Elucidation of the interaction mechanisms between Geotrichum candidum and Fusarium spp for a biocontrol optimization to reduce T-2 toxin contamination in the brewing process

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Elucidation of the interaction mechanisms between Geotrichum candidum and Fusarium spp for a biocontrol optimization to reduce T-2 toxin contamination in the brewing process

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List of Abbreviations

AAA: Aromatic Amino Acid
AAT: Aromatic Amino acid Transferase
ABC transporters: ATP Binding Cassettes
ADON: Acetyldeoxynivalenol
AFB1: Aflatoxin B1
AFB2: Aflatoxin B2
AFG1: Aflatoxin G1
AFG2: Aflatoxin G2
AFM1: Aflatoxin M1
AFM2: Aflatoxin M2
ATP: *Adenosine triphosphate*
A_w: Water Activity
BCAs: Biocontrol Agents
CAC: Cereal Agricultural Cooperative
CMC: Carboxymethylcellulose
Da: Dalton
DAS: Diacetoxyscirpenol
DMSO: Dimethylsulfoxyd
DON: Deoxynivalenon
EC: European Commission
EOs: Essential Oils
EU: European Union
FAO: Food and Agriculture Organization of the United Nations
FHB: *Fusarium* Head Blight disease
FPP: Farnesyl Pyrophosphate
FPW: Food Poisoning Whitefly
FUM: Fumonisin
GAPs: Good Agricultural Practices
GRAS: Generally Recognized As Safe (GRAS microorganisms)
HACCP: Hazard Analysis of Critical Control Points
HPLC: High Performance Liquid Chromatography
HPLC-DAD: High Performance Liquid Chromatography equipped with Diode-Array Detector
IARC: International Agency of Research on Cancer
IFBM: French Institute of Brewing and Malting
IgA: Immunoglobulin A
IgG: Immunoglobulin G
IgM: Immunoglobulin M
ILA: Indole Lactic Acid

ITTA: International Institute of Tropical Agriculture
LAB: Lactic Acid Bacteria
LDH: Lactate Dehydrogenase
MEB: Malt Extract Broth
MIC: Minimal Inhibitory Concentration
NIV: Nivalenol
NRPS: Non-Ribosomial Peptide Synthetase
OTA: Ochratoxin A
PAT: Patulin
PDA: Potato Dextrose Agar
PEA or 2-PE: Phenylethyl Alcohol
pH: Hydrogen Potential
Phe: Phenylalanine
PKS: Polyketide synthetase
PKS-NRPS: Polyketide synthetase–Non Ribosomal Peptide Synthetase
PLA: Phenyllactic Acid
PPA: Phenylpyruvic acid
RPM: Rotation Per Minute
RT-PCR: Real-Time Polymerase Chain Reaction
SD: Standard Deviation
SM: Synthetic Medium
Trp: Tryptophan
Tyr: Tyrosine
Ym: Yeast and Malt medium
ZEN: Zearalenone

General Introduction

« Some men crumbled in the street groaning and writhing, others fell down, drooling in fits of epilepsy, others vomited and showed signs of madness. Many of them shouted "Fire! I'm burning!". It was like an invisible fire that separated flesh from bone and consumed it. Men, women and children were dying in excruciating pain. »

Thus was the testimony of a disease, described as "burning sickness", which spread in European countries in 943 (Eskola et al. 2019). It was caused by the consumption of rye flour products that were later found to be contaminated by secondary metabolites produced by the mold *Claviceps purpurea* (van Dongen and de Groot 1995). These secondary metabolites were belatedly designated as mycotoxins.

Mycotoxins are toxic compounds produced by filamentous fungi that can contaminate food and feedstuffs in the field and during storage when the surrounding environmental factors are in optimal conditions (Bennett and Klich 2013).

They have received considerable attention from many scientists over the past decades especially after the “TurkeyX” disease burst out in the United Kingdom in the early 1960s as a result of aflatoxin-contaminated peanut consumption. Cereals and cereal products, milk and dairy derivatives, coffee beans, fresh and dried fruits, nuts and seeds, beverages such as juice, beer and wine are all matrices susceptible to be mold-damaged. This puts both human and animal health in a critical position since the contamination affects the basic elements of their nutrition(Zain 2011; Negedu et al. 2011; Fink-Gremmels 1999; Placinta et al. 1999; Magan and Aldred 2008; Medina et al. 2010; Sanchis and Magan 2004).

Thus, fighting against mycotoxin contamination appears to be a must-win battle. Control attempts have been developed: they range from the implementation of good agricultural practices in the field, the implementation of strategies before and post-harvest, through the application of chemical and physical treatments directly on crops, raw materials or final products. The development of biological approaches has aroused more and more interest since the emergence of the ecological concept. As a matter of fact, several approaches were proved to be effective against fungi spread and consequently mycotoxin production such as the use of essential oils and phenolic compounds and the appliance of none toxigenic microorganisms.

In Europe, regulations defined by the European Commission have been set for mycotoxins of most concern including aflatoxins (B1, B2, G1, G2 and M1), ochratoxin A, deoxynivalenol, fumonisins, zearalenone and patulin in certain food and feedstuff to protect human and animal health (European Commission 2006). However, other mycotoxins such as T-2 and HT- 2 toxins, the main interest of this study are not yet regulated, they are under a European recommendation (2013/165/EU 2013).

T-2 and HT-2 toxins are two of the most toxic members of type-A trichothecenes, mainly produced by *Fusarium* species, aggressive phytopathogenic fungi towards a wide range of

cultivated crops, including barley (Zakaria 2017; "Mycotoxins , Economic and Health Risks, Report No.116" 1989). Over the years, studies have determined seven species mainly present on malting barley: *Fusarium graminearum, F. culmorum, F. poae, F. langsethiae, F. sporotrichioides, F. avenaceum* and *F. tricinctum*. Others *Fusarium* species such as *F. proliferatum, F. equiseti, F. oxysporum and F. sambucinum* can also be found in relatively small amounts, but seem to have less affinity for barley. It is from 2003 to 2007 that populations of *F. poae* decreased sharply, suggesting an exchange of occupation of the biotope. Indeed, according to the observations of the French Institute of Brewing and Malting (IFBM), *F. langsethiae* and *F. sporotrichioides* were gradually established on all french barley from early 2003 until 2007 (Boivin 2009). Up until now, all barley samples contained T-2 and HT-2 toxins at a detectable level (Balzer et al. 2004).

Barley is considered to be the main ingredient used during the beer process. Its quality will systematically affect the quality of the final product. Thus, contamination of barley with *Fusarium* species and T-2/HT-2 toxins implies that the beer is most likely contaminated (Pascari et al. 2018; Morcia et al. 2016a; Bauer et al. 2016).

Fungal contamination on barley can put both human and animal health in danger and is also responsible for gushing; an uncontrolled leakage of wet foam when opening a beer bottle. This phenomenon is due to the presence of fungal hydrophobins produced by *Fusarium species.* Consequently, the economic losses that occur due to contaminated beer products are still incrementing (Fisher et al. 2012).

To reduce the concentration of T-2 and HT-2 toxins during the malting step of the beer production process, the IFBM has developed a biocontrol method. This method is based on the addition of a none harmful filamentous yeast, *Geotrichum candidum,* during malting step to ensure a safer final product (Boivin 1999).

To this day, the full understanding of the interaction mechanisms between *Geotrichum candidum* and *Fusarium* species involved in the toxin production is far from being conclusive. Thus, several questions still arise:

- What is the nature of the interactions between the *G. candidum* and *Fusarium species* leading to the reduction of T-2 toxin concentration?
- Does it affect the toxins' biosynthesis pathway? If not, how does the reduction occur?
- What are the ultimate conditions to cultivate *G. candidum* to be the biocontrol able to reduce the toxin concentration as efficiently as possible?
- How to improve the protocol for adding *G. candidum* during the malting step in order to optimize the reduction of the T-2/HT-2 concentration in the final product?

This project is the continuum of a previous ANR BARESAFE project held in the Chemical Engineering Laboratory based in Toulouse, France from 2009 till 2012 (Gastélum-Martinez

2012). During the previous work, the focus was held on T-2 toxin solely and several elements were demonstrated, the main ones being the following:

- During co-cultures between *G. candidum* and *F. langsethiae*, the T-2 toxin concentration drastically decreases but the fungal growth was not affected
- The interaction mechanisms probably occur indirectly. Indeed, in sequential cultures, *G. candidum* produces in the culture medium an agent that reduces the toxin concentration produced by *Fusarium* spp.
- Several thermal and enzymatic tests showed that the concerned agent is thermostable and of none protein nature.

Based on these data, the current study aims to elucidate the interaction mechanisms between two *Fusarium* species, *F. langsethiae* and *F. sporotrichioides* and *G. candidum* to better understand and improve this approach. To bring more elements to this survey, the effectiveness of this biocontrol was tested against two main barley contaminants of the *Fusarium* genus, *F. langsethiae* and *F. sporotrichioides.* Testing the direct and indirect interactions will help to better acknowledge the inhibition phenomenon of *G. candidum* on fungi. In the case of *G. candidum*, very few studies focused on the production of such metabolites. Thus, researching the presence of these or similar molecules with similar effects consists of an important task to clarify the interaction phenomenon. Subsequently, it is important to identify the culture conditions most favorable to *G. candidum* which allow a better use of this agent and guarantee the reduction of T-2 toxin concentration.

All these elements of the study will assist to comprehend the components of this preventive approach and hence contribute to optimizing the implementation of this biocontrol on an up-scaled level. The conditions of the micro-malting will be simulated to provide an actual malting environment where the presence of the two *Fusarium* species, *G. candidum*, the levels of the biocontrol agent and T-2 toxin will be monitored.

Bibliographic Review

To better orient the objectives and the experimental work of this project, a study of the various bibliographical studies was carried out.

The scientific context is divided into three chapters. The first chapter contains an introduction to the mycotoxin world and will firstly collect general data on the mycotoxins of greatest concern, their structure, the type of foodstuffs that each molecule contaminates, and will slightly present their impact on both health and economy. In addition, it will address strategies for combating these mycotoxins by exposing the various means of control already in place to prevent or reduce their occurrence in foodstuffs.

In chapter two, the focus will then be narrowed down to one particular food commodity, barley kernels used as a main ingredient in the brewing process and their contamination with two *Fusarium* species, *F. langsethiae* and *F. sporotrichioides*. These filamentous fungi are T-2 and HT-2 toxins producers, the most studied type A trichothecene. The beer making process will be first presented, then, each step having a potential risk of fungal and mycotoxin contamination, will be reported.

The last chapter will highlight the filamentous yeast *G. candidum,* previously identified as a biocontrol agent used during the malting process. It will also present the characteristics of this microorganism, and its ability to reduce T-2 toxin concentration in beer and more specifically during the malting step. The use of *G. candidum* as an alternative strategy to control T-2 toxin is the core of this thesis.

Chapter 1 - Introduction to the mycotoxin issue and its management system

Part I - Filamentous fungi and their mycotoxins: frequent food contaminants

A. Fungi: microscopic filamentous microorganisms

Filamentous fungi are eukaryotic microorganisms belonging to the *Fungi* kingdom, which means mushroom in Latin. This kingdom includes several species of the Ascomycetes *phylum* such as *Aspergillus*, *Fusarium* and *Penicillium*. Reproduction in these species can be sexual or asexual and the life cycle pattern in fungi is represented in figure 1. The asexual reproduction is characterized by the formation of conidia (also called spores) capable of germinating into the mycelium form. Sexual reproduction involves mycelium of opposite sexual types which contribute to the formation of hyphae containing ascospores. These ascospores, once mature, are secreted and germinate to form the mycelium.

These filamentous fungi grow on a wide range of foods and need a range of nutrients and growth factors to be able to proliferate. The presence of carbon and nitrogen sources is therefore essential, as well as a certain quantity of minerals (sulfur, potassium, magnesium, etc.), for the proper functioning of cell metabolism. Indeed, fungi ensure their subsistence by degrading the organic carbon present in the medium to transform it into a mineral substance which will be directly assimilated by absorption. Besides, the accessibility of the water in the environment plays a key role in the fungal growth: a minimum water activity of 0.65; a favorable temperature specific to each fungus and an isotonic pH are also required. Several vectors (air, animals, humans, agricultural products easily transport spores…) leading their wide occurrence in nature.

