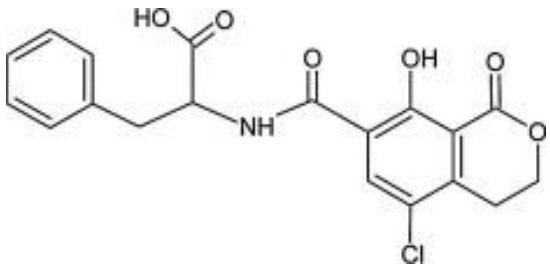


Figure 2.5. Chemical structure of ochratoxin A.

Although there are no conclusive reports of the effect of OTA in human health, a great number of animal and cell experiments have reported that the exposure to OTA can result in various toxicological effects, including the disruption of the gut microbiota homeostasis, teratogenicity, carcinogenicity, mutagenicity, hepatotoxicity, genotoxicity, immunotoxicity, embryotoxicity, developmental toxicity, neurotoxicity, testicular toxicity, blood-brain barrier damage, and especially nephrotoxicity (Pfohl-Leszkowicz and Manderville 2007, 2011; Qi et al. 2014; Liang et al. 2015; Marin and Taranu 2015; Bhat et al. 2016; Liew and Mohd-Redzwan 2018). OTA has been classified by IARC as a possible human carcinogen (group 2B), but its mode of action is still not clearly established. Several developments in the study of OTA genotoxicity; its contribution to oxidative stress; and the identification of epigenetic factors involved in OTA carcinogenesis, provides evidence of OTA carcinogenicity that could lead to its re-classification by IARC (IARC 1993; Pfohl-Leszkowicz and Manderville 2007; Ostry et al. 2017). OTA acts by disturbing cellular physiology in several ways. OTA is a structural analogue of phenylalanine and thus can act on metabolic systems involving phenylalanine. Major effects seem to be related to the inhibition of phenylalanine-tRNA synthase and inhibition of phenylalanine hydroxylase. Additionally, since OTA interrupts protein synthesis, it consequently impairs the activity of cellular enzymes causing, indirectly, alterations in the carbohydrates metabolic pathways. OTA can also inhibit mitochondrial ATP production and increases lipid peroxidation (Ringot et al. 2006).

Apart from health problems, OTA also causes enormous monetary losses by decreasing the productivity of livestock and farm crops, by increasing medical costs in humans and animals,

increasing mortality rate of farm animals and by increasing the costs related to prevention, control and detoxification of OTA (Duarte et al. 2010).

Similarly to other mycotoxins, OTA production depends on the moisture levels, temperature and presence of other microbiota in the substrate in which the producing fungus grows. OTA generally contaminates unprocessed products, and can be found in cereals (such as barley, oat, rye and wheat) (Lund and Frisvad 2003; Araguás et al. 2005; Duarte et al. 2010), cocoa (Serra 2004), spices (Ozbey and Kabak 2012), herbs (Efuntoye 1999), coffee (Studer-Rohr et al. 1995), dry fruits (Bircan 2009), grapes (Bellí et al. 2002; Serra et al. 2003) and animal feed (Dalcero et al. 2002; Fraga et al. 2007). Since OTA is stable during normal food processing operations, it can also contaminate final food products, such as bread and wine (Otteneder and Majerus 2000; Serra et al. 2006; González-Osnaya et al. 2007; Juan et al. 2008). Additionally, it has a cumulative effect on animals that have consumed contaminated feed, which leaves residues of the mycotoxin in products of animal origin (Piotrowska and Zakowska 2005). Ochratoxin A has been detected in pork and chicken meat intended for human consumption, as well as in other animal tissues and in milk, (Elling and Moller 1973; Elling et al. 1975; Hult et al. 1992; Marquardt and Frohlich 1992; Iqbal et al. 2014). OTA is also implicated in the Balkan nephropathy, a chronic nephritis found in some populations in the margins of the Danube River. This hypothesis is based on the fact that residents in these regions are exposed to relatively high concentrations of OTA. However, chronic nephrotoxicity associated with OTA consumption has never been proved (De Broe 2012; Stiborová et al. 2016).

According to the latest studies, levels of OTA in food products should raise concern. Lee and Ryu (2017) reviewed data from 4000 samples described in 33 different works. Incidence of OTA in raw cereals and processed cereals were 29 and 38%, respectively. The highest level of OTA (1164 μg/kg) was detected in a sample of rice from Africa (Makun et al. 2007). Additionally, this paper reported the incidence of OTA in retailed breakfast cereal samples was of 42% (205 of 489 samples), with 59% (30 of 51 samples) of oat based infant cereals being contaminated in the range of 0.6 to 22.1 μg/kg, well above EU maximum limit for OTA in infant food, 0.5 μg/kg (EC 2018).

Based on several studies conducted to determine the maximum levels for human exposure to OTA, the EC recommends that maximum OTA levels should be below 5 μg/kg in unprocessed

cereal products, to 3 μg/kg in cereal-derived processed products and 10 μg/kg in dried vine fruits (EC 2018).

2.2.3 Fumonisin

Fumonisins were originally isolated in South Africa in 1988 from *Fusarium* species (Gelderblom et al. 1988). There are several types of fumonisins divided into four groups: A, B, C and P (Rheeder et al. 2002). Fumonisins B are the most relevant in terms of toxicity with fumonisin B1 (FB1) being the most toxic and the most often detected in maize (Gelderblom et al. 1992a; Gutleb et al. 2002). Fumonisins are biosynthetized by condensation of alanine into an acetate-derived precursor. FB1 is a diester of propane-1,2,3-tricarboxylic acid and 2S-amino-12S,16R-dimethyl-3S,5R,10R,14S,15R-pentahydroxyeicosane in which the C-14 and C-15 hydroxy groups are esterified with the terminal carboxy group of propane-1,2,3 tricarboxylic acid, its chemical structure can be seen in Figure 2.6 (Sweeney and Dobson 1999). Fumonisins are produced mainly by *F. verticillioides* and *F. proliferatum* (Marasas 2001; Rheeder et al. 2002), with *F. verticillioides* being of more relevant since it contaminates corn worldwide, often without visible symptoms to the plant (Nelson et al. 1993).

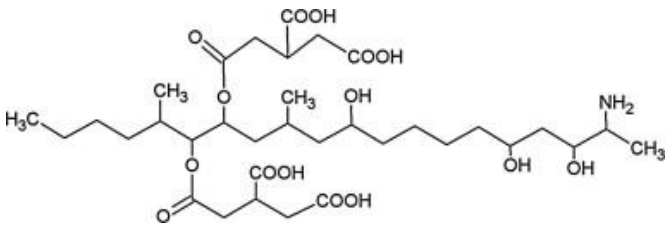


Figure 2.6. Chemical structure of fumonisin B1.

Besides corn and corn-derived products (such as corn flour), the presence of fumonisins has been reported in dried figs, tea and medicinal plants, among others (Martins et al. 2001; Omurtag and Yazicioğlu 2004; Karbancıoglu-Güler and Heperkan 2009). Currently, there is no proven evidence of the adverse health effects of fumonisins in humans. Nonetheless, there is an inconclusive association between the ingestion of fumonisin-contaminated food and oesophageal and liver cancers. For this reason, fumonisins have been classified by IARC as a group 2B

carcinogenic, *i.e.* a possible carcinogen to humans (Gelderblom et al. 1992b; Yoshizawa et al. 1994; IARC 2002). This toxin is also immonosuppressive, and its consumption has been related to neural tube defects in human babies (FAO/WHO 2002; Marasas et al. 2004). In animals, FB1 has hepatotoxic and nephrotoxic effects and has been linked to several diseases in domestic animals, such as pulmonary edema in swine (Haschek et al. 2001) and leukoencephalomalacia in horses (Marasas et al. 1988; FAO/WHO 2002).

Regulations in the EU recommends a maximum level for FB1 and fumonisin B2 (FB2) combined of 4000 μg/kg in unprocessed maize for animal feed and limits fumonisins to a maximum of 1000 μg/kg for human consumption, with this value being considerably reduced when maize is to be consumed by children (200 μg/kg) (EC 2018).

2.2.4 Patulin

Patulin (4-hydroxy-4H-furo[3,2c]pyran-2[6H]-one) was first isolated from *A. clavatus*, in the 1940s, initially creating interest for its antimicrobial effect, and then followed by the discovery of its toxicity in the 50s (Wiesner 1942). Its chemical structure is represented in Figure 2.7. Patulin is mainly produced by *P. expansum* in contaminated fruits, though other species of *Penicillium*, *Aspergillus, Paecilomyces* and *Byssochlamys* genera are recognized producers of patulin in other substrates (Morales et al. 2007). Patulin is the most common mycotoxin found in apples and apple processed products, such as juice, cider and compotes. Patulin is classified as a group 3 carcinogen (no evidence of causing cancer in humans) (IARC 1993). Nonetheless, it has mutagenic properties, acts as a strong antibiotic, is immunosuppressive and causes ulceration, congestion, haemorrhagic lesions and kidney damage (Restani 2004). Patulin toxic effect is caused by the inhibition of DNA translation/transcription and by enzyme alkylation (Puel et al. 2010). A Provisional Maximum Tolerable Daily Intake (PMTDI) of 0.4 μg/kg bw/day (and of 0.2 μg/kg bw/day for children) has been established by the EC for patulin (EC 2018).

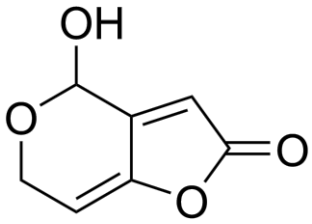


Figure 2.7. Chemical structure of patulin.

2.2.5 Zearalenone

Zearalenone is a non-steroidal mycotoxin produced by *Fusarium* species (the chemical structure of ZEN is shown in Figure 2.8). This mycotoxin is biosynthesized through a polyketide pathway by several *Fusarium* species, particularly by *F. graminearum*, *F. culmorum*, *F. equiseti*, *F. cerealis*, *F. verticillioides* and *F. crookwellense*. These species are common contaminants of crops worldwide, generally in moist and cool environmental conditions. Zearalenone production occurs during post-harvest but can also take place after harvest or during storage (Marin et al. 2013). This toxin is present worldwide and can contaminate different cereal crops (such as barley, maize, oats, among others) and has also been detected in cereal-derived products like flour, malt and beer.

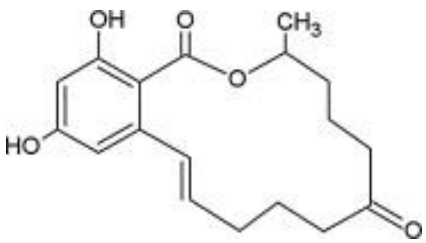


Figure 2.8. Chemical structure of zearalenone.

ZEN toxicity is associated with reproductive and endocrine problems in animal and probably in humans, since it binds competitively to oestrogen receptors (Zinedine et al. 2007). IARC reports concluded that ZEN is not classifiable as a carcinogen to humans (group 3) (IARC 1993). However, some studies indicate that ZEN may have a role in the development of breast cancer, and it has also been found in endometrial tissue of women with adenocarcinoma (Tomaszewski

et al. 1998; Ahamed et al. 2001). ZEN and its derivative zearalenol have also been reported as responsible for an epidemic episode of precocious puberty in Puerto Rico (de Rodriguez et al. 1985). In animals, the prolonged consumption of contaminated feed can lead to genotoxicity, reproductive toxicity, endocrine effects and immunotoxicity. Studies show that a dietary concentration of 1.0 mg/kg of ZEN may lead to problems in animal production, mainly embryo survival, reduced weight of the fetus, absence of milk and rectal prolapse. These effects have been reported in cattle and sheep (El-Nezami et al. 2002; Zinedine et al. 2007; Minervini and Dell'Aquila 2008).

Regulations in the EU define a maximum level for ZEN of 75 μg/kg in cereals intended for direct human consumption, 50 μg/kg for bakery products and 20 μg/kg for processed maize-based food for infants (EC 2018).

2.2.6 Trichothecenes

There are approximately 170 identified trichothecenes (Krska et al. 2001), which have been grouped in four types according to differences in the functional hydroxyl and acetoxy side groups. Most relevant trichothecenes are of type A: HT-2 toxin and T-2 toxin, and type B: DON. Their chemical structures are shown in Figure 2.9.

Trichothecenes are very stable and are able to maintain their toxic properties during milling and processing, as well as during cooking (Hazel and Patel 2004). These mycotoxins act by inhibiting eukaryotic protein synthesis by binding to the 60S ribosomal subunit and by interacting with the enzyme peptidyltransferase (Cundliffe and Davies 1977).

Regarding their toxicity, T-2 toxin has immunosuppressive and cytotoxic effects and it is known to inhibit protein synthesis and mitochondrial function. Since T-2 is rapidly metabolized to HT-2, it is assumed that toxic effects of T-2 include those of HT-2 (Marin et al. 2013). Regarding carcinogenicity, T-2 and HT-2 are classified as group 3 (non-carcinogenic to humans) (IARC 1993). Tolerable daily intake (TDI) for the combine consumption of these toxins has been limited to 0.06 μg/kg bw/day.

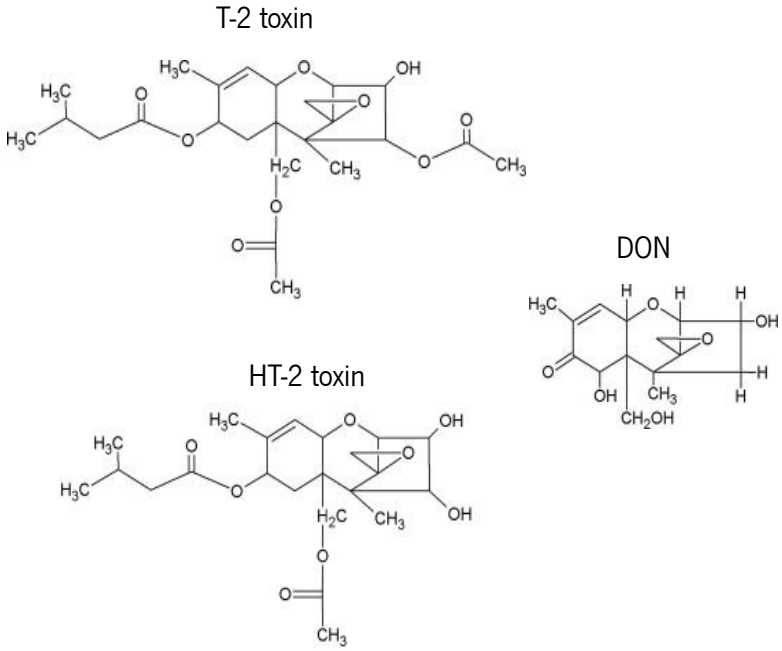


Figure 2.9. Chemical structure of trichothecenes: T-2 toxin, HT-2 toxin and DON.

For DON there is no evidence for mutagenic or carcinogenic effect and it is, as well, classified as a group 3 carcinogen, with a TDI below 1 μg/kg bw/day being defined (SCF 2002). Although DON is not as toxic as T-2 and HT-2, it is a very common contaminant of cereals worldwide. Acute effects of DON consumption cause abdominal pain, headache, nausea, vomiting, diarrhoea and bloody stools (Rotter 1996). Prolonged exposure to trichothecenes can also cause a disease known as alimentary toxic aleukia, which can ultimately lead to death (Marin et al. 2013).

2.2.7 Ergot Alkaloids

Ergot alkaloids are tryptophan-derived alkaloids characterized by a tetracyclic ergoline ring system. These compounds are produced in the sclerotia of *Claviceps* species and can affect grain cereals. In Europe, *Claviceps purpurea* is the primary fungal species responsible for the production of several ergot alkaloids, namely ergometrine, ergotamine, ergosine, ergocristine, ergocryptine and ergocornine; however, more than 50 different ergot alkaloids have been identified (Maruo et al. 2018). Due to their structural similarity with dopamine, noradrenaline,

adrenaline and serotonin, ergot alkaloids interrupt neurotransmission. Exposure to these toxins can result in ischemia, abortion, decreased body weight gain, dizziness, convulsions and alterations in hormone levels (Maruo et al. 2018).

An increase in the presence of ergot alkaloids in food products has been noticed in Europe and USA in recent years (Maruo et al. 2018). Currently, regulations are based on the quantities of ergot sclerotia, with maximum levels for sclerotia of *C. purpurea* in wheat and durum wheat, for human consumption, being 0.5 g/kg and 5 g/kg, respectively. Recently, EFSA proposed a TDI of 0.6 μg/kg bw/day (EFSA 2012a).

2.2.8 Co-occurrence of Mycotoxins

Recently, the co-occurrence of more than one mycotoxin in food or feed products, produced by the same or different fungus has been gaining attention, since this can result in a synergistic or additive toxic effect of the mycotoxins (Stoev 2008; Stoev and Denev 2013).

Synergistic effect has been suggested between OTA and penicillic acid (Stoev et al. 2001), FB1 and citrinin (Ostry et al. 2013), OTA and FB1 (Klaric et al. 2008; Stoev et al. 2012) or OTA and citrinin (Krogh et al. 1973; Vrabcheva et al. 2000).

Co-occurrence of mycotoxins can be a result of the production of multiple mycotoxins by one fungi, or the presence of several mycotoxigenic fungi, or even the mixture of various commodities (Streit et al. 2012). A three-year worldwide survey concluded that 48% of the analysed feedstuffs samples (total of 7049) were contaminated with two or more mycotoxins (Rodrigues and Naehrer 2012).

The presence of multiple mycotoxins in food and feed products presents an additional challenge since toxicological effects of simultaneous exposure is still scarce (Stoev 2008) and toxicity of mycotoxin combination cannot be predicted based on their individual toxicities (Smith et al. 2016). Also, there is a limited understanding of the effects caused by the exposure to several mycotoxins in low concentrations, during extensive time periods, that occurs due to the consumption of a diversified diet. Still, it is believed that exposure to these mycotoxins might have a role in the development of chronicle diseases in animals and humans.

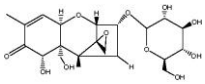
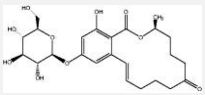
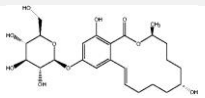
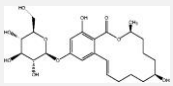
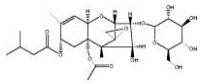
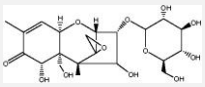
Therefore, in the future, regulations might consider co-occurrence of several mycotoxins and their possible interaction and synergistic effects because they can increase the risk for human and animals.

2.2.9 Modified Mycotoxins

The term "masked mycotoxins" was first used in the mid-1980s, due to some cases of mycotoxicoses in animals that when observed clinically did not correlate with the low content of mycotoxins found in the feed ingested by those animals. The unpredicted high toxicity was attributed to undetected forms of mycotoxins that were modified from the precursor toxin (Berthiller et al. 2005).

Plants can alter the chemical structure of mycotoxins as part of their defence mechanisms against xenobiotics. Generally, plant enzymes involved in detoxification processes catalyse chemical alterations that originate masked mycotoxins. These mycotoxin derivatives are not detected by conventional analytic techniques, due to their changed structure, and thus are named masked mycotoxins (Berthiller et al. 2013). New nomenclature has currently been proposed since the term "masked mycotoxins" only includes conjugated mycotoxins generated by plants' defence mechanisms, and does not include mycotoxins altered biologically by animals or fungi or altered chemically (either by thermal processes, UV light or chemical treatments). These metabolites have now been renamed as modified mycotoxins (Rychlik et al. 2014). Principal modified mycotoxins, their origin mycotoxin and products and regions where they have been detected are shown in Table 2.2.

Table 2.2. Masked Mycotoxins found in different food matrices and respective regions. Adapted from (Zhang et al. 2019)

Chemical structure	Modified Mycotoxin	Mycotoxin	Matrix	Region
	DON-3G	DON	Feed, maize, silage, flour, breakfast cereal, wheat, durum wheat, beer, barley, oats	Europe
			Maize, silage	South Africa
			Maize, silage	South America
			Wheat germplasm, wheat, corn, swine and poultry feed, corn, corn products	Japan and China
	ZEN-14G	ZEN	Barley, oat, wheat, bread, breakfast cereals	Finland, Belgium and Germany
	α-ZEL-14G	α-ZEL	Barley, oat, wheat, bread, breakfast cereals	Finland and Belgium
	β-ZEL-14G	β-ZEL	Barley, oat, wheat, bread, breakfast cereals	Finland and Belgium
	HT2-3G	HT-2	Barley, oat, wheat	Finland
	NIV-3G	NIV	Barley, oat, wheat	Finland and Japan

DON-3G: Deoxynivalenol-3-glucoside; ZEN-14G: zearalenone-14-glucoside; α-ZEL-14G**:** α-zearalenol-14-glucoside; β-ZEL-14G: β-zearalenol-14-glucoside; HT2-3G: HT-2-toxin-3-glucoside; NIV-3G: nivalenol-3-glucoside

Modified mycotoxins also include both extractable conjugated and non-extractable or bond varieties. Extractable conjugated mycotoxins can be detected by suitable analytical methods when their structure is known and analytical standards exist. Bound mycotoxins are covalently ("conjugated") or non-covalently ("hidden") attached to the carbohydrates or protein matrices in the contaminated plant, these have to be detached from the matrix by chemical or enzymatic treatments before analysis (Freire and Sant'Ana 2018).

Modified mycotoxins are not currently routinely screened for food nor regulated by legislation and analysis of samples containing these compounds leads to the underestimation of mycotoxin content. For example, although glucosides of zearalenone and deoxynivalenol possess toxic capability, toxicological reference values as well as maximum levels in food and feed are based

exclusively on the original compounds (ZEN and DON, respectively) (Rychlik et al. 2014). Difficulties in analysing modified mycotoxins are due to physicochemical alterations, which changes chromatographic behaviour, modify the epitopes recognized by antibodies used for detection, or causes low extraction efficiency due to changes in the polarity of the molecule. Other major concern is the possibility of hydrolytic deconjugation after ingestion or processing, that could bioconvert masked mycotoxin into the parent compound (Berthiller et al. 2013). Also, there is a lack of official standards and methodologies for detection of modified mycotoxins, with only deoxynivalenol-3-glusoside standard currently existing commercially (Freire and Sant'Ana 2018).

Currently, there is not enough toxicokinetic and toxicodynamic investigation to estimate the risk or hazard of modified mycotoxin for human and animal health when compared with their original form (Stoev 2015).

2.2.10 Influence of Climate Change in Mycotoxin Contamination

The research on fungal and mycotoxin contamination should also be discussed under the perspective of climate change, which may strongly affect mycotoxins production and change their patterns of occurrence in food and feed. Climate change is defined as "*a change of climate which is attributed directly or indirectly to human activity that alters the composition of the global atmosphere and which is, in addition to natural climate variability, observed over comparable time periods*" (UN 1994). Previsions for the next 10 to 20 years point to a rise in atmospheric CO_2 at the rate of 1.5 μmol/year. Due to this increase and the increase of other greenhouse gases, the global temperature is expected to rise 0.03 °C/year. In a 50 years scenario it is expected, in a situation where no preventive measures are implemented, that temperatures will increase in the range of 2-4.5 °C, with a best estimate value of 3 °C. Also, projected tendencies indicate a higher risk of drought in mid-continental areas and alterations in precipitation levels (Solomon et al. 2007).

Crop growth and its interaction either with beneficiary, pathogenic or toxigenic microorganisms varies with weather, making the agricultural sector specially affected by climate change (Battilani et al. 2016). Many reports demonstrated that the ability of fungi to produce

mycotoxins is particularly influenced by environmental factors, mainly temperature, drought, and relative humidity. Stress conditions in plants and presence of insect damage that create infection sites for fungal contamination, also contribute to the increase of mycotoxin content in agriculture commodities.

In wheat, dissemination of Fusarium Head Blight (FHB) disease depends primarily of climate conditions and in particular temperature and intense rain during anthesis period that disperse *Fusarium* inoculums, with warm and humid conditions favouring *Fusarium* infection of cereals. Thus is expected that fumonisin contamination may increase in the future (Miraglia et al. 2009). In maize, *A. flavus* contamination, the main source of aflatoxin, is increasing in temperate areas causing, in the year 2003 (a year of prolonged hot and dry weather) in South Europe, an alarming level of aflatoxins (Miraglia et al. 2009). Aflatoxins are expected to become more prevalent with the predicted climate change (Bunyavanich et al. 2003). Simulation of risk maps for aflatoxin contamination in Europe is shown in Figure 2.10.

Effect of climate change on fungal contamination and mycotoxins production should be studied for each occurrence since every fungi species has its optimal temperature and humidity conditions for the production of mycotoxins.

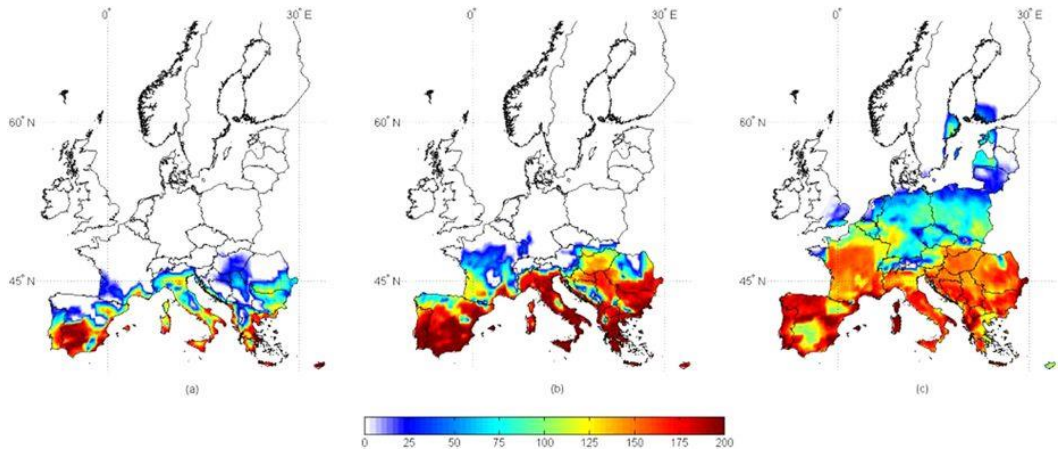


Figure 2.10. Risk maps for aflatoxin contamination in maize in different climate scenarios: present, + 2 °C and + 5 °C. The scale refers to the aflatoxin risk index, with 0 representing no risk and 200 the highest risk of contamination. Adapted from Battilani et al. (2016).

2.3 PREVENTION AND ELIMINATION OF FUNGI AND MYCOTOXIN CONTAMINATION

Due to all the negative effects of fungal and mycotoxigenic contamination, several strategies have been developed to prevent the growth of toxigenic fungi, to inhibit the synthesis of mycotoxins and to decontaminate commodities already affected by these toxins. Strategies of prevention of fungal and mycotoxin contamination can be applied during pre-harvest, harvest and post-harvest.

If contamination still prevails after harvest, several techniques for removal of contaminated commodities should be employed. If, still, the contamination persists, physical, chemical or biological methods to eliminate the fungal contamination or inactivate mycotoxins can also be used. Table 2.3 summarizes the strategies for prevention and elimination of fungal and mycotoxin contamination.

Table 2.3. Prevention, removal and inactivation of fungal and mycotoxin contaminated commodities

Prevention of Mycotoxin Contamination	
Pre-harvest control strategies	Resistance varieties: Use of plants less susceptible to contamination. Even though, obtaining high levels of native genetic resistance is difficult to achieve.
	Field management: Appropriate practices such as: crop rotation, deep ploughing (to remove residual fungi from the surface), fertilization, proper irrigation, and avoiding nutritional stress that are proven to reduce mycotoxin presence in crops.
	Chemical agents: The use of fungicides and other agents of pest management control reduces fungal contamination since insect damage to plant promotes fungal infection (Torres et al. 2014). Fungicides have to be proven safe both for humans and the environment. Still, in some conditions, for *Fusarium* species, there is evidence that fungicide may increase mycotoxin production, while reducing the presence of the fungi. Some synthetic pesticides have also been proven to reduce levels of OTA contamination in experimental viticultures, with a reduction of OTA of 97% for treatments with azoxystrobin (Lo Curto et al. 2004).

	Biological agents: Biocontrol microorganisms (bacteria, yeast and filamentous fungi) have been reported to reduce fungal infections, which lead to reduction in mycotoxin contamination. It has been demonstrated that soil inoculation with nontoxigenic fungi will compete with mycotoxin producing strain for infection sites and nutrients (Dorner 2004).
Harvest management	During harvest there should be an efficient separation of contaminated material from healthy commodities. Plants should be harvest at optimum growth and physical damage should be avoided. Also, moisture control of the harvested products is of primary importance, to avoid fungal development.
Post-Harvest control strategies	Improving drying and storage conditions: Storage should maintain an adequate aeration and moisture, since fungal development is facilitated with increased moisture levels. Drying should take place shortly after harvest. Aflatoxins can be produced at a_w values of at least 0.82, while for OTA production minimum a_w is 0.85. Temperature is also a main factor for fungal and mycotoxin contamination. Maintaining low temperatures in storage facilities is a suitable strategy to reduce fungal contamination (Sweeney and Dobson 1998; Milani 2013).
	Use of chemical agents: The application of fungicides after harvesting can be used to reduce or eliminate fungal contamination. The effect of fungicide on fungal growth and mycotoxin production is affected by amount of fungicide used, crop type, fungal species and storage conditions. Fungicides such as iproidine, dichlorvos, landrin, malathion, and diazinon are effective in inhibition aflatoxin formation (D´Mello et al. 1998). Also, sorbic acid and its salts (sodium and potassium), propionic acid, nisin and ethyl or methyl benzoate are also effective in preventing fungal contamination during storage (Bullerman 1983; Paster et al. 1999).
	Use of biological agents: The use of antagonistic bacteria, yeast and filamentous fungi can control mycotoxigenic fungi contamination. Several studies reported the use of yeast and non-toxigenic filamentous fungi as being able to reduce mycotoxin production. Antagonistic bacteria, such as LAB and their metabolites have been studied recently as natural preservatives with the ability to inhibit fungal growth and mycotoxin production (El-Tarabily and Sivasithamparam 2006; Dalié et al. 2010). Some plant extracts and spices are also known to prevent fungal contamination (Bobbarala et al. 2009).

	Irradiation: Irradiation has been used to prevent mycotoxin synthesis during storage (Aziz and Moussa 2002). Radiation can be ionizing (X-rays, gamma (γ) rays and UV rays, microwaves, infrared rays and radio waves) or non-ionizing (Calado et al. 2014). Despite the concerns with food irradiation, the FAO/IAEA/WHO Expert Committee indicates that a 10 KGy dose causes no toxicological hazards or nutritional reduction (Molins 2001). Efficiency of radiation in the inhibition of fungal growth and mycotoxin production is strain and dose dependent, and is influenced by humidity and storage conditions (Zeinab et al. 2001; Aziz and Moussa 2002; Oviedo et al. 2011).
Removal of Contaminated Commodities	
Physical separation	Sorting: Sorting of damaged crops containing visible mould contamination can lead to the elimination of significant quantities of mycotoxins. Contaminated kernels are normally damaged, discoloured or shrivelled. Sieving and sorting can remove the majority of affected kernels. In some cases, electronic sorting devices can detect some parameters (i.e. florescence) that seems to correlate to the presence of mycotoxins, and therefore can be used to improve the process (Sinha 1998).
	Density segregation: Contaminated kernels exhibit different properties, allowing them to be separated by density segregation in some liquids or sorted by specific gravity tables (Bata and Lásztity 1999).
	Washing: Washing contaminated grains with a water and sodium carbonate solution have been reported to reduce DON, ZEN and fumonisins. However, this treatment is only feasible when used prior to wet milling or alcoholic fermentation (Sinha 1998).
	Milling: Milling can result in a reduction of mycotoxin levels in contaminated grains. This effect has been reported for OTA in the production of white flour, with a 66% reduction (Scudamore et al. 2003).
Extraction with solvents	Some solvents are able to extract mycotoxins from contaminated commodities, such as oilseed peanut and cottonseed. Most used solvents include ethanol, acetone, isopropanol, methanol-water, acetonitrile-water, acetone-hexane-water, hexane-methanol. This technique is limited by high cost and problems with waste disposal and are only suited for commodities used for animal feed (Rustom 1997).
Adsorption	Several adsorbents can bind and remove mycotoxins from aqueous solutions. Active charcoal and bentonite have been reported to bind efficiently to mycotoxins (Huwig et al. 2001).

Inactivation in Contaminated Commodities	
Physical methods	Heat-treatment: Mycotoxins are relatively heat-stable in the range of food processing temperatures (80-120 °C). However, roasting commodities for 30 minutes at high temperatures (above 150 °C) can result in reducing mycotoxin contamination by 20-60% (Kabak et al. 2006). The level of elimination of mycotoxins depends of the toxin type, concentration, binding of the toxin to the food product, heat penetration and temperature and time of the heat treatment (Rustom 1997).
	Ionizing Radiation: In addition to be useful in the post-harvest control of mycotoxins in storage, ionizing radiation has been studied for the degradation of mycotoxins. Inactivation of mycotoxins depends of level of radiations, mycotoxin and type of food product (Herzallah et al. 2008). Water also plays an important role in mycotoxins degradation, since radiolysis of water initiates free radical reactions resulting in aflatoxin degradation (Rustom 1997). Aflatoxins are reported to be sensible to some wavelengths of UV radiations (Samarajeewa et al. 1990). Also, OTA can be easily degraded by gamma radiation when dissolved in water (over 90% with ≥2.5 KGy) (Calado et al. 2018).
Chemical methods	Acid-treatments: Treatments with acids (HCl or H_2SO_4) have shown to reduce levels of aflatoxins. Aflatoxins B1 and G1 can be completely degraded after treatment with 1% solution of HCL or H_2SO_4 (Tabata et al. 1994). Also, lactic acid, under conditions simulating cooking (80 °C), has been shown to degrade AFB1 (85%) into aflatoxin B2 (AFB2) and mainly into AFB2a, which exhibited much less toxicity than AFB1 (Aiko et al. 2016).
	Treatment with bases: Contaminated commodities can be detoxified with organic or inorganic bases. Hydroxide or gaseous ammonia is reported to be effective to remove aflatoxins in peanut, cotton and maize (Samarajeewa et al. 1990; Burgos-Hernández et al. 2002). These treatments depend of quantity, time of treatment, temperature and pressure used in the treatment (Piva et al. 1995). However, the US Food and Drug Administration (FDA) and the EU do not allow the importation of ammoniated cottonseed or maize.
	Oxidizing agents: Ozone has been successfully used to detoxify mycotoxins in some commodities. Several studies reported the degradation of aflatoxins, CPA, FB1, OTA, patulin and ZEN (McKenzie et al. 1997). Hydrogen peroxide has also been used to detoxify citrinin and OTA (Fouler et al. 1994).
	Reducing agents: Sodium bisulfite has been reported to be able to detoxify mycotoxins and reduce the mutagenic potential of AFB1 (Samarajeewa et al. 1990; Piva et al. 1995).

	Chlorinating agents: Washing food products with chlorinated agents such as chlorine and sodium hypochlorite have been reported to degrade mycotoxins (Sen et al. 1988; Samarajeewa et al. 1990). Also, gaseous chlorine was able to reduce aflatoxin levels in peanuts (Kabak et al. 2006).
Biological methods	Biological methods have been developed to overcome some limitations of physical and chemical methods to detoxify mycotoxins, such as safety issues and losses in nutritional quality and limited efficiency. Non-toxigenic fungal cultures have been proven to degrade AFB1 and OTA (Shantha 1999; Abrunhosa et al. 2002). *Aspergillus* spp. were also able to degrade OTA in liquid media (Varga et al. 2000). Several fermentations processes, both with yeast and bacteria, also result in mycotoxin reduction (Bennett and Richard 1996; Cabañes et al. 2002; Abrunhosa et al. 2014). Also, some LAB strains can adsorb mycotoxins, reducing the gastrointestinal absorption of these compounds (Taheur et al. 2017).

Even though the numerous methods to deal with the mycotoxin problem, many of these methods have significant disadvantages. Preventive methods can only decrease fungal infections and are insufficient to eliminate the contaminant completely. Additionally, an increasing number of fungi are becoming resistant to both antifungals and preservatives.

When preventive measures are insufficient, commodities may need detoxification before being used for food and feed purposes. Detoxification is generally accomplished by removal of the contaminated commodities or by inactivation of mycotoxins. In general, detoxification methods have only reduced or partial efficiency, are time consuming, with limited applicability, sometimes are not economically feasible or create problems with waste disposable. Also, the available physical and chemical methods for detoxification of commodities is restricted by potential losses in nutritional quality, limited efficiency and safety issues. Additionally, detoxification of products by chemical treatment is not allowed in the EU and mixing of contaminated products with products with good quality is also prohibited (EC 2001).

In fact, it is in general very difficult to prevent the production of mycotoxins, since they are a natural contaminant of food and feed products. Nevertheless, mycotoxins levels should be reduced to the feasible minimum.

2.3.1 Biopreservation/Biocontrol

Preventive measures directed at the inhibition of mycotoxin formation in agricultural products are the most efficient approach to avoid the presence of these toxins in the food chain. Nowadays, the most common food preservation strategies involve chemical or physical techniques, which may only decrease fungal infection and eventually alter the characteristics of food and feed. Additionally, consumer's trends are moving towards a minimally processed, green-labelled and "chemical free" food products (Reis et al. 2012). Biopreservation refers to the use of a microorganism or its products to increase shelf life and food safety (Stiles 1996). This strategy is a biological method of prevention and control of contaminants and uses specific microorganisms to protect food from microbial spoilage. In addition to improve food safety, biopreservation can improve nutritional value and organoleptic properties of food products.

Many organisms have been tested for their capacity to reduce mycotoxin contamination in crops before harvest. Non-mycotoxigenic species of fungi have been tested in biocontrol strategies with success. Non-aflatoxigenic strains of *A. flavus* and *A. parasiticus* have been used to reduce the contamination of aflatoxins in commodities such as peanuts, maize and cottonseed. This inhibitory effect is thought to be linked to competition for nutrients and production of inhibitory compounds (Cotty 1994). Some *A. flavus* strains have been approved for the control of aflatoxigenic strains, such as *A. flavus* AF36 that has been considered as a biopesticide by the U.S. Environmental Protection Agency and is used for the control of aflatoxin producing strains in cotton production, and *A. flavus* NRRL 21882 that has been used in peanuts (Chang and Hua 2007). In 2004, a commercial biopesticide, Afla-guard®, was approved for use, for the first time, in peanut crops in the southeast of the United States (Dorner and Lamb 2006). Field studies showed that aflatoxins in farmers' stock peanuts were reduced by 80 to 90% (Dorner 2009). Also, early field studies in Nigeria using the biocontrol product Alfasafe™ demonstrated an 80 to 90% reduction in aflatoxin contamination of maize and groundnut (Imes 2011). Another commercial product named AF-X1™ is being developed in Italy for the prevention of aflatoxin contamination in maize (Mauro et al. 2018). Co-inoculation of non-toxigenic *A. flavus* A2085 with aflatoxin producing strains resulted in a reduction of over 90% in aflatoxin accumulation. In naturally contaminated maize reduction reached 92% (Mauro et al. 2018). Large-scale field trials are being

conducted to determine the extension of aflatoxin reduction that is practically feasible and how to best incorporate biocontrol in agriculture practice. However, multiple applications over the years of non-aflatoxigenic strains could cause alterations in the genetic composition of native strains due to gene transfer from the biocontrol strains. The capacity of these altered stains to produce mycotoxin and infect crops need assessment (Torres et al. 2014).

Non-pathogenic species *Phoma betea* and *Phytium ultimum* were also found to reduce development and severity of infection with different species of *Fusarium* in wheat ears (Diamond and Cooke 2003) and a *Fusarium* species, *F. equiseti*, was able to control other *Fusarium* species and reduce DON formation on wheat (Dawson et al. 2004).

Yeasts have also been employed in the control of mycotoxigenic fungal strains. Several saprophytic yeasts isolated from almond, pistachio and walnut exhibited the ability to interrupt aflatoxin formation in early steps of the polyketide pathway (Hua et al. 1999). Other yeasts have been identified as antagonists capable of inhibiting Fusarium head blight (a fungal disease of cereals caused by several *Fusarium* species that infects the head of the crop), especially in barley and wheat (Schisler et al. 2002; Schisler et al. 2011). Additionally, yeasts, including *Saccharomyces*, *Rhodotorula*, *Cryptococcus* and *Trichosporon mycotoxinivorans* are able to detoxify OTA by hydrolyzing the amide bond of OTA converting it into phenylalanine and ochratoxin α, both nontoxic products (Schatzmayr et al. 2003; Molnar et al. 2004; Abrunhosa et al. 2010).

Certain strains of bacteria can also be used for the control of mycotoxigenic fungi. Other works report that bacterial isolates were capable to suppress *F. graminearum* by 37 to 97% (Nourozian et al. 2006). Strains of *Streptomyces* sp. and *Pseudomonas* spp. were able to reduce the severity of Fusarium head blight disease (Nourozian et al. 2006), and some *Pseudomonas* reduced DON to up to 78% (Khan and Doohan 2009).

Biocontrol strategies can also reduce fungal proliferation and mycotoxin production during post-harvest. Antagonistic yeasts were found to reduce fungal growth both *in vitro* and in simulated storage conditions. *Pichia anomala* and *Saccharomyces cerevisiae* were capable of reducing OTA accumulation by *P. verrucosum* in wheat (Petersson et al. 1998). *Kluyveromyces* strains were able to inhibit the growth of some species of *Aspergillus* section *Flavi* and their aflatoxin production (La Penna and Etcheverry 2006). Other yeast species isolated from grapes,

reduced contamination of grapes by *A. niger* and *A. carbonarius*, the principal species responsible for the production of OTA in grapes and wine (Bleve et al. 2006).

Several species of fungi, including *Phoma*, *Mucor*, *Trichoderma*, *Rhizopus*, *Alternaria* species, were reported to inhibit AFB1 biosynthesis by over 90% (Shantha 1999). Also, *F. proliferatum* inhibited the production AFB1 by *A. flavus* when co-cultured under specific growth conditions, with inhibition being dependent of the inoculum concentration (Picco et al. 1999). Culture filtrates can also demonstrate antifungal potential. Supernatant from *Rhodococcus erythropolis* (25 mL/kg) totally inhibited AFB1 production by *A. flavus* in stored rice. This antimycotoxigenic effect is apparently enzymatically mediated since enzymes present in the extract were responsible for AFB1 degradation (Alberts et al. 2006).

Studies with bacteria showed promising results with *Bacillus amyloliquefaciens* and *Microbacterium oleovorans* being effective in reducing *F. verticillioides* counts in maize grains and also reducing the levels of FB1 and FB2 in grains from plants grown from those seeds (Pereira et al. 2007). A strain of *Propionibacterium freudenreichii* was able to suppress the production of DON and NIV by *F. graminearum*, with their supernatants being responsible for reductions of 38 and 35%, respectively. Also, two strains of *Paenibacillus polymyxa* inhibited *F. graminearum* growth and reduce production of DON. Fusarium head blight severity was reduced by 56%, *F. graminearum* colonization of wheat head by approximately 60% and DON production by up to 90% (He et al. 2009).

The control of fungal spoilage by antifungal microorganisms is complex and depends of the interaction between the food product, the fungal spoiler and the antifungal microorganisms. A scheme representing this interaction can be seen in Figure 2.11.

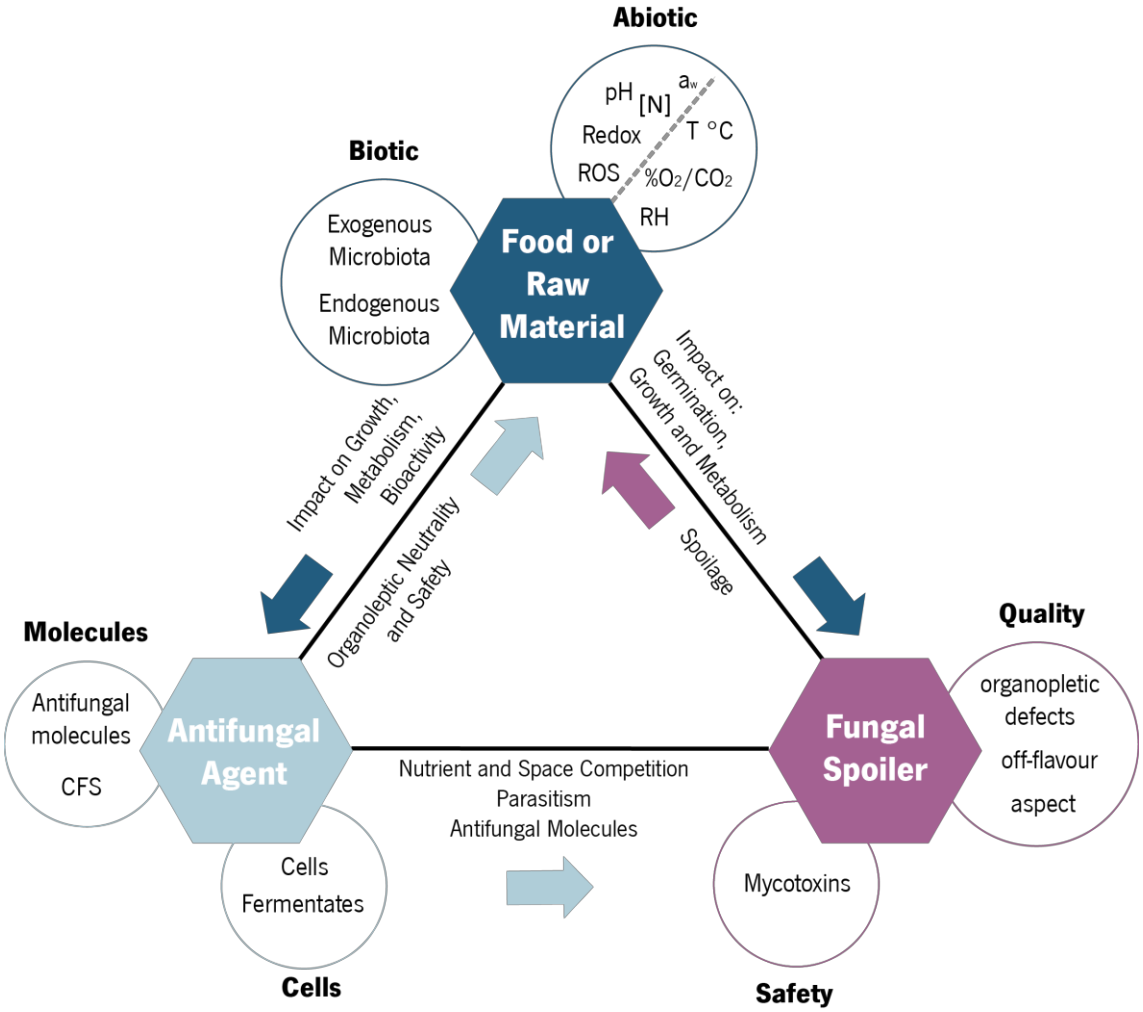


Figure 2.11. Scheme representing interactions occurring between food or raw material, antifungal agents and fungal spoilage in a biopreservation approach (CFS: culture free supernatant, ROS: reactive oxygen species, [N]: nutrient content, RH%: relative humidity, aw: water activity, T °C: temperature) Adapted from (Leyva-Salas et al. 2017).

2.3.2 Lactic Acid Bacteria as Biopreservation/Biocontrol Agents

A vast number of works regarding biopreservation/biocontrol strategies focus in the antifungal properties of lactic acid bacteria, which can exhibit inhibitory activity against a broad range of mycotoxigenic fungi.

Lactic acid bacteria are Gram-positive microorganisms that are tolerant to acid, non-motile, non-spore forming, aerotolerant, are rod- or coccus shaped, and produce lactic acid as a main end product during carbohydrate fermentation. They are a diverse group of bacteria connected by metabolic and physiological features. Initial taxonomy included four main genera involved in food fermentation, namely *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus* (Wessels et al. 2004). A more recent classification broadened this original group, and LAB currently include the following genera: *Aerococcus, Alloiococcus, Carnobacterium, Dolosigranulum, Enterococcus, Globicatella, Lactobacillus, Lactococcus, Lactosphaera, Leuconostoc, Mlissococcus, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weisella* (Crowley et al. 2013b).

Generally, LAB can be classified according to the resulting products of the fermentation of carbohydrates. Homofermentative LAB, such as *Enterococcus*, *Pediococcus*, *Streptococcus*, *Lactococcus* and some *Lactobacillus spp*. produce lactic acid as the main product, while heterofermentative LAB such as *Weissella*, *Leuconostoc* and some *Lactobacillus* produce as main products of fermentation, a part from lactic acid, ethanol, acetic acid and carbon dioxide. LAB can also produce other organic acids and aromatic compounds, that can contribute to the typical flavour of certain fermented products. Additionally, some strains of *Lactobacillus* and *Streptococcus* are able to convert complex sugars in lactic acid (Pawlowska et al. 2012).

For a long period of time, LAB have been used as a biopreservative microorganism, as they perform a critical role in several food fermentations, mainly involving milk, meat, vegetables and sourdoughs, by causing a rapid acidification of the food product. Also, lactic acid fermentation is commonly used as a simple and safe method to enhance properties of food (Oliveira et al. 2014). LAB are considered GRAS (Generally Regarded as Safe) organisms and Qualified Presumption of Safety (QPS) in the European Union, which make these microorganisms ideal candidates for biocontrol agents (Leroy and De Vuyst 2004). In recent years, their use has

become popular in the manufacture and storage processes, by prolonging shelf life, microbial safety, texture, sensory qualities and nutritional value (Oliveira et al. 2014).

Lactic acid bacteria also represent the major group of probiotic bacteria in the food industry. Species of the *Lactobacillus* and *Enterococcus* genera are commonly used for their probiotic abilities (Salminen et al. 1998). Probiotic bacteria can be defined as live microorganisms, which at a given concentration can confer health benefits to the host (Sanders 2008). Probiotics help to support the growth of preferred microorganisms, reduce potentially harmful bacteria and, reinforce the natural defence mechanisms of the body (Holzapfel et al. 1998; Gismondo et al. 1999). Suggested mechanisms for probiotic action of LAB include production of antimicrobial metabolites to inhibit pathogens, and modulation of immune response, among others (Dicks and Botes 2009).

The use of LAB in biocontrol strategies include inoculating protective cultures in the food matrix or incorporating purified microbial metabolites (Stiles 1996). Several screenings have been made to identify LAB with antifungal properties isolated from different sources, such as cereals (Rouse et al. 2008; Ndagano et al. 2011), sourdough (Corsetti et al. 1998; Lavermicocca et al. 2000; Coda et al. 2008; Gerez et al. 2009), vegetables (Magnusson and Schnurer 2001; Gerez et al. 2010), and dairy products (Schwenninger et al. 2005; Voulgari et al. 2010).

With growing pressure from consumers towards more natural food preservatives, LAB represent ideal candidates for commercial development. Compounds produced by LAB are considered “natural” and thus regarded as a biological method to ensure safety of food products, allowing the reduction of chemical preservatives or other antimicrobial treatments.

The most easily recognized antimicrobial activity of LAB is the production of organic acids, mainly lactic and acetic acid, but also formic, propionic, butyric, hydroxylphenyllactic and phenyllactic acids. LAB are also able to produce other antagonistic compounds such as fatty acids, hydrogen peroxide, ethanol, diacetyl, and proteinaceous compounds. Additionally many LAB strains produce bacteriocins, such as nisin, reuterin, reutericyclin, pediocin, lacticin and entorocin or bacteriocin-like inhibitory substances (Leroy and De Vuyst 2004; Schnurer and Magnusson 2005).

2.3.3 Antifungal Compounds Produced by LAB

2.3.3.1 Organic acids

Organic acids are end products of carbohydrate fermentation by LAB and are considered safe agents for the preservation of food. The ability of these weak acids to inhibit the growth of fungal cells is based on the reduction of pH, disruption of substrate transport and reduction of proton motive force (Campos et al. 2010). These compounds acidify the cell cytoplasm allowing the undissociated form of the acid to diffuse across the target organism membrane. The undissociated acid acts by collapsing the proton gradient or by altering membrane permeability, which creates unfavourable conditions for the growth of potentially pathogenic microorganisms in both food products and the human intestinal microflora (Ammor et al. 2006). Still, the full mechanisms by which organic acids inhibit fungal growth are not completely understood.

Organic acids that have been used as antifungal agents include acetic, benzoic, citric, lactic, malic, propionic, sorbic and tartaric acid, among others. Lactic, acetic acids and propionic acids have been extensively used as inhibitors of mould growth and it is believed that the effect of acetic and propionic acids is often dependent on the decrease of pH caused by presence of lactic acid (Schnurer and Magnusson 2005). Both acids have a higher pKa values than lactic acid, thus having a higher proportion of acid in the undissociated state at a given pH (Peláez et al. 2012). Both, propionic and acetic acids are able to reduce amino acid uptake at low pH, and affect fungal membranes at pH lower than 4.5 (Schnurer and Magnusson 2005).

In the last decades, other organic acids have been identified as antifungal compounds. Phenyllactic acid (PLA) effect against mould contamination has been first described by Lavermicocca et al. (2000). In this study, the authors showed that *Lactobacillus plantarum* 21B produced PLA and that this acid had strong antifungal activity against *P. expansum* IDM/FS2, *A. niger* strains FTDC3227 and IDM1, *A. flavus* FTDC3226 and *F. graminearum* IDM623 at a concentration of approximately 50 mg/mL. Moreover, in this study it was reported the production of 4-hydroxy-phenyllactic acid (OH-PLA) by the same LAB strain, that also demonstrated antifungal properties. Both PLA and OH-PLA are involved in phenylalanine metabolism, and are produced to avoid intracellular accumulation of phenylalanine. This aminoacid is hydroxylated to tyrosine or transaminated to phenylpyruvic acid, which is further metabolized to phenylactic and

hydroxyphenyllactic acids. When *L. plantarum* 21B was used in sourdough it delayed fungal growth for up to 7 days when compared with sourdough without this LAB strain (Lavermicocca et al. 2000). Also, *L. plantarum* MiLAB 393, a strain isolated from grass silage, was shown to produce PLA and it was suggested that PLA might act in a synergistic way with other compounds produced by this strain (Ström et al. 2002).

Several other organic acids produced by LAB have as well demonstrated antifungal activity. Corsetti et al. (1998) determined that the broad antifungal effect of the strain *Lactobacillus sanfrancisco* CB1 was due to the production of a combination of acetic, formic, propionic, butyric and *n*-valeric acids, and also the fatty acid caproic acid, with all the compounds possibly acting in a synergic way.

Later, Valerio et al. (2004) studied the ability of 29 LAB isolates, belonging to species used in food fermentation to produce PLA and OH-PLA. A total of 15 strains produced both metabolites (0.16-0.46 mM for PLA and 0.07-0.29 mM for OH-PLA) and 5 strains only accumulated PLA (0.17-0.57 mM). The authors were able to increase the production of PLA of one of the *L. plantarum* strains with the addition of phenylalanine and the reduction of tyrosine in the culture medium. The authors also found that PLA is involved in the formation of flavours.

