DOCTORAL (PHD) DISSERTATION

Emelin Leandro Rodrigues

Budapest

2025

HUNGARIAN UNIVERSITY OF
AGRICULTURE AND LIFE SCIENCES

EVALUATION OF PATULIN PRODUCTION BY *ASPERGILLUS* STRAINS FROM HUNGARIAN APPLE-GROWING REGIONS AND YEAST-BASED DEGRADATION STRATEGIES

Emelin Leandro Rodrigues

Budapest

2025

The PhD School

Name: Doctoral School of Food Science

Discipline: Food Science

Head: **Dr. Livia Simon-Sarkadi**
Professor, DSc
Hungarian University of Agriculture and Life Sciences
Institute of Food Science and Technology
Department of Nutritional Science

Supervisors: **Ildikó Batáné Dr. Vidács**
Senior researcher, PhD
Eszterházy Károly Catholic University
Centre for Research and Development

Dr. Zsolt Zalán
Senior research fellow, PhD
Hungarian University of Agriculture and Life Sciences,
Institute of Food Science and Technology
Department of Bioengineering and Alcoholic Drink Technology

....

Approval of the Head of Doctoral School Approval of the Supervisor(s)

TABLE OF CONTENT

1 INTRODUCTION 1

2 OBJECTIVES 4

3 LITERATURE REVIEW 5

3.1 Apples 5

3.2 Molds attacking apples 7

3.3 Mycotoxins 9

3.4 Patulin 12

3.5 Occurrence of patulin in apples and other agricultural products 15

3.6 Current methods for controlling patulin 17

3.7 Biocontrol 18

3.8 Mechanism of yeast action against molds and mycotoxins 20

3.9 *Wickerhamomyces anomalus* and its applications 22

4 MATERIALS AND METHODS 24

4.1 Isolation and identification of molds 24

4.1.1 Apples 24

4.1.2 Isolation of molds 25

4.1.3 Determination of morphological characteristics of mold isolates 26

4.1.4 Molecular microbiological identification of selected mold isolates 26

4.2 Restriction enzyme screening for mold identification using RFLP- PCR 27

4.2.1 Strains 27

4.2.2 Culture conditions and DNA extraction 28

4.2.3 PCR Amplification 28

4.2.4 *In silico* restriction enzyme analysis 30

4.2.5 Restriction fragment length polymorphism (RFLP) analysis 30

4.2.6 Data analysis and species identification 30

4.3 Occurrence of patulin-related genes and mycotoxin production 30
4.3.1 Evaluation of the occurrence of the patulin gene (*idh*) in isolated *Aspergillus* and *Penicillium* strains 30
4.3.2 Confirmation of mycotoxin production by thin layer chromatography 31
4.4 Influence of environmental factors on patulin production 31
4.4.1 Effects of physicochemical parameters, temperature and pH, on the patulin production by *A. clavatus* B9/6 31
4.5 Yeast-based biocontrol of *A. clavatus* B9/6 32
4.5.1 Yeast screening for potential growth inhibition of *A. clavatus* B9/6 32
4.5.2 Patulin degradation by *Wickerhamomyces anomalus* strains 33
4.5.3 Kinects of patulin degradation by *W. anomalus* 01499 and 1655 34
4.5.4 Co-cultivation of *W. anomalus* 01499 and 1655 and *A. clavatus* B9/6 34
4.5.5 Evaluation of *A. clavatus* B9/6 inhibition by *W. anomalus* 01499 *in vivo* 34
4.5.6 Assessment of patulin degradation by intracellular/extracellular enzymes and patulin binding of *W. anomalus* 01499 35
4.6 Patulin extraction 36
4.7 High-performance liquid chromatography (HPLC) method 36
4.8 Statistical evaluation 37
5 RESULTS AND DISCUSSION 38
5.1 Mold isolation and identification 38
5.2 Occurrence of patulin-related genes and mycotoxin production 47
5.2.1 Detection of the patulin gene in *Aspergillus* and *Penicillium* strains 47
5.2.2 Confirmation of patulin production by thin-layer chromatography 50
5.2.3 Molecular microbiological identification of selected mold isolates 52
5.3 PCR-RFLP method development for rapid identification of *Aspergillus* species 54
5.3.1 Restriction enzyme screening for mold identification (RFLP-PCR) 54
5.3.2 RFLP-PCR analysis of mold isolates from apples 57

5.4 Effect of pH and temperature on patulin production by *A. clavatus* B9/6 59

5.5 Yeast-based biocontrol of *A. clavatus* B9/6 62

5.5.1 Screening of yeast strains for antifungal activity 62

5.5.2 Patulin degradation by *Wickerhamomyces anomalus* strains 67

5.5.3 Kinetics of patulin degradation *by W. anomalus* 01499 and 01655 69

5.5.4 Co-cultivation of *W. anomalus* 01499 and 01655 with *A. clavatus* B9/6 70

5.5.5 *In vivo* evaluation of *A. clavatus* B9/6 inhibition using *W. anomalus* 01499..... 72

5.5.6 Intracellular and extracellular enzymatic degradation of patulin and PAT binding capacity of *W. anomalus* 01499..... 74

6 CONCLUSION AND RECOMMENDATIONS 77

7 NEW SCIENTIFIC RESULTS 80

8 REFERENCES..... i

9 SUMMARY OF FIGURES..... xvii

10 SUMMARY OF TABLES..... xix

1 INTRODUCTION

Apples are one of the most widely cultivated and consumed fruits worldwide, deeply embedded in cultural traditions, literature, and dietary habits. Their widespread popularity and economic significance make them an essential crop in many regions, including Hungary, where they represent the most-produced fruit. However, maintaining apple orchards free from hazards is a critical challenge faced by producers. Environmental conditions, soil quality, and exposure to pests and pathogens can significantly impact fruit yield and quality. Among the various threats to apple production, fungal contamination and mycotoxin accumulation are among the most critical concerns, particularly during storage and post-harvest handling.

Fungal pathogens pose a major risk to apple production, affecting not only fruit appearance but also its nutritional and commercial value. Molds such as *Venturia inaequalis*, *Podosphaera leucotricha*, *Colletotrichum* spp., *Penicillium expansum*, and *Botrytis cinerea* are well-documented problems in reducing apple quality and causing significant post-harvest losses. These fungi succeed under specific environmental conditions, and their proliferation is often exacerbated by improper handling and storage practices. The consequences of fungal contamination extend beyond economic losses; in certain cases, fungal metabolites known as mycotoxins can pose serious health risks to consumers.

Among the various mycotoxins associated with apples, patulin (PAT) stands out as the most concerning. Patulin is a secondary metabolite produced predominantly by *Penicillium expansum*, though research has increasingly highlighted the potential role of *Aspergillus* species in its synthesis. This toxin is highly stable and can persist in processed apple products such as juices and purees, raising significant food safety concerns.

Regulatory bodies worldwide have established strict limits on patulin levels in food products to mitigate health risks. However, despite these regulations, numerous studies have documented patulin contamination in apple-derived products, emphasizing the need for more effective control strategies.

The presence of patulin in apples and their derivatives presents a dual challenge: preventing fungal contamination at the source and mitigating mycotoxin accumulation during post-harvest processing. Conventional control methods, such as synthetic fungicides, have been widely employed

to combat fungal infections. However, the overuse of these chemical agents has led to increasing concerns regarding environmental impact, consumer health, and the development of fungal resistance. Consequently, alternative, sustainable strategies for controlling fungal contamination and mycotoxin production are gaining attention within the scientific community.

One promising approach involves the use of biocontrol agents, particularly yeasts, to inhibit fungal growth and degrade mycotoxins. Certain yeast species have demonstrated strong antagonistic properties against toxigenic molds, effectively reducing their proliferation through competition for nutrients, production of antifungal compounds, and direct interactions. Moreover, specific yeast strains possess enzymatic mechanisms that facilitate the degradation or adsorption of patulin, thereby contributing to food safety. Understanding the mechanisms triggering yeast-fungal interactions is crucial for developing viable biocontrol strategies in apple production and post-harvest management.

Despite increasing interest in biocontrol methods, significant gaps remain in our understanding of the role of *Aspergillus* species in patulin contamination, as well as the most effective yeast strains for mitigating its impact. Investigating the occurrence of *Aspergillus* species in apples, their potential for patulin production, and the environmental factors influencing toxin synthesis can provide critical insights for risk assessment. Additionally, identifying and characterizing yeast strains capable of inhibiting patulin-producing fungi and neutralizing patulin itself could demonstrate the way for safer, more sustainable approaches to apple preservation.

Understanding how environmental conditions, such as humidity, temperature, pH and oxygen availability, influence patulin-producing molds could lead to the development of predictive models for contamination risk. Moreover, advancements in molecular biology and genomics could provide insights into genetic resistance mechanisms in apples, aiding in the breeding of cultivars with improved resistance to fungal infection.

Given the increasing consumer demand for organic and minimally processed food products, the importance of sustainable and non-chemical interventions in food safety cannot be exaggerated. Biocontrol strategies align well with global efforts to reduce chemical use in agriculture while maintaining high food quality standards. The integration of yeasts and other microbial antagonists into apple storage and processing environments offers a natural and eco-friendly approach to mitigating patulin contamination, potentially reducing reliance on synthetic fungicides and chemical

preservatives. Exploring the biochemical interactions between yeasts, molds, and apples at the cellular and molecular levels will further enhance the effectiveness of biocontrol interventions.

This study aims to address these gaps by exploring the interaction between fungal pathogens, mycotoxin contamination, and biocontrol strategies in apple production. Through the isolation and characterization of *Aspergillus* species, the evaluation of their patulin-producing potential, and the screening of yeast strains for antagonistic activity, this research seeks to contribute to the development of innovative, eco-friendly solutions for maintaining apple quality and ensuring food safety, promoting safer and more sustainable apple production and post-harvest management practices.

2 OBJECTIVES

This study was designed to explore critical aspects of fungal contamination and mycotoxin production in apples, with a focus on *Aspergillus* species, investigate the occurrence and patulin production potential of *Aspergillus* species isolated from apples in Hungary, analyze the genetic determinants and environmental factors influencing patulin synthesis, and evaluate the effectiveness of yeast strains in inhibiting patulin-producing *Aspergillus* species through antagonistic interactions and patulin binding capabilities.

Specific Objectives

- Isolation of *Aspergillus* species from apples originated from regions of Hungary and selection of isolates that belong to molds theoretically able to produce patulin.
- Detection of the gene responsible for patulin production in *Aspergillus* isolates of molds theoretically able to produce patulin.
- Evaluation of patulin production of *Aspergillus* isolates that possess the gene responsible for patulin production.
- Study of the effects of physico-chemical factors on the patulin production of *Aspergillus* spp.
- Screening the potential of yeast strains by antagonistic culturing for the best yeasts to hinder the growth of patulin producing *Aspergillus* spp.
- Study of the patulin binding ability of selected yeasts

3 LITERATURE REVIEW

3.1 Apples

Apples are among the most known fruits worldwide, not only viewed as food and feed, but it is also a part of the culture of many peoples. From child fairytales as Snow White to biblical references this crop has been under the spotlight for many centuries. This fruit is originally from Kazakhstan but was spread to Eurasia along the silk road, nowadays it is cultivated in over 90 countries and consumed worldwide (Cornille et al., 2014). Besides its consumption *in-natura* this fruit is also used as base for a variety of products such as fruit juices, purees, and ciders as well as a sweetener agent for green label products (Zhang et al., 2021).

Apple is the third most produced fruit globally per kilogram, preceded only by banana and watermelon, according to the Food and Agriculture Organization of the United Nations (FAO). Currently, its production is focused on temperate climates and is produced in both hemispheres, its stability when stored in a cold environment allows the apple availability almost everywhere during the whole year.

Apple composition is primarily carbohydrate-based, with over 90% of dry matter consisting of sugars and dietary fiber. The predominant sugar in apples is fructose, followed by glucose and sucrose. As apples ripen, the sugar concentration increases, enhancing their sweetness.

Moisture content in apples typically ranges from 82.5% to 86.2%, with some varieties reaching up to 88.1%. Protein content varies between 1.42% and 4.35%, while fat content is relatively low, ranging from 0.28% to 3.62%. The ash content, which reflects the presence of minerals, ranges from 1.32% to 2.08%. Among minerals, potassium and phosphorus are the most abundant, playing vital roles in cellular functions and metabolic processes (OECD, 2015). Table 1 shows the average composition of these fruits according to different regulatory agencies.

Table 1 - Proximate and Carbohydrate Composition of Apple Fruit (% dry matter, edible portion) extracted from (OECD, 2015)

Nutrient	USDA 2015	Germany 2014	Denmark 2019	UK 2015	Switzerland 2015	China 2009
Moisture (% fresh weight)	85.56	82.47	84.9	86.2	85	85.9
Protein (% dry matter)	1.8	1.93	2	4.35	2	1.42
Fat (% dry matter)	1.18	0.28	1.3	3.62	2	1.42
Ash (% dry matter)	1.32	1.82	2	-	-	1.42
Total carbohydrates (% dry matter)	95.63	-	94.7	-	-	95.74
Available carbohydrates	-	81.63	80.1	83.04	77.99	-
Total dietary fiber (% dry matter)	16.62	11.43	14.6	9.42	14	8.52
Total sugars (% dry matter)	71.95	58.65	72.2	83.04	77.33	-
Sucrose (% dry matter)	14.35	14.48	20.5	19.27	-	-
Glucose (% dry matter)	16.83	11.54	11.7	15.22	-	-
Fructose (% dry matter)	40.86	32.63	40.1	48.55	-	-
Starch (% dry matter)	0.35	3.41	0	0	0.67	-

Dietary fiber is a crucial component of apples, with the highest concentration found in skin, where it can be up to 30% higher than in the flesh. Pectin, a type of non-starch polysaccharide, constitute a major part of apple fiber. These compounds contribute to the firmness of the fruit and undergo structural changes during ripening, leading to texture modifications. The consumption of apples has been linked to several health benefits, including improved digestion, enhanced gut microbiota, and better glycemic control due to their high fiber content.

Apples also contain bioactive compounds such as flavonoids and polyphenols, which act as antioxidants, reducing oxidative stress and inflammation in the body. These compounds contribute to

the protective effects of apples against chronic diseases such as cardiovascular conditions and certain types of cancer. Overall, apples are a rich source of essential nutrients while being low in fat and calories, making them a valuable addition to a balanced diet. Their versatility allows them to be consumed fresh, processed into juices, or incorporated into a variety of food products, ensuring their continued popularity in global nutrition.

China was the biggest producer of this crop in 2021, producing over 45 million tons according to FAO. Türkiye, the USA, and Poland were the next in the ranking, producing approximately 4 million tons each, ten times less than the largest producer (FAOSTAT, 2021).

In the Hungarian context, apples also play a key role being the most-produced fruit with over 350 ton per year according to the (Hungarian Central Statistical Office, n.d.). Therefore, keeping the orchards and consequently the fruits free of potential hazards is essential to avoid economic and health-related losses. The main concerns regarding apple cultivars are their possible yield reduction due to unexpected environmental effects such as excessive amount of water/sun and soil aspects, besides their contamination with parasites and diseases (Cornille et al., 2014; Delgado et al., 2021; Hou et al., 2021).

3.2 Molds attacking apples

Among the main factors that can influence the quality of the apples, and a successful harvest is the presence of molds in the orchard or during the storage, since these microorganisms can cause multiple damages. The most common is the apple scab caused by *Venturia inaequalis.* This mold has the potential to affect not only the fruit but also the leaves and flowers of the tree causing dark spots and fruit deformation. Its presence is ubiquitous in the producing regions, but it is more prevalent in areas with wet and cold springs (Bowen et al., 2011).

Another common disease is powdery mildew, caused by *Podosphaera leucotricha*, its principal characteristic is the presence of a white powder mainly on the leaves and shoots as well into some young fruits. As the previous disease, it can be found in any environment that has apple cultivation, but it prevails in warm and dry conditions (Strickland et al., 2021).

The bitter rot, caused by *Colletotrichum* spp., is presented as dark spots that are sunken into the fruit. The lesions are circular and grow with time under specific weather conditions with higher moisture, and small black or orange spots are formed due to the sporulation of different species. Some studies showed that the spores can remain in a dormant state even in severe winter conditions,

exemplifying how robust this mold is. The disease can also show up after the harvest, during the fruit storage, if it is not done properly (Ali et al., 2024).

These diseases represent a major challenge to maintaining fruit quality and minimizing losses. In fact, studies estimate that 20-25% of apples are lost after storage, primarily due to fungal infections caused by molds such as *Penicillium expansum* and *Botrytis cinera* (Khadiri et al., 2024). These pathogens thrive under certain storage conditions, particularly when humidity is high, temperatures are not well-regulated, or when apples are bruised or damaged during handling.

Molds take advantage of these favorable environments to grow and spread, affecting not only the external appearance of the apples but also their internal quality, rendering them unfit for consumption or sale. This deterioration directly impacts the profitability of apple production and contributes to significant food waste. Table 2 presents an overview of the main fungal diseases affecting apples, indicating whether the pathogens are associated with mycotoxin production and whether they primarily impact the fruit before or after harvest.

Table 2 - Major fungal diseases affecting apples: toxin production and pre- or postharvest incidence

Scientific name	**Common disease name**	**Produces toxin**	**Pre/post-harvest**	**Reference**
Fusarium oxysporum	Apple replant disease (ARD)	No	Pre-harvest	(Jiang et al., 2025)
Botrytis cinerea	Gray mold	No	Post-harvest	(Yu et al., 2024)
Penicillium expansum	Blue mold	Yes (Patulin)	Post-harvest	(Wang et al., 2025)
***Alternaria* spp.**	Moldy core	Yes (Alternaria toxins)	Both	(Patriarca, 2019)
Podosphaera leucotricha	Powdery mildew	No	Pre-harvest	(Paz-Cuadra et al., 2014)
***Colletotrichum* spp.**	Bitter rot	No	Both	(Andrello et al., 2024)
Venturia inaequalis	Apple scab	No	Pre-harvest	(Bowen et al., 2011)

Monilinia fructigena	Brown rot	No	Both	(Madbouly et al., 2020)

Another significant concern for this crop is contamination with mycotoxins. These toxic compounds represent a unique challenge as they do not alter the organoleptic attributes of the fruit, such as taste, smell, or appearance (Gonçalves et al., 2019; A. Gong et al., 2024; Vidal et al., 2019). As a result, unless the mold has grown enough to become visibly apparent, the presence of mycotoxins can only be detected through specialized analytical methods (Dias et al., 2019; MacDonald et al., 2000; C. Yang et al., 2021). This makes their detection particularly problematic in post-harvest management.

3.3 Mycotoxins

Mycotoxins are toxic secondary metabolites produced by fungi that contaminate agricultural products, posing severe health risks to humans and animals. The history of mycotoxins dates back centuries, though scientific recognition of their dangers emerged only in the 20th century. Their presence in crops has led to significant agricultural challenges and notable outbreaks of mycotoxicosis.

The presence of mycotoxins possible began with the advent of grain storage in early agricultural societies. Molds would grow under humid and warm conditions, contaminating stored grains. One of the earliest suspected mycotoxin-related diseases was ergotism, caused by *Claviceps purpurea* in rye, which led to mass poisoning incidents in medieval Europe. Symptoms included convulsions, hallucinations, and gangrene.

In 18th-century England, mold-contaminated rye was suspected in high child mortality rates, with symptoms including intestinal ulceration and bloody diarrhea (Matossian, 1981). Another major outbreak occurred in Russia during World War II, known as Alimentary Toxic Aleukia (ATA), caused by *Fusarium* species producing T-2 toxin. The disease led to high mortality rates due to hemorrhagic symptoms and immune suppression (Richard, 2007).

The field of mycotoxin research took a pivotal turn in the early 1960s when a massive outbreak of “Turkey X Disease” in England killed over 100,000 turkeys. The cause was traced to *Aspergillus*

flavus-contaminated peanut meal from Brazil, leading to the identification of aflatoxins, one of the most potent carcinogenic mycotoxins (Richard, 2008).

Further research established links between aflatoxins and liver cancer, prompting global food safety regulations. Additional studies in Africa highlighted the presence of mycotoxins in staple foods like maize and peanuts, exacerbating malnutrition and health crises (Williams, 2004).

Today, scientists have identified between 300 and 400 different mycotoxins, produced by over 200 fungal species. Among the most concerning are aflatoxins, which are highly carcinogenic and commonly found in corn, peanuts, and cottonseed. Ochratoxins, present in cereals, coffee, and wine, are known to cause kidney damage. Trichothecenes, which affect wheat, barley, and maize, can lead to vomiting and immune suppression. Zearalenone, found in corn and wheat, disrupts reproductive health, while fumonisins, commonly detected in maize, have been linked to esophageal cancer and neural tube defects (Murugesan et al., 2015).

Crop contamination varies based on climatic conditions, with humid and warm environments being most conducive to fungal growth. The globalization of trade has exacerbated mycotoxin spread, as contaminated grains move across borders.

Several large-scale mycotoxin outbreaks have highlighted the severe risks associated with these toxins. Between 2004 and 2006, Kenya experienced a devastating outbreak of aflatoxicosis caused by the consumption of contaminated maize, leading to over 300 deaths. In 1974, India faced a similar crisis when aflatoxin-contaminated maize resulted in more than 100 fatalities, underscoring the dangers of mycotoxin exposure in staple foods. In the United States, the 2012 drought created ideal conditions for increased aflatoxin contamination in corn, causing widespread crop losses and economic damage exceeding $1 billion (Wu, 2022).

The economic burden of mycotoxins is immense, affecting food security, trade, and livestock productivity. Regulatory agencies like the FDA, EFSA, and FAO set permissible mycotoxin limits in food and feed to mitigate risks.

A major challenge in mycotoxin analysis is their ability to bind with other food components, forming masked or modified mycotoxins that evade traditional detection methods. These modified forms can still be toxic upon digestion, complicating risk assessments and requiring advanced analytical techniques such as mass spectrometry and biomarker-based evaluations (Vidal et al., 2018).

Mycotoxins remain a persistent challenge in global agriculture, requiring continuous monitoring and management. While technological advances have improved detection and mitigation, climate change and agricultural practices will continue to influence mycotoxin prevalence. Ongoing research is essential to safeguard food security and public health. Table 3 summarizes the most prevalent mycotoxins found in food commodities, including their main sources, health effects and their regulatory limits by the EU.

Table 3 - Overview of major mycotoxins: sources, health effects, and regulatory limits

Mycotoxin	Main foods affected	Health effects	Regulatory limits in the EU	Reference
Aflatoxin B1	Maize, peanuts, rice, wheat, cereal-based products	Hepatotoxic, carcinogenic (Group 1 IARC), immunosuppressive, mutagenic	2 µg/kg (AFB1 in cereals), 4 µg/kg (total aflatoxins in cereals)	(Kousar et al., 2024)
Fumonisin B1	Maize and maize-based products	Neurotoxic, hepatotoxic, nephrotoxic, immunotoxic, associated with neural tube defects and esophageal cancer	1000 µg/kg in unprocessed maize, 200-4000 µg/kg in food (varies by product)	(Obafemi et al., 2025)
Ochratoxin A	Cereals, coffee, cocoa, dried fruits, wine, beer	Nephrotoxic, hepatotoxic, immunotoxic, possibly carcinogenic (Group 2B IARC), teratogenic	5 µg/kg in cereals, 3 µg/kg in processed products, 2 µg/kg in wine	(Banahene et al., 2024)
Zearalenone	Maize, wheat, barley, oats, sorghum, animal feed	Estrogenic, reproductive disorders, hepatotoxic, immunotoxic, teratogenic	100 µg/kg in cereals, 20 µg/kg in baby food	(Cai et al., 2024)
Deoxynivalenol (DON)	Wheat, maize, barley, oats, rye, processed cereal products, infant food, beer	Vomiting, anorexia, immune suppression, intestinal and liver toxicity	750 µg/kg in unprocessed cereals, 200 µg/kg in infant foods	(Zhao et al., 2025)
Patulin	Apples and apple-based products	Gastrointestinal distress, immunotoxic, genotoxic, possibly carcinogenic	10-50 µg/kg depending on product	(Zheng et al., 2021a)

The presence of mycotoxins in food is a serious issue, as these substances can cause a range of harmful effects on both human and animal health, including acute toxicity, immune suppression, and long-term carcinogenic risks. Although toxins such as aflatoxin B1 and fumonisins can be found to a very low extent in apples, undoubtedly patulin (PAT) is the most problematic and thus most studied for this crop (Zhong et al., 2018).

3.4 Patulin

Patulin is a polyketide lactone (4-hydroxy-4H-furo [3,2-c] pyran-2(6H)-one), which under environmental conditions appears as a crystalline substance and is soluble in water. It is produced as a secondary metabolite by fungi such as *Penicillium*, *Aspergillus,* and *Byssochlamys* species (Vidal et al., 2019). It was first reported in the early 40s as a promising medicine for the common cold, and sometimes it is even claimed to be a properly controlled multicenter trial (Chalmers & Clarke, 2004).

Unfortunately, it was later established that patulin not only failed to show a significant effect in controlling the common cold but also posed serious risks to human and animal health. Studies revealed its toxic nature, including potential genotoxic and carcinogenic effects, which raised concerns about its presence in food products. This shifted the perception of patulin from a promising therapeutic agent to a harmful contaminant that requires strict monitoring and control.

Today, patulin is recognized as a significant food and feed contaminant with well-documented toxic effects. Its primary impact is genotoxicity, driven by oxidative damage that results in mutagenicity, DNA base modifications, inter-strand crosslinks, and ultimately, direct damage to the DNA strand. In addition to its genotoxic effects, patulin is associated with immunotoxicity, hepatotoxicity, and gastrointestinal and neurological issues, making it a broad-spectrum threat to health. The kidneys, immune system, and intestinal tissues are particularly vulnerable, as they are most significantly impacted by exposure to this mycotoxin (Mahato et al., 2021).

Due to its potential health risks, regulatory agencies have established limits on its presence in food and food products. The World Health Organization (WHO) has determined the maximum tolerable intake for humans to be 0.4 µg/kg of body weight per day. The European Food Safety Authority (EFSA) sets three different limits depending on the product type: 50 µg/kg for juices, ciders, and spirits; 25 µg/kg for solid apple-based products; and 10 µg/kg for apple products intended for babies, infants, and lactating women (EUROPEAN UNION, 2023). These variations reflect the higher vulnerability of certain populations, such as infants and pregnant or nursing women, to potential toxic effects. Other countries, such as Brazil and China, have adopted the 50 µg/kg limit for juices and apple beverages but do not specify limits for other types of food products (Iha & Sabino, 2008).

The majority of scientific research on patulin-producing microorganisms has focused on species within the genus *Penicillium*, particularly *P. expansum*, *P. griseofulvum*, and *P. crustosum*, which are well-documented as primary contaminants in fruits and fruit-derived products (Coelho et

al., 2007; Csernus et al., 2015; Reddy et al., 2010; Rodrigues et al., 2022). However, there is growing concern regarding the potential contribution of *Aspergillus* species to patulin contamination, a topic that remains comparatively underexplored.

Among these, *Aspergillus clavatus* has garnered increasing attention due to its ability to produce patulin under certain environmental conditions. Additionally, other *Aspergillus* species, such as *A. terreus*, *A. giganteus*, and *A. longivesica*, have also been reported as potential patulin producers. Given the limited studies on *Aspergillus* spp. in this context, further investigation is warranted to better understand their role in patulin contamination and assess their significance in food safety (Tóth et al., 2012; Varga et al., 2007).

Aspergillus clavatus, a less studied but significant species within the *Aspergillus* genus, has been identified as a patulin producer, further emphasizing the importance of understanding its metabolic pathways. This emphasizes the necessity of including *A. clavatus* in monitoring and control strategies, particularly in agricultural and food-processing settings where fungal contamination is prevalent. Given the health risks associated with patulin exposure, the identification of additional fungal sources, such as *A. clavatus*, is crucial for improving food safety protocols and mitigating potential health hazards.

Regarding its morphology, *A. clavatus* is a filamentous fungus characterized by its distinctive tall conidiophores, which can reach up to several hundred micrometers in height. The conidiophores terminate in a large, elongated, club-shaped (clavate) vesicle, a key morphological feature that distinguishes this species from other members of the genus *Aspergillus*. Its conidia are smooth-walled or finely roughened, typically forming compact chains that give the colony a bluish green to grayish appearance. Colonies of *A. clavatus* grow rapidly on common mycological media, such as Czapek yeast extract agar, where they develop a powdery to velvety texture. Additionally, the species produces characteristic elongated phialides that radiate from the vesicle, further contributing to its unique morphology (Figure 1) (Tannous et al., 2014; Varga et al., 2003).

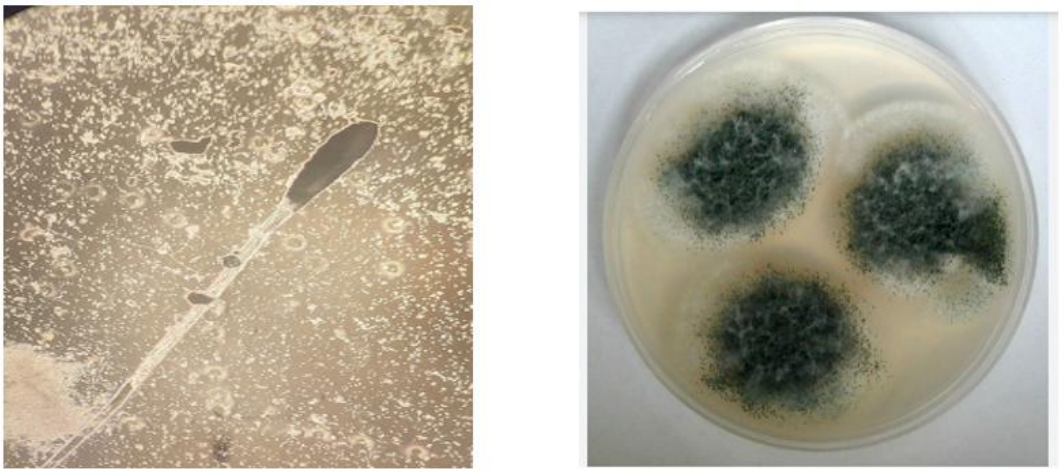


Figure 1 – *Aspergillus clavatus* under microscope (100x magnification) and on PDA culture medium after 7 days incubation at 37 °C

In filamentous fungi, the production of secondary metabolites like patulin is controlled by gene clusters, where multiple genes work together to create the final compound. These genes encode enzymes that function in a step-by-step manner, transforming initial molecules into the final toxin. A specific transcription factor, usually found within the cluster, activates these genes, while global regulators influence its activity. In the case of patulin, biochemical studies and mutant analysis suggest a biosynthetic pathway involving about 10 steps, with key enzymatic reactions requiring cofactors like NADPH and oxygen. One important step is the hydroxylation of m-hydroxybenzyl alcohol to gentisyl alcohol, which involves cytochrome P450 enzymes.