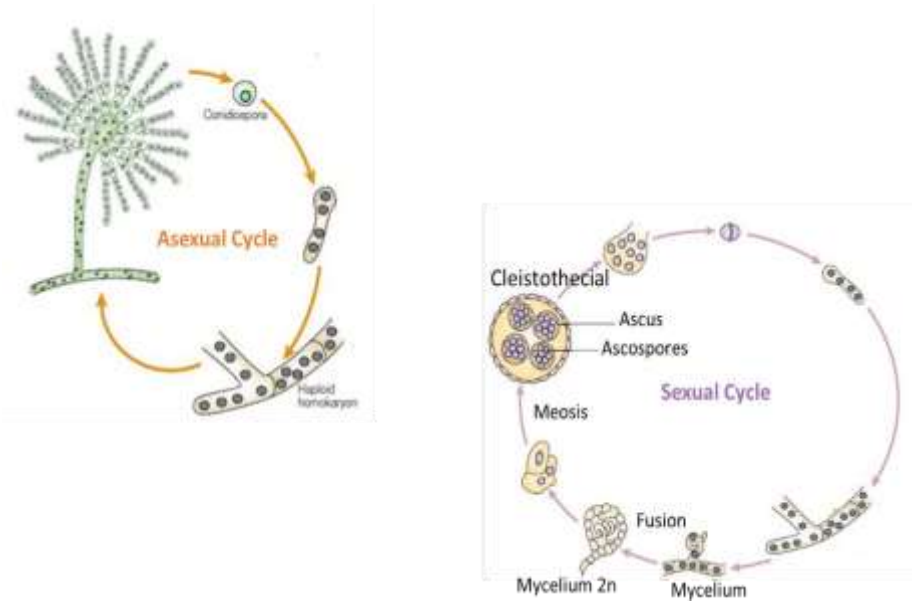


Figure 1 - Life cycle pattern in fungi

A remarkable metabolite diversity characterizes these microorganisms hence the interest of their use in different industrial fields: food, pharmacological, cosmetics ... Depending on the availability of nutritional elements and environmental conditions, molds are capable of producing many secondary metabolites. The role of these secondary metabolites is not still elucidated but they could facilitate the substrate colonization by the fungus when it competes with other species in the surrounding environment. These secondary metabolites can have beneficial effects for human health, such as antibiotics, but can also exhibit toxic properties such as mycotoxins. Under favorable conditions, filamentous fungi can grow on a wide variety of substrates, thus they can contaminate a wide range of matrices foodstuffs (vegetable products such as fruits, juices, wines, cereal products and their derivatives such as barley, beer and bread, products of animal origin such as meat and milk).

B. Occurrence of the main mycotoxins, toxic effects and economic impact

a. Mycotoxins and their occurrence

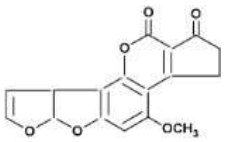
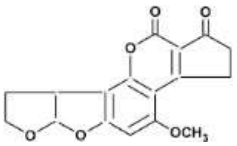
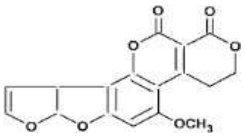
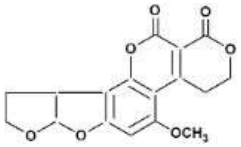
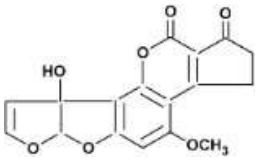
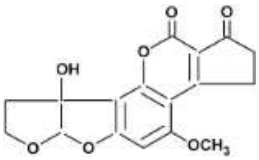
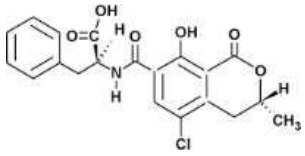
The term mycotoxin reveals in itself its definition: *mykos* in Greek means fungus and *toxicum* in Latin gives it a toxic appearance and therefore refers to poison (Jouany et al. 2005).

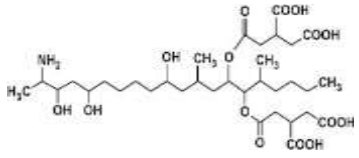
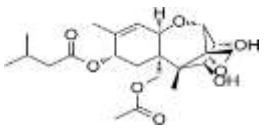
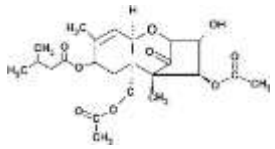
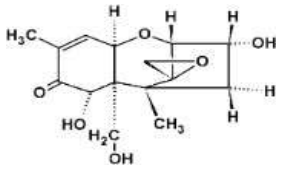
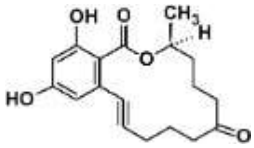
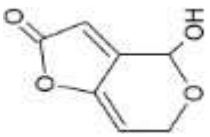
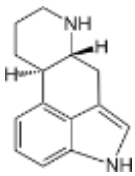
These toxic molecules are characterized by a low molecular weight, approximately less than 1000 Da and may have different chemical structures depending on the type of producing microorganism and its surrounding environment. To date, there are four different types of structures, polyketides (PKS), terpenes, alkaloids (NRPS) and hybrids (PKS-NRPS) (Boettger and Hertweck 2013). This depends mainly on the metabolic pathway from which it originates.

Their presence in raw materials is mainly due to bad agricultural practices associated with favorable climatic conditions (especially temperature and high humidity) leading to fungal development. Mycotoxin contamination can occur directly in the fields, during harvest or post-harvest stages (such as storage and processing). This implies that the presence of mycotoxins can be detected in raw materials, processed products and their derivatives. Indeed, mycotoxins have a very high thermal resistance, which explains their persistence in foodstuffs even after treatments at high temperatures (cooking, sterilization). Despite the diversity of contaminated substrates, each region is characterized by different climate conditions, cultivation techniques and agricultural customs, and thus by different types of contaminating molds.

Regardless of the diversity of mycotoxins (more than 300 mycotoxins identified to date), the regulations concern only a few, classified as the most toxic for humans and animals: aflatoxins, ochratoxin A (OTA), fumonisins, T-2/HT-2 toxins (type A trichothecenes) and deoxynivalenol (DON - type B trichotecenes), zearalenone (ZEN) and patulin (Paterson and Lima 2010). Their chemical structures are shown in table 1 along with the filamentous fungi that are susceptible to produce them.

Table 1 - The Chemical structures of major concern mycotoxins and their respective producing fungi

Mycotoxins of greatest concern		Molecule structure	Major producing fungi
Aflatoxins	Aflatoxin B1 (AFB1)		*Aspergillus flavus* *A. parasiticus* *A. nomius*
	Aflatoxin B2 (AFB2)		
	Aflatoxin G1 (AFG1)		
	Aflatoxin G2 (AFG2)		
	Aflatoxin M1 (AFM1)		
	Aflatoxin M2 (AFM2)		
Ochratoxin A			*A. westerdijkiae* *A. carbonarius* *Penicillium verrucosum*

Fumonisin B1			*Fusarium verticillioides* *F. proliferatum*
Tricothecenes	Type A T-2/HT-2	 	*F. poae* *F. sporotrichioides* *F. langsethiae* *F. tricinctum*
	Type B Deoxynivalenol (DON)		*F. graminearum* *F. culmorum* *F. crookwellens*
Zealarenone (ZEN)			*Fusarium species*
Patulin (PAT)			*P. expansum* *A. clavatus* *Byssochlamys nivea*
Ergot alkaloids **(exemple de l'ergoline)**			*Claviceps* spp, *Neotyphodium* spp *Aspergillus* spp *and* *Penicillium* spp

The world map presented in figure 2 demonstrates the occurrence of mycotoxins in each region of the world in 2020. Results on the occurrence of mycotoxins in 9,618 in finished feed and raw commodities samples from 68 countries based on 42,505 analyses conducted between January and June 2020. The distribution and occurrence of various mycotoxins differ from one region to another due to different weather conditions and agricultural practices. For instance, North America recorded the highest amounts of DON (81%), whereas FUM (97%) remains the main threat in Central and South America, Southern Europe, East Asia including China and Taiwan. DON is the major mycotoxin detected in Northern and Central Europe (85%) along with the Middle East and North Africa. Finally, OTA and ZEN were detected in most Middle Eastern African cultivars respectively. Each of these mycotoxins is associated with its

own food matrices and its consumption affects both animal and human health.

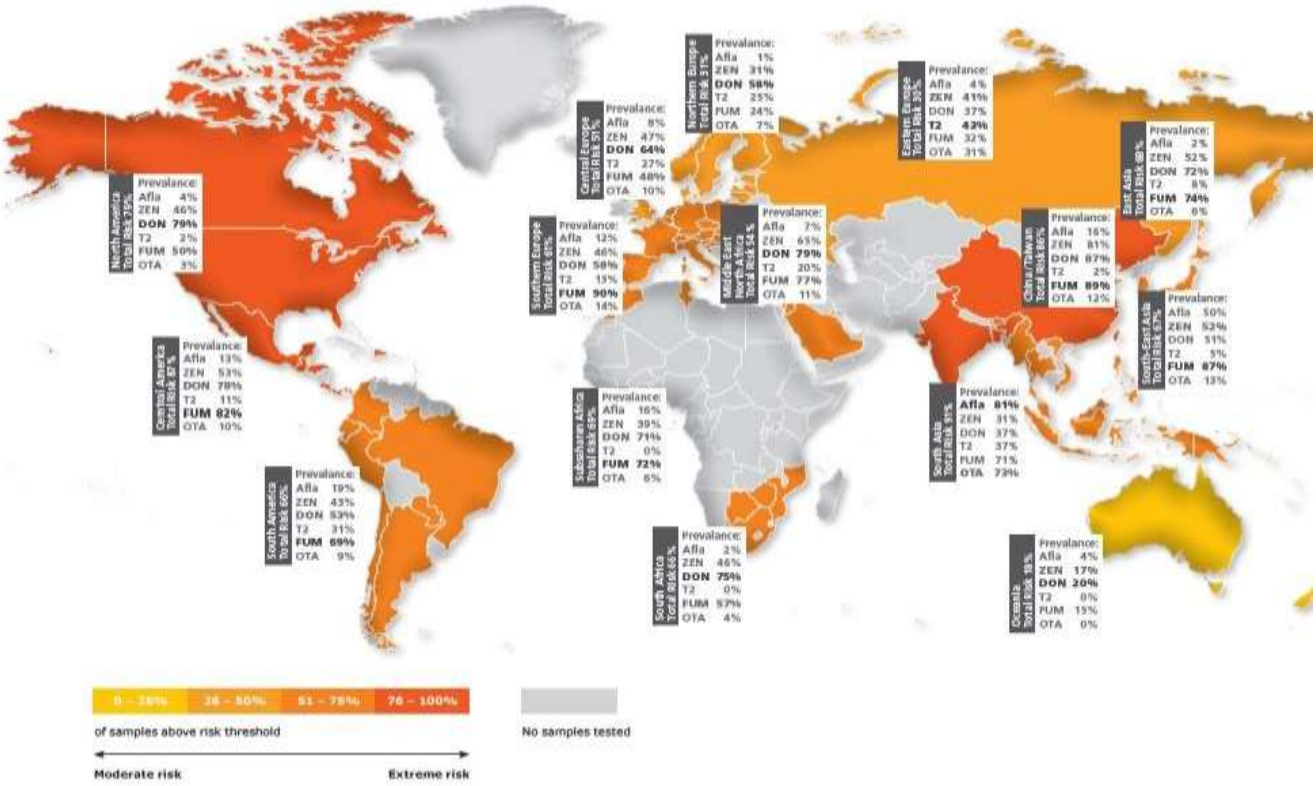


Figure 2 - Map of mycotoxin occurrence worldwide (BIOMIN Mycotoxin Survey - Worldwide mycotoxin threats in 2020). None tested regions are represented in grey.

b. Contaminated food matrices and toxic effects

Accumulation of mycotoxins in foodstuffs can affect the health of humans as well as animals. Indeed, exposure to mycotoxins occurs as a result of consumption of food contaminated with molds and/or persisting mycotoxins of the authorized doses or as a result of the consumption of products derived from contaminated animals, in particular meat, milk and eggs, containing mycotoxins ingested by these animals. Syndromes caused by inhalation or ingestion of mycotoxins by humans or animals, are called "mycotoxicoses" (AFSSA 2009). One of the most important mycotoxicosis is known as "ergotism", defined as a result of poisoning, following long-term ingestion of the alkaloids derived from rye ergot, *Claviceps purpurea*, a fungus that infects the rye and other cereals. The ergotism is known since the 1750s as the "St. Anthony's Fire", became epidemic in Central Europe during the Middle Ages. Another example of mycotoxicosis is the food poisoning whitefly (FPW), a historical disease causing the death of thousands of Russians in 1940, severe fevers, bleeding from the skin, nose and gums, as well as necrosis and suppression of the immune system with mortality rates as high as 80% (Nelson et al. 1994). Later, in the 1960s, aflatoxin B1 was discovered as a result of the death of 100,000 turkeys in Europe (Bennett and Klich 2013). In table 2 is presented the diversity of mycotoxins

associated with its targeted food matrices, its effect on human health and their tolerated limits according to the European regulations (Commission and Communaut 2006).