Other researchers investigated the role of PLA in LAB antifungal effect. This effect has been demonstrated against several fungi isolated from food or feed products. In two different studies, Gerez et al. (2009; 2013) reported that the major responsible for the antifungal ability of several LAB strains (*Lactobacillus casei*, *L. plantarum*, *Lactobacillus fermentum*, *Lactobacillus reuteri*. and *Lactobacillus acidophilus*) were acetic, lactic and phenyllactic acids. It was also showed that the inclusion of antifungal LAB strains in bread starter cultures allowed the reduction of calcium propionate use (a commercial antifungal) by 50%, while still achieving a similar shelf life when compared with traditional bread (Gerez et al. 2009). PLA was also found to be the main responsible for the broad antifungal spectrum of a *L. plantarum* strain (Prema et al. 2010). In another study, Oliveira et al. (2015a) analysed the production of antifungal compounds by *Lactobacillus amylovorius* and *L. reuteri* strains. Up to 13 different antifungal metabolites were identified, with lactic, acetic, phenyllactic, hydroxyphenyllactic, and benzoic acids playing an essential role in the antifungal ability of these strains supernatants. Later the same authors applied the same strains in the early stages of malting and verified that *L. reuteri* supernatants

were able to reduce *F. culmorum* growth by 23% and mycotoxin contamination by 83% (Oliveira et al. 2015b).

Other less reported carboxylic acids produced by LAB have also demonstrated antifungal properties. Several carboxylic acids, including D-glucoronic acid, salicylic acid and three derivatives of cinnamic acid (3-phenylpropanoic, *p*-coumaric and (E)-2-methylcinnamic acids) among other compounds, were reported as antifungal metabolites from *L. amylovorus* DMS 19280 (Ryan et al. 2011). Numerous antifungal carboxylic acids were also detected in silos inoculated with *L. plantarum* MiLAB 14 and MiLAB 393, including 3-hydroxydecanoic, *p*-hydrocoumaric, hydroferrulic, hydrocaffeic, *p*-coumaric, ferrulic, salicylic, 4-hydroxybenzoic, azelaic, 3,4-dihydroxycylohexane-1-carboxilic, 2-hydroxy-4-methylpentanoic, vanillic and benzoic acids. These compounds demonstrated activity against *P. anomala*, *Penicillium roqueforti* and *A. fumigatus.* Against all three fungi, *3*-hydroxydecanoic acid had the lowest Minimum Inhibitory Concentration (MIC) (0.1 mg/mL) (Broberg et al. 2007). Brosnan et al. (2012) have also isolated several organic acids from *L. amylovorus*, *Lactobacillus arizononas*, *L. plantarum*, *L. reuteri* and *Weisella cibaria*. The authors were able to detect some of the acids that were also reported by Ryan et al. (2011) and Broberg et al. (2007), namely D-glucoronic, vanillic, p-coumaric, azeilaic, ferulic, benzoic, salicylic, caffeic and hydrocinnamic acids, among other carboxylic acids (Brosnan et al. 2012). Similarly, Oliveira et al. (2015a) detected in the supernatant of *L. amylovorus* and *L. reuteri* strains, with inhibitory ability against *F. culmorum*, similar profile of carboxylic acids, namely hydroxybenzoic, benzoic, caffeic, coumaric, ferullic, hydrocaffeic, hydrocinnamic, hydroferellic, phloretic and vanilic acids.

2.3.3.2 Proteinaceous compounds and cyclic dipeptides

Many microorganisms are capable of synthetizing antimicrobial peptides. These peptides are, generally, 20 to 50 amino acids long; have a hydrophobic and a hydrophilic end and cationic properties (Nissen-Meyer and Nes 1997). Several authors have reported that some LAB strains are able to produce antifungal compounds that loose activity in the presence of proteolytic enzymes (Gourama and Bullerman 1997; Magnusson and Schnurer 2001; Atanassova et al. 2003; Voulgari et al. 2010).

Batish et al. (1989) reported that the antifungal ability of a LAB isolate disappeared after proteinase treatment, concluding that the compound responsible for antifungal activity was of proteinaceous nature. Similarly, a *Lactococcus lactis* subsp. *lactis* lost its antifungal activity after enzymatic treatment with chemotrypsin, trypsin and pronase E (Roy et al. 1996). Gourama and Bullerman (1997) reported that the antifungal activity of a *L. casei* subsp. *pseudoplantarum* strain was sensitive to trypsin and α-chymotrypsin, which suggests the presence of an antifungal protein compounds in the medium, that the authors concluded that it was a peptide of low molecular mass. Also, Magnusson and Schnürer (2001) reported that the compounds involved in the antifungal activity of a *Lactobacillus coryniformis* subsp. *coryniformis* strain were heat stable, small (3 kDa), and sensitive to proteolytic enzymes, thus suggesting that the antifungal activity was related to proteinaceous compounds. Supplementation with ethanol, formic or acetic acid increased the amount of antifungal peptide produced.

Atanassova et al. (2003) reported a proteinaceous compound (43 kDa) produced by *Lactobacillus paracasei* subsp. *paracasei* that demonstrated broad antimicrobial activity. Also, Voulgari et al. (2010) found that proteinaceous compounds were responsible for the antifungal activity of LAB strains isolated from dairy products.

Cyclic-dipeptides are the simplest peptide derivatives and most of them appear to be by-products of fermentation; several works report the antifungal ability of these compounds. Rouse et al. (2007) isolated a possible cyclic dipeptide from *Pediococcus pentosaceus* fermentation with activity against *P. expansum*. Yang and Chang (2010) and Dal Bello et al. (2007) were the first to report antifungal activity of cyclo (Leu-Leu) and cyclo (L-Leu-L-Pro) from LAB, respectively. Kwak at al. (2018) isolated 16 cyclic-dipeptides from *L. plantarum* LBP-K10 culture filtrate, and the combination of this compounds exhibited a strong antimicrobial activity, inhibiting bacteria, fungi and virus. Other cyclic dipeptides have also been isolated from LAB cultures and identified as antifungals; these include cyclo (Phe-Pro), cyclo (Phe-OH-Pro), cyclo (L-Phe-L-Pro) and cyclo (Gly-L-Leu) (Niku-Paavola et al. 1999; Ström et al. 2002; Magnusson et al. 2003; Ryan et al. 2011; Li et al. 2012).

2.3.3.3 Reuterin

Reuterin is a low molecular weight, broad-spectrum antimicrobial and water soluble compound originally isolated from *L. reuteri* culture medium, and it is one of the most studied low-molecular mass compounds produced by LAB (Axelsson et al. 1989; Chung et al. 1989; Talarico and Dobrogosz 1989). However, its antifungal activity is still poorly documented (Vimont et al. 2019). Reuterin acts by suppressing ribonuclease activity, the enzyme which catalyses the first step in DNA synthesis (Dobrogosz et al. 1989).

Reuterin is produced from glycerol by starving cells under anaerobic conditions and active reuterin is a mixture of 3-hydroxypropionaldehyde (3-HPA), its hydrate, its cyclic dimeric form and acrolein (Engels et al. 2016). Generally, LAB lack the oxidative pathway for glycerol degradation, therefore glycerol cannot be metabolized as a sole carbon source (Schnurer and Magnusson 2005). The only pathway for glycerol degradation existing in LAB goes through the intermediate state of 3-HPA. An increased antifungal activity towards several food-spoilage fungi was verified with the addition of glycerol to the growth medium of several strains of *L. coryniformis.* Glycerol/diol dehydratase enzymes catalysed the conversion of glycerol to 3-HPA in *L. coryniformis*, suggesting that the increase in inhibition resulted from production of reuterin from the breakdown products of glycerol degradation. 1,3-propanediol and 3-hydroxypropionic acid were also detected in the culture supernatant of the cells (Magnusson 2003).

The production of reuterin has been reported for some LAB strains from *L. reuteri*, *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus collinoides* and *L. coryniformis* (Schütz and Radler 1984; Claisse and Lonvaud-Funel 2000; Magnusson 2003).

Reuterin antimicrobial activity was proven against Gram-positive and Gram-negative bacteria, yeasts and filamentous fungi and this activity has been demonstrated against species of *Candida*, *Saccharomyces*, *Clostridium*, *Salmonella*, *Listeria*, *Aspergillus* and *Fusarium* (Chung et al. 1989; El-Ziney and Debevere 1998; Ávila et al. 2014; Langa et al. 2018). A recent work reported that reuterin was able to reduced fungal growth at a MIC of 1.38 mM and exert fungicide effect at a MIC of 6.9 mM (inhibition tested against *Penicillium* and *Aspergillus* spp.) (Vimont et al. 2019).

Reuterin has a high potential to be used as a food preservative since it is soluble in water, resistant to proteolytic and lipolytic enzymes, has antimicrobial activity at low pH and high NaCl concentrations and does not lose activity at refrigerated temperatures.

The use of reuterin-producing LAB, together with glycerol, in food products can be a feasible alternative for the direct addition of reuterin in food products (Martín-Cabrejas et al. 2017; Langa et al. 2018).

2.3.3.4 Fatty acids

Fatty acids have been reported to have both antibacterial and antifungal effect (Bergsson et al. 2001). Some LAB strains have the ability to produce these fatty acids, that additionally can improve sensory properties of fermented food (Leroy and De Vuyst 2004). Decades ago, Rao and Reddy (1984) found several fatty acids in LAB cultures from fermented milk, but only more recently, in a study by Sjögren et al. (2003), fatty acids were pointed as antifungal compound. In this work, several 3-hydroxylated fatty acids (3-(R)-hydroxydecanoic acid, 3-hydroxy-5-cis-dodecenoic acid, 3-(R)-hydroxydodecanoic acid and 3-(R)-hydroxytetradecanoic acid) were isolated from *L. plantarum* MiLAB 14 supernatant and their broad antifungal activity was demonstrated against several moulds and yeasts. This was the first study to report the antifungal activity of hydroxylated fatty acids produced by LAB.

Previous studies, which focused on antibacterial activity of fatty acids, reported that the antagonistic activity of straight-chained fatty acids increased with their chain length, with caprylic (C8) acid being the most effective (Woolford 1975). Fatty acids longer than 10 carbons were not as effective as antimicrobial compounds due to low solubility in aqueous solutions. However, Kabra (1983) reported that fatty acids with 12-16 carbons were the most effective and demonstrated detergent-like properties. Other work, showed that lauric (C_{12}) and capric (C_{10}) acids were the most potent fatty acids against *Candida albicans* (Bergsson et al. 2001). Differences in these reports may be due to different experimental conditions and different target microorganisms.

Corsetti et al. (1998) discovered that a *L. sanfrancisco* strain CB1 produced caproic (C_6) acid, which played a role in the antifungal effect of this LAB strain. Ndagano et al. (2011), reported that *L. plantarum* and *Weissella paramesenteroides* strains, isolated from cassava, showed antifungal activity against *Aspergillus* and *Penicillium* species and that this activity was attributed to the production of a hydroxyl fatty acid, 2-hydroxy-4-methylpentatoic acid and PLA, in a synergy with other metabolites. Broberg et al. (2007) also detected high levels of two hydroxyl

fatty acids (3-hydroxydecanoic acid and 2-hydroxy-4-methylpentanoic acid) in silages inoculated with *L. plantarum* strains that exerted antifungal activity in combination with other antifungal compounds. Also, Brosnan et al. (2012) detected six fatty acids, including 3-hydroxydecanoic acid and DL-β-hydroxymyristic acid, in the supernatant of a LAB strain with antifungal effect. Ryan et al. (2011) concluded that sodium decanoate was the most active compound produced by *L. amylovorus* against *A. fumigatus* with a MIC of 0.05 mg/mL. Furthermore, Kishimoto et al. (2003) documented the transformation of linoleic acid into hydroxyl fatty acids by several strains of LAB. Later, Black et al. (2013) studied the conversion of linoleic acid into fatty acids with antifungal properties. *Lactobacillus hammesi* DMS 16381 was able to produce coriolic (13-hydroxy-9,11-octadecadienoic) acid, and ricinoleic (12-hydroxy-9-octadecenoic) acid, both demonstrating antifungal activity against *A. niger*, with a MIC of 0.7 mg/mL and 2400 mg/L, respectively. The use of sourdough fermented with *L. hammesi* DMS 16381 was able to delay the growth of *A. niger* and *P. roqueforti* and increase bread shelf life for up to 3 days. It was also observed that the fatty acids structure is an important factor for antifungal activity with at least one double bond and one hydroxyl group along a C_{18} aliphatic chain being needed for antifungal activity.

In general, hydroxylated fatty acids have a strong antifungal activity against a large number of moulds with their MIC ranging from 0.01 to 0.1 mg/mL, which are similar with common antifungal drugs (such as amphotericin B) (Sjögren et al. 2003). Fatty acids production kinetics follows bacterial growth, suggesting that these compounds do not result from cell lysis (Sjögren et al. 2003).

Still, the metabolic role of hydroxylated fatty acids is not well defined and there is limited information about the mode of action of these acids. One of the proposed mechanisms states that antifungal fatty acids separate the lipid bilayers of fungal membranes compromising membrane integrity, resulting in an uncontrolled release of intracellular electrolytes and proteins, eventually leading to cytoplasmatic disintegration of fungal cells (Avis and Bélanger 2001).

2.3.3.5 Hydrogen peroxide

Most LAB are able to produce hydrogen peroxide (H_2O_2) in the presence of oxygen, by oxidizing lactate (Kandler 1983). The production of hydrogen peroxide results in a inhibitory

effect, since LAB do not produce catalase, hydrogen peroxide accumulates in the environment, and oxidizes the lipid membrane and cellular proteins of target organisms (Condon 1987).

In some foods, the effect of hydrogen peroxide at non-inhibitory concentrations is potentiated by lactoperoxidase and thiocyanate, both present in milk and saliva (Condon 1987). Lactoperoxidase catalyses the oxidation of thiocyanate using hydrogen peroxide as a substrate, which results in the formation of highly reactive oxidizing agents (Seifu et al. 2005). These reaction products oxidise the sulfhydryl groups in enzymes and proteins, damaging the cytoplasmic membranes of target microorganism, which results in the eventual death of the cells (Kussendrager and van Hooijdonk 2000). Venturini et al. (2002) determined the antimicrobial activity of hydrogen peroxide against *P. expansum* and assessed a minimum lethal concentration of 0.025% of hydrogen peroxide, when evaluating inhibition of mycelia growth in liquid broth. In solid medium, 5% of hydrogen peroxide was needed to completely inhibit the growth of *P. expansum*. In other work, Ponts et al. (2006) found that hydrogen peroxide may affect spore germination of *F. graminearum*. Furthermore, the incubation of spores inoculated with a 0.5 mM hydrogen peroxide solution resulted in a reduction of trichothecenes accumulation (Gil-ad and Mayer 1999).

2.3.3.6 Other compounds

Several other antifungal compounds have been isolated from LAB supernatants. Ryan et al. (2011) described the isolation of two nucleosides (cytidine and deoxytidine) from the culture filtrate of *L. amylovorus* DMS 19280. These compounds were identified among many other antifungal compounds. Their MIC values against *A. fumigatus* were above 200 mg/mL, making these compounds the less active between the other detected antifungal compounds.

Niku-Paavola et al. (1999) first reported lactones as antifungal compounds produced by LAB. Mevanolactone, produced by *L. plantarum* VTT E-78076, caused approximately 15% inhibition in *F. avenaceum* growth and showed a synergistic effect with lactic acid. Also, Yang et al. (2011) reported the production of δ-dodecalactone by *L. plantarum* AF1, which demonstrated antifungal activity against *Aspergillus* spp. and *P. roqueforti* with MIC values ranging from 350 to 6250 μg/mL Mandal et al. (2007) reported the involvement of a phenolic compound produced by *Pediococcus acidilactici*, that showed antifungal activity against several food and feed moulds.

However, this compound remains unidentified. Another antifungal compound, 3,6-bis(2-methylpropyl)-2,5-piperazinedion, was identified in *L. plantarum* AF1 (isolated from kimchi) supernatant. The study concluded that fungal inhibition was caused by this and other compounds in addition to organic acids (Yang and Chang 2010).

Aunsbjerg et al. (2015) studied the role of volatile compounds in the antifungal ability of *L. paracasei* DGCC 2132 in defined medium and yogurt. They identified diacetyl as the main volatile produced by the *L. paracasei* strain and reported its antifungal activity against *Penicillium* spp. While studying the composition of the exametabolome of three *L. paracasei* strains, Honoré et al. (2016) reported for the first time the production of indolelactic acid, 2-hydroxy-(4-methylthio)butanoic acid, and 2-hydroxy-3-methylbutanoic acid by *L. paracasei* and their inhibitory activity against *Penicillium* strains.

Chemical structures of several antifungal compounds produced by LAB are shown in Figure 2.12 and Table 2.4 summarizes LAB strains, fungal strains inhibited by those LAB, inhibitory compounds produced and application in food products.

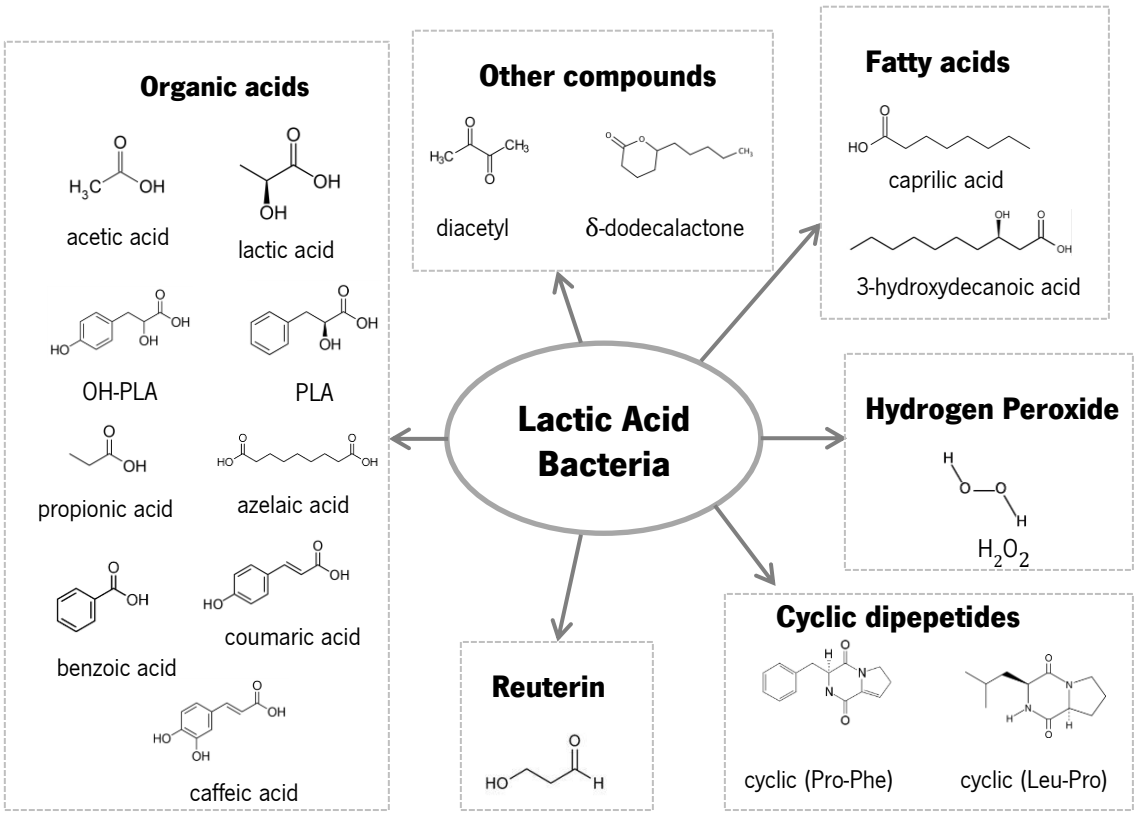


Figure 2.12. Chemical structure of several antifungal compounds produced by LAB (adapted from Crowley et al. (2013b)).

Table 2.4. Summary of LAB strains, their source, fungal activity, produced inhibitory compounds and food application

LAB strain	Source	Activity against	Inhibitory Compounds	Application	Reference
L. casei subsp. *pseudoplantarum 371*	Silage	*A. flavus parasiticus*	Possible proteinaceous	Rice	Gourama and Bullerman (1995a)
L. sanfrancisco CB1	Sourdough	*Penicillium* spp., *Fusarium* spp., *Aspergillus* spp., and *Monilia* spp.	Caproic, acetic, formic, propionic, butyric, *n*-valeric acids	*In vitro*	Corsettii et al. (1998)
Lactobacillus pentosus TV35	Vaginal secretions	*Candida albicans*	Pentocin	*In vitro*	Okkers et al. (1999)
L. plantarum VTT E-78076	Beer	*Pantoea agglomerans* and *F. avenaceum*	Benzoic acid, methylhydantoin, mevalonolactone and cyclo(glycyl-l-leucyl)	*In vitro*	Niku-Paavola et al. (1999)
L. plantarum 21B	Sourdough	*Eurotium repens*, *Eurotium rubrum*,*Penicillium corylophilum*, *P. roqueforti*, *P. expansum*, *Endomyces fibuliger*, *A. niger* and *A. flavus*, *Monilia sitophila* and *F. graminearum*	PLA and OH-PLA	Bread	Lavermicocca et al. (2000)
L. coryniformis subsp. coryniformis Si3	Grass silage	*A. fumigatus*, *Aspergillus nidulans*, *P. roqueforti*, *Mucor hiemalis*, *Talaromyces flavus*, *F. poae*, *F. graminearum*, *F. culmorum* and *Fusarium sporotrichoides*	Low molecular weight proteinaceous compound	*In vitro*	Magnusson and Schnürer (2001)
L. plantarum MiLAB 393	Grass silage	*F. sporotrichioides*, *A. fumigatus*, *Kluyveromyces marxianus* and *P. roqueforti*	Cyclo(L-Phe-L-Pro), Cyclo(L-Phe-trans-4-OH-L-Pro) and 3-PLA	*In vitro*	Ström et al. (2002)
L. plantarum MiLAB 14	Lilac flowers	*P. roqueforti*, *P. commune*, *A. nidulans*, *A. fumigatus*, *K. marxianus*, *Rhodotorula mucilaginosa* and *P. anomala*	3-(R)-hydroxydecanoic acid, 3-hydroxy-5-*cis*-dodecanoic acid, 3-(R)-hydroxydodecanoic acid and 3-(R)-hydroxytetradecanoic acid	*In vitro*	Sjögren et al. (2003)
L. coryniformis Si3	Grass	*A. fumigatus*, *A. nidulans*, *P. commune*, *F. sporotrichioides*, and *Rhodotorula mucilaginos*	Lactic, acetic and phenyllactic acids, cyclo(Phe-Pro), cyclo(Phe-4-OH-Pro) and reuterin	*In vitro*	Magnusson et al. (2003)

LAB strain	Source	Activity against	Inhibitory Compounds	Application	Reference
L. plantarum MiLAB 14 and *L. plantarum* MiLAB 393	Lilac flowers and grass silage	*P. anomala*, *P. roqueforti*, and *A. fumigatus*	3-hydroxydecanoic acid, 2-hydroxy-5methylpentanoic acid, benzoic acid, catechol, hydrocinnamic acid, salicylic acid, 3-PLA, 4-hydroxybenzoic acid, (trans, trans)-3,4-dihydroxycyclohexane-1-carboxylic acid, *p*-hydrocouramic acid, vanillic acid, azealic acid, hydroferulic acid, p-coumaric acid, hydrocaffeic acid, ferulic acid and caffeic acid	*In vitro*	Broberg et al. (2007)
Pediococcus pentosaceus R47	Malted cereals	*P. expansum*	Possibly antifungal peptides	Apple	Rouse et al. (2007)
L. plantarum FST 1.7	Malted barley	*F. culmorum* and *F. graminearum*	Lactic acid, PLA and the two cyclic dipeptides cyclo(L-Leu-L-Pro) and cyclo(L-Phe-L-Pro)	Wheat bread	Dal Bello et al. (2007)
P. pentosaceus, *W. paramessenteroides*, *L. plantarum* and *Lactobacillus paracollinoides*	Fresh vegetables	*A. flavus, F. graminearum*, *Rhizopus stolonifer* and *Botryris cinerea*	Non identified	Fresh vegetables	Sathe et al. (2007)
L. brevis AM7	Sourdough	*P. roqueforti, Penicillium polonicum, Penicillium chrysogenum, Penicillium paneum, Penicillium bialowiezense, Penicillium brevicompactum, Penicillium albocoremium, Penicillium chermesinum, Penicillium carneum, Eurotium herbarioum, E. rubrum, A. parasiticus* and *A. versicolor*	Possible proteinaceous	Bread	Coda et al. (2008)
L. plantarum CRL 778, *L. reuteri* CRL 1100, and *L. brevis* CRL 772 and CRL 796	Sourdough	*Aspergillus*, *Fusarium*, and *Penicillium*	Acetic acid and PLA	Bread	Gerez et al. (2009)
L. plantarum	Grass silage	*A. niger*, *A. fumigatus*, *A. flavus*, *Aspergillus terreus*, *P. roqueforti*, *P. camembertii*, *P. corylophilum*, *A. nidulans*, and *P. expansum*	3-PLA	*In vitro*	Prema et al. (2010)

LAB strain	Source	Activity against	Inhibitory Compounds	Application	Reference
L. plantarum LB1, *Lactobacillus rossiae* LB5	Raw wheat germ	*P. roqueforti, P. polonicum, P. chrysogenum, P. paneum, P. albocoremium, P. chermesinum, P. carneum, E. herbarioum, E. rubrum, A. parasiticus, A. versicolor and P. bialowiezense*	PLA, formic acid and small peptides. Synergy from organic acids mixture (oxalic, lactic, acetic, citric, phenyllactic and valeric acids)	Bread	Rizzello et al. (2011)
L. plantarum PCS 20 and *Leuconostoc pseudomesenteroides* PCK 18	Different food matrices	*P. nordium*	Lactic, acetic acid and non identified compounds	*In vitro*	Schillinger and Villarreal (2010)
L. plantarum AF1	Kimchi	*A. flavus*	Cyclo(Leu–Leu), δ-dodecalactone	Soybeans	Yang and Chang (2010)
L. plantarum VE56, *W. paramesenteroides* LC11	Fermented cassava	*A. niger, C. albicans, Aspergillus tubingensis, Penicillium crustosum*	PLA, 2-hydroxy-4 methylpentanoic acid	*In vitro*	Ndagano et al. (2011)
L. amylovorus DSM 19280	Cereal environment	*A. fumigatus, F. culmorum, A. niger, P. expansum, P. roqueforti*	Lactic acid, acetic acid, salicylic acid, d-glucuronic acid, cytidine, 2′-deoxycytidine, sodium decanoate, p-coumaric acid, 3-phenylpropanoic acid, (E)-2-methylcinnamic acid, 3-PLA, 3-(4 hydroxyphenyl)lactic acid, cyclo(L-Pro-L-Pro), cyclo(L-Leu-L-Pro), cyclo(L-Try-L-Pro), cyclo(L-Met-L-Pro) and cyclo(L-His-L-Pro)	Bread	Ryan et al. (2011)
L. casei AST18	Dairy products	*Penicillum* sp.	Lactic acid, cyclo-(Leu-Pro), 2,6-diphenyl-piperidine, and 5,10-diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo[1,2-a;1′,2′-d]pyrazine.	*In vitro*	Li et al. (2012)
L. hammesii DSM 16381	French wheat sourdough	*A. niger*, *Mucor plumbeus* and *P. roqueforti*	Mono-hydroxy C18 fatty acid	Sourdough	Black et al. (2013)
Lactobacillus sakei KTU05-6, *P. acidilactici* KTU05-7, *P. pentosaceus* KTU05-8, KTU05-9 *and* KTU05-10	Rye sourdough	*F. culmorum*, *P. chrysogenum*, *A. fumigatus*, *A. versicolor*, *P. expansum*, *A. niger*, *Debaryomyces hansenii* and *Candida parapsilosis*	Organic acids and bacteriocins-like inhibitory substances	Bread	Cizeikiene et al. (2013)

LAB strain	Source	Activity against	Inhibitory Compounds	Application	Reference
L. fermentum CRL 251	Non-identified	*A. niger*, *F. graminearum* and *Penicillium digitatum*	Lacic, acetic and phenyllactic acids, small peptides	*In vitro*	Gerez et al. (2013)
L. plantarum K35	Fermented rice noodle	*A. flavus* and *A. parasiticus*	Lactic acid, 1H-Indene,2-butyl-4-hexyloctahydro, PLA, 2,3-Di-tert-butylphenol, pyroglutamic acid, 5-methyl-l-phenylbicyclo, palmitic acis, stearic acid, oleic acid, linoleic acid	*In vitro*	Sangmanee et al. (2014)
L. amylovorus DSM19280 and *L. reuteri* R29	Cereal and human	*F. culmorum*	Acetic, lactic, hydroxybenzoic, phenyllactic, OH-phenylactic, benzoic, caffeic, catechol, coumaric, ferullic, hydrocaffeic, hydrocinnamic, hydroferellic, phloretic and vanilic acids	Malting process	Oliveira et al. (2015b)
L. plantarum UFG 108 and UFG 121	Different food matrices	*A. niger*, *A. flavus*, *F. culmorum*, *P. roqueforti*, *P. expansum*, *P. chrysogenum*, and *Cladosporium* spp.	Lactic acid and PLA	Oat beverages	Russo et al. (2017)
L. plantarum UM55	Milk	*A. flavus*	Lactic, phenyllactic, hydroxyphenyllactic and indole lactic acids	*In vitro*	Guimarães et al. (2018b)
L. plantarum UM55 and *L. buchneri* UTAD104	Milk and silage	*P. nordicum*	Lactic,acetic, phenyllactic, hydroxyphenyllactic and indole lactic acids	*In vitro*	Guimarães et al. (2018c)

2.3.4 Inhibition of Mycotoxin Biosynthesis by Lactic Acid Bacteria

Despite the wide diversity of antifungal metabolites that are produced by LAB, the influence of these compounds in the production of mycotoxins is still underexploited. Some works have reported the reduction of accumulation of mycotoxins when filamentous fungi were in the presence of LAB or LAB metabolites. The production of mycotoxins does not necessarily depend on fungal growth; in fact, fungi can present a normal radial growth and with an impaired or reduced mycotoxin production (Gourama and Bullerman 1995b). Examples of works where the production of mycotoxins was inhibited by LAB or their metabolites are reported in the following sections.

2.3.4.1 Inhibition of aflatoxin production

Most of the data concerning the effects of LAB in the production of mycotoxins are related to aflatoxigenic moulds, and the effect of LAB in the inhibition of the production of aflatoxins has been reported in several studies. Wiseman and Marth (1981) found a inhibitory relationship between *L. lactis* and *A. parasiticus*., by observing that when fungal spores were added to a 3-day culture of *L. lactis*, the production of aflatoxins was reduced. However, when spores and *L. lactis* were incubated at the same time, aflatoxin production increased. Contrary to these findings, Coallier-Ascah and Idziak (1985), reported an inhibition in aflatoxin production when spore solution of *A. flavus* and *L. lactis* were simultaneously inoculated, however the growth of the fungus was not hindered. They concluded that suppression of mycotoxin production was not a result of drop in pH or reduction of available nutrients, but was related to the production of a low-molecular weight metabolite in the beginning of *L. lactis* exponential growth phase. Also, Karunaratne et al. (1990) described the inhibition of aflatoxin by three species of *Lactobacilli* (*L. acidophilus, Lactobacillus bulgaricus and L. plantarum*). LAB cells were effective in reducing *A. parasiticus* NRRL 2999, while LAB supernatants reduced the amount of aflatoxins produced; though fungal growth was not affected.

Gourama and Bullerman (1995b) used a mixture of *Lactobacillus* species, isolated from silage, to reduce mould growth and aflatoxin production by *A. parasiticus*. Viable cells of *Lactobacillus* spp. totally inhibited germination of mould spores. When the authors tested the cell free supernatants, they also

inhibited fungal growth but did not reduce mould spore viability. When grown in a dialysis sacks, *Lactobacillus* spp. also inhibited the production of mycotoxins by *A. flavus* growing outside the sack, but did not inhibited fungal growth. It was concluded that LAB produce molecules that potentially inhibit production of aflatoxins. In a following study, Gourama and Bullerman (1997) determined that the inhibition of mould growth and aflatoxin (B1 and G1) by *L. casei pseudoplantarum* was probably caused by proteinaceous compounds produced by LAB. In another study, Onilude et al. (2005) reported the inhibition of aflatoxin production by several species of *Lactobacillus*. Also, Petchkongkaew et al. (2008) reported that *Bacillus* species, isolated from soybeans, inhibit aflatoxin B1 production up to 85%. Gerbaldo et al. (2012) tested the ability of *L. fermentum* and *Lactobacillus rhamnosus* against several strains of *Aspergillus* section *Flavi*. Fungal growth rate was inhibited from 77 to 96%, by *L. rhamnosus* and 36 to 50% by *L. fermentum*, when compared with the control, and aflatoxin production was diminished by the reduction of fungal growth, due to the lower mycelial biomass formation. Decrease in aflatoxin accumulation varied between 73 and 99%, with *L. rhamnosus* strains being more effective in the control of aflatoxin production. Sangmanee et al. (2014) reported that *L. plantarum* K35 supernatant caused significant reduction of *A. flavus* and *A. parasiticus* growth and aflatoxin production. The LAB supernatant caused damage to the fungal cell wall and cytoplasmatic membrane, leading to the leaking of cytoplasmatic content and destruction of membranous organelles. Recently, Kaktcham et al. (2018) reported that a *L. plantarum* strain isolated from food exhibited a broad antifungal activity and ability to inhibit aflatoxin production. Moreover, this strain showed the ability to prevent aflatoxigenic mould growth in tomato paste, without altering its organoleptic properties.

2.3.4.2 Inhibition of ochratoxin A production

Only a few studies have dealt with the LAB efficiency in the control of OTA production. Studies in this area are important since OTA can occur in many foods and feed products and the isolation of LAB strains and their metabolites that can reduce OTA production would be important to improve OTA control.

Still, some studies on antifungal LAB against ochratoxigenic fungi are available. Kapetanakou et al. (2012) developed a work in which a mixture of six LAB strains were tested as antagonists to *A. carbonarius*. Both growth and OTA production in culture medium and beverages were not reduced. Contrary, Gerez et al. (2014) tested *L. plantarum* CRL 778 as a biopreservative for packed bread.

Inhibitory activity of *L. plantarum* against *A. niger* resulted in a reduction of 60% of OTA content in bread inoculated with this strain. Belkacem-Hanfi et al. (2014) tested 54 LAB isolated from wheat against 8 moulds species isolated from durum wheat. Three of those species (*L. plantarum, Lactobacillus graminis and P. pentosaceus*) demonstrated a high inhibitory effect against an *A. carbonarius* strain and OTA production on liquid medium. OTA content was reduced by over 97% by *L. plantarum* and *L. graminis* and by 82% by *P. pentosaceus*. Authors also concluded that production of OTA is affected by factors such as temperature, pH and bacterial biomass. In fact, it was observed that OTA reduction by these LAB was due to pH variation on media, which might be a result of fermentative compounds accumulation, such as organic acids. It was also noted that highest bacterial counts did not show highest OTA reduction, which could be in part explained by reversible antifungal mechanisms that did not depend of cell viability. These mechanisms include mycotoxin binding to peptidoglycans in the cell wall or to surface proteins.

2.3.4.3 Inhibition of fumonisin production

There are also few works that described the ability of LAB to directly affect the production of fumonisins. Dalié et al. (2012) tested, *in vitro,* the capacity of a *P. pentosaceus* strain and its metabolites to modulate fumonisin biosynthesis by *F. verticillioides*. Authors found that when *F. verticillioides* and *P. pentosaceus* were co-inoculated in a glucose-yeast extract-peptone (GYEP) medium supplemented with amylopectin the production of fumonisins increased slightly. The same occur when the fungus was inoculated in a three-day-old culture of *P. pentosaceus*. However, extracellular metabolites (not specified) produced by *P. pentosaceus* in Mann-Rogosa-Sharpe (MRS) broth were able to reduce fumonisin significantly both in liquid medium and in maize kernels. In maize kernels, fumonisin levels were reduced by approximately 80% after 20 days of incubation. This work also showed a potential risk of the use of antagonistic bacteria in fumonisin contamination and hypothesized that under certain conditions, the presence of bacteria could be perceived as a stressful signal, inducing mycotoxin production.

A study by Deepthi et al. (2016) determined the antifungal activity of a strain of *L. plantarum* against *F. proliferatum.* This LAB strain demonstrated a probiotic potential and showed inhibitory activity on conidia germination and biomass growth, with its activity resulting also in alteration of fungal structures. Additionally, *L. plantarum* supernatants were able to inhibit fungal development in maize

kernels. The major antifungal compounds produced by this isolate were 10-octadecenoic acid, methyl ester, palmitic acid, heptadecanoic acid, stearic acid and lauric acid. Both *L. plantarum* and its supernatant reduced fumonisin content in a feed model up to 0.505 and 0.313 mg/kg, respectively when control sample had 0.870 mg/kg of fumonisins.

2.3.4.4 Inhibition of patulin production

Antimicotoxigenic activity of LAB against the production of patulin has been reported in a work by Gourama (1997). The author tested LAB isolated from dairy products, vegetables and fruits, and selected two isolates with activity against *Penicillium citrinum* and *P. expansum*. The two antifungal LAB were identified as *L. casei,* and their supernatants were able to inhibit the production of patulin and citrinin. Compounds responsible for the inhibition were probably of proteinaceous nature. Similarly, Florianowicz (2001) reported the potential of three strains of *Lactobacillus* to inhibit *P. expansum*. The species, *L. casei*, *L. delbrueckii* ssp. *bulgaricus* and *L. lactis* ssp. *Cremoris,* were able to reduce *P. expansum* growth and patulin production, both on liquid and solid media. The inhibition was more pronounced when LAB were cultured with spores in comparison with mycelia themselves. This strains can have an additional an advantage in fungal control since the antagonistic microorganisms should act in the first stages of infection.

2.3.4.5 Inhibition of zearalenone production

Few studies to evaluate the inhibitory activity of LAB on the growth of ZEN producing fungi and consequent ZEN production have been reported in the literature. Sellamani et al. (2016) evaluated the efficiency of *Pediocuccus pentosaceus* (isolated from traditional dairy products in India) against *F. graminearum* and ZEN production. Analysis of the cell free supernatants (CFS) of *P. pentosaceus* indicated the presence of phenolic antioxidants, antimicrobial peptides and carboxylic acids. The MIC of *P. pentoseceus* supernatant was 66 µL/mL and caused alterations of the micromorphology of mycelia of *F. graminearum*. Supernatants were also effective in reducing ZEN production. The effect of supernatant both on mycelial biomass and ZEN production was observed in a dose-dependent way. A qPCR analysis of ZEN metabolic pathway genes (PKS4 and PKS13) showed that there is a dose

dependent down-regulation of target gene expression when samples were in the presence of the *P. pentosaceus* supernatants.

2.3.4.6 Inhibition of trichothecenes production

The inhibition of *F. culmorum* and the reduction of DON production has been evaluated in malting processes in the presence of LAB supernatants (Oliveira et al. 2015b). *L. amylovorus* and *L. reuteri* supernatants were produced in a worth based substrate and used in the early stages of malting. *L. reiteri* supernatants effectively inhibited *Fusarium* growth by 23% and DON by 83%. Analysis of the supernatants showed that phenyllactic, lactic, acetic and benzoic acids played an essential role in the antifungal and antimycotoxigenic activity of the supernatants. This biocontrol method resulted in a higher extract yield. No significant changes in the quality of malt due to LAB inoculum, were detected and the pH decrease caused by the presence of organic acids stimulated the enzymatic activity during malting and fermentation (Oliveira et al. 2015b).

Hassan and Bullerman (2008) reported that *L. paracasei* subsp. *tolerans* (isolated from sourdough bread) inhibit the growth of *F. proliferatum* and *F. graminearum* in a liquid medium. When inoculated in a medium that supports *Fusarium* growth and trichothecenes production, this LAB strain was found to reduce fungal growth but increase mycotoxin production. Antifungal activity seems to be related to the production of organic acids. Mycotoxin increased production might be caused by stress due to exposure to LAB strain, as it was also reported by Dalié et al. (2012).

2.3.5 Detoxification by Binding of Mycotoxins to Lactic Acid Bacteria

Mycotoxin reduction can also be accomplished by using additives that adsorb this toxins. This process should inactivate mycotoxins without creating other toxic products and while maintaining the nutritional value of the food product. LAB can be used as mycotoxin binding agents, thus reducing their bio-availability, and consequently reducing toxin uptake and subsequent distribution in blood and target organs. It is important that the mycotoxin-LAB complex is stable throughout the digestive track, so that mycotoxins can be eliminated (Jard et al. 2011). For a microorganism to be used in the detoxification of mycotoxins it must follow certain requirements: (i) derived products resultant from mycotoxin

decontamination must be non-toxic; (ii) biotransformation should be fast and efficient under different conditions (especially pH and oxygen levels); (iii) microorganism must be non-pathogenic; and (iv) microorganism must be active in a complex environment, such as the digestive track (Jard et al. 2011). Detoxification by binding to LAB cells of aflatoxins (El-Nezami et al. 1998; Haskard et al. 2000; Hernandez-Mendoza et al. 2009) OTA (Turbic et al. 2002; Fuchs et al. 2008; Kabak et al. 2009), ZEN (El-Nezami et al. 2002; El-Nezami et al. 2004; Sangsila et al. 2016) and fumonisins (Niderkorn et al. 2006; Niderkorn et al. 2007; Pizzolitto et al. 2012) by LAB has been reported in several works.

El-Nezami et al. (1998) used two *L. rhamnosus* strains to remove AFB1 from contaminated liquid media. Approximately 80% of aflatoxin was removed within 24 hours. Hernandez-Mendoza et al. (2009) conducted a similar experiment using 8 strains of *L. casei* to bind AFB1, and studying the effect of bile salts in binding ability concluding that the exposure to bile increased aflatoxin binding. Haskard et al. (2000) discovered that hydrophobic interactions play a major role in AFB1 binding to *L. rhamnosus* strain GG, and that binding occurs predominantly to the carbohydrate components of bacteria. Aflatoxin binding is a reversible reaction and seems to be dependent of strains, matrix, temperature, pH, ionic strength and incubation time, thus specific conditions and strains are needed for each particular application. LAB potential to bind aflatoxins has been recently reviewed elsewhere (Ahlberg et al. 2015).

There are also several studies describing the detoxification of OTA by lactic acid bacteria. Piotrowska et al. (2005) studied the effect of several *Lactobacillus* species, showing that *L. acidophilus*, *L. rhamnosus* GG and *L. plantarum* are capable of reducing OTA by 50% from liquid media, and that this process is partially reversible. In a study carried out by Fuchs et al. (2008), it was reported a decrease in OTA of more than 95% when *L. acidophilus* was added to the contaminated medium. Following experiments determined that binding of OTA was dependent of toxin concentration, cell density, cell viability, and pH. Also Turbic et al. (2002) reported that *L. rhamnosus* (viable and non-viable) was able to remove OTA by 36 to 76%, without affecting significantly the presence of other aromatic dietary substances (caffeine, vitamin B12 and folic acid). A reduction of 73% of OTA was reported when probiotic bacteria were used under *in vitro* gastrointestinal conditions (Kabak et al. 2009).

Niderkorn et al. (2006) was the first to propose the use of LAB to remove fumonisins from a liquid medium. Fumonisin removal reached 82% and 100%, for FB1 and FB2, respectively. The authors concluded that binding ability of LAB seems to be the mechanism involved in the removal, since most common degradation products (aminopentols) were not detected and removal levels were not reduced

when non-viable bacteria were used. For fumonisins, it was also reported, that the pH of the medium may have influence on the binding process, with binding decreasing to almost 0% at neutral pH. In another study, Niderkorn et al. (2007) tested approximately 200 strains of fermentative bacteria to detoxify corn silage contaminated with *Fusarium* toxins and reported binding of 24 and 62%, for FB1 and FB2, respectively. Other study by Pizzolitto et al. (2012) demonstrated that *L. acidophilus* was able to remove FB1 and the amount of fumonisin removed was dependent of toxin and bacteria concentration. Cell viability was not a condition for the removal, however cell wall integrity was required. Physical adsorption was the mechanism proposed for the removal of fumonisin.

Detoxification of ZEN and its derivative α-zearalenol (α-ZOL) was attempted, by El-Nezami et al. (2002) using two strains of *L. rhamnosus*. A substantial part of both mycotoxins (38 to 46%) was recovered with the bacterial pellet, with no degradation products being detected in the supernatants or when pellets were extracted with methanol. ZEN and α-ZOL were completely recovered from bacterial cells and culture medium. Both viable and non-viable bacteria were able to remove the toxins, indicating that no metabolic mechanism is involved. The simultaneous incubation of both ZEN and α-ZOL affected the individual binding of the toxins, indicating that they may share the same binding sites on the cell surface. In a subsequent study, El-Nezami et al. (2004) suggested that ZEN binds mainly to carbohydrate components of the cell wall, and that hydrophobic interactions are important in this binding. Also, Mokoena et al. (2005) documented a reduction of 68 to 75% of ZEN in spiked maize meal, after 4 days of incubation with LAB strains. More recently, several strains of *Lactobacillus pentosus* were reported to remove 83% of ZEN when compared with control. The results showed that the detoxification ability of LAB was strain and toxin dependent (Sangsila et al. 2016).

2.3.6 Application of Antifungal LAB as Biocontrol Agents in Food Systems

The use of LAB as biocontrol method to prevent or delay fungal contamination has been described for multiple food systems, including fruits, vegetables, bakery products, and dairy products among others. Testing *in situ* is important to validate LAB application against fungal spoilage, as well as their safety and organoleptic implications.

2.3.6.1 Fresh fruit and vegetables

Fresh fruits and vegetables are products that can highly benefit from LAB application in a biocontrol approach. These food products have high water availability which makes them susceptible for fungal contamination, and the periods of storage during transportation gives fungi the opportunity to proliferate. *Penicillium*, *Fusarium*, *Alternaria* and *Botrytis* species, among others, are major fungal spoilers of fruits and vegetables. Several author describe the use of LAB strains to delay the proliferation of spoilage fungi in fresh fruit and vegetables obtaining promising results.

Sathe et al. (2007) used a *L. plantarum* strain, isolated form vegetables, to inoculate (either cell suspension or CFS) fresh cucumber, resulting in the delay of *A. flavus*, *F. graminearum*, *R. stolonifer* and *B. cinerea* contamination. Rouse et al. (2008) used apples as a model to determine antagonistic ability of *P. pentosaceus* against *P. expansum*. No mould growth was detected after 14 days of storage. Another study tested the LAB isolates for the inhibition of *P. expansum* infection wounds of Golden Delicious apples, with a *W. cibaria* strain decreasing infection by 50%. Antifungal activity was attributed to organic acid and hydrogen peroxide production by LAB (Mansilla et al. 2008). Biocontrol of *P. expansum* by LAB isolated from food sources (*P. pentosaceus*) was also described for application in pear, plum and grape models with good results (Crowley et al. 2013a). Grapes were also used as a test model by Lan et al. (2012) that demonstrated that *W. cibaria* inhibited *Penicillum oxalicum* from the surface of the grapes for up to 6 days of storage (fungi growth was noticeable after 2 days in control samples). Other fruit application involved the genome shuffling (by UV radiation and diethyl sulphate mutagenesis and protoplast fusion with *Lactobacillus* IMAU40097) of *L. plantarum* IMAU10014 to improve its antifungal activity. This methodology improved antifungal ability against several fungi up to 200%, compared with the wild-type strain. The modified *L. plantarum* culture (strain F3A3) was sprayed on the surface of kumquats that were infected with *P. digitatum*. Spoilage tests indicated an excellent ability to prevent the fungal spoilage, adding 5 days to shelf life of kumquats. Antifungal activity of strain F3A3 seemed to be a combination of several metabolites produced by this strain (Wang et al. 2013). *L. lactic* subsp. *lactis* was effective in the control of *R. stolonifer* in jackfruit, either reducing or delaying the disease incidence (Ghosh et al. 2015). LAB strains (non-identified) were used to treat wounded apples exposed to *P. expansum*, resulting in reduced infection when compared with the controls (Matei et al. 2015). Lv et al. (2018) studied the application *of L. plantarum* C10 CFS on muskmelon fruit against pink rot caused by *Trichothecium roseum.* The CFS reduced the contamination zone in muskmelon by

enhancing enzyme activity (namely phenylalanine ammonialyase, peroxidase and polyphenoloxidase) and by promoting the production of phenolics and flavonoids.

The previous reports indicate that LAB or their metabolites can be used to delay or prevent fungal growth in fresh fruits and vegetables. However, only a limited number of studies are available on this topic. More studies are needed to elucidate antifungal mechanisms *in situ*, application methods, determine safety and quality issues and access consumers' acceptance of products with biocontrol agents.

2.3.6.2 Bakery products

Bakery products are frequently spoiled by fungal contamination, leading to waste and eventually health problems. For this reason, several authors developed biocontrol methods with suitable LAB strains for bakery products. The use of antifungal LAB strains as sourdough starters have been extensively investigated with the majority of reports highlighting *L. plantarum* isolates as efficient in increasing shelf life and the quality of the products (Crowley et al. 2013b). Since active compounds produced by LAB are released *in situ*, the incorporation of these bacteria in sourdough can replace chemical preservatives and have additional positive effects such as improved flavour, texture and nutritional properties (Pawlowska et al. 2012). Lavermicocca et al. (2000) conducted one of the earliest studies of application of a *L. plantarum* antifungal strain as a sourdough starter. The use of *L. plantarum* 21B in co-fermentation with *S. cerevisiae* was effective in delaying *A. niger* contamination for over seven days of storage. Another *L. plantarum* strain (FST1.7) that produced antifungal compounds, was used in sourdough and resulted in a good quality bread with the ability to retard the growth of *F. culmorum* and *F. graminearum* (two fungi commonly found in bread) (Dal Bello et al. 2007). Ryan et al. (2008) also used *L. plantarum* FST1.7 in the fermentation of sourdough and verified that the use of this sourdough affected negatively the growth of *A. niger*, *F. culmorum* and *P. expansum* in bread. A strong synergistic effect was found when combining this antifungal sourdough with calcium propionate, allowing the reduction of this preservative in bread. In another work, *L. plantarum* CRL778 was used to inhibit *Penicillium* sp.. The use of this strain was as effective as 0.2% calcium propionate and increased shelf life for 5 days. When combining *L. plantarum* SL778 with 0.4% of calcium propionate, shelf life of bread was extended to 24 days (2.6 fold longer than bread with only 0.4% calcium propionate) (Gerez et al. 2010). In a follow up study, this strain was inoculated into a mixture of

water, wheat and quinoa flour, resulting in the inhibition of growth of *A. niger* and OTA in the bread slices by 60%, independently of the a_w (Gerez et al. 2014). Elsanhoty et al. (2013) tested the antimycotoxigenic potential of *L. rhamnosus* TISTR541 (combined with a bakery yeast) by inoculating wheat grains with *A. flavus*. Resulting bread had lower levels of aflatoxin, than breads fermented only with yeast. DON content was also reduced (by approximately 60%) in sourdough fermentation using a commercial starter mix (*L. plantarum* and *L. brevis*) in artificially contaminated wheat flour with *F. graminearum* (Banu et al. 2014).

The combination of *L. plantarum* with *Wickerhamomyces anomalus* (a yeast commonly found in artisan bakery sourdoughs) as starter cultures resulted in a bread with extended shelf life (28 days). Sensory analysis of this bread was positive for elasticity, colour and overall taste (Coda et al. 2011). Ryan et al. (2011) fermented wheat dough with *L. amylovorus* DSM19280, obtaining good quality breads (by rheofermentometer and texture analysis measurements) that showed a retarded growth of *F. culmorum*, *A. niger*, *P. expansum*, *P. roqueforti* and other fungal species. Breads fermented with *L. amylovorus* had a longer shelf life than breads with 0.3% of calcium propionate. Garofalo et al. (2012) identified two strains, *L. rossiae* (LD108) and *Lactobacillus paralimentarius* (PB127), with antifungal properties against *Aspergillus japonicus* and used them in sourdough for the production bread. Obtained bread had an increased shelf life (from 19 to 32 days, comparing with 11 days of the control bread loafs). Rizzello et al. (2011) used sourdough fermentation to stabilize wheat germ (a by-product of the milling process, rich in vitamins, proteins, fibres, fatty acids and minerals) and fermented it with a co-culture of *L. plantarum* LB1 and *L. rossiae* LB5. The resulting bread slices remained fungal free for 28 days.

Several other authors tested different species such as *L. buchneri,* (Zhang et al. 2010), *Leuconostoc citreum* (Choi et al. 2012), *L. acidipholus, L. mesenteroides* (Najafi et al. 2012), *P. acidilactici*, *P. pentosaceous* (Cizeikiene et al. 2013), *L. hammesii* (Black et al. 2013), *L. amylovorus,* (Axel et al. 2015), *Lactococcus* sp. (Varsha et al. 2014)*, L. fermentum* (Barman et al. 2017) and *L. paracasei* (Ouiddir et al. 2019) for their antifungal properties against numerous fungal strains, that can be present in bakery products. In general, antifungal effect can be obtained due to the pH drop associated with the production of organic acids, especially lactic and acetic acids. However, chemical acidification of sourdough does not result in fungal inhibition (Ryan et al. 2011; Axel et al. 2015). This suggests that other compounds (such as PLA, OH-PLA, benzoic acid, cyclic dipeptides, fatty

acids or peptides) produced by LAB contribute to this antifungal activity and that these compounds may act in synergy (Corsetti et al. 1998).

Overall the use of LAB has great potential for the improvement of quality and shelf life of bread.

2.3.6.3 Dairy products

Dairy products (mainly yogurt and cheese) are frequently susceptible to fungal contamination. LAB are used often as starter cultures in the manufacturing and as natural biopreservatives of these products. Yogurt has gain importance in consumers eating habits due to its high nutritional value and health benefits, with its consumption increasing over the last 30 years (Li et al. 2013). Currently there are several commercial antifungal bioprotective cultures for dairy products, such as HoldBac, FreshQ® and Befresh™ (Garnier et al. 2017a). Still, research for new LAB with antifungal properties and their metabolites raises interest. In a study developed by Delavenne et al. (2013), a strain of *Lactobacillus harbinensis* K.V9.3.1Np (isolated form milk) showed a very strong antifungal effect in yogurt, completely inhibiting several fungi commonly responsible for yogurt spoilage. The addition of *L. harbinensis* did not alter the growth or acidification rates of yogurt starter culture, nor affected the pH and acetic and lactic acid levels. Also, *L. paracasei* SYR90 and *L. rhamnosus* $BIO_{III}28$ were reported to retard the growth of *R. mucilaginosa* and *P. brevicompactum* in yogurt, during a period of 5 weeks at 10 °C (Lacanin et al. 2017). Another study determined that *L. casei* AST18 could be used as a bioprotective culture against *Penicillium* sp., since it completely inhibited mould growth in yogurt, at 4 °C. The addition of *L. casei* did not significantly influence the viscosity, sensory quality, pH and titratable acidity of the product (Li et al. 2013).

Cheeses are also vulnerable to spoilage by moulds. LAB species such as *L. plantarum*, *L. paracasei*, *L. casei*, *L. rhamnosus* and *L. brevis* are often found in cheese as non-starter cultures, that are capable of growing during cheese ripening and contribute to flavour development and additionally, can suppress the growth of undesirable moulds. Lynch et al. (2014) demonstrated that *L. amylovorus* DMS 19280 could be used as an adjunct culture in Cheddar cheese. When inoculated with *P. expansum*, cheeses with *L. amylovorus* present a four-day delay in the contamination, when compared with control. When exposed to airborne fungi, the delay in fungal growth increased for 6 days. Additionally, *L. amylovorus* did not have a negative impact on cheese characteristics. Cheong et al. (2014) isolated 897 LAB, 12 of each (all *L. plantarum* strains) demonstrated a strong antifungal

activity. LAB isolates were inoculated into cottage cheese, which was then contaminated with *P. commune.* All strains delay fungal growth by 14 to at least 25 days, when compared with controls. Salas et al. (2018) selected LAB isolates as adjunct cultures against common dairy pathogens (*P. commune*, *M. racemosus* and *R. mucilaginosa*). Most effective combinations were composed of *L. plantarum* L244 and *L. harbinensis* L172 or *L. rhamnosus* CIRM-BIA1113. First combination delay fungal contamination for 2-14 days on sour cream, depending on LAB inoculum. Both combinations delayed the growth of *P. commune* in semi-hard cheese for 1-6 days. Antifungal LAB at lower concentrations (10^6 CFU/mL) did not affect starter cultures, or pH of both products.