A cluster of 15 genes responsible for patulin biosynthesis has been identified in *Aspergillus clavatus*, covering a 40 kb region. This cluster includes genes encoding enzymes, transporters, and one transcription factor. Some of the key enzymes include a putative carboxyl esterase (PatB), cytochrome P450 enzymes (PatH and PatI), and an isoepoxydon dehydrogenase (PatN). Interestingly, related gene clusters exist in fungi like *Penicillium chrysogenum*, *Talaromyces stipitatus*, and *Aspergillus terreus*, but these species do not produce patulin due to missing essential genes (Puel et al., 2010; Snini et al., 2015).

A deeper understanding of this metabolic pathway (Figure 2) can provide insights into potential intervention strategies, such as targeted inhibition of specific biosynthetic enzymes, to reduce or eliminate patulin contamination in food products.

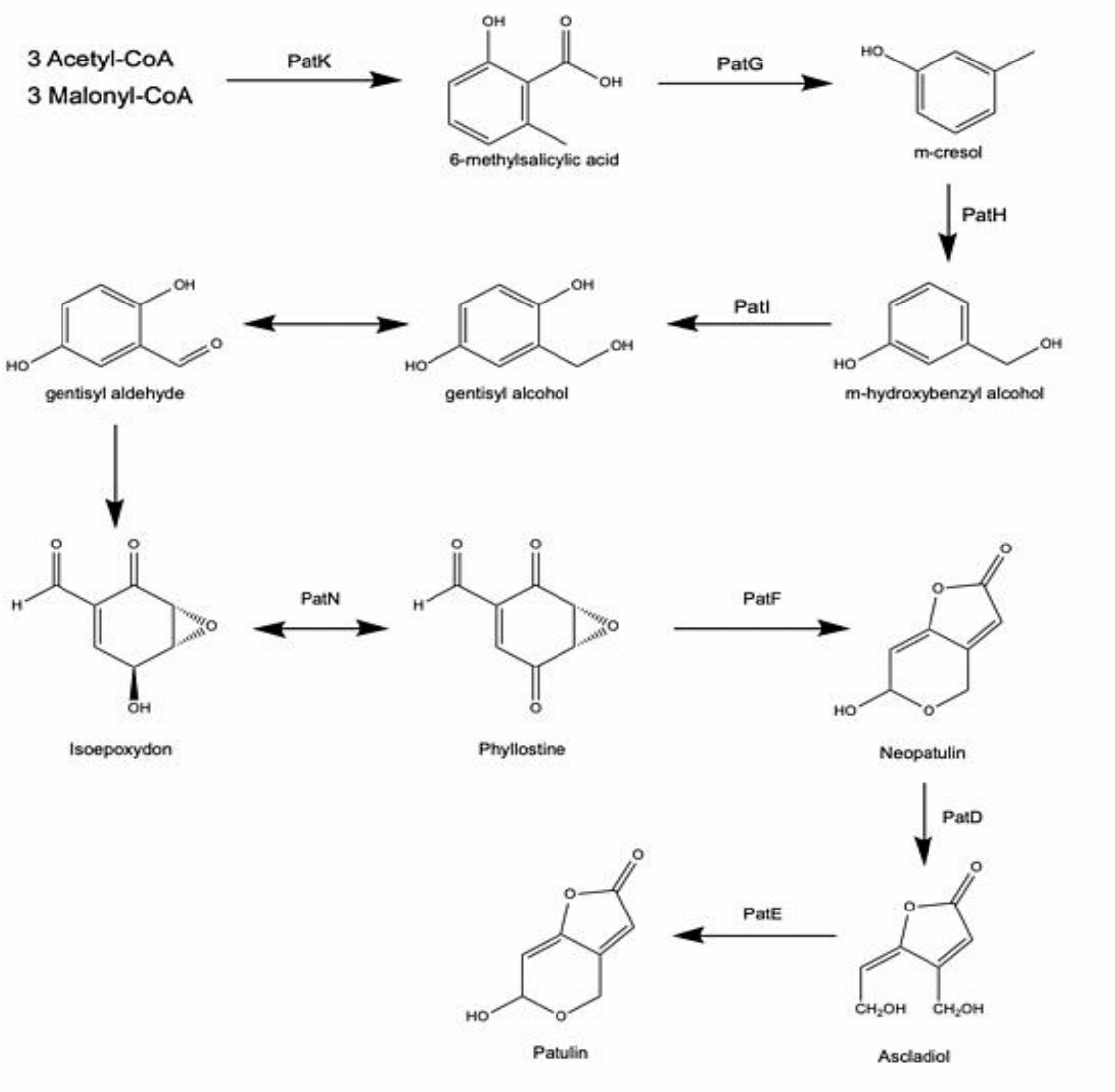


Figure 2 - Metabolic pathway of patulin synthesis for *P. expansum* (Puel et al., 2010).

3.5 Occurrence of patulin in apples and other agricultural products

Improper storage conditions, such as high temperatures and humidity, create an ideal environment for mold proliferation, increasing the risk of contamination. Additionally, cross-contamination can spread fungi to healthy apples, further compounding the problem. When

contaminated apples are processed into juice or puree, patulin levels can concentrate, posing a greater risk since the toxin remains stable even after pasteurization (Vidal et al., 2019).

Although the limits for patulin are established, many researchers over the years revealed the occurrence of this contaminant in different products worldwide as it is presented in Table 4.

Table 4 - Summary of patulin contamination in different sample types, maximum levels, and previous study locations

Sample	Maximum contamination level (µg/kg or µg/L)	Location	Reference
Apple juice	82.2	Qatar	(Hammami et al., 2017)
Baby apple juice	61.3		
Baby apple compote	24.57		
Apple juice	190.7	Iran	(Rahimi & Rezapoor Jeiran, 2015)
Apple juice	167	Tunisia	(Zaied et al., 2013)
Mixed fruits juice	125		
Apple baby food	165		
Apple juice	376	Turkey	(Gökmen & Acar, 2000)
Apple juice	153		
Apple juice	103		
Apple juice	119		
Pear juice	126	Spain	(Marín et al., 2011)
Organic apple juice	50.2	Spain	(Piqué et al., 2013).
Full apple juice	566	Brazil	(Dias et al., 2019)
Apple nectar	197		
Soybean drink with apple	195		
Mixed juice apple +pear	524		
Apple juice	42	Portugal	(Barreira et al., 2010)
Apple purees	5.2		
Cherry tomato	116.2	Brazil	(Rodrigues et al., 2022)
Fresh-cut apple	192.78	China	(Q. Gong et al., 2024)
Apple juice	140.44		
Apple cider	87.14		

In the majority of the studies samples above the tolerance limit were found, poising serious risks to the consumer's health, especially that in many cases even if the product is not labeled for infants and babies, they can be consumed anyway for these groups that are more susceptible to the damage of this toxin (Bonerba et al., 2010).

It is necessary to highlight that even though patulin is already regulated in many countries, this compound is still neglected and unknown to a big part of the population, even more when compared to more discussed toxins such as aflatoxins, ochratoxins, and fumonisins, found mainly in grains and their products (Gonçalves et al., 2019; Juodeikiene et al., 2018; Perrone et al., 2007). In this sense, it is necessary to inform and educate the general population about the risks, and regulatory bodies should be obliged to take stricter actions.

As observed in the table above, although most studies focus on apples and apple products, patulin (PAT) can also be found in other products such as pears and tomatoes. In fact, climate change has become a key factor to consider, as rising temperatures are causing fungal populations to shift and migrate. As a result, more species are being detected in regions or agricultural systems where they were previously absent or isolated (Bui et al., 2020; Pec et al., 2021; X. Yang et al., 2021).

3.6 Current methods for controlling patulin

The mechanisms for controlling patulin can be broadly categorized into two main groups: pre-harvest and post-harvest. Pre-harvest methods are applied in the orchard during apple cultivation, mainly focusing on preventing the development of toxin producing molds. Post-harvest methods, on the other hand, are implemented after the apples have been harvested, aiming to reduce or eliminate the mycotoxin, especially in processed products (Zhong et al., 2018).

Among the pre-harvest methods we can refer to cultivation control, such as Good Agricultural Practices (GAP) with proper fruit handling, correct plant spaces, and controlled irrigation. Although this can offer some improvement in regions with high humidity and temperature, it can also be ineffective in other places. One of the most commonly used methods for controlling patulin is the application synthetic fungicides, with thiophanate-methyl and iprodione being the most prevalent. However, these fungicides can lead to fungal resistance and may be avoided by consumers, especially those seeking greener and more sustainable products. Another pre-harvest alternative is the use of resistant cultivars, but their market acceptance is still lower than of the regular apples.

When we talk about post-harvest methods, another subdivision can be made: physical, chemical, and biological. At this stage the main goal is to degrade and remove the patulin already existing in the crop.

Some of the possibilities for physical methods are cutting the contaminated and adjacent parts of the fruits, which can be ineffective, since studies have already shown that the toxin can migrate deeper into the fruit. Thermal methods can also be ineffective in removing patulin, as they not only alter the organoleptic characteristics of the product but also fail to eliminate patulin as it is thermo-stable. Filtration and clarification can also be applied; they have better potential than the thermal methods but also possess a higher cost and reduce the product yield. More modern technologies such as UV and gamma radiation can also be applied, and while sensory aspects might be changed, the major challenge is consumers' acceptance in this case (Moake et al., 2005; Zheng et al., 2021b)

Chemical methods, the same way as physical ones, also present as a big challenge due to alteration of sensorial characteristics of the products. The most common compound widely used in food industry is the ascorbic acid, but to be used as a reducing agent, a high concentration is needed, which enhances the acidity of the juice. Sulfur dioxide and sulfites can also be used, their reaction with PAT neutralizes its toxicity, but the usage of these compounds is highly regulated, since their consumption can also cause damage to health. Adsorbents are also a potential method for controlling patulin, but their major challenge lies in optimizing the dosage. The goal is to ensure that while the patulin is effectively removed, the beneficial nutrients in the product are not also adsorbed and lost (Moake et al., 2005).

In recent years, a new alternative has gained attention. Biocontrol utilizes microorganisms and/or enzymes, as well as essential oils and plant extracts to degrade the patulin. Since, in this research this was the selected approach, a more detailed information will be presented in the next chapter.

3.7 Biocontrol

The biocontrol approach has gained increasing attention over the years, driven by the appeal of providing cleaner and healthier solutions to the consumer, as well as the necessity to be sustainable and to avoid microbial resistance. Among the possibilities are the use of essential oils and plant extracts. Although the potential of these compounds are being known for many years, new technologies are helping to surpass the challenges such their volatile characteristics, sensorial impacts, as well their phytotoxicity (Khadiri et al., 2024). The use of smart packaging that releases the

components slowly, the microencapsulation of the oils that can help not only with the volatility but also to enhance nutritional characteristics, and the use of edible films enriched with diverse compounds have also presented good results with oils as: *Mentha pulegium, Citrus aurantium, Thymus vulgaris, Origanum compactum, Syzygium aromaticum,* and *Cinnamomum zeylanicum* (Haro-González et al., 2021).

The potential use of enzymes is also presented in many studies, their high specificity being the most prominent advantage, with a minimal impact on the product characteristics. Enzymes used for mycotoxin control include oxidoreductases, hydrolases, and esterases, each playing a crucial role in detoxifying fungal metabolites (Lyagin et al., 2025).

Laccases and peroxidases, found in molds, bacteria, and yeasts, degrade mycotoxins through oxidative reactions that break down toxic molecular structures. Hydrolases, such as lactonases, carboxypeptidases, and ochratoxinases, catalyze the hydrolysis of specific bonds, making toxins inactive. Esterases, like fumonisin esterases, act on ester bonds within mycotoxin molecules, leading to their detoxification (Ouyang et al., 2024) .

These enzymes can be applied *via* postharvest coatings, washing treatments, or during apple juice processing, offering an eco-friendly and efficient alternative to chemical fungicides. However, challenges related to enzyme stability, cost, and large-scale implementation must be addressed for their widespread adoption in food safety management (Sun et al., 2023).

The use of microorganisms for patulin degradation has emerged as an effective and environmentally friendly approach to mitigating mycotoxin contamination in apples and apple-based products. Among the microbial candidates, lactic acid bacteria (LAB) such as *Lactobacillus casei YZU01* (currently named *Lacticaseibacillus* casei) have shown great potential in reducing patulin levels in fruit juices. *L. casei YZU01* primarily degrades patulin by secreting extracellular metabolites and adsorbing the toxin onto its cell wall, leading to its neutralization. Studies have demonstrated that this strain can completely degrade 10 μg/mL of patulin in raw apple juice within 36 hours and effectively reduce patulin levels in commercial juices within 48 hours (Zheng et al., 2020, 2022). The ability of LAB to detoxify patulin without affecting juice quality makes them promising agents for industrial applications.

Additionally, some other LAB strains, such as *Lactobacillus plantarum* (currently named *Lactiplantibacillus plantarum*), have been shown to secrete enzymes capable of transforming patulin

into less toxic metabolites. However, further research is needed to fully understand the enzymatic pathways and optimize bacterial formulations for large-scale use (Lai et al., 2022a).

Apart from bacterial strains, some fungi and actinobacteria have also been investigated for their patulin-degrading capabilities. Certain former *Rhodotorula* and *Candida* species (Shruthi et al., 2022; Zhu et al., 2015), while primarily classified as yeasts, have demonstrated significant enzymatic activity in breaking down patulin into non-toxic derivatives. Overall, microbial degradation of patulin presents a viable alternative to chemical detoxification methods, offering a natural and safe approach to reducing mycotoxin contamination in apples and apple-based products.

This study evaluates the potential of various yeasts against PAT and the inhibition of *Aspergillus clavatus*, the mycotoxin-producing mold. The following section will focus on their action mechanisms.

3.8 Mechanism of yeast action against molds and mycotoxins

The use of yeast in food and feed technology is not new to humanity. Basic products like bread and wine rely on these microorganisms as their primary agents of transformation. However, new applications are discovered each year, including their potential as biocontrol agents.

The modes of action of these microorganisms are still being studied, but they primarily function in three keyways. First, they compete for nutrients, as yeasts can rapidly develop in environments with various nutrient sources, preventing pathogenic molds from obtaining the resources needed to establish themselves. Additionally, some strains have the potential to produce antifungal substances, such as volatile organic compounds (VOC) and organic acids, and as they grow, they lower the pH of the environment, making it more difficult for toxigenic molds to develop (He et al., 2024).

The second mechanism involves the production of enzymes with the potential to degrade the patulin already formed. These enzymes can be secreted into the medium or the microorganisms can absorb the patulin and endoenzymes will modify the mycotoxin structure making it less toxic or completely harmless. Figure 3 below shows the principal compounds found after the enzymatic degradation of patulin.

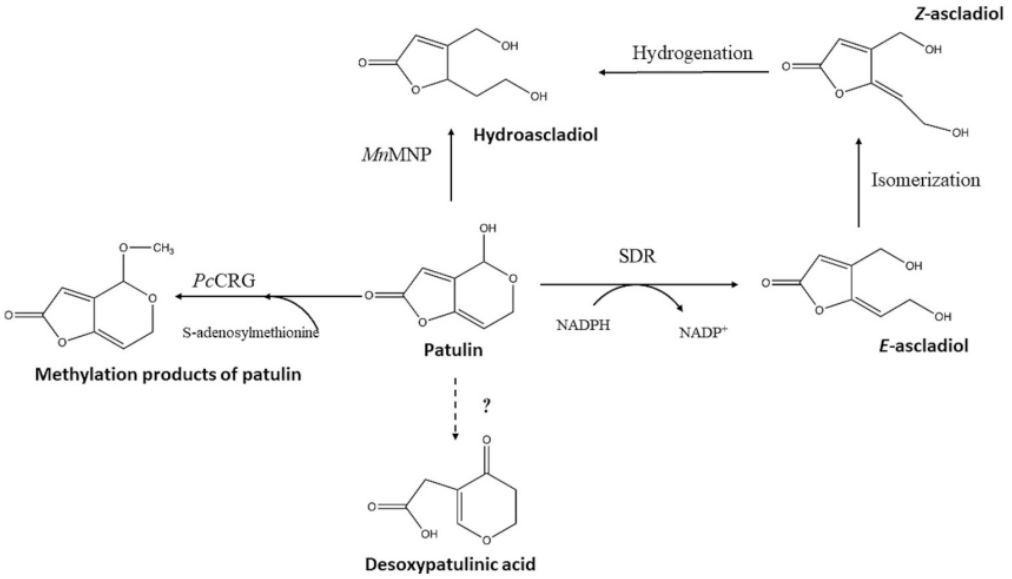


Figure 3 - Main compounds identified after enzymatic degradation of patulin. In which MnP indicates manganese peroxidase, SDR indicates peroxidase short-chain dehydrogenase/reductase, and pcCRG indicates S-adenosylmethionine-dependent methyltransferase (Modified from Pan et al., 2025)

The principal mechanism is the opening of the furan or the pyran ring, and although for the other compounds the enzymes are already clarified as recombinant manganese peroxidase (*Mn*MnP) for the hydroascladiol pathway, short-chain dehydrogenase/reductase (SDR) for ascladiol formation, and S-adenosylmethionine-dependent methyltransferase (pcCRG) for the methylation products of patulin, the enzyme responsible for converting PAT into deoxypatulinic acid is still undiscovered (Pan et al., 2025).

Diverse studies have shown that compounds derived from PAT degradation are less harmful or even do not present any risk, proving that the bio enzymatic degradation has a great potential in patulin detoxification (Castoria et al., 2011; Pinedo et al., 2018; Q. Yang et al., 2021).

The third mechanism is related to the potential of the yeast to bind the toxin onto their cell wall, although this phenomenon has largely been studied for LAB and less information is available for yeasts. Studies using *Saccharomyces cerevisiae* were performed to evaluate the mitigation of PAT through adsorption mechanisms involving the yeast cell wall. Both viable and heat-treated *S. cerevisiae* cells have demonstrated the ability to bind patulin, with no significant difference in efficacy — viable cells achieved a 53.28% reduction, while heat-treated cells achieved 51.71%. This suggests

that the cell wall components are primarily responsible for patulin removal (Guo et al., 2012; Ul Hassan et al., 2021).

The yeast cell wall is composed mainly of polysaccharides and proteins, which play crucial roles in patulin adsorption. Chemical and enzymatic treatments that modify these components, such as protease E, methanol, formaldehyde, periodate, or urea treatments, have been shown to significantly decrease the yeast's ability to bind patulin. Additionally, hydrophobic interactions between patulin and the cell wall contribute to the binding process.

However, the exact molecular interactions and binding sites involved in patulin adsorption remain unclear, necessitating further research to fully elucidate the underlying mechanisms.

3.9 *Wickerhamomyces anomalus* and its applications

Wickerhamomyces anomalus, formerly known as *Pichia anomala*, is among the promising candidates for the use as a biocontrol agent for a variety of molds. This yeast belongs to the ascomycetes and presents oval/ellipsoidal shaped cells. They form creamy white cells in the most traditional culture media for yeasts, such as yeast extract peptone dextrose (YPD). This microorganism is very versatile and is able to ferment a wide range of carbohydrates. It is a facultative anaerobe, surviving even in environments with low pH and high concentrations of salt, sugar, and other components that can be toxic for microorganisms, all these features make *W. anomalus* a promising candidate for industrial processes (Godana et al., 2024).

In the field of biofuels and biocompounds, it demonstrated the ability of converting multiple substrates into ethanol and ethyl acetate with high conversion rates, some strains are also able to produce biosurfactants, detergents, and bioremediation with applicability in the food industry (Qin et al., 2024; Teixeira Souza et al., 2018). In the food industry, this yeast is used in bakery goods, enhancing the flavor due the production of higher alcohols and esters that also act as antimicrobial agents prolonging the shelf-life of the goods.

The *W. anomalus* is also used in production of wines and fermented beverages, usually co-fermenting with *S. cerevisiae*, and it improves the aroma and flavor complexity, reduces alcohol content, and also the presence of its killer toxins helps prevent the spoilage by other microorganisms (Izquierdo Cañas et al., 2014; Lee & Park, 2020)

Besides all the applications mentioned above, this species is gaining even more attention in the context of biocontrol. As previously mentioned, its ability to produce VOCs, biosurfactants, and killer

toxins makes it a promising candidate as a key agent against mold spoilage and mycotoxin production. Its mechanisms against molds are similar as the ones previously discussed in chapter 3.7, *W. anomalus* growth is rapid, outcompeting pathogenic fungi for nutrients, as well it has the potential to produce enzymes that degrade mycotoxins, although the mechanism has less studies for PAT. The VOCs also act as inhabitants for PAT-producing molds such as *Aspergillus* and *Penicillium* spp. This group of yeasts is also capable of forming biofilms, creating a physical barrier and inducing host mechanisms of defense (Hua et al., 2014; Oufensou et al., 2023)

Figure 4 below summarizes the mechanisms of action of *W. anomalus* strains against pathogens and molds. In the context of biocontrol, the pathogen refers to the fungal organism causing disease, while the host is the plant or fruit colonized by *W. anomalus* to enhance resistance against infection.

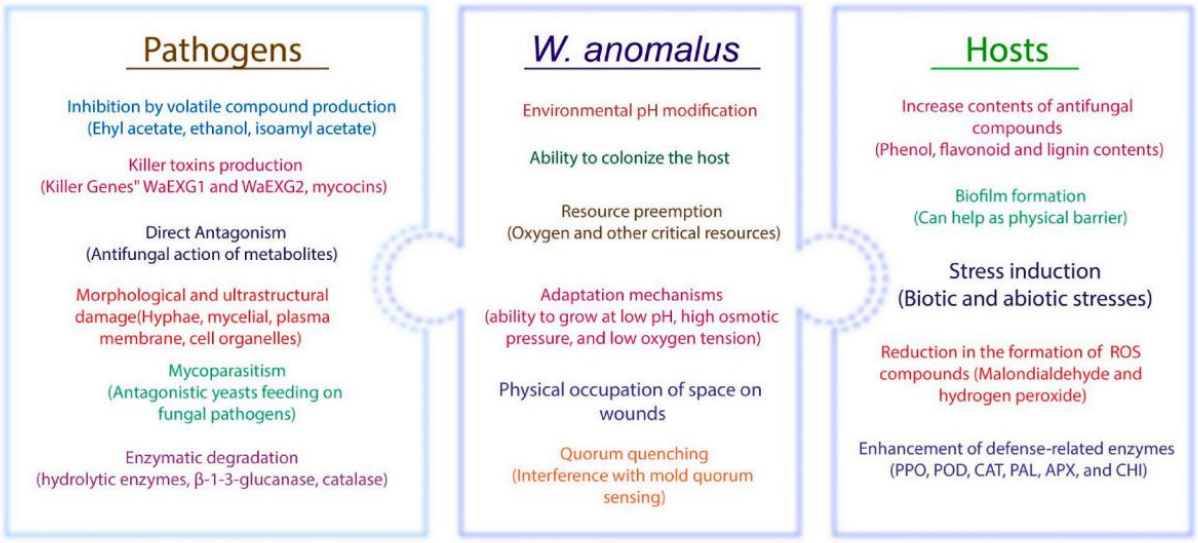


Figure 4 - Mechanisms by which *W. anomalus* controls the growth of pathogenic microorganisms and molds**.** (Obtained from Godana et al., 2024)

In this study, this species provided the best results against *A. clavatus* growth as well as in PAT degradation.

4 MATERIAL AND METHODS

4.1 Isolation and identification of molds

4.1.1 Apples

In September 2022, 40 apple samples were obtained from the FruitVeB Hungarian Vegetable and Fruit Association and Product Council with the cooperation of the Institute of Horticulture of the Faculty of Agricultural and Food Sciences and Environmental Management of the University of Debrecen. One sample consisted of 3 apple fruits in a paper bag, harvested the day before. The apples have arrived from 7 locations: Csenger (47°50’6.11”N, 22°40’56.46”E), Damak (48°19’1.24”N, 20°49’19.63”E), Pallag (47°36'0"N, 21°40’0”E), Lövőpetri (48°10’55.2”N, 22°11’56.4”E), Nagykálló (47°52’59”N, 21°51’0”E), Nyírtass (48°6’50.98”N, 22°1’29.32”E), and Újfehértó (47°47’55.64”N, 21°40’59.88”E), all located in the north-eastern region of Hungary.

Additionally, apples from Újfehértó were sourced from both traditional, long-established orchards and from trees cultivated under protective ice-net systems, which safeguard the fruit from frost damage and adverse weather conditions. Apple cultivars, farming methods and locations are summarized in Table 5.

Table 5 - Apple cultivars, cultivation methods, and locations used in this study.

Intensive	**Integrated**			**Organic**		**Old**	**Ice-net**
Csenger	**Damak**	**Pallag**	**Nyírtass**	**Lövőpetri**	**Nagykálló**	**Újfehértó**	
C.C. Golden Jeromine Pinova	Idared Jonathan M41	Golden Reinders Orion Pinova Red Idared Red Jonaprince Red Topaz Royal Gala	Gala Golden Jeromine Red Jonaprince	Florina Freedom FRW-12 FRW-16 Húsvéti Rozmaring Liberty Melody Red Love Topaz	Freedom Goldrush Pinova Reanda Reno Rewena Topaz	Golden Jonathan Pinova	Golden Red Delicious

4.1.2 Isolation of molds

Culture media for the isolation and cultivation of molds were Potato Dextrose Agar (PDA), Czapek medium, and Malt Extract Agar (MEA) that have been proven to support patulin production (Varga et al., 2003). MEA (Malt Extract Agar, VWR) was chosen for further experiments, as it proved to be favorable in the preliminary experiments for species differentiation and patulin production.

Three apple fruits were selected from all 40 samples, so a total of 120 apples were tested for the study. The apples were individually packed in sterile plastic bags and kept in a refrigerator at 4 °C until processed.

To the whole apples in the sterile Stomacher® bags, 90 mL sterile peptone water (1 g peptone (VWR), 9 g NaCl (VWR), and 1 L distilled water) were added. The bags were shaken vigorously by hand for 2 minutes (Csernus et al., 2015). No previous treatment such as sanitation was performed. After that, a tenfold dilution series was prepared with peptone water. One hundred microliters of the dilutions were spread onto MEA plates, and then the plates were incubated for 7 days at 37 °C.

Normally molds are cultivated at room temperature, but the aim of this study was to investigate the prevalence of aspergilli, so a higher temperature was selected that suits *Aspergillus* spp. but hinders the growth of *Penicillium*, *Fusarium*, etc. species (Ghosal et al., 2020; Gougouli & Koutsoumanis, 2010; Nazari et al., 2018).

Following the completion of the incubation period, fungal colonies that had developed on the surface of the agar plates were carefully examined and selected. The selection process focused on identifying molds that exhibited distinct morphological characteristics, such as variations in texture, color, and growth patterns. Once selected, the colonies were meticulously picked from the agar plates using sterile loop to prevent contamination. To facilitate later calculations and analysis, the colonies were counted and categorized into groups based on their morphological similarities. This grouping helped in organizing the isolates for further study.

After classification, the selected fungal isolates were transferred onto fresh MEA plates to promote further growth. These plates were then incubated under the same controlled conditions as the initial incubation. To ensure the purity of each fungal strain and eliminate any potential cross-contamination, this transfer and incubation process was repeated twice.

From the plates containing pure isolates, small pieces of the mold colonies were cut out with sterile scissors. A piece of colony was placed into an Eppendorf tube containing 0.5 mL of MEB (Malt

Extract Broth, VWR) and 0.5 mL of 43.5% sterile glycerol, then the tube was placed to -20 °C for preservation as a stock collection. Another piece of colony was placed into an Eppendorf tube containing 1.5 mL of MEB broth, and it was incubated at 37 °C for 3 days, centrifuged, and then placed at -20 °C as biomass for further molecular microbiological identification.

4.1.3 Determination of morphological characteristics of mold isolates

Mold isolates placed in the stock collection were inoculated at three points on the surface of MEA plates using a sterile inoculating needle. As the aim of the study was to find *Aspergillus* species able to produce patulin, and according to the preliminary experiments increased temperatures favor this genus, among them possible patulin producer *A. clavatus*, over penicillia (penicillia usually being dominant on apples at environmental temperatures), the plates were incubated at 37 °C for 7 days.

After the incubation period, the morphological features of the colonies, the hyphae, spore-holding formula, and spore characteristics of the isolates, examined under a light microscope (KERN OBN 147 fluorescence microscope) at 400x magnification, made it possible to group the isolates into four clusters: *Aspergillus*, *Alternaria*, *Penicillium*, and other.