Table 2 - Main mycotoxins and their associated effects on health, and contaminated foodstuffs

Mycotoxin	Effects on human health	Contaminated food matrices	European regulation (µg/kg)	References
Aflatoxins	Liver-related diseases (hepatotoxic, hepato-carcinogenic), carcinogenic and teratogenic effects, haemorrhages (intestinal tract, kidney), immune suppression.	Peanuts, hazelnuts, cereals and cereal products, milk and milk products, spices…	2–12 for AFB1 4–15 for total 0.05 in milk and 0.025 for AFM1 in infant formula and infant milk	(Lunn et al. 1999; Leong et al. 2010)
Ochratoxin A	Nephrotoxic, carcinogenic, immune suppression	Cereals (wheat, corn), wine, grape berries, grape juice	2 – 10	(Heussner and Bingle 2015; Studer-Rohr et al. 1995)
Fumonisins	Pulmonary epiedemas, nephro- and hepatotoxic, immune suppression	Corn Maize, maize, products,sorghum, asparagus	200 – 1000	(Li and Guo 1994)
Tricothecenes	Digestive disorders (vomiting, diarrhea), weight loss, bleeding (stomach, heart, intestine, lungs, bladder), oral lesions, dermatitis, infertility, bone marrow degeneration, immune suppression.	Cereals (wheat, barley)	Type A: No regulations for T-2/HT-2 toxins, only recommendations (2013/165/UE) Type B: 200 – 50 (DON)	(Arunachalam and Doohan 2013)

Zearalenone	Oestrogenic, vulvar edema, vaginal prolapse, uterine enlargement, testicular atrophy, ovarian atrophy, infertility, abortion	Cereals, cereal products,maize, wheat, barley	20 – 100	(Arunachalam and Doohan 2013; Zinedine et al. 2007)
Patulin	Mutagenic, genotoxic, carcinogenic	Fruits (apple, pear) Apples, apple juice,and concentrate	10 – 50	(Ramos and Sanchis 2009; Mahfoud et al. 2002)
Rye Ergots	Gangrene, hallucinations, convulsions...	Rye	0.5g/kg (2015/1940/UE)	(Alderman et al. 2007)

Mycotoxin toxicity can be of two types: acute toxicity or chronic toxicity. Acute diseases are due to the consumption of one high dose of mycotoxin leading to clinical symptoms whereas chronic diseases are due to regular consumption of a minor amount of mycotoxin over a long period time, causing over time, the appearance of the disorders insidiously (Alderman et al. 2007). Certainly, the acute and chronic diseases caused by these mycotoxins depend on the concentration and exposure frequency of the latter and especially on the degree of toxicity specific to each mycotoxin. In animals, acute diseases are related to kidney and liver damage, attacks on the central nervous system, and skin and hormonal system diseases. However, chronic diseases manifested mainly by genotoxicity, cytotoxicity, mutagenicity and immunotoxicity, present real concerns for human health. To better assess the risks of each mycotoxin, the International Agency for Research on Cancer (IARC) has classified mycotoxins into several groups according to the degree of carcinogenicity of each. Thus, AFB1 has been classified as a human carcinogen in Group 1. OTA and fumonisins are part of Group 2B, which includes "potentially carcinogenic" agents. Trichothecenes, patulin and zearalenone, are classified in Group 3 as non-carcinogenic to humans (IARC 1993b).

c. Economic impact

The economic impact of the contamination of foodstuffs with mycotoxins is considerable in both industrialized and developing countries and is particularly significant in warm and humid regions (Maaroufi et al 1995; Miraglia and Brera, 2000; Beretta et al, 2002) where fungal contaminations are a major concern. It results in a decrease in the monetary value of contaminated crops. The existence of maximum authorized levels in foodstuffs, set by more

than 100 nations in the world and intended to protect consumers against the harmful effects of these mycotoxins, leads to a very significant decrease (or even destruction) of the market value of lots identified as contaminated by these toxins. According to the Food and Agriculture Organization of the United Nations (FAO), world agricultural production does not meet the needs of the population, and contamination by mycotoxins accentuates this deficiency.

Indeed, a study carried out in 2003 estimates that a loss of US$ 392 million is recorded in the world's agricultural production in the United States each year due to the presence of aflatoxins, fumonisins and DON (CAST 2003; Jouany 2011). Mycotoxin contamination is correlated with temperature and reach their maximum in years considered warm, such as 2012 (Mitchell et al., 2016). At the global level, the FAO estimates that about a quarter of the world's agricultural production is contaminated by mycotoxins (Eskola et al. 2019). According to the International Institute of Tropical Agriculture (ITTA 2013), annual losses due to aflatoxins alone reach 1.2 billion US dollars, with African countries suffering 38% of these losses, i.e. 450 million dollars (Marechera, 2015). Several food chains are affected, including the cereal industry; fruits, nuts and dried fruits; coffee; and their derivatives. Besides, there are also indirect losses, which are more difficult to assess and which are related to the reduced productivity of animals receiving mycotoxin contaminated feed (CAST 2003).

Given the extent of the impact on human and animal health and the economic losses caused by mycotoxins contamination, it is important to control their presence in the food chain. Various strategies can be adopted at different steps of the processing chain to keep mycotoxin risks at their lowest thresholds. Thus, the management of mycotoxin contamination is presented in the following part.

Part II - Management of mycotoxin contamination

To reduce mycotoxin contamination, several strategies can be implemented in fields, during the storage of harvested goods or during industrial processes. These strategies can be of two types. First, preventive methods can act directly on fungal development and/or on mycotoxin production. On the one hand, in the case where mycotoxin production is correlated to the fungal growth, the reduction of fungal growth can lead to the reduction of mycotoxin concentration. On the other hand, the reduction of mycotoxin concentration without impacting fungal growth involves regulatory mechanisms that lead to the down expression of genes encoding the enzymes involved in the mycotoxin biosynthesis pathway. The second category gathered curative methods that consist to reduce mycotoxin concentration in contaminated matrices. Apart from good cultivation, harvesting and storage practices which are exclusively preventive strategies, other preventive and curative methods can be classified in 3 categories: physical, chemical or biological methods. A classification of such strategies is proposed in figure 3.

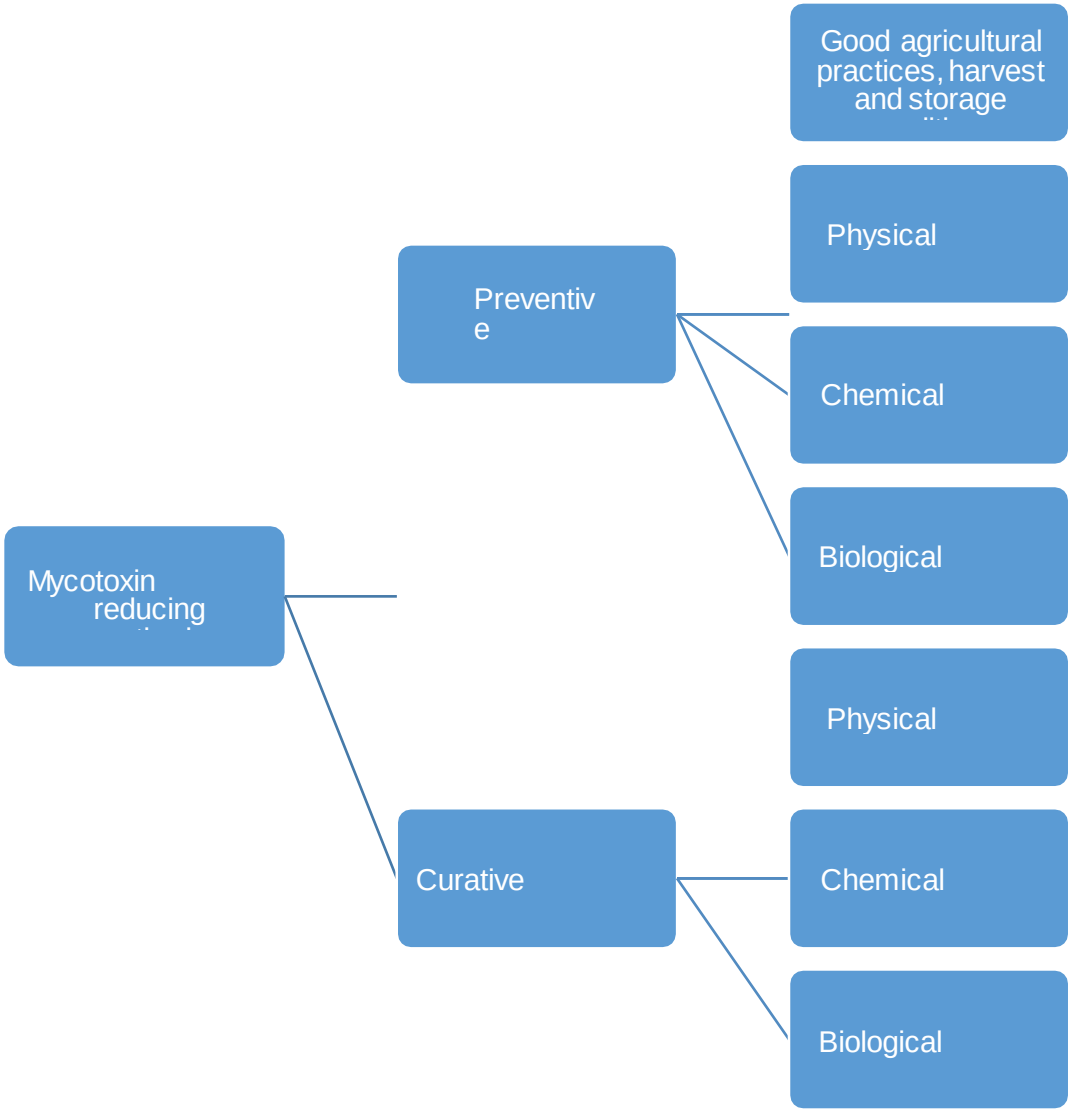


Figure 3 - Mycotoxin reduction strategies (pattern adapted and modified from Mielniczuk andSkwaryło-Bednarz (2020)

A. Preventives approaches

a. Good agricultural practices, harvest and storage conditions

The first critical point in limiting the incidence of filamentous fungi and their toxins is to adopt good agricultural practices to limit the proliferation of fungal spores. Those strategies include pre-plowing the soil before sowing, weeding, respecting the specific sowing period for each type of crop and the optimal time for harvesting, fertilizing, amending and enriching the soil, managing irrigation and rotating crops that are less sensitive to fungal growth (Waliyar et al. 2008; Diao et al. 2014).

The harvest period also plays a key role in preventing fungal development. According to the Cereal Agricultural Cooperative (CAC), it is recommended to collect grains when their physiological maturity has been reached to limit fungal infection (CAC 2004). Otherwise, commodities harvested just after maturity have high moisture levels, which allows fungal development during subsequent storage (Diao et al. 2014; Gerez et al. 2010; Waliyar et al. 2008).

Although agricultural practices in the field and at harvest time are essential to limit contamination by toxinogenic fungi and the production of toxins, storage conditions are also key factors in limiting fungal contamination and mycotoxin production during this period, which can be prolonged. It has even been suggested that outbreaks of aflatoxicosis in Kenya have occurred as a result of post-harvest contamination (Kumar et al. 2017). Indeed, if fungi contaminate crops in the field, under favorable conditions during storage it can rapidly grow and produce significant amounts of mycotoxins (Cardwell 1998). To limit the risk, storage of dried grains should be in a clean, dry place at moderate temperatures and protected from insect infestation. Re-wetting during storage should also be avoided. Re-wetting may result from leaking storage roofs, poor ventilation of silos causing water condensation, or too high an initial moisture content of the grain before storage (Bryła et al. 2016). Similarly, the duration of storage in silos should be as short as possible. Mycotoxin levels can increase by a factor of several times over a period of days under favorable temperature and humidity conditions during storage (Hell et al. 2008).

In recent years, the introduction of the HACCP approach (Hazard Analysis of Critical Control Points) has become widespread to prevent and limit the risks associated with mycotoxins. Since the factors influencing toxin contamination in the field are difficult to predict and control, the critical control points are instead located during the harvesting, drying and storage of agricultural products (CAC 2004).

b. Physical methods

There are not many preventive physical methods. However, the most recommended and practiced is the sorting of kernels before storage (Torres et al. 2014). Indeed, damaged kernels are more vulnerable and thus susceptible to fungal contamination and insect entry (Diao et al. 2014). The insects increase the surface area of the ear susceptible to fungal infection, moisten the kernels through their metabolic activity and thus promote fungal development (Udoh, Cardwell, and Ikotun 2000). The fungus can then grow indoors and is thus sheltered and in direct contact with nutrients (Torres et al. 2014). Several sorting methods can be used. The traditional sorting method is the manual sorting based on the visible abnormalities of kernels, like smaller size, mold spots or color changes, which could be associated with a higher possibility of fungal contamination (Kaaya and Kyamuhangire 2006; Xu et al. 2014). Other sorting methods based on electronic and optical technologies have been also reported as mycotoxin-reducer methods. After sorting methods, the bleaching can also reduce mycotoxin contamination (Njoroge et al. 2016). Bleaching consists of removing the pericarp of the peanuts as well as dust, mold, and other foreign matter and allows better detection of color. The latter technique could reduce aflatoxin levels by up to 80% (Dorner 2008). However, discoloration of kernels does not always imply the presence of contamination (Torres et al. 2014). The greatest limitation of this technique remains its high cost (Dorner 2008).

c. Chemical methods

As chemical methods used as preventive approach to reduce mycotoxin contamination, the use of chemical inputs such as fungicides or insecticides is widespread. Indeed, the obvious effectiveness of certain chemical inputs in controlling the development of various agricultural pathogens and thus increasing productivity has led to the massive use of these products since the 1950s. It now appears that this strategy is reaching its limits for several reasons: environmental contamination and adverse effects on animal and plant biodiversity; loss of effectiveness through the appearance of resistance in target organisms and finally, the specific toxicity of these compounds in mammals during prolonged exposure (Carvalho 2017; Zubrod et al. 2019).

Fungicides have been considered for years as essential to maintain healthy crops with good productivity. The mode of action of these fungicides is mainly to inhibit the fungal growth. In this case, the total or partial elimination of the targeted microorganisms unbalance the natural microbial ecosystem, causing the potential emergence of new microorganisms may be even more dangerous. However, an increasing limitation to the use of fungicides is their inherent toxicity, especially when their use is inappropriate or exceeds recommended standards. For example, a moderately innocuous chemical at low doses could easily become toxic and oncogenic at higher doses (Bawa and Anilakumar 2013). The acute toxicity of fungicides to humans is low. The most common effects of fungicides used in agricultural processes are allergic dermatitis and irritation of the skin and mucous membranes (Schneider and Dickert

2010). In addition, many different fungicide residues can be detected in human serum, urine and adipose tissue (Bawa and Anilakumar 2013).