The use of LAB isolates can be considered as a suitable alternative to the use of preservatives such as sodium benzoate, sorbic acid and natamycin in yogurts and cheeses (Crowley et al. 2013b).

2.3.6.4 Other food products

Antifungal LAB have also been tested as antifungal agents in other food products. Muhialdin (2011) demonstrated that LAB (*L. fermentum* Te007, *P. pentosaceus* Te010, and *L. pentosus* G004) cells or their CFS inhibited conidia germination for 23-40 days at 4 °C and 5-6 days at 20 and 30 °C in tomato puree. Similarly, the use of a strain of *L. fermentum* (YML014) against *P. expansum* in tomato puree, extended its shelf life for 9 days at 4 °C and 12 days at 25 °C (Adedokun et al. 2016). Garcha et al. (2012) evaluated the effect of *L. acidophilus* in a raw poultry meat. This LAB strain inhibited the growth of *A. alternata,* leading to a decrease of 26.7% log units of the fungi*. L. lactis* ssp. *lactis* 194 and K-205 was effective in the inhibition of *E. repens* on smoked sausages, increasing the product's shelf life (Stoyanova et al. 2010). *L. citreum* C5 and *W. confuse* HO24 and D2-96 were also showed to retard fungal growth when used as starters in rice cakes (Baek et al. 2012).

Despite an extensive research on LAB as biopreservatives, there are still many challenges in their application in the food industry. Addition of LAB strain should not alter flavours or textures, and even though LAB have GRAS status, it is necessary to conduct rigorous safety assessments (e.g. biogenic amines production and antibiotic resistance patterns).

2.4 EDIBLE FILMS AND COATINGS FOR LACTIC ACID BACTERIA

Lactic acid bacteria can see their efficiency as antifungals in food products compromised by several factors; thus new strategies for the incorporation of LAB in food products need to be explored to increase their functionality. The use of edible films or coatings is one of these strategies, since they can reduce the diffusion of active compounds produced by LAB, therefore maintaining a suitable concentration on the surface of the food product, while also guaranteeing a higher stability of the microorganisms that are entrapped in the film or coating matrix. Additionally, when LAB have probiotic properties, they can be added to films and coatings, allowing them to reach the consumer´s gut in adequate amounts to confer health benefits.

In the last decades, there has been a growing interest in edible films and coatings. This interest has been driven by the consumer demand for high quality and healthy food, by environmental concerns, and by the need of new preservation techniques able to answer to new products requirements. These new preservation techniques, through edible films and coatings, can improve food quality and safety, while providing a semi-permeable barrier to water, vapour and gases between the food and the surrounding atmosphere (Donhowe and Fennema 1994). They also meet environmental concerns since they are usually produced with by-products from agricultural and marine sources, replacing food packaging made of non-renewable sources and consequently reducing waste products (Tharanathan 2003).

Although the terms films and coatings are sometimes used as synonyms, they represent different concepts. Films are usually defined as a thin layer of material that can be used as a cover or wrap, while coatings are directly formed on the surface of the product they are intended to protect (Gennadios 2002), as represented in Figure 2.13.

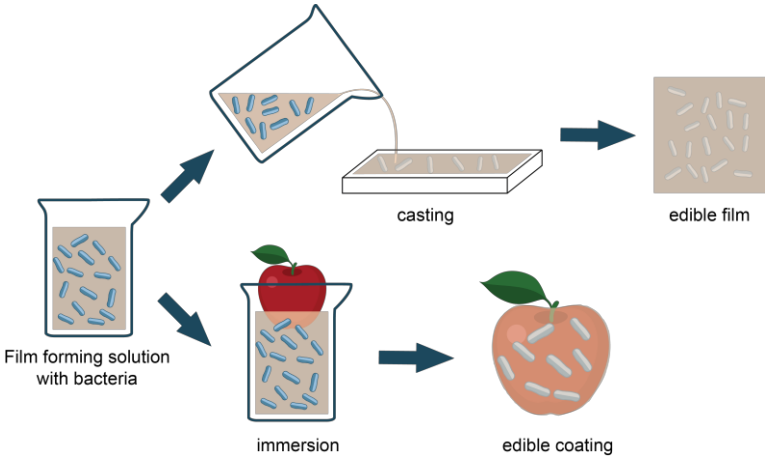


Figure 2.13. Production of bioactive edible films and edible coatings.

Due to the fact of being consumed altogether with the food product, edible films and coatings are simultaneously a packaging and a food component; therefore, they must meet some specifications. For example, they should have good sensory quality, good barrier and mechanical properties, good physicochemical and microbiologic stability, be organoleptically and functionally compatible with food, be non-toxic and safe for health, and have a low production cost (Debeaufort et al. 1998). Moreover, they should fit on the legislation requirements (Cerqueira et al. 2016). The EU considers that edible films and coatings are a special part of the food product, and for legislative point of view, have to fulfil the general requirements for food. In USA legislation, edible films and coatings are regarded as part of the food, and consequently their ingredients should comply with the Code of Federal Regulations (Campos et al. 2010).

The main materials used for the production of edible films and coatings are polysaccharides, proteins and lipids or a combination of these materials. Individually, each material can form films or coatings with specific properties, but the use of combinations of materials and the addition of plasticisers and surfactants are normally one of the approaches to enhance their final properties (i.e. flexibility, barrier and optical properties) (Campos et al. 2010). The selection of materials is based on their physical and chemical characteristics, which influence the final properties of films and coatings. Generally, it is desirable to have a film with good water-vapour barrier properties to retard surface dehydration, especially in fresh products; but also with good gas control, especially for oxygen, to reduce oxidation and rancidity. Hydrophobic substances or lipids are practical barriers against moisture transfer, whereas polysaccharides and proteins show poorer barrier properties against moisture transfer. Polysaccharides and proteins based films also have low permeability to gases and better

mechanical properties than lipid-based films. The appearance of the films is also dependent of the film forming material (Debeaufort et al. 1998). The optimisation of film or coating composition is crucial to their successful application since they must be formulated according to their purpose and the properties of the food product in which they are going to be applied.

Films and coatings have been successfully used in several food products, acting as a way to decrease the moisture loss and the gas transfer, and thus delay the ripening of food products, and thus increase the shelf life of the coated food product. Other possibility to add value to food products through films and coatings is by the incorporation of active ingredients. Addition of nutrients and minerals, antioxidants, antimicrobials, flavours, colorants and probiotics can improve food nutritional value, safety and product shelf life (Falguera et al. 2011). The incorporation of active ingredients in edible films and coatings has the advantage of protecting the additive from environmental conditions and from interactions with the food components, helping to maintain a suitable concentration of the active compound where it is needed (Quirós-Sauceda et al. 2014).

The use of antimicrobial packaging can have clear advantages over direct addition of microbial agents onto food. Efficiency of antimicrobial compounds can be compromised by several factors when added directly to the food product. Diffusion of antimicrobial agents to the bulk of the food, temperature, processing, alterations of pH, enzymatic degradation and inactivation by interaction with food ingredients may decrease antimicrobial activity during storage. The use of edible coatings or films can improve antimicrobial efficiency by reducing the diffusion of active compounds, thus maintaining a suitable concentration on the surface of the food product to be effective against foodborne pathogens, over extended periods of storage (Quintavalla and Vicini 2002; Gyawali and Ibrahim 2014). Incorporation of antimicrobial compounds would also be shield from alterations in the environment and could be important in preserving organoleptic properties of the food product (Campos et al. 2010).

The first author to incorporate living bacteria into edible coatings was Tapia et al. (2007), with the goal of conferring probiotic properties to fresh fruits. Authors reported that alginate- and gellan-based edible coatings were efficient in supporting probiotics on fresh-cut fruits, since *Bifidobacterium lactis* Bb-12 population remained viable during 10 days of storage at 2 °C, with values between 10^6 and 10^7 CFU/g. Following works by Altamirano-Fortoul et al. (2012), López de Lacey et al. (2012; 2014), Kanmani and Lim (2013), Romano et al. (2014), Tavera-Quiroz et al. (2015), Pereira et al. (2016) and Soukoulis et al. (2014b; 2014c; 2016; 2017) have also described the incorporation of probiotic LAB in edible films or coatings with good results.

A few works describe the incorporation of LAB into edible films or coatings in a biocontrol perspective. The inclusion of viable LAB can lead to the inhibition of pathogens through competition for space and nutrients, and/or through the production of antimicrobial substances. Incorporating cells into edible films and coatings may result in a high spread and concentration of viable cells on the food product surface and, consequently, in a high biopreservation effect. This fact can be due to the decrease in diffusion rate of the cells from the packaging to the product (Gialamas et al. 2010). Also, the use of films and coatings helps in binding the biocontrol agent to the food product, thus maintaining a suitable cell concentration to exert an antimicrobial effect. Despite the large number of publications on antimicrobial activity of edible films, only few works explored the antimicrobial activity of live bacteria cells when immobilized in edible films or coatings (Gialamas et al. 2010; Concha-Meyer et al. 2011; Sánchez-González et al. 2013; Sánchez-González et al. 2014; Pereira et al. 2018; Ye et al. 2018; Bambace et al. 2019; Settier-Ramírez et al. 2019). Figure 2.14 depicts a summary of benefits of incorporating living bacteria into edible films and coatings and Table 2.5 summarizes works where LAB were incorporated into edible films and coatings for an antimicrobial effect.

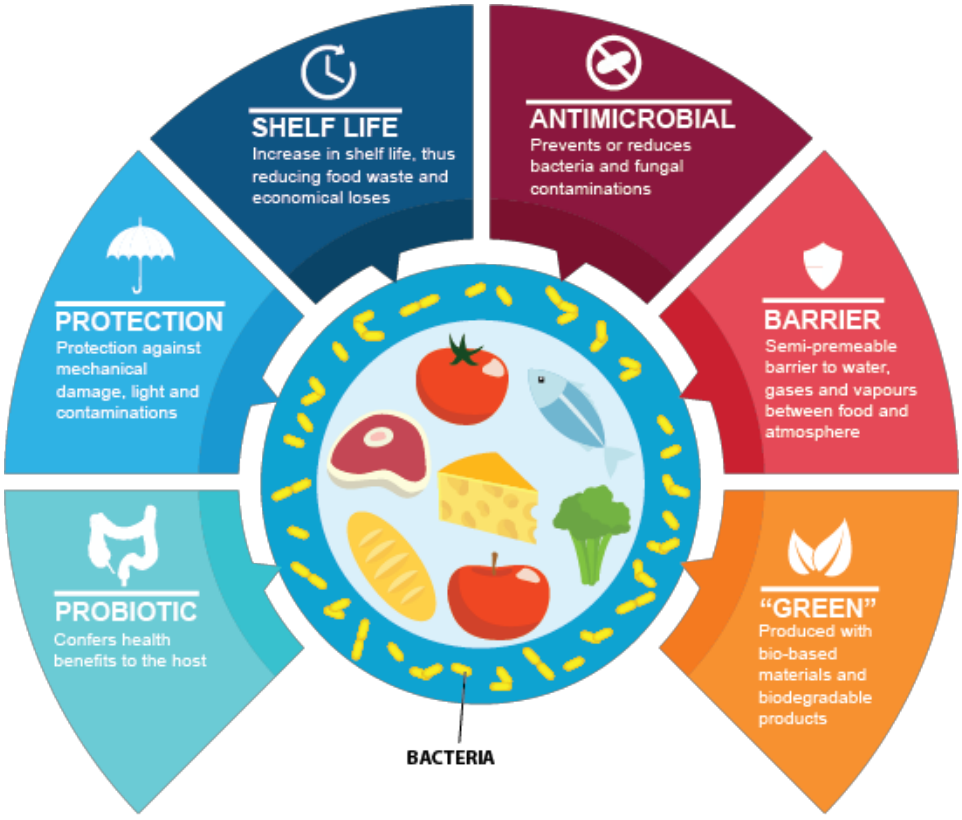


Figure 2.14. Benefits of edible films and coatings containing living bacteria. Adapted from Guimarães et al. (2018a).

Table 2.5. Summary of works that incorporate LAB with the goal of biopreservation in edible films or coatings

Microorganism	Polymeric matrix	Additives	Food application	Inhibition of pathogens	Main conclusions	Reference
Lactobacillus sakei (by incorporation in the film or spraying)	Sodium caseinate	Sorbitol	Fresh beef	*Listeria monocytogenes*	Sorbitol increased the viability of the cells, up to 90%, for 30 days. Incorporation of the cells did not affect the mechanical and physico-chemical properties of the film. The use of the antimicrobial films against *L. monocytogenes* (inoculated on laboratory) in media and food model systems resulted in a significant inhibition of the pathogen.	Gialamas et al. (2010)
Carnobacterium mataromaticum (isolated from smoked salmon)	Alginate (4%), starch (16%)	Nisin and glycerol as plasticizer (20%)	Cold-smoked salmon	*L. monocytogenes*	Smoked salmon covered with a film containing 2 strains of LAB and sprayed with nisin showed a bacteriostatic effect on the development of *L. monocytogenes* for 20 days at 4 °C. Inhibitory effect was attributed to a bacteriocin-like substance.	Concha-Meyer et al. (2011)
Lactobacillus acidophilus and *Bifidobacterium bifidum*	Gelatin	Glucose (2%), cysteine (0.05%). Sorbitol and glycerol as plasticizers	Fish (hake)	H_2S producing and luminescent microorganisms	Gelatin edible films and coatings are suitable to incorporate LAB, since *L. acidophilus* and *B. bifidum* remained viable for 13 days. H_2S-producing microorganisms were reduced by to 2 log units. These films and coatings are promising for fish preservation, especially in combination with high-pressure treatment.	López de Lacey et al. (2012)
Lactobacillus plantarum	Sodium caseinate, pea protein, methylcellulose and hydroxypropylmethylcellulose	Glycerol as plasticizer	-	*Listeria innocua*	Optical and mechanical properties of the films were not altered by LAB, but WVP values increased for all biopolymers. Viability of the *L. plantarum* was lower in cellulose derivative matrices than in protein films. Significant antimicrobial activity against *L. innocua* was observed for polysaccharide matrices, but not for protein films, where the bacteriocin production was delayed.	Sánchez-González et al. (2013)

Microorganism	Polymeric matrix	Additives	Food application	Inhibition of pathogens	Main conclusions	Reference
Lactobacillus paracasei L26 and *Bifidobacterium lactis* B94	Agar	Green tea. Glycerol as plasticizer	Fish (hake)	H_2S-producing bacteria and luminescent bacteria. *Pseudomonas* spp., *Enterobacteriaceae*	The green tea and/or probiotic film caused a reduction of H_2S-producing bacteria counts and total viable bacteria. The probiotic strains added to the film could pass to the fish resulting in an increment of LAB counts. Films with green tea and probiotic were able to extend shelf life of hake at least for a week and increase the beneficial LAB in fish.	López de Lacey et al. (2014)
Lactobacillus acidophilus and *Lactobacillus reuteri*	Sodium caseinate and methylcellulose	Glycerol as plasticizer	-	*L. innocua*	Incorporation of LAB altered some of the film's physical properties, mainly resulted in the increase of WVP. Viability of LAB was higher in sodium caseinate films, although bacteriocin production was greater in the MC matrix. Both formulations were able to inhibit the growth of *L. innocua*, with best results in MC films.	Sánchez-González et al. (2014)
Lactobacillus paracasei	Hydroxypropyl cellulose (HPC) (0.5%)-konjac flour (1.5%)	Glycerol as plasticizer	Strawberries	*L. monocytogenes*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhimurium*	Mechanical, crystalline and colour properties, and oxygen permeability were not notably altered; however, the gloss and WVP were weakened by the incorporation of cells. The HPC–konjac flour–*L. paracasei* films were effective in inhibiting Gram-positive and Gram-negative pathogens, and the films were retained physical property and antibacterial activity within a storage period of 30 days. When applied to strawberries, films sustained the storage quality, value and extend the fruit shelf life.	Dai et al. (2018)
Bifidobacterium animalis and *Lactobacillus casei*	Whey protein isolate	Glycerol as plasticizer	Sliced ham	*Enterobacteriaceae*, *Staphylococcus* spp. and mesophilic aerobic bacteria, yeast and moulds	Coatings decreased water and weight loss on sliced ham. No colour change was noticeable, comparatively to uncoated slices. Viable cell numbers were maintained at 10^8 CFU/g during 45 days at 4 °C. The coatings with probiotic strains inhibited the detectable growth of *Staphylococcus* spp., *Pseudomonas* spp., *Enterobacteriaceae* and yeasts/moulds for 45 days of storage at 4 °C. The sensory evaluation showed a preference for the sliced coated ham.	Pereira et al. (2018)

Microorganism	Polymeric matrix	Additives	Food application	Inhibition of pathogens	Main conclusions	Reference
Lactococcus lactis	Alginate	Glycerol as plasticizer and sodium carboxymethyl cellulose as modifier	-	*S. aureus*	*L. lactis* did not change thickness of the films, decreased transparency and increased red and yellow hues of the film. Incorporation of cells slightly decreased the tensile strength and significantly increased WVP of the films. The films with *L. lactis* (1.5g/100g) showed bacteriostatic activity against *S. aureus* by at least four logarithmic units.	Ye et al. (2018)
L. rhamnosus	Alginate	Glycerol as plasticizers. Supplemented with inulin and oligofrutose	Fresh blueberries	*L. innocua,* aerobic mesophilic microorganisms, psychrotrophic bacteria, yeast and moulds	Prebiotic compounds improved probiotic viability maintaining counts at 6.2 log CFU/g for the 21-day storage period. Native microbiota counts remained under safe levels. Odour and flavour of blueberries were acceptable up to days 14 of storage. Films containing *L. rhamnosus* were able to reduce *L. innocua* counts from 4.46 to 2.76 log in inoculated blueberries.	Bambace et al. (2019)
Lactococcus lactis subsp. *lactic*	Poly(vinyl alcohol)	Gelatin, sodium caseinate, casein, gelatin hydrolysates and yeast extract as nutritional supplements	-	*L. monocytogenes*	The thickness, colour and morphology of the films with LAB and nutritional supplements did not show significant differences when compared with control PVOH films. *L. lactis* remained viable for 4 weeks, but viability depended on the matrix composition. The highest antimicrobial activity was observed in PVOH matrices with hydrolyzed gelatin or casein, and the lowest in control PVOH films. The films exerted antilisterial activity, which increased with protein hydrolysates.	Settier-Ramirez et al. (2019)

CHAPTER 3

LACTIC ACID BACTERIA AS BIOPRESERVATIVES FOR THE CONTROL OF MYCOTOXIGENIC FUNGI

3. LACTIC ACID BACTERIA AS BIOPRESERVATIVES FOR THE CONTROL OF MYCOTOXIGENIC FUNGI

3.1 INTRODUCTION

Contamination by moulds is a problem that affects food and feed, making them unsuitable for consumption. *Aspergillus* and *Penicillum* are two of the most relevant genera, since they are able to contaminate numerous environments, causing health and economic issues. Additionally, many of the species in these genera can produce mycotoxins.

A. flavus has a vast economic impact. This species is a known pathogen in plants that causes the rot of many important agricultural crops (such as maize, rice, nuts, peanuts and cotton) and it also produces aflatoxins. Due to health hazard of this toxin, regulation regarding the levels of aflatoxin in food and feed is currently implemented in approximately 100 countries (van Egmond et al. 2007).

The application of good agricultural practices in crop fields, as well as proper harvest techniques and correct storage conditions, are critical for reducing *A. flavus* contamination in commodities. Several physical and chemical methods are also employed during pre and post-harvest to control the development of fungi and formation of mycotoxins (Schnurer and Magnusson 2005).

In the last decades there was an extensive development of biocontrol agents for the management of fungal contamination. This has been mainly driven by the ban of many chemical compounds for crop protection by the European Union (Medina et al. 2017), and also by consumer's demands for more natural alternatives to chemical pesticides. Biocontrol of fungal contaminants is achieved by diverse mechanisms of action including competitive exclusion of the pathogen, production of inhibitory metabolites, hyperparasitism and production of volatile organic compounds (Medina et al. 2017). The use of biocontrol agents to restrict mycotoxigenic fungi is focused mainly on two aspects: i) the control

of fungal growth/colonization in the plant or commodity; ii) the reduction/inhibition of mycotoxin production.

A vast range of bacteria, yeast and filamentous fungi has been tested with success as biocontrol agents against *A. flavus* and several non-toxigenic species have been used in field trials, such as the commercial biocontrol *A. flavus* strains, Afla-Guard® and Aflasafe™.

Bacterial strains have also been proved to be effective in inhibiting *A. flavus*. Several species such as *Bacillus* spp., *Lactobacillus* spp., *Pseudomonas* spp. and *Ralstonia* spp. have shown ability to inhibit *A. flavus* growth and aflatoxin production (Nesci et al. 2005; Palumbo et al. 2006; Reddy et al. 2009b; Mannaa et al. 2017). Also, several saprophytic yeasts (*Candida* spp. and *Pichia* spp.) have shown antagonistic activity towards *A. flavus* in laboratory conditions (Paster et al. 1993; Hua et al. 2014).

Penicillium nordicum is a common contaminant in protein-rich foods, like dry-cured meat, salted fish and cheeses, and several isolates have been shown to produce OTA in these food matrices (Lund and Frisvad 2003; Bogs et al. 2006; Battilani et al. 2007; Sonjak et al. 2011). *P. nordicum* can growth well at low temperatures and it is adapted to high concentrations of this salt (5%), which is found in cured meats and cheeses. High concentrations of NaCl (20 g/L) can promote the synthesis of OTA, possibly as a way to remove chlorine from inside the cell (Geisen 2004).

OTA has been classified by IARC in group 2B (possible human carcinogen) suspected to be involved in nephropathies and tumours (IARC 1993). Because of its high stability, OTA is difficult to eliminate from food products, thus strategies for preventing this mycotoxin from entering the food chain should be focused primarily on inhibiting fungal growth and OTA production. Some physical treatments (such as thermal, UV and radiation treatments, among others) can be effective against fungal contamination; however, these methods can destroy endogenous enzymes and microbiota, thus being inappropriate for dry-ripened foods, and chemical treatments go against the claims of traditional products, and can raise concerns about health risks. Additionally, mycotoxins may not be efficiently destroyed by these methods.

Biological control of *P. nordicum* contamination provides a method to improve food safety without affecting the properties of commodities. For this purpose, bacteria (Schillinger and Villarreal 2010), yeast (Virgili et al. 2012; Iacumin et al. 2017; Meftah et al. 2018) and moulds (Rodríguez et al. 2015) were used in several works.

Lactic acid bacteria can be good candidates in biocontrol approaches since they have been proven to have antifungal properties. LAB have also the additional advantage of their extensive use in

fermentation processes, their GRAS and QPS classification, and also some strains act as probiotics, that confer benefits to the host. LAB group includes bacteria that have morphological, metabolic and physiologic similarities, and that are also phylogenetically closed. Classification of LAB genera is based on morphology, type of glucose fermentation, growth temperature and sort of sugar utilization.

The antagonistic properties of LAB starter cultures on spoilage organisms found in food and beverages has been exploited throughout history, since the origins of agriculture and brought significant benefit to human health and to the industry involved in fermented food. LAB play an important role in the world's food production, being used in the fermentation processes of dairy products, meats and vegetables, but are also necessary for the production of wine, coffee, silage, cocoa, sourdough and other foods (Makarova et al. 2006). LAB are able to thrive in food-related habitats, since they require a nutrient rich environment that includes amino acids, peptides, salts and vitamins among others (Carr et al. 2002). Additionally, they can colonize mucosal surfaces of animals (such as colon, small intestine and vagina). Isolates of the same species are usually found in the same environments, suggesting that there is an adaptation mechanism to different environments (Magnusson 2003; Makarova et al. 2006).

Antifungal activity of LAB is a complex process that has been associated with the end products of their metabolism (such as organic acids, cycle dipeptides, fatty acids and bacteriocins), as well as from a combination of other factors, such as competitive growth and decrease in environmental pH.

The most studied antifungal activity of LAB is generally associated with the production of organic acids, which result from the primary metabolism from carbohydrates fermentation. Numerous works have reported the production of organic acids with antifungal ability; mainly lactic and acetic acid, but also formic, propionic, butyric, phenyllactic, hydroxyphenyllactic acids, among others (Corsetti et al. 1998; Lavermicocca et al. 2000; Ström et al. 2002; Magnusson et al. 2003; Broberg et al. 2007; Gerez et al. 2009; Prema et al. 2010; Yang and Chang 2010; Özcelik et al. 2016). Inhibition of fungal growth by LAB results typically from the reduction of pH, which is a inhibiting factor for many microorganisms.

The focus for biocontrol agents should be both on fungal growth and mycotoxin control. Still, only few reports acknowledge the effect of LAB and their metabolites in the production of mycotoxins (Karunaratne et al. 1990; Gourama and Bullerman 1995b; Gourama 1997; Onilude et al. 2005; Petchkongkaew et al. 2008; Gerbaldo et al. 2012; Sangmanee and Hongpattarakere 2014; Oliveira et al. 2015b; Marie et al. 2018).

In this chapter, 98 strains of LAB isolated from milk, wine, silages, elderberry and sausages were screened for antifungal ability against *Aspergillus* spp., namely *A. flavus*, *A. carbonarius* and *A. parasiticus*. Further selection included determination of fungal growth inhibition and mycotoxin production, using selected LAB cell-free supernatants. Following work aimed at demonstrating the potential of LAB supernatants to control the growth of mycotoxigenic fungi, mainly *A. flavus* and *P. nordicum*, and mycotoxin production. Metabolites responsible for that antifungal effect were determined, as well as their individual ability to affect fungal growth and mycotoxin production.

3.2 MATERIALS AND METHODS

3.2.1 Chemicals and Culture Media

Aspergillus spp. fungal cultures were grown using malt extract agar base (MEA) with mycological peptone (supplied by Himedia). *P. nordicum* was cultured in YES (yeast extract sucrose) agar, consisting in 150 g/L of sucrose, 20 g/L of yeast extract, 15 g/L of agar, 0.5 g/L of $MgSO_4$, 0.01 g/L of $ZnSO_4$ and 0.005 g/L of $CuSO_4$.

Lactic acid bacteria were cultivated in MRS broth (Oxoid) or in MRS agar for cultures in solid media. Both broth and solid MRS were supplemented with 20% of Tomato Juice broth (Fluka) and 1 g/L of L-cysteine (Fisher-Chemical). For the screening of LAB antifungal ability Malt Soft Agar (MSA) composed by 2.5% malt extract (Himedia), 0.1% bacteriological peptone (Himedia), 2.5% glucose (Fisher Scientific) and 1% bacteriological agar (Oxoid) was used.

For mycotoxins extraction and preparation of mobile phase, methanol, acetonitrile (both HPLC grade) and acetic acid were used. Standards for aflatoxins B1, B2, G1 and G2 (Sigma-Aldrich) and OTA (Sigma-Aldrich) were used as standards for mycotoxin quantification.

Acetic acid (Fisher Scientific), butyric acid (Fluka), lactic acid (Fluka), DL-3-phenyllactic acid (PLA) (Acros Organics), propionic acid (Sigma-Aldrich), DL-p-hydroxyphenyllactic acid (OH-PLA) (Sigma-Aldrich) and DL-Indole-3-lactic acid (ILA) (Sigma-Aldrich) were used as organic acid standards. Additionally, for the analysis of organic acids present in LAB CFS, the following reagents were used:

magnesium sulphate (Sigma-Aldrich), sodium chloride (VWR), ethyl acetate (VWR), DMSO (VWR) and formic acid (Sigma-Aldrich).

3.2.2 Biological Cultures and Growth Conditions

Aspergillus flavus MUM 17.14, *Aspergillus carbonarius* MUM 04.52, and *Aspergillus parasiticus* MUM 92.02 were the mycotoxigenic strains used in the preliminary screening study to identify antifungal potential of LAB isolates. Aflatoxigenic strains, in particular *A. flavus* MUM 17.14, were used in further inhibition studies. Aflatoxins produced by selected fungal species are shown in Table 3.1. *P. nordicum* MUM 08.16 (CBS 112573) was used for inhibition studies of fungal growth and OTA production.

Table 3.1. Fungal strains used and their ability to produce aflatoxins. Where + corresponds to production and - to no production of that type of aflatoxin

Species	MUM code	Other code	Aflatoxins			
			AFB1	AFB2	AFG1	AFG2
A. flavus	MUM 17.14	-	+	+	-	-
A. flavus	MUM 10.237	CBS 100927	+	+	-	-
A. parasiticus	MUM 92.02	NRRL 3386	+	+	+	+
A. arachidicola	MUM 10.238	CBS 117610	+	+	+	+
A. nomius	MUM 09.01	NRRL 3353	+	+	-	-
A. minisclerotigenes	MUM 10.240	CBS 117635	+	+	+	+

Aspergillus strains were revived in MEA for seven days in the dark at 25 °C, while *P. nordicum* was revived in YES agar, in the same conditions. Spore solutions for inocula were prepared by adding a 0.1% peptone solution with 0.05% of Tween 80 (Fisher Scientific) to the fully-grown fungi, therefore suspending the spores. Spores were counted in a microscope using a Neubauer cell chamber, and concentration of the spore solution was adjusted to 10^6 spore/mL. Aliquots of spore solutions were store at – 20 °C.

LAB strains were isolated from milk, wine, silages and sausages, by Inês (Inês 2007) and Rodrigues (Rodrigues 2011) and phenotypical characterization of the strains showed that all the samples had coccus or coccobacillus or bacillus morphology and were Gram-positive.

Identification of LAB strains was carried on by PCR identification of rDNA 16S using universal primers PA (5' AGAGTTTGATCCTGGCTCAG 3') and PH (5´ AAGGAGGTGATCCAGCCGCA 3'). Amplification products were purified and sent to sequencing at STABVIDA where they were sequenced using the primer 907R (5' CCGTCAATTCCTTTGAGTTT 3'). Resulting partial sequences of rDNA 16S were compared with sequences available in public databases (GenBank). Strain code, species and source of LAB are shown in Table 3.2.

Table 3.2. LAB isolates, attributed strain code and corresponding specie and isolation source

Strains Nº	Species	Source	Strains Nº	Species	Source
UM1	*L. plantarum*	Cow milk	UM 334	*P. parvulus*	Red wine
UM2	n. i.	n. i.	UM 335	*P. parvulus*	Red wine
UM3	*L. casei*	Cow milk	UM 346	*L. plantarum*	Red wine
UM4	n. i.	n. i.	UM 350	*L. plantarum*	Red wine
UM5	*E. durans*	Cow milk	UM 473	*P. parvulus*	Red wine
UM6	*L. casei*	Cow milk	UTAD5	n. i.	Elderberry
UM7	*L. plantarum*	Cow milk	UTAD7	n. i.	Elderberry
UM8	*L. mesenteroides*	Cow milk	UTAD8	n. i.	Elderberry
UM10	*L. casei*	Cow milk	UTAD11	n. i.	Elderberry
UM13	*L. casei*	Cow milk	UTAD12	n. i.	Elderberry
UM17	*L. casei*	Cow milk	UTAD18	n. i.	Elderberry
UM25	n. i.	n. i.	UTAD20	n. i.	Elderberry
UM34	n. i.	n. i.	UTAD22	n. i.	Elderberry
UM41	*L. casei*	Cow milk	UTAD25	n. i.	Elderberry
UM53	*L. casei*	Cow milk	UTAD27	n. i.	Elderberry
UM54	*L. casei*	Cow milk	UTAD37	*E. faecium*	Pig sausage
UM55	*L. plantarum*	Cow milk	UTAD47	n. i.	Elderberry
UM56	n. i.	n. i.	UTAD48	n. i.	Elderberry
UM59	n. i.	n. i.	UTAD49	n. i.	Elderberry
UM61	*L. plantarum*	Cow milk	UTAD50	n. i.	Silage
UM62	*L. casei*	Cow milk	UTAD52	n. i.	Silage
UM64	*L. pentosus*	Cow milk	UTAD55	n. i.	Elderberry
UM65	n. i.	n. i.	UTAD57	n. i.	Elderberry
UM66	*L. paraplantarum*	Cow milk	UTAD58	n. i.	Elderberry
UM67	*L. plantarum*	Cow milk	UTAD62	n. i.	Elderberry
UM69	*L. brevis*	Cow milk	UTAD73	n. i.	Silage

Strains Nº	Species	Source	Strains Nº	Species	Source
UM70	*L. brevis*	Cow milk	UTAD78	n. i.	Elderberry
UM72	*L. casei*	Cow milk	UTAD79	n. i.	Elderberry
UM73	*L. brevis*	Cow milk	UTAD89	n. i.	Elderberry
UM74	*L. mesenteroides*	Cow milk	UTAD90	n. i.	Elderberry
UM75	*L. paracasei*	Cow milk	UTAD93	n. i.	Elderberry
UM76	*L. casei*	Cow milk	UTAD94	n. i.	Elderberry
UM77	*L. plantarum*	Cow milk	UTAD95	n. i.	Elderberry
UM81	*L. casei*	Cow milk	UTAD100	n. i.	Elderberry
UM82	*L. casei*	Cow milk	UTAD101	n. i.	Silage
UM83	*L. casei*	Cow milk	UTAD102	n. i.	Silage
UM87	*L. plantarum*	Cow milk	UTAD103	n. i.	Silage
UM88	*L. paracasei*	Cow milk	UTAD104	*L. buchneri*	Silage
UM89	*L. casei*	Cow milk	UTAD118	n. i.	Elderberry
UM111	*P. parvulus*	Red wine	UTAD121	n. i.	Elderberry
UM141	n. i.	n. i.	UTAD125	n. i.	Elderberry
UM168	*P. parvulus*	Red wine	UTAD131	n. i.	Elderberry
UM224	*O. oeni*	Red wine	UTAD158	n. i.	Elderberry
UM228	*O. oeni*	Red wine	UTAD206	n. i.	Silage
UM244	*O. oeni*	Red wine	UTAD210	n. i.	Silage
UM271	n. i.	n. i.	UTAD212	n. i.	Silage
UM279	*O. oeni*	Red wine	UTAD214	n. i.	Silage
UM296	*O. oeni*	Red wine	UTAD218	n. i.	Silage
UM333	*P. parvulus*	Red wine	UTAD220	n. i.	Silage

n. i.: not identified

3.2.3 Screening of Lactic Acid Bacteria for Antifungal Properties

LAB strains were screened for antifungal activity using a "dual-culture overlay" method (Magnusson and Schnurer 2001). Briefly, bacteria were inoculated as 2 cm long line in MRS agar plates, and grown at 30 °C for 72 h to allow a proper grown bacteria line to form. The plates were then covered with 10 mL of MSA containing a 10^4 spores/mL concentration of either *A. flavus*, *A. carbonarius* or *A. parasiticus* and incubated aerobically for 7 days at 25 °C. During the incubation period, inhibition of fungal growth (inhibition zones around the bacterial streaks) was assessed qualitatively. The following scale was used: -, no inhibition (bacteria were completely overlaid by fungal growth); +, slight inhibition (bacteria were not overlaid by the fungi); ++, inhibition (formation of

inhibition zones around the bacteria culture) and +++ strong inhibition (formation of strong inhibition zones around the bacteria culture).

Most active strains were re-evaluated using the "poisoned food technique" (Balouiri et al. 2016), in order to quantify inhibition ability on the fungal radial growth and production of mycotoxins. LAB were cultivated in MRS broth at 30 °C for 2 days. Cell culture was then centrifuged (10 min at 7000 RCF), and the resulting supernatant was filtered through a 0.2 μm pore size sterile syringe filter (cellulose acetate, Whatman). The cell free supernatant was added to MEA at a 10% proportion and poured into petri plates (20 mL per plate). Plates were inoculated with 10 μL of the fungal spore solution (10^6 spores/mL) on the centre of the plate. In control plates, CFS were replaced by MRS broth. Plates were incubated at 25 °C, in the dark, for 7 days. Diameters of the fungal colonies were measured daily. After 7 days of growth, mycotoxins were extracted from the fungal colonies as described in section 3.2.5. All experiments were done in triplicate.

3.2.4 Characterization of Antifungal Properties of Lactic Acid Bacteria Cell-free Supernatants

In order to determine the nature of the compounds responsible for the antifungal activity of the selected LAB strains against *A. flavus* and *P. nordicum*, the CFS of those strains were subjected to treatments with heat, proteases and pH neutralization. The sensitivity to heat was evaluated by subjecting one fraction of the CFS to 121 °C for 20 minutes. The susceptivity to proteases was assessed by subjecting another fraction of CFS to a proteolytic enzyme (Pepsin, Amresco) at a final concentration of 2 mg/mL. After the addition of the protease, the CFS was incubated at 37 °C for 3 hours, and boiled for 5 min to inactivate the enzyme. Finally, another fraction of CFS was used to test the effect of pH on the antifungal activity. The pH of CFS was adjusted to 7 with 1 M NaOH before the antifungal assays. The antifungal activity of treated CFS was determined also by the "poisoned food technique" as described previously.

The effect of CFS concentration in the inhibition of *A. flavus* MUM 17.14 growth and in the production of aflatoxins was also determined. Concentrations of 5, 10, 15, 20 and 25% of supernatant in the MEA culture media were tested as described above. The composition of MEA was adjusted accordingly in order to maintain the nutritional composition of culture media consistent for each

percentage of supernatant used. The inhibitory effect of CFS over an extended period of incubation (22 days) was also tested against *A. flavus* MUM 17.14 using 10% of CFS. The growth of fungal colonies was measured daily and aflatoxins were extracted from the MEA plates after 3, 6, 9, 12, 16, 19 and 22 days of growth at 25 °C in the dark. The effect of LAB CFS on other aflatoxigenic species (listed in Table 3.1) was also evaluated. Experiments were performed as previously described with 10% of CFS and aflatoxins were extracted after 7 days of growth at 25 °C in the dark.

3.2.5 Mycotoxin Extraction and Quantification

To determine the mycotoxin content present in MEA plates inoculated with mycotoxigenic species the following protocol was used: at the end of the incubation period, the total content of plates was cut and placed into falcon tubes and extracted with 30 mL of an organic solvent solution of acetonitrile/methanol/acetic acid (78/10/2, v/v/v) by strong vortex agitation. Extracts were left overnight at room temperature in the dark, then filtered using 0.45 µm syringe filters (polypropylene, Whatman) and analyzed by HPLC and fluorescence detection with previously implemented methods (Soares et al. 2010). Briefly, the HPLC system is comprised of a Varian Prostar 210 pump, a Varian Prostar 410 autosampler, a photochemical post-column derivatization reactor (PHRED unit, Aura Industries, USA) and a Jasco FP-920 fluorescence detector. The instrument and the chromatographic data were managed by a Varian 850-MIB data system interface and a Galaxie chromatography data system, respectively. The chromatographic separation was performed on a C18 reversed-phase YMC-Pack ODS-AQ analytical column (250 x 4.6 mm I.D., 5 µm) that was fitted with a pre-column with the same stationary phase. The mycotoxins were eluted using a flow rate of 1.0 mL/min for 30 or 15 min isocratic run, for aflatoxins and OTA, respectively.

For aflatoxins the mobile phase was a mixture of water/acetonitrile/methanol (3:1:1, v/v/v) that was filtered and degassed with a 0.2 µm membrane filter (GHP, Gelman), the fluorescence detector was set to λ_{ex}= 365 nm and λ_{em} = 435 nm and injection volume was of 30 µL. Calibration curves were constructed using certified aflatoxins standard solution mixture (46304-U, Sigma-Aldrich) at defined concentrations in the respective mobile phase (calibration curves are shown in appendix A Figure A.1, Figure A.2, Figure A.3 and Figure A.4). The limit of detection (LOD) and limit of quantification (LOQ) were calculated from the standard deviation of the response signal (SD) and the slope (b) of the

calibration curves. LOD was calculated by the formula: 3×(SD/b) and LOQ by 10×(SD/b) (Épshtein 2004). Values for LOD and LOQ for aflatoxins are shown in Table 3.3.

For OTA, the mobile phase was a mixture of water/acetonitrile/acetic acid (99:99:2, v/v/v) that was also filtered and degassed with a 0.2 µm membrane filter (GHP, Gelman) and the fluorescence detector was set to λ_{ex_c}= 333 nm and λ_{em} = 460 nm, and an injection volume of 50 µL. Calibration curves were constructed using certified OTA standard solution (CRM46912, Sigma-Aldrich) at defined concentrations in the respective mobile phase (calibration curve is shown in appendix A Figure A.5). Mycotoxins were quantified by comparing the peak areas with the calibration curves. The LOD and LOQ for OTA were calculated as explained before for aflatoxins, and are presented in Table 3.3.

Table 3.3. Limits of detection and limits of quantification (ng/mL) of aflatoxins (AFB1, AFB2, AFG1, AFG2) and OTA

Limits (ng/mL)	**Aflatoxins**				**OTA**
	AFB1	**AFB2**	**AFG1**	**AFG2**	
LOD	14	2	8	11	0.18
LOQ	42	6	26	33	0.53

3.2.6 HPLC Analysis of Cell-free Supernatants

A protocol adapted from Brosnan et al. (2014) was used for the extraction of organic acids from LAB CFS. Briefly, 10 mL of the CFS obtained as described before were added to 10 mL of ethyl acetate with 1% formic acid, 4 g of $MgSO_4$ and 1 g of NaCl. The mixture was vortexed for 1 min and centrifuged for 10 min at 3000 RCF and the organic solvent fraction was collected. To 5 mL of that fraction, 100 µL of DMSO was added. The resultant solution was dried under nitrogen, reconstituted to 1 mL with H_2O/acetonitrile (90/10, v/v) and filtered with a 0.45 µm membrane filter (Nylon, Whatman) into HPLC amber vials. For controls, unfermented MRS broth was treated under the same conditions.

A Shimadzu Nexera LC system with a photodiode array detector (SPD-M20A) was used to detect and quantify the organic acids. The chromatographic separation was performed on a Brisa LC2 C18 column (250 x 4.6 mm I.D., 5 µm; Teknokroma) that was fitted with a pre-column with the same stationary phase. The organic acids were eluted under gradient conditions using H_2O with 0.1% formic

acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). Both solvents were filtered with a 0.2 µm membrane filter and degassed before use. Gradient was as follows: 0 min – 5% B; 9 min – 10% B; 16 min – 30% B; 32 min – 30% B; 48 min – 40% B; 56 min – 40% B; 65 min – 95% B; 73 min – 95% B; 80 min – 95% B. The flow rate was set to 1 mL/min, the temperature to 30 °C, and a volume of 20 µL of each sample was injected. Organic acids were detected at a UV wavelength of 210 nm. Chromatographic data were analysed with the software LabSolutions version 5.71 (Shimadzu Corporation).

Individual stock solutions of each organic acid tested were prepared by dissolving the compounds in H_2O. Seven points calibration curves were prepared at concentrations ranging from 0.1 to 2 mg/mL for PLA; from 1 to 20 mg/mL for lactic acid; from 0.01 to 0.5 mg/mL for OH-PLA and from 0.01 to 0.15 mg/mL for ILA. Organic acids were quantified by comparing the peak areas with the calibration curves (shown in appendix A Figure A.6, Figure A.7, Figure A.8 and Figure A.9). The LOD and LOQ were calculated as described before for mycotoxins (Épshtein 2004). Values for LOD and LOQ for organic acids are shown in Table 3.4.

Table 3.4. Limits of detection and limits of quantification (mg/mL) of lactic acid, PLA, OH-PLA and ILA

Limits (mg/mL)	**Lactic acid**	**PLA**	**OH-PLA**	**ILA**
LOD	0.810	0.045	0.006	0.010
LOQ	2.454	0.136	0.018	0.031

3.2.7 Antifungal Properties of Organic Acids Produced by Lactic Acid Bacteria

Organic acids that were detected in LAB CFS were tested individually for the inhibition of fungal growth and mycotoxin production in *A. flavus* MUM 17.14 and *P. nordicum* MUM 08.16. Lactic acid, PLA, OH-PLA and ILA were tested using the "poisoned food technique" as described previously, by adding solutions of the organic acids to MEA or YES (for *A. flavus* and *P. nordicum*, respectively) in the same volume proportion previously used for LAB CFS (10%, v/v) in order to achieve the desired concentration. Concentrations of organic acids selected to be added to the culture medium were 0.1, 0.25, 0.5, 1.0, 1.5, 2.0, 4.0 and 8.0 mg/mL. As in the previous experiments, fungal diameters were

measured daily and aflatoxin contents analysed after 7 days of incubation at 25 °C in the dark. All experiments were done either in duplicate or in triplicate. The concentration of each organic acid, which inhibited by 50% (IC_{50}) and 90% (IC_{90}) the fungal growth and the production of mycotoxins, was derived from non-linear fit of log doses versus normalized inhibitions against a four-parameter logistic model (Hill equation, showed in Figure A.12) using the software GraphPad Prism version 7.02 for Windows (GraphPad Software, USA).

3.2.8 Antifungal Properties of Lactic Acid Bacteria Cells

The antifungal ability of selected LAB cells was, as well, tested by the "poisoned food technique". Isolates from both strains were grown in MRS supplemented with tomato juice and 1 g/L of cysteine, for 2 days at 30 °C. Cells were centrifuged at 7000 RCF for 10 minutes, and the supernatant was discarded. The bacterial pellet was resuspended in phosphate buffered saline (PBS) prepared in the following way: 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 0.24 g of KH_2PO_4 were added to 1 L of distilled water, and the final pH set to 7.4. The number of cells per mL was standardised by measuring optical density (OD) at 600 nm. Suspensions of cells in PBS with OD_{600} of 1, 2.5 and 5 (which correspond to approximately to 1×10^9; 2.5×10^9; 7×10^9 CFU/mL respectively for *L. plantarum* UM55 and to 6.1×10^9; 1.7×10^{10}; 3.6×10^{10} CFU/mL respectively for *L. buchneri* UTAD104) were prepared. Cell suspensions in PBS were incorporated into YES agar at the proportion of 10% (v/v) and poured into plates. Then plates were centre inoculated with 10 µL of the fungal (*A. carbonarius* in the screening procedure and *P. nordicum* in further studies) spore suspension (10^6 spores/mL). In the control experiments, PBS replaced cell suspensions. Again, plates were incubated for 7 days at 25 °C in the dark. Diameters of the growing fungal colonies were measured daily. OTA was extracted after 7 days of growth and quantified as described previously. All experiments were done in triplicate.

3.2.8.1 HPLC Analysis of Lactic Acid and Acetic Acid Extracted from Plates Containing Lactic Acid Bacteria Cells

Organic acids were determined in the solid medium (YES agar) incorporated with LAB cells, and PBS (control), as stated in the previous section (section 3.2.8). After 7 days of incubation, plates were cut and placed in a tube with 20 mL of distillate water. Tubes were vortexed vigorously and left overnight. Solutions were filtered through a syringe 0.22 µm filter (PES, Whatman) into vials and analysed by HPLC with UV detection. An HPLC method adapted from datasheet T910226A from YMC Separation Technology was used to determine lactic and acetic acid in samples. The HPLC system consisted of a Varian Prostar 210 pump, a Varian Prostar 410 autosampler and a Varian ProStar 325 UV-Vis detector. The interface between HPLC system and computer was established by a Varian 850-MIB data system, and Galaxie software managed chromatographic data. A C18 reversed-phase YMC-Pack ODS-AQ analytical column (250 x 4.6 mm I.D., 5 µm) was used for the chromatographic separation. The mobile phase was a solution of 10 mM of NaH_2PO_4 and 10 mM of 85% orto-phosphoric acid, which pH was set to 2.8 with 1 M of NaOH. The mobile phase was filtered with a 0.45 µm membrane filter (MCE, Whatman) and sonicated by 20 minutes. Chromatographic runs were of 50 minutes with a flow rate of 0.7 mL/min. The injection volume was of 20 µL, and the temperature was set to 30 °C. Detection of organic acids was made at a wavelength of 220 nm. Calibration curves were made with standards of lactic and acetic acids in distilled water with a concentration of 0.5 to 10 mg/mL and 1 to 10 mg/mL, respectively (shown in appendix A Figure A.10 and Figure A.11). The LOD and LOQ for lactic and acetic acids were calculated, as well, from de SD of the response signal and slope of calibration curves. For lactic acid LOD and LOQ were, respectively, 0.303 and 0.918 mg/mL; and for acetic acid LOD and LOQ were 0.341 and 1.038 mg/mL, respectively.

3.2.9 Data Analysis

Three independent replicates were performed in all experiments and results were expressed as mean values ± standard deviation. Data were compared by analysis of variance (ANOVA) with Tukey's or Sidak's multiple comparisons test at $p < 0.05$ using the software GraphPad Prism version 7.02 for Windows (GraphPad Software, USA).

3.3 RESULTS AND DISCUSSION

3.3.1 Screening of Lactic Acid Bacteria for Antifungal Properties

This preliminary study highlights the antifungal activity of LAB isolated from food and feed sources. LAB antifungal activity, measured qualitatively by the "dual-culture overlay" assay, against *A. flavus* MUM 17.14, *A. carbonarius* MUM 04.52 and *A. parasiticus* MUM 92.02 is shown in Table 3.5, Table 3.6 and Table 3.7, respectively.

Table 3.5. Antifungal activity of screened LAB against *A. flavus* MUM 17.14. Table shows antifungal activity of LAB isolates at 3, 5 and 7 days of *A. flavus* growth. Where - corresponds to no inhibition; + some inhibition; ++ inhibition and +++ strong inhibition

	A. flavus				***A. flavus***		
Strain Nº	**3 days**	**5 days**	**7 days**	**Strain Nº**	**3 days**	**5 days**	**7 days**
UM1	+	+	+	UM 334	++	+	+
UM2	+	-	-	UM 335	+++	+++	++
UM3	-	-	-	UM 346	+	+	+
UM4	-	-	-	UM 350	+++	++	+
UM5	+	-	-	UM 473	-	-	-
UM6	-	-	-	UTAD5	++	++	++
UM7	**+++**	**+++**	**+++**	UTAD7	++	+	+
UM8	++	+	-	UTAD8	+++	++	++
UM10	++	-	-	UTAD11	+	-	-
UM13	+	-	-	UTAD12	++	+	+
UM17	++	+	-	**UTAD18**	**+++**	**+++**	**+++**
UM25	++	+	+	UTAD20	++	-	-
UM34	-	-	-	UTAD22	+++	++	++
UM41	+	+	-	UTAD25	++	+	+
UM53	+	+	-	UTAD27	-	-	-
UM54	++	+	-	UTAD37	-	-	-
UM55	**+++**	**+++**	**+++**	UTAD47	+++	+	+
UM56	++	+	+	UTAD48	++	++	+
UM59	-	-	-	UTAD49	++	++	++
UM61	++	+	+	UTAD50	-	-	-
UM62	+	-	-	UTAD52	+	+	-
UM64	++	+	-	UTAD55	++	-	-
UM65	-	-	-	UTAD57	-	-	-
UM66	++	+	+	UTAD58	++	++	+

	A. flavus				*A. flavus*		
Strain Nº	**3 days**	**5 days**	**7 days**	**Strain Nº**	**3 days**	**5 days**	**7 days**
UM67	++	+	+	UTAD62	++	++	+
UM69	-	-	-	UTAD73	++	++	++
UM70	++	+	+	UTAD78	+++	++	++
UM72	-	-	-	UTAD79	+	-	-
UM73	+	-	-	UTAD89	++	+	+
UM74	+	-	-	UTAD90	++	+	+
UM75	-	-	-	UTAD93	+++	++	++
UM76	-	-	-	UTAD94	++	+	+
UM77	+	+	+	UTAD95	++	+	+
UM81	-	-	-	UTAD100	++	+	+
UM82	+	+	-	UTAD101	++	+	+
UM83	-	-	-	UTAD102	++	+	+
UM87	+++	+++	++	UTAD103	+++	++	++
UM88	-	-	-	**UTAD104**	**+++**	**+++**	**+++**
UM89	-	-	-	**UTAD118**	**+++**	**+++**	**+++**
UM111	++	-	-	UTAD121	+	-	-
UM141	+++	+++	++	UTAD125	++	+	-
UM168	++	+	+	UTAD131	++	+	+
UM224	++	++	+	UTAD158	+++	++	+
UM228	++	++	++	UTAD206	+++	++	++
UM244	++	+	+	UTAD210	++	+	-
UM271	+++	+++	++	UTAD212	++	+	+
UM279	++	++	++	UTAD214	+++	+++	++
UM296	++	+	+	UTAD218	+++	++	+
UM333	-	-	-	UTAD220	++	+	+

Table 3.6. Antifungal activity of screened LAB against *A. carbonarius* MUM 04.52. Table shows antifungal activity of LAB isolates at 3, 5 and 7 days of *A. carbonarius* growth. Where - corresponds to no inhibition; + some inhibition; ++ inhibition and +++ strong inhibition

	A. carbonarius				*A. carbonarius*		
Strain Nº	**3 days**	**5 days**	**7 days**	**Strain Nº**	**3 days**	**5 days**	**7 days**
UM1	+	+	-	UM 334	++	+	-
UM2	-	-	-	UM 335	+	-	-
UM3	-	-	-	UM 346	+	-	-
UM4	-	-	-	UM 350	++	+	+
UM5	-	-	-	UM 473	-	-	-
UM6	-	-	-	UTAD5	+++	++	+
UM7	**++**	**++**	**++**	UTAD7	+++	+	+
UM8	+	+	+	UTAD8	++	+	+
UM10	+	+	+	UTAD11	++	+	+
UM13	-	-	-	UTAD12	+	-	-
UM17	+	-	-	**UTAD18**	**+++**	**++**	**++**

	A. carbonarius		
Strain Nº	**3 days**	**5 days**	**7 days**
UM25	+	+	-
UM34	+	+	+
UM41	-	-	-
UM53	-	-	-
UM54	+	-	-
UM55	**++**	**++**	**++**
UM56	-	-	-
UM59	-	-	-
UM61	+	-	-
UM62	+	+	+
UM64	++	+	+
UM65	-	-	-
UM66	++	+	+
UM67	+	-	-
UM69	+	+	+
UM70	+	+	+
UM72	-	-	-
UM73	-	-	-
UM74	-	-	-
UM75	-	-	-
UM76	+	-	-
UM77	+	-	-
UM81	-	-	-
UM82	-	-	-
UM83	-	-	-
UM87	**+++**	**++**	**++**
UM88	-	-	-
UM89	++	-	-
UM111	-	-	-
UM141	++	++	+
UM168	+	-	-
UM224	++	++	+
UM228	++	+	+
UM244	++	++	+
UM271	**++**	**++**	**++**
UM279	++	+	-
UM296	+	+	+
UM333	-	-	-

	A. carbonarius		
Strain Nº	**3 days**	**5 days**	**7 days**
UTAD20	-	-	-
UTAD22	**+++**	**+++**	**++**
UTAD25	**++**	**++**	**++**
UTAD27	++	++	+
UTAD37	-	-	-
UTAD47	**+++**	**++**	**++**
UTAD48	++	++	+
UTAD49	**+++**	**+++**	**++**
UTAD50	+	+	+
UTAD52	-	-	-
UTAD55	-	-	-
UTAD57	-	-	-
UTAD58	**++**	**++**	**++**
UTAD62	-	-	-
UTAD73	-	-	-
UTAD78	**+++**	**++**	**++**
UTAD79	++	++	+
UTAD89	**+++**	**++**	**++**
UTAD90	+++	++	+
UTAD93	++	+	+
UTAD94	**+++**	**++**	**++**
UTAD95	**++**	**++**	**++**
UTAD100	+++	++	+
UTAD101	**+++**	**++**	**++**
UTAD102	++	++	+
UTAD103	+	-	-
UTAD104	**+++**	**++**	**++**
UTAD118	**++**	**++**	**++**
UTAD121	**+++**	**+++**	**++**
UTAD125	**++**	**++**	**++**
UTAD131	**++**	**++**	**++**
UTAD158	-	-	-
UTAD206	**++**	**++**	**++**
UTAD210	**+++**	**++**	**++**
UTAD212	+	+	+
UTAD214	+	+	+
UTAD218	+	+	+
UTAD220	+++	+	+

Table 3.7. Antifungal activity of screened LAB against *A. parasiticus* MUM 92.02. Table shows antifungal activity of LAB at 3, 5 and 7 days of *A. parasiticus* growth. Where - corresponds to no inhibition; + some inhibition; ++ inhibition and +++ strong inhibition

	A. parasiticus				***A. parasiticus***		
Strain Nº	**3 days**	**5 days**	**7 days**	**Strain Nº**	**3 days**	**5 days**	**7 days**
UM1	++	+	+	UM334	++	++	+
UM2	+	-	-	UM335	++	+	+
UM3	-	-	-	UM346	+++	++	-
UM4	-	-	-	UM350	++	++	-
UM5	-	-	-	UM473	+++	+++	+
UM6	+++	++	-	UTAD5	++	+	+
UM7	**++**	**++**	**++**	UTAD7	+++	++	+
UM8	+	+	+	UTAD8	+++	+++	+
UM10	+++	+++	+	UTAD11	+++	++	+
UM13	++	+	-	UTAD12	++	+	+
UM17	+	+	+	UTAD18	+++	++	+
UM25	++	-	-	UTAD20	++	-	-
UM34	-	-	-	UTAD22	+++	++	+
UM41	++	+	-	UTAD25	++	+	+
UM53	+++	++	-	UTAD27	++	+	+
UM54	++	-	-	UTAD37	-	-	-
UM55	**++**	**++**	**++**	UTAD47	++	+	+
UM56	+++	++	-	UTAD48	+++	+++	+
UM59	-	-	-	UTAD49	++	+	+
UM61	+	+	+	UTAD50	++	-	-
UM62	++	+	-	UTAD52	+++	++	+
UM64	**++**	**++**	**++**	UTAD55	-	-	-
UM65	-	-	-	UTAD57	-	-	-
UM66	++	+	+	**UTAD58**	**++**	**++**	**++**
UM67	++	+	-	UTAD62	++	-	-
UM69	+	+	+	UTAD73	++	+	+
UM70	+	+	+	**UTAD78**	**++**	**++**	**++**
UM72	++	+	-	UTAD79	++	+	+
UM73	-	-	-	**UTAD89**	**++**	**++**	**++**
UM74	++	+	-	**UTAD90**	**++**	**++**	**++**
UM75	+++	+	-	**UTAD93**	**+++**	**++**	**++**
UM76	-	-	-	**UTAD94**	**+++**	**++**	**++**
UM77	**+++**	**+++**	**++**	**UTAD95**	**++**	**++**	**++**
UM81	+++	+	-	**UTAD100**	**+++**	**+++**	**++**
UM82	+	-	-	**UTAD101**	**++**	**++**	**++**
UM83	-	-	-	**UTAD102**	**++**	**++**	**++**
UM87	++	+	+	**UTAD103**	**++**	**++**	**++**
UM88	-	-	-	**UTAD104**	**+++**	**+++**	**++**
UM89	++	+	+	**UTAD118**	**++**	**++**	**++**
UM 111	++	+	+	UTAD121	+	+	+
UM 141	+++	++	+	**UTAD125**	**++**	**++**	**++**

	A. parasiticus		
Strain Nº	**3 days**	**5 days**	**7 days**
UM 168	+++	++	+
UM 224	+++	++	+
UM 228	**++**	**++**	**++**
UM 244	+++	+++	+
UM 271	+++	+++	+
UM 279	+++	+++	+
UM 296	+++	+++	+
UM 333	++	+	-

	A. parasiticus		
Strain Nº	**3 days**	**5 days**	**7 days**
UTAD131	-	-	-
UTAD158	**+++**	**++**	**++**
UTAD206	++	+	+
UTAD210	**+++**	**++**	**++**
UTAD212	+++	++	+
UTAD214	-	-	-
UTAD218	+++	++	+
UTAD220	**+++**	**++**	**++**

Among the 98 strains tested, 43, 46 and 36 strains showed no antifungal activity against *A. flavus*, *A. carbonarius* and *A. parasiticus* growth, respectively. In these cases, bacterial streaks were completely covered by the fungal growth as it can be seen Figure 3.1 B and Figure 3.2 A. From the remaining strains, only 5 demonstrated a strong activity against *A. flavus* MUM 17.14 growth. An example of the formation of an inhibition halo around the bacterial lines is demonstrated in Figure 3.1 A. Qualitative evaluation for ´+` and ´++` inhibitions are shown in Figure 3.2 B and C.