4.1.4 Molecular microbiological identification of selected mold isolates

Starting from the mold biomass stored at -20 °C, the MasterPure™ Yeast DNA Purification Kit (Epicentre Biotechnologies) was used to extract DNA according to the manufacturer's instructions. The success of DNA isolation was checked on a 1% agarose gel with the composition of 0.25 g of agarose and 25 mL of TBE buffer (10.8 g of Tris, 5.5 g of boric acid, 0.93 g of Na_2EDTA, 1000 mL of distilled water). To the gel 1.25 µL ECOSafe nucleic acid staining solution (Pacific Image Electronics Co., Taiwan) was added. Pockets were created in the gel using a comb. 2 µL Loading Dye was pipetted onto a parafilm, into which 5 µL sample was mixed, and then the mixture was loaded into the pockets. GeneRuler™ DNA Ladder Mix (Thermo Fisher Scientific Inc., Waltham, MA USA) was run parallel to the samples for 20 minutes at 90 V. The gel image was checked under UV light.

For identification of the mold isolates, extracted DNA was used as templates in ITS PCR. Composition for the ITS reaction was as follows for a final volume of 50 µL: MQ water (Lonza, Belgium): 26,3 µL, dNTP (Thermo Scientific cc. 1 mM): 10 µL, Dream Taq buffer (Thermo Scientific): 5 µL, DMSO (VWR Chemicals): 3 µL, $MgCl_2$ ($x7H_2O$, 0.1M): 1.5 µL, BSA (bovine serum albumin (VWR Chemicals), concentration: 0.1 µg/µL): 0.5 µL, ITS1 (5′ TCCGTAGGTGAACCTGCGG 3′) primer: 0.5 µL, ITS4 (5′ TCCTCCGCTTATTGATATGC 3′) primer: 0.5 µL, Dream Taq polymerase (Thermo Scientific 1U/ µL): 0.2 µL, template: 3 µL containing

at least 35 ng of DNA template. The reaction was done in a PCR Cycler (Mastercycler Nexus Gradient Thermal Cycler, Eppendorf), the heat profile of the reaction was as follows: denaturation: 5 minutes at 95 °C, annealing and polymerization: 30 s at 94 °C -> 30 s at 48 °C -> 40 s at 72 °C (these three steps were repeated 35 times), final extension: 10 minutes at 72 °C, storage at 4 °C.

The sequencing was done in forward direction at the BaseClear B.V., the Netherlands, and the Institute of Aquaculture and Environmental Safety of the Hungarian University of Agriculture and Life Sciences. The isolates were identified by comparing the received sequences with the Blast and the ISHAM Barcoding online databases.

4.2 Restriction enzyme screening for mold identification using RFLP- PCR

4.2.1 Strains

The microorganisms used as reference strains were obtained from the Hungarian National Collection of Agricultural and Industrial Microorganisms (Table 6).

Table 6 - Reference *Aspergillus* strains utilized in this study, with source and corresponding NCBI access numbers.

Strain name	Source	NCBI Code
***Aspergillus ochraceus* F.00850**	NCAIM	PV833264.1
***Aspergillus ochraceus* F.00919**	NCAIM	PV833270.1
***Aspergillus parasiticus* F.00899**	NCAIM	PV833256.1
***Aspergillus parasiticus* F.00960**	NCAIM	PV833254.1
***Aspergillus clavatus* F.00817**	NCAIM	PV833265.1
***Aspergillus clavatus* B9/6**	Isolated from Hungarian apples	PV833267.1
***Aspergillus clavatus* BR1**	Isolated from Hungarian apples	PV833266.1
***Aspergillus flavus* B10/4**	Isolated from Hungarian apples	PV833263.1
***Aspergillus flavus* B14/18**	Isolated from Hungarian apples	PV833259.1
***Aspergillus niger* B5/5**	Isolated from Hungarian apples	PV833268.1

***Aspergillus niger* B10/3**	Isolated from Hungarian apples	PV833269.1
***Aspergillus fumigatus* B1/8**	Isolated from Hungarian apples	PV833257.1
***Aspergillus fumigatus* B6/13**	Isolated from Hungarian apples	PV833261.1

A group of twenty-one fungal isolates from the previous essays at this work was used to evaluate the robustness of the methodology.

4.2.2 Culture conditions and DNA extraction

Fungal strains were cultured in malt extract broth at 25 °C for 48 hours. Mycelia were collected using a sterile platinum loop and transferred to 1.5 mL microtubes. DNA extraction was performed using the Genomic DNA Mini Kit (Plant) from Geneaid® with minor modifications to the manufacturer's protocol, replacing the cell disruption, originally done with the use of nitrogen, for mechanical disruption using a pestle (SP Bel-art®, Sigma-Aldrich, USA) and silica sand (274739, white quartz, 50-70 mesh, Sigma-Aldrich, USA) and homogenizing the mixture for 2 minutes. The purity and integrity of the extracted DNA were assessed by electrophoresis in a 1% agarose gel, run at 90 V for 20 minutes, and visualized under UV light. The DNA concentration of the samples was evaluated using the Nanodrop spectrophotometer (Thermo Fisher Scientific). Samples with a DNA concentration higher than 35 ng/µL and acceptable purity were considered suitable for use in subsequent steps.

4.2.3 PCR Amplification

PCR amplification targeted the β-tubulin (*Bt2a* and *Bt2b*) and internal transcribed spacer (ITS) regions. The reaction mixture (50 µL) contained 35 ng of DNA template, 5 µL of 10X DreamTaq Green Buffer (Thermo Fisher Scientific, EUA), 0.2 mM each of forward and reverse primers (Integrated DNA Technologies), 0.1 µmol of dNTP (Thermo Scientific), and 0.5 U of Taq DNA polymerase (Thermo Fisher Scientific). The final volume was adjusted to 50 µL with nuclease-free water.

PCR conditions followed the method of (Nasri et al., 2015), with an initial denaturation at 94 °C for 5 minutes, followed by 35 cycles of 94 °C for 30 seconds, 60 °C for 45 seconds, and 72 °C for 1 minute, concluding with a final extension at 72 °C for 5 minutes. For ITS amplification the

protocol is described at 4.1.4. The quality of the PCR reaction was evaluated as mentioned in chapter 4.1.4. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Germany) according to the manufacturer's instructions. The primer sequences can be found in Table 7.

Table 7- Primer sequences used in the study

	Sequence
Forward β-tubulin (Bt2a)	5'-GGT AAC CAA ATC GGT GCT GCT TTC-3'
Reverse β-tubulin (Bt2b)	5'-ACC CTC AGT GTA GTG ACC CTT GGC-3'
Forward ITS1	5'-TCC GTA GGT GAA CCT GCG G-3'
Reverse ITS4	5'-TCC TCC GCT TAT TGA TAT GC-3'

Amplicon sizes obtained for each *Aspergillus* species targeted in this study are summarized in the Table 8 below, alongside their expected lengths based on NCBI references.

Table 8- Comparison of expected and observed amplicon sizes for *Aspergillus* species based on *β*-tubulin gene amplification

	β-tubulin gene size (bp)		
Aspergillus species	**NCBI**	**Real (1)**	**Real (2)**
A. ochraceus	529	544	542
A. parasiticus	372	499	503
A. clavatus	456	522	520
A. flavus	595	496	500
A. niger	413	505	512
A. fumigatus	552	506	503

4.2.4 *In silico* restriction enzyme analysis

To determine appropriate restriction enzymes, β-tubulin gene sequences of target *Aspergillus* species were obtained from National Center for Biotechnology Information (NCBI). Virtual restriction digestion was performed using A Plasmid Editor (APE software) to predict polymorphic sites. Based on literature and *in silico* results, XhoI, KpnI, AlwI, HpaII, and AluI enzymes (Thermo Fisher Scientific and New England Biolabs) were selected for experimental validation.

4.2.5 Restriction fragment length polymorphism (RFLP) analysis

PCR products were digested using the selected restriction enzymes in a reaction mixture containing: 200 ng of PCR product; 1 U of restriction enzyme (Thermo Fisher Scientific and New England Biolabs); 2.5 µL of 10x enzyme-specific buffer (Thermo Fisher Scientific and New England Biolabs), and nuclease-free water to achieve a final volume of 25 µL (Lonza, Belgium).

Digestion was conducted at 37 °C for 2 hours for enzymes AlwI and HpaII and for 15 minutes for FastDigest AluI, XhoI, and KpnI. The digested fragments were separated on a 1.5-4% agarose gel, electrophoresed at 90 V for 20-25 minutes, and visualized under UV light.

4.2.6 Data analysis and species identification

All strains listed in the first step were sequenced for ITS and β-tubulin genes to establish reference profiles. These strains served as patterns for evaluating fungal isolates previously identified from Hungarian apples. Fragment sizes obtained after digestion were compared with expected *in silico* digestion patterns and reference RFLP profiles. The RFLP results of the Hungarian apple isolates were then assessed against the established standards to confirm their species identity.

4.3 Occurrence of patulin-related genes and mycotoxin production

4.3.1 Evaluation of the occurrence of the patulin gene (*idh*) in isolated *Aspergillus* and *Penicillium* strains

At least 10 genes are known to be involved in patulin biosynthesis (Gaucher, G.M. and Fedeshko, 1997), however, the nucleotide sequences of only two *P. griseofulvum* patulin biosynthesis genes, 6-methylsalicylic acid synthase (*6msas*) (Genebank accession number X55776), and the *idh* gene (Genebank accession number AF006680) are available in the GenBank. The *idh* gene is the seventh enzyme of the patulin biosynthesis pathway and can be used as a marker for potential patulin-producing microorganisms.

Previous studies attested that all tested microorganisms that were negative for this gene were negative for patulin, and 66% of the microorganisms that tested positive were also positive for patulin production (Paterson et al., 2003). The primers used to amplify 600 base pairs of the *idh* gene (GIBCO BRL, Paisley, Scotland) had the following sequences: 5' - CAA TGT GTC GTA CTG TGC CC-3' and 5' -ACC TTC AGT CGC TGT TCC TC-3'. Varga et al. (2003) detected the *idh* gene in various *Aspergillus* species such as *A. clavatus, A. pallidus, A. clavatonanica*, and *A. longivesica* using the primers described by Paterson et al. (2003).

To evaluate the presence of the *idh* gene, the PCR reaction aimed at amplifying the *idh* gene was performed according to the protocol described by Paterson et al. (2003).

4.3.2 Confirmation of mycotoxin production by thin layer chromatography

The mold isolates were cultured in malt extract broth (20 mL) with shaking at 35 °C for 7 days. After the incubation period, PAT was extracted as described in chapter 4.6. Using a Hamilton pipette, 10 µL of the sample was slowly pipetted onto silica gel (TLC Silica Gel 60, 25 aluminum sheets 20x20, Merck). The running buffer was toluene: ethyl acetate: formic acid in a ratio of 6:3:1. For reference, 50 µL of patulin standard was applied.

The silica gel was placed vertically in the running buffer and ran. The gel was dried under the fume hood. To identify patulin, slides were sprayed with 0.5% aqueous 3-methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate (MBTH; Aldrich, St. Louis, MO, USA) and heated at 130 °C for 15 min. Patulin appears as a yellow-orange, fluorescent spot under UV light (366 nm) (Northolt et al., 1979).

4.4 Influence of environmental factors on patulin production

4.4.1 Effects of physicochemical parameters, temperature and pH, on the patulin production by *A. clavatus* B9/6

The effects of physicochemical factors, temperature and pH, on the patulin production of *Aspergillus clavatus* B9/6 were evaluated in apple juice 100%. The juice was obtained from a local supermarket, 20 mL was transferred to Erlenmeyer flasks and subsequently the pH was adjusted with NaOH and HCl accordingly to the desired values of 2.5, 4, and 5.5. The flasks then were inoculated with 1 mL of a spore suspension with 10^7 spores/mL of *A. clavatus* B9/6.

The flasks were incubated under two different temperatures of 25 °C and 35 °C for 7 days with no agitation, since a preliminary assay showed there was no significant difference among patulin production with or without agitation. After incubation, the whole volume was centrifuged and filtered

through a 0.22 µm filter to guarantee the removal of any cell. The samples were stored at -4 °C for the patulin extraction and quantification using HPLC as described in chapters 4.6 and 4.7.

4.5 Yeast-based biocontrol of *A. clavatus* B9/6

4.5.1 Yeast screening for potential growth inhibition of *A. clavatus* B9/6

Spores of *Aspergillus clavatus* B9/6 were inoculated from an initial suspension of 10^7 spores/mL into malt extract agar (MEA) cooled to 45 ºC to achieve a final concentration of 10^3 spores/mL The medium was distributed into sterile Petri dishes and allowed to solidify. Using a loop, a streak was made at the center of each plate with a considerable amount of yeast biomass, previously grown on PDA plates for 24 h. The plates were then incubated at 25 °C and evaluated after 48 hours and 7 days.

The strains screened are listed in Table 9. Three groups of yeasts were analyzed: commercial ones were obtained from local suppliers, mainly belonging to *S. cerevisiae* and *S. bayanus* species, and the groups named *Saccharomyces* and *Wickerhamomyces* were obtained from the National Collection of Agricultural and Industrial Microorganisms (NCAIM). Although the names *C. robusta* and *S. willians* were maintained to facilitate the workflow, these 2 strains were reevaluated and now are classified under the group of *S. bayanus***.**

Table 9 - List of yeasts screened for potential antagonistic effect against *A. clavatus* B9/6

Commercial strains	***Saccharomyces* strains**	***Wickerhamomyces* strains**
HD A 54®	*S. cerevisiae* 0220	*W. anomalus* 00927
HD 562®	*S. cerevisiae* 0223	*W. anomalus* 01109
HD S135®	*S. cerevisiae* 00225	*W. anomalus* 00548
GV S107®	*S. cerevisiae* 00164	*W. anomalus* 00213
Uvaferm®	*S. cerevisiae* 00206	*W. anomalus* 00545
Lalizyme®	*S. cerevisiae* 00224	*W. anomalus* 00717
Uvaferm Danstil A®	*S. cerevisiae* 00171	*W. anomalus* 00935
Uvaferm 228®	*S. cerevisiae* 00205	*W. anomalus* 00367
Jazz®	*S. cerevisiae* 00204	*W. anomalus* 00459
Safcider AS-2/ AB-1/ TF- 6®	*S. cerevisiae* 00677	*W. anomalus* 01655
Craft series MO2®	*S. cerevisiae* 00151	*W. anomalus* 00170
Safcider®	*S. cerevisiae* 00210	*W. anomalus* 00961
Safbrew LA-01®	*S. cerevisiae* 00208	*W. anomalus* 01499
Spiriferm®	*S. cerevisiae* 00164	
Oenoferm Xtreme/X-thiol®	*S. cerevisiae* 0219	
CM®	*C. robusta/ S. cerevisiae 00134*	
Flavia®	*S. willianus*	
	S. bayanus 01201	

A second experiment was conducted to evaluate the potential of *W. anomalus* to inhibit the spread of the mold, a central streak of *A. clavatus* B9/6 suspension containing 10^7 spores/mL was done using a sterile inoculation loop into a PDA plate. This fungal streak extended from one edge of the plate to the other. Subsequently, the yeast strains under evaluation at a minimum concentration of 1×10^8 CFU/mL were inoculated in parallel streaks on either side of the fungal streak, maintaining a uniform distance from the central streak. The plates were then incubated at 25 °C and evaluated after 48 hours and 7 days.

4.5.2 Patulin degradation by *Wickerhamomyces anomalus* strains

The four best-performing *Wickerhamomyces anomalus* strains, 01499, 01655, 0961, and 0170, were selected for the patulin degradation experiment. 1 mL with a concentration of 10^8 CFU/mL of each strain was inoculated into 20 mL of apple juice spiked with 10 ppm of patulin. The inoculated

flasks were incubated at 25 °C under constant agitation (150 rpm) for 24 hours to ensure homogeneous conditions for yeast growth and patulin interaction.

Following incubation, patulin was extracted from the samples using the method described in chapter 4.6. The extracted samples were then analyzed using HPLC as specified in chapter 4.7 to quantify the remaining patulin. The results were compared to the initial patulin levels to assess the degradation efficiency of each *W. anomalus* strain.

4.5.3 Kinects of patulin degradation by *W. anomalus* 01499 and 1655

To evaluate the kinetics of patulin degradation, *Wickerhamomyces anomalus* strains 01499 and 01655 were cultivated in apple juice under controlled conditions. 1 mL at a concentration of 1×10^8 CFU/mL of each strain was inoculated into 20 mL of apple juice spiked with 10 ppm of patulin. The cultures were incubated at 25 °C with constant agitation for 48 hours.

Patulin amounts at a total of seven time points were collected throughout the 48-hour period, including the initial time point (T0) to assess patulin degradation over time. At each time point, an aliquot of 2 mL of the culture was taken, and patulin was extracted using the methodology described in chapter 4.6. The remaining patulin concentration in each sample was quantified *via* HPLC according to chapter 4.7.

4.5.4 Co-cultivation of *W. anomalus* 01499 and 1655 and *A. clavatus* B9/6

To evaluate the interaction between *Wickerhamomyces anomalus* and *Aspergillus clavatus* B9/6, separate flasks were prepared for *W. anomalus* strains 01499 and 01655. Each flask containing 20 mL of apple juice was inoculated with 1 mL of *W. anomalus* suspension of an initial concentration of 1×10^7 CFU/mL, and 1 mL of a spore suspension of *A. clavatus* B9/6 were added to the same flasks of a concentration of 1×10^7 spores/mL A negative control containing only *A. clavatus* B9/6 was also included to assess patulin production in the absence of yeast.

The flasks were incubated under continuous agitation at 150 rpm for 15 days at 25 °C. At the end of the incubation period, an aliquot was taken from each flask, and patulin was extracted using the method described in chapter 4.6. The extracted samples were then analyzed by HPLC to quantify the patulin concentration following the method described in chapter 4.7.

4.5.5 Evaluation of *A. clavatus* B9/6 inhibition by *W. anomalus* 01499 *in vivo*

To evaluate the inhibition of *A. clavatus* B9/6 growth by *W. anomalus* 01499, an *in vivo* assay was conducted using apples as a substrate. The apples were first surface sterilized by immersion first

in a sodium hypochlorite 2.5% solution followed by 70% ethanol. After drying, 0.3 mL of inoculum was injected into each apple using a 30×7 mm sterile syringe. The fungal suspension contained *A. clavatus* B9/6 at a concentration of 10^7 CFU/mL, while the yeast suspension contained *W. anomalus* at 10^7 CFU/mL

A negative control was included, in which only a saline solution of 0.9 % was applied, along with a positive control, where only *A. clavatus* B9/6 was inoculated. Additionally, three experimental conditions were tested: 1. where the mold was inoculated 24 hours before the yeast, 2. where the yeast was inoculated 24 hours before *A. clavatus*, and 3. in which both microorganisms were inoculated simultaneously. The apples were then stored under ambient conditions for 20 days. After this period, they were cut in half and qualitatively evaluated for fungal growth through direct observation.

4.5.6 Assessment of patulin degradation by intracellular/extracellular enzymes and patulin binding of *W. anomalus* 01499

In this essay, only the strain *W. anomalus* 01499 was used. This yeast was cultured in apple juice at 25 °C with constant agitation (150 rpm) until reaching the desired concentration of $1x10^8$ CFU/mL

Following incubation, the culture was centrifuged at 4,000 rpm for 40 minutes to separate the yeast cells from the supernatant. The supernatant was collected, filtered through a 0.22 µm filter, and stored in a sterile Falcon tube at 4 °C for subsequent enzymatic activity evaluation. The pelleted yeast cells were washed twice with sterile saline solution of 0.9% and divided into two portions for further analysis.

For extracellular enzyme evaluation, 10 mL of the collected supernatant was incubated with patulin at a final concentration of 10 ppm and incubated at 25 °C for 24 h. After the incubation period, patulin was extracted according to chapter 4.6 and quantified using HPLC according to the method in chapter 4.7.

A part of the yeast cells pellet was subjected to mechanical disruption using a FRENCH® Press (Thermo Electron Corporation) at 850 psi. The lysate was centrifuged at 3,000 rpm for 5 minutes to remove debris, and 5 mL of the intracellular extract was incubated with patulin at a final concentration of 10 ppm. The mixture was incubated at 25 °C for 24 h. Samples were collected and

the remaining patulin content was extracted as described in chapter 4.6 for posterior evaluation with HPLC as described in chapter 4.7.

The other portion of the yeast cells pellet was used to evaluate patulin adsorption on the cell wall. The protocol was adapted from a previous study (Bata-Vidács et al., 2020) performed with lactic acid bacteria. The culture medium and time were adapted to yeasts. The cell concentrations were adjusted to 10^8 CFU/mL and a volume of 10 mL was used. PAT was added to obtain a final concentration of 10 ppm.

The mixture was incubated for 10 minutes at room temperature, followed by centrifugation at 4,000 rpm for 40 min, then the supernatant was discarded, and 2 mL of dichloromethane was added. The mixture was vortexed for 20 minutes in the dark. The mixture was centrifuged one more time for 10 minutes at 3,000 rpm. One milliliter of the sample was evaporated and then resuspended in 10% acetonitrile.

4.6 Patulin extraction

After centrifuging the fermented samples (containing yeasts and/or molds) at 4000 rpm for 10 minutes four milliliters of supernatant were transferred into a 15 mL Falcon tube, 2 mL of dichloromethane was added, the tube was shaken vigorously and then left to stand in the dark for 20 minutes. One milliliter of the lower phase was pipetted into an Eppendorf tube and centrifuged at 14,000 rpm for 1 minute, then 0.5 mL of the supernatant was pipetted into a clean Eppendorf tube. The dichloromethane was evaporated at 40 °C in a thermoshaker (BIOSAN TS-100) under a fume hood. The dried material was dissolved in 1 ml of 10% acetonitrile.

4.7 High-performance liquid chromatography (HPLC) method

The quantification of patulin was performed using High-Performance Liquid Chromatography (HPLC) under isothermal and isocratic conditions. A Kinetex 2.6 µm XB-C18 100A column (150 × 4.6 mm) was used. The mobile phase consisted of acetonitrile and water in a 10:90 (v/v) ratio, with a constant flow rate of 0.3 mL/min. Detection was carried out using a UV detector set at a wavelength of 276 nm.

4.8 Statistical evaluation

All experiments were conducted in triplicates. Statistical analyses were performed using STATISTICA software, with significance set at $p < 0.05$. Differences between groups were assessed using one-way ANOVA followed by t-test where applicable.

5 RESULTS AND DISCUSSION

5.1 Mold isolation and identification

Five different farming methods were applied in the cultivation of apples. For apples intended for consumption in the fresh market, two primary cultivation approaches were employed: intensive (conventional) farming and organic (bio) farming. Intensive farming refers to modern, highly maintained apple plantations, where plant protection measures are carried out with precision to ensure optimal fruit quality. This method is primarily used for cultivating widely known apple cultivars such as Gala, Golden Delicious, Red Delicious, Jonagold, Idared, and Pinova.

In contrast, organic apple production is far less common, with only a limited number of plantations dedicated to this method. While organic cultivation also includes some of the popular cultivars mentioned above, it places a stronger emphasis on scab-resistant cultivars. These include Freedom, Florina, Prima, Pinova, various cultivars from the Re-series, CrimsonCrisp (C.C), Bonita, and others, which are better suited to organic farming practices due to their natural resistance to common apple diseases.

For industrial apple production, plantations typically follow an integrated plant protection approach. In these plantations, plant protection measures are applied more sparingly compared to intensive farming, primarily because the lower market price of industrial apples incentivizes producers to minimize costs. Additionally, the processing industry has less stringent quality requirements for industrial apples than those imposed by the fresh market, making a more restrained approach to plant protection both practical and economical.

Altogether 183 mold strains were isolated from 40 apple samples of 28 apple cultivars from 7 locations and 5 cultivation methods. The isolates were clustered according to morphological features (Figure 5) to groups *Aspergillus*, *Alternaria*, *Penicillium*, and Other.

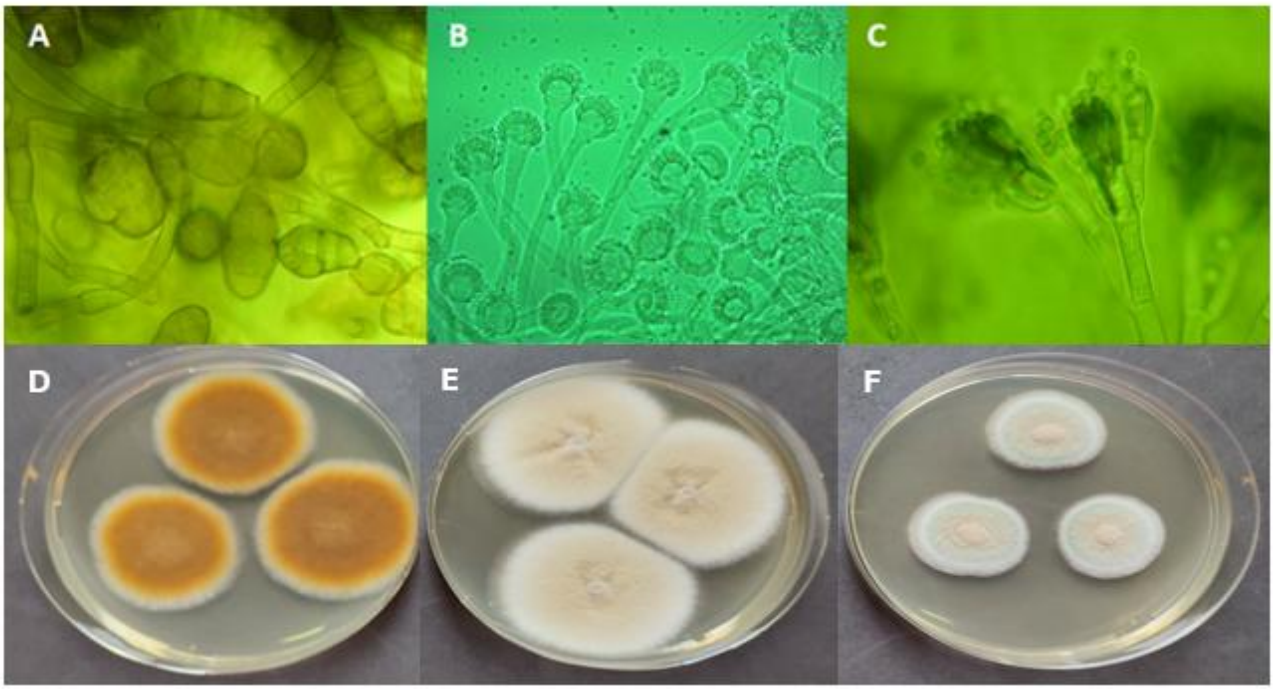


Figure 5 - Morphological features of mold isolates for clustering: (A) *Alternaria,* (B) *Aspergillus,* (C) *Penicillium* (microscopic view)*;* (D) *Alternaria,* (E) *Aspergillus,* (F) *Penicillium* (colony morphology on PDA)

According to the clustering, 67 isolates were in the *Alternaria* group, 45 were in the *Aspergillus* group, 13 in the *Penicillium* group, and the rest were other kinds of mold.

Aspergillus, Alternaria, and *Penicillium* are filamentous fungi with distinct morphological characteristics. Under the microscope, *Aspergillus* features a round, vesicle-like structure at the tip of its conidiophore, from which conidia are produced in chains. *Alternaria* presents long, branching hyphae with conidia that appear in a beaded or stacked formation, often with both transverse and longitudinal septa. *Penicillium*, in contrast, has conidiophores that form brush-like arrangements, giving it a characteristic appearance similar to a paintbrush. Their colony colors also vary, with *Aspergillus* typically green or yellow, *Alternaria* dark brown to black, and *Penicillium* often blue/green with a velvety or powdery texture (Amalaradjou & Venkitanarayanan, 2008).

Although this study focuses on patulin, which is primarily produced by *Penicillium* species, both *Alternaria* and *Aspergillus* also have the ability to produce mycotoxins. *Alternaria* is known to produce alternariol (AOH) and tenuazonic acid (TeA), which have been associated with cytotoxic and

genotoxic effects, posing potential health risks. *Penicillium*, on the other hand, beside patulin, produces a variety of mycotoxins, including ochratoxin A (OTA) and citrinin, both of which can be harmful to human and animal health, with OTA being particularly concerning due to its nephrotoxic and carcinogenic properties (Bacha et al., 2023; Navale et al., 2021).

Table 10 presents the number of mold isolates for each apple growing region, grouped by the farming method, as well as the sum of colonies and their distribution.