The grain is frequently infested with insects that spread spores of toxigenic fungi belonging to *Fusarium, Penicillium, Rhizoctonia* and *Aspergillus* species (Hell et al. 2008; Khan et al. 2016). By preventing insect attacks on crops and subsequently in storage, pesticides can reduce the occurrence of spores of fungal species and subsequently the production of mycotoxins (Torres et al. 2014). Several pesticides (Spinosad, Thiamethoxam, Imidacloprid, Indoxacarb…) have been used to protect crops from insects and the correlation with the levels of mycotoxins produced has been assessed. In the different combinations of treatments tested, mycotoxin concentration was correlated with insect survival rates (Khan et al. 2010). A study monitored by Vayias et al. (2010), showed that the efficacy of Spinosad is less important on maize than on the other crops such as wheat and barley, which shows the limits of Spinosad's spectrum of action. Its long-term efficacy is questioned since its effects decrease with storage time. It seems therefore necessary to consider combinations of insecticides, especially if several insect species can coexist in a region, to improve crop protection. However, this raises the question of the combined toxicity of the different compounds used.
Awareness of the possible toxic effects, cost and environmental risk represented by the intensive use of chemical inputs has justified certain measures and limitations to the use of these compounds in Europe in favor of more organic and sustainable crops (Council of the European Communities 1991).

Therefore, it seems important to find alternatives described below to the exclusive use of pesticides to control fungal growth and mycotoxin contamination and thus guarantee food safety while developing sustainable and more environmentally friendly agricultural practices.

d. Biological methods

Biological methods to reduce mycotoxin contamination include the use of natural compounds, macroorganisms and microorganisms. Among them, the use of microorganisms in preventive approaches is of great importance. These microorganisms are called biocontrol agents (BCAs) and can be applied directly in the field to protect crops as a preharvest strategy, during the storage of raw materials or during the production process as a postharvest strategy. These microorganisms, such as lactic acid bacteria, yeasts, actinobacteria or fungi are able to reduce fungal growth but they can also act on mycotoxin biosynthesis pathway to reduce mycotoxins production. Among bacteria, ubiquitous soil bacteria can be used for their antagonistic effects on toxigenic fungi. These bacteria, belong mainly to Bacillus, Pseudomonas, Agrobacterium and Streptomyces genera. They are able to produce a wide number of active compounds reducing fungal growth and mycotoxin production (Ren et al. 2020). Other bacteria, belonging to the lactic acid bacteria (LAB) have also demonstrated effectiveness in inhibiting mycotoxin

production. These bacteria are involved in several food fermentation processes and can act as biocontrol agent in reducing fungal growth and mycotoxin production (Gerez et al. 2010). Concerning the use of fungal strain as preventive methods to reduce mycotoxin contamination, non-toxigenic fungal strains (i.e. mycotoxin non-producer fungal strains) can be applied directly in the field to protect crops. However, this kind of strategy is well described and used only in the management of aflatoxins contamination (Abbas et al. 2006).

In biocontrol strategies, the use of natural compounds from plants as preventive approach is also well described. Green plants produce various secondary metabolites such as phenolic compounds, terpenoids and alkaloids, in order to protect against external aggression (mechanical, biological or climatic). Over the years, these compounds have demonstrated their usefulness against fungal and/or mycotoxin contamination.

For example, essential oils (EOs) discovered and used for centuries in traditional medicine, are plant extracts that can be applied as food preservation and flavoring, as well as in perfumery (Kalemba and Kunicka 2005). Since the Middle Ages, the EOs have been widely used in bactericidal, virucidal, fungicidal, pesticide and insecticide applications (Bakkali and Idaomar 2008). Their antifungal properties have been widely studied over the years. Indeed, in-depth studies are on their ability to inhibit fungal growth and the toxic molecules produced by filamentous fungi. The assessment of the antifungal effect of EOs against toxinogenic fungi has been conducted on several fungal species such as *A. ochraceus* and *A. carbonarius* both OTA producers. The active components of EOs responsible for their antifungal activity are of different chemical natures, such as phenolic compounds (eugenol, dill, carvacrol), terpenes (carvone), aldehydes (cinnamaldehyde) and ethers (thymol) (Nilgun et al. 2004).

Despite their fungicidal and inhibitory properties against many fungal species, the mode of action of EOs is poorly described. However, several potential cellular targets have been identified. Given their richness in bioactive molecules, EOs can act simultaneously on several cellular targets. Their actions can be located at the plasma membrane level of the fungi, at the level of the intracellular enzymes involved in respiration cell and structural protein synthesis, or leading to the repression of genes responsible for mycotoxin biosynthesis (Prakash et al. 2011; Manfredini et al. 2015; Jeršek et al. 2014). The structure of phenolic compounds allows them to penetrate the cell membrane of fungi and interfere with the biosynthetic pathways for ergosterol, glucan, chitin, and structural proteins and glucosamines in filamentous fungi (Sparringa and Owens 1999).

Furthermore, the phenolic compounds can affect fungi that have acquired resistance to antifungal treatments. Indeed, flavonoids, catechin, the chalconoids, xanthones, stilbenes, anthocyanins and tannins, may cross the cell membrane and inhibit the activity of ABC transporters (ATP binding cassettes, which give the fungi their ability to resist the antifungals) (Ansari et al. 2013; Meschini et al. 2003). Other studies show that polyphenols can attach to cellular proteins and subsequently change their tertiary structures, thus reducing the activity of ABC (Wink 2015; Wink 2013).

Medicinal plants and herbs, used as remedies for benign disorders, have been the subject of several studies to better understand the action of their various active compounds against the presence of mycotoxins. Among these active compounds, phenolic compounds appear to have antifungal potential and most interesting anti-toxinogen (Olyneux 2004). In addition, vanillic, gallic and hydroxybenzoic acids, which are considered as antioxidant agents, are capable to reduce mycotoxin production as well as fungal growth. The effect of these phenolic compounds were tested on seven ochratoxinogenic *Aspergillus* species, including *A. alliaceus, A. lanosus, A. albertensis, A. melleus, A. sulphureus, A. elegans,* and A. *sclerotiorum*, and have shown a drastic reduction of OTA concentration. However, mechanisms of action leading to the OTA concentration reduction is not yet elucidated (Palumbo and O'Keeffe 2008).

B. Curative approaches

Curative approaches can be of two types: First, decontamination methods that aim to eliminate or degrade the targeted mycotoxin and second, detoxification methods that correspond to the transformation of the targeted mycotoxin leading to a reduced toxicity. In both cases, these curatives approaches can involve physical, chemical or biological processes.

a. Physical methods

Several physical treatments can be applied to contaminated matrices to reduce mycotoxin concentration. These physical treatments can denature mycotoxins by heat or irradiation or they can eliminate mycotoxin by binding it.

With respect to heat treatment, mycotoxins are heat stable molecules that can survive food processing, including roasting, stirring and cooking (Krogh 1979). For example, a temperature of 100°C reduces OTA contaminating wheat by only 50% even after 10 hours of cooking (Valle-Algarra et al. 2009). UV treatments and γ-irradiation can destroy the conidia of filamentous fungi, but only γ-irradiation is capable of destroying OTA (a dose of 10.0 KGy eliminates 88% of the OTA present in grapes) (Valle-Algarra et al. 2009; Aravantinos and Markaki 2014; Aziz et al 2004). However, irradiation with γ-rays is only authorized if doses do not exceed 10 KGy (Calado and Ven 2014; Stefano et al. 2014).

Other strategies, targeting only animal foodstuff, may aim to reduce the availability of toxins after the ingestion of contaminated feed. Reducing the bioavailability of the toxin in the gastrointestinal tract will prevent toxic effects from occurring. This blockage is most often based on the property of certain molecules to adsorb toxins, i.e. to bind to them through non-covalent bonds. The European Commission (EC), in its Regulation No. 386/2009 of 12 May 2009, has defined these adsorbents as a new functional group of feed additives (European Commission, 2009). They are substances with a high affinity for mycotoxins and capable of reducing their availability in the body. Adsorbents are generally high molecular weight

compounds that are able to bind mycotoxins in the animal gut, thus preventing their absorption and resulting in fecal excretion (Carraro et al. 2014). The efficiency of the adsorption mechanism is largely dependent on the physical properties and structure of the adsorbent and the adsorbed molecule (size, solubility, polarity, pore size for the adsorbent, accessible surface area and, if applicable, charge distribution) (Gouda et al. 2019). There are several types of adsorbents (mineral, organic and synthetic) such as active carbon, bentonite (Mg, Na, et Ca), zeolite, kaolinite, aluminosilicates, montmorillonite (Nyankson et al. 2018).

b. Chemical methods

Mycotoxin detoxification strategy by chemical means is based on the use of chemical compounds able to convert toxins into other metabolites, less harmful. The best-known methods are ammonization, alkaline hydrolysis, as well as the use of bisulfites, acids and oxidizing agents. Indeed, ammonization has been adopted by several countries due to its ability to completely break down some toxins in crops such as OTA in maize, wheat and barley (Los et al. 2020; Varga et al. 2015). Although this technique did not result in the formation and the accumulation of toxic by-products, its use alters organoleptic properties of treated foodstuffs (browning of cereals, loss of lysine and other amino acids) (Varga et al. 2010). Therefore, nowadays, due to its side effects on the quality of processed foods, its use is no longer recommended (FAO 2002). In addition, alkaline hydrolysis by hydrogen peroxide (H_2O_2) and sodium hydroxide (NaOH) can also break down mycotoxins. Formic, propionic and sorbic acids, and sodium hypochlorite degrade OTA after 3-24 hours incubation (Sueck et al. 2019).

Ozonation is a simple chemical treatment that is progressively used to inhibit the growth of pathogenic molds to provide good storage conditions. Ozonation was proven to reduce drastically deoxynivalenol content in wheat grain. Usually, mycotoxin reduction rate is directly related with ozone fixation and time of grain introduction to ozone (Afsah-Hejri 2020). In comparison with chemical treatments, applying ozone to limit fungal growth has numerous advantages, such as fast ozone disintegration to atomic oxygen, no toxic residues and its easy- use and accessibility. A 15-second ozonation treatment (10% O3) was proven to reduce OTA levels in hazelnuts and plants to levels not detectable by HPLC (Uka et al. 2019).

Although these chemical compounds decrease mycotoxin levels in food matrices, their use on products intended for human consumption is prohibited by the European Union (European Commission 2006).

c. Biological methods

Microorganisms can also be used as a curative approach to reduce mycotoxin contamination. They can adsorb, degrade and/or transform mycotoxins.

Mycotoxin Adsorption

An interesting solution is using mycotoxin-binding microorganisms, particularly lactic acid bacteria (LAB) (Vanhoutte et al. 2016). According to the literature, the application of these microorganisms is diagnosed as safe (GRAS microorganims) (Niderkorn et al. 2011). It has been proven that using diverse *Lactobacillus rhamnosus* strains can lead to the removal of zearalenone (ZEN). It appears that this mycotoxin is adsorbed to the bacterial cell wall, and not absorbed into the cell. Indeed, the study of the interaction between ZEN, and two *Lb. rhamnosus* strains used as nutritional supplements, showed that a substantial ZEN percentage ranging from 38 to 46% was recovered from the bacterial fraction, and not ZEN degradation product was detected. After being treated at high-temperature conditions and acid conditions, ZEN concentration increased, indicating that it was initially adsorbed by cell walls and not metabolized (Niderkorn et al. 2011). In another study, *Pediococcus acidilactici*, *Lb. sake* and *P. pentosaceus* strains decreased DON content in malting wheat grain samples by 47%, and *P. acidilactici* and *P. pentosaceus* reduced ZEN content by around 38% (Juodeikiene et al. 2018). However, it is important to note that the physiological state and the environmental conditions can highly affect mycotoxin reduction through microbiological binding (Kagot et al. 2019).

During winemaking, the OTA content increases up during the alcoholic fermentation stage before bottling. Normally, during this phase, OTA level decreases, probably due to its adsorption on the surface of the *Saccharomyces* cell yeast. The fact that no degradation metabolite was detected proves the adsorption phenomena (Bueno and Oliver 2007). As a result, recent winemaking technologies focus on the use of dead yeast fractions to accelerate the process of wine detoxification without affecting its physico-chemical characteristics. Following up on this, Petruzzi et al (2014b) have tested two dead strains of *S. cerevisiae* (thermal inactivation), as well as commercial cell walls, to compare their ability to adsorb OTA. This author found that cell walls can adsorb up to 50% of OTA, whereas dead yeast adsorbed only 43% of this toxin. Besides, Bizaj et al. (2009) found that there is no significant difference between dead and live *S. cerevisiae* yeasts in wine detoxification, proving that this process relies only on the adsorption of OTA and not on its degradation by this yeast.

Mycotoxin degradation or transformation

Another type of treatment is based on the use of microorganisms is the degradation or transformation of mycotoxins. Some microorganisms are characterized by an important metabolic activity through a huge enzyme production that leads to mycotoxin degradation and thus the formation of none toxic or less toxic compounds (Vanhoutte et al 2016). Many examples of mycotoxin biodegradation by microorganisms have been described. Pfliegler et al. (2015) highlights the effectiveness of several genera of yeast *(Saccharomyces cerevisiae, Geotrichum candidum, Kluyveromyces marxianus* and *Metschnikowia pulcherrima)* in the degradation of mycotoxins. Verheecke et al. (2016) reported the microbial degradation of the

aflatoxin B1 by *Actinobacteria, Bacillus, ɣ-Proteobacteria* and *α- Proteobacteria* (Verheecke et al. 2016).