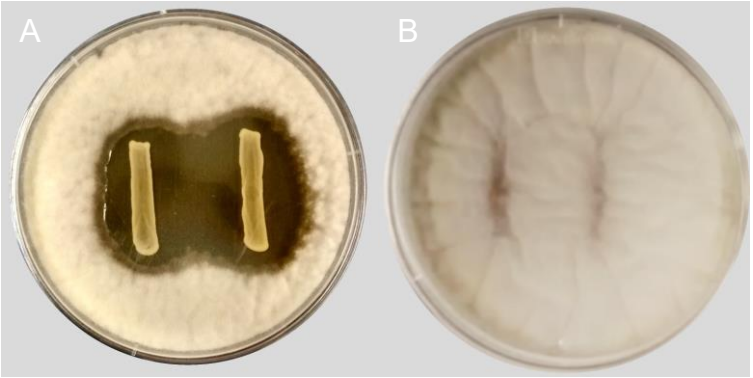


Figure 3.1. Example of antifungal activity by the "dual-culture overlay" method of (A) *L. plantarum* UM55 and (B) *L. casei* UM3, after 7 days of growth. An inhibition halo is visible around *L. plantarum* UM55 lines while no effect is noticeable for *L. casei* UM3.

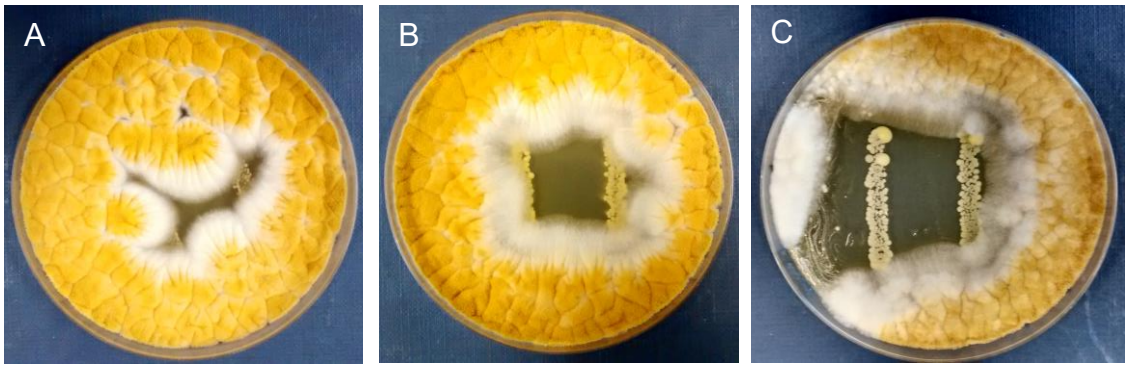


Figure 3.2. Example of antifungal activity against *A. parasiticus* MUM 92.02 by the "dual-culture overlay" method of (A) LAB UM333; (B) LAB UM 296 and (C) LAB UTAD158, after 7 days of growth. Figures represent qualitative inhibitions of -, + and ++, respectively.

For *A. carbonarius* and *A. parasiticus* none of the LAB tested indicated a strong inhibitory activity; however, 22 of these strains demonstrated substantial inhibition potential against these strains. The percentage of LAB strains that inhibit *A. flavus*, *A. carbonarius* and *A. parasiticus* and their level of inhibitory effect (no inhibition, mild inhibition, inhibition and strong inhibition) are shown in Figure 3.3. Thirteen of these strains showed considerable antifungal activity against the three fungal strains tested. Four strains demonstrated a strong antifungal activity against *A. flavus* MUM 17.14 and high inhibitions against *A. carbonarius* and *A. parasiticus*. These strains are *L. plantarum* UM7, *L. plantarum* UM55, *L. buchneri* UTAD104 and LAB UTAD118 (not identified).

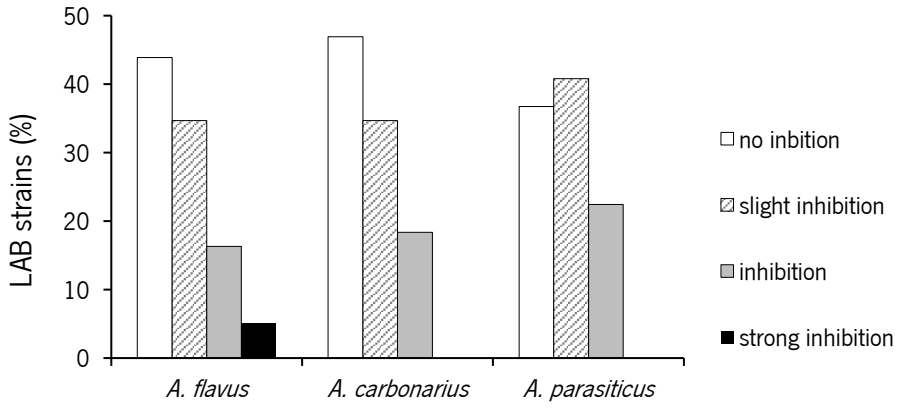


Figure 3.3. Antifungal activity of LAB strains by "overlay dual culture" method. Percentage of LAB strains showing no inhibition (white bar); slight inhibition (light grey bar); inhibition (grey bar) or strong inhibition (black bars) against tested fungal species.

Moreover, these 13 LAB isolates were selected for further tests using the "poisoned food technique". Fungal colonies were measured during 7 days of incubation, and at the end of this period mycotoxins were extracted and quantified by HPLC. Results of fungal growth, mycotoxin production and their inhibition, in percentage relative to controls (MRS agar without LAB CFS) are shown in Table 3.8 and Table 3.9.

Table 3.8. Fungal colony growth after 7 days of incubation in plates supplemented with 10% CFS from different LAB. Results are expressed as fungal colony diameter (mm). Corresponding percentage of inhibition relative to controls (MRS agar plates) are shown between brackets

	A. flavus	***A. carbonarius***	***A. parasiticus***
UM7	52.7 (29%)	65.8 (6%)	62.7 (15%)
UM55	51.0 (35%)	79.8 (4%)	63.7 (12%)
UM87	not tested	65.8 (11%)	not tested
UTAD8	53.2 (30%)	not tested	not tested
UTAD18	51.0 (31%)	75.8 (0%)	not tested
UTAD78	52.7 (29%)	70.7 (5%)	not tested
UTAD89	not tested	73 (4%)	not tested
UTAD93	52.7 (31%)	not tested	not tested
UTAD94	not tested	73.3 (4%)	not tested
UTAD95	not tested	73.5 (4%)	not tested
UTAD104	not tested	67.2 (10%)	not tested
UTAD118	52.0 (32%)	not tested	not tested
UTAD206	46.5 (37%)	82.7 (3%)	not tested

Tested strains were able to inhibit *A. flavus* radial growth by approximately 30% (Table 3.8) and its aflatoxin production by over 65% (Table 3.9), when compared with control. Results for *A. carbonarius* colony growth were less remarkable, since maximum growth inhibition obtained was 11% and most strains did not affect OTA production. Strains UM7 and UM55 were the only tested for *A. parasiticus* inhibition and while growth reduction was modest (bellow 15%), both strains inhibited aflatoxin production by over 90%. Results indicate that different fungal species respond differently to the presence of the antifungal strains, with *A. carbonarius* being more resistant to the CFS antifungal effect. Additional, these preliminary results show that reduction of mycotoxin production is not directly correlated with the inhibition of the fungal growth.

From Table 3.8 and Table 3.9, it is observed that, *L. plantarum* UM55 exhibited a distinct antifungal activity against the three tested fungal species, especially in reducing mycotoxin production. Also, the isolate *L. plantarum* UM7 showed consistent results for the tested fungal strains, even though it was not able to reduce OTA production.

Table 3.9. Mycotoxin content produced by fungal colonies, after 7 days, in plates supplemented with 10% of CFS from different LAB. Results are expressed as mycotoxin content in μg/plate. Corresponding percentage of inhibition respective to controls (MRS agar plates) is shown between brackets. For aflatoxins, results express the sum of all aflatoxin types detected

	A. flavus **(AF)**	***A. carbonarius*** **(OTA)**	***A. parasiticus*** **(AF)**
UM7	6.9 (72%)	32.4 (0%)	3.5 (95%)
UM55	0.6 (97%)	31.1 (15%)	6.4 (92%)
UM87	not tested	45.2 (0%)	not tested
UTAD8	7.6 (67%)	not tested	not tested
UTAD18	4.6 (71%)	46.3 (0%)	not tested
UTAD78	7.2 (78%)	33.0 (28%)	not tested
UTAD89	not tested	66.9 (0%)	not tested
UTAD93	5.7 (72%)	not tested	not tested
UTAD94	not tested	67.8 (0%)	not tested
UTAD95	not tested	68.1 (0%)	not tested
UTAD104	not tested	42.3 (0%)	not tested
UTAD118	5.8 (70%)	not tested	not tested
UTAD206	1.7 (85.6%)	45.0 (0%)	not tested

Further fungal inhibition tests were performed using living LAB cells instead of CFS. Since *A. carbonarius* was the most resistant species to the antifungal ability of the CFS, this fungus was used in this assay. Cell cultures were incorporated in MEA plates after 48 h of growth in MRS medium and at the same proportion used for CFS (10%). Cell culture was replaced with MRS broth in the control experiments. Results for fungal growth inhibition and OTA accumulation are shown in Table 3.10. The tested LAB strains demonstrated the capacity of inhibiting *A. carbonarius* growth between 24 and 34%, with the exception of strain UTAD104, which was more effective, reducing the fungal growth by approximately 80%, when compared with the control. The inhibitory capacity of this strain was further confirmed in the production of OTA since it was almost totally inhibited (99%). Among the other strains, only the LAB UM7 and UM87 were able to reduce OTA content, but inhibitions reached only

approximately 30%. Strain UTAD104, which CFS did not have a significant inhibition ability (Table 3.8), demonstrate to be efficient in reducing *A. carbonarius* growth (79%) and OTA production (99%) in these assays.

Table 3.10. *A. carbonarius* growth and OTA content produced by fungal colonies, after 7 days, in plates supplemented with 10% of cell culture from different LAB. Growth results are expressed in colony diameter (mm) and OTA content in μg/plate. Corresponding percentage of inhibition respective to controls are shown between brackets

	***A. carbonarius* growth (mm)**	**OTA (μg/plate)**
UM7	49.0 (34%)	23.9 (27%)
UM87	56.8 (24%)	22.5 (31%)
UTAD89	54.3 (29%)	70.1 (0%)
UTAD94	53.8 (29%)	62.1 (0%)
UTAD95	57.7 (24%)	87.3 (0%)
UTAD104	15.8 (79%)	0.4 (99%)
UTAD206	58.2 (24%)	71.5 (0%)

From the preliminary selection of 13 isolates that showed a pronounced antifungal activity, *L. plantarum* UM55 and *L. buchneri* UTAD104 were selected for further studies for potentially having a broad-spectrum antifungal activity.

L. plantarum is one of the most studied LAB, this species is flexible and versatile and it is found in a variety of environments including dairy, meat and vegetables (Martino et al. 2016). Additionally, *L. plantarum* is frequently encountered as a natural inhabitant of the human gastrointestinal tract and has also been found in breast milk (de Vries et al. 2006; Jiang et al. 2016). This ecological flexibility is reflected by the fact that this species has one of the largest genomes among LAB.

L. plantarum has a long story of safe use in a variety of foods, including cheese (Hynes et al. 2001; Manolopoulou et al. 2003), green olives (Ruiz-Barba et al. 1994; Ercolini et al. 2006), sauerkraut (Beganovic et al. 2011), pickles and other fermented vegetables, wine (Pozo-Bayón et al. 2005), fermented sausages (Enan et al. 1996; Cocolin et al. 2000) and sourdough (Leroy and De Vuyst 2004). Furthermore, *L. plantarum* has demonstrated probiotic properties. Studies have documented that specific *L. plantarum* strains seem to have beneficial effects in patients with irritable bowel syndrome (Ducrotté et al. 2012), that this species show a high tolerance to exposure to hydrochloric acid and bile

salts (Haller et al. 2001; Zago et al. 2011), and that it displayed a high survival *in vivo* following human ingestion, and the ability to colonize the gut with apparent safety to the consumer (Vesa et al. 2000). Other studies show that administration of *L. plantarum* reduces the carriage of faecal enterobacteriacea (Kingamkono et al. 1999), and reduces cardiovascular disease risk factors (Zapolska-Downar et al. 2002). However, studies describing probiotic benefits of *L. plantarum* have a great variability in the experimental setup and quality. Still, probiotic claims of *L. plantarum* allowed the development of several commercial probiotic formulations (de Vries et al. 2006).

L. plantarum is also recognized by its antimicrobial activity mainly through the production of organic acids, but also compounds of proteinaceous nature and fatty acids, with numerous authors reporting the production of this metabolites and their antifungal effects (Niku-Paavola et al. 1999; Lavermicocca et al. 2000; Ström et al. 2002; Sjögren et al. 2003; Prema et al. 2010; Arasu et al. 2014; Russo et al. 2017). Several authors also emphasised its capability for production of bacteriocins (Ogunbanwo et al. 2003; Todorov and Dicks 2005). *L. plantarum* strains have previously been shown to have effect on the growth and mycotoxin production of *A. flavus* and *A. parasiticus* (Sangmanee and Hongpattarakere 2014), and also have been described has having a broad antifungal spectrum and inhibitory effect in OTA production (Prema et al. 2010; Belkacem-Hanfi et al. 2014).

Lactobacillus buchneri is a Gram-positive obligate heterofermentative and facultative anaerobic bacteria, capable of growth by metabolizing hexoses and pentoses to lactic acid, acetic acid and traces of ethanol and carbon dioxide (Elferink et al. 2001). It has been isolated from a vast range of foods and feeds due to its wide metabolic ability. Isolation environments include human intestines, fermented sorghum, wine, beer wort, tomato pulp, Swiss cheese and silages (Daughtry et al. 2018).

This *Lactobacillus* species has the particular ability to metabolize lactic acid into acetic acid and 1,2-propanediol and, due to this distinctive metabolic activity, it has been proposed for several industrial applications (Elferink et al. 2001). One specific *L. buchneri* strain, isolated from pickles, showed potential probiotic ability (Zeng et al. 2010), another strain, isolated from sauerkraut, produced bacteriocins against several Gram-positive bacteria (such as *Listeria*, *Bacillus*, *Enterococcus*, *Streptococcus* and *Pediococcus* (Yildirim and Yildirim 2001)) and another *L. buchneri* strain (NRRL B-30929) has been exploited for bioethanol production and was the first strain of this species to have its genome fully sequenced (Liu et al. 2011).

Still, currently, *L. buchneri* main application is as started cultures in the fermentation of silage. Lactic acid conversion in acetic and propionic acids by *L. buchneri* in silage, acts as a natural

antimicrobial against aerobic spoilage yeast and fungi that are often responsible for the degradation of dry matter in silage. The ability of *L. buchneri* to produce propionate can also be used to extent food products shelf life without the addition of preservatives (Zhang et al. 2010).

The selection of antagonistic strains has largely been studied because of their possible application in food preservation with the aim to extend shelf life and reduce or even replace "chemical" additives, as requested by the consumers.

3.3.2 Anti-aflatoxigenic Effect of Organic Acids Produced by *Lactobacillus plantarum*

3.3.2.1 Characterization of antifungal properties of Cell-free supernatants from L. plantarum UM55

In order to identify the nature of the antifungal activity of *L. plantarum* UM55 against *A. flavus* MUM 17.14, the effect of CFS after its treatment by heat, proteases and pH neutralization was tested. Without any treatment, antifungal activity of *L. plantarum* UM55 was moderate for fungal growth since a reduction of about one third was reached, however reduction in aflatoxins production was considerable, reaching an inhibition higher than 90%. It was observed that *L. plantarum* UM55 CFS retained its inhibitory properties against fungal growth and aflatoxins production after being autoclaved and treated with proteases since the reduction of fungal colony size remained around 30% and the reduction of AF production around 90% (Figure 3.4).

Several authors have described the potential of LAB to inhibit the growth of different fungi (Niku-Paavola et al. 1999; Lavermicocca et al. 2000; Ström et al. 2002; Magnusson et al. 2003; Sjögren et al. 2003; Gerez et al. 2009; Prema et al. 2010; Yang and Chang 2010; Cortés-Zavaleta et al. 2014; Sangmanee and Hongpattarakere 2014) and others reported its influence in the inhibition of aflatoxins (Gourama and Bullerman 1995b; Gourama and Bullerman 1997; Chang and Kim 2007; Fernández-Juri et al. 2011; Bayankaram and Sellamuthu 2016).

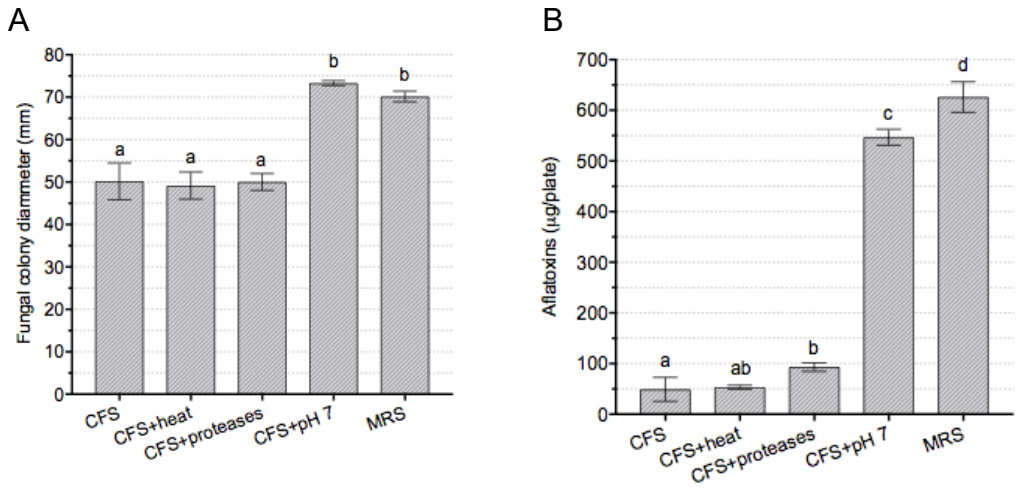


Figure 3.4. Effect of *L. plantarum* UM55 CFS in: (A) growth and (B) aflatoxin production of *A. flavus* MUM 17.14. Error bars indicate a standard deviation of n=3. Statistically significant differences for each condition are indicated with different letters, $p<0.05$ (one-way ANOVA and Sidak's Post-hoc test). Data used for the construction of this figure can be seen in Table A.1 and Table A.2 of appendix A).

When *L. plantarum* UM55 CFS was submitted to pH neutralization the antifungal activity was lost. The inhibitory effect on fungal growth dropped to 0% and on AF production to 13%. Gerez et al. (2009) reported a similar effect to *L. plantarum* UM55 for other *L. plantarum* strains, observing that CFS was not affected by the heat treatment or the enzymatic action of proteinase K, but that pH adjustment to 7 severely reduced the antifungal activity.

Because the pH greatly influenced the CFS activity of *L. plantarum* UM55, it was hypothesized that organic acids could be the main element responsible for the antifungal and anti-aflatoxigenic effect observed. Organic acids act by diffusing through the membrane of target organisms in the undissociated state, reducing the intracellular pH, thus causing the stop of metabolic activities (Brul and Coote 1999). Lactic and acetic acids are the most commonly reported organic acids in LAB supernatants once they are the main products of carbohydrate fermentation. Other organic acids such as propionic, formic, butyric, phenyllactic, hydroxyphenyllactic, coumaric, benzoic, salicylic, vanillic, and azealic acids are also produced by LAB, and some have been reported as potential antifungal agents (Corsetti et al. 1998; Niku-Paavola et al. 1999; Lavermicocca et al. 2000; Magnusson and Schnurer 2001; Ström et al. 2002; Magnusson et al. 2003; Broberg et al. 2007; Gerez et al. 2009; Prema et al. 2010; Rizzello et al. 2011; Ryan et al. 2011; Gerez et al. 2013). Since CFS antifungal effect has been

fully reverted when pH was adjusted to 7, in the following experiments neutralized CFS was used as control.

When the effect of different concentrations of CFS in culture medium was tested, a decrease of aflatoxin production with increasing concentrations of *L. plantarum* UM55 CFS (Figure 3.5 B) was observed. Even though the complete inhibition of aflatoxin production was not achieved, it reached a maximum of 95% with 25% of CFS. When 5% of CFS was used, the inhibition of AF was limited to 50%. Fungal growth was less affected, but followed the same trend exhibiting percentages of reduction that varied between 24 and 28% (Figure 3.5 A). Sangmanee and Hongpattarakere (2014) reported similar results when they increased the concentration of *L. plantarum* supernatant in media.

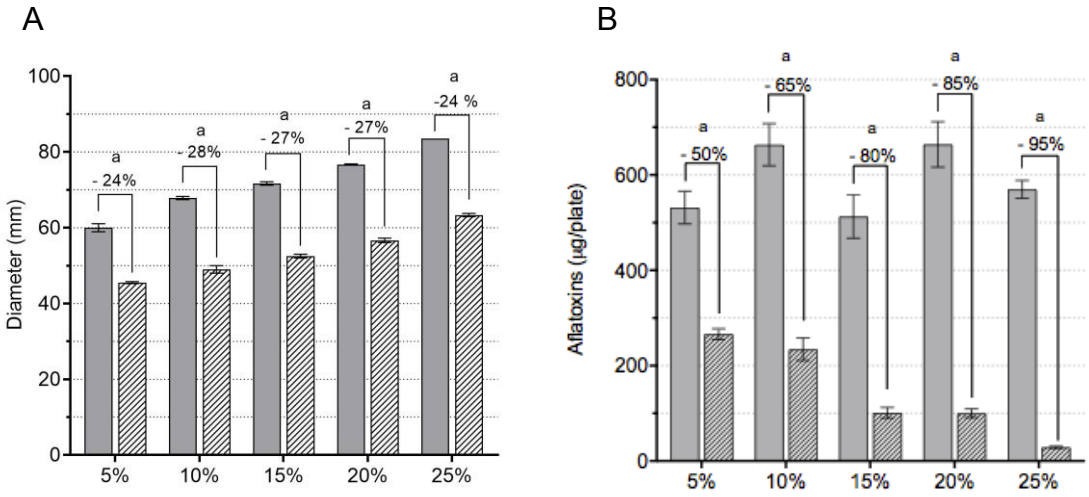


Figure 3.5. Fungal colony diameter (A) and aflatoxins content (B) in the presence of 5 to 25% of *L. plantarum* UM55 CFS. Bars represent control experiments (light grey) and *L. plantarum* UM55 CFS (stripe-pattern). Percentages represent differences between control and CFS. All were significantly different at $p<0.05$ (two-way RM ANOVA and Sidak's Post-hoc test). Data used for the construction of this figure can be seen in Table A.3 and Table A.4 in appendix A)

Antifungal activity of *L. plantarum* UM55 was also tested over an incubation period of 22 days. The growth of fungal colonies and aflatoxin production are shown in Figure 3.6. In the control experiments, colonies covered the entire area of petri dish after 9 days, and the production of aflatoxins stabilized after this point most probably because of depletion of nutrients (black symbols in Figure 3.6). In plates supplemented with *L. plantarum* UM55 CFS (open symbols in Figure 3.6), fungal growth as well as aflatoxin production increased much more slowly than in controls and stopped approximately after 15 days. In this case, colonies never reached the entire area of the petri dish and production of aflatoxins

stabilized at a much lower value. After 22 days, a reduction of aflatoxin production of approximately 70% was observed. On the other hand, it was also observed that colonies cultivated in the CFS supplemented medium that possessed the same size of control colonies, produced much less aflatoxins (e.g. CFS-colonies of day 9 with 57 mm produced less 63% of aflatoxins than control colonies of day 6 with 58 mm). This experiment demonstrates that the reduction of aflatoxin production induced by *L. plantarum* UM55 CFS does not depend only on colony growth decrease. So, it can be assumed that aflatoxin biosynthesis was disturbed by some compound present in *L. plantarum* UM55 CFS.

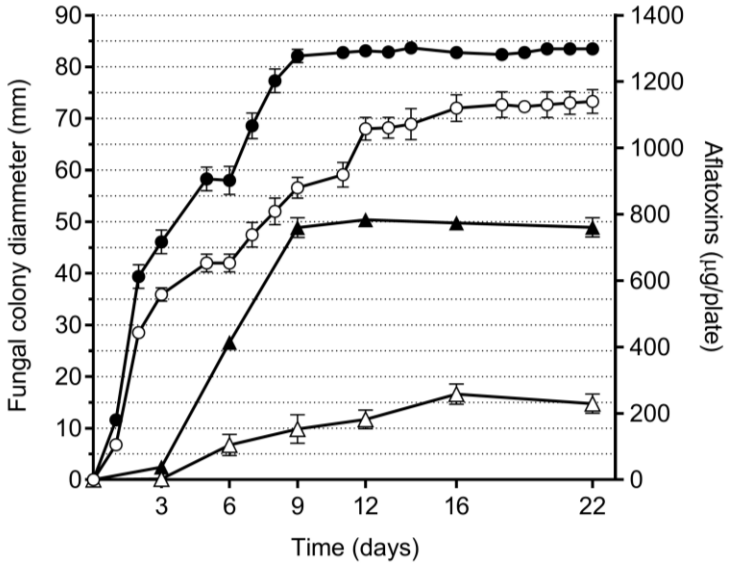


Figure 3.6. Inhibition effect of *L. plantarum* UM55 CFS over an extended period of incubation (22 days). *A. flavus* MUM 17.14 growth (circles) and production of aflatoxins (triangles) in the presence of 10% of CFS (open symbols) and control experiment with CFS with pH adjusted to 7 (closed symbols). In both graphs, error bars indicate a standard deviation of n=3. Data used for the construction of this figure can be seen in Table A.5 and Table A.6 in appendix A).

Antifungal activity of *L. plantarum* UM55 was also tested against other aflatoxigenic species. *L. plantarum* UM55 CFS at 10% in culture medium inhibited the fungal growth of all fungal strains tested. Growth inhibition of fungal colonies was between 4 and 26%, with the higher inhibition being observed for *A. flavus* MUM 17.14 (Figure 3.7 A). Furthermore, all fungal strains produced less aflatoxins in the presence of CFS than in controls (Figure 3.7 B).

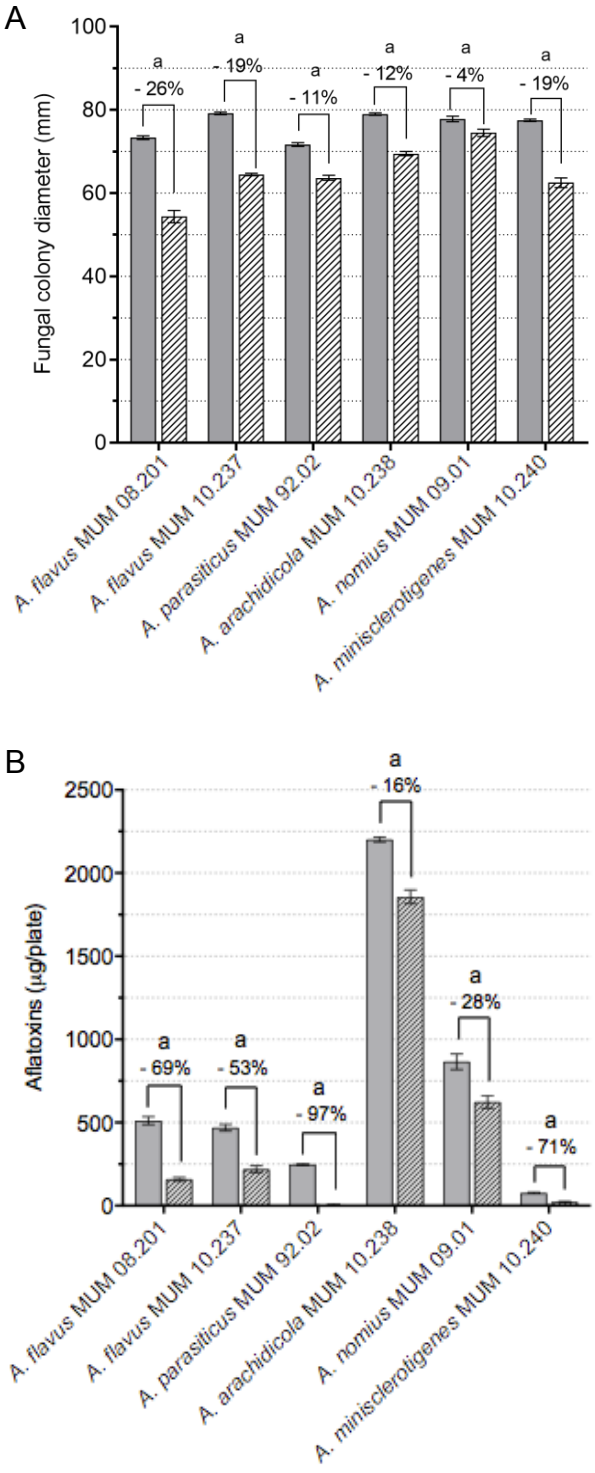


Figure 3.7. Fungal colony diameter (A) and aflatoxin produced (B) by several aflatoxigenic species in the presence of 10% of CFS from *L. plantarum* UM55 (stripe-pattern) and control done with CFS with pH adjusted to 7 (light grey). Error bars indicate a standard deviation of n=3. Data used for the construction of this figure can be seen in Table A.7 and Table A.8 in appendix A).

The anti-aflatoxigenic effect of *L. plantarum* UM55 CFS was stronger for *A. parasiticus* since a 97% decrease in aflatoxin production was observed. In plates supplemented with *L. plantarum* UM55, *A. flavus* MUM 17.14, *A. flavus* MUM 10.237 and *A. minisclerotigenes* MUM 10.240 reduced their aflatoxin production in 69%, 53% and 71%, respectively. Two species, *A. arachidicola* MUM 10.238 and *A. nomius* MUM 09.01 were less inhibited since a reduction in aflatoxins production of only 16 and 28% were respectively observed (Figure 3.7 B).

3.3.2.2 Antifungal properties of individual organic acids

Since it was observed that neutralization of CFS reverted its anti-aflatoxigenic effects, the presence of organic acids in *L. plantarum* UM55 CFS was investigated by HPLC. Organic acid profiles of *L. plantarum* UM55 were compared with those of *L. casei* UM3 (one of the strains that did not demonstrate antifungal activity) and MRS broth. Main differences in HPLC chromatograms between *L. plantarum* UM55 CFS and controls were detected at retention times corresponding to peaks of lactic acid, OH-PLA, PLA and ILA (Figure 3.8).

In MRS broth none of these acids were detected while in *L. plantarum* UM55 CFS their concentration was significantly higher than in CFS from *L. casei* UM3 (Table 3.11). The amounts of lactic acid, PLA, OH-PLA, and ILA in *L. plantarum* UM55 CFS were 4-fold, 2-fold, 10-fold, and 7-fold higher than in LAB UM3 CFS, respectively. Acetic, propionic and butyric acids, which may also be produced by LAB and are described as also having antifungal properties, were not found in the supernatant of tested the strains, when using this detection method.

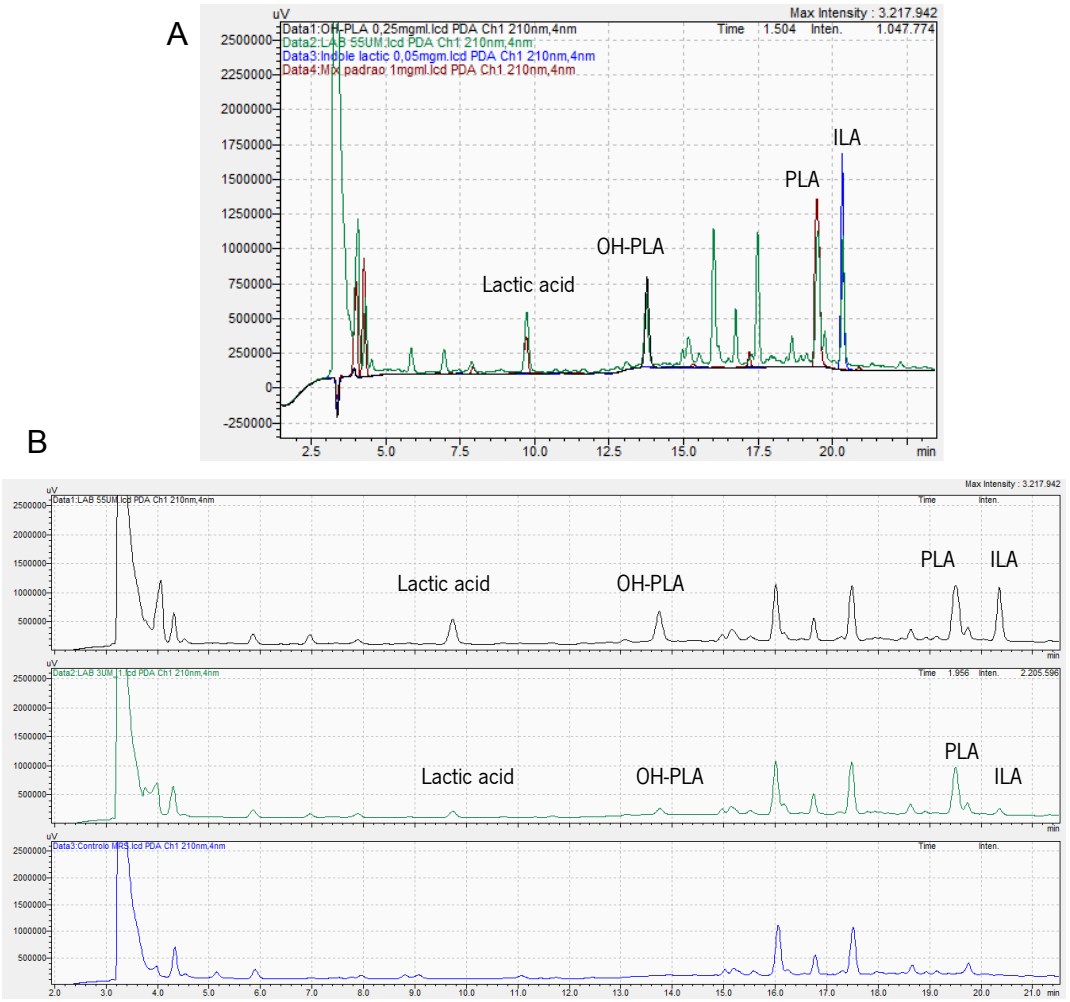


Figure 3.8. Chromatograms of *L. plantarum* UM55 CFS (A) overlayed with standards (where the green line represents *L. plantarum* UM55 CFS; brown line represents lactic acid and PLA standards; black line represents OH-PLA standard and blue line represents ILA standard); (B) compared to MRS broth (where the black chromatogram line represents *L. plantarum* UM55 CFS green chromatogram line represents *L. casei* UM3 and the blue chromatogram line the MRS broth).

Table 3.11. Concentration of lactic acid, OH-PLA, PLA and ILA present in *L. plantarum* UM55 CFS, *L. casei* UM3 CFS and MRS broth.

	Lactic acid (g/L)	PLA (mg/L)	OH-PLA (mg/L)	ILA (mg/L)
L. plantarum UM55 CFS	4.3 ± 0.2	74.8 ± 0.4	30.8 ± 1.5	6.7 ± 0.1
L. casei UM3 CFS	1.0 ± 0.1	40.9 ± 1.0	3.1 ± 0.5	0.9 ± 0.1
MRS broth	0	0	0	0

Data for the construction of this table can be seen in Table A.9.

Following, the antifungal properties of these acids were tested individually using the "food poisoning" method (shown in Table 3.12). Overall, all tested acids were able to inhibit to some extent the fungal growth and aflatoxin production. However, the minimum concentration at which they had their maximum effect was different for each one.

Table 3.12. Inhibition (%) of *A. flavus* MUM 17.14 fungal growth and aflatoxin production with different organic acids (lactic acid, PLA, OH-PLA and ILA) at different concentrations and pH of the media at those concentrations.

		Organic acid concentration (mg/mL)								
	Inhibition (%)	**0**	**0.1**	**0.25**	**0.5**	**1**	**1.5**	**2**	**4**	**8**
Lactic acid	Fungal growth	0.0	3.2	10.6	17.7	19.1	21.9	22.6	23.9	25.8
	AF	0.0	1.3	3.27	24.1	50.7	59.3	69.3	96.7	99.6
	pH	5.4	4.6	4.9	4.2	4.0	3.7	3.5	3.2	2.9
PLA	Fungal growth	0.0	4.2	6.3	11.3	12.0	14.5	15.5	36.5	82.3
	AF	0.0	9.5	28.8	68.0	94.7	99.6	99.8	100.0	100.0
	pH	5.4	4.5	4.8	4.6	4.3	4.1	4.0	3.6	3.3
OH-PLA	Fungal growth	0.0	1.0	12.0	15.5	17.7	16.4	15.3	14.3	15.3
	AF	0.0	40.8	59.5	71.9	83.2	85.9	88.5	97.2	99.9
	pH	5.4	5.1	4.8	4.5	4.2	4.0	3.9	3.5	3.2
ILA	Fungal growth	0.0	0.0	0.0	0.0	0.0	0.0	4.7	33.6	47.5
	AF	0.0	7.5	36.0	52.4	84.7	92.3	98.3	99.9	99.9
	pH	5.4	5.1	4.7	4.6	4.3	4.1	3.7	3.6	4.1

Data for the construction of this table can be seen in Table A.10, Table A.11, Table A.12, Table A.13, Table A.14, Table A.15, Table A.16 and Table A.17 from appendix A

As for *L. plantarum* UM55 CFS, all organic acids tested inhibited to a greater extent the production of aflatoxin than the fungal growth. At the maximum concentration used (8 mg/mL), PLA was the most effective acid at inhibiting fungal growth with an inhibition of 82%, followed by ILA, lactic acid, and OH-PLA with inhibitions of 48%, 26%, and 15%, respectively. Concerning the production of aflatoxins, an inhibition >99% was achieved with 1.5 mg/mL of PLA, 4 mg/mL of ILA, 8 mg/mL of lactic acid, and 8 mg/mL of OH-PLA. At those concentrations, the growth of the fungus was inhibited respectively by 15%, 34%, 26% and 15%. The pH of the culture media was 4.1, 3.6, 2.9 and 3.2 for PLA, ILA, lactic acid and OH-PLA, respectively. Thus, the stronger effect of PLA on aflatoxin is not a result of pH drop, neither dependent only on fungal growth. At those pH, and considering the pKa values of 4.02, 4.06, 3.86 and

4.00 for PLA, ILA, lactic acid and OH-PLA, respectively, the percentage of the undissociated form of each acid was 45%, 74%, 90% and 86%, respectively. Therefore, the stronger effect of PLA also appears not to be dependent on the percentage of its undissociated form in the medium since it has the lowest of them.

IC_{50} and IC_{90} calculated for each organic acid are shown in Table 3.13 (graphic representation of IC_{50} and IC_{90} can be seen in appendix A in Figure A.13, Figure A.14 and Figure A.15).

Table 3.13. Inhibitory concentrations (IC_{50} and IC_{90} in mg/mL) of lactic acid, OH-PLA, PLA and ILA for *A. flavus* MUM 17.14.

	Fungal growth			**Aflatoxins production**		
	IC_{50} (mg/mL) (95% CI)	IC_{90} (mg/mL) (95% CI)	R^2	IC_{50} (mg/mL) (95% CI)	IC_{90} (mg/mL) (95% CI)	R^2
Lactic acid	n.a.	n.a.	n.a.	1.09 (1.01 - 1.16)	3.92 (3.41 - 4.5)	0.9890
PLA	4.37 (3.98 - 4.79)	11.91 (10.04 - 14.36)	0.9799	0.36 (0.35 - 0.37)	0.87 (0.81 - 0.93)	0.9967
OH-PLA	n.a.	n.a.	n.a.	0.18 (0.16 - 0.19)	1.80 (1.51 - 2.15)	0.9908
ILA	n.a.	n.a.	n.a.	0.40 (0.37 - 0.43)	1.47 (1.27 - 1.70)	0.9904

IC_{50} - Concentration of organic acid which produced an inhibition of 50%; IC_{90} - Concentration of organic acid which produced an inhibition of 90%; 95% CI - Confidential interval of calculated values; R^2 - R-square of fitted curves; n.a. - not applicable, the data does not allow us to calculate the ICs. Data for the construction of this table can be seen in Table A.10, Table A.11, Table A.12, Table A.13, Table A.14, Table A.15, Table A.16 and Table A.17 in appendix A

Concerning fungal growth, ICs were only calculated for PLA because, for the other acids, the percentages of inhibition achieved were insufficient to fit the model. IC_{50} and IC_{90} of PLA were of 4.37 and 11.91 mg/mL, respectively. PLA has been first appointed as a LAB antifungal compound by Lavermicocca et al. (2000) and in this study, PLA and OH-PLA have been identified in an active fraction of *L. plantarum* strain 21B CFS. When used individually, both compounds exhibited antifungal activities. PLA had the highest activity and inhibited the growth of several fungi at 50 mg/mL in a disk assay. Later, the same authors concluded that less than 7.5 mg/mL of PLA was enough to obtain a 90%

growth reduction in 14 fungal species and that PLA had a synergetic effect when combined with lactic or acetic acid (Lavermicocca et al. 2003). More recently, Cortés-Zavaleta et al. (2014) also associated the production of PLA with the antifungal properties of several LAB. In this case, growth inhibition of tested fungi ranged from 3 to 67% and PLA produced by LAB between 3.5 and 45.7 mg/L. The strain that resulted in the highest inhibition of fungal growth, also produced the highest amounts of PLA, even though authors suggested that a synergistic effect between PLA and other organic acids was responsible for the inhibition observed. These authors also reported a minimal inhibitory concentration of 6 mg/mL for PLA. Comparing this data with the data from the current thesis, it can be considered that our IC_{90} of 11.91 mg/mL is almost 2 times higher than values reported in mentioned works, however different inhibition responses are expected from different fungal strains.

Concerning aflatoxins inhibition, the lowest IC_{50} was obtained for OH-PLA (0.18 mg/mL), followed by PLA (0.36 mg/mL), ILA (0.40 mg/mL), and lactic acid (1.09 mg/mL). Differently, the lowest IC_{90} was obtained for PLA (0.87 mg/mL), followed by ILA (1.47 mg/mL), OH-PLA (1.80 mg/mL) and lactic acid (3.92 mg/mL). Therefore, compared to PLA, OH-PLA showed a stronger effect at lower concentrations (0.1 mg/mL of OH-PLA was sufficient to inhibit the production of aflatoxins in 48% without inhibiting the growth of the fungus) but less potency at the highest ones. This may result from the effect of PLA on fungal growth, which is approximately 5.4 times stronger than the one observed for OH-PLA at 8 mg/mL. From ICs obtained it can also be observed that lactic acid is the less effective of the organic acids in inhibiting the production of aflatoxins. To obtain a reduction of 90%, it was necessary almost 4.5 times more concentration of lactic acid than PLA, 2.2 times more than OH-PLA, and 2.7 times more than ILA. When comparing these data with the concentration of organic acids in *L. plantarum* UM55 CFS (Table 3.11), it can be concluded that the antifungal and antiaflatoxigenic effects of CFS result mostly from the high quantity of lactic acid (4.3 g/L) produced by the strain since in the individual assays, 4 g/L of lactic acid inhibited the growth of *A. flavus* in 25% and the production of aflatoxins in 97%. Even so, PLA, OH-PLA and ILA (total of 0.112 g/L) may, probably, also be involved in the inhibitory properties of CFS since we observed that 0.1 g/L of PLA inhibited the fungal growth in 4.2% and the production of aflatoxins in 9.5%. At the same concentration, OH-PLA and ILA did not inhibit fungal growth but inhibited the production of aflatoxins in 40.8% and 7.5%, respectively. The synergism between some of these acids has been observed by Lavermicocca et al. (2003) for the inhibition of fungal growth, with a mixture of lactic acid (15.8 mg/mL) with PLA (5 mg/mL) causing an increase of about 30% in PLA inhibitory activity against *A. niger*; when lactic acid was tested alone, it

caused a reduced inhibitory effect; and similar results were obtained by Ndagano et al. (2011) and Axel et al. (2016).

Despite the many studies describing the capacity of PLA and OH-PLA to inhibit fungal growth, to date, no study analysed the influence of these compounds on the production of mycotoxins. PLA and OH-PLA are both involved in the phenylalanine and tyrosine metabolism. Accumulation of phenylalanine is avoided by the hydrolysation of this amino acid to tyrosine or transamination to phenylpyruvic acid, which is consequently metabolized to PLA and OH-PLA. These metabolites have also been found to be involved in the formation of cheese flavours (Gummalla and Broadbent 2001). Furthermore, it is the first time that ILA antifungal properties are studied. ILA is a product of tryptophan catabolism in lactobacilli through successive transamination and dehydrogenation reactions. Gummalla and Broadbent (1999) described the accumulation of ILA in supernatants of *L. casei* grown in media supplemented with tryptophan and concluded that this conversion might restrict the synthesis of indole-3-acetic acid, a compound that produces off-flavours in cheese. Indole-3-acetic acid was not detected in CFS of *L. plantarum* UM55, which can indicate that the metabolic pathway that produced ILA is favoured. ILA has been found to have antimicrobial activity against Gram-negative and Gram-positive bacteria (Rao and Reddy 1984) but the influence of ILA in fungi has not, to my knowledge, been described before. Here, it is shown that at a concentration of 8 mg/mL can inhibit the growth of *A. flavus* by 48%.

These properties can make *L. plantarum* UM55 a promising biological solution to reduce aflatoxin levels in food or feed systems in order to comply with limits imposed by global regulations. For example, this strain (or other strains with similar organic acid profile) can be useful for application in silages, feed or even in crop fields. In silage, it is well known that the addition of LAB favours its stability because of rapid pH decrease. The acidic conditions of silage have been considered unfavourable for *A. flavus* growth and aflatoxin production in some reports (Scudamore and Livesey 1998). This may explain why aflatoxins are not detected frequently or detected at low levels on well-preserved silage (Garon et al. 2006). Therefore, the use of LAB strains with well-studied anti-mycotoxin properties and higher production capabilities of PLA, OH-PLA and ILA can be an important advantage to take into account in future strain selection programs of silage inoculants. In crop fields, *L. plantarum* UM55 and/or their organic acids could be used as a biocontrol agent to prevent the production of aflatoxin by aflatoxigenic species in the soil and/or in plants. Besides controlling the growth of aflatoxigenic fungi, they would be able to inhibit the production of aflatoxin, reducing the levels in harvested commodities.

Still, further studies of how this organic acids act, both together and individually, on fungal development and aflatoxin production pathways are needed, as are further studies for their application in silage, feed or crop fields or cultures.

3.3.3 Antifungal Effect of Organic Acids from Lactic Acid Bacteria on *Penicillium nordicum*

3.3.3.1 Characterization of antifungal properties of cell-free supernatants from lactic acid bacteria strains

The inhibitory effect of *L. plantarum* UM55 and *L. buchneri* UTAD104 CFS (at a 10% concentration) from on the growth of *P. nordicum* MUM 08.16 was weak since reductions of only 17% and 10% were achieved, respectively. Nonetheless, the effect in OTA production was considerably higher, as it reached inhibitions of 63% and 61% for *L. plantarum* UM55 and *L. buchneri* UTAD104 CFS, respectively.

Cell-free supernatants were further subjected to treatments by heat, proteases and pH neutralisation, in order to determine the nature of the compounds responsible for the antifungal activity. It was observed that both *L. plantarum* UM55 and *L. buchneri* UTAD104 CFS retained their inhibitory properties after being autoclaved and treated with proteases, but not after the pH neutralisation (Figure 3.9). Results show that *L. plantarum* UM55 CFS treated with pepsin and heat reduced fungal colony size in 16% and 24%, respectively, and OTA production in approximately 57% and 53%, respectively. Similar results were obtained with *L. buchneri* UTAD104 CFS. Inhibition values for both of these treatments were not significantly different from inhibition caused by untreated CFS showing that they were not able to revert CFS effects. Contrariwise, for both LAB strains, the CFS that was submitted to pH neutralisation lost its antifungal activity. The growth of fungi achieved similar values to MRS control and OTA produced by the fungal colonies increased. Other authors reported the same effect in *Penicillium* species (Belkacem-Hanfi et al. 2014).

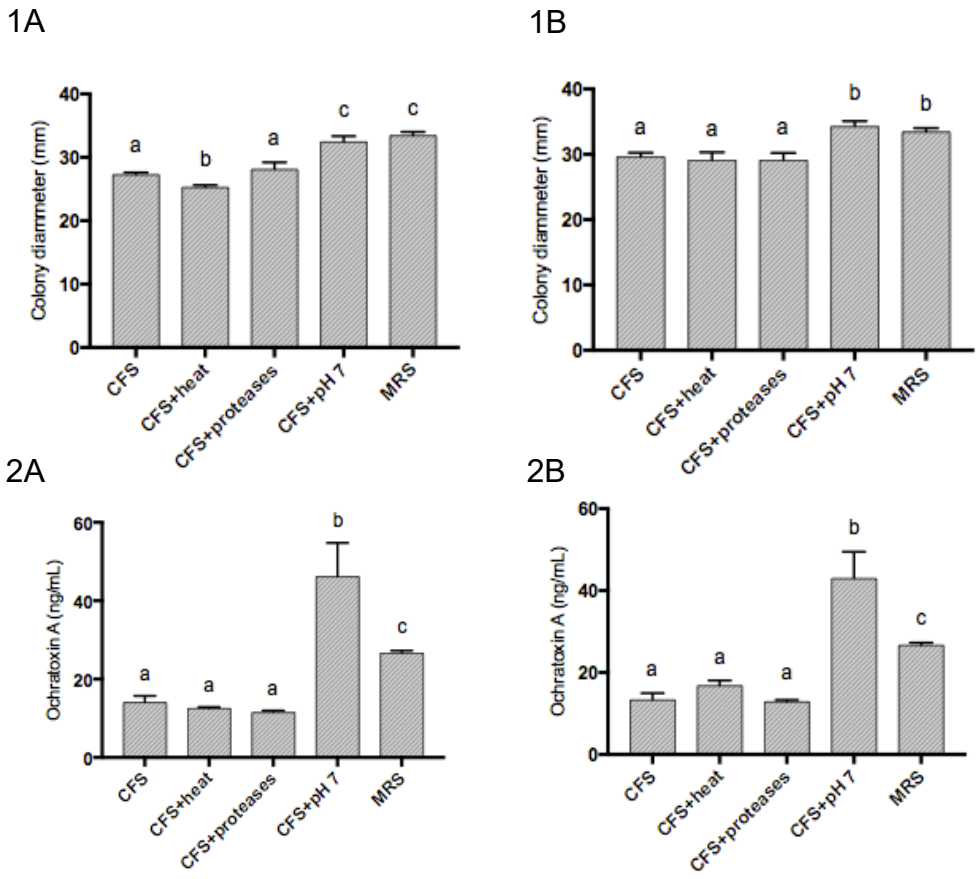


Figure 3.9. Effect of CFS on fungal growth (1) and ochratoxin A production (2) of *P. nordicum* MUM 08.16. Effect of *L. plantarum* UM55 (A) and *L. buchneri* UTAD104 (B). Error bars indicate a standard deviation of n=3. Statistically significant differences for each condition are indicated with different letters, $p<0.05$ (one-way ANOVA and Tukey's post-hoc test). Data for the construction of this figure is shown in Table A.18, Table A.19, Table A.20 and Table A.21 in appendix A.