Table 10 - Mold distribution regarding type of cultivation, location and apple cultivar

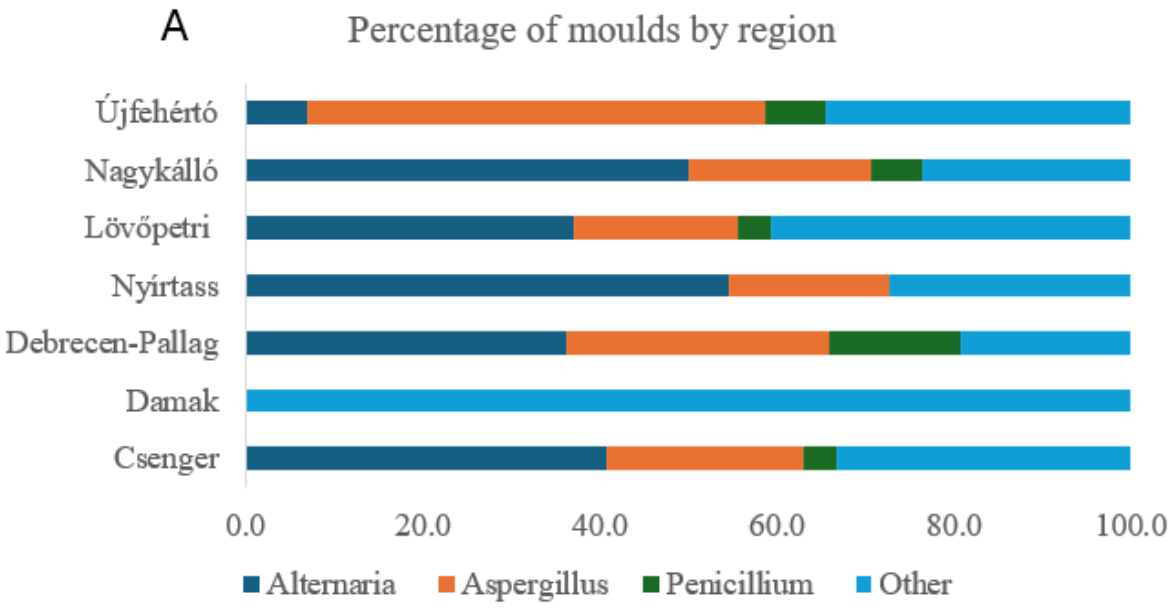
Type of cultivation	Location	Apple cultivar	*Aspergillus*	*Alternaria*	*Penicillium*	Other	Total number of isolates
Intensive	Csenger	C.C Jeromine (I. class) Jeromine scabbed Golden scabbed Pinova	22.2% (n=6)	40.7% (n=11)	3.7% (n=1)	33.3 (n=9)	27
Integrated	Damak	Idared Jonathan M41				100% (n=8)	8
	Debrecen-Pallag	Golden Reinders Pinova Orion Red idared Red Idared Red Topaz Royal Gala	29.8% (n=14)	36.2% (n=17)	14.9% (n=7)	19.1% (n=9)	47
	Nyírtass	Gala Schnitzer-Schiniga Golden Jeromine Red Jonaprince	18.2% (n=2)	54.5% (n=6)		27.3% (n=3)	11
Bio	Lövőpetri	Freedom FRW-12 FRW-13 FRW-14 FRW-15 FRW-16	18.5 (n=5)	37.0% (n=10)	3.7% (n=1)	40.7% (n=11)	27

		FRW-17 FRW-18 FRW-19 FRW-20 Húsvéti Rozmaring Liberty Melody Red love					
	Nagykálló	Freedom Goldrush Pinova Reanda Reno Rewena Topáz	20.6% (n=7)	50.0% (n=17)	5.9% (n=2)	23.5% (n=8)	34
Old cultivar	Újfehértó	Golden Jonathan Pinova	47.4% (n=9)		10.5% (n=2)	42.1% (n=8)	19
Ice net	Újfehértó	Golden Red Delicious	20.0% (n=2)	60.0% (n=6)		20.0% (n=2)	10
Sum			45	67	13	58	183

The highest number of isolates was obtained for Debrecen-Pallag with integrated farming method, in absolute terms (n=47) and when colonies were evaluated per apple (n=6.7). The second highest was obtained for Újfehértó with 6.3 per apple. Interestingly this last group was the only one in which *Alternaria* was not among the dominant specie.

The lowest number of colonies per apple was found in apples from Lövőpetri. Despite evaluating 14 different apple cultivars, only 27 fungal colonies were isolated in total, averaging approximately two colonies per apple. This result is unexpected, as these apples are grown using a bio-based approach that prohibits the use of conventional chemical fungicides.

A possible explanation for the low fungal presence is the cultivation of more resistant apple cultivars, which may naturally inhibit mold growth. Additionally, factors such as favorable environmental conditions during the growing season, lower initial fungal contamination, proper orchard management practices, or the presence of beneficial microbial communities could have contributed to this reduced fungal load.



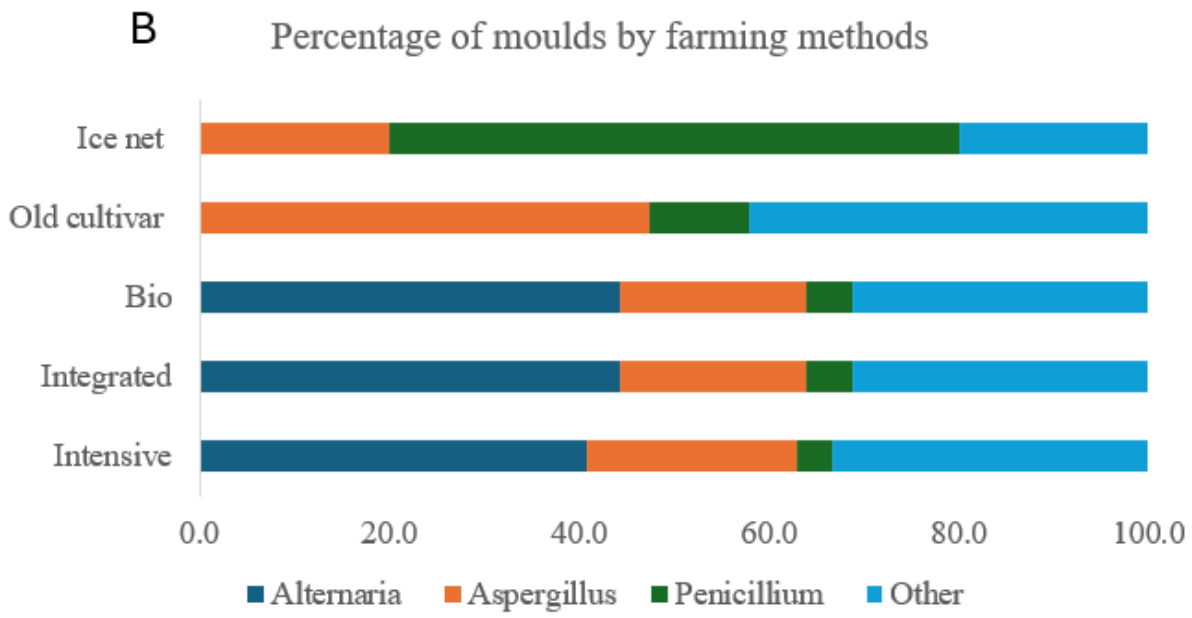


Figure 6- Percentage of contamination by *Aspergillus, Alternaria, Penicillium,* and other molds for A - location and B - farming method

In Figure 6, the percentage occurrences of the four mold clusters are shown for each location (Fig. 6A) and farming method (Fig. 6B). Due to the higher temperature used for mold isolation, the number of samples containing *Penicillium* was low, with only 10 different apples presenting it. This result was expected as the temperature used partially inhibited the development of this genus (Gougouli & Koutsoumanis, 2010). These results also draw attention to *Penicillium* spp. that are able to grow at elevated temperatures.

Location possesses a higher influence on the distribution of mold genera than farming method, as "ice-net" and "old cultivars" results showed a different trend compared to results of other farming methods, both located in the Újfehértó region. Despite the use of higher temperature, *Aspergillus* species dominated only for the region of Újfehértó with approximately 50% of the isolates belonging to the genus.

Four of the seven locations assessed: Csenger, Debrecen-Pallag, Nyírtass, and Nagykálló, were dominated by *Alternaria* species. This result agrees with the findings of other studies, where *Alternaria* was also the predominant genus when isolating potential mycotoxin producers from apples in Ontario, Canada (Soliman et al., 2015).

No significant trends were observed for apples of the same cultivar. It is necessary to highlight that the composition of microbiota is multifactorial, and not exclusively dependent on the environmental factors but also anthropogenic effects, as the use of pesticides or other agrichemicals, can implement changes to the biodiversity in the short and long term.

No difference in mold counts between bio and non-bio farming methods was observed, the sum of colonies was slightly higher for the bio apples, the most common molds belonged to the cluster named Other followed by *Alternaria* and *Aspergillus* (Figure 7).

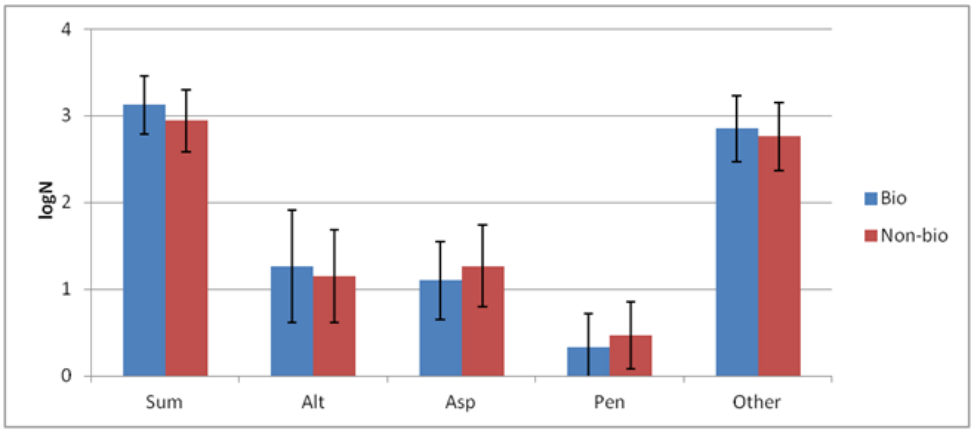


Figure 7 - Comparison of the mold loads of bio and non-bio apples regarding the colony numbers (sum), *Alternaria* spp. (Alt), *Aspergillus* spp. (Asp), *Penicillium* spp. (Pen), and other mold species of apple

Weather data for the apple cultivation locations were obtained from the online database **metnet.hu** and included parameters such as annual mean temperature (°C), average minimum and maximum temperatures (°C), highest daily temperature fluctuation (°C), total annual precipitation (mm), number of days with precipitation, days of heat, and days of frost. Since all locations are situated in the northeastern region of Hungary, significant differences in temperature parameters were not expected. However, annual precipitation varied among locations: Csenger, Damak, Pallag, and Újfehértó received approximately 500 mm, while Lövőpetri and Nyírtass had lower levels, ranging

between 200-300 mm. Precipitation data for Nagykálló were unavailable. A summary of the weather conditions is presented in Figure 8.

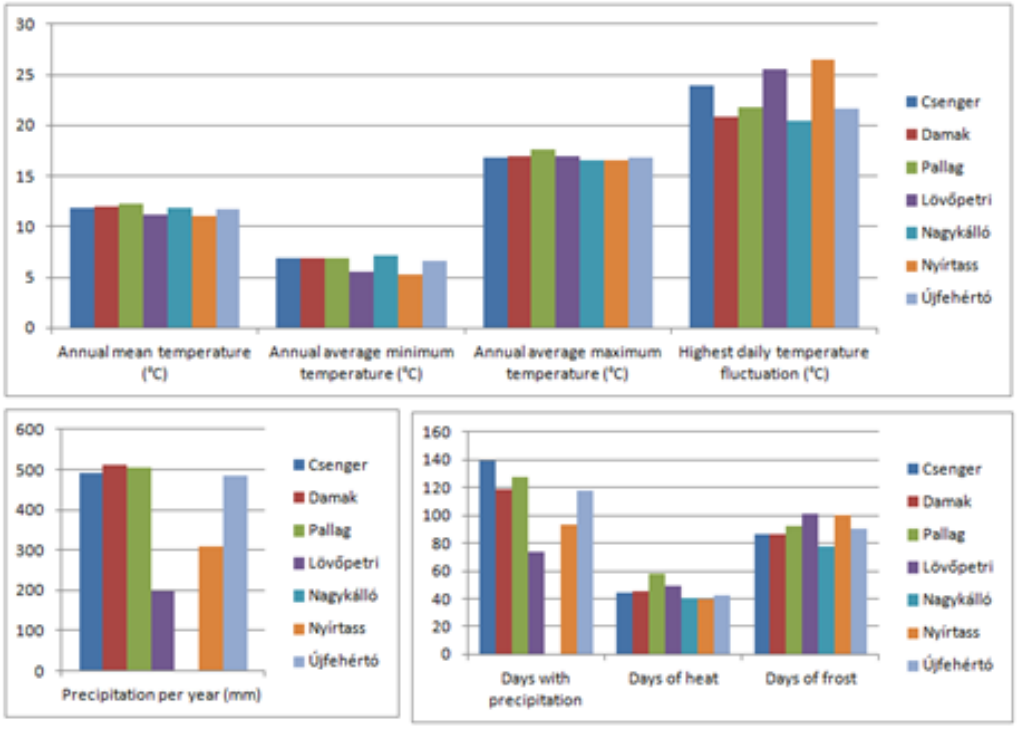


Figure 8 - Weather parameters of the apple cultivation locations in 2022

To investigate whether the annual precipitation differences had any effect on the mold contamination, total mold, *Alternaria*, *Aspergillus*, *Penicillium*, and other mold (not belonging to the ones listed) species contamination of the apples were compared from locations with higher and lower annual precipitation (Figure 9).

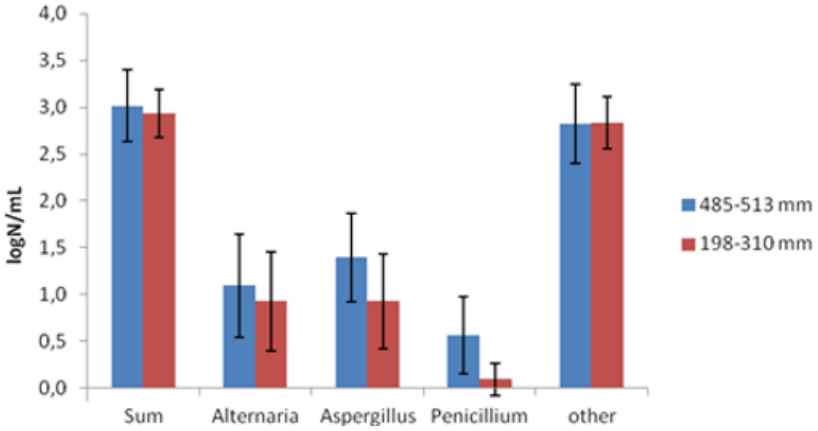


Figure 9 - The effect of annual precipitation on the mold contamination of apples (n = 60 at higher precipitation and n= 39 at lower precipitation)

Though the number of molds was lower from locations of lower precipitation, significant difference was only found for *Penicillium* numbers. Studies performed in China evaluating bacterial and fungal community differences between years (2016 and 2017) and different seasonal precipitation showed that even though the changes in total counts of microorganisms were not significant during more humid weather, the prevalence of *Alternaria* spp. increased (X. Yang et al., 2021).

5.2 Occurrence of patulin-related genes and mycotoxin production in isolated *Aspergillus* and *Penicillium* strains

5.2.1 Detection of the patulin gene in *Aspergillus* and *Penicillium* strains

The *idh* gene can be used as a marker to possible patulin producer microorganisms (Paterson et al., 2000), however, Varga *et al.* (2003) showed that even if a mold strain has the complete sequence of the genes that code patulin production, the mycotoxin might not be produced due to some post-transcriptional processes. The main techniques to evaluate the presence of patulin and to quantify its amount are thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC).

All isolates belonging to the genus *Aspergillus* were tested for the presence of the *idh* gene. The only strain that presented a positive result (Figure 10) was strain B9/6, originated from the apple cultivar Golden Reinders grown in Debrecen-Pallag by integrated cultivation.

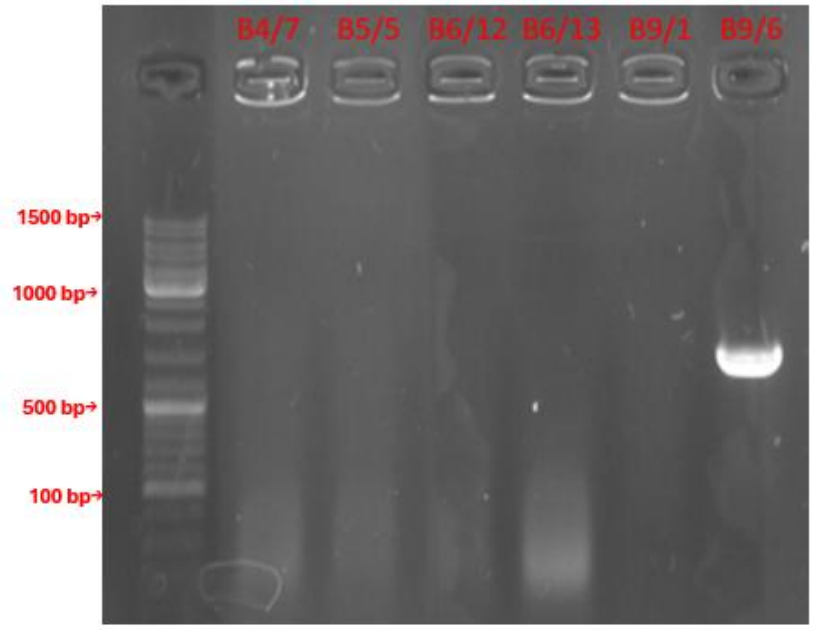


Figure 10 - Presence and absence of the *idh* gene in *Aspergillus* isolates B4/7, B5/5, B6/12, B6/13, and B9/6

The presence of the gene is proved by the bright band located between 600 and 500 bp. As the presence of the gene does not guarantee patulin production, TLC assays were conducted to observe the patulin production of this strain.

Figure 11 shows the morphology of the *Aspergillus clavatus* B9/6 strain on malt extract agar medium and its microscopic image.

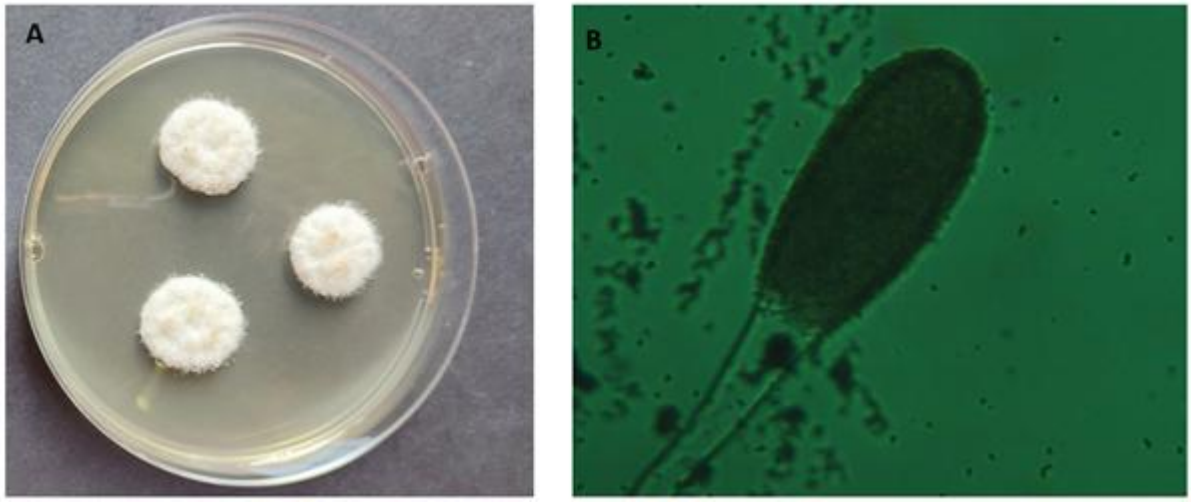


Figure 11 - *Aspergillus clavatus* B9/6 colony morphology on malt extract agar (A) and its microscopic image 100x (B)

Based on morphological characteristics and ITS sequencing, this strain was identified as *Aspergillus clavatus.* This species is characterized by large-sized, elongated, club-shaped vesicles as it is shown in Figure 11. This result is in accordance with the literature, as from the different species of the genus *Aspergillus*, *A. clavatus*, *A. longivesica*, and *A. giganteus* are among the ones that produce patulin (Bacha et al., 2023; Varga et al., 2007).

The isolated molds belonging to the genus *Penicillium* were also tested for the presence of the *idh* gene. The results are shown in Figure 12.

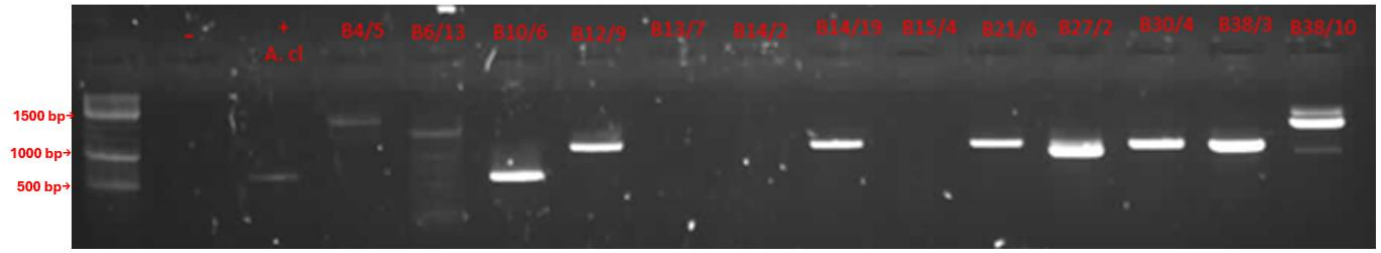


Figure 12 - Presence and absence of the *idh* gene in *Penicillium* isolates. From left to right: ladder, no template control, positive control (A. clavatus B9/6), B4/5, B6/13, B10/6, B12/9, B13/7, B14/2, B14/19, B15/4, B21/6, B27/2, B30/4, B38/3, and B38/10

Unlike in case of *Aspergillus* spp., of the *Penicillium* isolates more than one strain presented bands in the gel, however, only for B10/6 had the band the expected size of the *idh* gene (500-600

bp). Other works have already reported longer sequences of this gene, these codes can be found under NCBI GenBank: AF006680.1 and DQ084388.1 (Gaucher, G.M. and Fedeshko, 1997; White, S.I. and Dobson, 2005).

Although isolate B10/6 was morphologically characterized as part of the *Penicillium* cluster, the ITS sequencing results showed that this strain should be classified as *Talaromyces pinophilus.* The high similarity between these two genera is well documented, and till recent years *Talaromyces* spp. were classified as a sexual state from the genera *Penicillium* (Yilmaz et al., 2014), recurrently these species are cited as penicillium-like.

Figure 13 shows the morphology of the *Talaromyces pinophilus* B10/6 strain on Malt Extract Agar plate and its microscopic image.

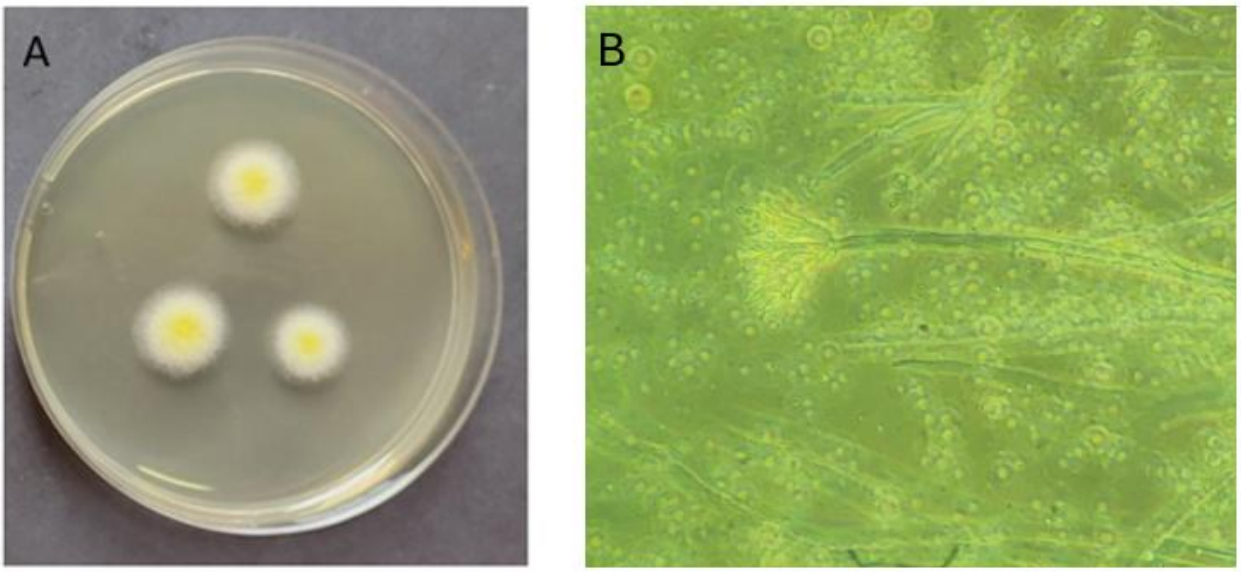


Figure 13 - *Talaromyces pinophilus* B10/6 colony morphology on malt extract agar (A) and its microscopic image 400x (B)

5.2.2 Confirmation of patulin production by thin-layer chromatography

As mentioned previously, the positive results for the *idh* gene do not guarantee mycotoxin production, therefore, more assays were needed to evaluate the production of PAT. Confirming the PCR results of the *idh* gene, the only strain that tested positive for patulin production among the *Aspergillus* isolates (yellow mark) was *A. clavatus* B9/6 (Figure 14), demonstrating that under the conditions of the evaluation this strain was able to produce patulin.

It is even possible to notice that the sample presented a brighter spot than the control, indicating a possible PAT content above 50 ppm.

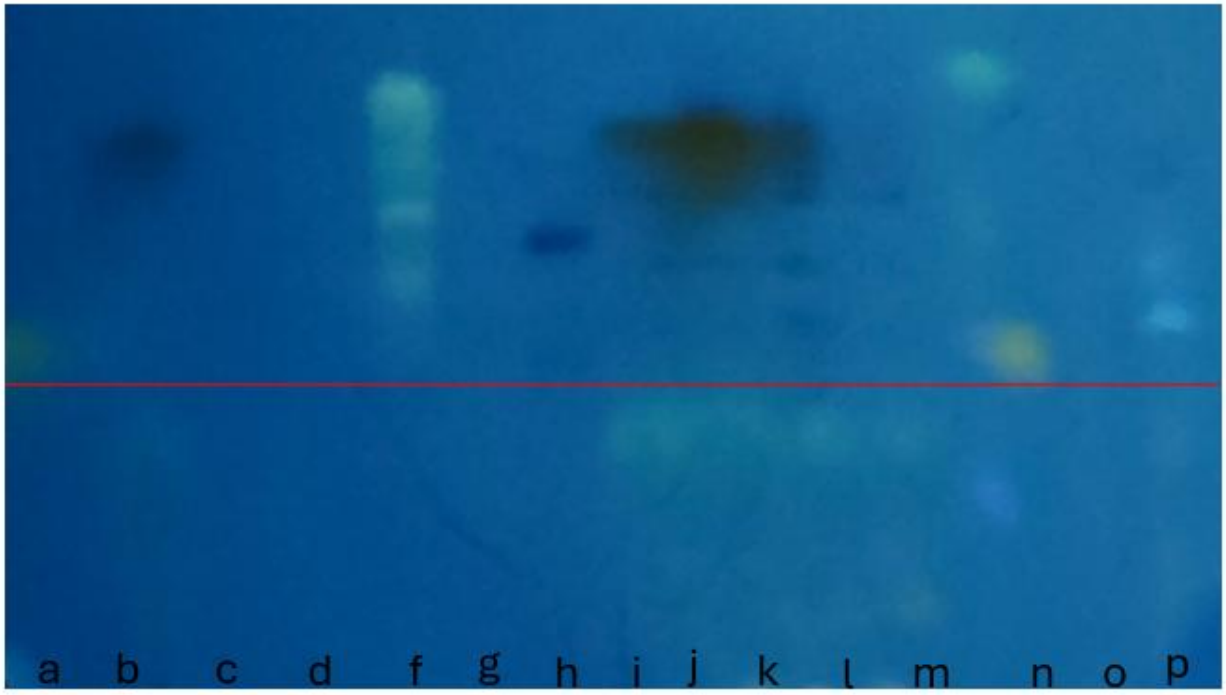


Figure 14- Thin layer chromatography for patulin detection – a - patulin standard 50 ppm; b - patulin standard 5 ppm; c -B1/2; d - B1/8; e - B4/7; f - B5/6; g - B6/7; h - B6/12; i - B6/12a; j -B6/13; k - B7/5; l - B7/6; m - B9/1; n - B9/6; o - B10/4; p -B10/5; the red line indicates the band height for patulin

For the *Penicillium*-like group, according to the TLC tests, B10/6 was the only strain producing patulin under the studied conditions as shown in Figure 15, confirming the results obtained in the *idh* PCR assay.

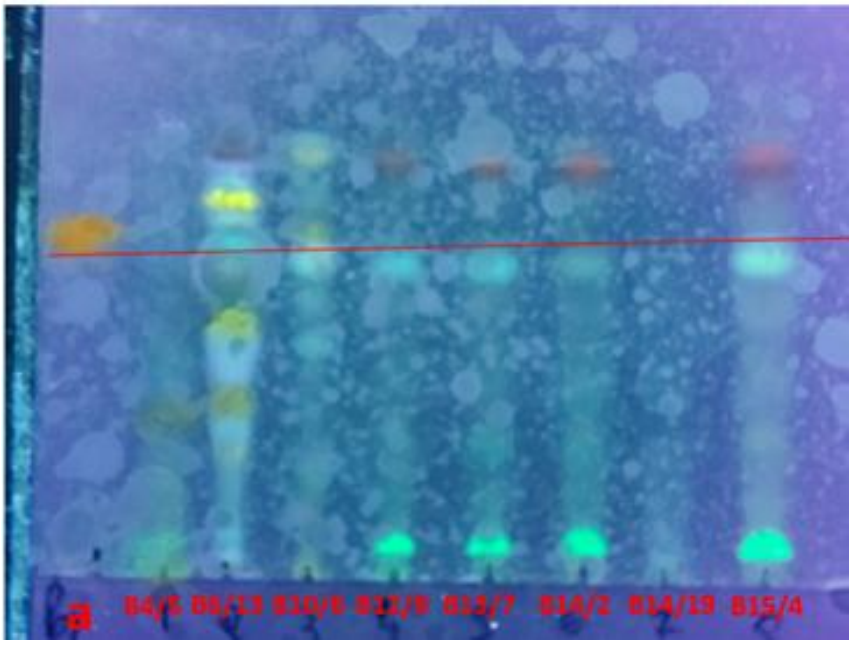


Figure 15 - Thin layer chromatography for patulin detection. a - patulin standard 50 ppm, isolates B4/5, B6/13, B10/6, B12/9, B13/7, B14/2, B14/19, B15/4. The red line indicates the band height for patulin.