Numerous studies have also demonstrated that microorganism are also able to transform mycotoxin in metabolites less harmful. In fact, zearalenone can be transformed to other by-products such as ZEA-glycosides or ZEA-sulfates, that were proven to be less toxic to animals (Plasencia and Mirocha 1991). Others studies showed that the degradation of trichothecenenes can occur by microorganisms isolated from the digestive tract of cattle and pigs. It occurs through de-acylation or de-epoxidation phenomena. For instance, *Curtobacterium* spp is capable of deacetylating T-2 toxin to form HT-2 toxin and then to T-2 triol. Subsequently, triol T-2 can be deacetylate to T-2 tetraol. T-2 triol toxicity is 23 times less toxic T-2 toxin and 13 times lower than toxin HT-2 (Ueno 1984). Other microorganisms such as bacteria from the *Agrobacterium-Rhizobium* group are involved in the transformation of trichothecenes by oxidating the hydroxyl group position C3 of DON to ketone leading to the formation of less toxic compounds (Yu et al. 2014). Studies conducted on *Acinetobacter calcoaceticus* have shown that it can degrade OTA into OTα under the effect of an extracellular esterase produced by this bacterium (Anga and Draughonz 1994). In fact, the majority of OTA-degrading microorganisms are capable of cleaving phenylalanine from this toxin, causing OTα accumulation in the medium. The similarities in the degradation profiles of OTA by *Bacillus* strains and non-ochratoxicogenic *A. niger* strains and the detection OTα, suggest the involvement of a carboxypeptidase A responsible for this biotransformation (Petchkongkaew et al. 2008).

After overviewing mycotoxin characteristics, and going through the most substantial elements in managing their occurrence in cultures and food products, this survey will thereafter shed lights on one particular commodity affected by mycotoxins: barley kernels used during beer production. As mentioned above, mycotoxins are thermostable. Thus, if the main ingredient (barley cereals) is initially infected, these toxic elements will persist in the final product even after processing. What are the main fungi that contaminate barley grains? What kind of mycotoxins do they produce? What is their fate during the brewing process? These queries will be deeply discussed further all along chapter 2.

Chapter 2 – The beer industry threatened by T-2/HT-2 toxins producing *Fusarium* species

Part I – *Fusarium* spp., main barley contaminant

A. Characteristics of *Fusarium* spp.

The genus *Fusarium* belongs to the *Deuteromycetes* class, more commonly known as imperfect fungi, comprising more than 100 species. The term *Fusarium* is of Latin origin *fucus* which means spindle, referring to the shape of these compartmentalized spores and fusiform spores. These spores can be in three forms: macroconidia, microconidia and chlamydospores (Nelson et al. 1994). The figure 4 presents *Fusarium langsethiae* morphology on both macroscopic and microscopic levels as an example (Kaukoranta et al. 2019). The characteristics of these spores are shown in table 3 and figure 5. These microorganisms reproduce asexually (vegetative reproduction) by producing exogenous spores. Due to their great diversity and variability in cultivation, the identification and classification of such fungi depend on several characteristics. These criteria are divided into two major tabs: the macroscopic and the microscopic planes (Manandhar and Maragos 2000; Schmidt et al. 2016).

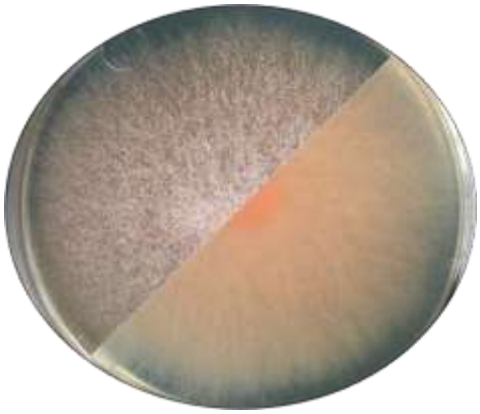
Macroscopic observation	Microscopic observation
	
The appearance and color of the colonies characterize each species. Indeed, the mycelium of *Fusarium* spp. is rather aerial, most often with a fairly light color, remarkable pink, red or purple. The white, creamy or ochraced colors are less recurring. Some cases present, at the level of the thallus, gelatinous masses of conidiophores called sporodochia (conidia attached to each other by filamentous protuberances). In addition, the smell of the crop and its speed of growth allows a subsequent recognition of the species.	At the microscopic level (25µm), differentiation is made according to the macroconidia shape, the presence or not of chlamydospores, the presence or not or even the microconidia shape and finally the form of phialides.

Figure 4 - Macroscopic and microscopic F. langsethiae morphology on PDA medium after 7 days at 20°C (Torp and Nirenberg 2004)

Table 3 - Characteristics of the three *Fusarium* spores shapes (Nelson, Dignani, and Anaissie 1994)

Macronidia	Micronidia	Chlamydospores
○ Produced in a sporodochium (cluster of conidiophores arising from stroma to form a cushion-like mass) ○ Produced on mono-phialides and poly-phialides on aerial mycelium. ○ Vary in size and shape.	○ Produced in the aerial mycelium. ○ Produced on mono-phialides or poly-phialides ○ Different shapes and sizes.	○ Thick-walled spores filled with lipid-like material that carries the spores overwinter in the soil. ○ Airborne occurring in pairs, in clumps, or in chains. ○ Smooth or rough outer wall

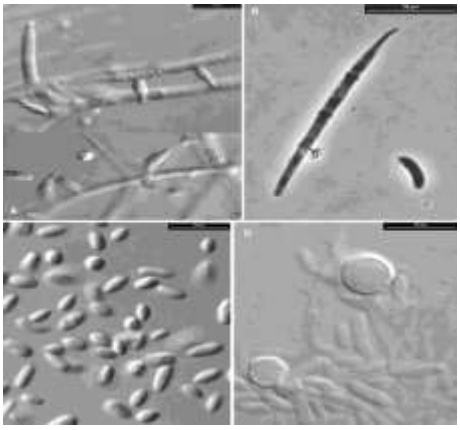


Figure 5 - Microscopic features of Fusarium acutatum. (A) conidiogenous cells from aerial mycelium ; (B) macroconidium and microconidium; (C) microconidia; (D) chlamydospores and microconidia (Taj-Aldeen et al. 2006)

This thesis focuses on two *Fusarium* species: *F. langsethiae* and *F. sporotrichioides*, and they display rather divergent morphological characteristics.

Indeed, *F. langsethiae* grows with a powdery appearance on a potato-based medium (PDA). This odorless species is characterized by a mycelium whitish airborne of 1-2 mm in height, and the production of napiform conidia (in the form of a turnip) or globular conidia when cultured on PDA medium. Monophialides, responsible for the production of spores, are cylindrical and curved (9-15 µm long and three µm wide) at the ends of which are found conidia. Sporulation is then triggered after 5-7 days of incubation in the mycelium giving rise to short and simple conidiophores at first which will later branch out into arrangements. *F. langsethiae* does not have macroconidia, chlamydospores or sclerotia (a preservative of clustered mycelial filaments whose function is to store nutrients and energy necessary for the survival of the microorganism under unfavorable environmental conditions) (Torp and Nirenberg 2004).

F. sporotrichioides is characterized by a fairly developed aerial mycelium (at least 5 mm in height) and a reddish complexion on the PDA medium. Microscopically, the phialides (which may be monophialidic or polyphialidic) hold at their ends conidia varying between the round shape and the falciform shape. This species has chlamydospores and macroconidia (Varga et al. 2015).

B. *Fusarium* spp. life cycle

Fusarium species are phytopathogenic and toxigenic fungi and are commonly found in soils (Oldenburg 2017). They contaminate numerous kinds of crops, particularly cereals including wheat, barley, maize, rice and oats in temperate and semi-tropical areas (Alshannaq and Yu 2017; Saeger 2016). The fungi get contact with targeted organs of plants when the plants suffered from specific symptoms of rot diseases related to ears, kernels, rudimentary ears, roots, stem, leaves, seed and seedlings. *Fusarium* species can infect cereal crops over-cultivation and post-harvest period, including during storage (Oldenburg 2017; Saeger 2016).

Fusarium infection of cereal plants, presented in figure 6, leads to a disease called "Fusarium Head Blight" (FHB) (Arseniuk et al. 1993). It can easily turn into an epidemic due to the high propagation capacity of *Fusarium* species. Under unfavorable conditions (elevated temperature and humidity), *Fusarium* species, mainly *F. langsethiae* and *F. sporotrichioides* behave like saprophytes and their spores remain in the soil and form the major source of inoculum (Thammavongs et al. 2008; Manova and Mladenova 2009; Pietri et al. 2010; Tabuc et al. 2009). In wheat and barley, during vegetative ear growth and when environmental conditions become favorable, the spores reach the ears and cause infection (Spanic et al. 2018). The critical period for ear infection begins at heading and extends over the next few days. During this period, climatic factors such as rain or humidity, combined with heat, will have the greatest impact on the level of infection (Elad 2014; Doohan et al. 2003). Infection occurs mainly for a very short period time, when the stamens come out. At this stage of development, the plant is subject to invasion by fungi. Fungal infection at this stage has the greatest impact on grain yield. Indeed, several studies have shown a yield loss of between 15% and 60% following infection (Arseniuk et al. 1993). FHB is manifested by discoloration of the spikelets with pink or orange spots indicating the presence of the fungus (Nielsen et al. 2011; Champeil et al. 2004). Mature fusarium-damaged kernels are small and wrinkled, often with a chalky appearance. Blockage of the steam vessels by the mycelium blocks the supply of the nutrients to the spikelets above the point of infection and leads to the bleaching of this part of the ear. During infection and development on the spike, some *Fusarium* species can produce mycotoxins.

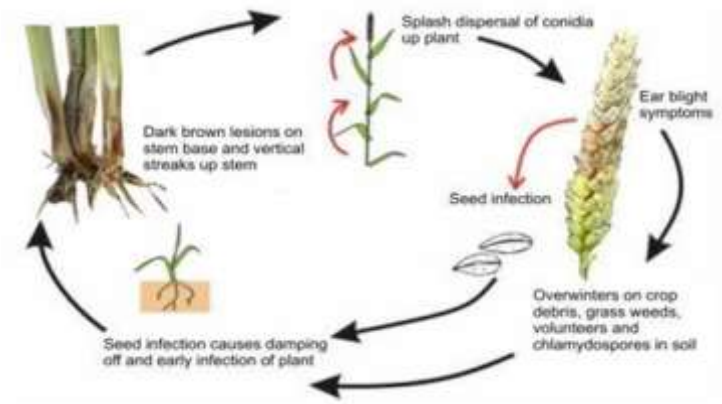


Figure 6 - The typical life cycle of Fusarium in wheat and barley leading to the Fusarium Head Blight Disease (HGCA, UK) (N Matny 2015a)

In France, the appearance of *F. langsethiae* on malting barley has become a source for concern for several years now. This species has replaced other *Fusarium* species such as *F. poae* in the early 2000s and co-exists now with *F. sporotrichioides* (Nielsen et al. 2011). The latter is a major concern especially when the cereal is intended for malting (Wolf-Hall 2007). The risk of establishment of this species is not only linked to the contamination of barley grains in the field but often triggers the production of mycotoxins consisting of trichothecenes, fumonisins and others (Krska et al. 2001). According to Pestka et al. (2007), DON and T-2/HT-2 toxins are the most frequently dominant, which is health threatening (Pestka 2007 ; Jansen et al. 2005). Indeed, both mycotoxins and *Fusarium* spores can persist even after food processing in barley- based products (Kostelanska et al. 2011)

The T-2 toxin belonging to the trichothecenes family is the main interest of this thesis, therefore, the following part will mainly be developing this mycotoxin family.

C. The trichothecenes family

a. Trichotecenes classification: Group A, B, C and D

In the last 40 years, more than 200 types of trichothecenes have been identified (Grove 1986). This family is composed of more than sixty metabolites of chemical structure belonging to sesquiterpenes produced by several fungal genera including *Fusarium, Myrothecium, Phomopsis, Trichoderma, Trichothecium* and *Stachybotrys* (Ueno 1984; Kostelanska et al. 2009). Sesquiterpenes compounds share a common core comprised of a rigid tetracyclic ring system consisting of a cyclohexene, A-ring with a double C-C bond occurring between C-9 and C-10; a tetrahydropyranyl B-ring; a cyclopentyl C-ring, and an epoxide at C-12/13 as shown in

figure 7. The rigidity of this structure results in a distinct stereochemistry for the A- and B-rings (Shank et al. 2011).

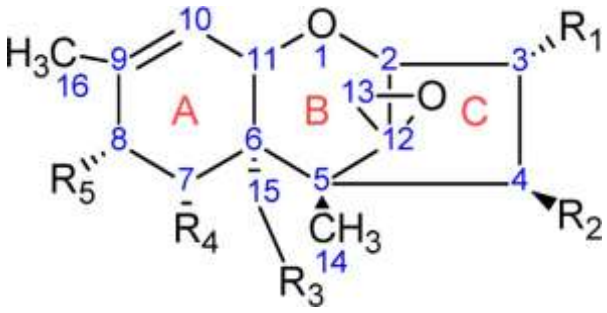


Figure 7 - Chemical structure of trichothecenes common skeleton (Shank et al. 2011)

Tricothecenes vary according to the presence or absence of hydroxide and/or acetone groups and their numbers and position on substituants R1 to R5. In 1977, trichothecenes have been classified into four groups according to these substituents. There are non-macrocyclic trichothecenes (Groups A, B and C) and macrocyclic trichothecenes (Group D). Carbon, hydrogen and oxygen are the major elements forming the backbone of trichothecenes. The latter is characterized by a double bond linking the carbon 9 to carbon 10; and by the presence of an epoxide group in the C12 position, responsible for the toxicity of these molecules (Kistler 2004). Depending on the type of toxins produced, hydroxyl, acyl or epoxides can be found in positions C3, C4, C7, C8 and C15. The carbon 8 can also have a ketone function (Ueno 1984).