Again, since the pH greatly influenced the CFS activity of these strains, it was assumed that the secreted organic acids were the primarily responsible for the described antifungal effect. In following experiments, CFS with pH 7 were used as the control because antifungal effects were reverted when pH of CFS was neutralised.

3.3.3.2 Characterization of antifungal properties of organic acids present in cell-free supernatants from lactic acid bacteria

Since neutralisation of CFS reverted its anti-ochratoxigenic effect, the presence of organic acids in CFS of *L. buchneri* UTAD104 was investigated by HPLC. Organic acid profiles of both LAB were

compared with MRS broth. As stated before, differences between *L. plantarum* UM55 and MRS control were detected at peaks of lactic acid, OH-PLA, PLA and ILA. In the case of *L. buchneri* UTAD104, differences between CFS and controls were found in lactic acid and PLA. In MRS broth none of these acids was detected (Figure 3.8, Figure 3.10, Table 3.11 and Table 3.14).

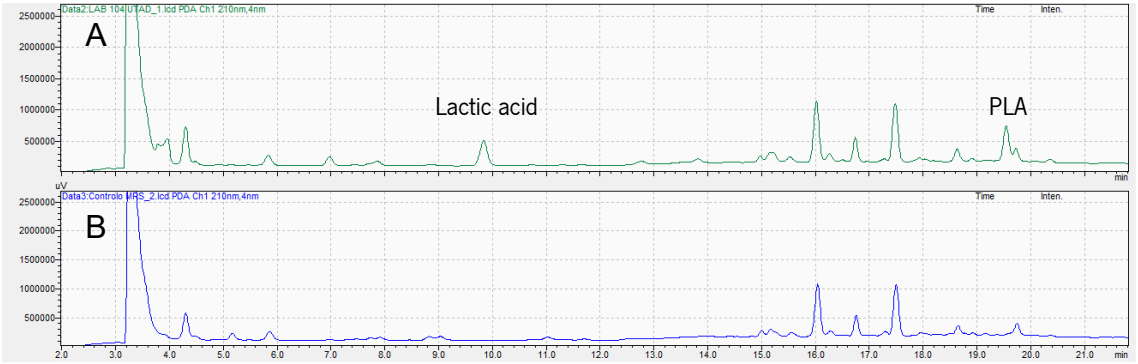


Figure 3.10. Chromatograms of *L. buchneri* UTAD104 CFS (A) (green line chromatogram) and MRS broth (B) (blue line chromatogram).

Table 3.14. Concentration of lactic acid, PLA, OH-PLA and ILA present in *L. plantarum* UM55 CFS, *L. buchneri* UTAD104 CFS and MRS broth.

	Lactic acid (g/L)	PLA (mg/L)	OH-PLA (mg/L)	ILA (mg/L)
L. plantarum UM55 CFS	4.3 ± 0.2	74.8 ± 0.4	30.8 ± 1.5	6.7 ± 0.1
L. buchneri UTAD104 CFS	3.4 ± 0.1	48.1 ± 2.2	0	0.6
MRS broth	0	0	0	0

Data for the construction of this table can be seen in Table A.9

Organic acids profile for *L. plantarum* UM55 are presented in Table 3.11 and their concentration were found to be similar to concentrations previously described by other authors (Valerio et al. 2004; Gerez et al. 2014). For example, Valerio et al. (2004) reported that strains of *L. plantarum* produced between 35 and 46 mg/L of PLA and 40 and 50 mg/L of OH-PLA. Also, Geréz (2014) reported that dough with *L. plantarum* produced 4.25 mg/g (4.25 g/L) of lactic acid, 0.013 mg/g (13 mg/L) of PLA and 0.01 mg/g (10 mg/L) of OH-PLA, while in dough without this LAB strain none of these acids where detected. Concerning *L. buchneri* UTAD104, the production of lactic acid and PLA was identified but the method used in these quantifications did not allow us to detect any acetic acid, though *L. buchneri* is an

obligate heterofermentative microorganism which produces, under aerobic as well as anaerobic conditions, both lactic and acetic acid (Heinl et al. 2012).

Organic acids produced by LAB exert their antimicrobial action after penetrating the cell's membrane in their undissociated form, leading to a drop in the intercellular pH and to the disruption of metabolic activities (Brul and Coote 1999). Besides the production of lactic acid, *L. plantarum* has been described as a producer of several organic acids with antimicrobial effect, such as acetic, propionic, phenyllactic, hydroxyphenyllactic, indole lactic, benzoic, coumaric, vanillic, butyric, formic, salicylic, succinic and azealic acids, among others (Niku-Paavola et al. 1999; Lavermicocca et al. 2000; Ström et al. 2002; Valerio et al. 2004; Broberg et al. 2007; Gerez et al. 2009; Prema et al. 2010; Dallagnol et al. 2011; Rizzello et al. 2011; Özcelik et al. 2016), while *L. buchneri* is mainly known for its capacity of producing lactic and acetic acid, and in some conditions propionic acid (Driehuis et al. 1999; Kung and Ranjit 2001; Johanningsmeier et al. 2012).

Next, the antifungal properties of these acids were tested individually against *P. nordicum* using the "food poisoning" method. An example of the fungal colonies obtained in this experiment can be seen in Figure 3.11.

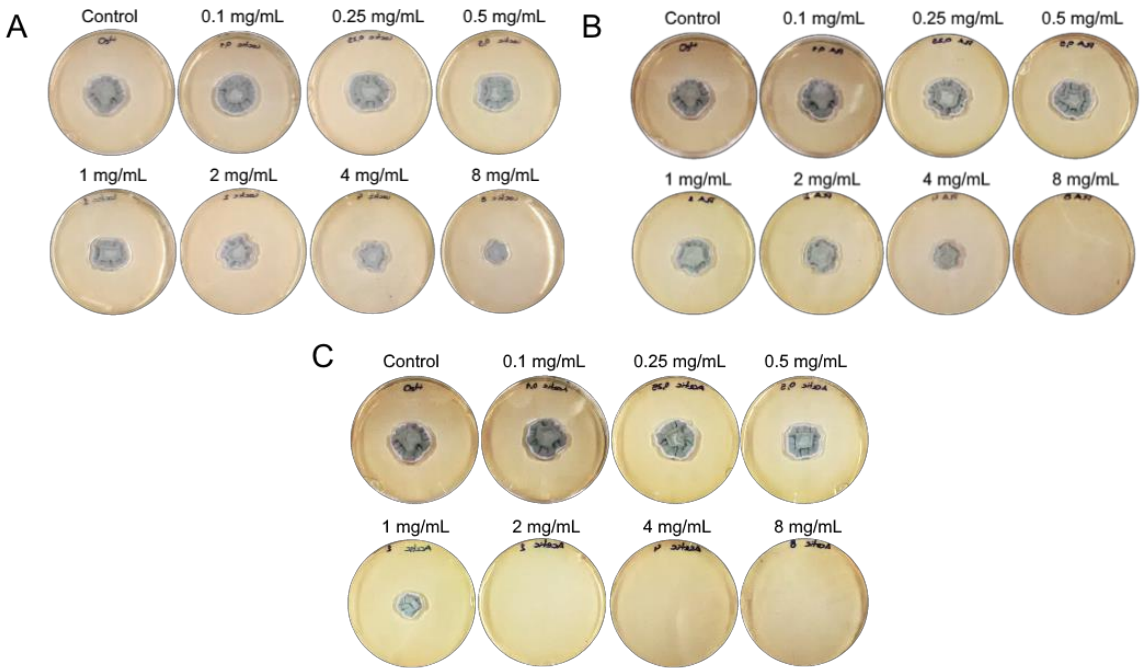


Figure 3.11. Example of the inhibition of *P. nordicum* growth by lactic acid (A), PLA (B), and acetic acid (C) determined by the "poisoned food technique".

In general, all of the mentioned acids inhibited to some degree the fungal growth of *P. nordicum*, and more substantially OTA production. Again, the minimum concentration at which each acid had the maximum effect was different. Similarly to what was observed for *A. flavus* inhibition, all tested organic acids inhibited to a greater extent the production of OTA than the fungal growth. Acetic acid and PLA were able to produce total inhibition of fungal growth at concentrations of 2 and 8 mg/mL, respectively.

Lactic acid, OH-PLA and ILA produced an inhibition of approximately 50% in fungal growth at the maximum tested concentration (8 mg/mL), as it can be seen in Table 3.15.

Concerning the production of OTA, since there was no fungal growth at 2 mg/mL of acetic acid, 8 mg/mL of PLA and 4 mg/mL of ILA, there was no production of OTA. Maximum inhibition obtained with lactic acid and OH-PLA was of 60% and 72%, respectively, at a concentration of 8 mg/mL. The pH of the culture media in which the maximum OTA inhibition was achieved was 4.5, 3.7, 4.6, 3.6 and 3.7, for acetic acid (2 mg/mL), PLA (8 mg/mL), ILA (4 mg/mL), lactic acid (8 mg/mL) and OH-PLA (8 mg/mL), respectively. Therefore, the stronger effects were obtained with acetic acid, PLA and ILA.

Table 3.15. Inhibition (%) of *P. nordicum* MUM 08.16 fungal growth and OTA production with different organic acids (lactic acid, PLA, OH-PLA and ILA) at different concentrations and pH of the media at those concentrations.

		Organic acid concentration (mg/mL)							
	Inhibition (%)	**0**	**0.1**	**0.25**	**0.5**	**1**	**2**	**4**	**8**
Lactic acid	Fungal growth	0.0	0.0	1.7	10.1	21.0	24.4	31.9	48.7
	OTA	0.0	12.9	16.2	30.5	40.0	49.9	56.1	59.9
	pH	6.5	6.23	5.98	5.62	5.2	4.52	3.98	3.57
PLA	Fungal growth	0.0	0,0	2.5	7.6	15.1	16.8	33.6	100.0
	OTA	0.0	11.8	20.3	35.9	51.0	71.0	94.2	100.0
	pH	6.5	6.38	6.11	5.57	5.06	4.59	4.22	3.7
OH-PLA	Fungal growth	0.0	2.5	7.6	7.6	13.5	21.0	28.6	42.9
	OTA	0.0	2.4	18.4	41.0	46.1	68.4	68.7	72.1
	pH	6.5	6.34	6.12	5.82	5.39	4.81	4.19	3.66
ILA	Fungal growth	0.0	0.8	0.0	8.4	12.6	20.2	50.4	-
	OTA	0.0	2.3	17.5	40.6	46.4	74.3	99.5	-
	pH	6.5	6.35	6.26	5.86	5.38	4.8	4.59	-
Acetic acid	Fungal growth	0.0	2.5	6.7	14.3	30.3	100.0	100.0	100.0
	OTA	0.0	0.0	0.0	10.3	22.1	100.0	100.0	100.0
	pH	6.5	6.44	5.84	5.4	4.8	4.49	4.13	3.96

IC_{50} and IC_{90} were calculated for each organic acid based on the data available in appendix A Table A.22, Table A.23, Table A.24, Table A.25, Table A.26, Table A.27, Table A.28, Table A.29, Table A.30 and Table A.31; and are shown in Table 3.16. Graphic representation of IC_{50} and IC_{90} can also be seen in appendix A in Figure A.16, Figure A.17, Figure A.18 and Figure A.19. Concerning fungal growth, IC_{50} of the selected acids were (in crescent order): acetic acid (1.6 mg/mL), ILA (3.6 mg/mL), PLA (4.4 mg/mL), lactic acid (7.4 mg/mL) and OH-PLA (8.9 mg/mL). The calculated values for IC_{90} follow the same trend, with IC_{90} for acetic acid, ILA, PLA, lactic acid and OH-PLA being 1.7, 8.2, 8.4, 58.4 and 68.1 mg/mL, respectively.

Concerning OTA, the lowest IC_{50} was obtained for ILA (1.2 mg/mL), followed by PLA (1.3 mg/mL), acetic acid (1.6 mg/mL), OH-PLA (1.7 mg/mL), and lactic acid (2.9 mg/mL). Except for lactic acid, all acids showed similar concentrations at which they produced an inhibition of approximately 50% in OTA production. Results of IC_{90} showed a different trend. Acetic acid presented the lowest value (1.7 mg/mL), followed by ILA and PLA (3.2 and 4.5 mg/mL, respectively), and OH-PLA and lactic acid with a considerably higher concentration (13.1 and 45.9 mg/mL, respectively).

When comparing ICs values for growth and OTA inhibition of all organic acids tested, acetic acid had the lowest IC_{50} and IC_{90} for fungal growth and IC_{90} for OTA and is probably the primary responsible for the strong antifungal ability of *L. buchneri* UTAD104 against *P. nordicum* MUM 08.16. Acetic acid anti-ochratoxigenic activity appears to be caused by the inhibition of the fungal growth since low concentrations (0.1 and 0.25 mg/mL) produce no inhibition whatsoever of both growth and OTA, intermediate concentrations inhibit growth and OTA similarly (approximately 15% and 30% of inhibition for 0.5 and 1 mg/mL, respectively) and high concentrations inhibit the growth of *P. nordicum* entirely, therefore no OTA could be produced.

Table 3.16. Inhibitory concentrations (IC_{50} and IC_{90} in mg/mL) of lactic acid, PLA, OH-PLA, ILA and acetic acid against *P. nordicum* MUM 08.16

	Fungal growth			**Ochratoxin A production**		
	IC_{50} (mg/mL) (95% CI)	IC_{90} (mg/mL) (95% CI)	R^2	IC_{50} (mg/mL) (95% CI)	IC_{90} (mg/mL) (95% CI)	R^2
Lactic acid	7.4 (6.4 - 8.5)	58.4 (40.8 - 87.0)	0.9845	2.9 (2.3 - 3.8)	45.9 (24.1 - 98.6)	0.9561
PLA	4.4 (3.7 - 4.9)	8.4 (6.2 - 12.3)	0.9621	1.3 (1.2 - 1.4)	4.5 (3.6 - 5.8)	0.9838
OH-PLA	8.9 (7.8 - 10.4)	68.1 (48.3 - 98.8)	0.9868	1.7 (1.3 - 2.2)	13.1 (6.8 - 19.4)	0.9346
ILA	3.6 (3.3 - 3.9)	8.2 (6.9 - 9.9)	0.9852	1.2 (1.0 - 1.4)	3.2 (2.4 - 4.5)	0.9599
Acetic acid	1.6 (1.5 - 1.7)	1.7 (1.5 - 1.9)	0.9854	1.6 (1.4 - 1.8)	1.7 (1.6 - 1.8)	0.9918

IC_{50} - Concentration of organic acid which produced an inhibition of 50%; IC_{90} - Concentration of organic acid which produced an inhibition of 90%; 95% CI - Confidential interval of calculated values; R^2 - R-square of fitted curves. Data for the construction of this table can be seen in Table A.22, Table A.23, Table A.24, Table A.25, Table A.26, Table A.27, Table A.28, Table A.29, Table A.30 and Table A.31from appendix A

IC_{50} for OTA are relatively similar for all acids (approximately 1.5 mg/mL) except for lactic acid, which IC_{50} is approximately 2 times higher (2.9 mg/mL). Although lactic acid was the less effective in inhibiting OTA production and the second less effective in inhibiting *P. nordicum* growth (after OH-PLA), lactic acid is probably the primary responsible for *L. plantarum* UM55 CFS inhibitory effects, since a concentration of 4 g/L inhibited the growth of *P. nordicum* in 32% and the production of OTA in 56%, which corresponds approximately to the inhibitions obtained with *L. plantarum* UM55 CFS. Still, PLA, OH-PLA and ILA may also have been involved in the antifungal ability against *P. nordicum* of this strain. From the data, it can also be concluded that a relatively low concentration (0.25 mg/mL) of PLA, OH-PLA and ILA result in a 20% inhibition of OTA production and that a concentration as low as 0.1 mg/mL of PLA allows a reduction of 12%. Additionally, it has been mentioned that synergistic effects may explain the antifungal effect of some of these acids (Lavermicocca et al. 2003; Belguesmia et al. 2014). However, these synergisms remains unclear, and this type of interactions have not been proven rigorously for most of the compounds (Ström et al. 2002).

The antimicrobial ability of PLA, OH-PLA and ILA have already been described (Narayanan and Rao 1976; Lavermicocca et al. 2000; Lavermicocca et al. 2003), and have been confirmed for the inhibition of *A. flavus*, as showed before, but this is the first time that their antifungal ability against an OTA producing fungus (*P. nordicum*) has been characterised.

3.3.3.3 Antifungal properties of lactic acid bacteria cells

Besides the effects observed with CFS of *L. plantarum* UM55 and *L. buchneri* UTAD104, also viable LAB cells of these strains have shown antifungal ability (Figure 3.12). The incorporation of viable cells in culture media resulted in *in situ* production of antifungal compounds, increasing the antifungal potential of these biocontrol agents. *L. plantarum* UM55 and *L. buchneri* UTAD104 cells were very effective in inhibiting both growth and OTA production of *P. nordicum* MUM 08.16 when they were incorporated into YES culture medium. At OD_{600} of 5, the strain *L. plantarum* UM55 inhibited fungal growth in 93% and the production of OTA in 100%, while at OD_{600} of 5 *L. buchneri* UTAD104 completely prevented the fungus from growing and consequently no OTA was produced. With an OD_{600} of 2.5, the addition of *L. buchneri* UTAD104 to the medium continued to produce the total inhibition of *P. nordicum* and production of OTA, while the addition of *L. plantarum* UM55 resulted in inhibitions of only 70% and 84%, for fungal growth and OTA production, respectively. As expectable a lower cell concentration, OD_{600} 1, resulted in lower inhibitions for *L. plantarum* UM55, resulting in inhibitions of 46% and 65%, for fungal growth and OTA production, respectively. For *L. buchneri* UTAD104, antifungal activity of OD_{600} 1 remained high with the inhibition of growth reaching 81%, which resulted in no production of OTA.

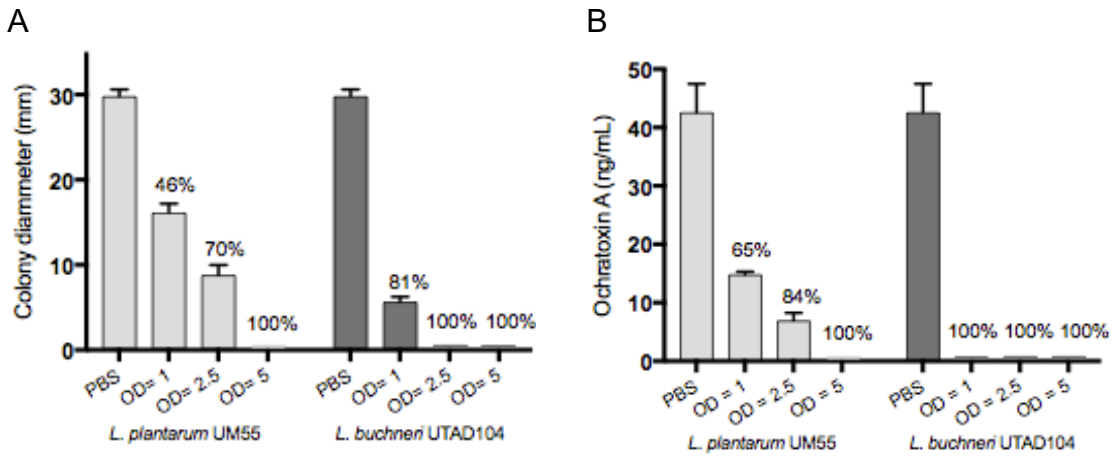


Figure 3.12. Fungal growth (A) and ochratoxin A production (B) of *P. nordicum* in YES media incorporating suspensions of cells of *L. plantarum* UM55 and *L. buchneri* UTAD104 with OD_{600} of 1, 2.5 and 5. Error bars indicate a standard deviation of n=3. The percentages express the inhibition of growth and OTA production relatively to control experiments conducted with PBS. Data for the construction of this figure can be seen in appendix A in Table A.32 and Table A.33.

3.3.3.4 Organic Acids Extracted from Plates Containing Lactic Acid Bacteria Cells

The quantities of organic acids produced in YES agar plates by *L. plantarum* UM55 and *L. buchneri* UTAD104 cells was analysed to determine the content of acetic and lactic acids. Results can be seen in Figure 3.13.

The lactic acid content was higher in plates containing *L. plantarum* UM55 (approximately 30% higher) than in *L. buchneri* UTAD104 ones, except for plates containing cells at a concentration of OD_{600} of 2.5. In these conditions, *L. buchneri* UTAD104 was confirmed as an acetic acid producer, with levels of this acid being significantly higher in plates containing this strain than in *L. plantarum* UM55 plates. Values were 6, 5 and 4 times higher for OD_{600} of 1, 2.5 and 5, respectively.

The production of relatively high quantities of acetic acid is certainly responsible for the stronger inhibitory ability of *L. buchneri* UTAD104. As stated before, acetic acid has shown an inhibitory ability substantially superior to lactic acid, 27 times higher in what concerns the inhibition of OTA production.

Antifungal properties of these bacteria and their metabolites can make them a promising biological solution to reduce *P. nordicum* contamination and OTA levels in food and feed systems. Also, this knowledge can be used by LAB starter cultures companies to select new strains with improved properties (higher secretion levels of acetic acid, PLA or ILA), improve their products and deliver solutions to control ochratoxigenic fungi, e.g. in silages, cheese and dry-sausages.

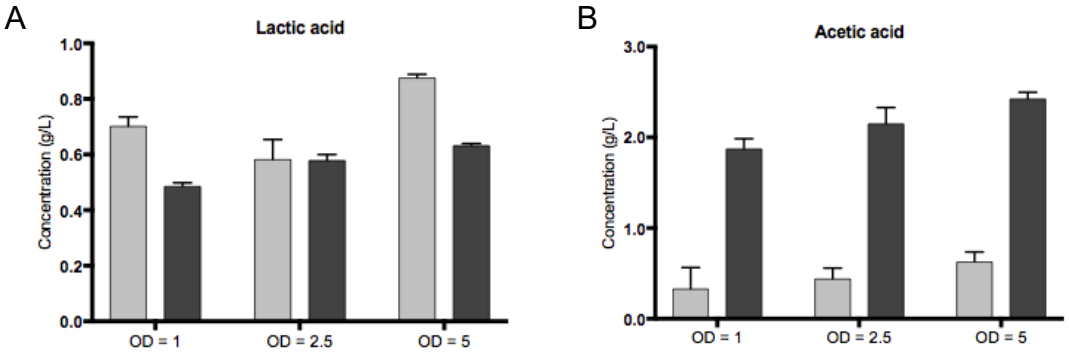


Figure 3.13. Concentration of lactic acid (A) and acetic acid (B) extracted from YES agar plates containing 10% of cells suspensions of *L. buchneri* UTAD104 and *L. plantarum* UM55 in PBS with OD_{600} of 1, 2.5 and 5. Grey bars represent plates containing *L. plantarum* UM55 and black bars represent plates containing *L. buchneri* UTAD104. Data for the construction of this figure can be seen in Table A.34 and Table A.35.

The use of *L. buchneri* strains has been extensively described for applications in silages (Filya 2003). Silage conservation is dependent on the absence of oxygen and maintenance of low pH. The occurrence of natural LAB populations in crops is responsible for their acidification (McDonald 1981). Traditionally, homofermentative bacteria have been employed to achieve a faster production of lactic acid, to improve the effectiveness of the fermentation processes and increase dry matter recovery (Tabacco et al. 2011). However, the aerobic spoilage increases the risk of proliferation of undesirable microorganisms (Driehuis and Elferink 2000). The deterioration of silages is triggered by the degradation of organic acids, generally by yeasts, which causes a rise in pH and an increase in temperature, leading to the proliferation of spoilage microorganisms, such as moulds (Elferink et al. 2000). The use of *L. buchneri*, an obligate heterofermentative LAB, increases the content of acetic acid, keeps the pH at low values, and restrains the proliferation of pathogens, resulting in a 20-fold increase in aerobic stability when compared with the untreated silages (Driehuis et al. 1999).

As mentioned before, acetic acid is a more potent antifungal metabolite than lactic acid (Ranjit and Kung 2000). Acetic acid has a higher pKa than lactic acid (4.75 and 3.86, respectively), which results in the stronger antimicrobial activity of acetic acid in low pH environments, because a high proportion of acetic acid is in the undissociated state, and this acid is capable of penetrating in higher quantities the cell membrane of spoilage microorganisms. The production of acetic acid is probably the main reason why heterofermentative strains have a higher antimicrobial activity than homofermentative LAB (Holzer et al. 2003; Voulgari et al. 2010; Kharazian et al. 2017), thus explaining the results with the cells of *L.*

buchneri UTAD104, which presented a higher inhibitory capacity than the cells of *L. plantarum* UM55. Still, some studies also suggest that *L. buchneri* can produce other metabolites with antifungal properties (Driehuis et al. 1999). For example, *L. buchneri* is capable of converting part of the lactic acid formed into acetic acid (Elferink et al. 2001), and during this process produce propionic acid and 1,2-propanediol (Driehuis et al. 1999). Propionic acid can also contribute to the antifungal ability of this LAB species, according to Gerez et al. (2009), however, in our study, we were not able to detect propionic acid in CFS of *L. buchneri* UTAD104.

Despite the antifungal potential of *L. buchneri*, for example, Kharazian et al. (2017) reported recently that *L. buchneri* was able to inhibit *Fusarium verticillioides*, *Penicillium* sp. and *Verticillium dahlia*; only a few studies describe the inhibition of mycotoxins production in silages inoculated with this strain. For example, Teller et al. (2012) reported that *L. buchneri* added to silages decreased the concentration of ZEN in silage made from unspoiled corn plants. Therefore, *L. buchneri* UTAD104 and other strains that may have a similar profile of organic acids can be of interest for silage inoculation, since acetic acid is a strong inhibitor of the ochratoxigenic fungi (Gallo et al. 2015).

Besides silages, application of *L. buchneri* has been tested in bread to control moulds. In one study, the shelf life of bread prepared with sourdough incorporating *L. buchneri* and *L. diolivorans* was increased, when compared with bread prepared with traditional sourdough. This sourdough also demonstrated better inhibitory ability than traditional sourdough supplemented with acetate (Zhang et al. 2010). Le Lay et al. (2016), also reported that *L. buchneri* isolates affected the growth of spoilage fungi from bakery products. *In vitro*, the isolates had inhibited to some extent the growth of *Aspergillus niger*, *Eurotium repens*, *P. corylophilum*, *Cladosporium sphaerospermum* and *Wallemia sebi*. Additionally, in sourdough bread, some authors consider that acetic acid production improves the qualitative characteristics of the final product (Vernocchi et al. 2004) and is responsible for some of its typical flavours (Brandt 2007).

L. buchneri can also be beneficial in cheese ripening. Fröhlich-Wyder et al. (2013) determined that *L. buchneri* and *L. parabuchneri* isolated from cheese did not affect cheese quality or its flavours, but improve its chemical composition and eye formation by enhancing gas production. Several authors have reported the presence of OTA in different types of cheeses - blue cheese, hard cheese, traditional Italian cheese, Gorgonzola, Roquefort and white cheeses (Dall'Asta et al. 2008; Pattono et al. 2013; Benkerroum 2016; Decontardi et al. 2017). Others, associate *P. nordicum* with the production of OTA in cheeses (Larsen et al. 2000). Despite the contaminations being relatively low, it would be important

to eliminate totally the presence of OTA in these products. This work shows that *L. buchneri* UTAD104, or other strains with similar organic acids profiles, could be beneficial to control *P. nordicum* growth and, consequently the production of OTA in this type of food.

L. plantarum strains have been also used broadly in several fermentation processes. In silage*, L. plantarum* has been employed in conjugation with other LAB, mainly *L. buchneri* (Weinberg et al. 2002; Filya 2003). In cheese, and similarly to what has been stated for *L. buchneri*, *L. plantarum* can be used as an adjunct culture in cheese formation and simultaneously as a biopreservative (Milesi et al. 2008; Cheong et al. 2014). Also in bread, *L. plantarum* was able to produce antifungal compounds during sourdough fermentation, retarding the growth of *Fusarium* species (Dal Bello et al. 2007). Again, the application of *L. plantarum* UM55 in these food products would be beneficial to control *P. nordicum* and OTA production, though it is a weaker inhibitor than *L. buchneri* UTAD104.

P. nordicum raises concerns in fermented meats such as dry-cured ham or dry-fermented sausages, because of the environmental condition in which the ripening takes place that favours the growth of moulds indiscriminately (Sonjak et al. 2011). However, the growth of moulds on the surface of dry-cured meat is essential for the development of flavour and aromas, thus being a desirable event in many traditional products produced in Hungary, Italy, Spain, Greece, France, Austria, Belgium, among others (Berni 2014). Therefore, strategies in which some fungal growth is allowed, but the accumulation of OTA is prevented could be of great value in the production of traditional dry-cured meats. Several authors reported biopreservation methods for cured meats using microorganisms. For example, using yeasts such as *Debaryomyces hansenii* positive results for inhibition in fungal growth and OTA production were obtained by Simoncini et al. (2014). Virgili et al. (2012) also reported *Hyphopichia burtonii* and *Candida zeylanoides* as effective inhibitors of *P. nordicum* and OTA production. Similarly, *L. plantarum* UM55 and/or *L. buchneri* UTAD104 could be applied in cured meats production processes as biopreservatives. The use of LAB is especially advantageous because they have a long history of use in fermented foods, but also because they can grow in a great variety of conditions; they do not need oxygen for growth, they are resistant to inhibition by carbon dioxide, nitrites and smoke and can grow at relatively high concentrations of salt (Egan 1983). The application of these bacteria can be an alternative solution to avoid the occurrence of OTA in these products without reducing significantly fungal growth and thus the beneficial characteristics that they confer to the final product, e.g. flavours and aromas (Iacumin et al. 2009). Pre-treatments, where dry sausages were soaked in a solution of acetic acid, have also been described to prevent mould growth during the

maturation of sausages (Holley 1981). In the present work, it is proved that the use of acetic and lactic acids are indeed beneficial for mould inhibition and most important for OTA production control since they have a considerable inhibitory effect in the production of this mycotoxin. Also, the ability of LAB strains to produce PLA, OH-PLA and ILA can be relevant to the antifungal ability of the strains and should be taken into consideration when selecting strains for biopreservation proposes.

Application of these antifungal LAB strains could be made directly, either by dipping or spreading the LAB cultures onto food products or by incorporation into films and coatings that would enclose the food product. These films and coatings can improve the shelf life of the products, by providing a barrier against physical and mechanical damage, and by creating a controlled atmosphere around the food product (Hassan et al. 2018).

3.4 CONCLUSIONS

Fungal contamination and consequent mycotoxin production can cause significant health and economic losses. Biocontrol can be introduced as an alternative to conventional physical and chemical methods to minimize those losses. LAB have demonstrated high potential for such strategy.

Results obtained have shown that CFS of *L. plantarum* UM55 can inhibit *A. flavus* growth as well as aflatoxin production, and that *L. plantarum* UM55 and *L. buchneri* UTAD104 can also reduce *P. nordicum* growth and OTA contamination. For both fungi results indicate that inhibition of aflatoxin and OTA production was not dependent on the reduction of fungal growth and was reverted when the pH of CFS was neutralized. The main substances responsible for the inhibitory activity were organic acids, with particular relevance to acetic acid, ILA, PLA, OH-PLA and lactic acid. When these compounds were tested individually, all exhibited the ability to inhibit the production of aflatoxins and OTA; and to a lesser extent, the growth of both fungal species.

Concerning the inhibition of *A. flavus*, PLA, OH-PLA and ILA showed the stronger effects at the lowest concentrations. About 1 mg/mL of these acids was sufficient to inhibit the production of aflatoxins by 90%. While for *P. nordicum*, acetic acid, ILA and PLA showed the stronger effects at the lowest concentrations, with concentrations of 2 mg/L for acetic acid and 4 mg/L for ILA and PLA, producing an inhibition of OTA production of over 90%.

For *L. plantarum* UM55, lactic acid seems to be the primary responsible for the antifungal activity, since its concentration in CFS is substantially higher than for the other acids detected. For *L. buchneri* UTAD104, acetic acid seems to have the preponderant role in the fungal inhibition. The incorporation of living cells in the culture media resulted in a potent inhibition of *P. nordicum*.

L. plantarum UM55 and *L. buchneri* UTAD104 have the potential to be used in a perspective of biopreservation to prevent *A. flavus* and *P. nordicum* contamination and the consequent production of mycotoxins.

CHAPTER 4

ACTIVE WHEY PROTEIN EDIBLE FILMS AND COATINGS INCORPORATING LACTIC ACID BACTERIA FOR CHEESE APLICATION

4. ACTIVE WHEY PROTEIN EDIBLE FILMS AND COATINGS INCORPORATING LACTIC ACID BACTERIA FOR CHEESE APPLICATION

4.1 INTRODUCTION

Moulds are the main spoilage organism in various food products, such as cereals, bread and fermented dairy products (Schnurer and Magnusson 2005). Cheeses are particularly vulnerable to the growth of filamentous fungi due to their pH, water activity, and nutritional profile (Hymery et al. 2014). Fungal contamination can produce discolouration, off-flavours, alterations in texture and appearance leading to loss of quality, waste and consequently, economic losses. Additionally, some of the spoilage fungi can produce mycotoxins. These toxins have been proved to cause mutagenic, carcinogenic, toxigenic, neurotoxic and immunogenic effects, among others, and are a serious concern to public health (Hymery et al. 2014).

Current strategies to reduce or eliminate fungal contaminants in cheese consist in incorporating chemical antifungal agents, such as natamycin, sodium benzoate, potassium sorbate and benzoic acid, or in the use of modified atmosphere packaging (Davidson et al. 2013). However, an increasing number of fungi are becoming resistant to conventional chemical antifungal agents and preservatives (Garnier et al. 2017b). Additionally, consumer demands is moving towards safer, natural and high-quality food products with reduced content or even absence of "chemical" preservatives, and with extended shelf life time (Lucera et al. 2012b; Varsha and Nampoothiri 2016).

The use of lactic acid bacteria in a perspective of biopreservation is considered as an alternative to overcome the problems of chemical methods for fungal inhibition (Reis et al. 2012). Several LAB present antifungal properties that are, generally, the result of a synergetic effect of several factors combined: e.g., decrease of environmental pH, competitive growth and production of antifungal substances such as organic acids, proteinaceous compounds, fatty acids, cyclic dipeptides, bacteriocin-like substances, hydrogen peroxide and others (Corsetti et al. 1998; Niku-Paavola et al. 1999; Lavermicocca et al. 2000; Ström et al. 2002; Bianchini and Bullerman 2009; Rizzello et al. 2011; Gerez et al. 2013; Özcelik et al. 2016).

LAB present several advantages for this biologic strategy: they occur naturally in many foods, have a long history of usage in the manufacture and fermentation of dairy products, are considered GRAS, and some strains have probiotic properties (Naidu et al. 1999; Giraffa et al. 2010; Rhee et al. 2011; Varsha and Nampoothiri 2016). However, in some particular cases, LAB may not be able to maintain their numbers (i.e., concentrations above 10^6 CFU/g) and metabolism to exert their antifungal ability or probiotic effect (Burgain et al. 2011). The use of active edible films and coatings is a novel strategy that can overcome this problem since they can maintain the viability of microorganisms and keep an adequate concentration of active compounds in the surface of the food products (Soukoulis et al. 2014c).

Edible films and coatings can be effective carriers of LAB or many different compounds (such as antimicrobials, nutraceuticals, anti-browning and antioxidant agents). Besides, they can also improve food quality by acting as semipermeable barrier to oxygen, carbon dioxide, and water vapour transfer; by controlling lipid oxidation, loss of volatile aromas and flavours; and by improving the mechanical integrity of the food product (Hassan et al. 2018). Also, edible films and coatings meet environmental concerns since they are usually produced with food-grade by-products from agricultural and marine sources, thus contributing to actively reduce the required amount of synthetic packaging material for each product and thus the environmental impact of waste from non-renewable sources (Tharanathan 2003).

Edible films and coatings can be made with polysaccharides, proteins, and lipids or a combination of these materials; plasticisers and surfactants are also usually added to enhance their final properties (Bourtoom 2008). Protein based films and coatings have also been gaining interest due to their functional and nutritional properties (Ozdemir and Floros 2008; Ramos et al. 2012a; Ramos et al. 2016). Whey proteins represent 20% of the total protein content in bovine milk, being a by-product of cheese production that can be used for the production of edible films and coatings (Siso 1996; Ramos et al. 2012a). Films produced with whey protein are transparent, flavourless and flexible, with good oxygen and aroma barrier properties and also good mechanical properties; however, due to the whey protein hydrophilic nature they have poor moisture barrier properties (Pérez-Gago et al. 1999; Pérez-Gago and Krochta 2001; Khwaldia et al. 2004; Ramos et al. 2013). Previous studies already reported that whey protein films (in conjugation with soluble prebiotic fibres) promote the stability of *Lactobacillus rhamnosus* GG (Soukoulis et al. 2014b; Soukoulis et al. 2014c) and that these films can be effective carriers of this microorganism in pan bread (Soukoulis et al. 2014c).

Additionally, some published works are combining edible films and coatings with LAB, most of them as probiotics. The use of an edible matrix as a carrier of viable microorganisms can improve their survival rates during food storage. This can result in an increased antimicrobial action since, when viable, cells will be able to produce antimicrobial compounds and in the case of probiotics, they will be delivered more efficiently and with a better dosage control. Studies show positive results in the inhibition of bacterial pathogens and concluded that incorporation of cells did not significantly compromised the physicochemical properties of films, with exception to barrier properties. (Gialamas et al. 2010; Concha-Meyer et al. 2011; Sánchez-González et al. 2013; Sánchez-González et al. 2014; Dai et al. 2018; Pereira et al. 2018; Ye et al. 2018; Bambace et al. 2019; Settier-Ramírez et al. 2019).

Gialamas et al. (2010) was the first author to use LAB in edible films and coatings with the purpose of biopreservation. In this work, a sodium caseinate edible film incorporating *L. sakei* was able to significantly reduce *L. monocytogenes* contamination in the beef, when compared to the control. *L. sakei* was capable of growing within the film reaching a count of 10^7 CFU/cm^2 after 4 days and its antimicrobial effect was attributed to the production of lactic acid and competition for nutrients.

In another study, Concha-Meyer et al. (2011) tested films incorporating two strains of *Carnobacterium maltaromaticum* and nisin to the control *L. monocytogenes* in smoked salmon. The combination of these strains was effective in reduction *L. monocytogenes* contamination, which was associated to the production of bacteriocin-like substance. However, after 21 days, the antimicrobial activity was significantly decreased, which was attributed to cell death.

Anti-*Listeria* effect of films containing LAB was also studied by Sánchez-González et al. (2013; 2014). Both studies report the incorporation of a bacterial strain in different film matrices to improve the production of bacteriocins by those strains. Polysaccharide films containing *L. plantarum* were effective in controlling *L. innocua* growth, reducing it by 3 log units after 3 days, with bacteriocin production being higher in these films than in protein films. When *L. acidophilus* and *L. reuteri* were tested, bacteriocin production was also influenced by film matrix, with methylcellulose films presenting the best results, leading to a reduction of *L. innocua* by 1.5 log units after 12 days of storage.

More recently, Dai et al. (2018) reported that hydroxypropyl cellulose films with konjac flour incorporating *L. paracasei* were effective in inhibiting Gram-positive and Gram-negative pathogens during a period of 30 days. These films maintained strawberries quality while extending shelf life. Pereira et al. (2018) developed whey protein isolate coatings containing the probiotic strains *B. animalis* or *L. casei* for sliced ham application. Films inhibited growth of *Staphylococcus* spp., *Pseudomonas*

spp., *Enterobacteriaceae* and yeasts/moulds for 45 days of storage at 4 °C, with probiotics maintaining cell counts of 10^8 CFU/g during storage. Coatings contributed to the decrease of weight loss and to maintain the product freshness. Also, Ye et al. (2018) showed that sodium alginate films with *L. lactis* (at a concentration of 1.5 g per 100 g) had bacteriostatic activity against *S. aureus* for one week at 4 °C, thus reducing the pathogen by 4 log units. Bambace et al. (2019) determined that the addition of prebiotics to alginate films with *L. rhamnosus* improved probiotic viability for 21 days. When applied to blueberries, antimicrobial coatings reduced *L. innocua* counts by 1.7 log units. Finally, Settier-Ramírez et al. (2019) studied the effect of several additives in poly(vinyl alcohol) films incorporating *L. lactis* subs *lactis*, and concluded that viability of this strain was influenced by film matrix composition. Films with *L. lactis* subs *lactis* and containing hydrolyzed gelatin or casein showed the highest anti-*listeria* activity.

Active films incorporating LAB with antimicrobial activity applied in food products is also limited. These films and coatings have being tested in beef (fresh and ham) (Gialamas et al. 2010; Pereira et al. 2018), fish (fresh and smoked) (Concha-Meyer et al. 2011; López de Lacey et al. 2012; López de Lacey et al. 2014) and berries (strawberries and blueberries) (Dai et al. 2018; Bambace et al. 2019), still, they have not been tested in cheese products. Additionally, to our knowledge, only a few works regard the incorporation of living bacteria in edible films and coatings against fungal contamination, and none has evaluated how these films influence the production of mycotoxin.

The present work aimed to investigate the antifungal properties of whey protein based films incorporating LAB and to study the influence of cells addition on films physicochemical and structural properties. Finally, the antifungal coatings were also tested in a cheese matrix against *P. nordicum*.

4.2 MATERIALS AND METHODS

4.2.1 Biological Materials and Growth Conditions

Penicillium nordicum MUM 08.16 was revived in YES agar in the dark at 25 °C. After 7 days of growth, fungal spores were suspended in a 0.1% peptone solution with 0.05% Tween 80. The spore concentration was determined by counting the spores in a Neubauer cells chamber and concentration

was adjusted to 10^6 spores/mL, and aliquots of spore solutions were stored at -20 °C, during experimental work.

L. buchneri UTAD104 was previously selected for its antifungal ability against *P. nordicum* MUM 08.16 and *L. casei* UM3 (which showed no antifungal activity) was used as a negative control strain. For the production of antifungal films and coatings, LAB were cultivated in MRS broth supplemented with 20% of tomato juice and 1 g/L of cysteine, at 30 °C for 48 h. A 5% aliquot from the culture was transferred into the 30 mL of modified MRS broth and grown overnight at 30 °C. The cell cultures were concentrated by centrifugation (10 min at 7000 RCF), and cell number was adjusted with culture medium for a concentration of approximately 1×10^{11} CFU/mL.

4.2.2 Production of Films and Coatings

Whey protein concentrate (WPC) (supplied by Armor Proteines) was used as the base material for film and coating formulations. Glycerol (87% purity, Panreac) was used as a plasticizer, guar gum (Sigma-Aldrich) as a thickener, and Tween 20 (Acros Organic) as a surfactant. All the materials, with the exception of WPC, were sterilized prior to use at 121 °C for 15 min.

Film forming solutions were prepared by adapting the protocol reported by Ramos et al. (2012b). Briefly, WPC (10%, w/w) was dissolved in distillate water, and glycerol (5%, w/w) was added to the solution, which was magnetically stirred for 2 h. The solution was then heated in a water bath at 80 °C under agitation for approximately 5 min. This heating step is crucial for the formation of intermolecular bonds that originates the protein cross-linking, which is necessary for the film formation (Le Tien et al. 2000). The solution was allowed to cool down at room temperature to approximately 30 °C. Then, Tween 20 (0.2%, w/w) was added and the solution was magnetically stirred for 20 min. After this, guar gum (0.7%, w/w) was added and the solution was magnetically stirred for approximately 16 h. This agitation period is necessary to hydrate completely the guar gum in order to form more homogenous films. Afterwards, solutions were adjusted to pH 6 using pure glacial acetic acid (Fisher Scientific).

L. buchneri UTAD104 and *L. casei* UM3 were individually added at a 10, 20 or 30% (w/w) (approximately 1×10^{10}; 2×10^{10} and 3×10^{10} CFU/mL for film solution) concentration to the film forming solution and homogenized. Finally, the film forming solution was poured onto Teflon coated plates. To achieve similar film thickness, the same quantity of solution (approximately 1 g for 5 cm^2) was poured

onto each Teflon plate. Solutions were allowed to dry in an oven with ventilation at 25 °C for 24 h. Once dried, films were peeled off and stored at room temperature in a sealed compartment.

For antifungal coatings, *L. buchneri* UTAD104 was added at a 30% (w/w) concentration to the film forming solution and homogenized. The coatings were applied directly onto the cheese surface, with the aid of a sterile syringe, to ensure that the entire surface was covered. Residual coating solution was allowed to drip off.

4.2.3 In vitro Antifungal Activity of Films against *Penicillium nordicum*

Three sets of films were tested to assess its activity against *P. nordicum*: i) films incorporating *L. buchneri* UTAD104, ii) films incorporating *L. casei* UM3, and iii) films without any LAB (the two last formulations were tested as negative controls). The antifungal activity of these films was evaluated by agar diffusion assay as follows. Films were cut into circles with 20 mm of diameter and centrally placed into Petri plates containing YES agar previously surface spread with 100 μL of a spore solution (10^6 spore/mL) of *P. nordicum*. Additionally, the ability of *P. nordicum* to grow on films surface was also evaluated. Films circles (20 mm of diameter) were placed in petri plates with YES agar and 10 μL of a spore solution (10^6 spore/mL) of *P. nordicum* was centre inoculated in the films surface. Plates were incubated at 25 °C for 7 days. Visual inspection was conducted daily until 7 days of growth. All experiments were done in duplicate.

4.2.4 Enumeration of Lactic Acid Bacteria in Films

The viability of incorporated LAB was determined after film formation. Films with *L. buchneri* UTAD104 and *L. casei* UM3 were analysed. Each film was cut into circular discs with 20 mm of diameter (approximately 0.1 g) and placed in a sterile falcon with 9.9 mL of PBS. The falcons were strongly vortexed until the film was completely dissolved. Sequential 10-fold dilutions were done in PBS and plated on MRS agar. Plates were incubated at 30 °C until colonies were visible. Films were stored in sterile petri plates sealed with parafilm at room temperature and analysed every 3 to 5 days. Films were analysed until 30 days after casting.

4.2.5 Film Characterization

Physicochemical and mechanical properties of control films without LAB and films incorporating *L. buchneri* UTAD104 at 30% (w/w) (films with stronger fungal inhibition) were fully characterized as detailed below. Prior to characterization, films were conditioned at approximately 54 ± 1% RH and 25 ± 2 °C for at least 24 h.

4.2.5.1 Morphologic characterization of films

The surface morphology of the samples was examined using a desktop Scanning Electron Microscope (SEM) (Phenom ProX, Netherlands). All results were acquired using the ProSuite software. Non-conductive and uncoated samples of control films and films containing *L. buchneri* UTAD104 were added to aluminium pin stubs with electrically conductive carbon adhesive tape (PELCO Tabs™) on a Phenom Charge Reduction Holder (CRH) at 5KV and a spot size of 3.3. All SEM observations were performed in duplicate in both sides of the films.

4.2.5.2 Thickness

Film thickness was measured with a hand-held digital micrometer (Mitutoyo, Japan) with 0.001 mm accuracy. Thickness was measured randomly at five different points in each sample. Two samples for each film were analysed. Mean values were used for further calculations.

4.2.5.3 Moisture content and water solubility

Moisture content was determined gravimetrically by drying film samples (discs of 20 mm diameter) at 105 °C for 24 h. Moisture content was given by the percentage of water removed from the initial film sample. For water solubility evaluation, film portions of 20 mm diameter were placed in beakers with 40 mL of distilled water, sealed with parafilm and shaken at 60 rpm at 25 °C for 24 h. Undissolved film samples were recovered and desiccated at 105 °C for 72 h. The non-soluble part of each film was weighted and solubility, expressed as a percentage, was calculated by the Equation 4.1:

Equation 4.1: Film solubility $$Fs(\%) = \left(\frac{Wi - Wf}{Wi}\right) \times 100$$

where, *Fs* was the film solubility, W_i was the initial weight of film as dry matter and W_f was the dry weight of the undissolved film residue. All experiments were performed in triplicate for each film sample and two samples of each film were tested.

4.2.5.4 Water vapour permeability

Water vapour permeability (WVP) was determined gravimetrically according to the American Society for Testing and Materials (ASTM) E 96-92 method as described by Cerqueira et al. (2012). Three samples from each film (with or without *L. buchneri* UTAD104) were cut and placed in a permeation cell (cup with water at 100% RH; 2.337×10^3 Pa vapour pressure at 20 °C). Cups with a diameter of 4 cm and depth of 7 cm were filled with 50 mL of distilled water. The films were mounted with the upper side facing the controlled environment (desiccator with silica gel 0% RH, 0 Pa, 20 °C). The weight loss of the cups was measured at 2 h intervals for a total of 10 h, to determine water-transferred through the samples films. Water vapour transmission rate (WVPR) was calculated by dividing the slope of the linear regression of weight loss versus time by the film area (g/(day m^2)). Afterwards, WVTR was multiplied by film thickness and divided by vapour pressure differential across the sample film to obtain WVP (g mm/(day m^2 kPa)).

4.2.5.5 Mechanical properties: tensile strength and elongation at break

Tensile strength (TE) and elongation at break (EB) were determined with a texture analyser TA.XT Plus (Model TA.HD Plus, Stable Micro Systems, Surrey, UK) according to the ASTM Standard Method D 882-91. Film samples were cut into 120×20 mm^2 strips and placed between the tensile grips, a load cell capacity of 5000 g was used. The initial grip separation and crosshead speed were set to 100 mm and 5 mm/min, respectively. Tensile strength (MPa) was determined by dividing maximum load (N) by cross-sectional area of the film (m^2), and EB (%) was calculated as a ratio of the final length (cm) at the point of sample rupture to the initial length (cm) of the sample. At least eight strips of each film sample were analysed.

4.2.5.6 Surface hydrophobicity

Surface hydrophobicity of the films was evaluated by measuring the contact angle of a water droplet with the surface of the film, with an optical contact angle measuring system (OCA20, DataPhysics, Germany) equipped with the C20 software and with CCD video camera (resolution of 752 × 582 pixel) at 23 °C. Measurements were performed according to sessile drop method using a 500 μL syringe filed with ultra-pure water. Film samples were tape-glued to the centre of the equipment stand and a drop of 2 μL was placed on the film surface. Measurements were made from 0 to 60 s and 10 replicates (5 from each film sample) were analysed for each film type.

4.2.5.7 Fourier Transform Infrared Spectroscopy

The Fourier transform infrared spectroscopy (FTIR) spectra of the films with and without cells were recorded with a Bruker FT-IR VERTEX 80/80v (Boston, USA) using Attenuated Total Reflectance mode (ATR) with a platinum crystal accessory. Film spectra result from 16 scans in the wavelength range 4000-400 cm^{-1}, at a resolution of 4 cm^{-1}. Before analysis, an open bean background spectrum was recorded as a blank.

4.2.5.8 X-Ray diffraction

Crystallinity phases of the sample films were evaluated by X-ray diffraction (XRD). XRD patterns of each film were analysed between $2\theta=10°$ and $2\theta=50°$ with a step size $2\theta=0.02°$ and recorded 14 pts/s with a Cu source, X-ray tube ($\lambda=1.54056$ Å) at 45 kV and 40 mA in an X-ray diffraction instrument (X'Pert³ Powder, Panalytical, Almelo, Netherlands).

4.2.5.9 Optical properties

The colour of the films was measured using a colorimeter CR 400 (from Konica Minolta, Japan). Colour coordinates *L** (black to white hue component), *a** (red to green hue component) and *b** (yellow to blue hue component) of CIELab colour scale were measured in 5 random points of each sample and

two samples from each film were tested. Films were measured against the surface of a white standard plate with defined colour coordinates ($L_{standard}$= 97.7, $a_{standard}$=0.04 and $b_{standard}$=1.47). The total colour difference (ΔE) was calculated according to the Equation 4.2:

Equation 4.2. Total colour difference $$\Delta E = \sqrt{(\Delta L*)^2 + (\Delta a*)^2 + (\Delta b*)^2}$$

Where ΔL^*, Δa^* and Δb^* are the differentials between the film colour parameter and the colour parameter of a standard used as the film background.

Opacity of each sample was determined according to the Hunterlab method, as a relationship between opacity of the sample on a black standard and the opacity of the samples in a white standard. Five measurements were made for each sample, and two samples of each film were tested.

4.2.6 Antifungal Activity of Films and Coatings Against *Penicillium nordicum* in Cheese

4.2.6.1 Cheese

The cheese used for this study was a sliced, semi-hard flamengo cheese, obtained commercially and manufactured by Terra Vento (São Miguel, Portugal). This cheese is produced with pasteurized bovine milk, with addition of calcium chloride and packed in protective atmosphere. It requires refrigeration at low temperature (0 to 10 °C). The typical composition of this cheese is 23.6% of protein, 27.8% of fat and 1.4% of salt.

4.2.6.2 Antifungal activity of films and coatings in cheese

To determine the antifungal activity of WPC based films in cheese, films were produced as described above, placed on top of the cheese surface (with an area of 10.5 cm^2) and centre inoculated with *P. nordicum* (10^6 spores/mL). Films incorporating *L. buchneri* UTAD104 and *L. casei* UM3 at 30% (w/w), films without cells and untreated cheese were tested in duplicate.

The same WPC solutions were used to directly coat the cheese samples, thus avoiding the film-forming step to simulate better the application conditions of the industry. The coatings were applied to the entire surface of the cheese, with a sterile syringe and residual coating solution was allowed to drip off. Cheese samples were left to dry in a vertical flow chamber for 1 h at room temperature. After that period, they were transferred to an incubator with air circulation at 25 °C for approx. 24 h, to ensure that the coating was completely dry. Cheeses samples were then centre inoculated with *P. nordicum* (10^6 spores/mL).

All cheese manipulation and films and coating application were performed in a sterile environment. After inoculation, cheeses samples were placed in an incubator at 25 °C and approx. 90% RH. The growth of the fungi and other microbial contaminations were observed regularly for a storage period of 30 days.