5.2.3 Molecular microbiological identification of selected mold isolates

Although morphological identification can be used for genus differentiation, molecular identification is still needed for accurate species determination. According to colony morphology characteristics and microscopic images of the sporangiophores of the isolated molds, groups were formed. From each group, representatives were selected for molecular identification. According to the results, the 45 *Aspergillus* isolates belonged to *Aspergillus fumigatus* (28), *Aspergillus flavus* (15), *Aspergillus nomius* (1), and *Aspergillus clavatus* (1).

The presence of *Aspergillus nomius* drew significant attention, as this is, to our knowledge, the first reported case of *A. nomius* in Hungary, isolated from a crop. This species is known for its ability to produce aflatoxins, highly toxic secondary metabolites that can contaminate food and feed, posing serious health risks. *A. nomius* is typically found in warm, humid environments and is commonly associated with soil, decaying vegetation, and various agricultural products, particularly oilseeds and nuts (Kurtzman et al., 1987).

The morphology of this strain is presented in Figure 16, in different culture media cultivated for 7 days at 25 °C.

Aspergillus nomius B39/1

Malt Extract Agar (MEA) Potato Dextrose Agar (PDA) Czapek Agar (CZA)

Figure 16 - Colony morphology of *Aspergillus nomius* B39/1 on different culture media

To confirm the aflatoxin production of *A. nomius* B39/1, a TLC test was performed using standard mixture of aflatoxin B1 and G1. The result is presented in Figure 17.

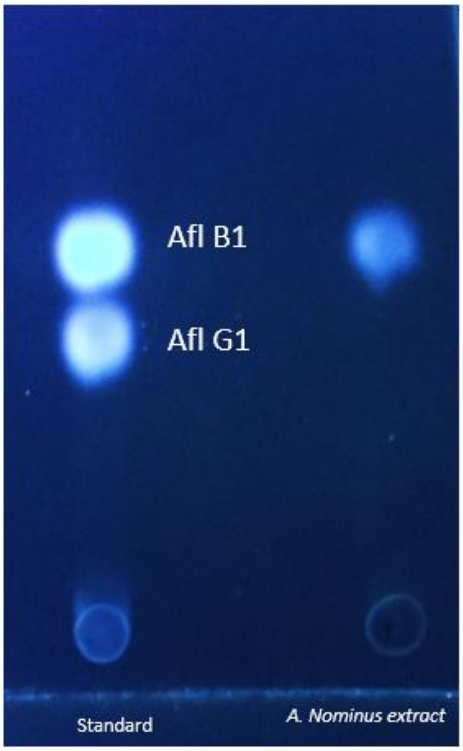


Figure 17 – Thin layer chromatography for aflatoxin production of *A. nomius* B39/1

The *A. nomius* B39/1 strain isolated from apple grown in Újfehértó, cultivated with ice-net was able to produce aflatoxin B1 but did not produce aflatoxin G1.

5.3 PCR-RFLP method development for rapid identification of *Aspergillus* species

5.3.1 Restriction enzyme screening for mold identification (RFLP-PCR)

During the development of this study, the need for a faster method to identify *Aspergillus* strains at the species level has arisen, and the advancement of molecular identification techniques presents a promising solution (Hinrikson et al., 2005).

PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) is a molecular technique that combines PCR amplification and restriction enzyme digestion to identify genetic variation within DNA samples. In this method, specific regions of DNA are first amplified through PCR to increase the quantity of targeted genetic material. These fragments are then treated with restriction enzymes that cut the DNA at species-specific sites, generating unique patterns of DNA fragments.

Each *Aspergillus* species might exhibit distinctive RFLP patterns, allowing for reliable identification based on genetic differences. In the literature, this method was mainly developed and optimized for species with medical interest (Abastabar et al., 2022; Nasri et al., 2015), but the method can also be a valuable tool in agricultural and food safety contexts, as it enables rapid and accurate detection of contaminating *Aspergillus* species in crops and food products.

To evaluate and define the restriction enzymes that would be further applied for actual digestion, *β*-tubulin gene sequence of the strains of interest were obtained from NCBI and applied to APE software, the predicted size of the fragments for the enzymes studied are listed in Table 11.

Table 11 - *In silico* fragment profile of enzyme digestion for *Aspergillus* spp.

β-tubulin gene size (bp)		Fragment profile after enzyme digestion (bp)				
		XhoI	KpnI	AlwI	HpaII	AluI
A. ochraceus	529	529	453,76	295,184,50	254,152,54,45,24	283,207,39
A. parasiticus	372	372	218,154	187,185	208,144,14,6	302,70
A. clavatus	456	278,130,48	417,39	242,214	415,41	251,180,25
A. flavus	595	459,136	595	216,204,104,41,30	192,116,95,83,74,16,15,4	595
A. fumigatus	552	434,118	443,109	478,74	465,51,36	185,148,78,72,44,25
A. niger	413	368,45	377,36	261,152	250,125,24,14	209,183,21

Two strains from the same species were used for the digestions assays, to ensure they were correctly identified, sequencing of the *β*-tubulin gene and ITS were performed and compared to the NCBI database. The digestion profiles can be observed in Figures 18 to 22.

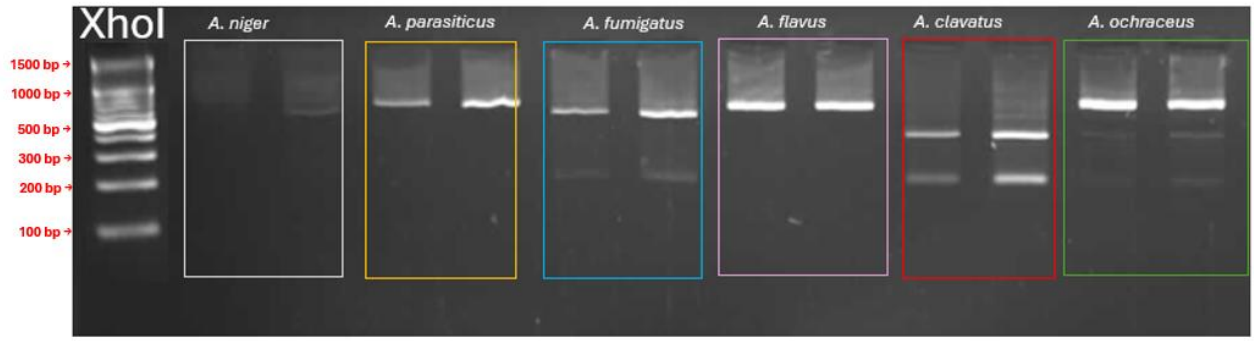


Figure 18 - Fragment profile of *β*-tubulin gene from *Aspergillus* spp. digested with XhoI enzyme

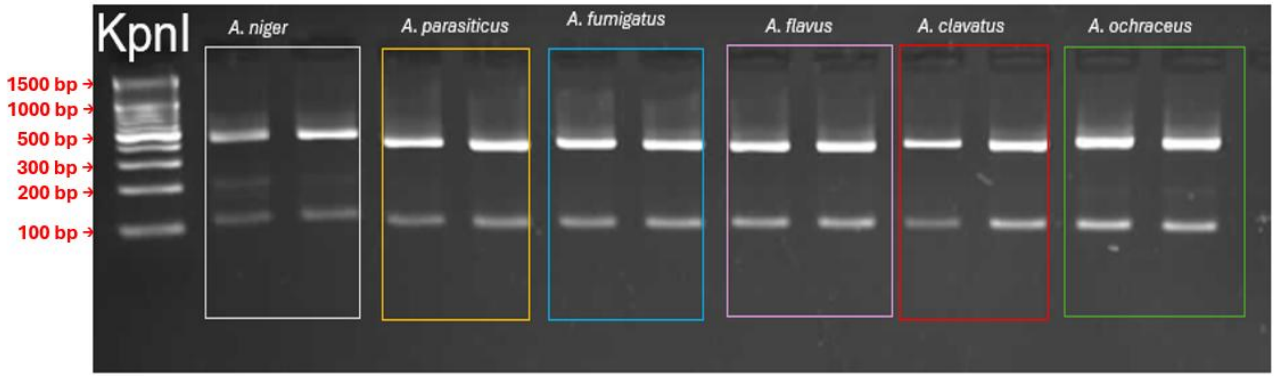


Figure 19 -Fragment profile of *β*-tubulin gene from *Aspergillus* spp. digested with KpnI enzyme

For the KpnI enzyme, no difference among the samples was observed. Notably, both *A. flavus* isolates exhibited two bands instead of a single one, as expected from the *in-silico* tests. It is important to highlight that the *β*-tubulin gene can vary to different extent among species, which could explain the two-band profile. Regarding XhoI, *A. fumigatus*, *A. clavatus*, and *A. ochraceus* displayed distinct digestion patterns, whereas *A. niger*, *A. parasiticus*, and *A. flavus* shared the same banding profile. Consequently, these enzymes were not suitable candidates for differentiating *Aspergillus* species.

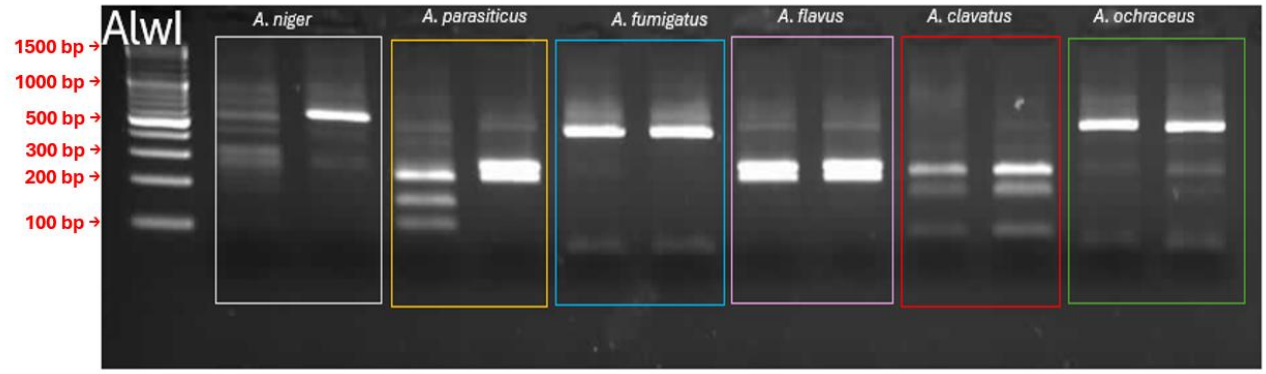


Figure 20 - Fragment profile of *β*-tubulin gene from *Aspergillus* spp. digested with AlwI enzyme

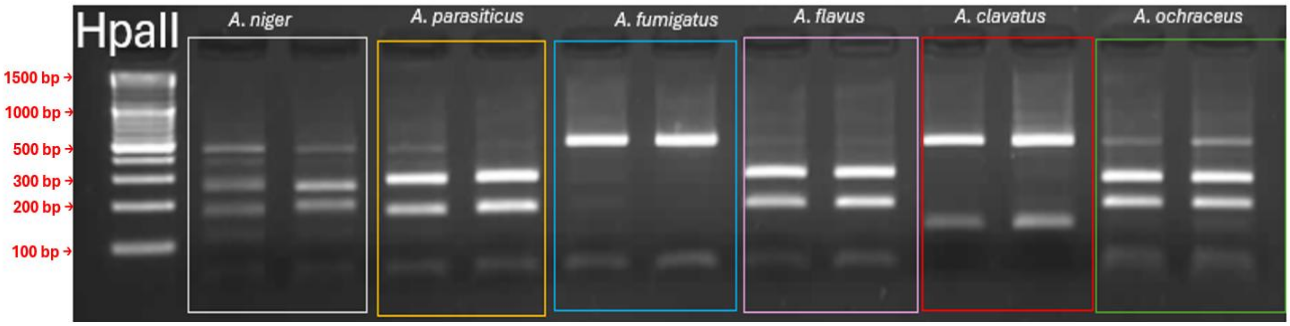


Figure 21 - Fragment profile of *β*-tubulin gene from *Aspergillus* spp. digested with HpaII enzyme

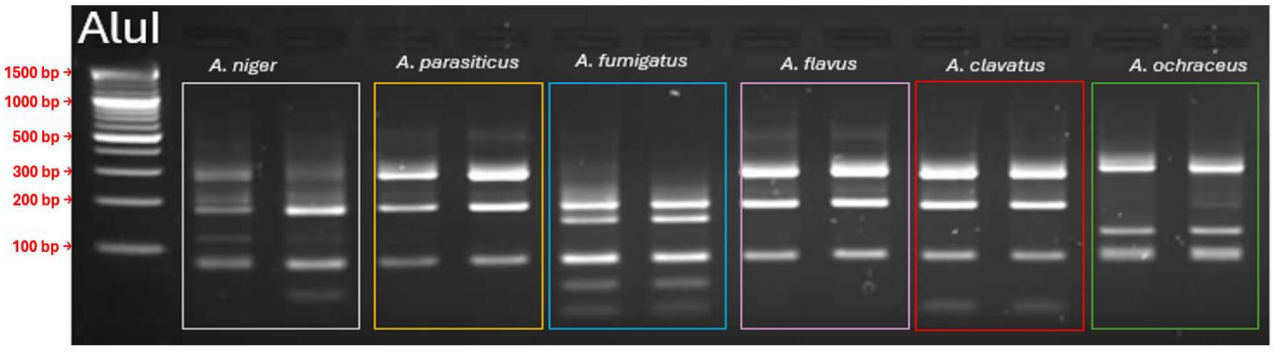


Figure 22 - Fragment profile of *β*-tubulin gene from *Aspergillus* spp. digested with AluI enzyme

The restriction digestion patterns obtained using AluI, AlwI, and HpaII enzymes revealed distinct profiles among the analyzed *Aspergillus* species. Contrary to expectations, AlwI digestion

produced fewer bands, with most species displaying a single prominent fragment, thereby limiting its effectiveness for species-level discrimination. Furthermore, even within the same species group, *A. parasiticus* exhibited inconsistent profiles between strains, indicating a lack of reproducibility.

In contrast to previously published findings, the obtained results suggest that AlwI may have limited utility for *Aspergillus* differentiation. While earlier studies reported that this enzyme effectively discriminates among species, these digestion patterns showed a lower degree of variation than anticipated (Nasri et al., 2015).

AluI digestion produced well-defined restriction profile, with *A. fumigatus, A. clavatus, A. niger,* and *A. ochraceus* displaying unique profiles. In contrast, *A. parasiticus* and *A. flavus* exhibited similar patterns, indicating limited differentiation potential for these species. However, optimizing electrophoresis conditions, such as adjusting agarose concentration and running time, could enhance band resolution and improve species discrimination, making AluI a more effective tool for *Aspergillus* differentiation.

HpaII digestion resulted in more distinct profiles, particularly differentiating *A. fumigatus*, *A. parasiticus*, and *A. clavatus*, indicating its potential utility for species identification as showed previously for AluI. *A. parasiticus* and *A. flavus* presented a similar pattern, therefore, for the following experiments the running time was increased to 5 minutes and the gel percentage was increased from 1.5% to 4% allowing a better differentiation among the species.

5.3.2 RFLP-PCR analysis of mold isolates from apples

After the initial screening, the two enzymes with the best potential, AluI and HpaII, were applied to a larger group of samples, previously isolated from Hungarian apples and morphologically classified, to assess their applicability to environmental samples from agricultural sources. The results are presented in Figures 23 and 24.

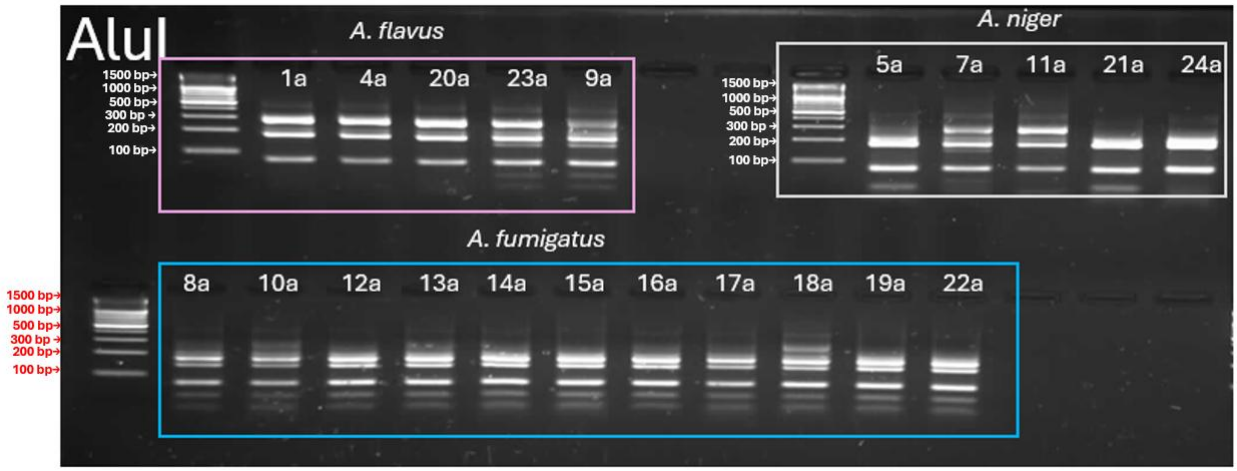


Figure 23 - Fragment profile of *β*-tubulin gene digested with AluI enzyme for enviromental samples

The samples were grouped based on their morphological characteristics. For *A. fumigatus*, all samples presented the same profile when digested with AluI. When compared to the previously established references (Figure 22), the same profile can be observed, but with different distances between the bands. This discrepancy can be explained by changes in electrophoresis conditions, as mentioned earlier. To confirm that the entire group belonged to the corresponding species, the ITS gene of sample 22a was sequenced, confirming the efficacy of the clustering for this species.

A similar observation can be made for the *A. flavus* cluster. However, in samples 23a and 9a, in addition to the three strong bands seen in all samples, two faint bands appear near the lower edge of the gel. These bands may be due to the high variability of this species or limitations of the method used. Even so, the method proves to be effective, since the bands in characteristic positions, just like in the standard electrophoresis gel, are observed in all samples.

For *A. niger*, as expected with AluI, some extra bands appeared in samples 7a and 11a. In this cluster, along with these two strains, sample 5a was also sequenced for the ITS region. All strains showed high similarity not only to *A. niger* but also to *A. tubingensis*, both belonging to the *Nigri* section. Studies have reported the challenge of distinguishing these two species using RFLP due to their highly similar molecular characteristics (Kizis et al., 2014). However, an important advancement is that AluI proves effective in differentiating *Aspergillus* species from the *Nigri* section from other agriculturally relevant *Aspergillus* spp. This highlights the enzyme's potential as a valuable candidate tool for identifying a broader range of fungal species.

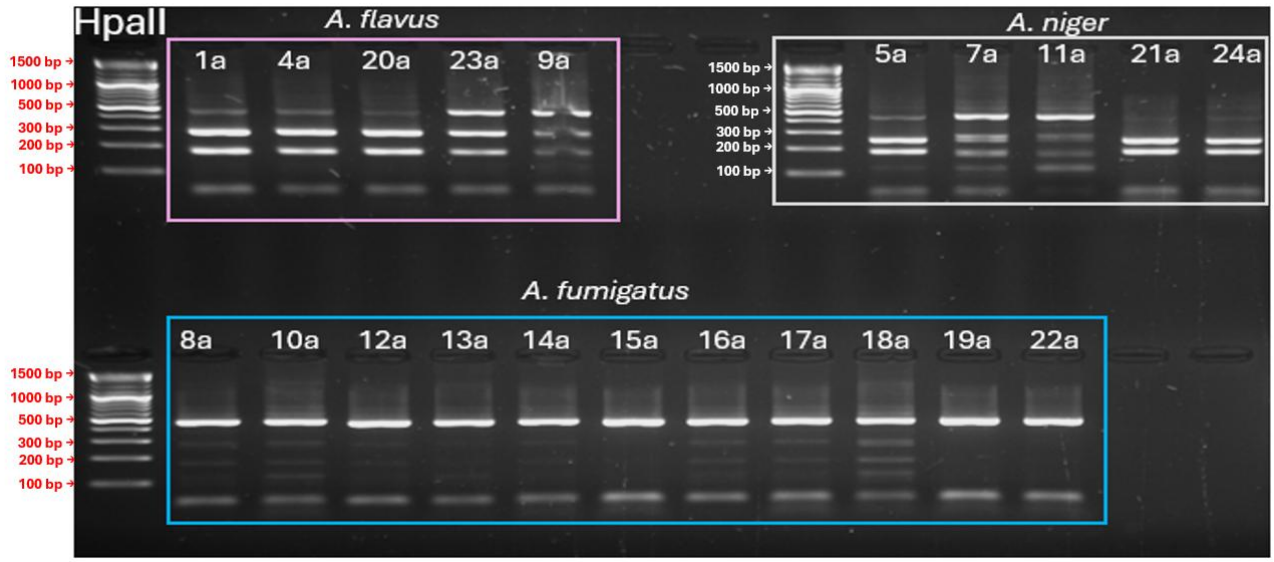


Figure 24- Fragment profile of *β*-tubulin gene digested with *HpaII* enzyme for environmental samples

In *A. flavus*, differently from the digestion with AluI, all samples show nearly identical patterns when digested with HpaII, with 23a and 9a having a brighter first band (around 500bp), but this could be the result of method limitations such as partial digestion or slightly different initial concentration of the PCR product.

For *A. niger* samples, 7a and 11a have similar banding, but for this enzyme, sample 5a has a similar profile, with the first band weaker, this is in accordance with previous results, which suggest a possible incomplete enzymatic digestion or the presence of intraspecific polymorphisms that may favor distinct cleavage patterns in each analyzed strain.

A. fumigatus samples are the most uniform, showing a brighter band at 500 bp and the second brightest lower than 100 bp. These findings reinforce the complexity of enzymatic digestion patterns and highlight the importance of considering intraspecific variability when selecting restriction enzymes for species differentiation.

5.4 Effect of pH and temperature on patulin production by *A. clavatus* B9/6

Figure 25 below shows the growth of *A. clavatus* B9/6 under different pH and temperature conditions after 7 days of incubation in apple juice.

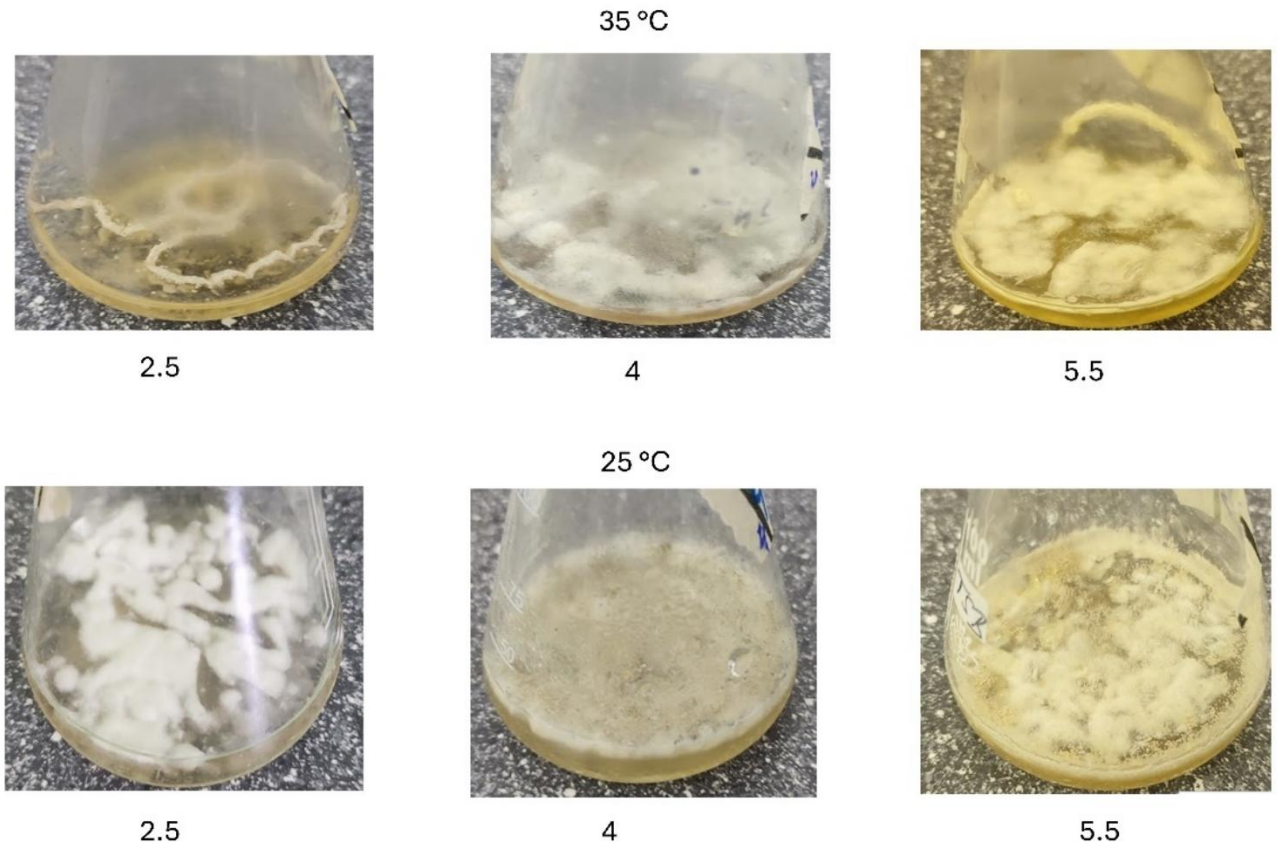


Figure 25 – Morphological characteristics of *A. clavatus* grown in apple juice under different pH and temperature conditions

It is possible to observe that for both temperatures studied, at pH of 2.5 the morphology was different from those at pH 4 and 5.5. For the assay at 35 °C, almost no growth could be observed compared to the other samples, and for the assay at 25 °C, although the growth was considerable, no sporulation was observed at pH 2.5. A high amount of spores was observed in the experiments at pH 4 and 5.5 at 25 °C. This corroborates with the PAT results presented below.

The quantification of PAT for the assays with different pH and temperature values are shown in Table 12.

Table 12 - Patulin production (ppm) by *A. clavatus* at different pH and temperature values

Patulin production (ppm)		
	Temperature	
pH	**25 °C**	**35 °C**
2.5	<LOQ[a]	<LOQ[a]
4	2754 ± 462[b]	3.6 ±1[a]
5.5	6061 ± 756[c]	<LOQ[a]

(Superscripts a, b and c, represent statistically significant differences based on Tukey's test ($p < 0.05$)).

As expected, samples at 25 °C produced higher concentrations of patulin, this temperature is heavily reported as the optimal temperature of toxin production not only for *Aspergillus* but for other molds as well (Hassane et al., 2017; Northolt et al., 1979). The growth at 35 °C reduced patulin production to below the limits established by the majority of the regulatory agencies for apple juice (50 µg/L).

The decrease in patulin production at 35 °C compared to 25 °C was significant. One possible reason is that PAT production is related to secondary metabolism (Snini et al., 2014). Therefore, when the conditions are not optimal, the microorganism may prioritize resources for primary pathways essential for survival. The only presence of PAT at that temperature was observed at pH 4, where 11.8 µg/L was observed, highlighting the optimal conditions, since this pH is considered optimal for fungal development.

For the assays conducted at the pH of 2.5, the growth was heavily affected, and therefore, the production of patulin was below the limits of quantification at both temperatures. Additionally, such a low pH can negatively affect enzymes activity and modify metabolic pathways.

The production of PAT in the assays conducted at 25 °C under pH conditions of 4 and 5.5 was remarkably high, reaching 2754 ppm at pH 4 and an even more impressive 6061 ppm at pH 5.5. The significant difference in PAT concentrations between the two pH levels highlights the influence of pH on the metabolic activity of this strain.

Although a study performed with *Penicillium* strains achieved values up to 13,200 µg/kg of PAT when apricots were inoculated with spore solutions and incubated at 25 °C, the level of contamination

was strain sensitive, and the values for the same experiment ranged between 1,400 to 13,200 µg/kg (Reddy et al., 2010).

Another study demonstrated that the genes involved in PAT synthesis showed their highest expression at pH 5, corroborating with the findings in this experiment, as the largest amount of PAT was found at that pH. The sporulation and mycelium growth were also weaker at pH 2.5 than when the pH was set to 5 (Jimdjio et al., 2021).

These findings highlight the importance of proper storage conditions and preservation techniques for ensuring the safety of food and food products. This is particularly relevant considering that the average pH of apple juice falls between 3.5 and 4.5, and these products are often stored under environmental conditions — typically around 25 °C. Such conditions create an ideal environment not only for mold growth but also for toxin production. The results demonstrate that both low and elevated temperatures can play a role in reducing PAT contamination. Additionally, acidification emerges as an effective strategy to mitigate the formation of this toxin, reinforcing the need for careful control of storage parameters in food safety management.

5.5 Yeast-based biocontrol of *A. clavatus* B9/6

5.5.1 Screening of yeast strains for antifungal activity

Yeast-based solutions are broadly studied in many fields. The genus *Saccharomyces* is used in many branches of the food industry, such as for bakery products and fermented beverages, and is recognized as Generally Recognized as Safe (GRAS). The potential of *Saccharomyces* species in PAT detoxification has been studied, and promising results were obtained by (M. Li et al., 2018) when using strain *S. cerevisiae* CITCC 93161. The endoenzymes were able to degrade 100% of PAT at a concentration of 100 mg/L. Another study, evaluating the potential enzymes that can be responsible for PAT degradation, showed the potential of YKL069W enzyme of *S. cerevisiae* CICC 31084 in binding and degrading patulin into E-ascladiol in 48 h from an initial concentration of 10 µg/mL (Chao Yang et al., 2024).

In this work, twenty different commercial yeast strains were tested for their potential to inhibit *Aspergillus clavatus* B9/6. These products included cultures intended for the production of beer, wine, and cider. Although the exact strains were not disclosed by the manufacturers, they belonged to the *S. cerevisiae* and *Saccharomyces* groups. The selection of these yeasts based on their availability and widespread use in the food and beverage industry. If any of them had demonstrated antifungal activity,

their application on an industrial scale would have been easily facilitated due to their existing market presence.