- Group A: characterized by an absence of ketone function at their C8 (R5), including T-2 toxin, HT-2 toxin, and diacetoxycirpenol (DAS).

- Group B: characterized by the presence of ketone function at their C8 (R5), of which nivalenol, deoxynivalenol and fusarenone-X.

- Group C: characterized by the presence of an epoxide moiety at the level between the C7 and C8 (R4 and R5), including crotocin.

- Group D: characterized by the presence of a macrocycle between the C4 and the C15, including verrucarins, roridins and satratoxins.

b. Physical and chemical proprieties

The molecular weight of these molecules ranges from 154 Daltons to 697 Daltons (generally between 300 and 600) and are generally found under form of colorless crystalline powders (Doyle et al. 2009). Initially neutral, trichothecenes can be dissolved in polar solvents such as alcohols, chlorinated solvents, ethyl acetate and ethyl ether. Trichothecenes are characterized by high thermal stability: they can be stored at room temperature as they can withstand sterilization conditions. Hydrolysis of T-2 toxin results in the formation of two molecules of acetic acid, one molecule of isovaleric acid and molecule of T-2 tetraol.

c. Trichothecenes biosynthesis pathway

The trichothecenes biosynthesis pathway, presented in figure 8 was first determined in *F. sporotrichioides* and *F. graminearum* species, allowing the characterization of the different biochemical and molecular steps leading to the production of these molecules from isoprenoid metabolism (Kimura et al. 2007). From a molecular point of view, 15 *Tri* genes, located at three different loci on different chromosomes involved in the trichothecenes biosynthesis pathway have been identified (Alexander et al. 2009). Indeed, 12 of these genes are located on the main cluster called "cluster *Tri5*", the *Tri1* and *Tri16* genes are located on another locus and the *Tri101* gene is located on a third locus (Gale et al. 2005). The figure 9 shows a schematic representation of the Tri5 cluster and the Tri genes leading to the production and regulation of trichothecenes biosynthesis. The trichothecene skeleton is derived from farnesyl pyrophosphate (FPP) which, in a first enzymatic step, is cyclized to form trichodiene, the first intermediate of the trichothecene biocynthesis pathway (Grünler et al. 1994). This step is under the action of the trichodiene synthase encoded by the *Tri5* gene (Hohn and Beremand 1989). The activation of this enzyme depends on the presence of Mg^{2+} and its inhibition depends on the phosphate concentration (Brown et al. 2001). The *Tri4*, *Tri101*, *Tri11* and *Tri3* genes code for enzymes responsible for nine enzymatic reactions, leading to the formation of the calonectrin (Alexander et al. 2009). These steps are common for the formation of trichothecenes type A (T-2 toxin) and type B (NIV and DON) in *F. sporotrichioides* and *F. graminearum* producing fungi (Brown et al. 2001). The *Tri7* and *Tri13* genes are only functional in *F. sporotrichioides* and *F. graminearum* strains which can produce the NIV toxin, and code for enzymes responsible for the two subsequent steps (Brown et al. 2004; Lee et al. 2002). In *F. sporotrichioides* species, the *FsTri1*, *FsTri16* and *FsTri8* genes encode enzymes responsible for the formation of the T-2 toxin (Mccormick and Alexander 2002; Wiemann et al. 2010; Peplow et al. 2003a; Peplow et al. 2003b). For *F. graminearum* producing NIV, the *FgTri1* gene code for the enzyme responsible for the formation of 4ANIV which is transformed into NIV under the action of the enzyme encoded by the *FgTri8* gene (Brown et al. 2004). In contrast, DON-producing strains have non-functional *FgTri7* and *FgTri13* genes (Lee et al. 2002). Nevertheless, the action of the enzymes encoded by the *FgTri1* and *FgTri8* genes results in the formation of either 3ADON or 15ADON, which are acetylated derivatives of DON (depending on the sequence of the esterase encoded by the *Tri8* gene) and then possibly DON (McCormick et al. 2011).

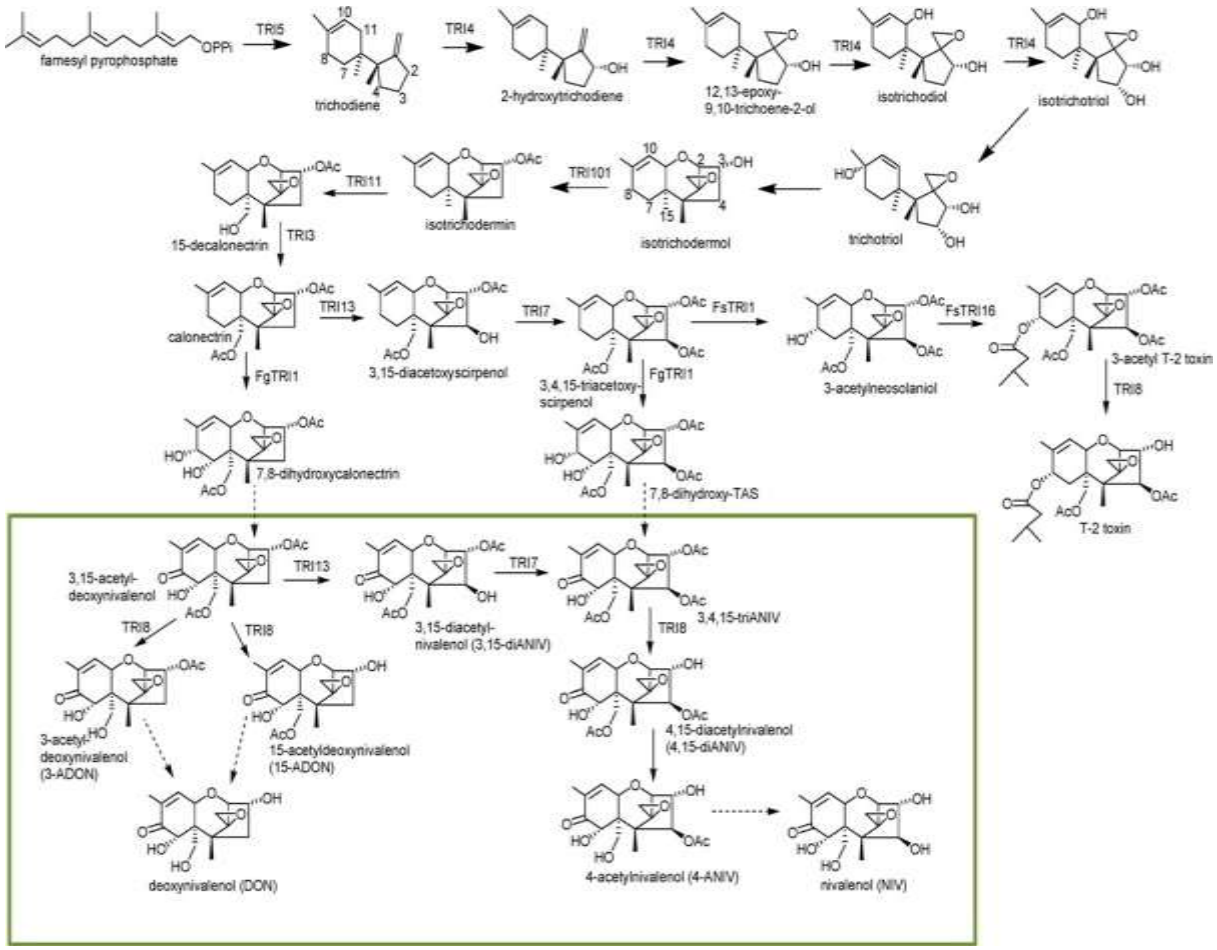


Figure 8 - Proposed trichothecene biosynthetic pathway in Fusarium spp. Genes encoding an enzymatic step are identified near the arrow indicating the step. Dashed arrows indicate steps for which a gene has not been assigned. Green box indentifies Type B trichothécènes (McCormick et al. 2011 - Trichothecenes: From Simple to Complex Mycotoxins)

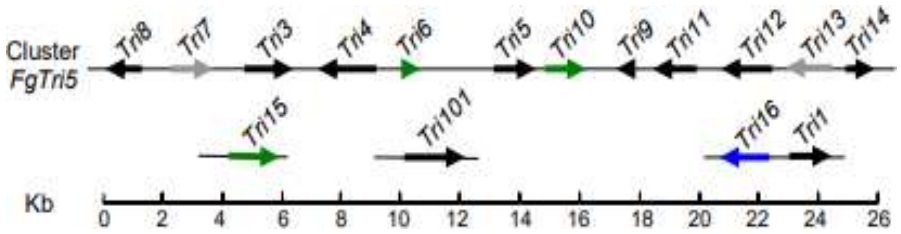


Figure 9 - Schematic representation of the Tri5 cluster and genomic regions containing Tri genes leading to the production or regulation of production of trichothecenes. The Tri7 and Tri13 genes (in grey) are not functional in DON producers. The Tri16 gene (shown in blue) is only functional in producers of trichothecenes A. the Tri6, Tri10 and Tri15 genes (green) play a regulatory role (Alexander et al., 2009).

d. Specific regulation of trichothecenes biosynthesis

Among the *Tri* genes of the cluster responsible for the production of trichothecenes, *Tri6* codes for a Cys_2His_2 zinc finger transcription factor capable of increasing the expression rate of several *Tri* genes in *F. sporotrichioides*. The absence of this gene reduces the expression of *Tri5* and *Tri4*, resulting in a lack of T-2 toxin production (Hohn, Krishna, and Proctor 1999). In fact, the protein encoded by *Tri6* binds to the TNAGGCCT consensus region present in front of promoters of the other *Tri* genes and stimulates their transcription (Hohn, Krishna, and Proctor 1999). Later, the *Tri10* gene has been identified as a positive regulator of the *Tri* genes, acting upstream of *Tri6*. Indeed, the deletion of the *Tri10* gene in *F. sporotrichioides* completely inhibits the production of T-2 toxin and drastically reduces the expression of the genes of the *Tri* family, in particular, that of *Tri6* (Tag et al. 2001; Peplow et al. 2003b). Seong et al. have tested *F. graminearum* mutants where *Tri6* and *Tri10* genes have been deleted (Seong et al. 2009). Moreover, these species exhibited lower virulence. Gene expression analyzes show a much lower disruption of their expression, especially those of the *Tri* family. Tri6 is therefore not only a positive regulator of the genes of the trichothecene biosynthetic pathway but also acts as an upstream regulator of the isoprenoid pathway, having a role in the accumulation of these toxins in these species. In addition, Tri6 negatively regulates the expression of *Tri10* but the deletion of the latter has no noticeable effect on the transcription rate from *Tri6*. On the other hand, located in the cluster of the biosynthesis pathway of these toxins, the *Tri14* gene plays a key role in the synthesis of these toxins and the role of a specific regulator. Similarly, its deletion affects the rate of trichothecenes produced and then it contributes to the reduction of the virulence of producing fungi, especially in *F. graminearum*. Like *Tri6*, the *Tri15* gene present at a fourth locus suspected of being involved in the regulation of the biosynthesis pathway, codes for a zinc finger transcription factor which negatively regulates the production of certain trichothecenes produced by *F. sporotrichioides* (Alexander et al. 2009). However, it does not affect the production of *F. graminearum* (Dyer et al. 2005).

e. Regulation of trichothecenes biosynthesis by environmental factors

Several environmental factors contribute to the regulation of trichothecenes biosynthesis, as pH and light exposure. Moreover, contaminated plants can alter fungal growth and mycotoxin production to defend themselves.

pH regulation

The pH is an essential factor in the regulation of trichothecenes biosynthesis. Indeed, fungi tend to acidify the surrounding environment to trigger mycotoxigenesis. The expression of *Tri* genes and the production of toxins coincide with this phenomenon (Gardiner et al. 2009; Merhej et al. 2010). Acidification (reaching a pH 3) is therefore essential to promote the expression of the *Tri5* gene in *F. graminearum* in order to produce DON. Otherwise, if the surrounding environment is neutral or alkaline, gene expression is repressed and thus,

mycotoxin production is inhibited. Acidification of the medium may be due to the consumption of ammonium during fungal growth. Thanks to their pH regulation system, the fungus can survive even under extreme conditions.

The regulation is based on a signaling cascade involving the family of Pal proteins that activate the PacC regulation at alkaline pH. The active form PacC bind to the GCCARG consensus region of the promoters of the so-called acidic and other so-called alkaline genes. It represses the expression of acidic genes and activates the others (Tilburn et al. 2018; Espeso et al. 2018). The *PacC* gene in *A. nidulans* or its ortholog in different species is responsible for regulating the production of a variety of secondary metabolites: PacC regulates the production of sterigmatocystin, aflatoxins and penicillin in *Aspergillus* species, the production of cephalosporin C in *Acremonium chrysogenum* and penicillin in *P. chrysogenum* (Keller et al. 2007). In *F. verticillioides*, Pac1 regulates fumonisin production (Flaherty et al, 2003). The same gene is present in *F. graminearum* where it is directly involved in the regulation of trichothecenes according to the variation in pH. Indeed, the active form of Pac1 represses the expression of *Tri* genes leading to a decrease in the level of trichothecenes at acidic pH (Merhej et al. 2010; Merhej et al. 2011). The GCCARG sequence corresponding to the consensus region on which the PacC in *A. nidulans* was found in the promoter of the *Tri* genes of the cluster and the *Tri6* regulator gene (Bailey and Elkan 1994).