4.2.6.3 Mycotoxins analysis

Production of ochratoxin A in cheeses inoculated with *P. nordicum* was evaluated after 30 days of inoculation. Pieces of cheese involved in the edible coatings were cut in small pieces, placed into falcons and extracted with 20 mL extraction solution (acetonitrile/methanol/acetic acid (78/20/2, v/v/v)). Then, falcons were subjected to vigorous vortexing to extract the mycotoxin. The content of the falcons was filtered through 0.45 μm syringe filters into amber vials. OTA content was analysed by HPLC and fluorescence detection as described in section 3.2.5. Similarly, the flow rate was set to 1.0 mL/min for a 15 min isocratic run, and the injection volume was 50 μL. Calibration curves were done with a certified OTA standard solution at concentrations between 0.05 and 100 ng/mL in the mobile phase (Figure A.5 from appendix A). The LOD and LOQ for OTA were 0.18 ng/mL and 0.54 ng/mL, respectively.

4.2.7 Data analysis

At least three independent replicates were performed in all experiments and results were expressed as mean values ± standard deviation. Data were compared by mean analysis (t-test) at $p <$

0.05 using the software Microsoft Excel version 14.6.0 for Apple Macintosh (Microsoft Corporation, USA).

4.3 RESULTS AND DISCUSSION

4.3.1 Film Appearance

Film formulations with and without cells were flexible, slightly heterogeneous and transparent, with the films incorporating the cells showing a more yellow-brownish colour (Figure 4.1). All films could be easily peeled from the casting plates.

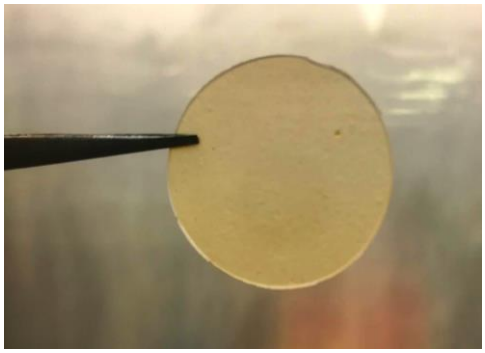


Figure 4.1. WPC based film

4.3.2 Antifungal Activity of WPC-based Films

Two qualitative assays were used to evaluate the inhibitory activity of WPC based films incorporated with LAB: one based on the fungal growth on the surface of the films and the other one based on the formation of an inhibition halo around the circular film disc (agar diffusion assay).

Figure 4.2 and Figure 4.3 show the antifungal capacity after 7 days of inoculation with *P. nordicum*. As expected, strain *L. casei* UM3 had no antifungal effect, in all the concentrations (10, 20 and 30% (w/w)) tested. On the other hand, *P. nordicum* was not able to grow on the surface of WPC based films incorporating *L. buchneri* UTAD104, whatever the cell concentration used (Figure 4.2).

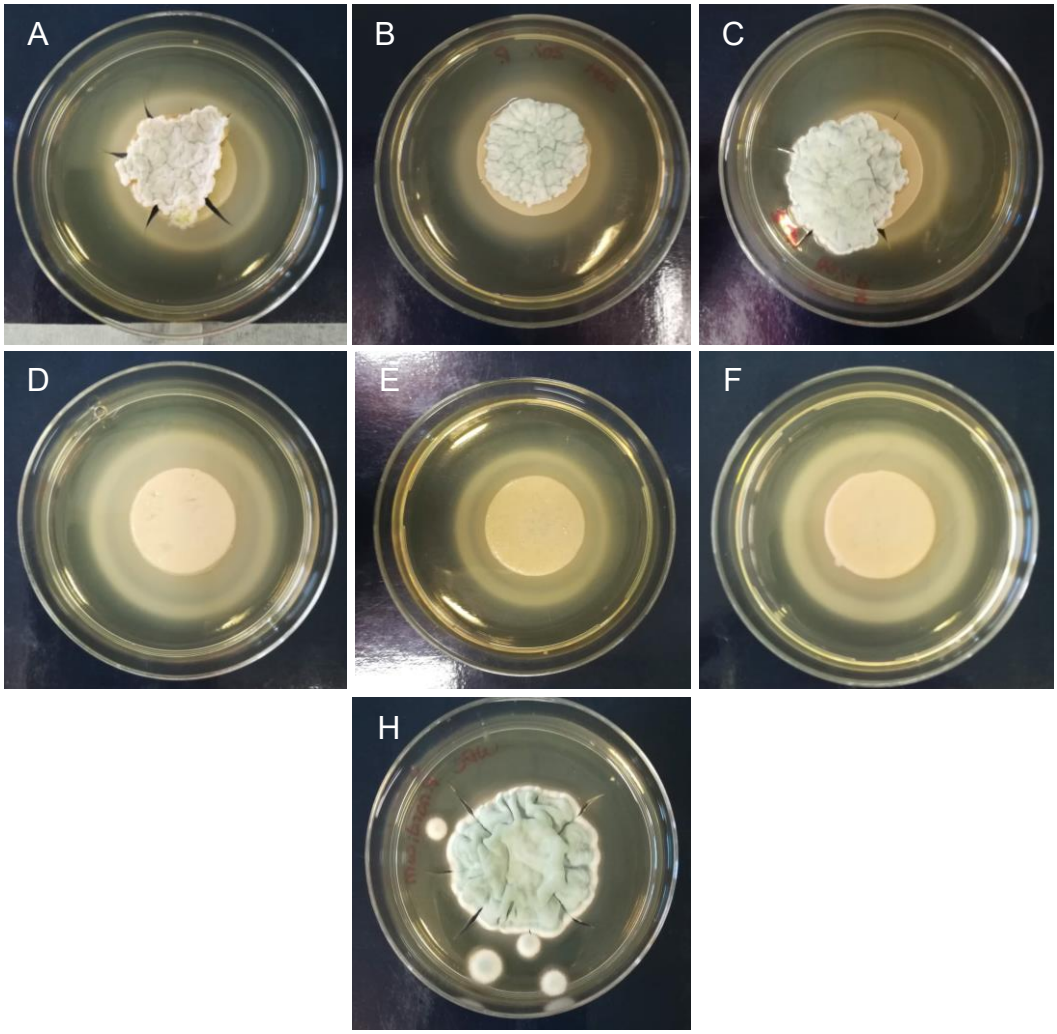


Figure 4.2. Antifungal activity against *P. nordicum* MUM 08.16 by inoculating the spore solution on the surface of WPC based films incorporating *L. casei* UM3 at 10% (A), 20% (B) and 30% (w/w) concentration (C); and incorporating *L. buchneri* UTAD104 at 10% (D), 20% (E) and 30% (w/w) concentration (F) and *P. nordicum* control (without film) (G).

Similar results were obtained with the agar diffusion assay (Figure 4.3). No inhibition halo was formed around the films incorporating *L. casei* UM3 at any of tested concentrations. In this case, the fungus has even managed to cover the surface of the films. The same of observed for films without cells. For *L. buchneri* UTAD104 containing films, an inhibition halo was clearly visible in films containing 20 and 30% (w/w) of cells. These inhibition halos were maintained for at least 30 days.

The antifungal capacity of *L. buchneri* UTAD104 has already been reported and is mainly due to organic acids, such as, acetic, lactic and phenyllactic acids, as it was described in chapter 3 of this thesis. When LAB are incorporated in the films, produced organic acids are probably diffusing into the culture medium, inhibiting *P. nordicum* growth around the film margin. This is probably the main

inhibitory mechanism responsible for the inhibition of *P. nordicum*, since if other inhibitory mechanisms were involved (e.g. competitive growth), an inhibitory effect in fungal growth in films incorporating *L. casei* UM3 would had been observed.

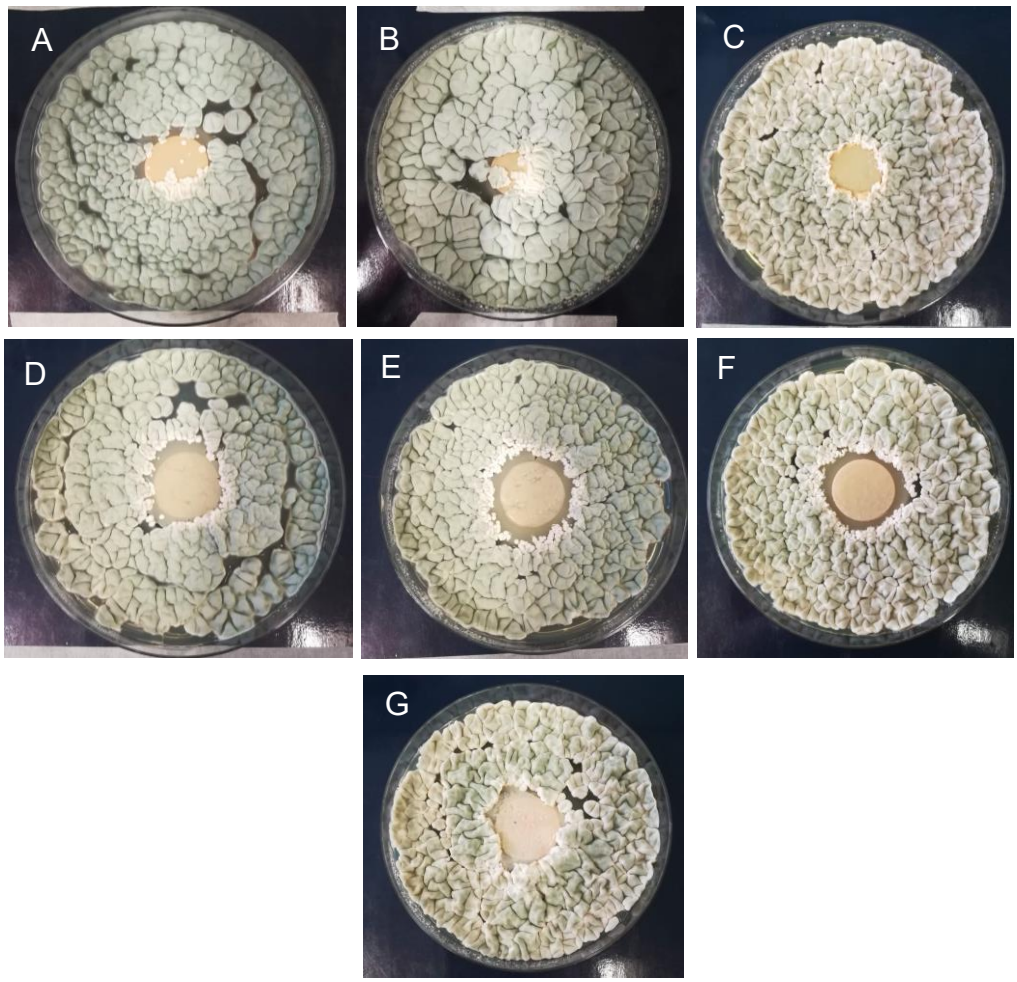


Figure 4.3. Antifungal activity against *P. nordicum* MUM 08.16 of WPC based films with and without LAB by agar diffusion assay. Films incorporating *L. casei* UM3 at 10% (A), 20% (B) and 30% (w/w) concentration (C); and incorporating *L. buchneri* UTAD104 at 10% (D), 20% (E) and 30% (w/w) concentration (F) and films without cells (G).

4.3.3 Lactic Acid Bacteria Survival in Films

Viability of *L. buchneri* UTAD104 and *L. casei* UM3 incorporated in WPC based films was assessed during a storage period of 30 days at room temperature in order to evaluate films capacity to maintain

cell survival. Changes in the count of viable cells over time are shown in Figure 4.4 (data for the construction of this figure can be seen in Table B.1 and Table B.2 in appendix B).

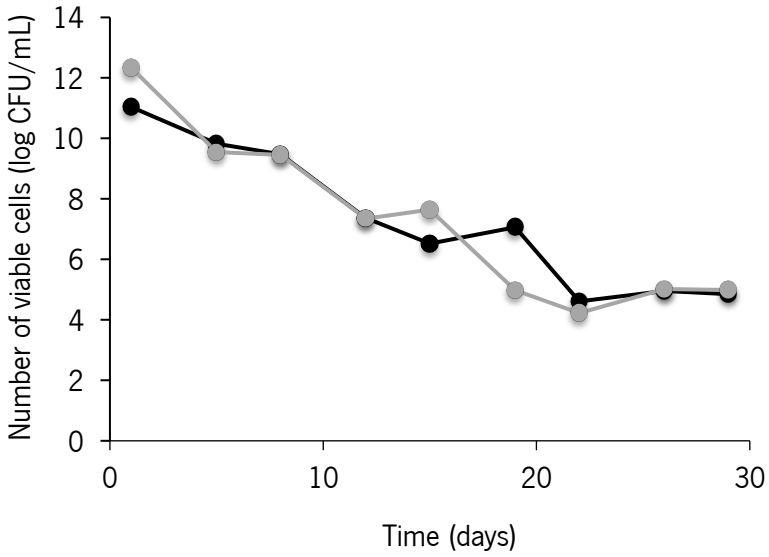


Figure 4.4. Number of viable LAB (expressed in log CFU/mL) present in the films over time (days). *L. buchneri* UTAD104 represented by the black line and *L. casei* UM3 represented by the grey line.

For both strains, there is a consistent decrease in the number of viable LAB in the films throughout the 30 days of storage. In the first 15 days of storage, there was a more accentuated decrease in viable LAB in films, of approximately 5 log CFU/mL. After that period, and until 30 days of storage, the decrease in viable LAB was less accentuated (approximately 1 to 2 log). Nonetheless, between day 20 and 30, the films were able to maintain a concentration of bacteria of ca. 1×10^5 CFU/mL for both LAB, with *L. buchneri* UTAD104 maintaining 1×10^7 CFU/mL around 20 days of storage.

In edible films, the presence of relatively high amounts of solutes combined with rubbery physical state leads to the occurrence of enzymatic and chemical reactions which can cause damage in essential cellular structures (Soukoulis et al. 2014b), and consequently affect cell survival within those films. Still, nutrients present in the films can, normally, support cells to maintain their viability (Soukoulis et al. 2014a). However, loss of cellular viability throughout time still occurred, this can be mainly attributed to film dehydration and further depletion of nutrients.

Similar results for cell survival in films were obtained by Fan et al. (2009), which reported the capability of sodium alginate films to maintain half of the *Cryptococcus laurentii* population after a 20-day storage period. Sánchez-González et al. (2013) also reported the ability of protein films, made of

pea protein, methylcellulose and sodium caseinate, to maintain at least half of the initial viable counts of *L. plantarum,* reducing from the initial 5 log CFU/g to 3 and 2.5 log CFU/g for pea protein and methylcellulose, respectively. *L. plantarum* did not lost viability in sodium caseinate films after 30 days of storage at 5 °C. Differences in cell survival in film matrix may be attributed to the tolerance of entrapped bacterial strain, composition and structure of the film and drying kinetics and storage conditions (Soukoulis et al. 2014b).

4.3.4 Morphological Characterization of Films

An extensive characterization was made to films containing *L. buchneri* UTAD104 (30% w/w), since this formulation presented the best antifungal ability. Surface structure of the films were analysed using Scanning Electronic Microscopy (SEM). Images of the surface of control film and films with *L. buchneri* UTAD104 are shown in Figure 4.5. The less magnified images (Figure 4.5 A and B) show a heterogeneous surface, which was probably cause by a deficient dissolution of the guar gum. Fissures are present at the surface, especially in films containing *L. buchneri* UTAD104. These fissures are only superficial and do not go through the film. The structural integrity was not significantly affected as seen in the mechanical analysis (presented further in this section). Water evaporation, during the film drying, in conjugation with the presence of cells may be responsible for the higher number of fissures in the film, when compared with control films. Other authors have reported a similar effect when incorporating essential oils in protein-based films (Broumand et al. 2011; Bahram et al. 2014), which lead to the presence of fissures caused by evaporation during drying of the film. Bacterial cells are clearly visible embedded in film, as seen in Figure 4.5 D and F. Bacterial morphology was apparently not altered by the incorporation in the film.

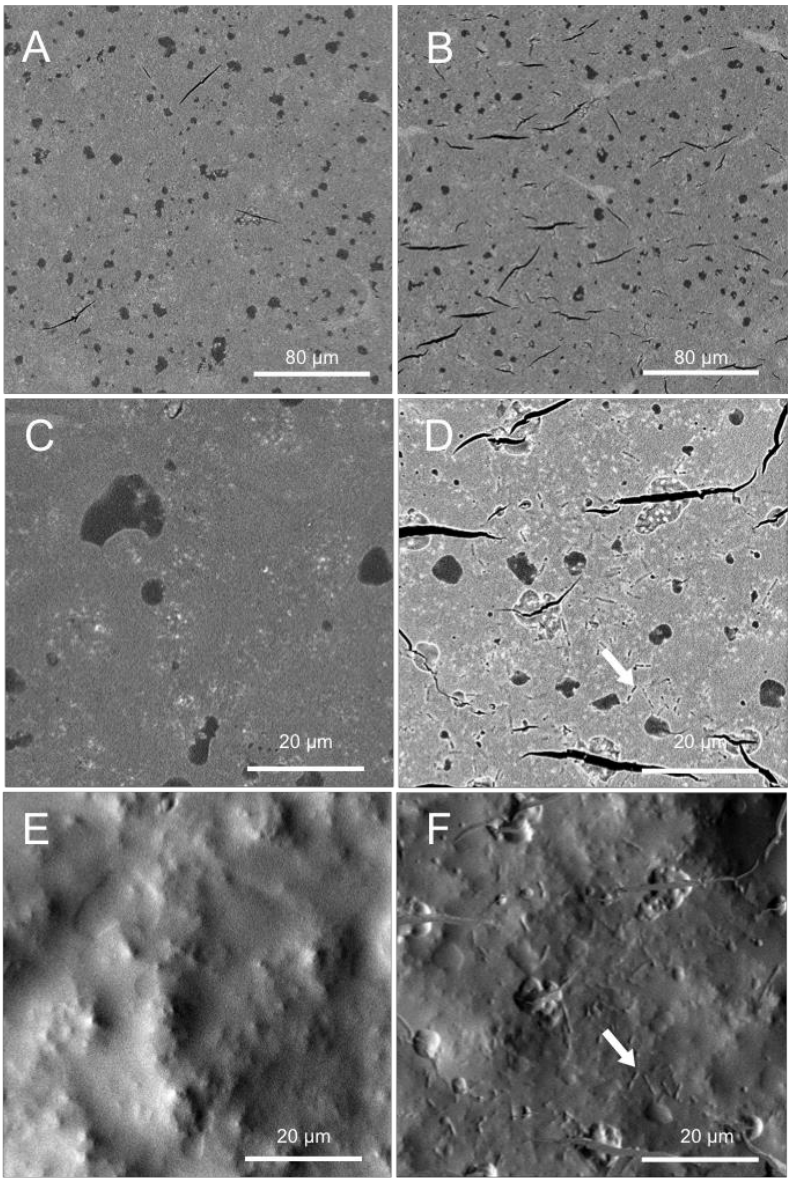


Figure 4.5. Scanning electron microscopy image of the film surface without (A); and with *L. buchneri* UTAD104 (B), at magnification of 1000x. Image of film surface without (C and E); and with *L. buchneri* UTAD104 (D and F), at magnification of 4000x. Images E and F show the topography of the films. Arrows indicate the presence of bacteria cells.

4.3.5 Film Characterization

4.3.5.1 Thickness, moisture content, water solubility and WVP

Table 4.1 shows the values obtained for thickness, moisture content, water solubility and WVP of films with and without the incorporation of *L. buchneri* UTAD104 (30% w/w) (original data for this table can be seen in Table B.3, Table B.4, Table B.5, Table B.6 and Table B.7 in appendix B). In general, incorporation of LAB did not affect significantly (p<0.05) the moisture content and WVP values in comparison to the plain WPC films (control films). However, the addition of cells decreased significantly the thickness of the films and increased slightly their solubility in water (Table 4.1).

Table 4.1. Thickness, moisture content, water solubility and WVP of films without and with *L. buchneri* UTAD104

WPC based films	Thickness (mm)	Moisture content (%)	Solubility (%)	WVP (g mm/(day m^2 kPa))
Without cells	0.23 ± 0.02[a]	26.2 ± 0.9[a]	43.7 ± 1.6[a]	9.6[a]
With *L. buchneri*	0.17 ± 0.03[b]	25.0 ± 1.2[a]	45.1 ± 0.9[b]	10.0[a]

[a-b] Different letters within the same column indicate significant differences (p<0.05). Results are presented as mean ± standard deviation

The thickness of edible films can vary with the preparation method and drying conditions (Kokoszka et al. 2010), and can affect their optical properties, permeability and mechanical properties. The thickness of the WPC based films was significantly affected by the incorporation of cells with values decreasing from 0.23 mm (control film) to 0.17 mm (film incorporating *L. buchneri* UTAD104). Thickness of the obtained control films was in general higher than those described in literature for whey protein films by Pérez-Gago et al. (1999), Ramos et al. (2013) and Pérez et al. (2016), i.e. 0.115, 0.170 and 0.126 mm, respectively. Still, other works reported thicker whey protein films (0.31 mm) (Bahram et al. 2014). These differences in film thickness are probably due to different film formulations and differences in the film making procedure, even though examples given are whey protein based.

The moisture content of edible films is an important parameter to estimate other film properties, such as dissolution ability during human consumption and capacity to withstand probiotic survival (Kanmani and Lim 2013). The incorporation of cells has not affected significantly the moisture content

of the films with control films having slightly higher moisture content values (25.0% *versus* 26.2%, respectively). Similar values of moisture content (27.9%) for WPC based films, with glycerol as plasticizer, were obtain by other authors (Pérez et al. 2016).

Water solubility is an important property of films for food applications. Many food applications require insoluble films with water resistant properties. For example, when foods have high water activity or when food processing includes steps that promote films contact with water. However, solubility in water may have advantages in consumer's acceptance and is also an indication of films biodegrability when used as packaging wrap (Stuchell and Krochta 1994; Bourtoom and Chinnan 2008). In general, higher solubility indicates lower water resistance.

Both films (with and without cells) maintained their integrity during the dissolution procedure for 24 h, though films have swelled. Control films presented solubility in water of 43.7%, while films incorporating cells showed a significantly higher (p<0.05) solubility of 45.1% (Table 4.1). The solubility of the control films was relatively similar when compared with other similar films in literature (Pérez-Gago et al. 1999; Ramos et al. 2012c; Pérez et al. 2016). The partial insolubility of the films can be attributed to strong intermolecular bonds between the protein molecules within the film matrix (Ramos et al. 2013). The slightly higher solubility of films containing *L. buchneri* UTAD104 can be explained by their higher content of water due to the incorporation of the cell culture. It also may indicate lower cohesion of the film matrix, due to the presence of cells.

The WVP is an important characteristic of edible films, as it gives an indication of their barrier properties against water vapour. Permeability to gases is influenced by film composition, crystallinity, thickness, intermolecular interactions, plasticizer type and content, existence of fissures or pores; and environmental relative humidity and temperature (Bertuzzi et al. 2007). Production of edible films with low WVP is generally necessary for the control of detrimental reactions (such as lipid oxidation, browning and vitamin reactions), the control of structural and textural decay (which facilitates microbial spoilage) and decrease weight loss of the food product.

The WVP of control films and films with *L. buchneri* UTAD104 were 9.6 and 10.0 g mm/(day m^2 kPa), respectively. Thus, the incorporation of cells in the film did not significantly alter their WVP. For the control films, similar WVP results were obtained by Ramos et al. (2012c). Alike, Aloui et al. (2015) and Gialamas et al. (2010) reported that the incorporation of cells did not affect significantly the barrier properties of films. In contrast, Sánchez-Gonzalez et al. (2013) reported that the incorporation of *L. plantarum* increased WVP due to the discontinuities introduced in the film matrix. As

expected, the WVP of the produced WPC films is high as a result of their hydrophilic nature and a high content of glycerol. This is a consequence of free hydroxyl groups in the matrix that can interact with migrating water molecules, resulting in high WVP values of protein films, with a high content of glycerol (5%) (Fang et al. 2002; Bertuzzi et al. 2007; Ghanbarzadeh and Oromiehi 2008; Kokoszka et al. 2010). As reported by Rodríguez et al. (2006) tween 20, despite its hydrophobic nature, may have also contributed to increase permeability of the films.

4.3.5.2 Mechanical properties: tensile strength and elongation at break

Edible films should be able to maintain their integrity during processing, storage, shipping and handling. To do so, they must possess adequate tensile strength and elongation (Falguera et al. 2011). In Table 4.2 presents the results of tensile strength (TS) and elongation at break (EB) for control films and films with *L. buchneri* UTAD104 (complete data for the construction of this table can be seen in Table B.8). Tensile strength indicates the maximum stress that the films sustain during stretching and elongation at break indicates the increase in film length from the initial point to the break point, giving a measure of the film extensibility.

Table 4.2. Tensile strength and elongation at break of WPC films with and without cells

WPC films	Tensile Strength (MPa)	Elongation at break (%)
Without cells	0.6 ± 0.06[a]	53.8 ± 25.1 [a]
With *L. buchneri*	0.5 ± 0.11[a]	48.1 ± 21.3[a]

[a] no significant differences between film formulations (p<0.05). Results are expressed as mean ± standard deviation

In general, the results indicate that the presence of cells did not influence significantly the mechanical properties of the films. This is in agreement with results obtained by other authors (Gialamas et al. 2010; Kanmani and Lim 2013; Soukoulis et al. 2016). Still, films with cells showed a lower TS and EB than control films. This can be attributed to the discontinuities introduced in the film by the presence of cells and cracks visible in the surface of the films (as shown in Figure 4.5 B). These films are less stiff, less extensible and weaker than control films. Still, these differences are not

significant; thus, films incorporating *L. buchneri* UTAD104 should be able to stand the same procedures as control films. Values for TS are similar to those obtained by Ramos et al. (2012c) and Bahram et al. (2014); however, these values are relatively low when compared with other edible films (Pérez-Gago et al. 1999; Pérez et al. 2016). It is known that the content of plasticizer can influence the TS of films, with high contents of plasticizer (such as the used in these films), resulting in low of TS values, due to their ability to reduce polymer-polymer interactions that are needed for higher tensile strengths (Fang et al. 2002). Additionally, McHugh and Krochta (1994) reported that high contents of glycerol in whey protein films significantly decreased tensile strength and increased elongation at break. WPC films had a EB of almost 50% for both films; which are similar values to the ones reported for whey protein films (Pérez-Gago et al. 1999).

4.3.5.3 Optical properties

Colour and optical properties are important characteristics of edible films, since they can directly influence the consumer and affect product choice. Colour of the films is affected by several factors that include plasticizer addition, thermal treatments, production process, storage conditions and, in the case of protein films, the protein concentration (Su et al. 2012). In Table 4.3 are shown the hue values and opacity for each film (data for this table can be seen in appendix B, Table B.9 and Table B.10). All analysed parameters were significantly affected by the incorporation of cells. However, all samples showed a yellowish colour typical of WPC based films, which can be linked to the presence of fat and phospholipids (Ramos et al. 2013; Pérez et al. 2016). In general, films with cells have less luminosity (L^*) than control films (90.6 and 94.1, respectively); have a slightly red hue opposed to control films that show a more "neutral" hue (a^* values of -0.4 for control films and 0.8 for films incorporation cells); and are also more yellowish than control films (higher b^* values of -22.4 for films with cells than control film, -10.5). The addition of cells was also related to an increase in the opacity of the films, when compared to control films (13.4 and 12.6%, respectively). The total colour difference (ΔE) that includes the three colour parameters was calculated for both films. Values of 9.2 and 21.6 were obtained for films without and with *L. buchneri* UTAD104, respectively. This difference confirms that addition of cells alters the colour and optical properties of WPC based films and that this difference can be perceived clearly, as 3 is considered the threshold of human perceivable colour differences for ΔE values. The

incorporation of cells within culture media (MRS) is probably the main responsible for these differences of colour.

Table 4.3. Colour parameters *L**, *a** and *b** and opacity of WPC based films with and without *L. buchneri* UTAD104

WPC films	*L**	*a**	*b**	Opacity (%)
Without cells	94.1±0.45 [a]	-0.4±0.03[a]	10.5±0.33 [a]	12.6±0.30 [a]
With *L. buchneri*	90.6±0.65 [b]	0.8±0.56 [b]	22.4±0.79 [b]	13.4±0.24 [b]

[a-b] Different letters within the same column indicate significant differences between film formulations ($p<0.05$). Results are expressed as mean ± standard deviation

4.3.5.4 Surface hydrophobicity

Water contact angle measurement is used to determine the hydrophilicity ($\theta < 65°$) or hydrophobicity ($\theta > 65°$) of films surface. This can be used to estimate the resistance of film to moisture transfer (Phan et al. 2005).

The initial contact angle (time 0 s) and contact angles after 1, 5, 15, 30, 45 and 60 s of applied water droplets were measured. Upper side of the films were analysed and obtained values are shown in Table 4.4 (data for the construction of this table can be seen in Table B.11). In general, both films demonstrated a hydrophilic character ($\theta < 65°$) at 0 s, with similar θ values for control films and films with cells (51.06° and 51.13°, respectively). These values are similar to the ones obtained in other works (Kokoszka et al. 2010). Throughout the experiments contact angles values dropped until reaching 29.5° and 20.8°, for films without and with *L. buchneri* UTAD104, respectively. The control films showed lower θ values until 30 s after the water droplet application, however the differences between films were only statistically significant after 60 s.

With time, swelling of the films (more visible in films with *L. buchneri* UTAD104) and adsorption of water droplet were noticeable. Swelling effect was observed for times larger than 30 s. These effects are linked with the presence of plasticizers that lead to increasing swelling capacity of the films and with water binding mechanisms and interactions between water and the surface of the films (Galus and Kadzińska 2016).

Table 4.4. Measurements of contact angle (θ) in function of time (s) after water droplet application on films with and without cells of *L. buchneri* UTAD104

	Contact angle (θ)	
Time (s)	WPC films without cells	WPC films with *L. buchneri*
0	51.1 ± 2.86 [a]	51.1 ± 4.78 [a]
1	40.5 ± 2.24 [a]	41.1 ± 1.96 [a]
5	35.7 ± 2.17 [a]	36.6 ± 1.27 [a]
15	32.6 ± 2.23 [a]	34.2 ± 1.14 [a]
30	30.7 ± 1.69 [a]	31.7 ± 1.36 [a]
45	29.9 ± 1.47 [a]	25.8 ± 7.41 [a]
60	29.5 ± 1.39 [a]	20.8 ± 10.35 [b]

[a-b] Different letters within the same line indicate significant differences between film formulations ($p<0.05$). Results are expressed as mean ± standard deviation

4.3.5.5 Fourier Transform Infrared spectroscopy

FTIR spectrum of films was analysed in the region of 4000 to 400 cm^{-1} (Figure 4.6). Similar peaks can be observed for both samples, with and without cells. The strong absorption band at 3271 cm^{-1} is associated with the stretching vibration of free and bound OH groups and N-H groups, which can be intensified by the addition of glycerol (Cerqueira et al. 2012; Ballesteros et al. 2018). Literature indicate that the N-H band appears generally at 3253 cm^{-1} (Ramos et al. 2013). So, the presence of the other film components, in particular glycerol, may be responsible for the band shift seen in the obtained spectra. This band is also characteristic of moist materials. The bands at 2874 cm^{-1} and 2930 cm^{-1} correspond to stretching of –C-H groups. Bands at 1535 cm^{-1} and 1628 cm^{-1} are characteristic of primary amine group of proteins (-CO-NH_2) and the secondary amide group of proteins (-CO-NH), and are characteristic of whey proteins. The band around 1035 cm^{-1} corresponds to the C-O band stretching and can be related to the addition of glycerol (López-Rubio and Lagaron 2012; Pérez et al. 2016). Also, bands at 852 cm^{-1} and 924 cm^{-1} corresponds to asymmetric and symmetric stretching vibrations of alkoxyl group (C-O-C) and are also originated by the presence of glycerol in the films (Cerqueira et al. 2012). From this FTIR analysis it can be concluded that the presence of *L. buchneri* UTAD104 did not alter the chemical structure of the films.

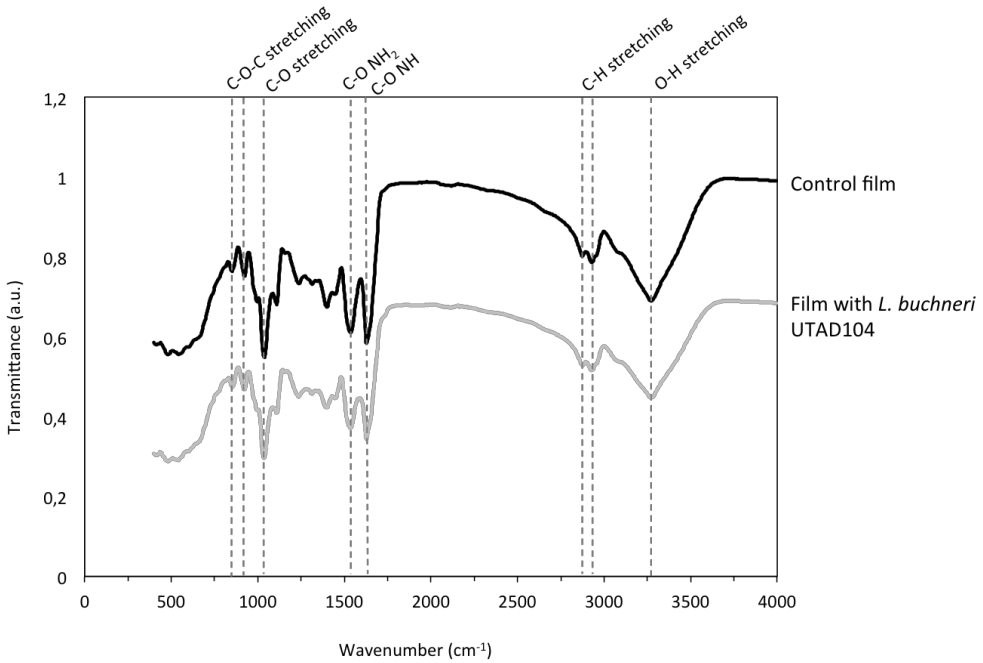


Figure 4.6. Fourier-transform infrared spectra obtained from control films and with *L. buchneri* UTAD104.

4.3.5.6 X-Ray diffraction

X-ray diffraction (XRD) pattern was used to perceive if the addition of cells to the film caused alterations in the film crystallinity. Generally, in a crystalline material spectra, several sharp peaks are present, while in amorphous substances a broad background pattern is shown (Fernandes et al. 2014). Figure 4.7 shows the XRD spectra of the analysed films, were the two films (with and without cells) showed similar profiles.

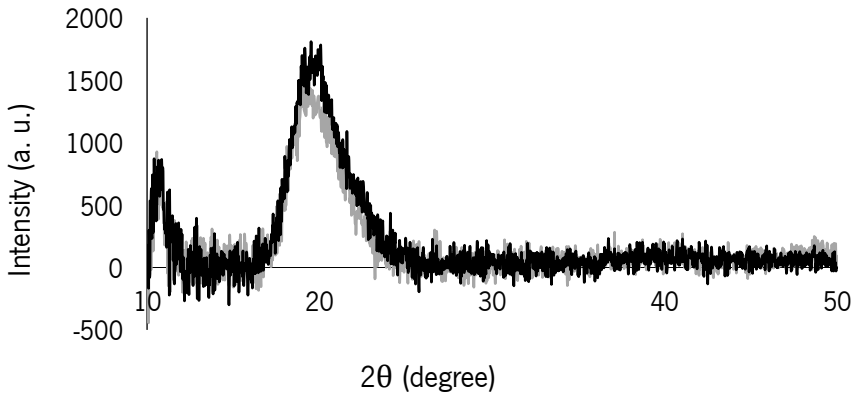


Figure 4.7. X-ray diffraction pattern of films without (black) and with *L. buchneri* UTAD104 (grey).

As expected, both films show a non-crystalline structure, presenting two broad peaks at approximately 2θ=11° and 2θ=20°. This second peak is present in the three raw materials (whey protein, glycerol and guar gum) that compose the tested films and is typical for amorphous structures (Martins et al. 2012), while 2θ =11° is typically present in whey protein XRD pattern (Le Tien et al. 2000; Li et al. 2011).

4.3.6 Antifungal Activity of the Bioactive Coatings against *P. nordicum* in Cheese

4.3.6.1 Cheese covered with films

The effect of the different WPC based coatings on fungal decay of cheese during storage at 25 °C and 90% RH are presented in Figure 4.8.

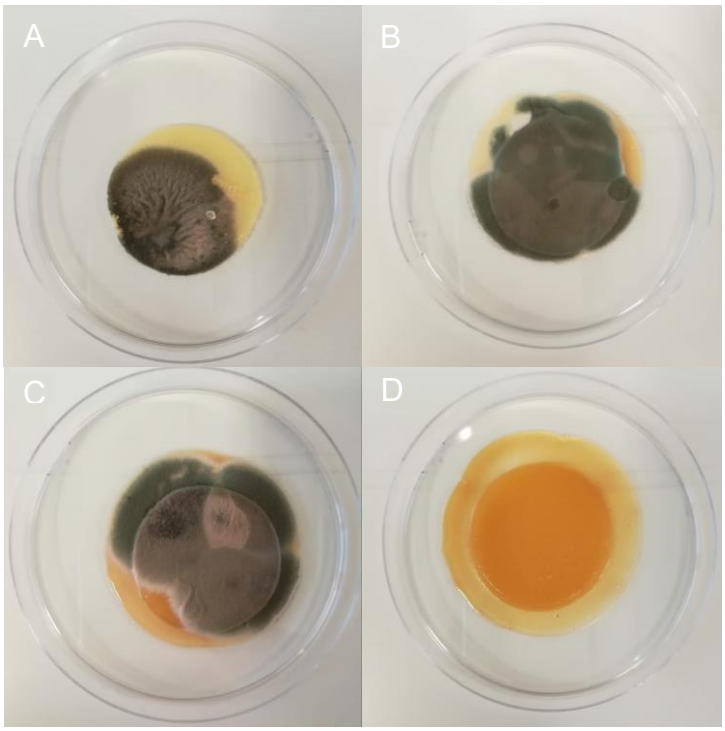


Figure 4.8. Growth of *P. nordicum* MUM 08.16 in uncoated cheese (A); cheese coated with WPC based film (B); cheese covered with WPC based film incorporating *L. casei* UM3 at 30% (C); cheese covered with WPC based film incorporating *L. buchneri* UTAD104 at 30% (D).

After being produced, films were placed on the top of cheese samples and inoculated with *P. nordicum*. After 30 days of storage, the total absence of fungal growth was only observed on cheese with films incorporating *L. buchneri* UTAD104 (Figure 4.8 D). In control films, the fungal colonies became visible with a delay of two days comparing to uncovered cheeses, and after 20 days of incubation, their surface was already entirely covered by the fungus (Figure 4.8 B and C). For the uncovered cheeses, this occurred after 7 days of incubation Figure 4.8 A).

4.3.6.2 Cheese coatings

The WPC solutions were used to coat the cheese samples directly, eliminating the film-forming step and thus simplifying the application process. Coated cheese samples were stored similarly at 25 °C and 90% RH. In general, after 5 days of storage, the uncoated cheese samples (Figure 4.9 A) were considerably infected by *P. nordicum,* with the fungi covering all the surface of the cheese sample. Cheeses coated with WPC without LAB and coated with WPC incorporating *L. casei* UM3, also developed fungal contamination. However, when compared to uncoated samples, there was a considerable delay in the infection process. Thus, it seems the coating alone affected the development of the fungus (Figure 4.9 B), probably, because it created a barrier between the fungus and the nutritive cheese surface. Cheese samples coated with WPC incorporating the antifungal strain *L. buchneri* UTAD104 remained free of fungal contamination for at least 30 days (Figure 4.9 D). Other authors reported similar results for different coatings materials. One work showed that chitosan coatings reduced infection in strawberries up to three days of storage when compared with non-coated ones (Perdones et al. 2012), while another presented that sodium alginate and locust bean gum coatings delayed infection in oranges for 5 days, when compared with uncoated fruits (Aloui et al. 2015).

When comparing both application strategies, the *P. nordicum* growth was less accentuated in coated cheese samples (Figure 4.9) than in film covered ones (Figure 4.8). The drying step required after the coating of the cheese can be responsible for this effect, since it lower samples a_w, thus making fungal germination and colonization more difficult.

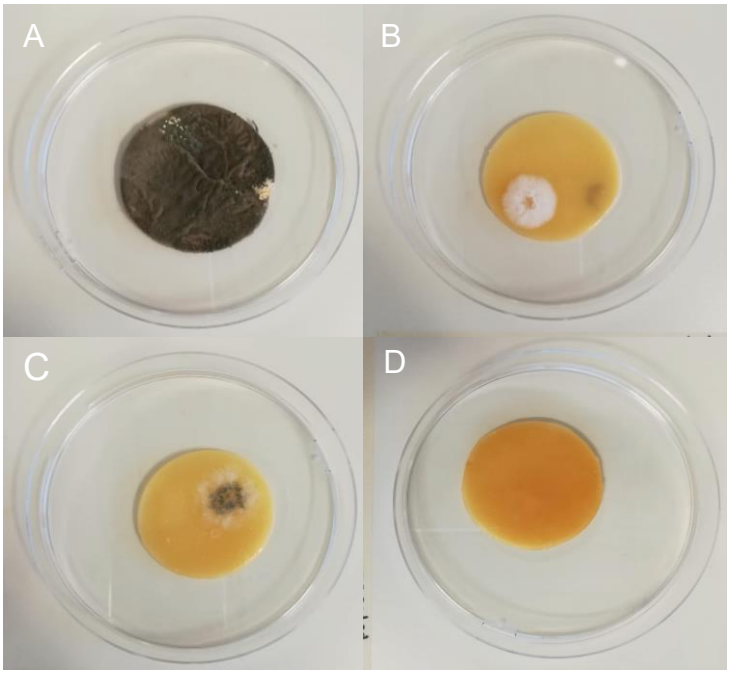


Figure 4.9. Growth of *P. nordicum* MUM 08.16 in uncoated cheese (A); cheese coated with WPC film forming solution (B); cheese coated with WPC incorporating *L. casei* UM3 at 30% (C); cheese coated with WPC incorporating *L. buchneri* UTAD104 at 30% (D).

4.3.6.3 Mycotoxin analysis in cheese covered with films and coated

OTA content was analysed in cheeses covered with WPC based films and coatings (Table 4.5) (data for the construction of this table can be seen in appendix B in Table B.12). All films were able to inhibit OTA production by over 83%, with films containing *L. casei* UM3 reaching a 94% inhibition. In cheese samples covered with WPC containing *L. buchneri* UTAD104, OTA was not detected since there was no fungal growth. Additionally, the size of fungal colonies on the surface of the films (as it can be seen in Figure 4.8) did not correlate with their OTA content. *P. nordicum* grew to similar sizes in uncovered cheeses and cheeses covered with WPC and WPC+*L. casei* UM3, but OTA detected on those samples was significantly different. This can indicate that the presence of WPC based films may exert, by itself, an inhibitory effect on the production of OTA. In coated cheeses, OTA was not detected in any case probably due to the weak fungal growth (as seen in Figure 4.9 B and C).

To our knowledge, none of the studies regarding the incorporation of LAB in edible films that reports the mycotoxin content of the product, thus this data cannot be compared with previous publications.

Table 4.5. OTA (ng/mL) detected on cheese samples covered with WPC based films and coatings

	Films		**Coatings**	
Cheese	OTA (ng/mL)	Inhibition (%)	OTA (ng/mL)	Inhibition (%)
Uncoated	7.5 ± 1.7	-	8.8 ± 1.5	-
WPC film	1.3 ± 0.3	83	0	100
With *L. casei*	0.4 ± 0.3	94	0	100
With *L. buchneri*	0.0 ± 0.0	100	0	100

OTA values are expressed as mean ± standard deviation

4.4 CONCLUSIONS

This study indicates that WPC based films incorporating LAB with antifungal properties could potentially be used in food packaging, contributing for an increased shelf life of cheese. Moreover, it is demonstrated that the presence of cells did not significantly affect moisture content, WVP and mechanical properties (TS and EB) of the films. However, the presence of cells had impact on the thickness of the films and colour of the films. SEM images show that cells are embedded in the film matrix. Despite LAB numbers keep diminishing during the experiment time, after 30 days, there was still a high number (1×10^5 CFU/mL) of viable cells in the films. FTIR and XRD data suggest that cells did not modify the films' chemical structure or crystallinity. Application of the active film to cheeses allowed the inhibition of *P. nordicum* growth for a storage period over 30 days. OTA was not present in cheeses covered or coated with *L. buchneri* UTAD104. Thus, the incorporation of LAB into edible films and coatings can represent a suitable natural strategy to control fungal proliferation in cheese products.

CHAPTER 5

CONCLUSION AND FUTURE PRESPECTIVES

5. CONCLUSION AND FUTURE PERSPECTIVES

The interest in biological preservation methods has increased in recent years, encouraged by scientific evidence that antagonistic microorganisms and their products of metabolism have potential to be used as natural preservatives, in the control of growth or in the elimination of pathogenic microorganisms in food. Lactic acid bacteria and their metabolites have been considered as a potential biopreservative method to improve food safety. Antimicrobial activity of LAB is mainly attributed to competition for nutrients and production of antimicrobial metabolites. This ability of LAB can be used to extend shelf life of food and improve food safety, without resorting to chemical additives that can affect nutritional and sensory properties of the food.

Biopreservation methods are considered to: i) not affect consumers health; ii) have a small impact of food nutritive proprieties; iii) being capable of reducing food processing costs; iv) not require advanced technology, and therefore could be used in undeveloped countries; and v) be a possible answer to the increased pesticides and fungicides resistances and for the control of emerging pathogens.

In this work, two LAB strains, *L. plantarum* UM55 and *L. buchneri* UTAD104, and their metabolites have shown to be effective against two economical and health impacting fungal species, *A. flavus* and *P. nordicum*, that contaminate mainly maize and high protein content foods, respectively. These species produce aflatoxins and OTA, two highly toxic mycotoxins. LAB CFS were able to reduce *A. flavus* growth by 30% and aflatoxins by 90%, while *P. nordicum* growth was reduced by 17% and OTA accumulation by 60%. Antifungal activity of LAB was a result of the production of organic acids, mainly lactic acid, acetic acid, PLA, OH-PLA and ILA. The efficiency of these acids was species dependent, with PLA being more effective in inhibiting *A. flavus*, while acetic acid was more effective against *P. nordicum*. ILA at 4 mg/mL was able to inhibit aflatoxins and OTA production by over 99%, and fungal

growth by 34% and 50% of *A. flavus* and *P. nordicum*, respectively. To our knowledge this was the first time that antifungal ability of ILA was described.

L. buchneri UTAD104 cells inhibited completely *P. nordicum* growth and OTA production, being these effects associated to the production of acetic acid. When this strain was incorporated into WPC based films and coatings, it did not affect significantly the structural properties of the films, and the films were able to maintain a reasonable number (1×10^5 CFU/mL) of viable cells for over 30 days. Films and coatings incorporating *L. buchneri* UTAD104 were effective in controlling the growth of *P. nordicum* in petri plate assays and when applied in cheese samples, with OTA being almost absent in these samples.

Further work regarding the biopreservation approaches and application of antifungal LAB strains and/or their metabolites in edible films still need to be conducted.

It would be interesting to elucidate why the mycotoxin contents are always more affected by the LAB CFS then fungal growth. The fact that CFS and tested organic acids inhibit more the production of the mycotoxins than the fungal growth seems to indicate that these compounds interfere with the metabolic pathways that lead to their biosynthesis. In the last decade, significant progress has been made in describing the aflatoxin biosynthesis pathway, genes involved and regulatory mechanisms (Bhatnagar et al. 2003). Studies in the expression of genes involved in mycotoxin biosynthesis could elucidate how LAB metabolites affect this pathway and if the inhibition of mycotoxins can cause the accumulation of other potential toxic products. For example, alterations in the synthesis of aflatoxin B1 can lead to the accumulation of its precursors (e.g. sterigmatocystin, an also toxic compound). Mycotoxin genes are generally clustered together, so approaches can be developed to inhibit specific regulatory or structural genes to stop mycotoxin production (Medina et al. 2017).

It would also be interesting to study the antimicrobial ability of *L. plantarum* UM55 and *L. buchneri* UTAD104 against other common food contaminants. In the screening part of this work, these strains showed to have antifungal ability also against *A. parasiticus* and *A. carbonarius*. As reported, *L. plantarum* UM55 also inhibited other aflatoxigenic *Aspergillus* species and *L. buchneri* UTAD104 was effective in inhibiting *Mucor racemosus* (data not shown). *L. plantarum* strains have been described to inhibit the growth of *Listeria* spp., *Enterococcus*, *Streptococcus* and *Staphylococcus* among other food spoilage and pathogenic organisms; and both *L. plantarum* and *L. buchneri* have been described as producers of bacteriocins, defined as proteinaceous compounds with inhibitory activity against related species (Rodríguez et al. 2000). Further screening for antifungal properties and compounds of *L.*

plantarum UM55 and *L. buchneri* UTAD104 could elucidate if these strains have a broader antimicrobial capacity and potential to be applied in other scenarios other than fungal and mycotoxin control. Additionally, a thorough investigation of all the compounds produced by LAB with antifungal ability could be preformed to find other inhibitory metabolites other than organic acids. Methodologies based on liquid chromatography coupled with mass spectrometry (LC-MS) conjugated with efficient extraction techniques could be useful to identify and isolate this compounds.

Even though synergetic behaviour between antifungal compounds produced by LAB has been speculated, until now synergetic mechanism between these compounds have not been elucidated (Lavermicocca et al. 2000). It is thought that the general antimicrobial effects of lactic and acetic acids, the main fermentation products, might act synergistically with other antifungal compounds. Each compound produced during LAB fermentation will act as an additional hurdle for spoilage microorganisms (Schnurer and Magnusson 2005). This is an important issue to account when developing biopreservation approaches. Also, many other low-molecular mass compounds with antimicrobial ability might be undetected due to the lack of efficient techniques to isolate trace levels of a substance in complex environments (Schnurer and Magnusson 2005).

Increased antifungal capacity could be achieved by supplementing culture media with compounds that lead to improved production of antifungal metabolites. Addition of phenylpyruvic acid, a precursor of PLA, allowed the increase up to 6 times of PLA, resulting in an increased antifungal activity against *A. niger* and *P. roqueforti* (Valerio et al. 2016). Comprehension of metabolic pathways of organic acids or other antifungal compounds produced by LAB could result in new strategies for the enhancement of their production. For example, supplementation with phenylalanine could lead to higher production of PLA and OH-PLA, and addition of tyrosine would increase ILA production. Other growth conditions and growth media could be further optimized to promote the production of these antifungal compounds.

Progresses in synthetic biology can conduct to the development of recombinant strains with enhanced ability of producing antifungal compounds. As example, a work using a recombinant *E. coli* was able to convert phenylalanine (a rather inexpensive precursor) into PLA with a yield of 81% (Zhao et al. 2018). Published genomic sequence of *L. plantarum* (strain WCFS1) can be an essential tool in increasing knowledge in the antimicrobial activity of these bacteria (Klaenhammer et al. 2002). Genome sequencing and DNA microarray analysis may also elucidate the differences between LAB strains that possess unique metabolic features and other without technological interest. Antifungal activity by LAB can often be altered by environmental conditions, and these technologies will allow comparing

transcription profiles of a given strain under producing and non-production conditions. This could provide important clues regarding the genes involved in the antimicrobial phenotype of these bacteria and provide a starting point for improving antimicrobial activity of a certain strain (Nes and Johnsborg 2004).

Also regarding these strains, it could be relevant to assess their probiotic potential. By definition "*Probiotic is a microbial dietary adjuvant that beneficially affects the host physiology by modulating mucosal and systemic immunity, as well as improving nutritional and microbial balance in the intestinal tract*" (Fuller 1989). LAB probiotics effects have been reported to improve nutritional quality of food, stimulate vitamin synthesis and enzyme production, stabilize gut microflora, enhance innate host defence by producing antimicrobial substances, and reduce of serum cholesterol (Naidu et al. 1999). To provide these health benefits, LAB must overcome acid and bile in the gastrointestinal track, inhibit the growth of pathogenic microorganisms, and reach gut in concentrations of at least 1×10^6 CFU/mL. Strains of *L. plantarum* (de Vries et al. 2006; Nguyen et al. 2007) and *L. buchneri* (Zeng et al. 2010) have demonstrated probiotic potential. It is accepted that probiotics are likely to provide general health benefits; however, many properties of probiotics are strain specific, and efficiency and safety associated with specific formulations should not be extrapolated to other probiotic products. In case of probiotic capability of *L. plantarum* UM55 and *L. buchneri* UTAD104, the combination of this ability with antifungal activity would add value to the food or feed.

New methods of application of LAB antimicrobial strains are also currently being developed. Most commonly used strategies incorporate LAB strains directly as starter cultures or use a biopreservative preparation in the form of previously fermented product or use the semi-purified or purified antimicrobial compound (Reis et al. 2012). Since viability of LAB is essential for their role as bioprotective cultures, several techniques have been developed to maintain cell viability and facilitate use of LAB in a wider number of food products. Microencapsulation has been consistently used and is recognized as an effective method, since immobilization of LAB in droplet matrix can protect cells against environmental conditions (heat, air, light, humidity), as well as competitors. To date, microencapsulation of cells in spray or freeze-dried matrices and in biopolymer-based microcapsules is the most common strategies to incorporate cells in real food systems. Encapsulation is usually applied to ensure the viability and activity of probiotics in the digestive tract, but the same concept can be extended to LAB as bioprotective cultures (Behboudi-Jobbehdar et al. 2013).

More recently, and as already described in this work, edible films have been applied for food preservation with relative success in several food products (Gialamas et al. 2010; Concha-Meyer et al. 2011; López de Lacey et al. 2014; Dai et al. 2018; Pereira et al. 2018). The application of edible films in food as proven to be effective in increasing shelf life and slow detrimental reactions by providing a barrier that retards water vapour, oxygen and solutes mobility (Falguera et al. 2011).

The use of microencapsulation and edible films might allow the controlled release of active substances. Small active antimicrobial molecules are often released completely even before spoilage microorganisms start to grow, and to be more effective antimicrobial molecules in edible films; they should be released gradually in time. The use of microencapsulation and edible films might allow the controlled release of antimicrobial substances, while providing an environment where cells can remain viable and further produce more metabolites. Complexity of this approach requires different methodologies of release control depending on the active compounds and final food application.

Research in edible films fields has largely increased in the last years, but there are still some drawbacks to be solved to allow their application in the packaging of foods, namely difficulties in up-scaling laboratory results to industrial production.

Fungal contamination of food and feed products is still a current worldwide problem and globalization of the food market makes harder to control the transmission of foodborne pathogens. New trends in food production and distribution, together with consumer habits and population susceptibility are also a motivation for new solution for fungal control. This work proposes an eventual alternative to other methods of fungal control, using two LAB strains with biocontrol properties, and elucidates the mechanisms of their antifungal and antimycotoxigenic ability, and suggests a method for their application in a vast consumed product.

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APPENDIX A

FROM CHAPTER 3

A. APPENDIX FROM CHAPTER 3

Material and Methods

Calibration curves

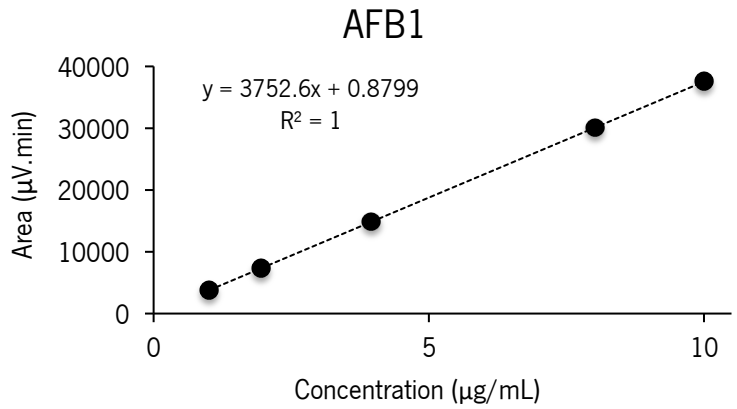


Figure A.1. Calibration curve for the quantification of aflatoxin B1.