However, none of the tested yeasts showed the expected inhibitory effect. After the incubation period, *A. clavatus* B9/6 continued to grow uninhibited, eventually spreading over the yeast colonies in the Petri dishes. This result suggests that the selected yeast strains lack the necessary antifungal properties to suppress the growth of this mold under the tested conditions.

The same results were observed for the *Saccharomyces* species obtained from the NCAIM collection. No inhibition of *Aspergillus clavatus* B9/6 was detected, despite initial yeast growth being visible within the first 24 hours of inoculation. However, by the end of the seven-day incubation period, the yeast colonies were completely overgrown by the fungal mycelium.

Figure 26 shows both sides of the Petri dish inoculated with *S. willians* 0034 (renamed *S. cerevisiae* 0034). On the reverse (A) side of the Petri dish, a distinct line can be seen in the center. This line represents the area where the yeast initially developed, confirming that the strain was able to grow. However, despite its faster growth rate, it lacked the necessary mechanisms to inhibit *A. clavatus*, allowing the mold to spread across the plate (B).

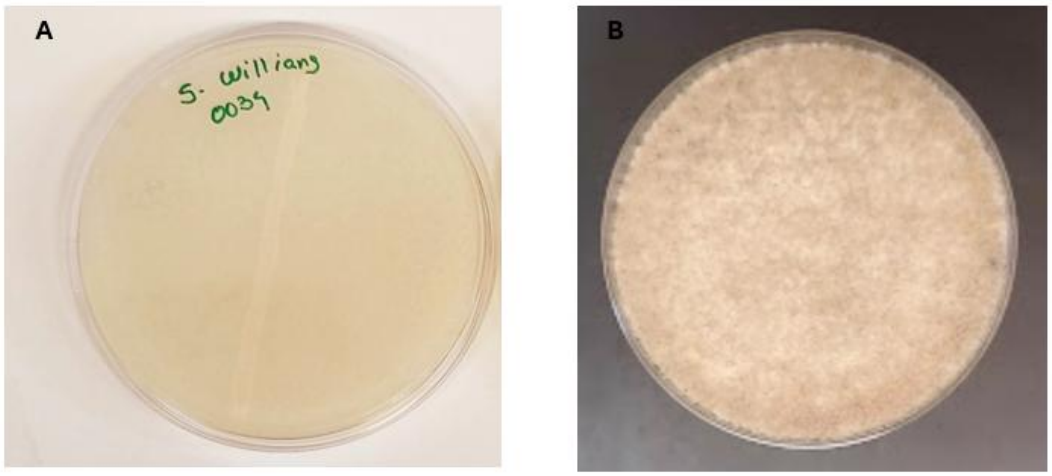


Figure 26 – Petri dishes containing *A. clavatus* and *S. willians* 0034. A- reverse, B- front

Since the results were negative with the genus *Saccharomyces*, the further assays were performed with strains of *Wickerhamomyces anomalus*, previously named *Pichia anomala,* as this species is also classified as GRAS and, therefore, can be applied in the food industry.

W. anomalus has been studied for its biocontrol potential against various fungal contaminants. Previous studies have demonstrated its effectiveness in inhibiting the proliferation of *Penicillium roqueforti*, *Aspergillus candidus*, *Penicillium italicum*, and *Aspergillus flavus* (Bjornberg & Schnurer, 1993; Hua et al., 2014; X. Li et al., 2025; Oufensou et al., 2023). However, to the best of my knowledge, this species has not yet been tested against *Aspergillus clavatus*. The primary mechanism of action of *W. anomalus* is attributed to the production of volatile organic compounds (VOCs), which have been shown to display antifungal activity. Additionally, in some cases, other bioactive compounds, such as killer toxins, have also been reported to contribute to its inhibitory effects.

In this study, all 13 strains of *W. anomalus* obtained from NCAIM showed some level of inhibition of *A. clavatus* B9/6, as presented in Figure 27.

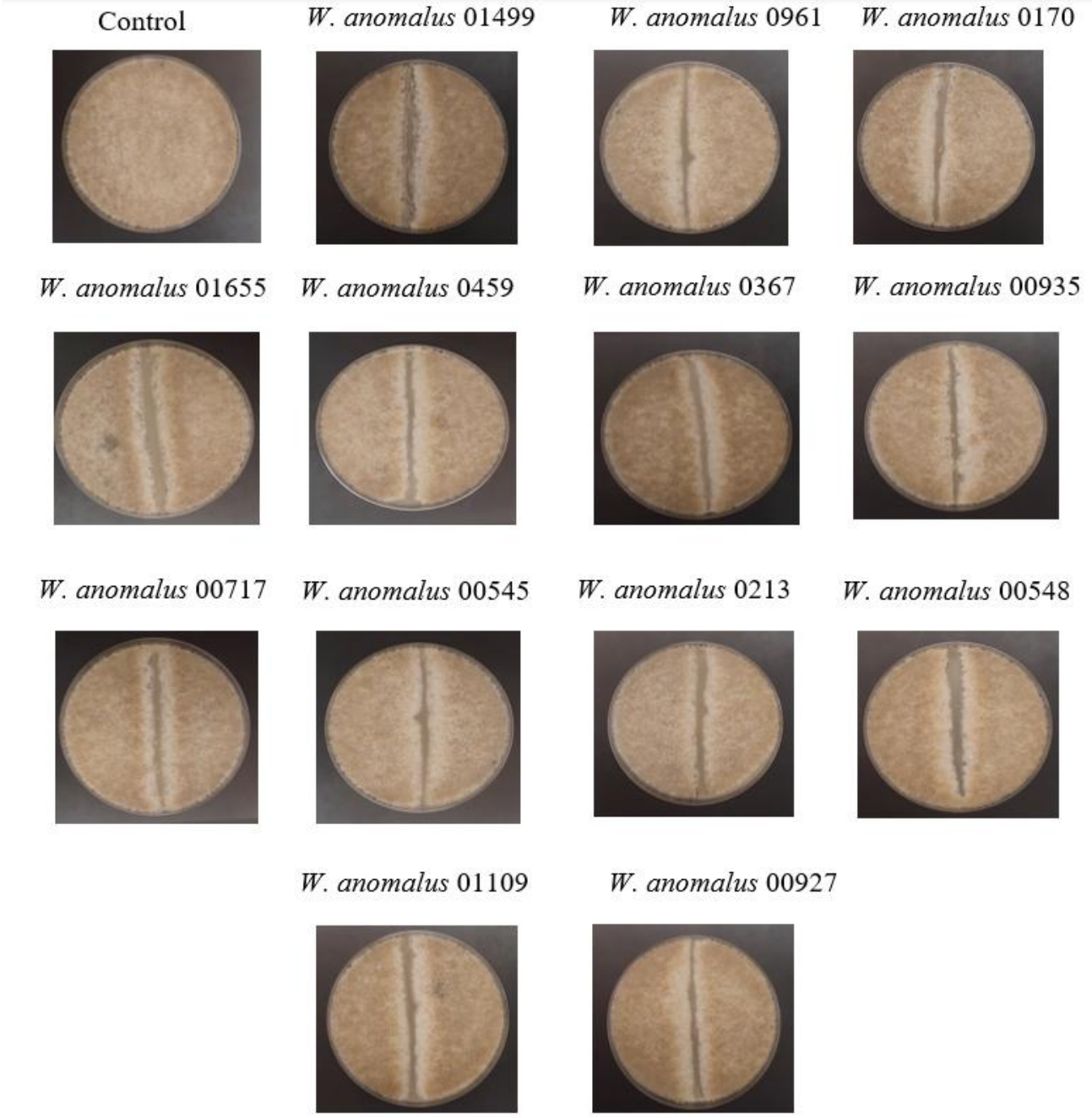


Figure 27 – Inhibition screening of *A. clavatus* B9/6 using different strains of *W. anomalus*

As it was not possible to define which strains presented the best inhibition, a second experiment was performed to evaluate the capacity of these yeasts to control the spread of *A. clavatus,* when its spores are not evenly distributed in the culture medium (Figure 28).

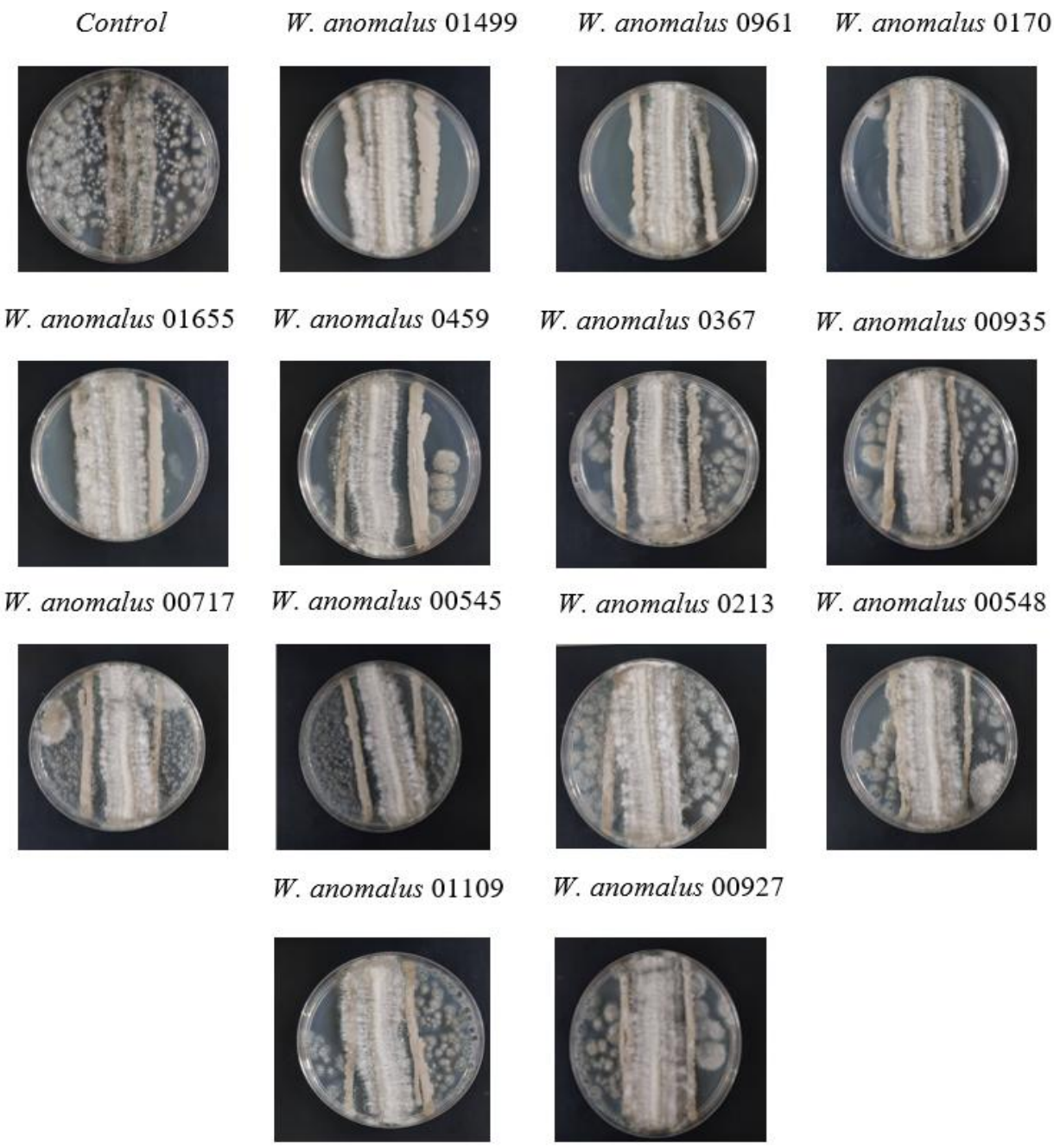


Figure 28 – Inhibition screening of *A. clavatus* B9/6 using different strains of *W. anomalus* by parallel streaks inoculation

Compared to the previous assay, more significant differences were observed in this experiment. *W. anomalus* 0213, 00717, and 00545 exhibited behavior very similar to control when a

saline solution was used. Although the lines where the yeasts had grown were still visible, *A. clavatus* colonies could spread across the entire surface of the dish.

W. anomalus 0459, 00935, and 0367 presented lower quantities of dispersed mold colonies, but some spreading was still observed. The best results were obtained with *W. anomalus* 01499, 0961, 0170, and 01655, as no mold colonies were observed beyond the area designated for yeast growth. Therefore, these four strains were selected for the next assay.

5.5.2 Patulin degradation by *Wickerhamomyces anomalus* strains

Since the goal of this study was not only to identify strains capable of inhibiting mold growth but also to degrade patulin if it was already present in the food product, a degradation assay was conducted. This experiment aimed to evaluate the ability of *W. anomalus* 01499, 01655, 0961, and 0170 to remove PAT from apple juice.

The decision to conduct the experiment using a real food product was based on findings from a previous study by (Ma et al., 2023)), which demonstrated that degradation time and efficiency can vary between culture media and actual fruit juices. Culture media provide all the necessary nutrients for optimal yeast growth, while fruit juices have a much more complex composition that can influence yeast metabolism. In the referenced study, the time required for complete degradation was 12 hours longer when using pear juice compared to NYBD culture medium.

Figure 29 shows the percentage of PAT degradation for each of the 4 strains studied after 24 h of incubation with the initial concentration of patulin of 10 ppm.

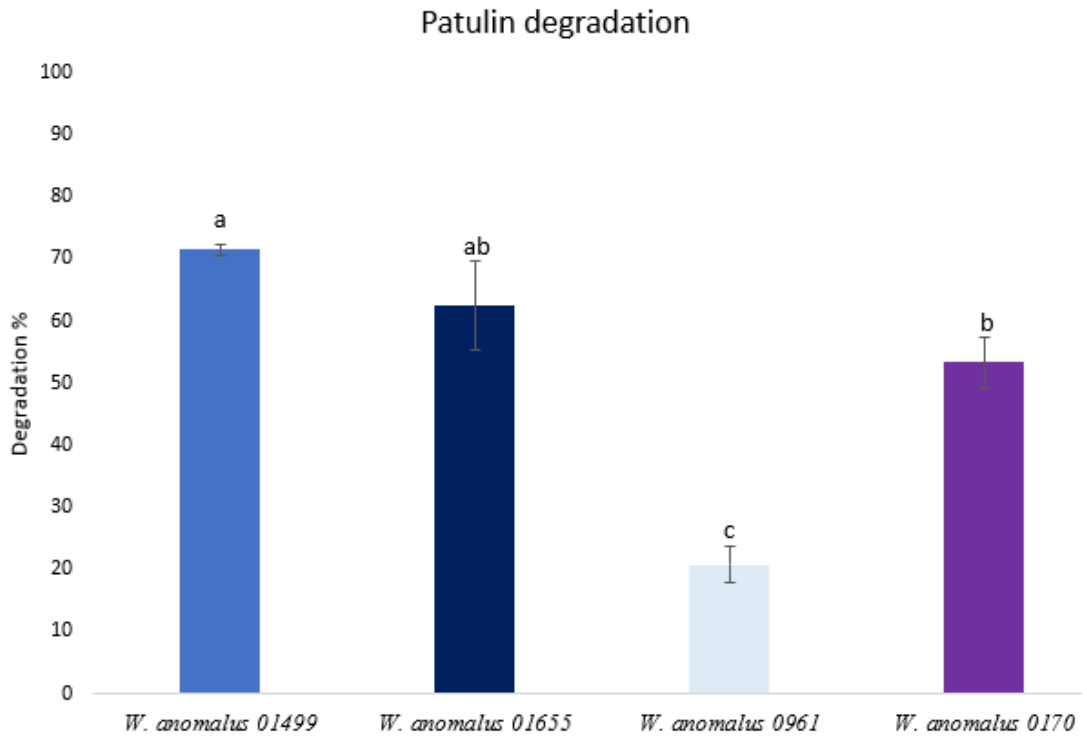


Figure 29 – Patulin degradation by different strains of *W. anomalus* (Superscripts a,b and c, represent statistically significant differences based on Tukey's test ($p < 0.05$)

Among the tested strains, *W. anomalus 01499* presented the highest degradation of PAT, achieving 71% in the first 24 hours, followed by *W. anomalus* 1655 with 62% and *W. anomalus* 0170 with 53%. The lowest results were obtained for *W. anomalus* 0961 with only 20% PAT degradation.

Comparing these results with previous studies, we find similarities when the yeast *Hannaella sinensis* was used, the degradation of PAT was around 70% in pear juice after 24 h (Ma et al., 2023). *Lactobacillus pentosus* (DSM 20314) in 24 h was able to degrade 53% of PAT in apple juice, followed by *Lactobacillus rhamnosus* DSM 6144, *Lactobacillus fermentum* DSM 21 and 828, *Lactobacillus paracasei* DSM 20 and 241, *Enterococcus faecium* DSM 20 and 420 and *Pediococcus acidilactici* (Lai et al., 2022b). Assays performed with yeast *Rhodosporidium paludigenum* showed after 24 h a decrease of around 70% in the PAT concentration when using viable cells and of 50% when using inactivated cells (Zhu et al., 2015).

As for strains *W. anomalus* 01499 and 01655 the degradation results were statistically equal, and therefore, both were evaluated in the next experiment.

5.5.3 Kinetics of patulin degradation *by W. anomalus* 01499 and 01655

The kinetics of PAT degradation were analyzed for *W. anomalus* strains 01499 and 01655. Since complete degradation was not achieved within 24 hours, the incubation period was extended to 48 hours to assess whether a longer duration would enhance the degradation process. Figures 30 and 31 illustrate the variations in toxin concentration over time when incubated with viable yeast cells.

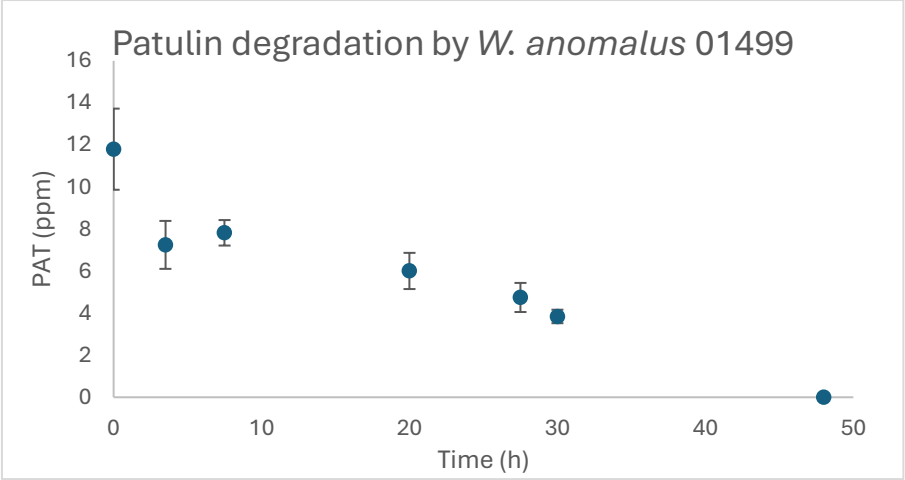


Figure 30 - Kinetics of patulin degradation by *W. anomalus* 01499

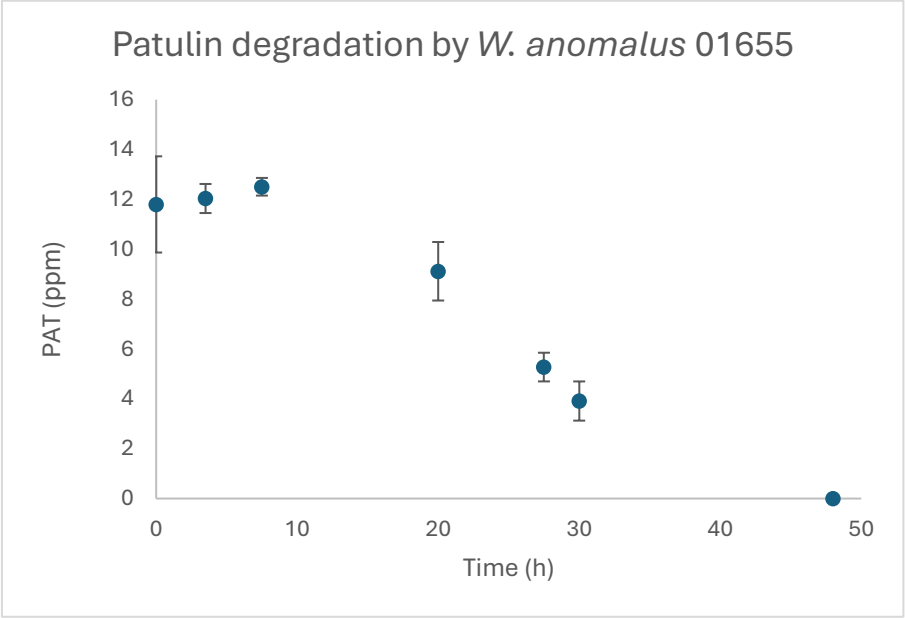


Figure 31 – Kinetics of patulin degradation by *W. anomalus* 01655

Both strains evaluated were able to degrade the total amount of PAT during 48 h under the studied conditions, however, the profiles of degradation are different for the two strains.

In the first 10 hours of the experiment no significant change in the patulin content was observed for *W. anomalus* 01655, the concentration started to fall from the 20th hour and presented a declining linear profile until reaching its lowest value at 48 h.

For the strain *W. anomalus* 01499, in the first 3.5 h of the experiment a reduction in the concentration of patulin of approximately 35% was already observed, but then until 10 h no significant change was noted. Between the hours 10 and 20, the concentration started to decrease again but in a less abrupt manner than for the strain 01655. From the 20th hour a slow decrease can be observed until total degradation by the end of the experiment.

These results indicate that for the strain *W. anomalus* 01499 it takes less time to adapt to the medium even in the presence of PAT. Studies performed with *Sporobolomyces* sp. showed that the mechanism for degrading PAT can be induced when this microorganism is cultivated with lower concentrations of toxin, however, total degradation was achieved after 4 days (Ianiri et al., 2017). Further improvements can be implemented to enhance the efficiency of the degradation. The study conducted with *L. casei* YZU01 showed that PAT degradation was directly affected by temperature, with the highest degradation observed when incubated at 37 °C (Zheng et al., 2020).

These findings suggest that optimizing environmental conditions, such as temperature and initial PAT concentration, could further enhance the degradation efficiency of *W. anomalus* 01499. Additionally, exploring potential metabolic pathways involved in PAT degradation may provide insights into improving its bioremediation capabilities.

5.5.4 Co-cultivation of *W. anomalus* 01499 and 01655 with *A. clavatus* B9/6

Since the strains of *W. anomalus* 01499 and 01655 were able not only to inhibit the growth of *A. clavatus* B9/6 in Petri dishes but also to degrade PAT when this mycotoxin was spiked in apple juice, a new experiment was designed to evaluate the performance of these yeasts when cultivated together with spores of the mold in apple juice and, if it was the case, to see whether they would be able to degrade patulin in these setups as well. The initial concentrations of *A. clavatus* and of the *W. anomalus* strains were the same. Figure 32 shows the flasks after 15 days of incubation.

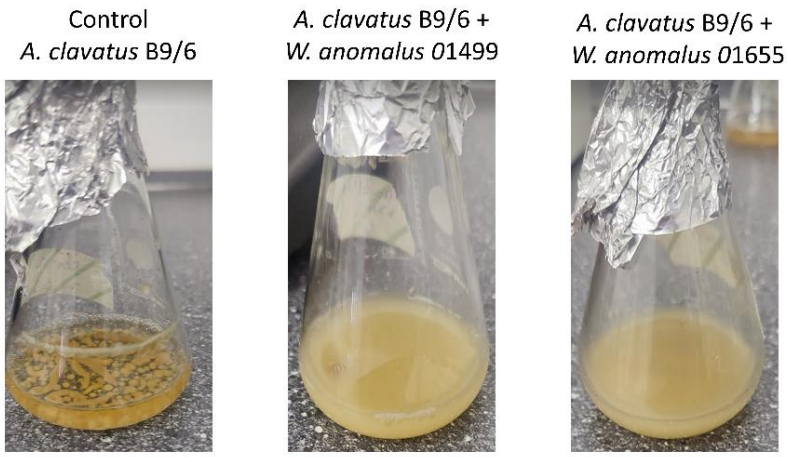


Figure 32 – Morphological characteristics of co-inoculation assays of *A. clavatus* B9/6 and *W. anomalus* 01499 and 01655

The difference between the assays with yeasts and the control only with *A. clavatus* B9/6 was clearly visible. While the control presented a clear medium containing spheres of mycelia and a line with spores adhered to the flask, the cultivation with yeasts showed cloudy characteristics. For the strain *W. anomalus* 01655 a small amount of spheres of mycelia was also observed, but in a considerably lower amount compared to the control.

The assay was conducted over a longer period to assess whether any viable *A. clavatus* spores could still germinate and survive in the environment despite the initial dominance of yeasts. This extended timeframe aimed to determine if a microbial shift would occur, where the yeast population is gradually replaced by the fungus, a phenomenon observed in certain fermentation processes. For instance, in some traditional cheese ripening and sourdough fermentation, initial yeast and bacterial populations can be outcompeted by filamentous fungi, which become dominant over time (Hirko et al., 2023; Hymery et al., 2014).

Despite the extended timeframe, this microbial shift was not observed in this study. *A. clavatus* B9/6 was unable to develop and establish itself in the environment, even after a prolonged period. The initial dominance of yeasts persisted throughout the experiment, indicating that the conditions did not favor fungal growth.

The PAT concentration followed the same profile for both yeasts inhibiting the growth of *A. clavatus* B9/6 and, therefore, the PAT production, the results are presented in Table 13.

Table 13 - Final patulin concentrations of the co-inoculation assays of *A. clavatus* B9/6 and *W. anomalus* 01499 and 01655

	PAT (ppm)
Control *A. clavatus* B9/6	21304 ± 515.0
***A. clavatus* B9/6 + *W. anomalus* 01499**	<LOQ
***A. clavatus* B9/6 + *W. anomalus* 01655**	<LOQ

As expected, the mycotoxin was only detected in the control group, as it was the only setup in which *A. clavatus* was able to grow properly. Interestingly, the PAT concentration in this experiment was even higher than in the experiments conducted under different pH and temperature conditions. This assay was performed at 25 °C and pH 3.6, which corresponds to the natural pH of the juice. However, the key factor contributing to the increased PAT levels was the extended incubation time, which was twice as long as that of the environmental assays.

The strains *W. anomalus* 01499 and 01655 demonstrated their effectiveness in controlling the growth of *A. clavatus* and inhibiting PAT production. In this case, the most probable mechanism of inhibition was competition for nutrients and rapid colonization of the medium, which limited the resources available for fungal development. Additionally, the ability of these yeast strains to establish dominance in the environment may have further restricted the survival and toxin production of *A. clavatus*. These findings highlight the potential of *W. anomalus* as a promising biocontrol agent for reducing mycotoxin contamination in food products (He et al., 2024; X. Li et al., 2025).

5.5.5 *In vivo* evaluation of *A. clavatus* B9/6 inhibition using *W. anomalus* 01499

To evaluate the potential of the yeast *W. anomalus* to inhibit *A. clavatus* B9/6 in actual fruits, tests were performed inoculating both microorganisms at different times and together beside a negative control injected only with water and a positive control in which only *A. clavatus* was injected. The results are presented in Figure 33.

Figure 33- *In vivo* assay of inhibition of *A. clavatus* B9/6 using *W. anomalus* 01499. A - Negative control, B- Positive control, C – *W. anomalus* 01499 injected 24 h before *A. clavatus* B9/6, D - *W. anomalus* 01499 injected at the same day as *A. clavatus* B9/6, E - *A. clavatus* B9/6 injected 24 h before *W. anomalus* 01499

In the assays where the yeast *W. anomalus* 01499 was inoculated 24 hours before the mold *A. clavatus* B9/6, almost no symptoms of infection were observed, suggesting a strong protective effect of the yeast. When both the mold and yeast were inoculated on the same day, a slight initial infection was noticeable, but it did not spread or penetrated the core of the apple.

In contrast, in the positive control where only the mold was injected, clear signs of fruit decay were observed, including a darkened lesion and the development of mycelial tissue at the center of the apple. Surprisingly, in the assay where the mold was injected 24 hours before the yeast, the symptoms of infection were even more severe. In addition to the dark spots, small holes appeared near the points where the needles were inserted.

This could be explained by that *A. clavatus* had already established an infection before the introduction of *W. anomalus*, potentially weakening the fruit tissue and making it more susceptible to structural damage. A previous study had a similar result when using *Rhodosporium paludigenum*: when its initial concentration increased, the virulence of the mold, in that case *P. expansum,* was enhanced and a higher concentration of PAT was observed (Zhu et al., 2015). In this sense, there is a need to study different concentrations of the yeast to possibly avoid this effect.

Finally, in the negative control, the needle mark was visible, but no microbial growth was observed.

5.5.6 Intracellular and extracellular enzymatic degradation of patulin and PAT binding capacity of *W. anomalus* 01499

This study evaluated the degradation potential of PAT through different enzymatic and cellular mechanisms. Exoenzymes of *W. anomalus* 01499 secreted into the culture medium after 24 hours of incubation were analyzed for their ability to break down PAT. Additionally, endoenzymes were extracted using the French press technique to disrupt cells and assess degradation activity of intracellular enzymes.

The capacity of the cell wall to bind these toxins was also investigated, considering its potential role in reducing toxin bioavailability. This integrated approach provides a broader understanding of the combined effects of extracellular, intracellular, and cell-wall-associated processes in PAT degradation. In Table 14, the results of PAT degradation are presented.