Light regulation and velvet complex

At the fungal level, light is seen as a source of informations rather than a source of energy. Indeed, fungal species react in different ways to this stimulus and can adapt its growth considerably depending on the presence or absence of light (Tisch and Schmoll 2010). The perception and transmission of the light signal in the fungal cell are initialized by two regulatory and photoreceptor proteins: the White collar-1 (WC-1) and the White collar-2 (WC-2) (Froehlich et al. 2002). Light influences the production of secondary metabolites in several fungi. The relationship between light and the synthesis of secondary metabolites was clarified following the identification of the velvet complex composed of three proteins: VelB, VeA, and LaeA in *Aspergillus* species (Kale et al. 2010; Woo Bok and Keller 2004). Several ortholog genes have been identified in different *Fusarium* species. As example, the deletion of *FvVE1* in *F. verticillioides* completely inhibits the production of fumonisins and fusarin C and suppresses the action of the specific regulator FUM21 of the fumonisin pathway (Roctor et al. 2009). Later, *VeA* and *LaeA* orthologs in *F. fujikuroi*, *FfVel1* and *FfLae1* respectively were characterized and subsequently the formation of a velvelt-like complex was identified (Wiemann et al. 2010). This acts as a positive regulator of gibberellins and fusarine C, and as a negative regulator on the bikaverinne. In 2011, Merhej et al. have identified the velvet gene *FgVe1* in *F. graminearum*. Deletion of this gene inhibits growth, conidiation and aurofusarin biosynthesis (Merhej et al. 2011). These modifications result in mycelium color change. Moreover, this deletion reduces also the virulence of *F. graminearum* on wheat.

Effect of plant defense mechanisms on fungal growth and trichothecene production

Host-microorganism exchanges lead to changes in the expression of the majority of genes, in particular *Tri* genes, responsible for the production of trichothecene in *F. graminearum* (Brown et al. 2004). Indeed, the virulence of the fungus increases radically once in contact with the plant: the toxin seems to inhibit the formation of callosis at the level of the cell membrane and thus blocks the formation of the defense barrier, which is supposed to protect the cell (Ilgen et al. 2009). The first contact between the two species stimulates the production of free radicals at the plant cell level such as H_2O_2 (Kachroo et al. 2003) which causes oxidative stress in the contact zone. The latter leads to an increase in the production of mycotoxins in certain cases, notably that of DON. The H_2O_2 molecule stimulates the expression of at least five *Tri* genes (Ponts et al. 2007). Moreover, the production of trichothecenes is also modulated by the presence or absence of antioxidants such as phenolic compounds. Ferulic acid, the most abundant compound in wheat, inhibits the biosynthesis of these toxins by repressing the expression of responsible genes in different *Fusarium* species (Boutigny et al. 2009). This implies that fungi react to oxidative stimuli from the environment and adapt their metabolism to external factors.

D. The T-2 toxin, a type A trichothecenes

Type A trichothecenes are distinguished by the absence of ketone grouping at the C8 position. The different substituents present on the main skeleton determine the nature of the toxin in question, the three main ones being T-2 toxin, HT-2 toxin and diacetoxyscirpenol (DAS). Being the primary concern of this investigation, the focus will be reduced to T-2 and HT-2 toxins.

a. T-2/HT-2 toxins chemical structure

Gilgan et al. were the first to determine the presence of the T-2 toxin isolated from *F. tricinctum* culture in 1966 (Gilgan et al. 1966). A method was subsequently developed in 1971 to produce and purify the T-2 toxin secreted by *F. tricinctum* culture on corn groats (Burmeister 1971). After the discovery of this toxin on corn, it was found to contaminate several other types of grains including wheat, oats, barley, soybeans, and rice. It can also originate from several species of *Fusarium* that can generally be phytopathogenic to the plant and can contaminate the resulting commodities.

The toxin 12,13-epoxytrichothec-9-ene-3α,4β,8α,15-tetraol,4,15-diacetate 8-isovalerate, commonly known as T-2 toxin, has the following formula $C_{24}H_{34}O_9$. Its molecular weight is 466.5 Da and is composed of 62.33% carbon, 6.54% hydrogen and 31.13% oxygen. Its melting point is 151-152°C. It is characterized by high solubility in polar organic solvents such as acetone, ethyl acetate, acetonitrile, chloroform, diethyl ether and dichloromethane (Taylor et al. 2010).

HT-2 toxin is the deacetylated form of T-2 toxin. HT-2 toxin or 12,13-epoxytrichothec-9-ene-3α,4ß,8α,15-tetraol,15-acetate 8-isovalerate has the crude formula $C_{22}H_{32}O_8$. Its molecular weight is 424.5 Da and it is stable in ethyl acetate. This toxin has been found in many raw materials. It is produced by many species of *Fusarium*, in particular *F. sporotrichioides, F. poae, F. langsethiae*. As a result, this toxin will be found in the same situations as those allowing the synthesis of T-2 toxin. Figure 10 presents the chemical structure of T-2 and HT-2 toxins.

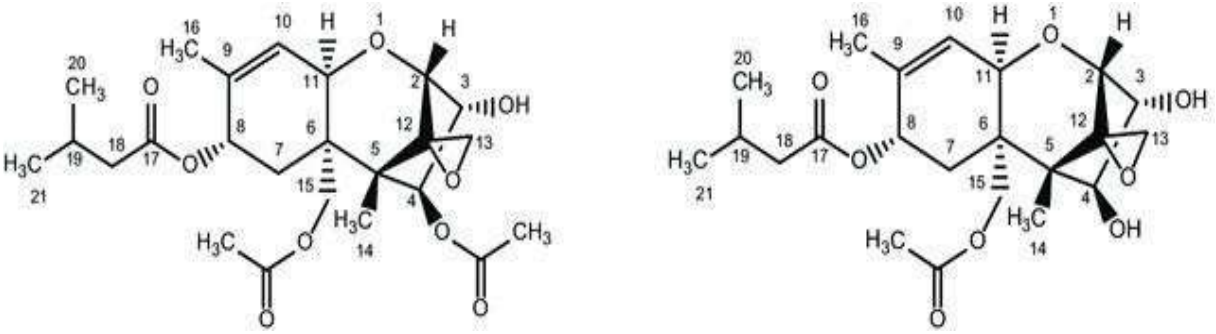


Figure 10 - Chemical structure of T-2 toxin (on the left) and HT-2 toxin (on the right)

b. T-2/HT-2 toxin toxicity

These mycotoxins may have adverse effects on cellular metabolism. Indeed, this results in inhibition of DNA and RNA synthesis (thymine alteration that will be incorporated during genomic synthesis), an inhibition of protein synthesis (since it has an affinity for the 60S ribosomal subunit and inhibits peptidyltransferase synthesis, which blocks the incorporation of amino acids). In addition, those toxins are suspected to be responsible for lipid peroxidation of cell membranes causing the formation of free radicals and therefore tissue damages. Alterations at the cellular level can lead the cells to apoptosis. In addition, T-2 and HT-2 toxins have been proved to be immunotoxic (inhibits 20 of the production of immunoglobulin IgA, IgG and IgM and impairs the proper functioning of human lymphocytes).

T-2 toxin is 1.5-1.7 times more toxic than its deacetylated form, HT-2 toxin. Its toxicity can vary depending on the duration and mode of exposure. It can be acute, manifested by weight loss, loss of appetite, vomiting, diarrhea, dermatitis, hemorrhages, necrotic lesions of the stomach and intestinal epithelium.

c. European recommendations

Official standards have not yet been set for T-2 toxin and HT-2 toxin levels in food. However, the recommendation 2013/165 on these toxins was published in the Official Journal of the European Union on April 4, 2013 specifying maximum levels in food products: wheat, amber

durum wheat, oats, barley, corn, infant food. The text also encourages, in case of repeated exceedances of these guideline levels, to investigate the factors leading to the presence of T- 2 and HT-2 toxins. It takes into consideration the large variation in the occurrence of T-2 and HT-2 toxins over the years, the different cereal matrices, agronomic factors and the effect of food manufacturing. Therefore, recommendation differs whether the cereal material is processed or not, intended for animal or human consumption. The table 4 shows the indicative levels of the sum of T-2 and HT-2 toxin in cereal and cereal products.

Table 4 - Recommendation (2013/165/EC) of 27 March 2013 concerning the presence of T-2 and HT-2 toxins in cereals and cereal products

	Cereals and cereals products	Indicative levels for the sum of T-2 and HT-2 toxin (µg/Kg)
Unprocessed cereal	Barley (including malting barley) and maize	200
	Oats (with husk)	1000
	Wheat, rye and other cereals	100
Cereal grain for direct human consumption	Oats	200
	Maize	100
	Other cereals	50
Cereal products for human consumption	Oat bran and flaked oats	200
	Cereal bran except oat bran, oat milling products other than oat bran and flaked oats, and maize milling products	100
	Other cereal milling products	50
	Breakfast cereals including formed cereal flakes	75
	Bread (including small bakery wares), pastries, biscuits, cereal snacks, pasta	25
	Cereal-based foods for infants and young children	15
Cereal products for feed and compound feed	Oat milling products (husks)	2000
	Other cereal products	500
	Compound feed, with the exception of feed for cats	250

Part II - Contaminated brewing barley alters the quality of the final product

Barley quality differs depending on several factors such as the variety, growing area, climate, harvesting conditions, and so on. During harvest, crop selection begins with a visual assessment. It assesses the smell, color, consistency, shine and husk quality. Adverse weather may cause grain defection such as early germination which will cause filtration problems and therefore decreases the beer quality. These grains are considered dead and are most likely infected by *F. langsethiae* and *F. sporotrichioides* (Esslinger 2009) The *Fusarium*-damaged barley grains shown in figure 11 have a pink, orange coloration.

Figure 11 - Fusarium infected barley grains (N Matny 2015b)

Harvested kernels are rarely directly delivered from fields to breweries. There is a storage period, which depending on conditions, can potentially increasing *Fusarium* species contamination.

Mycotoxin risk assessment at every step of the brewing process

Barley is the main ingredient in beer production along with water, yeasts and hops. This implies that its quality determines directly the quality of the final product. Therefore, beer is subject to mycotoxin contamination if the raw materials used are contaminated by fungal species and/or by mycotoxins (Malachova et al. 2010).

Concerning T-2 and HT-2 toxins, there is no regulation determining maximum levels of contamination. However, recommendations were set at 200 µg/kg for the sum of the two mycotoxins concerning barley cereals, even malting barley (2013/165/EU 2013). In the case of

heavy beer drinkers, this could have a serious health impact on their health. This has been proven by numerous surveys carried out around the world (Rodríguez-Carrasco et al. 2015; Piacentini et al. 2017a, 2015)

The production of beer, presented in figure 12 includes two main parts (part A and B) each composed of several steps: the malting (part A) is composed of steeping and germination followed by kilning and brewing (part B): wort preparation that includes mashing and boiling, fermentation, maturation, and filtration). Every step in the production chain has an impact on the quality properties of the resulting beer (Preedy 2009).

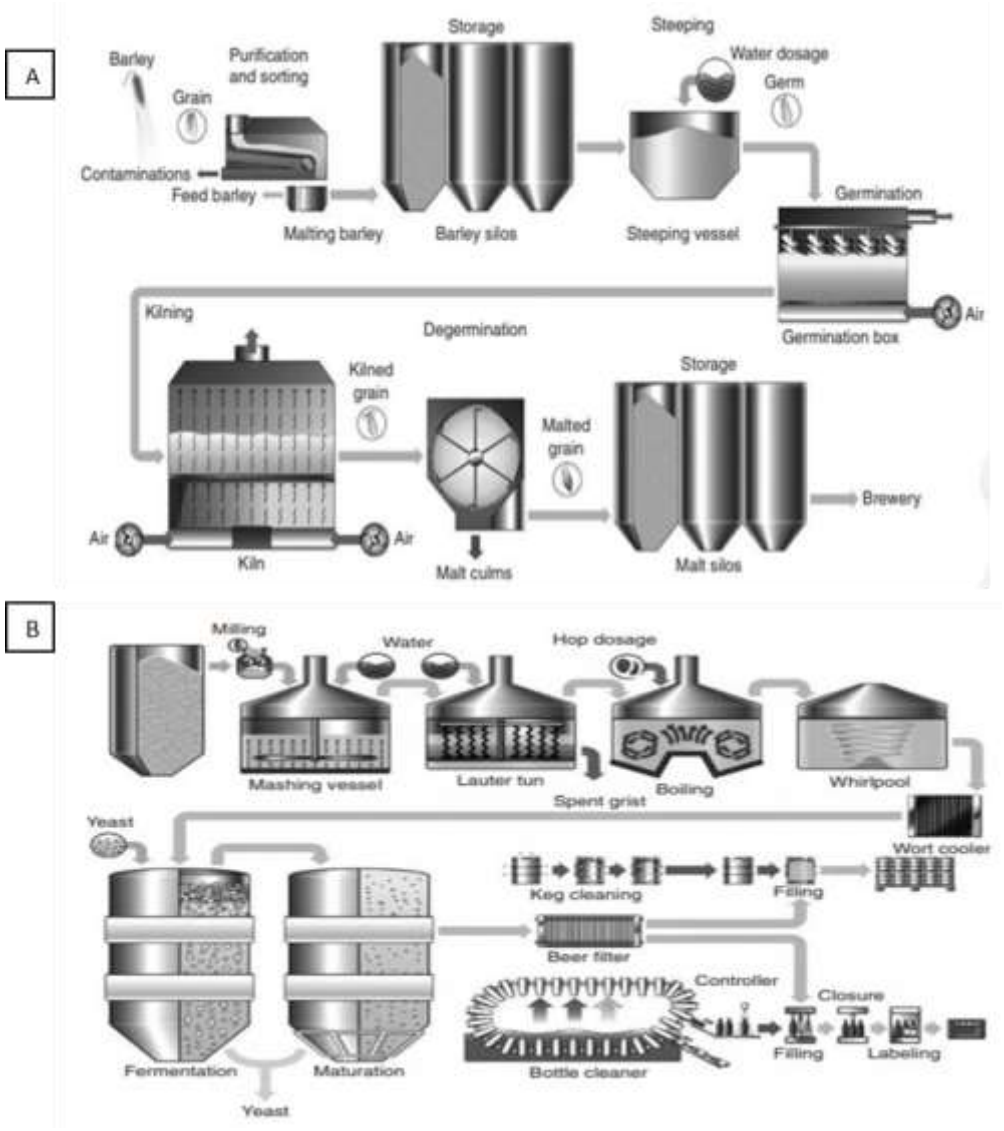


Figure 12 – Beer production process: Panel A shows the first part of the brewery process: the malting step composed of the steeping, the germination and kilning steps. Panel B shows the second part of the process, the brewing step composed of milling, mashing, lautering, boiling, fermentation, maturation and filtration (Preedy 2009).