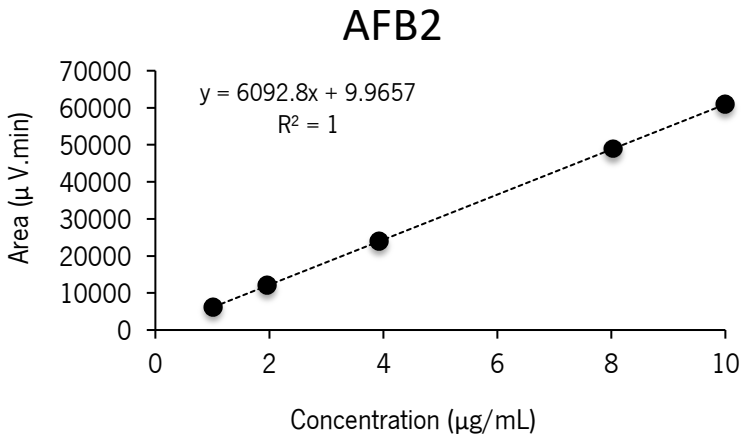


Figure A.2. Calibration curve for the quantification of aflatoxin B2.

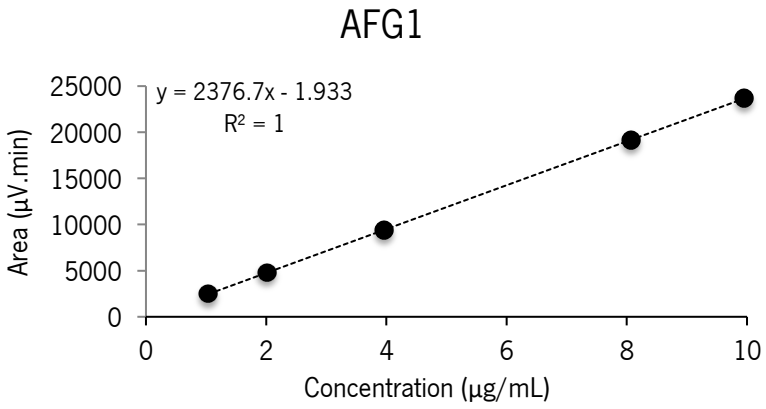


Figure A.3. Calibration curve for the quantification of aflatoxin G1.

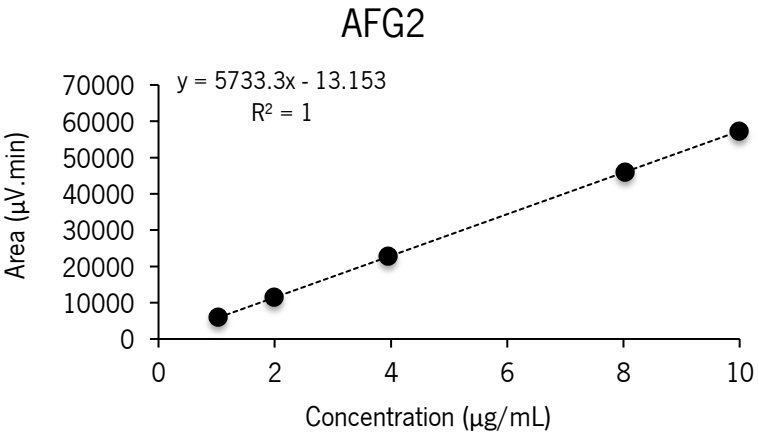


Figure A.4. Calibration curve for the quantification of aflatoxin G2.

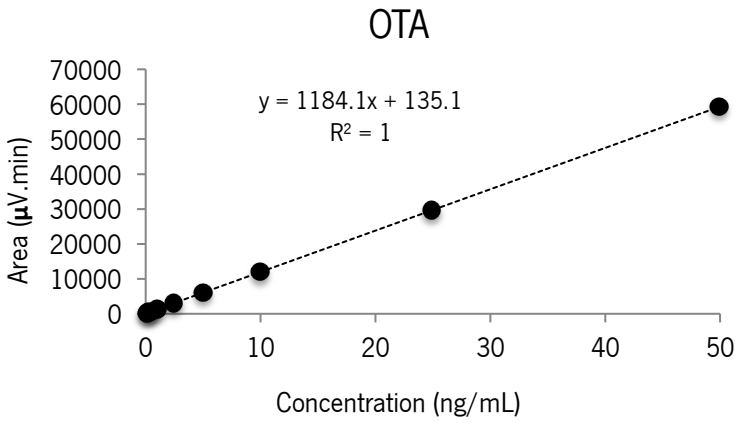


Figure A.5. Calibration curve for OTA quantification.

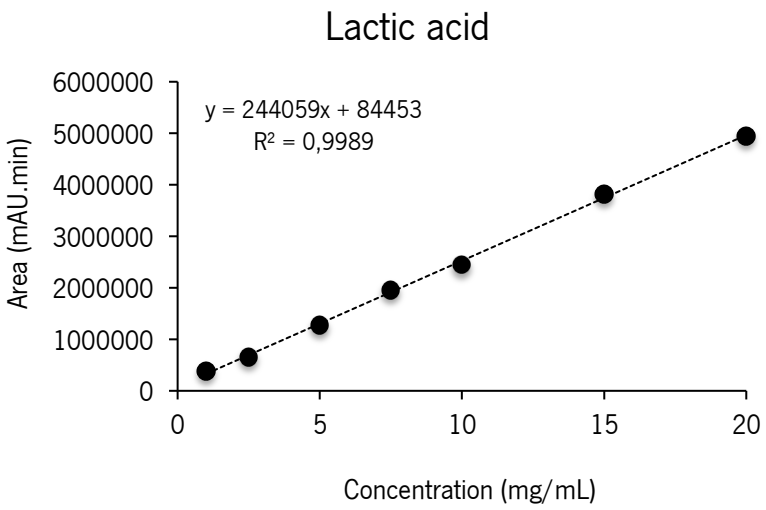


Figure A.6. Calibration curve for the quantification of lactic acid.

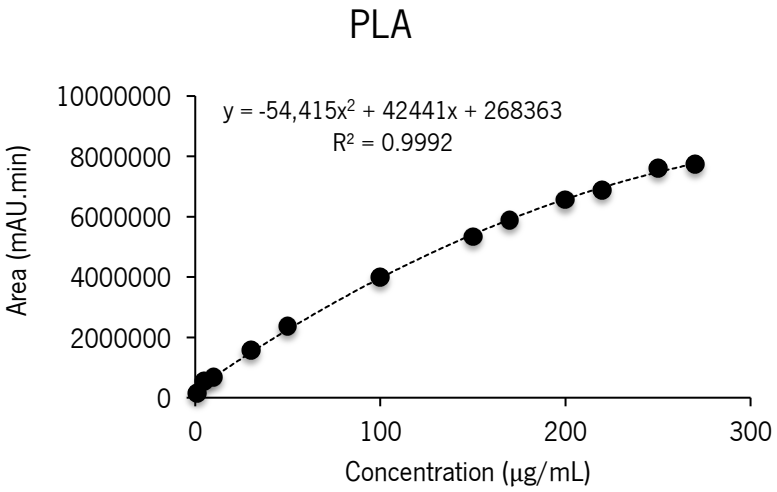


Figure A.7. Calibration curve for the quantification of PLA.

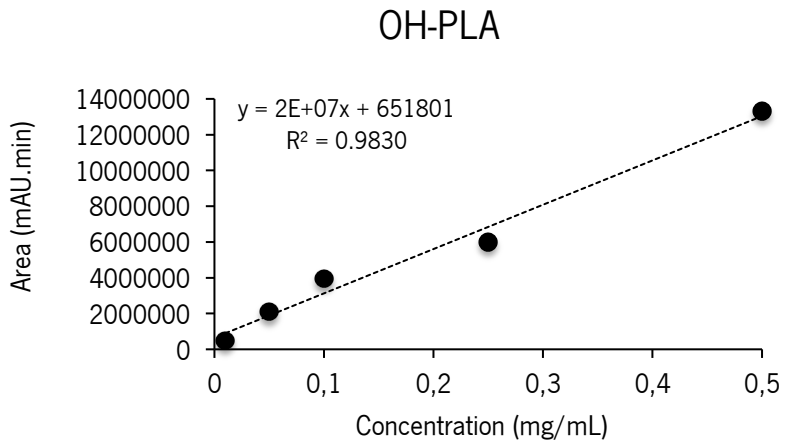


Figure A.8. Calibration curve for the quantification of OH-PLA.

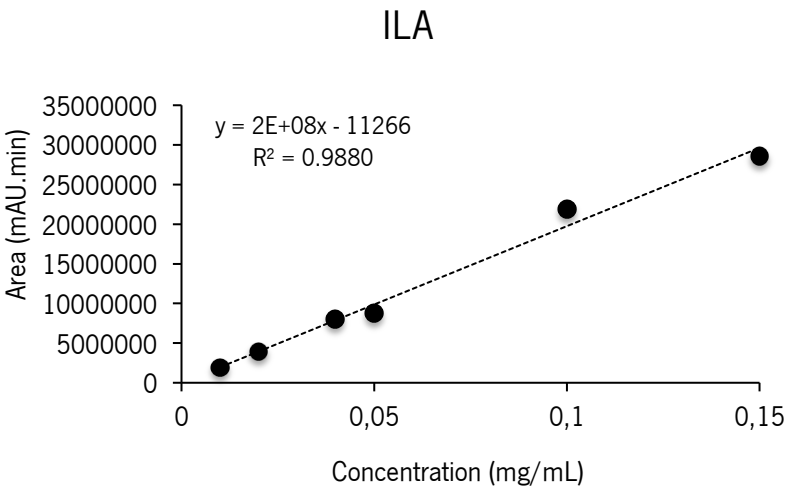


Figure A.9. Calibration curve for the quantification of ILA.

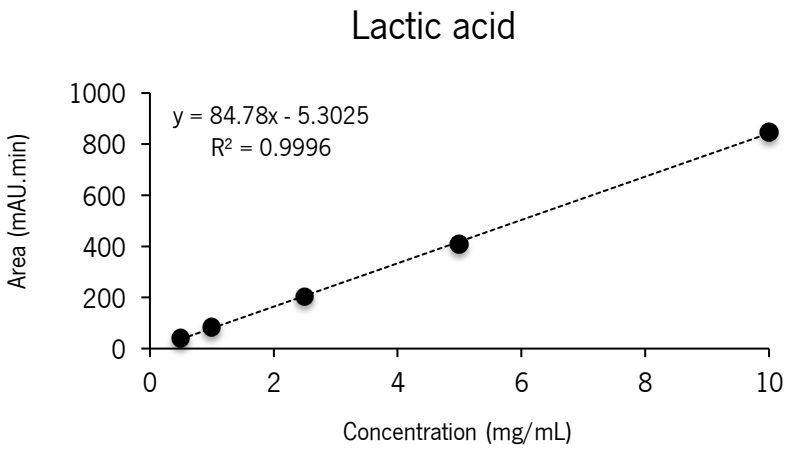


Figure A.10. Calibration curve for the quantification of lactic acid in MRS agar medium.

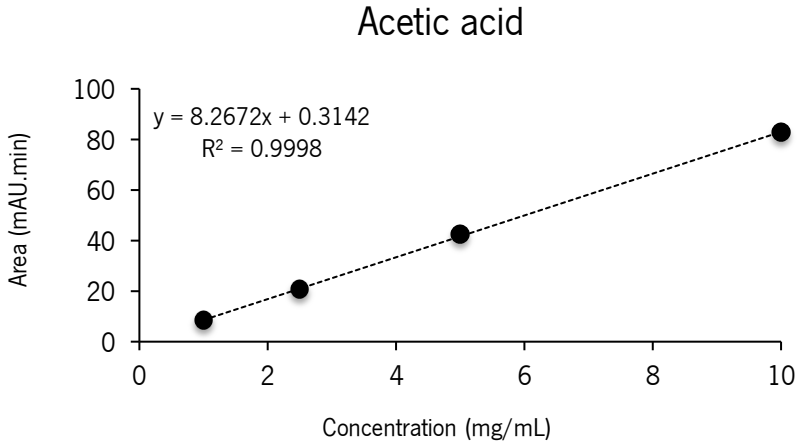


Figure A.11. Calibration curve for the quantification of acetic acid in MRS agar medium.

Hill Equation

Log(inhibitor) vs. response curves follow a symmetrical sigmoidal shape. This model does not assume a standard slope but rather fits the Hill Slope from the data, and is called a Variable slope model or a four-parameter dose-response curve.

The Model is given by the equation:

$$Y = Bottom + \frac{Top - Bottom}{1 + 10^{(LogIC50 - X) \times HillSlope}}$$

where IC50 is the concentration of agonist that gives a response half way between Bottom and Top; HillSlope is the steepness of the family of curves; and Top and Bottom are plateaus in the units of the Y axis.

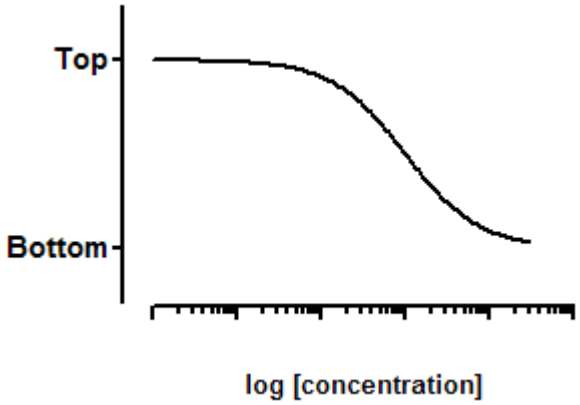


Figure A.12. Graphic representation of HillSlope. Adapted from GraphPad guide.

Anti-aflatoxigenic Effect of Organic Acids Produced by *Lactobacillus plantarum*

Characterization of Antifungal Properties of CFS from L. plantarum UM55

Table A.1. *A. flavus* MUM 17.14 growth (mm) in MRS broth supplemented with *L. plantarum* 55UM CFS subjected to different treatments: without treatment (CFS), heat treatment, enzymatic treatment, pH neutralization (CFS pH7) and without *L. plantarum* UM55 CFS (MRS control) and inhibition (%) for each of those treatments

***A. flavus* growth (mm) in MRS supplemented with *L. plantarum* UM55 CFS**

Day	CFS	Heat treatment	Enzyme treatment	CFS pH7	MRS control
	7.0	9.0	9.5	13.0	9.5
1	7.0	9.5	10.0	13.5	10.5
	7.0	10.0	8.0	12.5	13.0
Average	7.0	9.5	9.2	13.0	11.0
SD	0.0	0.5	1.0	0.5	1.8
Inhibition (%)	36.4	13.6	16.7	-18.2	0.0
	17.0	18.5	19.0	24.0	22.0
2	17.0	18.5	18.0	23.5	23.0
	17.0	17.5	18.5	24.0	20.5
Average	17.0	18.2	18.5	23.8	21.8
SD	0.0	0.6	0.5	0.3	1.3
Inhibition (%)	22.1	16.8	15.3	-9.2	0.0
	30.0	30.0	30.0	36.0	33.5
3	29.5	29.5	29.0	35.5	31.5
	28.0	27.5	28.5	35.0	30.5
Average	29.2	29.0	29.2	35.5	31.8
SD.	1.0	1.3	0.8	0.5	1.5
Inhibition (%)	8.4	8.9	8.4	-11.5	0.0
	43.5	42.0	44.0	64.0	61.5
6	48.5	47.5	47.0	65.5	61.0
	47.0	43.5	44.5	65.0	60.0
Average	46.3	44.3	45.2	64.8	60.8
SD	2.6	2.8	1.6	0.8	0.8
Inhibition (%)	23.8	27.1	25.8	-6.6	0.0
	54.0	45.5	50.0	73.0	71.5
7	51.0	51.5	52.0	73.0	70.0
	45.5	50.5	48.0	74.0	69.0
Average	50.2	49.2	50.0	73.3	70.2
SD	4.3	3.2	2.0	0.6	1.3
Inhibition (%)	28.5	29.9	28.7	-4.5	0.0

Table A.2. Aflatoxin (μg/plate) in *A. flavus* MUM 17.14 plates with *L. plantarum* UM55 CFS subjected to different treatments: without treatment (CFS), heat treatment, enzymatic treatment, pH neutralization (CFS pH7) and without *L. plantarum* UM55 CFS (MRS control) and inhibition (%) for each of those treatments

CFS			
	AFB2	AFB1	Total
	0.3	26.4	26.7
	0.6	46.2	46.8
	0.9	72.9	73.8
Average	0.6	48.5	49.1
SD	0.3	23.3	23.6
Inhibition (%)	98.2	91.8	92.2

CFS with heat treatment			
	AFB2	AFB1	Total
	1.5	47.4	48.9
	1.8	54.9	56.7
	1.8	52.8	54.6
Average	1.7	51.7	53.4
SD	0.2	3.9	4.0
Inhibition (%)	94.9	91.3	91.5

CFS with enzymatic treatment			
	AFB2	AFB1	Total
	3.0	84.0	87.0
	3.6	99.0	102.6
	3.0	86.7	89.7
Average	3.2	89.9	93.1
SD	0.3	8.0	8.3
Inhibition (%)	90.4	84.8	85.1

CFS at pH7			
	AFB2	AFB1	Total
	15.0	517.2	532.2
	15.9	528.9	544.8
	16.2	547.5	563.7
Average	15.7	531.2	546.9
SD	0.6	15.3	15.9
Inhibition (%)	52.7	10.5	12.7

MRS control			
	AFB2	AFB1	Total
	31.8	578.4	610.2
	31.2	576.6	607.8
	36.6	624.6	661.2
Average	33.2	593.2	626.4
SD	3.0	27.2	30.2
Inhibition (%)	0.0	0.0	0.0

Table A.3. *A. flavus* MUM 17.14 colony diameter (mm) in the presence of 5 to 25% of *L. plantarum* UM55 CFS over 7 days; and inhibition (%) for each concentration at each day

***A. flavus* growth (mm) in MRS supplemented with *L. plantarum* UM55 CFS**

	5% CFS		10% CFS		15% CFS		20% CFS		25% CFS	
Day	**CFS**	**CFS pH7**	**CFS**	**CFS pH7**	**CFS**	**CFS pH7**	**CFS**	**CFS pH7**	**CFS**	**CFS pH7**
	23.0	27.5	23.0	27.5	24.5	32.0	24.0	32.0	13.0	19.0
3	23.5	27.0	23.0	29.0	23.0	31.5	24.5	32.0	11.5	19.0
	23.0	27.0	23.5	28.5	24.5	30.0	24.0	32.0	12.0	19.0
Average	23.2	27.2	23.2	28.3	24.0	31.2	24.2	32.0	12.2	19.0
Stand. Dev.	0.3	0.3	0.3	0.8	0.9	1.0	0.3	0.0	0.8	0.0
Inhibition (%)	14.7	0.0	18.2	0.0	23.0	0.0	24.5	0.0	36.0	0.0
	29.5	31.0	31.0	40.0	32.5	41.0	32.5	47.0	22.0	30.5
4	30.0	30.5	31.5	39.0	31.5	41.0	33.0	48.5	22.0	30.5
	30.5	30.5	31.5	37.5	31.5	43.0	32.0	48.5	23.5	30.0
Average	30.0	30.7	31.3	38.8	31.8	41.7	32.5	48.0	22.5	30.3
Stand. Dev.	0.5	0.3	0.3	1.3	0.6	1.2	0.5	0.9	0.9	0.3
Inhibition (%)	2.2	0.0	19.3	0.0	23.6	0.0	32.3	0.0	25.8	0.0
	38.0	46.5	40.0	48.5	41.0	53.5	42.5	55.0	43.5	58.0
5	37.0	46.5	39.0	50.5	41.5	53.5	43.0	56.5	43.0	58.5
	37.0	45.0	38.0	51.0	41.5	53.5	43.5	58.5	44.0	59.0
Average	37.3	46.0	39.0	50.0	41.3	53.5	43.0	56.7	43.5	58.5
Stand. Dev.	0.6	0.9	1.0	1.3	0.3	0.0	0.5	1.8	0.5	0.5
Inhibition (%)	18.8	0.0	22.0	0.0	22.7	0.0	24.1	0.0	25.6	0.0
	40.5	50.5	44.5	57.5	45.5	62.0	50.0	67.5	53.0	69.5
6	40.5	52.5	44.0	58.0	46.0	62.0	49.5	68.5	51.0	70.5
	41.0	52.5	42.0	59.0	46.0	62.0	48.0	68.0	52.5	71.0
Average	40.7	51.8	43.5	58.2	45.8	62.0	49.2	68.0	52.2	70.3
Stand. Dev.	0.3	1.2	1.3	0.8	0.3	0.0	1.0	0.5	1.0	0.8
Inhibition (%)	21.5	0.0	25.2	0.0	26.1	0.0	27.7	0.0	25.8	0.0
	46.0	61.5	47.0	68.5	53.0	71.5	55.5	76.5	62.5	83.5
7	45.0	60.5	50.0	68.0	53.0	72.5	57.0	76.5	63.5	83.5
	45.5	58.0	50.0	67.0	51.5	71.0	57.5	77.0	64.0	83.5
Average	45.5	60.0	49.0	67.8	52.5	71.7	56.7	76.7	63.3	83.5
Stand. Dev.	0.5	1.8	1.7	0.8	0.9	0.8	1.0	0.3	0.8	0.0
Inhibition (%)	24.2	0.0	27.8	0.0	26.7	0.0	26.1	0.0	24.2	0.0

Table A.4. Aflatoxin (ng/plate) in *A. flavus* MUM 17.14 plates with *L. plantarum* UM55 CFS at different concentrations (5, 10, 15, 20 and 25%) and inhibition (%) for each of those concentrations

AF (ng/plate) in *A. flavus* plates supplemented with *L. plantarum* UM55 CFS

5% CFS						
	***L. plantarum* UM55 CFS**			***L. plantarum* CFS at pH7**		
	AFB2	AFB1	Total	AFB2	AFB1	Total
	5.1	278.1	283.2	10.8	565.2	576.0
	4.8	265.8	270.6	10.8	543.9	554.7
	4.2	240.6	244.8	7.8	456.6	464.4
Average	4.7	261.5	266.2	9.8	521.9	531.7
SD	0.5	19.1	19.6	1.7	57.5	59.2
Inhibition (%)	52.0	49.9	49.9	-	-	-
10% CFS						
	***L. plantarum* UM55 CFS**			***L. plantarum* CFS at pH7**		
	AFB2	AFB1	Total	AFB2	AFB1	Total
	4.5	266.7	271.2	15.9	694.5	710.4
	3.6	238.8	242.4	12.6	562.5	575.1
	3.0	186.6	189.6	15.0	690.0	705.0
Average	3.7	230.7	234.4	14.5	649.0	663.5
SD	0.8	40.7	41.4	1.7	74.9	76.6
Inhibition (%)	74.5	64.5	64.7	-	-	-
15% CFS						
	***L. plantarum* UM55 CFS**			***L. plantarum* CFS at pH7**		
	AFB2	AFB1	Total	AFB2	AFB1	Total
	1.2	80.7	81.9	10.8	420.0	430.8
	1.8	119.4	121.2	13.5	506.1	519.6
	1.8	98.7	100.5	15.0	573.6	588.6
Average	1.6	99.6	101.2	13.1	499.9	513.0
SD	0.3	19.4	19.7	2.1	77.0	79.1
Inhibition (%)	87.8	80.1	80.3	-	-	-
20% CFS						
	***L. plantarum* UM55 CFS**			***L. plantarum* CFS at pH7**		
	AFB2	AFB1	Total	AFB2	AFB1	Total
	1.5	87.6	89.1	18.6	657.6	676.2
	1.8	116.7	118.5	15.6	560.4	576.0
	1.8	91.2	93.0	21.0	719.1	740.1
Average	1.7	98.5	100.2	18.4	645.7	664.1
SD	0.2	15.9	16.0	2.7	80.0	82.7
Inhibition (%)	90.8	84.7	84.9	-	-	-
25% CFS						
	***L. plantarum* UM55 CFS**			***L. plantarum* CFS at pH7**		
	AFB2	AFB1	Total	AFB2	AFB1	Total
	0.6	32.4	33.0	9.0	572.9	581.9

	0.3	25.2	25.5	6.6	587.4	594.0
	0.6	27.6	28.2	9.0	524.0	533.0
Average	0.5	28.4	28.9	8.2	561.4	569.6
SD	0.2	3.7	3.8	1.4	33.2	32.3
Inhibition (%)	93.9	94.9	94.9	-	-	-

Table A.5. *A. flavus* MUM 17.14 growth (mm) in plates with *L. plantarum* UM55 CFS (10%) over 22 days

Day	CFS	CFS pH7	Day	CFS	CFS pH7
	5.5	10.5		52.0	76.0
	6.0	11.0		56.0	75.5
	5.5	12.5		50.0	76.5
	6.5	12.5		48.0	71.5
	7.5	12.0		54.0	77.0
	7.5	11.0		56.5	79.5
	6.5	11.5	**8**	52.5	77.0
	6.5	12.5		49.0	78.5
	8.5	11.5		50.5	78.0
1	6.5	11.0		51.0	79.5
	8.5	11.0		51.5	78.5
	8.0	11.5		52.5	80.0
	7.5	12.0	Average	52.0	77.3
	7.0	13.0	SD.	2.6	2.3
	5.0	11.5	Inhibition (%)	32.8	0.0
	6.5	13.0		57.5	81.5
	6.5	10.0		58.5	83.0
	7.5	10.0		54.0	81.0
Average	6.8	11.6		55.0	82.0
SD	1.0	0.9		56.0	82.0
Inhibition (%)	40.9	0.0		54.5	81.5
	27.5	39.5	**9**	57.0	79.5
	29.5	38.5		60.0	81.5
	30.0	39.5		56.5	81.5
	29.0	42.5		55.5	84.0
	30.0	40.0		60.0	83.5
	28.0	39.0		55.0	84.0
3	28.0	42.0	Average	56.6	82.1
	29.0	39.5	SD	2.0	1.3
	28.0	39.5	Inhibition (%)	31.0	0.0
	27.0	38.5		63.0	82.5
	27.0	38.5	**11**	60.5	82.0
	30.0	40.5		56.0	82.5

	28.0	40.5
	27.0	41.0
	27.5	40.0
	28.5	40.5
	30.0	38.0
	28.5	31.5
Average	28.5	39.4
SD	1.1	2.3
Inhibition (%)	27.7	0.0
	34.5	45.0
	36.0	48.0
	37.0	45.5
	35.0	47.5
	34.5	45.5
	34.5	46.5
	37.5	50.5
4	35.5	46.5
	38.5	44.5
	35.5	46.0
	35.0	39.5
	37.0	46.0
	38.0	45.5
	35.5	47.0
	35.0	47.5
Average	35.9	46.1
SD	1.3	2.3
Inhibition (%)	22.0	0.0
	44.0	52.0
	43.5	57.5
	40.0	57.5
	43.5	58.0
	44.0	58.5
	40.5	58.5
	39.0	57.5
5	40.0	58.5
	41.5	58.5
	41.0	59.5
	41.5	62.0
	42.0	59.5
	44.0	61.0
	41.5	60.0
	44.0	56.0
Average	42.0	58.3
SD	1.7	2.3

	57.5	81.0
	57.0	84.0
	63.0	83.0
	57.5	84.0
	58.0	83.5
	57.5	83.5
	57.5	83.5
	60.5	82.5
	61.5	82.0
Average	59.1	82.8
SD	2.4	0.9
Inhibition (%)	28.6	0.0
	70.0	82.5
	68.0	83.0
	66.5	84.0
	63.0	83.0
12	69.5	82.5
	68.0	83.5
	69.0	83.5
	68.0	82.5
	70.0	83.0
Average	68.0	83.1
SD	2.2	0.5
Inhibition (%)	18.1	0.0
	66.0	82.5
	68.5	83.0
	68.5	82.0
	69.0	83.0
13	67.0	83.0
	70.0	82.0
	69.0	83.0
	71.0	83.4
	64.5	84.0
Average	68.2	82.9
SD	2.0	0.6
Inhibition (%)	17.8	0.0
	67.0	83.0
	65.0	84.0
	67.0	84.0
14	73.0	83.0
	71.0	84.0
	70.5	84.0
Average	68.9	83.7
SD	3.0	0.5

Inhibition (%)	22.0	27.1
	44.0	52.0
	43.5	57.5
	40.0	52.5
	43.5	58.0
	44.0	58.5
	40.5	58.5
	39.0	57.5
6	40.0	58.5
	41.5	58.5
	41.0	59.5
	41.5	62.0
	42.0	59.5
	44.0	61.0
	41.5	60.0
	44.0	56.0
Average	42.0	58.0
SD	1.7	2.7
Inhibition (%)	27.5	0.0
	45.0	66.0
	50.0	70.5
	50.0	71.5
	48.5	69.0
	45.5	68.0
	46.0	68.5
	51.0	67.5
7	46.0	66.5
	52.0	61.5
	48.0	71.0
	45.0	70.5
	47.0	69.0
	45.5	70.0
	47.5	70.0
	45.0	69.5
Average	47.5	68.6
SD	2.4	2.5
Inhibition (%)	30.8	0.0

Inhibition (%)	17.6	0.0
	74.0	83.0
	69.5	82.5
	68.5	81.5
15	71.5	83.5
	74.0	83.0
	74.5	83.5
Average	72.0	82.8
SD	2.6	0.8
Inhibition (%)	13.1	0.0
	75.5	81.0
	76.0	81.5
	71.5	82.5
18	70.5	83.0
	70.5	83.5
	72.0	83.0
Average	72.7	82.4
SD	2.5	1.0
Inhibition (%)	11.8	0.0
	71.5	83.5
19	73.5	83.0
	72.0	82.0
Average	72.3	82.8
SD	1.0	0.8
Inhibition (%)	12.7	0.0
	71.0	83.0
20	75.5	84.0
	71.5	83.5
Average	72.7	83.5
SD	2.5	0.5
Inhibition (%)	13.0	0.0
	71.5	83.0
21	72.0	84.0
	75.5	83.5
Average	73.0	83.5
SD	2.2	0.5
Inhibition (%)	12.6	0.0
	72.0	83.0
22	72.0	84.0
	76.0	83.5
Average	73.3	83.5
SD	2.3	0.5
Inhibition (%)	12.2	0.0

Table A.6. Aflatoxin (μg/mL) in *A. flavus* MUM 17.14 plates with *L. plantarum* UM55 CFS (10%) over 22 days

Aflatoxins (μg/mL) in *A. flavus* plates with *L. plantarum* UM55 CFS

Days	CFS			CFS pH7		
	AFB1	AFB2	Total	AFB1	AFB2	Total
3	0.12	0.00	0.12	1.13	0.06	1.19
	0.04	0.00	0.04	1.09	0.06	1.15
	0.10	0.00	0.10	1.40	0.07	1.47
Average	0.10	0.00	0.10	1.20	0.10	1.30
SD	0.00	0.00	0.00	0.20	0.00	0.20
Inhibition (%)	92.8	100.0	93.2	-	-	-
6	3.40	0.11	3.51	13.54	0.49	14.03
	3.61	0.12	3.73	13.74	0.49	14.23
	3.18	0.10	3.28	12.68	0.46	13.14
Average	3.40	0.10	3.50	13.30	0.50	13.80
SD	0.20	0.00	0.20	0.60	0.00	0.60
Inhibition (%)	74.5	77.1	74.6	-	-	-
9	6.81	0.24	7.05	24.97	0.85	25.82
	4.47	0.14	4.61	23.17	0.78	23.95
	3.53	0.11	3.64	25.42	0.79	26.21
Average	4.90	0.20	5.10	24.50	0.80	25.30
SD	1.70	0.10	1.80	1.20	0.00	1.20
Inhibition (%)	79.9	79.8	79.9	-	-	-
12	7.13	0.24	7.37	25.96	0.88	26.84
	5.40	0.16	5.56	25.57	0.88	26.45
	5.08	0.16	5.24	24.26	0.86	25.12
Average	5.90	0.20	6.10	25.30	0.90	26.10
SD	1.10	0.00	1.10	0.90	0.00	0.90
Inhibition (%)	76.8	78.6	76.8	-	-	-
16	8.81	0.16	8.97	24.9	0.85	25.75
	7.10	0.12	7.22	24.77	0.81	25.58
	9.48	0.19	9.67	25.23	0.86	26.09
Average	8.50	0.20	8.60	25.00	0.80	25.8
SD	1.20	0.00	1.30	0.20	0.00	0.30
Inhibition (%)	66.1	81.3	66.6	-	-	-
22	7.08	0.15	7.23	24.96	0.90	25.86
	8.81	0.20	9.01	23.22	0.76	23.98
	6.60	0.13	6.73	25.31	0.90	26.21
Average	7.50	0.20	7.70	24.50	0.90	25.40
SD	1.20	0.00	1.30	0.20	0.00	0.30
Inhibition (%)	69.4	81.3	69.8	-	-	-

Table A.7. Growth (mm) of aflatoxigenic fungi (*A. flavus* MUM 17.14. *A. flavus* MUM 10.237, *A. parasiticus* MUM 10.238, *A. arachidicola* MUM 92.02, *A. nomius* MUM 09.01 and *A. minisclerotigenes* MUM 10.240) in MRS broth supplemented with *L. plantarum* 55UM CFS and growth inhibition (%) for each of those fungi

Growth (mm) of aflatoxigenic fungi in MRS supplemented with *L. plantarum* UM55 CFS

	***A. flavus* MUM 17.14**		***A. flavus* MUM 10.237**		***A. parasiticus* MUM 10.238**		***A. arachidicola* MUM 92.02**		***A. nomius* MUM 09.01**		***A. minisclerotigenes* MUM 10.240**	
Day	**CFS**	**CFS pH7**	**CFS**	**CFS pH7**	**CFS**	**CFS pH7**	**CFS**	**CFS pH7**	**CFS**	**CFS pH7**	**CFS**	**CFS pH7**
	6.5	9.0	6.5	7.5	6.0	11.0	7.5	10.0	7.0	8.0	5.0	8.0
1	6.5	9.0	5.0	8.0	6.0	11.5	6.0	9.0	6.0	8.0	6.0	9.0
	6.0	9.0	5.0	7.5	6.5	11.0	7.0	9.0	6.0	9.0	6.0	9.0
Average	6.3	9.0	5.5	7.7	6.2	11.2	6.8	9.3	6.3	8.3	5.7	8.7
SD	0.3	0.0	0.9	0.3	0.3	0.3	0.8	0.6	0.6	0.6	0.6	0.6
Inhibition (%)	29.6	0.0	28.3	0.0	44.8	0.0	26.8	0.0	24.0	0.0	34.6	0.0
	16.0	20.0	14.0	19.0	17.0	21.0	16.5	21.0	16.5	21.5	16.0	19.5
2	16.0	20.1	14.0	17.5	17.0	21.5	17.0	21.5	17.5	21.0	15.5	19.5
	16.0	20.1	14.0	18.0	18.5	21.0	17.5	20.1	17.0	20.1	15.0	19.5
Average	16.0	20.1	14.0	18.2	17.5	21.2	17.0	20.9	17.0	20.9	15.5	19.5
SD	0.0	0.1	0.0	0.8	0.9	0.3	0.5	0.7	0.5	0.7	0.5	0.0
Inhibition (%)	20.3	0.0	22.9	0.0	17.3	0.0	18.5	0.0	18.5	0.0	20.5	0.0
	42.5	55.5	46.0	57.0	48.0	53.5	48.0	57.5	52.0	57.0	47.0	57.5
5	44.0	55.0	45.0	55.5	46.0	53.5	47.0	57.0	53.5	57.5	45.5	57.5
	43.0	55.0	45.5	56.0	46.5	53.5	48.0	58.0	51.5	57.0	46.5	56.0
Average	43.2	55.2	45.5	56.2	46.8	53.5	47.7	57.5	52.3	57.2	46.3	57.0
SD	0.8	0.3	0.5	0.8	1.0	0.0	0.6	0.5	1.0	0.3	0.8	0.9
Inhibition (%)	21.8	0.0	19.0	0.0	12.5	0.0	17.1	0.0	8.5	0.0	18.7	0.0
	50.0	65.0	55.0	68.0	58.0	66.5	59.5	68.5	61.0	67.0	55.5	66.5
6	52.0	64.0	54.0	67.5	56.5	65.0	58.0	68.0	63.0	68.5	52.0	68.0
	47.5	64.0	54.0	68.5	60.0	66.0	59.0	68.0	62.0	68.0	55.0	68.5
Average	49.8	64.3	54.3	68.0	58.2	65.8	58.8	68.2	62.0	67.8	54.2	67.7
SD	2.3	0.6	0.6	0.5	1.8	0.8	0.8	0.3	1.0	0.8	1.9	1.0
Inhibition (%)	22.5	0.0	20.1	0.0	11.6	0.0	13.7	0.0	8.6	0.0	20.0	0.0
	51.5	73.5	64.5	79.5	64.5	71.5	69.0	79.0	76.0	78.5	64.5	77.5
7	56.5	72.5	64.0	78.5	62.5	71.0	69.0	78.5	74.5	78.5	60.5	78.0
	55.0	74.0	65.0	79.5	64.0	72.5	70.5	79.5	73.0	76.5	62.5	77.0
Average	54.3	73.3	64.5	79.2	63.7	71.7	69.5	79.0	74.5	77.8	62.5	77.5
SD	2.6	0.8	0.5	0.6	1.0	0.8	0.9	0.5	1.5	1.2	2.0	0.5
Inhibition (%)	25.9	0.0	18.5	0.0	11.2	0.0	12.0	0.0	4.3	0.0	19.4	0.0

Table A.8. Aflatoxins (ng/plate) produced by aflatoxigenic fungi (*A. flavus* MUM 17.14, *A. flavus* MUM 10.237, *A. parasiticus* MUM 10.238, *A. arachidicola* MUM 92.02, *A. nomius* MUM 09.01 and *A. minisclerotigenes* MUM 10.240) in MRS broth supplemented with *L. plantarum* 55UM CFS and production inhibition (%) for each of those fungi

AF (ng/plate) in aflatoxigenic fungi plates supplemented with *L. plantarum* UM55 CFS

***A. flavus* MUM 17.14**										
	UM55 CFS					**CFS pH7**				
	AFG2	AFG1	AFB2	AFB1	Total	AFG2	AFG1	AFB2	AFB1	Total
	0.0	0.0	3.3	173.4	176.7	0.0	0.0	12.0	461.4	473.4
	0.0	0.0	2.4	136.5	138.9	0.0	0.0	11.7	493.5	505.2
	0.0	0.0	3.3	159.6	162.9	0.0	0.0	13.8	542.1	555.9
Average	0.0	0.0	3.0	156.5	159.5	0.0	0.0	12.5	499.0	511.5
SD	0.0	0.0	0.5	18.6	19.1	0.0	0.0	1.1	40.6	41.6
Inhibition (%)	-	-	76.0	68.6	68.8	-	-	-	-	-
***A. flavus* MUM 10.237**										
	UM55 CFS					**CFS pH7**				
	AFG2	AFG1	AFB2	AFB1	Total	AFG2	AFG1	AFB2	AFB1	Total
	0.0	0.0	132.3	90.6	222.9	0.0	0.0	153.0	315.9	468.9
	0.0	0.0	66.0	188.4	254.4	0.0	0.0	153.3	350.1	503.4
	0.0	0.0	113.1	69.9	183.0	0.0	0.0	150.3	283.8	434.1
Average	0.0	0.0	103.8	116.3	220.1	0.0	0.0	152.2	316.6	468.8
SD	0.0	0.0	34.1	63.3	35.8	0.0	0.0	1.7	33.2	34.7
Inhibition (%)	-	-	31.8	63.3	53.1	-	-	-	-	-
***A. parasiticus* MUM 10.238**										
	UM55 CFS					**CFS pH7**				
	AFG2	AFG1	AFB2	AFB1	Total	AFG2	AFG1	AFB2	AFB1	Total
	0.0	2.9	0.0	3.5	6.4	2.4	152.4	2.4	94.5	251.7
	0.0	1.2	0.0	3.1	4.3	3.0	181.8	1.8	64.2	250.8
	0.0	2.9	0.0	5.6	8.5	3.0	173.1	1.8	63.0	240.9
Average	0.0	2.3	0.0	4.0	6.4	2.8	169.1	2.0	73.9	247.8
SD	0.0	1.0	0.0	1.4	2.1	0.3	15.1	0.3	17.9	6.0
Inhibition (%)	100.0	98.6	100.0	94.5	97.4	-	-	-	-	-
***A. arachidicola* MUM 92.02**										
	UM55 CFS					**CFS pH7**				
	AFG2	AFG1	AFB2	AFB1	Total	AFG2	AFG1	AFB2	AFB1	Total
	15.9	637.8	57.9	1074.0	1785.6	27.6	984.9	62.1	1103.1	2177.7
	15.9	676.8	60.6	1105.5	1858.8	25.5	977.7	60.3	1134.9	2198.4
	18.3	710.7	38.4	1160.7	1928.1	25.8	991.5	63.9	1146.3	2227.5
Average	16.7	675.1	52.3	1113.4	1857.5	26.3	984.7	62.1	1128.1	2201.2
SD	1.4	36.5	12.1	43.9	71.3	1.1	6.9	1.8	22.4	25.0
Inhibition (%)	36.5	31.4	15.8	1.3	15.6	-	-	-	-	-
***A. nomius* MUM 09.01**										
	UM55 CFS					**CFS pH7**				
	AFG2	AFG1	AFB2	AFB1	Total	AFG2	AFG1	AFB2	AFB1	Total

	6.9	517.5	5.1	169.5	699.0	15.3	850.2	0.0	86.1	951.6
	6.0	420.9	3.6	140.4	570.9	11.4	712.2	0.0	68.1	791.7
	5.7	448.5	3.9	143.1	601.2	12.9	763.5	0.0	77.4	853.8
Average	6.2	462.3	4.2	151.0	623.7	13.2	775.3	0.0	77.2	865.7
SD	0.6	49.8	0.8	16.1	66.9	2.0	69.8	0.0	9.0	80.6
Inhibition (%)	53.0	40.4	-	-95.6	28.0	-	-	-	-	-

***A. minisclerotigenes* MUM 10.240**

	UM55 CFS					**CFS pH7**				
	AFG2	AFG1	AFB2	AFB1	Total	AFG2	AFG1	AFB2	AFB1	Total
	0.0	13.8	0.0	12.0	25.8	0.0	64.8	0.0	14.4	79.2
	0.0	14.1	0.0	11.4	25.5	0.0	66.3	0.0	14.4	80.7
	0.0	8.7	0.0	7.8	16.5	0.0	59.4	0.0	12.9	72.3
Average	0.0	12.2	0.0	10.4	22.6	0.0	63.5	0.0	13.9	77.4
SD	0.0	3.0	0.0	2.3	5.3	0.0	3.6	0.0	0.9	4.5
Inhibition (%)	-	80.8	-	25.2	70.8	-	-	-	-	-

Antifungal Properties of Individual Organic Acids

Table A.9. Concentration (mg/mL) of lactic acid, PLA, OH-PLA and ILA in the CFS of *L. plantarum* UM55. *L. casei* UM3 and *L. buchneri* UTAD104

		Concentration (mg/mL)			
		Lactic acid	PLA	OH-PLA	ILA
L. plantarum UM55 CFS	R1	4.5164	0.0744	0.0294	0.0068
	R2	4.0000	0.0746	0.0302	0.0066
	R3	4.3228	0.0754	0.0328	0.0066
	Average	4.2797	0.0748	0.0308	0.0067
	SD	0.2130	0.0004	0.0015	0.0001

		Concentration (mg/mL)			
		Lactic acid	PLA	OH-PLA	ILA
L. casei UM3 CFS	R1	0.7882	0.0396	0.0024	0.0010
	R2	1.1018	0.0421	0.0004	0.0008
	R3	1.0876	0.0411	0.0064	0.0008
	Average	0.9925	0.0409	0.0031	0.0009
	SD	0.1446	0.0010	0.0025	0.0001

		Concentration (mg/mL)			
		Lactic acid	PLA	OH-PLA	ILA
L. buchneri UTAD104 CFS	R1	3.3012	0.0266	0.0000	0.0006
	R2	3.3998	0.0696	0.0000	0.0006
	R3	3.3958	0.0476	0.0000	0.0006
	Average	3.3656	0.0479	0.0000	0.0006
	SD	0.0558	0.0215	0.0000	0.0000

Table A.10. Growth (mm) of *A. flavus* MUM 17.14 in the presence of several concentration (mg/mL) of lactic acid and inhibition of growth (%) relatively to the control

	Growth *A. flavus* (mm)					Growth inhibition *A. flavus* (%)			
Lactic acid (mg/mL)	R1	R2	R3	Average	SD	R1	R2	R3	Average
0	47.2	46.9	46.7	46.96	0.24	-0.52	0.02	0.50	0.00
0.1	46.5	44.0	-	45.25	1.77	0.97	6.30	-	3.64
0.25	42.0	41.5	42.5	42.00	0.50	10.56	11.62	9.49	10.56
0.5	39.0	38.5	38.5	38.67	0.29	16.95	18.01	18.01	17.66
1	39.0	38.0	37.0	38.00	1.00	16.95	19.08	21.21	19.08
1.5	37.0	37.0	36.0	36.67	0.58	21.21	21.21	23.33	21.92
2	37.0	37.0	35.5	36.50	0.87	21.21	21.21	24.40	22.27
4	35.5	35.0	35.0	35.17	0.29	24.40	25.46	25.46	25.11
8	34.5	35.0	35.0	34.83	0.29	26.53	25.46	25.46	25.82

Table A.11. Aflatoxin (ng/plate) produced by *A. flavus* MUM 17.14 in the presence of several concentrations (mg/mL) of lactic acid and inhibition of their production (%) relatively to the control

	Aflatoxin (ng/plate)					Inhibition of aflatoxin production (%)			
Lactic acid (mg/mL)	R1	R2	R3	Average	SD	R1	R2	R3	Average
0	330343	318600	320743	323228.6	6253.6	-2.20	1.43	0.77	0.00
0.1	314130	324120	-	319125.0	7064.0	2.81	-0.28	-	1.27
0.25	310000	314100	313900	312666.7	2311.6	4.09	2.82	2.89	3.27
0.5	247900	236500	251200	245200.0	7713.0	23.31	26.83	22.28	24.14
1	156000	175800	146700	159500.0	14862.4	51.74	45.61	54.61	50.65
1.5	129400	132700	133000	131700.0	1997.5	59.97	58.95	58.85	59.25
2	99000	103800	95400	99400.0	4214.3	69.37	67.89	70.49	69.25
4	10382.7	10448.7	9254.4	10028.6	671.3	96.79	96.77	97.14	96.90
8	1335.9	1383.6	1425.9	1381.8	45.0	99.59	99.57	99.56	99.57

Table A.12. Growth (mm) of *A. flavus* MUM 17.14 in the presence of several concentration (mg/mL) of PLA and inhibition of growth (%) relatively to the control

	Growth *A. flavus* (mm)					Growth inhibition *A. flavus* (%)			
PLA (mg/mL)	R1	R2	R3	Average	SD	R1	R2	R3	Average
0	47.2	46.9	46.7	46.96	0.24	-0.52	0.02	0.50	0.00
0.1	44.5	45.5	-	45.00	0.71	5.23	3.10	-	4.17
0.25	44.5	43.0	44.5	44.00	0.87	5.23	8.43	5.23	6.30
0.5	41.0	42.0	42.0	40.17	0.58	12.69	10.56	10.56	11.27
1	42.0	41.5	40.5	39.67	0.76	10.56	11.62	13.75	11.98
1.5	40.0	40	40.5	41.67	0.29	14.82	14.82	13.75	14.46
2	38.5	40	40.5	41.33	1.04	18.01	14.82	13.75	15.53
4	29.5	30	30	29.83	0.29	37.18	36.11	36.11	36.47
8	10.0	8.0	7.0	8.33	1.53	78.70	82.96	85.09	82.25

Table A.13. Aflatoxin (ng/plate) produced by *A. flavus* MUM 17.14 in the presence of several concentrations (mg/mL) of PLA and inhibition of their production (%) relatively to the control

	Aflatoxin (ng/plate)					Inhibition of aflatoxin production (%)			
PLA (mg/mL)	R1	R2	R3	Average	SD	R1	R2	R3	Average
0	330343	318600	320743	323228.6	6253.6	-2.20	1.43	0.77	0.00
0.1	297000	288360	-	292680.0	6109.4	8.11	10.79	-	9.45
0.25	240000	219900	231000	230300.0	10069	25.75	31.97	28.53	28.75
0.5	97500	103800	108600	103300.0	5566.9	69.84	67.89	66.40	68.04
1	14728.5	17475.6	16662.3	16288.8	1411.1	95.44	94.59	94.85	94.96
1.5	1351.2	1349.4	1157.7	1286.1	111.2	99.58	99.58	99.64	99.60
2	522.9	268.8	492	427.9	138.6	99.84	99.92	99.85	99.87
4	24.6	30.0	26.7	27.1	2.7	99.99	99.99	99.99	99.99
8	33.0	31.2	30.9	31.7	1.1	99.99	99.99	99.99	99.99

Table A.14. Growth (mm) of *A. flavus* MUM 17.14 in the presence of several concentration (mg/mL) of OH-PLA and inhibition of growth (%) relatively to the control

	Growth *A. flavus* (mm)					Growth inhibition *A. flavus* (%)			
OH-PLA (mg/mL)	R1	R2	R3	Average	SD	R1	R2	R3	Average
0	47.2	46.9	46.7	46.96	0.24	-0.52	0.02	0.50	0.00
0.1	46.1	46.55	-	46.31	0.34	1.88	0.87	-	1.37
0.25	41.5	41.0	41.5	41.33	0.29	11.62	12.69	11.62	11.98
0.5	39.5	40.5	39.0	39.67	0.76	15.88	13.75	16.95	15.53
1	39.5	37.5	39.0	38.67	1.04	15.88	20.14	16.95	17.66
1.5	38.5	39.5	39.0	39.00	0.50	18.01	15.88	16.95	16.95
2	38.5	41.0	-	39.75	1.77	18.01	12.69	-	15.35
4	41.0	39.5	-	40.25	1.06	12.69	15.88	-	14.28
8	39.0	40.5	-	39.75	1.06	16.95	13.75	-	15.35

Table A.15. Aflatoxin (ng/plate) produced by *A. flavus* MUM 17.14 in the presence of several concentrations (mg/mL) of OH-PLA and inhibition of their production (%) relatively to the control

	Aflatoxin (ng/plate)					Inhibition of aflatoxin production (%)			
OH-PLA (mg/mL)	R1	R2	R3	Average	SD	R1	R2	R3	Average
0	330343	318600	320743	323228.6	6253.6	-2.20	1.43	0.77	0.00
0.1	189810	193140	-	191475.0	2354.7	41.28	40.25	-	40.76
0.25	134400	132600	126000	131000.0	4422.7	58.42	58.98	61.02	59.47
0.5	92100	85800	94800	90900.0	4618.4	71.51	73.46	70.67	71.88
1	54000	56100	53100	54400.0	1539.5	83.29	82.64	83.57	83.17
1.5	49900	40300	46000	45400.0	4828.0	84.56	87.53	85.77	85.95
2	36240.3	37938.3	-	37089.3	1200.7	88.79	88.26	-	88.53
4	9921.6	8226.9	-	9074.3	1198.3	96.93	97.45	-	97.19
8	278.1	301.5	-	289.8	16.5	99.91	99.91	-	99.91

Table A.16. Growth (mm) of *A. flavus* MUM 17.14 in the presence of several concentration (mg/mL) of ILA and inhibition of growth (%) relatively to the control

	Growth *A. flavus* (mm)					Growth inhibition *A. flavus* (%)			
ILA (mg/mL)	R1	R2	R3	Average	SD	R1	R2	R3	Average
0	47.2	47.0	46.7	47.0	0.2	-0.5	0.0	0.5	0.0
0.1	46.0	48.0	-	47.0	1.4	2.0	-2.2	-	-0.1
0.25	46.6	46.5	46.9	46.7	0.2	0.8	1.0	0.1	0.6
0.5	47.5	47.0	47.0	47.2	0.3	-1.2	-0.1	-0.1	-0.4
1	47.5	50.5	-	49.0	2.1	-1.2	-7.5	-	-4.3
1.5	51.0	49.5	47.0	49.2	2.0	-8.6	-5.4	-0.1	-4.7
2	44.5	45.0	-	44.8	0.4	5.2	4.2	-	4.7
4	31.4	31.0	-	31.2	0.3	33.1	34.0	-	33.6
8	24.8	24.5	-	24.7	0.3	47.1	47.9	-	47.5

Table A.17. Aflatoxin (ng/plate) produced by *A. flavus* MUM 17.14 in the presence of several concentrations (mg/mL) of ILA and inhibition of their production (%) relatively to the control

	Aflatoxin (ng/plate)					Inhibition of aflatoxin production (%)			
ILA (mg/mL)	R1	R2	R3	Average	SD	R1	R2	R3	Average
0	330343	318600	320743	323228.6	6253.6	-2.20	1.43	0.77	0.00
0.1	294576	303297	-	298936.5	6166.7	8.86	6.17	-	7.52
0.25	215700	190500	214500	206900.0	14215.5	33.27	41.06	33.64	35.99
0.5	166500	142500	152400	153800.0	12061.1	48.49	55.91	52.85	52.42
1	50400	48300	-	49350.0	1484.9	84.41	85.06	-	84.73
1.5	24600	25500	24900	25000.0	458.3	92.39	92.11	92.30	92.27
2	4200	6600	-	5400.0	1697.1	98.70	97.96	-	98.33
4	101.4	187.8	0.0	96.4	94.0	99.97	99.94	-	99.96
8	71.7	73.5	0.0	48.4	41.9	99.98	99.98	-	99.98

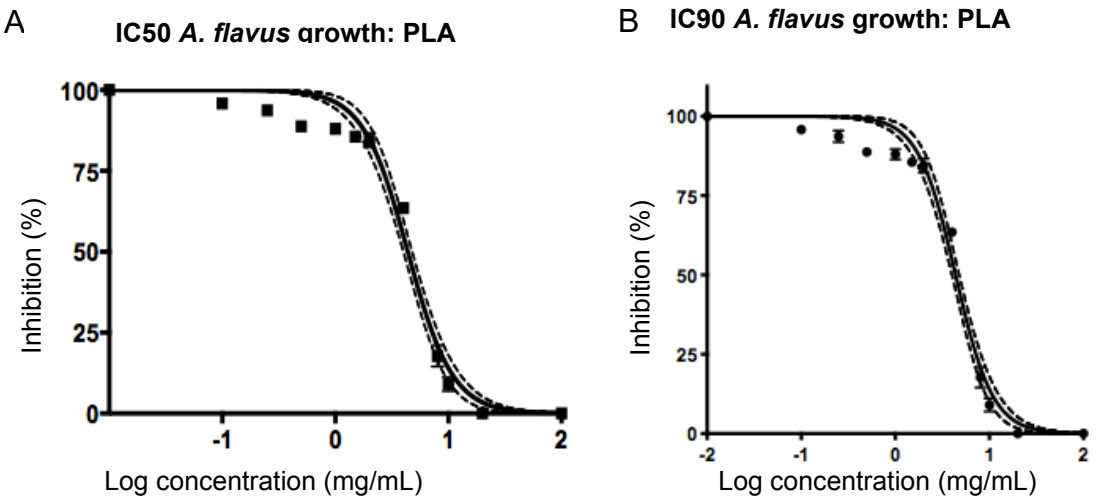


Figure A.13. Graphic representation of IC50 (A) and IC90 (B) of *A. flavus* MUM 14.17 growth with several concentrations (log mg/mL) of PLA.