Table 14 - Patulin detoxification by different cell components of *W. anomalus* 01499

	Remaining concentration of PAT (ppm)	**Reduction (%)**
Control	10.73 ±0.40	N/A
Supernatant	11.26 ±0.86	0
Cell-wall	9.55 ±0.18	11
Endoenzyme	7.23 ±0.12	33

The supernatant exhibited no effect on PAT degradation, indicating that no enzymes capable of degrading this mycotoxin were secreted during yeast fermentation under the tested conditions. This absence of enzymatic activity in the culture medium may be attributed to the lack of specific inducible enzymes necessary for PAT degradation or to insufficient expression and secretion of relevant exoenzymes. These findings suggest that the detoxification process primarily relies on intracellular mechanisms rather than extracellular enzyme activity. A similar result was observed for yeast *Hannaella sinesis*, where even after 30 hours of incubation no degradation of patulin could be observed using the supernatant (Ma et al., 2023),

In this study, an 11% reduction was observed when evaluating the potential of the cell wall of *W. anomalus* to detoxify patulin (PAT), demonstrating that PAT can partially bind to the cell wall. This finding contrasts with a previously mentioned study on *H. sinensis*, where no binding to the cell wall was observed. Conversely, the study by (Guo et al., 2012) reported the opposite, showing that the highest toxin reduction occurred when heat-treated cells of *S. cerevisiae* were used, highlighting the potential of toxin binding in this microorganism.

However, another mitigation mechanism exhibited an even greater effect. When evaluating the endoenzyme content, a reduction of 33% in PAT concentration was observed, suggesting that intracellular enzymes play a more significant role in PAT detoxification compared to cell wall binding. These findings indicate that enzymatic activity within the cell is a crucial factor in the detoxification process, contributing more substantially to PAT degradation.

These results suggest that while cell wall binding contributes to toxin removal, the primary mechanism for PAT removal in *W. anomalus* 01499 is the action of endoenzymes, which may actively break down the mycotoxin into less harmful compounds.

According to these findings, there is potential for further improvement in patulin detoxification by optimizing experimental conditions. Since the ideal parameters for enzymatic activity and expression were not established in this study, modifying factors such as pH, temperature, and nutrient availability could enhance both enzyme production and detoxification efficiency.

Additionally, strategies to increase enzymatic expression, such as adapting this yeast to the presence of the toxin, could be investigated. Previous studies have demonstrated that incubating *H. sinensis* with patulin can stimulate higher enzymatic activity, leading to greater degradation efficiency (Ma et al., 2023). Applying similar approaches, alongside genetic or metabolic engineering

techniques, could further enhance the yeast's detoxification capacity. Furthermore, prolonging the fermentation time or adjusting cultivation conditions to favor endoenzyme production may contribute to improved PAT removal, offering additional opportunities for optimization.

6 CONCLUSION AND RECOMMENDATIONS

In this study, the mold population of Hungarian apples was evaluated, with a particular focus on potential patulin-producing *Aspergillus* species. Research on *Aspergillus* species in this context has been limited compared to *Penicillium*, highlighting the need for further investigation. A total of 183 molds were identified from apples originating from seven different locations and cultivated using various farming methods. Among these, 67 isolates belonged to the *Alternaria* group, 45 to *Aspergillus*, and 13 to *Penicillium*, while the remaining were classified as other genera.

The results indicated that geographical location had a greater influence on the distribution of mold genera than farming methods. Despite higher temperatures generally favoring *Aspergillus* growth, species from this genus were dominant only in Újfehértó, where approximately 50% of the isolates belonged to *Aspergillus*. In contrast, four of the seven locations, Csenger, Debrecen-Pallag, Nyírtass, and Nagykálló, were dominated by *Alternaria* species.

Among the 45 *Aspergillus* isolates, species identification revealed the presence of *A. fumigatus* (28 isolates), *A. flavus* (15 isolates), *A. clavatus* (1 isolate), and *A. nomius* (1 isolate). Notably, *A. nomius* is reported in this work for the first time in Hungarian crops and is capable of producing aflatoxin B1.

The only *Aspergillus* strain that tested positive for the patulin gene was *A. clavatus* B9/6, which was isolated from the Golden Reinders apple cultivar grown in Debrecen-Pallag under integrated farming conditions. This strain's patulin-producing ability was confirmed using thin-layer chromatography (TLC) assays, leading to its selection for further experimentation.

To improve species-level differentiation among *Aspergillus* isolates, a rapid restriction fragment length polymorphism (RFLP) method was developed using the restriction enzyme *HpaII*, enabling the distinction of six different *Aspergillus* species.

Further studies on *A. clavatus* B9/6 assessed the effects of pH and temperature on patulin production. Results showed that the strain produced high levels of patulin at 25 °C, with the highest concentration (6,061 ppm) observed at pH 5.5. However, patulin production decreased significantly at 35 °C, and it was completely inhibited at pH 2.5, regardless of temperature.

A biocontrol approach using yeasts was also evaluated. Several commercial *Saccharomyces cerevisiae* and *S. bayanus* strains, along with strains from the NCAIM were tested, but none effectively inhibited *A. clavatus* B9/6 growth. Subsequently, 13 additional yeasts of *Wickerhamomyces anomalus* were screened, all of which showed some level of inhibition. The most promising results were obtained with *W. anomalus* strains 01499, 0961, 0170, and 01655.

These strains were further assessed for their ability to degrade patulin. *W. anomalus* 01499 achieved the highest degradation rate, reducing patulin levels by 71% within the first 24 hours, followed by *W. anomalus* 01655 (62%) and *W. anomalus* 0170 (53%). *W. anomalus* 0961 exhibited the lowest degradation rate at 20%. Kinetic studies showed that *W. anomalus* 01655 completely degraded patulin after 48 hours, with no significant change observed during the first 10 hours. In contrast, *W. anomalus* 01499 demonstrated a 35% reduction in patulin within the first 3.5 hours, followed by a slower decrease between 10 and 20 hours, ultimately leading to full degradation.

Visible differences were observed between yeast-treated and control samples when those were co-inoculated into apple juice. In the control assay with only *A. clavatus* B9/6, the culture medium remained clear, with fungal mycelia and spores adhering to the flask. In contrast, yeast-treated cultures appeared cloudy, and patulin was detected only in the control group, indicating that the presence of yeast prevented *A. clavatus* from growing properly.

To assess the *in vivo* protective potential of *W. anomalus*, assays were conducted on apples. When *W. anomalus* 01499 was inoculated 24 hours before *A. clavatus* B9/6, almost no infection symptoms were observed, demonstrating strong protective effects. When both were inoculated simultaneously, slight initial infection occurred but did not spread or penetrate the apple core. In contrast, apples inoculated solely with *A. clavatus* B9/6 showed clear signs of decay, including dark lesions and central mycelial growth. Interestingly, when the fungus was introduced 24 hours before the yeast, infection symptoms were even more severe.

Supernatant assays showed no effect on patulin degradation, suggesting that no extracellular enzymes capable of degrading patulin were secreted during fermentation. However, an 11% reduction in patulin was observed when evaluating yeast cell wall binding, indicating partial adsorption. Furthermore, assessment of endoenzyme activity revealed a 33% reduction in patulin, suggesting that intracellular enzymes play a more significant role in patulin detoxification.

Overall, this study provides valuable insights into the mold population of Hungarian apples, *Aspergillus* species capable of growing at elevated temperatures, potential for patulin production, and the efficacy of *W. anomalus* strains in both inhibiting fungal growth and degrading patulin. These findings highlight the potential of *W. anomalus* as a biocontrol agent, paving the way for further research into its application in food safety and post-harvest disease management.

Suggestions

- Study the effect of lower temperatures in the ability to produce PAT of *A. clavatus* B9/6
- Evaluate the potential of *W. anomalus* against other mycotoxin producer molds
- Study and identify the enzymes responsible for PAT degradation as well their optimal condition to enhance the applicability
- Creation of a fermented beverage using *W. anomalus* using apple juice as starter

7 NEW SCIENTIFIC RESULTS

- This study documents for the first time the occurrence of *Aspergillus nomius* in Hungarian crops, expanding the known distribution of this aflatoxin B1 producing species.

- Patulin-producing *A. clavatus* was identified in apples from Hungary, representing the first such report and revealing a previously unrecognized source of contamination.

- A restriction fragment length polymorphism (RFLP) method based on the enzyme *HpaII* was developed, providing a new and efficient tool to differentiate six mycotoxigenic *Aspergillus* species.

- Screening of 13 *Wickerhamomyces anomalus* strains revealed consistent antifungal activity against *A. clavatus* B9/6, with four strains showing particularly strong inhibition.

- W. *anomalus* 01499 was found to degrade patulin completely within 48 h, marking the first description of this detoxification capability in the species.

- In vivo assays showed that pre-inoculation of apples with *W. anomalus* 01499 prevented fungal infection, whereas delayed treatment increased symptom severity, underscoring the importance of application timing.

- Patulin detoxification by *W. anomalus* occurred through cell wall binding and intracellular enzymatic activity, with no extracellular involvement, offering novel insight into the underlying mechanisms.

- *W. anomalus* demonstrated a dual function in food safety by simultaneously inhibiting fungal growth and degrading patulin, highlighting its potential as a sustainable post-harvest biocontrol agent.

8 REFERENCES

Abastabar, M., Shabanzadeh, S., Valadan, R., Mayahi, S., Haghani, I., Khojasteh, S., Nargesi, S., Seyedmousavi, S., & Hedayati, M. T. (2022). Development of RFLP method for rapid differentiation of Aspergillus flavus and Aspergillus oryzae, two species with high importance in clinical and food microbiology. *Journal of Medical Mycology*, *32*(3), 17–20. https://doi.org/10.1016/j.mycmed.2022.101274

Ali, S., Abbasi, P., McLaughlin, M. S., & Abbasi, S. (2024). Apple Bitter Rot and Glomerella Leaf Spot: A Comprehensive Review of Causal Species and Their Biology, Fungicide Sensitivities, and Management Strategies. *Plant Disease*, *108*(12), 3432–3445. https://doi.org/10.1094/pdis-03-24-0552-fe

Amalaradjou, M. A. R., & Venkitanarayanan, K. (2008). Detection of Penicillium, Aspergillus and Alternaria Species in Fruits and Vegetables. In *Mycotoxins in Fruits and Vegetables* (pp. 225–247). Elsevier. https://doi.org/10.1016/B978-0-12-374126-4.00010-3

Andrello, A. J., Velho, A. C., Soares, V. F., de Godoy, R. S., de Freitas, M. B., Valdebenito-Sanhueza, R. M., Kulcheski, F. R., & Stadnik, M. J. (2024). Comparative virulence of Colletotrichum chrysophilum and Colletotrichum nymphaeae on apple leaves. *Physiological and Molecular Plant Pathology*, *131*(April), 102285. https://doi.org/10.1016/j.pmpp.2024.102285

Bacha, S. A. S., Li, Y., Nie, J., Xu, G., Han, L., & Farooq, S. (2023). Comprehensive review on patulin and Alternaria toxins in fruit and derived products. *Frontiers in Plant Science*, *14*(April), 1–32. https://doi.org/10.3389/fpls.2023.1139757

Banahene, J. C. M., Ofosu, I. W., Odai, B. T., Lutterodt, H. E., Agyemang, P. A., & Ellis, W. O. (2024). Ochratoxin A in food commodities: A review of occurrence, toxicity, and management strategies. *Heliyon*, *10*(20), e39313. https://doi.org/10.1016/j.heliyon.2024.e39313

Barreira, M. J., Alvito, P. C., & Almeida, C. M. M. (2010). Occurrence of patulin in apple-based-foods in Portugal. *Food Chemistry*, *121*(3), 653–658. https://doi.org/10.1016/j.foodchem.2009.12.085

Bata-Vidács, I., Kosztik, J., Mörtl, M., Székács, A., & Kukolya, J. (2020). Aflatoxin B1 and Sterigmatocystin Binding Potential of Non-Lactobacillus LAB Strains. *Toxins*, *12*(12), 1–12. https://doi.org/10.3390/toxins12120799

Bjornberg, A., & Schnurer, J. (1993). Inhibition of the growth of grain-storage molds in vitro by the yeast Pichia anomala (Hansen) Kurtzman. *Canadian Journal of Microbiology*, *39*(6), 623–628. https://doi.org/10.1139/m93-090

Bonerba, E., Conte, R., Ceci, E., & Tantillo, G. (2010). Assessment of dietary intake of patulin from baby foods. *Journal of Food Science*, *75*(7), 25–27. https://doi.org/10.1111/j.1750-3841.2010.01743.x

Bowen, J. K., Mesarich, C. H., Bus, V. G. M., Beresford, R. M., Plummer, K. M., & Templeton, M. D. (2011a). Venturia inaequalis: The causal agent of apple scab. *Molecular Plant Pathology*, *12*(2), 105–122. https://doi.org/10.1111/j.1364-3703.2010.00656.x

Bui, A., Orr, D., Lepori-Bui, M., Konicek, K., Young, H. S., & Moeller, H. V. (2020). Soil fungal community composition and functional similarity shift across distinct climatic conditions. *FEMS Microbiology Ecology*, *96*(12), 1–11. https://doi.org/10.1093/femsec/fiaa193

Cai, P., Liu, S., Tu, Y., & Shan, T. (2024). Toxicity, biodegradation, and nutritional intervention mechanism of zearalenone. *Science of the Total Environment*, *911*(November 2023), 168648. https://doi.org/10.1016/j.scitotenv.2023.168648

Castoria, R., Mannina, L., Durán-Patrón, R., Maffei, F., Sobolev, A. P., De Felice, D. V., Pinedo-Rivilla, C., Ritieni, A., Ferracane, R., & Wright, S. A. I. (2011). Conversion of the mycotoxin patulin to the less toxic desoxypatulinic acid by the biocontrol yeast Rhodosporidium kratochvilovae strain LS11. *Journal of Agricultural and Food Chemistry*, *59*(21), 11571–11578. https://doi.org/10.1021/jf203098v

Chalmers, I., & Clarke, M. (2004). Commentary: The 1944 patulin trial: The first properly controlled multicentre trial conducted under the aegis of the British Medical Research Council. *International Journal of Epidemiology*, *33*(2), 253–260. https://doi.org/10.1093/ije/dyh162

Coelho, A. R., Celli, M. G., Ono, E. Y. S., Wosiacki, G., Hoffmann, F. L., Pagnocca, F. C., & Hirooka, E. Y. (2007). Penicillium expansum versus antagonist yeasts and patulin degradation in vitro.

Brazilian Archives of Biology and Technology, *50*(4), 725–733. https://doi.org/10.1590/S1516-89132007000400019

Cornille, A., Giraud, T., Smulders, M. J. M., Roldán-Ruiz, I., & Gladieux, P. (2014). The domestication and evolutionary ecology of apples. *Trends in Genetics*, *30*(2), 57–65. https://doi.org/10.1016/j.tig.2013.10.002

Csernus, O., Beczner, J., Sebok, F., Tóth, M., & Dobolyi, C. (2015). Characterisation of moulds from apple fruit in Hungary. *Acta Alimentaria*, *44*(1), 150–156. https://doi.org/10.1556/AAlim.44.2015.1.16

Delgado, A., Dapena, E., Fernandez, E., & Luedeling, E. (2021). Climatic requirements during dormancy in apple trees from northwestern Spain – Global warming may threaten the cultivation of high-chill cultivars. *European Journal of Agronomy*, *130*(April). https://doi.org/10.1016/j.eja.2021.126374

Dias, J. V., da Silva, R. C., Pizzutti, I. R., dos Santos, I. D., Dassi, M., & Cardoso, C. D. (2019). Patulin in apple and apple juice: Method development, validation by liquid chromatography-tandem mass spectrometry and survey in Brazilian south supermarkets. *Journal of Food Composition and Analysis*, *82*(June). https://doi.org/10.1016/j.jfca.2019.103242

EUROPEAN UNION. (2023). *Regulation (EC) No 1881/2006 of the European Parliament and of the Council of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. EUR-Lex*. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A02006R1881-20230326

FAOSTAT. (2021). *Crops and livestock products. Available online.* https://www.fao.org/faostat/en/#rankings/countries_by_commodity

Gaucher, G.M. and Fedeshko, R. W. (1997). *Penicillium urticae isoepoxydon dehydrogenase (IDH) gene, complete cds. In: GenBank]. Bethesda (MD): National Center for Biotechnology Information*. https://www.ncbi.nlm.nih.gov/nuccore/AF006680

Ghosal, D., Tarafder, M., & Datta, B. (2020). Fusarium oxysporum isolates showed varied benzimidazole fungicides sensitivity under low temperature and heavy metal stress. *Rhizosphere*, *15*(June), 100227. https://doi.org/10.1016/j.rhisph.2020.100227

Godana, E. A., Edo, G. S., Yang, Q., Zhang, X., Zhao, L., Wang, K., Ngea, G. L. N., & Zhang, H. (2024). Wickerhamomyces anomalus: A promising yeast for controlling mold growth and diverse biotechnological applications. *Trends in Food Science and Technology*, *151*(April), 104649. https://doi.org/10.1016/j.tifs.2024.104649

Gökmen, V., & Acar, J. (2000). Long-term survey of patulin in apple juice concentrates produced in Turkey. *Food Additives and Contaminants*, *17*(11), 933–936. https://doi.org/10.1080/026520300750038117

Gonçalves, B. L., Coppa, C. F. S. C., de Neeff, D. V., Corassin, C. H., & Oliveira, C. A. F. (2019). Mycotoxins in fruits and fruit-based products: occurrence and methods for decontamination. *Toxin Reviews*, *38*(4), 263–272. https://doi.org/10.1080/15569543.2018.1457056

Gong, A., Song, M., & Zhang, J. (2024). Current Strategies in Controlling Aspergillus flavus and Aflatoxins in Grains during Storage: A Review. *Sustainability (Switzerland)* , *16*(8), 1–17. https://doi.org/10.3390/su16083171

Gong, Q., Zhang, Z., Huang, P., Wang, B., & Zheng, X. (2024). Assessment of Fungal and Contamination of Ochratoxin A and Patulin in Foods Susceptible to Contamination in the Yangzhou Market, China. *Foods*, *13*(19). https://doi.org/10.3390/foods13193205

Gougouli, M., & Koutsoumanis, K. P. (2010). Modelling growth of Penicillium expansum and Aspergillus niger at constant and fluctuating temperature conditions. *International Journal of Food Microbiology*, *140*(2–3), 254–262. https://doi.org/10.1016/j.ijfoodmicro.2010.03.021

Guo, C., Yuan, Y., Yue, T., Hatab, S., & Wang, Z. (2012). Binding mechanism of patulin to heat-treated yeast cell. *Letters in Applied Microbiology*, *55*(6), 453–459. https://doi.org/10.1111/j.1472-765x.2012.03314.x

Hammami, W., Al Thani, R., Fiori, S., Al-Meer, S., Atia, F. A., Rabah, D., Migheli, Q., & Jaoua, S. (2017). Patulin and patulin producing Penicillium spp. Occurrence in apples and apple-based products including baby food. *Journal of Infection in Developing Countries*, *11*(4), 343–349. https://doi.org/10.3855/jidc.9043

Haro-González, J. N., Castillo-Herrera, G. A., Martínez-Velázquez, M., & Espinosa-Andrews, H. (2021). Clove essential oil (Syzygium aromaticum l. myrtaceae): Extraction, chemical

composition, food applications, and essential bioactivity for human health. *Molecules*, *26*(21). https://doi.org/10.3390/molecules26216387

Hassane, A. M. A., El-Shanawany, A. A., Abo-Dahab, N. F., Abdel-Hadi, A. M., Abdul-Raouf, U. M., & Mwanza, M. (2017). Influence of Different Moisture Contents and Temperature on Growth and Production of Aflatoxin B1 by a Toxigenic Aspergillus flavus Isolate in Wheat Flour. *Journal of Ecology of Health & Environment*, *5*(3), 77–83. https://doi.org/10.18576/jehe/050302

He, Y., Degraeve, P., & Oulahal, N. (2024). Bioprotective yeasts: Potential to limit postharvest spoilage and to extend shelf life or improve microbial safety of processed foods. *Heliyon*, *10*(3), e24929. https://doi.org/10.1016/j.heliyon.2024.e24929

Hinrikson, H. P., Hurst, S. F., de Aguirre, L., & Morrison, C. J. (2005). Molecular methods for the identification of Aspergillus species. *Medical Mycology*, *43*(SUPPL.1), 129–137. https://doi.org/10.1080/13693780500064722

Hirko, B., Mitiku, H., & Getu, A. (2023). Role of fermentation and microbes in cacao fermentation and their impact on cacao quality. *Systems Microbiology and Biomanufacturing*, *3*(4), 509–520. https://doi.org/10.1007/s43393-023-00160-9

Hou, J., Cheng, X., Li, J., & Dong, Y. (2021). Magnesium and nitrogen drive soil bacterial community structure under long-term apple orchard cultivation systems. *Applied Soil Ecology*, *167*(April), 104103. https://doi.org/10.1016/j.apsoil.2021.104103

Hua, S. S. T., Beck, J. J., Sarreal, S. B. L., & Gee, W. (2014). The major volatile compound 2-phenylethanol from the biocontrol yeast, Pichia anomala, inhibits growth and expression of aflatoxin biosynthetic genes of Aspergillus flavus. *Mycotoxin Research*, *30*(2), 71–78. https://doi.org/10.1007/s12550-014-0189-z

Hungarian Central Statistical Office. (n.d.). *Fruit production (2000–)*. Retrieved May 20, 2024, from https://www.ksh.hu/docs/eng/xstadat/xstadat_annual/i_omn025b.html

Hymery, N., Vasseur, V., Coton, M., Mounier, J., Jany, J. L., Barbier, G., & Coton, E. (2014). Filamentous fungi and mycotoxins in Cheese: A review. *Comprehensive Reviews in Food Science and Food Safety*, *13*(4), 437–456. https://doi.org/10.1111/1541-4337.12069

Ianiri, G., Pinedo, C., Fratianni, A., Panfili, G., & Castoria, R. (2017). Patulin degradation by the biocontrol yeast Sporobolomyces sp. Is an inducible process. *Toxins*, *9*(2), 1–12. https://doi.org/10.3390/toxins9020061

Iha, M. H., & Sabino, M. (2008). Incidence of patulin in Brazilian apple-based drinks. *Food Control*, *19*(4), 417–422. https://doi.org/10.1016/j.foodcont.2007.05.001

Izquierdo Cañas, P. M., García-Romero, E., Heras Manso, J. M., & Fernández-González, M. (2014). Influence of sequential inoculation of Wickerhamomyces anomalus and Saccharomyces cerevisiae in the quality of red wines. *European Food Research and Technology*, *239*(2), 279–286. https://doi.org/10.1007/s00217-014-2220-1

Jiang, T., Ren, J., Li, D., Luo, Y., Huang, Y., Gao, T., Yang, J., Yu, J., Liu, L., & Yuan, H. (2025). Pseudomonas syringae exacerbates apple replant disease caused by Fusarium. *Microbiological Research*, *296*(February), 128124. https://doi.org/10.1016/j.micres.2025.128124

Jimdjio, C. K., Xue, H., Bi, Y., Nan, M., Li, L., Zhang, R., Liu, Q., & Pu, L. (2021). Effect of ambient ph on growth, pathogenicity, and patulin production of penicillium expansum. *Toxins*, *13*(8), 1–13. https://doi.org/10.3390/toxins13080550

Juodeikiene, G., Bartkiene, E., Cernauskas, D., Cizeikiene, D., Zadeike, D., Lele, V., & Bartkevics, V. (2018). Antifungal activity of lactic acid bacteria and their application for Fusarium mycotoxin reduction in malting wheat grains. *LWT - Food Science and Technology*, *89*, 307–314. https://doi.org/10.1016/j.lwt.2017.10.061

Khadiri, M., Boubaker, H., Lahmamsi, H., Taoussi, M., Ezzouggari, R., Askarne, L., Farhaoui, A., Barka, E. A., & Lahlali, R. (2024). Challenges in apple preservation: Fungicide resistance and emerging biocontrols. *Physiological and Molecular Plant Pathology*, *129*(October 2023), 102205. https://doi.org/10.1016/j.pmpp.2023.102205

Kizis, D., Natskoulis, P., Nychas, G. J. E., & Panagou, E. Z. (2014). Biodiversity and ITS-RFLP characterisation of Aspergillus section Nigri isolates in grapes from four traditional grape-producing areas in Greece. *PLoS ONE*, *9*(4). https://doi.org/10.1371/journal.pone.0093923

Kousar, M. ul, Yaseen, M., Yousouf, M., Malik, M. A., Mushtaq, A., Mukhtar, T., Javaid, R., Aijaz, A., Jabeen, A., & Amin, T. (2024). Aflatoxins in cereal based products-an overview of

occurrence, detection and health implication. *Toxicon*, *251*(October), 108148. https://doi.org/10.1016/j.toxicon.2024.108148

Kurtzman, C. P., Horn, B. W., & Hesseltine, C. W. (1987). Aspergillus nomius, a new aflatoxin-producing species related to Aspergillus flavus and Aspergillus tamarii. *Antonie van Leeuwenhoek*, *53*(3), 147–158. https://doi.org/10.1007/BF00393843

Lai, W., Cai, R., Yang, K., Yue, T., Gao, Z., Yuan, Y., & Wang, Z. (2022a). Detoxification of patulin by Lactobacillus pentosus DSM 20314 during apple juice fermentation. *Food Control*, *131*. https://doi.org/10.1016/j.foodcont.2021.108446

Lai, W., Cai, R., Yang, K., Yue, T., Gao, Z., Yuan, Y., & Wang, Z. (2022b). Detoxification of patulin by Lactobacillus pentosus DSM 20314 during apple juice fermentation. *Food Control*, *131*. https://doi.org/10.1016/j.foodcont.2021.108446

Lee, S. B., & Park, H. D. (2020). Isolation and investigation of potential non-saccharomyces yeasts to improve the volatile terpene compounds in korean muscat bailey a wine. *Microorganisms*, *8*(10), 1–17. https://doi.org/10.3390/microorganisms8101552

Li, M., Chen, W., Zhang, Z., Zhang, Z., & Peng, B. (2018). Fermentative degradation of Patulin by Saccharomyces cerevisiae in aqueous solution. *Lwt*, *97*(April), 427–433. https://doi.org/10.1016/j.lwt.2018.07.040

Li, X., Chen, O., Wang, W., Deng, L., Yao, S., Ming, J., Zhang, H., & Zeng, K. (2025). Advances and perspectives in biological control of postharvest fungal decay in citrus fruit utilizing yeast antagonists. *International Journal of Food Microbiology*, *432*(January). https://doi.org/10.1016/j.ijfoodmicro.2025.111093

Lyagin, I., Maslova, O., Stepanov, N., Senko, O., & Efremenko, E. (2025). Reassessing of enzymes degrading mycotoxins at acidic pH. *International Biodeterioration and Biodegradation*, *198*(August 2024), 105994. https://doi.org/10.1016/j.ibiod.2024.105994

Ma, J., Godana, E. A., Yang, Q., & Zhang, H. (2023). Effect of the antagonistic yeast Hannaella sinensis on the degradation of Patulin. *Biological Control*, *178*(November 2022), 105134. https://doi.org/10.1016/j.biocontrol.2022.105134

MacDonald, S., Long, M., Gilbert, J., Felgueiras, I., Brera, C., Jørgensen, K., Macho, M. L., Majerus, P., Martins, M. L., Mevissen, L., Michelet, J. Y., Nuotio, K., Pittet, A., Szymanski, L., Tucker,

N., Viladrich, R., Voogt, J., & Wennemar, A. (2000). Liquid chromatographic method for determination of patulin in clear and cloudy apple juices and apple puree: Collaborative study. *Journal of AOAC International*, *83*(6), 1387–1394.

Madbouly, A. K., Abo Elyousr, K. A. M., & Ismail, I. M. (2020). Biocontrol of Monilinia fructigena, causal agent of brown rot of apple fruit, by using endophytic yeasts. *Biological Control*, *144*(February), 104239. https://doi.org/10.1016/j.biocontrol.2020.104239

Mahato, D. K., Kamle, M., Sharma, B., Pandhi, S., Devi, S., Dhawan, K., Selvakumar, R., Mishra, D., Kumar, A., Arora, S., Singh, N. A., & Kumar, P. (2021). Patulin in food: A mycotoxin concern for human health and its management strategies. In *Toxicon* (Vol. 198, pp. 12–23). Elsevier Ltd. https://doi.org/10.1016/j.toxicon.2021.04.027

Marín, S., Mateo, E. M., Sanchis, V., Valle-Algarra, F. M., Ramos, A. J., & Jiménez, M. (2011). Patulin contamination in fruit derivatives, including baby food, from the Spanish market. *Food Chemistry*, *124*(2), 563–568. https://doi.org/10.1016/j.foodchem.2010.06.072

Matossian, M. K. (1981). Mold poisoning: An unrecognized english health problem, 1550–1800. *Medical History*, *25*(1), 73–84. https://doi.org/10.1017/S0025727300034116

Moake, M. M., Padilla-Zakour, O. I., & Worobo, R. W. (2005). Comprehensive review of patulin control methods in foods. *Comprehensive Reviews in Food Science and Food Safety*, *4*(1), 8–21. https://doi.org/10.1111/j.1541-4337.2005.tb00068.x

Murugesan, G. R., Ledoux, D. R., Naehrer, K., Berthiller, F., Applegate, T. J., Grenier, B., Phillips, T. D., & Schatzmayr, G. (2015). Prevalence and effects of mycotoxins on poultry health and performance, and recent development in mycotoxin counteracting strategies. *Poultry Science*, *94*(6), 1298–1315. https://doi.org/10.3382/ps/pev075

Nasri, T., Hedayati, M. T., Abastabar, M., Pasqualotto, A. C., Armaki, M. T., Hoseinnejad, A., & Nabili, M. (2015). PCR-RFLP on β-tubulin gene for rapid identification of the most clinically important species of Aspergillus. *Journal of Microbiological Methods*, *117*, 144–147. https://doi.org/10.1016/j.mimet.2015.08.007

Navale, V., Vamkudoth, K. R., Ajmera, S., & Dhuri, V. (2021). Aspergillus derived mycotoxins in food and the environment: Prevalence, detection, and toxicity. *Toxicology Reports*, *8*, 1008–1030. https://doi.org/10.1016/j.toxrep.2021.04.013

Nazari, L., Pattori, E., Manstretta, V., Terzi, V., Morcia, C., Somma, S., Moretti, A., Ritieni, A., & Rossi, V. (2018). Effect of temperature on growth, wheat head infection, and nivalenol production by Fusarium poae. *Food Microbiology*, *76*, 83–90. https://doi.org/10.1016/j.fm.2018.04.015

Northolt, M. D., Van Egmond, H. P., & Paulsch, W. E. (1979). Patulin Production by Some Fungal Species in Relation to Water Activity and Temperature. *Journal of Food Protection*, *42*(6), 485–490. https://doi.org/10.4315/0362-028x-42.6.485

Obafemi, B. A., Adedara, I. A., Delgado, C. P., Obafemi, O. T., Aschner, M., & Rocha, J. B. T. (2025). Fumonisin B1 neurotoxicity: Preclinical evidence, biochemical mechanisms and therapeutic strategies. *Toxicology Reports*, *14*(January). https://doi.org/10.1016/j.toxrep.2025.101931

OECD, O. for E. C. and D. (2015). Environment directorate. Joint meeting of the chemicals committee and the working party on chemicals, pesticides and biotechnology. In *OECD Environment, Health and Safety Publications Series on the Safety of Manufactured Nanomaterials* (Issue July).

Oufensou, S., Ul Hassan, Z., Balmas, V., Jaoua, S., & Migheli, Q. (2023). Perfume Guns: Potential of Yeast Volatile Organic Compounds in the Biological Control of Mycotoxin-Producing Fungi. *Toxins*, *15*(1), 1–22. https://doi.org/10.3390/toxins15010045

Ouyang, B., Zhang, W., Guang, C., Xu, W., & Mu, W. (2024). Opportunities and challenges in developing promising mycotoxin-degrading enzymes with high thermostability and strong pH activity/stability. *Food Bioscience*, *62*(October), 105239. https://doi.org/10.1016/j.fbio.2024.105239

Pan, C., Wei, C., Wang, X., Jin, Y., & Tian, F. (2025). Patulin-degrading enzymes sources, structures, and mechanisms: A review. *International Journal of Biological Macromolecules*, *291*(168), 139148. https://doi.org/10.1016/j.ijbiomac.2024.139148

Paterson, R. R. M., Archer, S., Kozakiewicz, Z., Lea, A., Locke, T., & O'Grady, E. (2000). A gene probe for the patulin metabolic pathway with potential for use in patulin and novel disease control. *Biocontrol Science and Technology*, *10*(4), 509–512. https://doi.org/10.1080/09583150050115098

Paterson, R. R. M., Kozakiewicz, Z., Locke, T., Brayford, D., & Jones, S. C. B. (2003a). Novel use of the isoepoxydon dehydrogenase gene probe of the patulin metabolic pathway and chromatography to test penicillia isolated from apple production systems for the potential to

contaminate apple juice with patulin. *Food Microbiology*, *20*(3), 359–364. https://doi.org/10.1016/S0740-0020(02)00087-4

Patriarca, A. (2019). Fungi and mycotoxin problems in the apple industry. *Current Opinion in Food Science*, *29*, 42–47. https://doi.org/10.1016/j.cofs.2019.08.002

Paz-Cuadra, A., Guzmán-Pantoja, L. E., Martínez-Peniche, R. Á., Caltzontzin-Fernández, C. K. K., Pacheco-Aguilar, J. R., & Arvizu-Medrano, S. M. (2014). Evaluation of the susceptibility of apple genotypes in Queretaro, Mexico to powdery mildew (Podosphaera leucotricha). *Scientia Horticulturae*, *170*, 53–60. https://doi.org/10.1016/j.scienta.2014.01.044

Pec, G. J., van Diepen, L. T. A., Knorr, M., Grandy, A. S., Melillo, J. M., DeAngelis, K. M., Blanchard, J. L., & Frey, S. D. (2021). Fungal community response to long-term soil warming with potential implications for soil carbon dynamics. *Ecosphere*, *12*(5). https://doi.org/10.1002/ecs2.3460

Perrone, G., Susca, A., Cozzi, G., Ehrlich, K., Varga, J., Frisvad, J. C., Meijer, M., Noonim, P., Mahakamchanakul, W., & Samson, R. A. (2007). Biodiversity of Aspergillus species in some important agricultural products. *Studies in Mycology*, *59*, 53–66. https://doi.org/10.3114/sim.2007.59.07

Pinedo, C., Wright, S. A. I., Collado, I. G., Goss, R. J. M., Castoria, R., Hrelia, P., Maffei, F., & Durán-Patrón, R. (2018). Isotopic Labeling Studies Reveal the Patulin Detoxification Pathway by the Biocontrol Yeast Rhodotorula kratochvilovae LS11. *Journal of Natural Products*, *81*(12), 2692–2699. https://doi.org/10.1021/acs.jnatprod.8b00539

Piqué, E., Vargas-Murga, L., Gómez-Catalán, J., Lapuente, J. de, & Llobet, J. M. (2013). Occurrence of patulin in organic and conventional apple-based food marketed in Catalonia and exposure assessment. *Food and Chemical Toxicology*, *60*, 199–204. https://doi.org/10.1016/j.fct.2013.07.052

Puel, O., Galtier, P., & Oswald, I. P. (2010). Biosynthesis and toxicological effects of patulin. *Toxins*, *2*(4), 613–631. https://doi.org/10.3390/toxins2040613

Qin, S., Hu, F., Kong, D., Zhao, T., Suo, Y., & He, F. (2024). Evaluation of a phenolic-tolerant Wickerhamomyces anomalus strain for efficient ethanol production based on omics analysis. *Energy*, *311*(October), 133479. https://doi.org/10.1016/j.energy.2024.133479

Rahimi, E., & Rezapoor Jeiran, M. (2015). Patulin and its dietary intake by fruit juice consumption in Iran. *Food Additives and Contaminants: Part B Surveillance*, *8*(1), 40–43. https://doi.org/10.1080/19393210.2014.967814

Reddy, K. R. N., Spadaro, D., Lore, A., Gullino, M. L., & Garibaldi, A. (2010). Potential of patulin production by Penicillium expansum strains on various fruits. *Mycotoxin Research*, *26*(4), 257–265. https://doi.org/10.1007/s12550-010-0064-5

Richard, J. L. (2007). Some major mycotoxins and their mycotoxicoses-An overview. *International Journal of Food Microbiology*, *119*(1–2), 3–10. https://doi.org/10.1016/j.ijfoodmicro.2007.07.019

Richard, J. L. (2008). Discovery of aflatoxins and significant historical features. *Toxin Reviews*, *27*(42067), 171–201. https://doi.org/10.1080/15569540802462040

Rodrigues, M. H. P., Marques, C. G., de Almeida Senna, C., Cerqueira, M. B. R., Kupski, L., & Furlong, E. B. (2022). Patulin in tomatoes and the inhibitory capacity of their phenolic extracts against Penicillium expansum. *Food Research International*, *162*(October), 1–7. https://doi.org/10.1016/j.foodres.2022.112085

Shruthi, B., Deepa, N., Somashekaraiah, R., Adithi, G., Divyashree, S., & Sreenivasa, M. Y. (2022). Exploring biotechnological and functional characteristics of probiotic yeasts: A review. In *Biotechnology Reports* (Vol. 34). Elsevier B.V. https://doi.org/10.1016/j.btre.2022.e00716

Snini, S. P., Tadrist, S., Laffitte, J., Jamin, E. L., Oswald, I. P., & Puel, O. (2014). The gene PatG involved in the biosynthesis pathway of patulin, a food-borne mycotoxin, encodes a 6-methylsalicylic acid decarboxylase. *International Journal of Food Microbiology*, *171*, 77–83. https://doi.org/10.1016/j.ijfoodmicro.2013.11.020

Snini, S. P., Tannous, J., Heuillard, P., Bailly, S., Lippi, Y., Zehraoui, E., Barreau, C., Oswald, I. P., & Puel, O. (2015). *Patulin is a cultivar-dependent aggressiveness factor favouring the colonization of apples by Penicillium expansum*. 1–11.

Soliman, S., Li, X. Z., Shao, S., Behar, M., Svircev, A. M., Tsao, R., & Zhou, T. (2015). Potential mycotoxin contamination risks of apple products associated with fungal flora of apple core. *Food Control*, *47*, 585–591. https://doi.org/10.1016/j.foodcont.2014.07.060

Strickland, D. A., Hodge, K. T., & Cox, K. D. (2021). An Examination of Apple Powdery Mildew and the Biology of Podosphaera leucotricha from Past to Present. *Plant Health Progress*, *22*(4), 421–432. https://doi.org/10.1094/PHP-03-21-0064-RV

Sun, H., He, Z., Xiong, D., & Long, M. (2023). Mechanisms by which microbial enzymes degrade four mycotoxins and application in animal production: A review. *Animal Nutrition*, *15*, 256–274. https://doi.org/10.1016/j.aninu.2023.09.003

Tannous, J., El Khoury, R., Snini, S. P., Lippi, Y., El Khoury, A., Atoui, A., Lteif, R., Oswald, I. P., & Puel, O. (2014). Sequencing, physical organization and kinetic expression of the patulin biosynthetic gene cluster from Penicillium expansum. *International Journal of Food Microbiology*, *189*, 51–60. https://doi.org/10.1016/j.ijfoodmicro.2014.07.028

Teixeira Souza, K. S., Gudiña, E. J., Schwan, R. F., Rodrigues, L. R., Dias, D. R., & Teixeira, J. A. (2018). Improvement of biosurfactant production by Wickerhamomyces anomalus CCMA 0358 and its potential application in bioremediation. *Journal of Hazardous Materials*, *346*(2018), 152–158. https://doi.org/10.1016/j.jhazmat.2017.12.021

Tóth, B., Török, O., Kótai, É., Varga, M., Toldiné Tóth, É., Pálfi, X., Háfra, E., Varga, J., Téren, J., & Mesterházy, É. (2012). Role of Aspergilli and Penicillia in mycotoxin contamination of maize in Hungary. *Acta Agronomica Hungarica*, *60*(2), 143–149. https://doi.org/10.1556/AAgr.60.2012.2.5

Ul Hassan, Z., Al Thani, R., Atia, F. A., Alsafran, M., Migheli, Q., & Jaoua, S. (2021). Application of yeasts and yeast derivatives for the biological control of toxigenic fungi and their toxic metabolites. *Environmental Technology and Innovation*, *22*. https://doi.org/10.1016/j.eti.2021.101447

Varga, J., Due, M., Frisvad, J. C., & Samson, R. A. (2007). Taxonomic revision of Aspergillus section Clavati based on molecular, morphological and physiological data. *Studies in Mycology*, *59*, 89–106. https://doi.org/10.3114/sim.2007.59.11

Varga, J., Rigó, K., Molnár, J., Tóth, B., Szencz, S., Téren, J., & Kozakiewicz, Z. (2003). Mycotoxin production and evolutionary relationships among species of Aspergillus section Clavati. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, *83*(2), 191–200. https://doi.org/10.1023/A:1023355707646

Vidal, A., Mengelers, M., Yang, S., De Saeger, S., & De Boevre, M. (2018). Mycotoxin Biomarkers of Exposure: A Comprehensive Review. *Comprehensive Reviews in Food Science and Food Safety*, *17*(5), 1127–1155. https://doi.org/10.1111/1541-4337.12367

Vidal, A., Ouhibi, S., Ghali, R., Hedhili, A., De Saeger, S., & De Boevre, M. (2019). The mycotoxin patulin: An updated short review on occurrence, toxicity and analytical challenges. In *Food and Chemical Toxicology* (Vol. 129, pp. 249–256). Elsevier Ltd. https://doi.org/10.1016/j.fct.2019.04.048

Wang, G., Luo, J., Liu, Y., Ouyang, A., Chen, J., & Hu, M. (2025). Assessment of physicochemical and optical properties changes in 'Fuji' apples during Penicillium expansum-induced disease development. *Food Bioscience*, *63*(September 2024), 105755. https://doi.org/10.1016/j.fbio.2024.105755

White, S.I. and Dobson, A. D. W. (2005). *Penicillium expansum isoepoxyedon dehydrogenase (idh) gene, partial cds. In: GenBank . Bethesda (MD): National Center for Biotechnology Information*. https://www.ncbi.nlm.nih.gov/nuccore/DQ084388

Williams, J. H. (2004). Human aflatoxicosis in developing countries_ a review of toxicology, exposure, potential health consequences, and interventions _ The American Journal of Clinical Nutrition _ Oxford Academic. *Am J Clin Nutr*, *February*, 1106–1122.

Wu, F. (2022). Mycotoxin risks are lower in biotech corn. *Current Opinion in Biotechnology*, *78*, 102792. https://doi.org/10.1016/j.copbio.2022.102792

Yang, C., Lingli, C., Meijin, G., Xu, L., jinsong, L., Xiaofeng, L., Zhongbing, C., Xiaojun, T., Haoyue, Z., Xiwei, T., Ju, C., & Yingping, Z. (2021). Application of near-infrared spectroscopy technology in the complex fermentation system to achieve high-efficiency production. *Bioresources and Bioprocessing*, *8*(1). https://doi.org/10.1186/s40643-021-00452-9

Yang, C., Zhang, Z., & Peng, B. (2024). New insights into searching patulin degrading enzymes in Saccharomyces cerevisiae through proteomic and molecular docking analysis. *Journal of Hazardous Materials*, *463*(June 2023), 132806. https://doi.org/10.1016/j.jhazmat.2023.132806

Yang, Q., Pang, B., Solairaj, D., Hu, W., Legrand, N. N. G., Ma, J., Huang, S., Wu, X., & Zhang, H. (2021). Effect of Rhodotorula mucilaginosa on patulin degradation and toxicity of degradation

products. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment*, *38*(8), 1427–1439. https://doi.org/10.1080/19440049.2021.1923821

Yang, X., Zhu, K., Loik, M. E., & Sun, W. (2021). Differential responses of soil bacteria and fungi to altered precipitation in a meadow steppe. *Geoderma*, *384*(October 2020), 114812. https://doi.org/10.1016/j.geoderma.2020.114812

Yilmaz, N., Visagie, C. M., Houbraken, J., Frisvad, J. C., & Samson, R. A. (2014). Polyphasic taxonomy of the genus Talaromyces. *Studies in Mycology*, *78*(1), 175–341. https://doi.org/10.1016/j.simyco.2014.08.001

Yu, X., Zhang, K., Liu, J., Zhao, Z., Guo, B., Wang, X., Xiang, W., & Zhao, J. (2024). Identification and evaluation of an endophytic antagonistic yeast for the control of gray mold (Botrytis cinerea) in apple and mechanisms of action. *Food Microbiology*, *123*(600), 104583. https://doi.org/10.1016/j.fm.2024.104583

Zaied, C., Abid, S., Hlel, W., & Bacha, H. (2013). Occurrence of patulin in apple-based-foods largely consumed in Tunisia. *Food Control*, *31*(2), 263–267. https://doi.org/10.1016/j.foodcont.2012.10.005

Zhang, F., Wang, T., Wang, X., & Lü, X. (2021). Apple pomace as a potential valuable resource for full-components utilization: A review. *Journal of Cleaner Production*, *329*(22), 129676. https://doi.org/10.1016/j.jclepro.2021.129676

Zhao, X., Guo, H., Huang, H., Zheng, M., Zhang, Z., Li, J., Li, C., Yuan, B., Pan, C., & Sun, Z. (2025). Contamination and biotransformation of deoxynivalenol (DON) in common commercial foods: Current status, challenges and future perspectives. *Green Synthesis and Catalysis*, *April*. https://doi.org/10.1016/j.gresc.2025.04.008

Zheng, X., Wei, W., Rao, S., Gao, L., Li, H., & Yang, Z. (2020). Degradation of patulin in fruit juice by a lactic acid bacteria strain Lactobacillus casei YZU01. *Food Control*, *112*(196), 107147. https://doi.org/10.1016/j.foodcont.2020.107147

Zheng, X., Wei, W., Zhou, W., Li, H., Rao, S., Gao, L., & Yang, Z. (2021a). Prevention and detoxification of patulin in apple and its products: A review. *Food Research International*, *140*(196), 110034. https://doi.org/10.1016/j.foodres.2020.110034

Zheng, X., Wei, W., Zhou, W., Li, H., Rao, S., Gao, L., & Yang, Z. (2021b). Prevention and detoxification of patulin in apple and its products: A review. In *Food Research International* (Vol. 140). Elsevier Ltd. https://doi.org/10.1016/j.foodres.2020.110034

Zheng, X., Zheng, L., Xia, F., Wei, W., Wang, S., Rao, S., Gao, L., & Yang, Z. (2022). In vivo evaluation of the toxicity of patulin degradation products produced by Lactobacillus casei YZU01. *Biological Control*, *169*(196), 104878. https://doi.org/10.1016/j.biocontrol.2022.104878

Zhong, L., Carere, J., Lu, Z., Lu, F., & Zhou, T. (2018). Patulin in apples and apple-based food products: The burdens and the mitigation strategies. *Toxins*, *10*(11), 1–30. https://doi.org/10.3390/toxins10110475

Zhu, R., Yu, T., Guo, S., Hu, H., Zheng, X., & Karlovsky, P. (2015). Effect of the yeast Rhodosporidium paludigenum on postharvest decay and patulin accumulation in apples and pears. *Journal of Food Protection*, *78*(1), 157–163. https://doi.org/10.4315/0362-028X.JFP-14-218

9 LIST OF PUBLICATIONS

IF Articles

Rodrigues, E. L., Zalán, Z., & Bata-Vidács, I. (2025). First report of *Aspergillus nomius* isolation from Hungarian apple: An emerging mycotoxigenic threat in Central Europe. *Acta Alimentaria*. https://doi.org/10.1556/066.2025.00158

Bata-Vidács, I., **Rodrigues, E. L**., Kosztik, J., Tóth, Á., Zalá., Zs., Csernus, O. & Kukolya, J. (2024). Molecular and chemical evaluation of patulin production of *Aspergillus* and *Penicillium*-like species isolated from Hungarian apples. *Food Additives and Contaminants - Part A*, 41(8), 990-1002. https://doi.org/10.1080/19440049.2024.2364364

Rodrigues, E. L., Fonseca, B. C., Gelli, V. C., de Carli, S., Meleiro, L. P., Furriel, R. D. P. M., & Reginatto, V. (2019). Enzymatically and/or thermally treated macroalgae biomass as feedstock for fermentative H2 production. *Revista Materia*, 24(2). https://doi.org/10.1590/s1517-707620190002.0678

Rodrigues, E. L., Csernus, O., Zalán Z., & Bata-Vidács, I. (under submission). Exploring yeast-based solutions for mycotoxin control: *Wickerhamomyces anomalus* as an effective agent against *Aspergillus clavatus* and patulin mitigation.

Rodrigues, E. L., Baka, E., Ábrahám, R., Suhajda, A., Csernus, O., & Bata-Vidács, I. (under submission). Swift and accurate detection of *Aspergillus* species in agricultural products *via* PCR-RFLP.

Abstract at international conferences

Rodrigues, E. L., Horváth-Szanics, E., Csernus, O., Bata-Vidács, I. (2025). Yeast-based biocontrol: *Wickerhamomyces anomalus* as an effective strategy for reducing patulin contamination. 46th Mycotoxin Conference, May 25-28, 2025, Martina Franca, Italy. Book of Abstracts p. 113.

Rodrigues, E. L., Horváth-Szanics, E., Csernus, O., Zalán, Zs., Bata-Vidács, I. (2025). Yeast-based biocontrol: *Wickerhamomyces anomalus* as an effective strategy for reducing patulin contamination. MTA, Élelmiszer-Biotechnológiai Munkabizottság, 6th June, 2025, Budapest, Hungary.

Rodrigues, E. L., Csernus, O., & Bata-Vidács, I. (2024). Isolation of moulds in Hungarian apples: Emerging contamination risks from mycotoxin producers uncommon in Central Europe CEFood 2024 Conference, 13-16. October 2024, Szeged, Hungary. Book of Abstracts, p. 28..

Rodrigues, E. L., Horváth-Szanics, E., Bata-Vidács, I., & Csernus, O. (2024). Evaluation of agitation effect on patulin production of two *Aspergillus clavatus* strains isolated from apples 45th Mycotoxin Workshop, 2-5. June 2024. Vienna, Austria. Book of Abstracts, p. 100.

Rodrigues, E. L., Kosztik, J., Tóth, Á., Csernus, O., & Bata-Vidács, I. (2024). Isolation of moulds and evaluation of their patulin production potential from Hungarian apples. FIBOK-2024, 6th National Conference of Young Biotechnologists, 4-5. April 2024, Martonvásárhely, Hungary. Book of Abstracts, p. 80.

Rodrigues, E. L., Kosztik, J., Tóth, Á., Kukolya, J., & Bata-Vidács, I. (2022). Evaluation of the presence of IDH gene and patulin production of *Aspergillus* and *Penicillium* strains isolated from Hungarian apples. *4th FoodConf - International Conference on Food Science and Technology*, 10-11. June 2022, Budapest, Hungary. Book of Proceedings, p. 85.

10 SUMMARY OF FIGURES

Figure 1 – *Aspergillus clavatus* under microscope (100x magnification) and on PDA culture medium after 7 days incubation at 37 °C 14
Figure 2 - Metabolic pathway of patulin synthesis for *P. expansum* (Puel et al., 2010). 15
Figure 3 - Main compounds identified after enzymatic degradation of patulin. In which *MnP* indicates manganese peroxidase, *SDR* indicates peroxidase short-chain dehydrogenase/reductase, and *pcCRG* indicates S-adenosylmethionine-dependent methyltransferase (Modified from Pan et al., 2025)..... 21
Figure 4 - Mechanisms by which *W. anomalus* controls the growth of pathogenic microorganisms and molds**.** (Obtained from Godana et al., 2024) 23
Figure 5 - Morphological features of mold isolates for clustering: (A) *Alternaria,* (B) *Aspergillus*, (C) *Penicillium* (microscopic view); (D) *Alternaria*, (E) *Aspergillus*, (F) *Penicillium* (colony morphology on PDA)..... 39
Figure 6- Percentage of contamination by *Aspergillus, Alternaria, Penicillium*, and other molds for A - location and B - farming method 44
Figure 7 - Comparison of the mold loads of bio and non-bio apples regarding the colony numbers (sum), *Alternaria* spp. (Alt), *Aspergillus* spp. (Asp), *Penicillium* spp. (Pen), and other mold species of apple 45
Figure 8 - Weather parameters of the apple cultivation locations in 2022 46
Figure 9 - The effect of annual precipitation on the mold contamination of apples (n = 60 at higher precipitation and n= 39 at lower precipitation) 47
Figure 10 - Presence and absence of the idh gene in *Aspergillus* isolates B4/7, B5/5, B6/12, B6/13, and B9/6 48
Figure 11 - *Aspergillus clavatus* B9/6 colony morphology on malt extract agar (A) and its microscopic image 100x (B) 49
Figure 12 - Presence and absence of the idh gene in *Penicillium* isolates. From left to right: ladder, no template control, positive control (*A. clavatus* B9/6), B4/5, B6/13, B10/6, B12/9, B13/7, B14/2, B14/19, B15/4, B21/6, B27/2, B30/4, B38/3, and B38/10 49
Figure 13 - *Talaromyces pinophilus* B10/6 colony morphology on malt extract agar (A) and its microscopic image 400x (B) 50
Figure 14- Thin layer chromatography for patulin detection – a - patulin standard 50 ppm; b - patulin standard 5 ppm; c -B1/2; d - B1/8; e - B4/7; f - B5/6; g - B6/7; h - B6/12; i - B6/12a; j -B6/13; k -

B7/5; l - B7/6; m - B9/1; n - B9/6; o - B10/4; p -B10/5; the red line indicates the band height for patulin 51
Figure 15 - Thin layer chromatography for patulin detection. a - patulin standard 50 ppm, isolates B4/5, B6/13, B10/6, B12/9, B13/7, B14/2, B14/19, B15/4. The red line indicates the band height for patulin. 52
Figure 16 - Colony morphology of *Aspergillus nomius* B39/1 on different culture media 53
Figure 17 - Thin layer chromatography for aflatoxin production of *A. nomius* B39/1 53
Figure 18 - Fragment profile of β-tubulin gene from Aspergillus spp. digested with XhoI enzyme . 55
Figure 19 -Fragment profile of β-tubulin gene from Aspergillus spp. digested with KpnI enzyme .. 55
Figure 20 - Fragment profile of β-tubulin gene from Aspergillus spp. digested with AlwI enzyme . 56
Figure 21 - Fragment profile of β-tubulin gene from Aspergillus spp. digested with HpaII enzyme 56
Figure 22 - Fragment profile of β-tubulin gene from Aspergillus spp. digested with AluI enzyme .. 56
Figure 23 - Fragment profile of β-tubulin gene digested with AluI enzyme for enviromental samples 58
Figure 24- Fragment profile of β-tubulin gene digested with HpaII enzyme for environmental samples 59
Figure 25 – Morphological characteristics of *A. clavatus* grown in apple juice under different pH and temperature conditions 60
Figure 26 – Petri dishes containing *A. clavatus* and *S. willians* 0034. A- reverse, B- front 63
Figure 27 – Inhibition screening of *A. clavatus* B9/6 using different strains of *W. anomalus* 65
Figure 28 – Inhibition screening of *A. clavatus* B9/6 using different strains of *W. anomalus* by parallel streaks inoculation 66
Figure 29 – Patulin degradation by different strains of *W. anomalus* (Superscripts a,b and c, represent statistically significant differences based on Tukey's test ($p < 0.05$) 68
Figure 30 - Kinetics of patulin degradation by *W. anomalus* 01499 69
Figure 31 – Kinetics of patulin degradation by *W. anomalus* 01655 69
Figure 32 – Morphological characteristics of co-inoculation assays of *A. clavatus* B9/6 and *W. anomalus* 01499 and 01655 71
Figure 33- In vivo assay of inhibition of *A. clavatus* B9/6 using *W. anomalus* 01499. A - Negative control, B- Positive control, C – *W. anomalus* 01499 injected 24 h before *A. clavatus* B9/6, D - *W. anomalus* 01499 injected at the same day as *A. clavatus* B9/6, E - *A. clavatus* B9/6 injected 24 h before *W. anomalus* 01499 73

11 SUMMARY OF TABLES

Table 1 - Proximate and Carbohydrate Composition of Apple Fruit (% dry matter, edible portion) extracted from (OECD, 2015) 6
Table 2 - Major fungal diseases affecting apples: toxin production and pre- or postharvest incidence 8
Table 3 - Overview of major mycotoxins: sources, health effects, and regulatory limits 11
Table 4 - Summary of patulin contamination in different sample types, maximum levels, and previous study locations 16
Table 5 - Apple cultivars, cultivation methods, and locations used in this study. 24
Table 6 - Reference *Aspergillus* strains utilized in this study, with source and corresponding NCBI access numbers. 27
Table 7- Primer sequences used in the study 29
Table 8- Comparison of expected and observed amplicon sizes for *Aspergillus* species based on β-tubulin gene amplification 29
Table 9 - List of yeasts screened for potential antagonistic effect against *A. clavatus* B9/6 32
Table 10 - Mold distribution regarding type of cultivation, location and apple cultivar 41
Table 11 - In silico fragment profile of enzyme digestion for *Aspergillus* spp. 54
Table 12 - Patulin production (ppm) by *A. clavatus* at different pH and temperature values 61
Table 13 - Final patulin concentrations of the co-inoculation assays of *A. clavatus* B9/6 and *W. anomalus* 01499 and 01655 72
Table 14 - Patulin degradation by different cell components of *W. anomalus* 01499 74

12 ACKNOWLEDGMENTS

It is impossible not to begin by thanking my family: my parents, Cida and Reginaldo, who have always encouraged me to follow my dreams and have been my greatest support throughout my life; my sisters, Ewelyn and Stephanie, who accompanied me through the entire process of change and offered a friendly shoulder whenever I needed it; and my niece Lara, who has always been the reason for my joy and brought light to my most difficult days. Even thousands of kilometers apart, between Brazil and Hungary, you were present in every achievement and every challenge, proving that love and true support know no boundaries.

To the friends I made along the way, Carol, Hanna, Lueji, Michelle, Monica,Viktor and Ester as well as many others whose names I will not mention here but who remain in my heart. You shared the past four years with me and became my family, embracing both joys and challenges, and making every moment lighter and more special.

To my supervisors, Dr. Ildikó, Dr. Zsolt, Dr. Ferenc, Dr. Olivia, and Dr. Ákos, I am grateful for the guidance and contributions offered throughout this journey. Even though I experienced different supervision changes along the way, each stage brought distinct perspectives that, in one way or another, contributed to the completion of this work.

To my lab friends, especially Sofia, Stefan and Kristian, I am thankful for the countless conversations, the attentive listening, and the laughter that helped ease the stress and pressure of a PhD. Your presence made the days lighter and the challenges of this journey far less heavy.

I am deeply grateful to my therapist, Gabi, whose guidance and support were fundamental in helping me navigate the emotional challenges of this journey. Her presence and encouragement made a profound difference in sustaining my motivation and well-being throughout the PhD.

I would also like to express my heartfelt gratitude to Dr. Erzsébet Baka, and to Renáta and Akos from the Institute for Aquaculture and Environmental Safety, for welcoming me and guiding me through every step of the molecular biology work. Their support, availability, and encouragement were invaluable during challenging moments, making this journey smoother and more rewarding.

Finally, I wish to thank FruitVeB and the University of Debrecen, especially Dr. Ferenc Apáti, for their support in providing the samples used in this research. I am also grateful to the Stipendium Hungaricum Program and the Tempus Public Foundation for the scholarship that made this work possible. My thanks extend to the Hungarian University of Agriculture and Life Sciences and its Department of Food Science, as well as to the NAIK Institute, where I began my PhD journey.