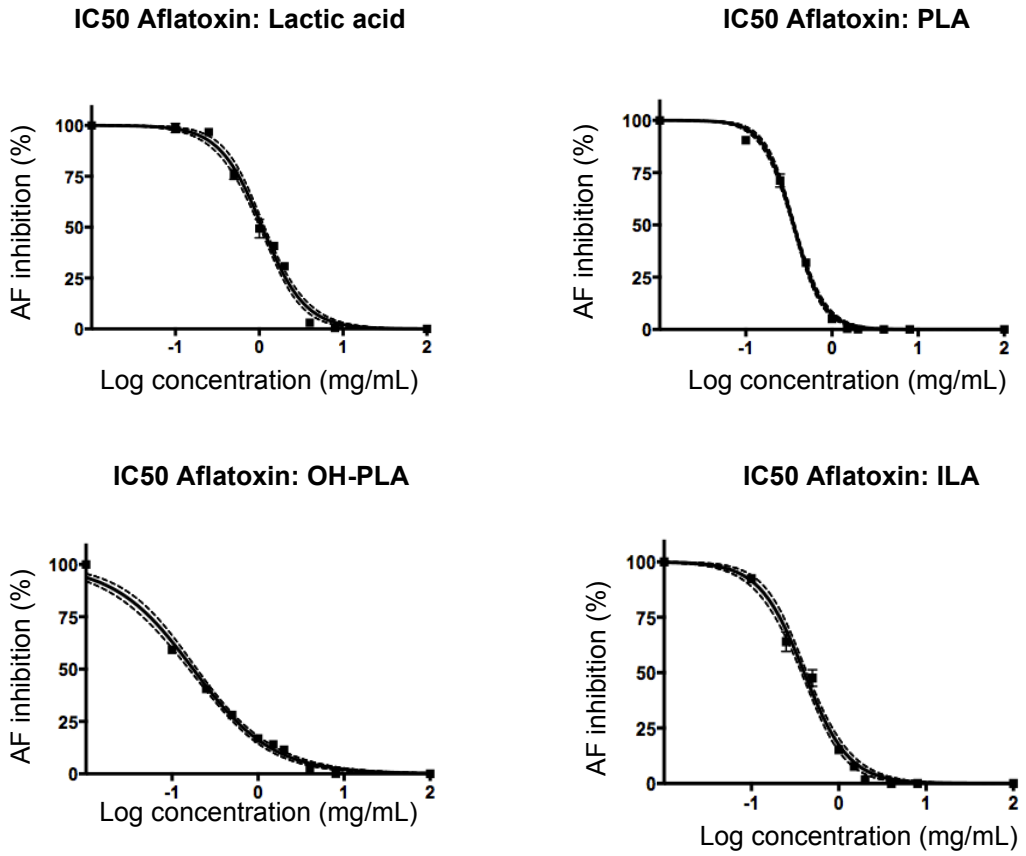


Figure A.14. Graphic representation of IC50 of aflatoxin production inhibition with several concentrations (log mg/mL) of lactic acid, PLA, OH-PLA and ILA.

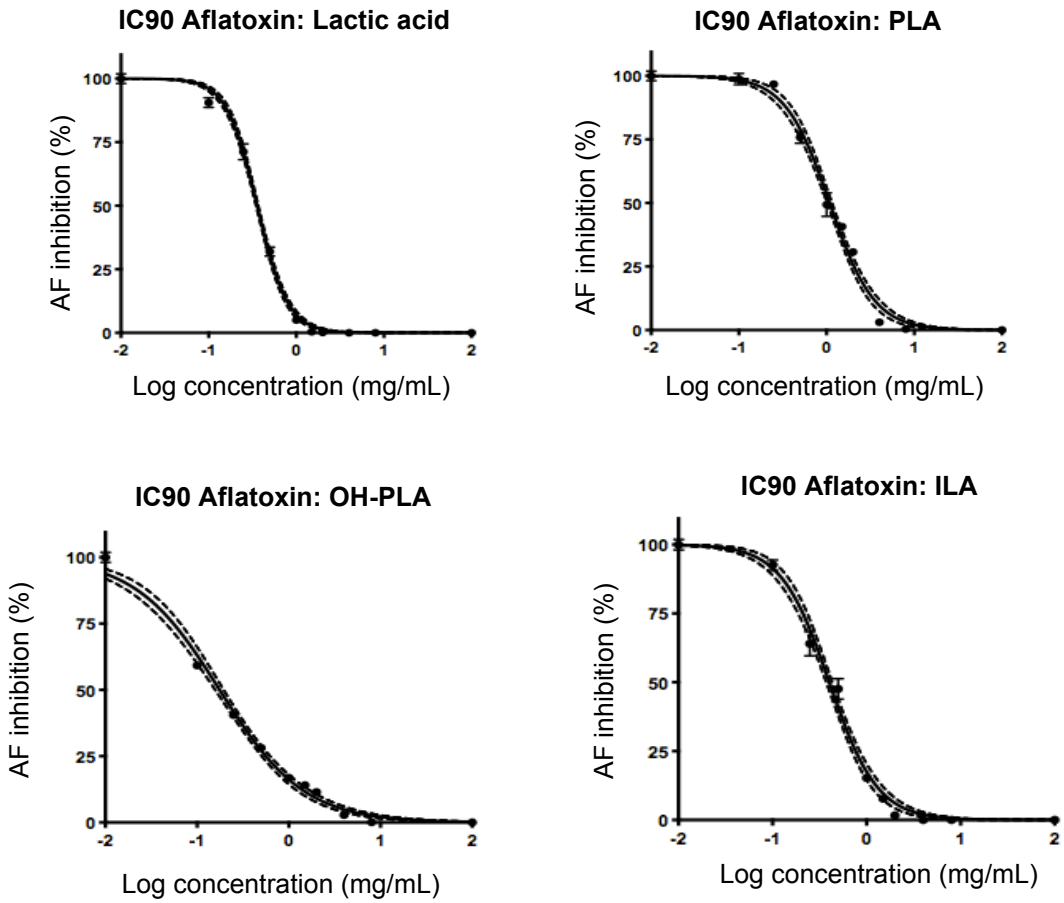


Figure A.15. Graphic representation of IC90 of aflatoxin production inhibition with several concentrations (log mg/mL) of lactic acid, PLA, OH-PLA and ILA.

Antifungal Effect of Organic Acids from Lactic Acid Bacteria on *Penicillium nordicum*

Characterization of Antifungal Properties of Cell-free Supernatants from LAB Strains

Table A.18. *P. nordicum* MUM 08.16 growth (mm) in MRS broth supplemented with *L. plantarum* UM55 CFS subjected to different treatments: without treatment (CFS). heat treatment, enzymatic treatment, pH neutralization (CFS pH7) and without *L. plantarum* UM55 CFS (MRS control) and inhibition (%) for each of those treatments

***P. nordicum* growth (mm) in MRS supplemented with *L. plantarum* UM55 CFS**

Day	CFS	Heat treatment	Enzyme treatment	CFS pH7	MRS control
	7.5	8.5	9.5	12.5	15.5
3	8.0	9.5	12.0	11.5	14.5
	7.5	9.5	11.0	12.0	15.5
Average	7.7	9.2	10.8	12.0	15.2
SD	0.3	0.6	1.3	0.5	0.6
Inhibition (%)	49.5	39.6	28.6	20.9	0.0
	15.5	13.0	15.5	18.5	20.5
4	14.5	13.0	16.5	18.5	19.5
	16.0	13.0	14.0	20.0	21.5
Average	15.3	13.0	15.3	19.0	20.5
SD	0.8	0.0	1.3	0.9	1.0
Inhibition (%)	25.2	36.6	25.2	7.3	0.0
	19.5	17.5	18.5	24.0	25.0
5	19.0	17.5	20.0	24.5	25.0
	18.5	16.5	18.5	23.0	25.5
Average	19.0	17.2	19.0	23.8	25.2
SD	0.5	0.6	0.9	0.8	0.3
Inhibition (%)	24.5	31.8	24.5	5.3	0.0
	22.0	20.5	23.0	27.0	29.0
6	23.00	20.5	24.0	27.5	28.5
	23.0	21.0	24.0	28.0	29.5
Average	22.7	20.7	23.7	27.5	29.0
SD	0.6	0.3	0.6	0.5	0.5
Inhibition (%)	21.8	28.7	18.4	5.2	0.0
	27.5	25.5	28.5	31.5	34.0
7	27.0	25.0	29.0	33.0	33.5
	27.5	25.5	27.0	33.0	33.0
Average	27.3	25.3	28.2	32.5	33.5
SD	0.3	0.3	1.0	0.9	0.5
Inhibition (%)	18.4	24.4	15.9	3.0	0.0

Table A.19. *P. nordicum* MUM 08.16 growth (mm) in MRS broth supplemented with *L. buchneri* UTAD104 CFS subjected to different treatments: without treatment (CFS), heat treatment, enzymatic treatment, pH neutralization (CFS pH7) and without *L. buchneri* UTAD104 CFS (MRS control) and inhibition (%) for each of those treatments

***P. nordicum* growth (mm) in MRS supplemented with *L. buchneri* UTAD104 CFS**

Day	CFS	Heat treatment	Enzyme treatment	CFS pH7	MRS control
	10.0	13.5	12.5	13.5	15.5
3	9.5	11.5	12.0	13.5	14.5
	9.0	12.5	12.0	14.5	15.5
Average	9.5	12.5	12.2	13.8	15.2
SD	0.5	1.0	0.3	0.6	0.6
Inhibition (%)	37.4	17.6	19.8	8.8	0.0
	19.0	18.5	18.0	20.5	20.5
4	19.5	17.5	18.0	20.0	19.5
	19.0	17.5	18.0	19.5	21.5
Average	19.2	17.8	18.0	20.0	20.5
SD	0.3	0.6	0.0	0.5	1.0
Inhibition (%)	6.5	13.0	12.2	2.4	0.0
	22.0	21.5	17.0	25.5	25.0
5	22.5	22.0	22.5	23.5	25.0
	22.5	23.0	21.5	24.5	25.5
Average	22.3	22.2	20.3	24.5	25.2
SD	0.3	0.8	2.9	1.0	0.3
Inhibition (%)	11.3	11.9	19.2	2.6	0.0
	26.0	26.5	26.0	28.0	29.0
6	25.5	24.0	26.0	27.5	28.5
	26.0	25.0	25.0	30.0	29.5
Average	25.8	25.2	25.7	28.5	29.0
SD	0.3	1.3	0.6	1.3	0,5
Inhibition (%)	10.9	13.2	11.5	1.7	0.0
	30.0	28.5	28.0	35.0	34.0
7	29.0	28.5	29.5	34.5	33.5
	30.0	30.5	30.0	33.5	33.0
Average	29.7	29.2	29.2	34.3	33.5
SD	0.6	1.2	1.0	0.8	0.5
Inhibition (%)	11.4	12.9	12.9	-2.5	0.0

Table A.20. OTA (ng/mL) in *P. nordicum* MUM 08.16 plates with *L. plantarum* UM55 CFS subjected to different treatments: without treatment (CFS), heat treatment, enzymatic treatment, pH neutralization (CFS pH7) and without *L. plantarum* UM55 CFS (MRS control) and inhibition (%) for each of those treatments

OTA (ng/mL) in *P. nordicum* plates supplemented with *L. plantarum* UM55 CFS

	CFS	Heat treatment	Enzyme treatment	CFS pH7	MRS control
OTA	12.40	12.78	11.61	43.2	26.43
	14.57	12.29	12.03	55.89	27.37
	15.40	12.78	11.25	39.66	26.58
Average	14.10	12.6	11.6	46.30	26.80
SD	1.5	0.3	0.4	8.5	0.5
Inhibition (%)	47.3	52.9	56.6	-72.6	0.0

Table A.21. OTA (ng/mL) in *P. nordicum* MUM 08.16 plates with *L. buchneri* UTAD104 CFS subjected to different treatments: without treatment (CFS), heat treatment, enzymatic treatment, pH neutralization (CFS pH7) and without *L. buchneri* UTAD104 CFS (MRS control) and inhibition (%) for each of those treatments

OTA (ng/mL) in *P. nordicum* plates supplemented with *L. buchneri* UTAD104 CFS

	CFS	Heat treatment	Enzyme treatment	CFS pH7	MRS control
OTA	14.55	16.06	12.53	50.49	26.43
	14.05	16.43	13.06	39.21	27.37
	11.72	18.25	13.29	39.51	26.58
Average	13.40	16.90	13.00	43.10	26.80
SD	1.5	1.2	0.4	6.4	0.5
Inhibition (%)	49.8	36.9	51.6	-60.7	0.0

Characterization of Antifungal Properties of Organic Acids Present in Cell-free Supernatants from Lactic Acid Bacteria Strains

Table A.22. Growth (mm) of *P. nordicum* MUM 08.16 in the presence of several concentration (mg/mL) of lactic acid and growth inhibition (%) relatively to the control

	Growth *P. nordicum* (mm)				Growth Inhibition *P. nordicum* (%)		
Lactic acid (mg/mL)	R1	R2	Average	SD	R1	R2	Average
0	29.5	30.0	29.8	0.4	0.8	-0.8	0.0
0.1	31.5	29.0	30.3	1.8	-5.9	2.5	-1.7
0.25	28.5	30.0	29.3	1.1	4.2	-0.8	1.7
0.5	27.0	26.5	26.8	0.4	9.2	10.9	10.1
1	23.5	23.5	23.5	0.0	21.0	21.0	21.0
2	22.5	22.5	22.5	0.0	24.4	24.4	24.4
4	19.5	21.0	20.3	1.1	34.5	29.4	31.9
8	15.5	15.0	15.3	0.4	47.9	49.6	48.7

Table A.23. OTA concentration (ng/plate) produced by *P. nordicum* MUM 08.16 in the presence of several concentrations (mg/mL) of lactic acid and inhibition of its production (%) relatively to the control

	OTA (ng/plate)				OTA inhibition (%)		
Lactic acid (mg/mL)	R1	R2	Average	SD	R1	R2	Average
0	877.2	767.4	822.3	77.6	-6.7	6.7	0.0
0.1	696.3	736.8	716.6	28.6	15.3	10.4	12.9
0.25	736.2	642.3	689.3	66.4	10.5	21.9	16.2
0.5	511.2	632.7	572.0	85.9	37.8	23.1	30.4
1	519.9	467.1	493.5	37.3	36.8	43.2	40.0
2	379.2	445.2	412.2	46.7	53.9	45.9	49.9
4	401.4	321.0	361.2	56.9	51.2	61.0	56.1
8	365.4	294.0	329.7	50.5	55.6	64.2	59.9

Table A.24. Growth (mm) of *P. nordicum* MUM 08.16 in the presence of several concentration (mg/mL) of acetic acid and growth inhibition (%) relatively to the control

	Growth *P. nordicum* (mm)				Growth Inhibition *P. nordicum* (%)		
Acetic acid (mg/mL)	R1	R2	Average	SD	R1	R2	Average
0	29.5	30.0	29.8	0.4	0.8	-0.8	0.0
0.1	29.0	29.0	29.0	0.0	2.5	2.5	2.5
0.25	28.0	27.5	27.8	0.4	5.9	7.6	6.7
0.5	24.5	26.5	25.5	1.4	17.6	10.9	14.3
1	22.5	19.0	20.8	2.5	24.4	36.1	30.3
2	0.0	0.0	0.0	0.0	100.0	100.0	100.0
4	0.0	0.0	0.0	0.0	100.0	100.0	100.0
8	0.0	0.0	0.0	0.0	100.0	100.0	100.0

Table A.25. OTA concentration (ng/plate) produced by *P. nordicum* MUM 08.16 in the presence of several concentrations (mg/mL) of acetic acid and inhibition of its production (%) relatively to the control

	OTA (ng/plate)				OTA inhibition (%)		
Acetic acid (mg/mL)	R1	R2	Average	SD	R1	R2	Average
0	877.2	767.4	822.3	77.6	-6.7	6.7	0.0
0.1	1125.6	862.2	993.9	186.3	-36.9	-4.9	-20.9
0.25	936.6	788.4	862.5	104.8	-13.9	4.1	-4.9
0.5	777.3	698.7	738.0	55.6	5.5	15.0	10.3
1	700.5	581.1	640.8	84.4	14.8	29.3	22.1
2	0.0	0.0	0.0	0.0	100.0	100.0	100.0
4	0.0	0.0	0.0	0.0	100.0	100.0	100.0
8	0.0	0.0	0.0	0.0	100.0	100.0	100.0

Table A.26. Growth (mm) of *P. nordicum* MUM 08.16 in the presence of several concentration (mg/mL) of PLA and growth inhibition (%) relatively to the control

	Growth *P. nordicum* (mm)				Growth Inhibition *P. nordicum* (%)		
PLA (mg/mL)	R1	R2	Average	SD	R1	R2	Average
0	29.5	30.0	29.8	0.4	0.8	-0.8	0.0
0.1	29.5	30.5	30.0	0.7	0.8	-2.5	-0.8
0.25	29.5	28.5	29.0	0.7	0.8	4.2	2.5
0.5	27.5	27.5	27.5	0.0	7.6	7.6	7.6
1	26.0	24.5	25.3	1.1	12.6	17.6	15.1
2	24.5	25.0	24.8	0.4	17.6	16.0	16.8
4	19.5	20.0	19.8	0.4	34.5	32.8	33.6
8	0.0	0.0	0.0	0.0	100.0	100.0	100.0

Table A.27. OTA concentration (ng/plate) produced by *P. nordicum* MUM 08.16 in the presence of several concentrations (mg/mL) of PLA and inhibition of its production (%) relatively to the control

	OTA (ng/plate)				OTA inhibition (%)		
PLA (mg/mL)	R1	R2	Average	SD	R1	R2	Average
0	877.2	767.4	822.3	77.6	-6.7	6.7	0.0
0.1	754.5	695.4	725.0	41.8	8.2	15.4	11.8
0.25	656.7	654.3	655.5	1.7	20.1	20.4	20.3
0.5	536.4	518.1	527.3	12.9	34.8	37.0	35.9
1	442.2	364.2	403.2	55.2	46.2	55.7	51.0
2	242.4	260.0	251.2	12.4	70.5	68.4	69.5
4	48.9	46.5	47.7	1.7	94.1	94.3	94.2
8	0.0	0.0	0.0	0.0	100.0	100.0	100.0

Table A.28. Growth (mm) of *P. nordicum* MUM 08.16 in the presence of several concentration (mg/mL) of OH-PLA and growth inhibition (%) relatively to the control

	Growth *P. nordicum* (mm)				Growth Inhibition *P. nordicum* (%)		
OH-PLA (mg/mL)	R1	R2	Average	SD	R1	R2	Average
0	29.5	30.0	29.8	0.4	0.8	-0.8	0.0
0.1	28.5	29.5	29.0	0.7	4.2	0.8	2.5
0.25	28.0	27.0	27.5	0.7	5.9	9.2	7.6
0.5	28.0	27.0	27.5	0.7	5.9	9.2	7.6
1	25.5	26.0	25.8	0.4	14.3	12.6	13.4
2	24.0	23.0	23.5	0.7	19.3	22.7	21.0
4	21.5	21.0	21.3	0.4	27.7	29.4	28.6
8	17.0	17.0	17.0	0.0	42.9	42.9	42.9

Table A.29. OTA concentration (ng/plate) produced by *P. nordicum* MUM 08.16 in the presence of several concentrations (mg/mL) of OH-PLA and inhibition of its production (%) relatively to the control

	OTA (ng/plate)				OTA inhibition (%)		
OH-PLA (mg/mL)	R1	R2	Average	SD	R1	R2	Average
0	761.7	895.2	828.5	94.4	8.1	-8.1	0.0
0.1	891.9	725.1	808.5	117.9	-7.7	12.5	2.4
0.25	694.5	658.2	676.4	25.7	16.2	20.6	18.4
0.5	486.6	490.5	488.6	2.8	41.3	40.8	41.0
1	390.3	503.1	446.7	79.8	52.9	39.3	46.1
2	287.7	281.7	284.7	4.2	65.3	66.0	65.6
4	296.1	268.8	282.5	19.3	67.2	70.2	68.7
8	441.6	312.0	376.8	91.6	66.0	76.0	71.0

Table A.30. Growth (mm) of *P. nordicum* MUM 08.16 in the presence of several concentration (mg/mL) of ILA and growth inhibition (%) relatively to the control

	Growth *P. nordicum* (mm)				Growth Inhibition *P. nordicum* (%)		
ILA (mg/mL)	R1	R2	Average	SD	R1	R2	Average
0	29.5	30.0	29.8	0.4	0.8	-0.8	0.0
0.1	29.0	30.0	29.5	0.7	2.5	-0.8	0.8
0.25	29.5	30.5	30.0	0.7	0.8	-2.5	-0.8
0.5	27.5	27.0	27.3	0.4	7.6	9.2	8.4
1	27.0	25.0	26.0	1.4	9.2	16.0	12.6
2	24.0	23.5	23.8	0.4	19.3	21.0	20.2
4	14.5	15.0	14.8	0.4	51.3	49.6	50.4
8	0.0	0.0	0.0	0.0	100.0	100.0	100.0

Table A.31. OTA concentration (ng/plate) produced by *P. nordicum* MUM 08.16 in the presence of several concentrations (mg/mL) of ILA and inhibition of its production (%) relatively to the control

	OTA (ng/plate)				OTA inhibition (%)		
ILA (mg/mL)	R1	R2	Average	SD	R1	R2	Average
0	761.7	895.2	828.5	94.4	8.1	-8.1	0.0
0.1	881.3	737.6	809.5	101.6	-6.4	11.0	2.3
0.25	628.5	739.2	683.9	78.3	24.1	10.8	17.5
0.5	473.6	510.2	491.9	25.9	42.8	38.4	40.6
1	399.3	489.1	444.2	63.5	51.8	41.0	46.4
2	117.9	128.1	123.0	7.2	86.9	85.8	86.4
4	2.1	9.0	5.6	4.9	99.8	99.0	99.4
8	0.0	0.0	0.0	0.0	100.0	100.0	100.0

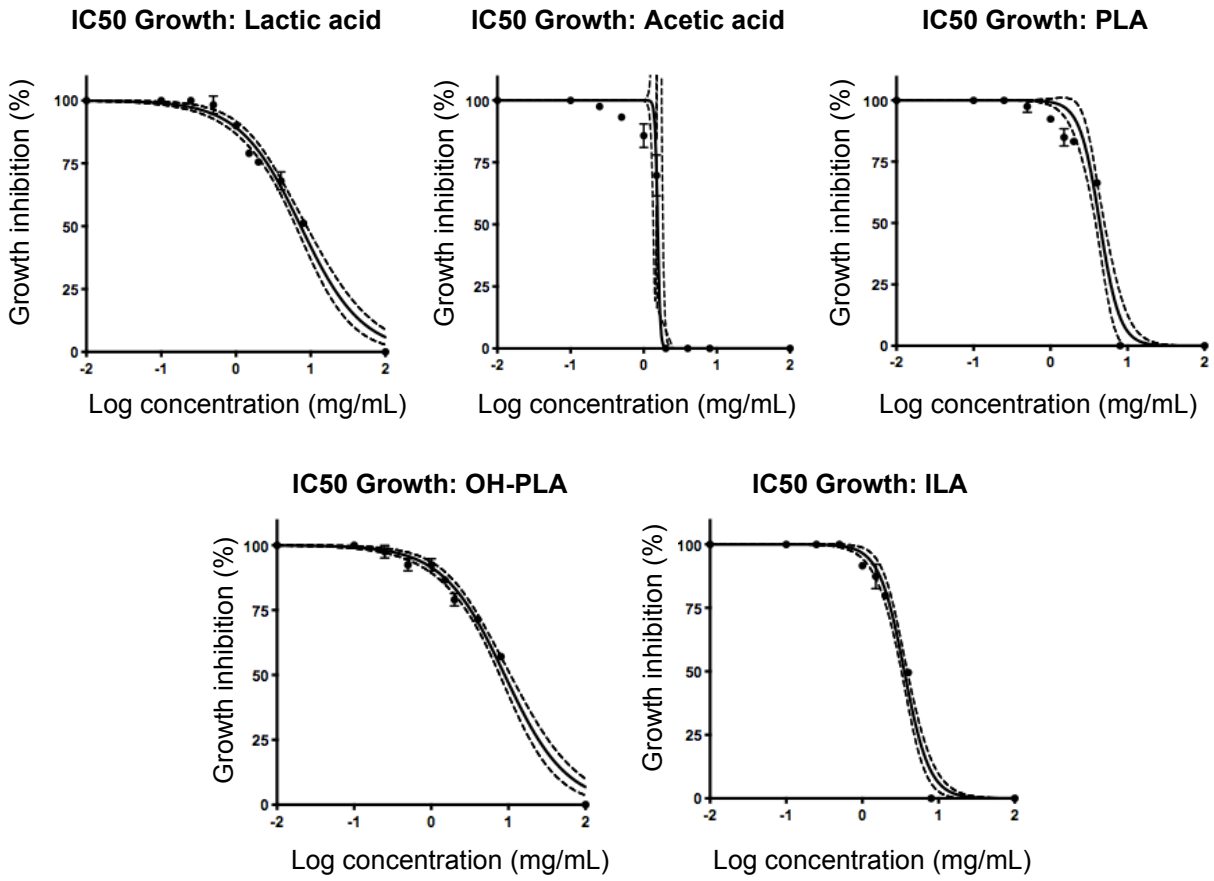


Figure A.16. Graphic representation of IC50 of *P. nordicum* MUM 08.16 growth inhibition with several concentrations (log mg/mL) of lactic acid, acetic acid, PLA, OH-PLA and ILA.

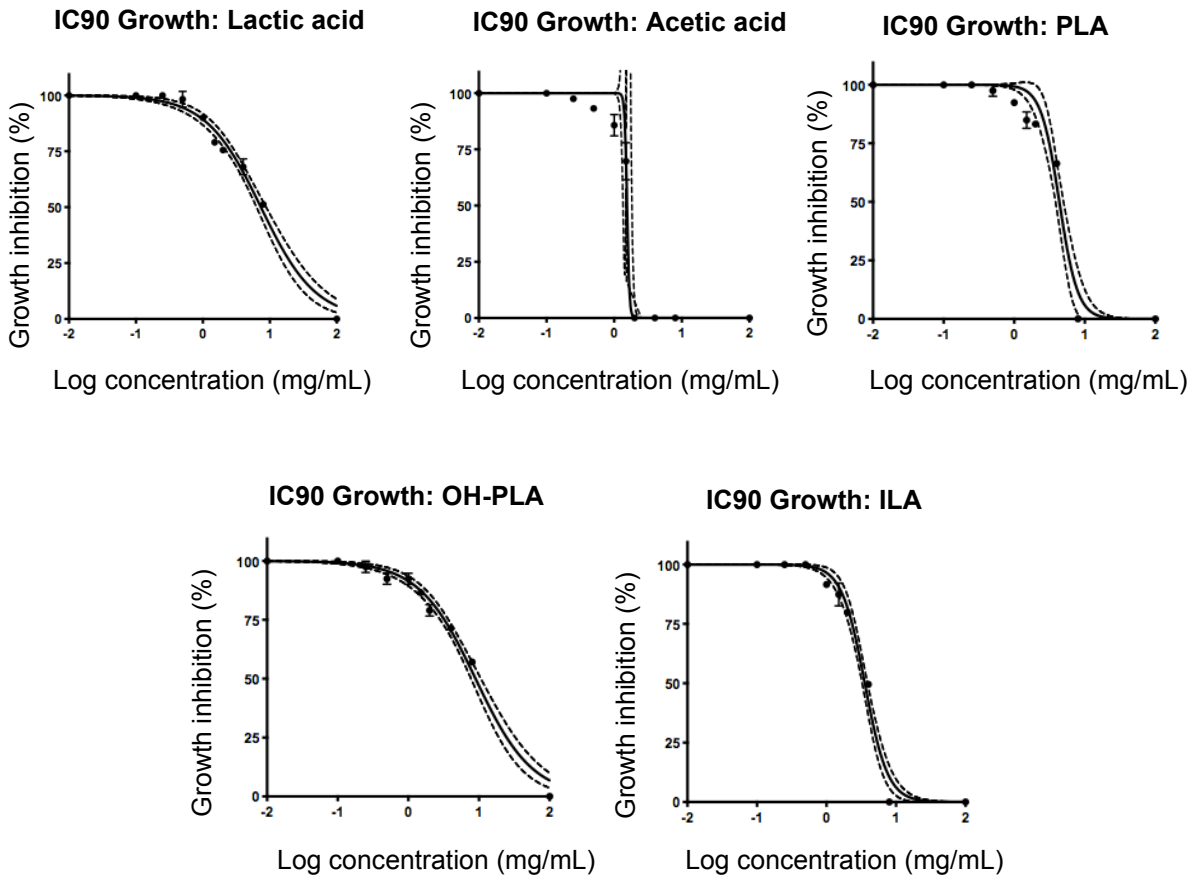


Figure A.17. Graphic representation of IC90 of *P. nordicum* MUM 08.16 growth inhibition with several concentrations (log mg/mL) of lactic acid, acetic acid, PLA, OH-PLA and ILA.

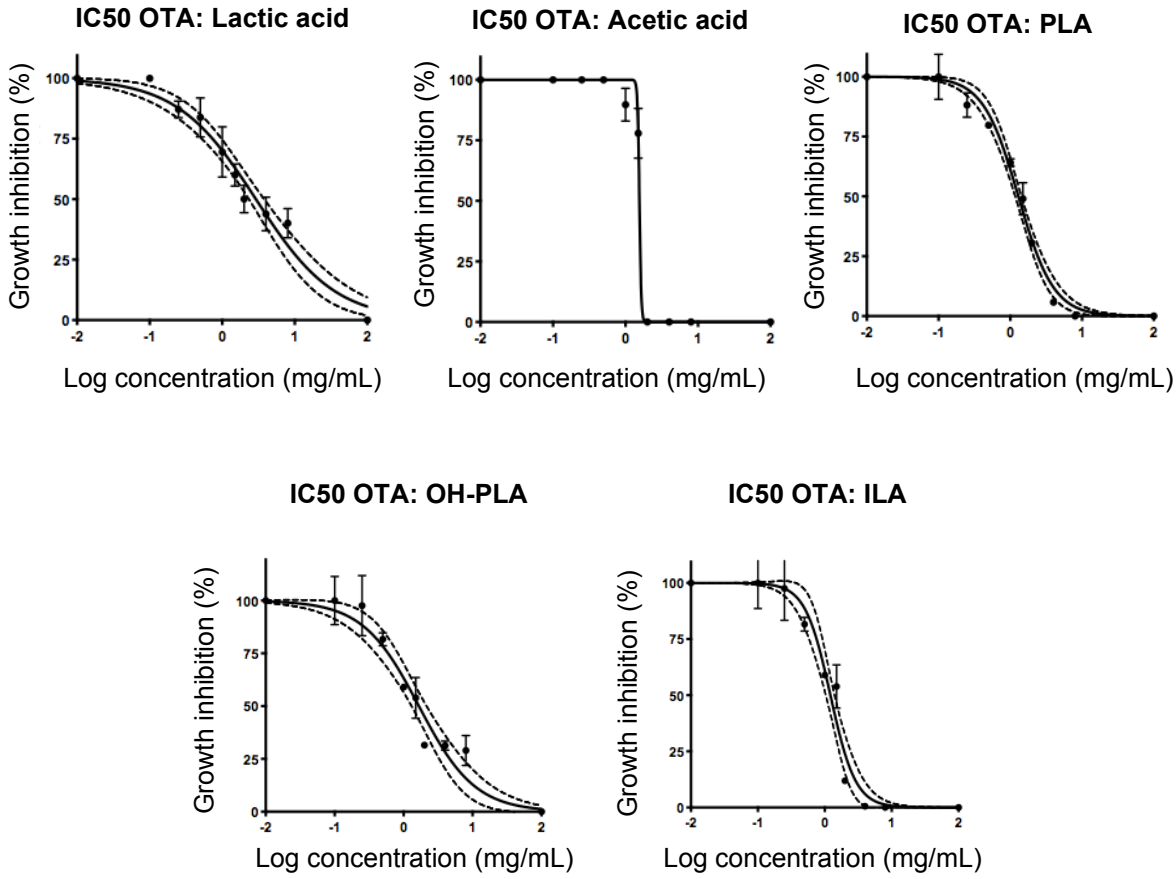


Figure A.18. Graphic representation of IC50 of OTA production inhibition with several concentrations (log mg/mL) of lactic acid, PLA, OH-PLA and ILA.

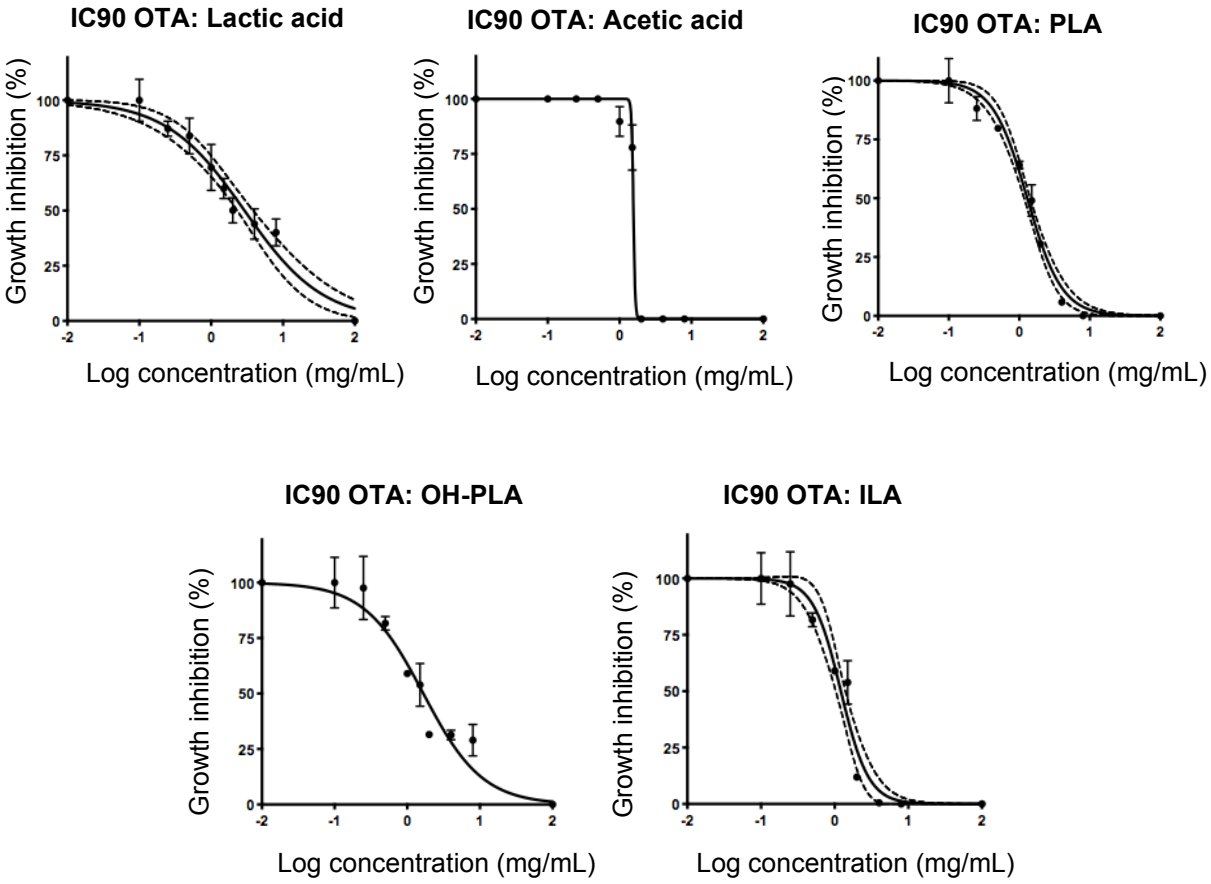


Figure A.19. Graphic representation of IC90 of OTA production inhibition with several concentrations (log mg/mL) of lactic acid, PLA, OH-PLA and ILA.

Antifungal Properties of Lactic Acid Bacteria Cells

Table A.32. *P. nordicum* MUM 08.16 growth (mm) in MRS broth supplemented with *L. plantarum* UM55 and *L. buchneri* UTAD104 cells at different concentrations (DO_{600} 1, 2.5, 5) and PBS control; and inhibition (%) for each of those treatments

***P. nordicum* growth (mm) in MRS supplemented LAB cells**

Days	*L. plantarum* UM55			*L. buchneri* UTAD104			PBS
	DO 1	DO 2.5	DO 5	DO 1	DO 2.5	DO 5	
	6.0	0.0	0.0	5.0	0.0	0.0	12.5
3	6.0	0.0	0.0	6.0	0.0	0.0	12.0
	5.0	0.0	0.0	5.5	0.0	0.0	12.2
Average	5.7	0.0	0.0	5.5	0.0	0.0	12.2
SD	0.6	0.0	0.0	0.5	0.0	0.0	0.3
Inhibition (%)	53.7	100.0	100.0	55.0	100.0	100.0	0.0
	8.0	5.0	0.0	5.0	0.0	0.0	17.5
4	8.0	5.0	0.0	6.0	0.0	0.0	17.5
	9.0	4.5	0.0	5.5	0.0	0.0	17.5
Average	8.3	4.8	0.0	5.5	0.0	0.0	17.5
SD	0.6	0.3	0.0	0.5	0.0	0.0	0.0
Inhibition (%)	52.4	72.4	100.0	68.6	100.0	100.0	0.0
	10.0	5.5	0.0	5.0	0.0	0.0	22.5
5	9.5	3.5	0.0	6.0	0.0	0.0	22.0
	10.0	5.5	0.0	5.5	0.0	0.0	22.0
Average	9.8	4.8	0.0	5.5	0.0	0.0	22.2
SD	0.3	1.2	0.0	0.5	0.0	0.0	0.3
Inhibition (%)	55.6	78.2	100.0	75.2	100.0	100.0	0.0
	13.5	7.5	0.0	5.5	0.0	0.0	25.5
6	12.5	5.5	0.0	6.0	0.0	0.0	26.0
	13.5	7.0	0.0	5.0	0.0	0.0	25.5
Average	13.2	6.7	0.0	5.5	0.0	0.0	25.7
SD	0.6	1.0	0.0	0.5	0.0	0.0	0.3
Inhibition (%)	48.7	74.0	100.0	78.6	100.0	100.0	0.0
	16.5	9.5	0.0	6.0	0.0	0.0	30.5
7	15.0	7.5	0.0	6.0	0.0	0.0	29.0
	17.0	9.5	0.0	5.0	0.0	0.0	30.0
Average	16.2	8.8	0.0	5.7	0.0	0.0	29.8
SD	1.0	1.2	0.0	0.6	0.0	0.0	29.6
Inhibition (%)	45.8	70.4	100.0	81.0	100.0	100.0	0.0

Table A.33. *P. nordicum* MUM 08.16 OTA production (ng/mL) in MRS broth supplemented with *L. plantarum* UM55 and *L. buchneri* UTAD104 cells at different concentrations (DO_{600} 1, 2.5, 5) and PBS control; and inhibition (%) for each of those treatments

OTA (ng/mL) in *P. nordicum* plates supplemented with LAB cells

	***L. plantarum* UM55**			***L. buchneri* UTAD104**			**PBS**
	DO 1	**DO 2.5**	**DO 5**	**DO 1**	**DO 2.5**	**DO 5**	
	15.3	8.2	0.0	0.0	0.0	0.0	39.69
OTA	14.4	7.1	0.0	0.0	0.0	0.0	39.9
	14.9	5.6	0.0	0.0	0.0	0.0	48.2
Average	14.9	7.0	0.0	0.0	0.0	0.0	42.6
SD	0.5	1.3	0.0	0.0	0.0	0.0	4.9
Inhibition (%)	65.2	83.7	100.0	100.0	100.0	100.0	0.0

Table A.34. Lactic and acetic acid (g/L) *p*resent in MRS agar medium, inoculated with *P. nordicum* MUM 08.16 supplemented with *L. plantarum* UM55 cells at different concentrations (DO_{600} 1, 2.5, 5) and PBS as control

Organic acids (g/L) in MRS plates supplemented with *L. plantarum* UM55 cells

	Lactic acid				**Acetic acid**			
	DO 1	**DO 2.5**	**DO 5**	**PBS**	**DO 1**	**DO 2.5**	**DO 5**	**PBS**
R1	0.68	0.66	0.87	0.05	0.12	0.52	0.55	0.45
R2	0.69	0.57	0.87	0.06	0.30	0.50	0.75	0.55
R3	0.74	0.52	0.89	0.08	0.58	0.31	0.59	0.54
Average	0.70	0.58	0.88	0.06	0.33	0.44	0.63	0.51
SD	0.03	0.07	0.01	0.02	0.23	0.12	0.11	0.06

Table A.35. Lactic and acetic acid (g/L) *p*resent in MRS agar medium, inoculated with *P. nordicum* MUM 08.16 supplemented with *L. buncheri* UTAD104 cells at different concentrations (DO_{600} 1, 2.5, 5) and PBS as control

Organic acids (g/L) in *P. nordicum* plates supplmented with *L. buchneri* UTAD104 cells

	Lactic acid				**Acetic acid**			
	DO 1	**DO 2.5**	**DO 5**	**PBS**	**DO 1**	**DO 2.5**	**DO 5**	**PBS**
R1	0.50	0.60	0.63	0.05	1.79	2.10	2.49	0.45
R2	0.48	0.58	0.63	0.06	2.00	2.01	2.35	0.55
R3	0.48	0.56	0.64	0.08	1.84	2.17	2.44	0.54
Average	0.49	0.58	0.63	0.06	1.88	2.09	2.43	0.51
SD	0.01	0.02	0.00	0.02	0.11	0.08	0.07	0.06

APPENDIX B

FROM CHAPTER 4

B. APPENDIX FROM CHAPTER 4

Lactic Acid Bacteria Survival in Films

Table B.1. Cell count (CFU/mL) of *L. buchneri* UTAD104 and *L. casei* UM3 during a period of 30 days

	Cell count (CFU/mL)								
Time (days)	**1**	**5**	**8**	**12**	**15**	**19**	**22**	**26**	**30**
L. buchneri UTAD104	1E+09	6E+07	3E+07	2E+05	4E+04	1E+05	4E+02	9E+02	7E+02
L. casei UM3	2E+10	3E+07	3E+07	3E+05	4E+05	1E+03	2E+02	1E+03	8E+02

Table B.2. Cell viability (log CFU/mL) of *L. buchneri* UTAD104 and *L. casei* UM3 during a period of 30 days. Values were correct for PBS dilution and weight of the films

	Cell viability (Log CFU/mL)								
Time (days)	**1**	**5**	**8**	**12**	**15**	**19**	**22**	**26**	**30**
L. buchneri UTAD104	11.0	9.8	9.5	7.4	6.5	7.1	4.6	4.9	4.8
L. casei UM3	12.3	9.5	9.4	7.4	7.6	5.0	4.2	5.0	5.0

Film Characterization

Thickness, Moisture Content, Water Solubility and Water Vapour Permeability

Table B.3. Thickness (mm) of WPC based films, with and without *L. buchneri* UTAD104 (at 30% w/w)

	Thickness (mm)			
	WPC film		WPC film + *L. bucnheri* UTAD104	
	Sample 1	Sample 2	Sample 1	Sample 2
R1	0.274	0.25	0.166	0.201
R2	0.214	0.197	0.159	0.16
R3	0.209	0.197	0.166	0.145
R4	0.214	0.197	0.243	0.147
R5	0.263	0.226	0.184	0.141
R6	0.268	0.203	0.179	0.144
R7	0.257	0.200	0.200	0.199
R8	0.228	0.186	0.162	0.151
R9	0.229	0.232	0.178	0.140
R10	0.243	0.202	0.180	0.162
R11	0.222	0.185	0.152	0.196
R12	0.238	0.237	0.178	0.246
R13	0.276	0.238	0.167	0.177
R14	0.244	0.210	0.145	0.174
R15	0.240	0.200	0.183	0.215
R16	0.228	0.248	0.182	0.185
R17	0.208	0.225	0.150	0.148
R18	0.226	0.22	0.170	0.143
Average	0.238	0.214	0.175	0.156
	0.226		0.166	
SD	0.022	0.021	0.022	0.031
	0.024		0.026	

Table B.4. Moisture content (%) calculated from sample weight (g) at 0h and after 24h of emersion in water

	Moisture Content (%)			
		Sample weight (g)		Moisture (%)
	Time (h)	0	24	
WPC films	Sample 1 (R1)	0.104	0.076	26.641
	Sample 1 (R2)	0.107	0.080	24.742
	Sample 1 (R3)	0.104	0.077	25.791
	Sample 3 (R1)	0.091	0.067	26.374
	Sample 3 (R2)	0.090	0.067	26.357
	Sample 3 (R3)	0.088	0.064	27.252
WPC films + *L. buchneri* UTAD104	Sample 1 (R1)	0.061	0.045	26.350
	Sample 1 (R2)	0.059	0.043	25.939
	Sample 1 (R3)	0.059	0.044	25.381
	Sample 3 (R1)	0.097	0.074	24.025
	Sample 3 (R2)	0.094	0.072	23.159
	Sample 3 (R3)	0.083	0.062	25.269

Table B.5. Solubility (%) calculated from sample weight (g)

	Solubility (%)			
		Sample weight (g)		Solubility (%)
		Non-dissolved	Dry weight	
WPC films	Sample 1 (R1)	0.045	0.076	41.130
	Sample 1 (R2)	0.044	0.080	44.931
	Sample 1 (R3)	0.045	0.077	41.473
	Sample 3 (R1)	0.037	0.067	44.560
	Sample 3 (R2)	0.038	0.067	43.628
	Sample 3 (R3)	0.036	0.064	43.701
WPC films + *L. buchneri* UTAD104	Sample 1 (R1)	0.025	0.045	43.430
	Sample 1 (R2)	0.024	0.044	44.725
	Sample 1 (R3)	0.025	0.044	44.318
	Sample 3 (R1)	0.041	0.074	45.418
	Sample 3 (R2)	0.039	0.072	45.961
	Sample 3 (R3)	0.035	0.063	45.151

Table B.6. Permeation cell and film sample weights (g) in period of 10h. Values were used for the calculation of WVP

		Sample weight (g)					
	Time (h)	0	2	4	6	8	10
WPC Film	Sample 1	197.017	196.973	196.931	196.897	196.866	196.839
	Sample 2	195.576	195.530	195.492	195.462	195.430	195.405
	Sample 3	184.478	184.424	184.375	184.343	184.307	184.278
WPC Film + *L. buchneri* UTAD104	Sample 1	195.576	195.523	195.486	195.452	195.422	195.397
	Sample 2	181.529	181.466	181.412	181.378	181.337	181.306
	Sample 3	197.712	197.660	197.618	197.583	197.552	197.525

Table B.7. Permeation cell and film sample weights weight loss (g) for each time point during 10h. to determine water loss transferred through film samples

		Sample weight variation (g)				
	Time (h)	2	4	6	8	10
WPC Film	Sample 1	-0.0440	-0.0422	-0.0338	-0.0314	-0.0270
	Sample 2	-0.0466	-0.0374	-0.0308	-0.0317	-0.0248
	Sample 3	-0.0535	-0.0497	-0.0321	-0.0360	-0.0286
	Average	-0.0480	-0.0431	-0.0322	-0.0330	-0.0268
WPC Film + *L. buchneri* UTAD104	Sample 1	-0.0527	-0.0371	-0.0336	-0.0303	-0.0248
	Sample 2	-0.0624	-0.0543	-0.0339	-0.0414	-0.0303
	Sample 3	-0.0520	-0.0418	-0.0350	-0.0315	-0.0269
	Average	-0.0557	-0.0444	-0.0342	-0.0344	-0.0273

Mechanical Properties

Table B.8. Tensile strenght (MPa) and Elongation at break (%) of WPC based films with and without *L. buchneri* UTAD104

		Mechanical Properties	
		Tensile Strength (MPa)	Elongation at break (%)
WPC films	Sample 1 (R1)	0.585	105.738
	Sample 1 (R2)	0.596	41.514
	Sample 1 (R3)	0.596	77.106
	Sample 1 (R4)	0.689	53.524
	Average (sample)	0.616	69.471
	SD (sample)	0.049	28.340
	Sample 2 (R1)	0.602	34.482
	Sample 2 (R2)	0.673	35.844
	Sample 2 (R3)	0.748	38.978
	Sample 2 (R4)	0.590	43.448
	Average (sample)	0.653	38.188
	SD (sample)	0.073	3.980
WPC films + *L. buchneri* UTAD104	Sample 1 (R1)	0.384	85.700
	Sample 1 (R2)	0.402	40.534
	Sample 1 (R3)	0.585	50.182
	Sample 1 (R4)	0.638	70.646
	Average (sample)	0.502	61.766
	SD (sample)	0.128	20.303
	Sample 1 (R1)	0.634	50.904
	Sample 1 (R2)	0.528	34.490
	Sample 1 (R3)	0.471	23.202
	Sample 1 (R4)	0.673	28.868
	Average (sample)	0.576	34.366
	SD (sample)	0.093	11.950

Optical Properties

Table B.9. Colour parameters (*L**, *a**, *b**) for WPC based films with and without *L. buchneri* UTAD104

		Colour Parameters		
		*L**	*a**	*b**
WPC films	Sample 1 (R1)	94.70	-0.37	10.94
	Sample 1 (R2)	94.48	-0.33	10.93
	Sample 1 (R3)	94.59	-0.34	10.81
	Sample 1 (R4)	94.47	-0.35	10.71
	Sample 1 (R5)	93.69	-0.36	10.56
	Sample 3 (R1)	93.73	-0.39	10.58
	Sample 3 (R2)	93.75	-0.42	10.09
	Sample 3 (R3)	94.00	-0.39	10.02
	Sample 3 (R4)	93.64	-0.42	10.28
	Sample 3 (R5)	93.55	-0.40	10.31
	Average	94.06	-0.38	10.52
	SD	0.45	0.03	0.33
WPC films + *L. buchneri* UTAD104	Sample 1 (R1)	90.62	0.08	21.56
	Sample 1 (R2)	90.24	0.12	22.73
	Sample 1 (R3)	90.69	0.22	21.77
	Sample 1 (R4)	89.87	0.32	22.55
	Sample 1 (R5)	89.52	0.55	23.14
	Sample 3 (R1)	90.96	1.52	22.38
	Sample 3 (R2)	91.43	1.27	21.74
	Sample 3 (R3)	91.48	1.11	21.44
	Sample 3 (R4)	90.78	1.25	22.30
	Sample 3 (R5)	90.04	1.28	23.99
	Average	90.56	0.77	22.36
	SD	0.65	0.56	0.79

Table B.10. Opacity for WPC based films with and without *L. buchneri* UTAD104

		Y (black backing)	Y (white backing)	Opacity (%)
WPC films	Sample 1 (R1)	85.59	11.10	12.97
	Sample 1 (R2)	85.73	10.94	12.76
	Sample 1 (R3)	85.95	11.15	12.97
	Sample 1 (R4)	86.14	11.15	12.94
	Sample 1 (R5)	86.15	11.12	12.91
	Sample 3 (R1)	84.65	10.51	12.42
	Sample 3 (R2)	84.70	10.35	12.22
	Sample 3 (R3)	85.27	10.49	12.30
	Sample 3 (R4)	84.44	10.42	12.34
	Sample 3 (R5)	84.22	10.62	12.61
	Average	85.28	10.79	12.64
	SD	0.73	0.34	0.30
WPC films + *L. buchneri* UTAD104	Sample 1 (R1)	77.65	10.35	13.33
	Sample 1 (R2)	76.81	10.14	13.20
	Sample 1 (R3)	77.81	10.18	13.08
	Sample 1 (R4)	76.01	10.22	13.45
	Sample 1 (R5)	75.27	10.00	13.29
	Sample 3 (R1)	78.4	10.51	13.41
	Sample 3 (R2)	79.44	10.63	13.38
	Sample 3 (R3)	79.54	10.61	13.34
	Sample 3 (R4)	78.00	10.56	13.54
	Sample 3 (R5)	76.39	10.68	13.98
	Average	77.53	10.39	13.40
	SD	1.41	0.24	0.24

Surface Hydrophobicity

Table B.11. Contanct angles (θ) for WPC based films wih and without L. *buchneri* UTAD104 during a period of 60s

	Contact angles (θ)							
	Time (s)	0	1	5	15	30	45	60
WPC films	Sample 1 (R1)	54.7	43.3	37.7	34.3	31.4	29.6	28.1
	Sample 1 (R2)	54.5	43.2	38.6	35.4	31.9	30.7	29.0
	Sample 1 (R3)	52.2	42.4	37.7	34.4	31.8	29.8	28.6
	Sample 1 (R4)	53.5	42.1	37.9	35.3	33.4	33.0	32.3
	Sample 1 (R5)	53.1	41.6	36.6	33.9	32.2	31.5	31.1
	Sample 3 (R1)	50.3	40.1	34.3	30.2	28.3	28.1	28.1
	Sample 3 (R2)	48.8	38.6	33.8	30.2	28.8	28.4	28.2
	Sample 3 (R3)	47.8	38.1	33.8	30.3	29.6	29.0	29.3
	Sample 3 (R4)	47.5	38.2	33.4	31.2	29.8	29.7	29.9
	Sample 3 (R5)	48.2	37.7	33.3	30.9	29.4	29.5	29.9
	Average	51.06	40.53	35.71	32.61	30.66	29.93	29.45
	SD	2.86	2.24	2.17	2.23	1.69	1.47	1.39
WPC films + *L. buchneri* UTAD104	Sample 1 (R1)	56.2	42.2	35.8	33.0	31.4	30.6	31.6
	Sample 1 (R2)	55.7	43.7	37.9	34.8	33.0	30.7	27.8
	Sample 1 (R3)	55.2	42.5	36.7	34.3	30.5	29.5	29.1
	Sample 1 (R4)	54.8	42.4	38.2	34.9	31.5	30.0	29.3
	Sample 1 (R5)	55.5	41.4	35.8	34.9	31.5	30.0	29.3
	Sample 3 (R1)	48.2	39.1	36.4	34.3	33.3	22.7	18.2
	Sample 3 (R2)	45.4	39.3	35.3	31.7	30.9	15.5	6.1
	Sample 3 (R3)	44.5	37.4	34.5	33.3	29.6	13.8	12.3
	Sample 3 (R4)	46.6	40.5	37.5	35.0	31.0	19.6	3.8
	Sample 3 (R5)	49.2	42.4	38.0	35.3	34.0	35.8	20.5
	Average	51.13	41.09	36.61	34.15	31.67	25.82	20.80
	SD	4.78	1.96	1.27	1.14	1.36	7.41	10.35

Mycotoxin Analysis of Coated Cheese

Table B.12. OTA (ng/mL) detected in cheese samples covered with WPC based films (without cells, with *L. casei* UM3 and with *L. buchneri* UTAD104) and uncoated

	OTA (ng/mL)			
	Uncoated control	WPC Film (without cells)	WPC + *L. casei* UM3	WPC + *L. buchneri* UTAD104
Sample 1	8.77	1.08	0.63	0.00
Sample 2	6.36	1.51	0.24	0.25
Average	7.57	1.30	0.44	0.13
SD	1.70	0.30	0.28	0.18