Table 5 summarizes the brewing process steps and highlights the conditions that might contribute to *F. sporotrichioides* and *F. langsethiae* growth and to increase T-2/HT-2 toxin concentration.

Table 5 - Brewing process steps and conditions leading to Fusarium growth or T-2/HT-2 toxin contamination (highlighted in red).

Step		Conditions	Consequences	References
Malting (part A)	Steeping: Starch dissolution (wetting grains, enzymes activation)	Controlled cycles of water spraying and immersion Aeration Grain water reaching 42% to 48% Temperature 10 - 25 °C Duration 30 -50 hours	Favorable humidity Enzyme activation Spread of *Fusarium* growth up to 90% *Fusarium* spores germination High levels of T-2/HT-2 toxins	(Kunze 2006; Vegi et al. 2011; Schwarz et al. 2018; Kirinčič et al. 2015)
	Germination	Water penetration in grains Liberation of growth promoter (gibberellic acid) Synthesis of amylases, dextrinases, cytolytic enzymes, proteolytic enzymes, lipases… Temperatures between 17 and 25 °C Duration 3-5 days	Activation of enzymes Degradation of starch reserves Degradation of proteins Formation of flavor and aromatic compounds *Fusarium* growth (infection increase by 40%), potential cross contamination from residual water and latent grain infection T-2/HT-2 toxin production	(Oliveira et al. 2012; Vegi et al. 2011)
	Kilning (drying grains)	Drying and roasting of grains Increase of temperature until reaching 80-90°C and 10% water content Duration 24-48 hours	Prevention of future structural changes of barley Degradation of minimal enzyme charge Flavor and color formation Promoting *Fusarium* growth at early stages (before reaching 50°C) and toxin production Potential degradation of fungi when reaching high	(Kunze 2006; Wolf-Hall 2007; Rodríguez-Carrasco et al. 2015)

			temperature; however, *Fusarium* spores persist Transfer thermostable T-2/HT-2 toxins from barley to malt	
Brewing (Part B)	Milling	Increase of contact surface between water and malt Breakdown of malt into fermentation factors: surgars, assimilable nitroge	No direct impact on mycotoxins Homogeneous spread of T-2/HT-2 mycotoxins in all batch	(Mousia et al. 2004)
	Mashing (activation of enzymes, dissolution of starch, conversion into sugars) and lautering (removal of solids)	Activation of enzymes Conversion of starch into fermentable sugars Controlled temperature (60-80°C), pH (5.2), oxygenation, steering speed and time allowing protein hydrolysation, maltose production, saccharification, amylases activation…	Further protein digestion in case of using *Fusarium* contaminated malting barley, thus flavor color and texture (gushing) alteration	(Inoue et al. 2013; Wolf-Hall 2007)
	Worth separation and boiling (thermal and enzymatic processes, addition of hops), fermentation (conversion of sugars into alcohol)	Addition of hops and flavor enhancing elements Hop boiling for 45-60 min above 100°C	Enzyme inactivation Evaporation of water and unwanted compounds Protein precipitation Maillard reactions Flavor enhancement T-2/HT-2 incorporation with added elements if not controlled (hop)	(Evans 2006; Kostelanska et al. 2009; Inoue et al. 2013)
	Fermentation	Yeast addition Temperature ranging from 15-25 °C Duration 7-9 days	Sugar conversion into alcohol and carbon dioxide Ester, higher alcohol and volatile compounds elaboration Adsorption of mycotoxins on beer fermentation residues due to toxin	(Piacentini et al. 2019a; Jouany et al. 2005; Kłosowski and Mikulski 2010;

			binding to beta-glucan (contained in barley grains) from yeast cell walls Inhibition of alcohol dehydrogenase, increase of unwanted volatile compounds (acetaldehyde) in case of presence of T-2/HT-2 toxins	Kłosowski et al. 2010)
	Maturation (degradation of undesirable side products), and storage (development and fixation of CO_2)	Duration 1-3 months Low temperature around 0°C May involve addition of sugars, secondary fermentation Potential use of inorganic adsorbent for clarification	Inorganic adsorbents show no significant effect on T-2/HT-2 toxins, depending on the polarity, water solubility and molecule size	(Belajová et al. 2007)

Since mycotoxins T-2 and HT-2 toxins are extremely stable compounds, even at the highest temperatures applied during beer processing, the toxins are not destroyed and can cause safety and organoleptic concerns in the finished product.

In their study in 2015, Rodríguez Carrasco et al. showed that T-2 and HT-2 toxins concentrations almost exceeded recommended levels (Rodríguez-Carrasco et al. 2015). Given all the data, works are continuously conducted on the development of rapid and reliable methods for the mycotoxin identification in raw materials and final products. The best preventive and corrective measures to avoid the accumulation of T-2/HT-2 toxins remain in reducing their concentration at the early stages of beer processing without altering its taste and texture.

Chapter 3 – *Geotrichum candidum*: a potential solution?

A. The French Institute of Brewing and Malting patent: the implementation of *Geotrichum candidum* as a biocontrol strategy

On the 21st of September 1999, the French Institute of Brewing and Malting (IFBM) filed a patent that sets up a biocontrol plan to decrease the concentration of T-2/HT-2 toxins by *Fusarium* species in brewery products. This biocontrol strategy involves the introduction of a filamentous yeast, *G. candidum*, during the steeping stage (first stage of malting, directly into the tank containing the barley or during the transport of the barley between the steeping unit and the germination unit either in the form of a pure culture or in association with other microorganisms, in particular lactic acid bacteria). The sowing proportions vary between 10^5 and 10^9 cfu/kg of barley. According to the patent, the use of *G. candidum* makes it possible to inhibit the production of T-2 toxin, but also contributes to the improvement of the organoleptic qualities of the beer.

According to the IFBM, the use of this strain during the malting step has an inhibitory effect on the undesirable flora (*Fusarium* spp., *Penicillium* spp., *Aspergillus* spp.) as well as the production of their secondary metabolites such as mycotoxins and/or products causing beer gushing. Moreover, the use of *G. candidum* also reduces the concentration of fatty acids in the must which are responsible for the oxidized taste and poor foam retention of a beer. The choice of this strain used in the malting process is based on the following criteria according to the patent:

- High sporulation capacity (around 10^7 spores/ml)
- Stable spore suspension up to 6 months
- Non toxinogenic/mutagenic character
- Low lipase activity (enzyme responsible for the oxidation of beer)
- No modification of the organoleptic profile of the product.

Nowadays, this strain is used during the malting process, but the mechanisms of interaction between the filamentous yeast *G. candidum* and the mycotoxin-producing fungus leading to a decrease in the level of mycotoxins in beer products are unknown.

B. *Geotrichum candidum*

a. *Geotrichum candidum* taxonomy

Due to the implementation of molecular methods and analyses of whole chromosomes, taxonomic classification of yeasts was modified over the years (Baroiller, Schmidt, and Lapadu-Hargues 1990) . Formerly named *Oidium lactis*, the species *G. candidum* was identified in soil and was therefore assigned to the genus *Geotrichum* in 1809 to designate fungi with "truncate spores" (Pottier et al. 2008; Carmichael 1957). As most yeasts are likely to be described as unicellular microorganisms that reproduce by budding or fission, *G. candidum* is now considered an exception. Its genotype and phenotype categorize the filamentous microorganism as a yeast-like fungus rather than a none filamentous yeast (Belin 1981; Hudecová et al. 2009b, 2009a; Wouters et al. 2002; Smith et al. 2000). *G. candidum* previously belonged to *Deuteromycetes* due to its asexual reproduction and the fact that *Galactomyces geotrichum* was considered to be the teleomorphic state of *G. candidum* (Groenewald et al. 2012).

For over 20 years, Smith and De Hoog have studied the genus *Geotrichum* in order to be able to propose a concrete classification of the species belonging to it using molecular methods. In December 2004, they proposed the most recent taxonomic revision of *Geotrichum* and its teleomorphs. Jacques and Casaregola added some slight changes in 2008, adding *G. candidum* to the *Heniascomycetes* class and the final classification goes as shown in table 6 (Chamba and Jamet 2008; Pottier et al. 2008; Polev et al. 2014).

Table 6 - Nomenclature of Galactomyces candidus/Geotrichum candidum.

	Anamorphic state	Telemorphic state
Phylum	*Ascomycota*	
Class	*Heniascomycetes*	
Order	*Saccharomycetales*	
Family	*Candidaceae*	*Dipodascaceae*
Genus	*Geotrichum*	*Galactomyces*
Species	*Geotrichum candidum*	*Geotrichum candidus*

b. *G. candidum* sources and applications

G. candidum is commonly found in a wide range of habitats such as soil, grass, silage, plants, fruits, feeding stuff, insects, man and other mammals (Welthagen and Viljoen 1999). For instance, *G. candidum* is an important microorganism used in the agro-food industry as it takes part of certain foodborne microflora. Its presence was detected among other microflora in dairy elements, especially raw milk and could be used as a starter in dairy products (Desmasures et al. 1997; Jakobsen and Narvhus 1996; Martin et al. 2001). This implies that *G. candidum* is also detected in raw milk cheeses regardless of the type of the milk origin such as cow milk, ewe milk and goat milk (Wojtatowicz et al. 2001; Cosentino et al. 2001; Tornadijo et al. 1998). However, its presence is barely noticeable in pasteurized kinds of cheese and whey or curd of Cheddar cheese during manufacture or ripening or at least quantified at very low concentrations (Welthagen and Viljoen 1999). Commercial *G. candidum* strains are most commonly employed as starter cultures for cheese ripening by adding it directly to the milk, to the brine, or sprayed on the cheese surface after processing (Larpin et al. 2006; Martin et al. 1999). *G. candidum* can also be used as a starter culture during the brewing process, giving to the beer a distinctive floral taste (Piegza et al. 2014; Dziuba et al. 2000). On another level, it could also be used in biodegradation and depollution treatments, such as decolorization of olive mill wastewaters (Gente et al. 2006; Assas et al. 2002).

c. Physiology, sporulation and germination mechanisms of *G. candidum*

In their work, G. De Hoog and M. Smith dedicated an entire chapter to discuss the genus *Geotrichum* (De Hoog and Smith 2011). *G. candidum* represents the asexual form, its reproduction results in the development of white, farinose, or hairy, usually dry colonies. The later shapes true hyphae and establishes round apices that divides into arthroconidia, also known as arthrospores (figure 13). It appears that hyphae are divided into cellular compartments by walls called septa that are perforated by microspores allowing molecules, ribosomes, mitochondria and other organelles to flow between cells (Fungi 1967). According to Caldwell and Trinci's survey, the yeast fragments its hyphae and septa during the growing phase, releases chains of athrospores to allow the generation of new growing particles (figure 14) (Caldwell and Trinci 1973).

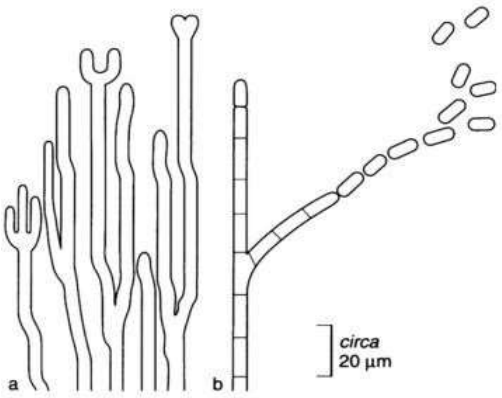


Figure 13 - Typical structures formed by Geotrichum candidum (Galactomyces candidus) on general purpose media: (a) expanding hyphae during active growth; and (b) older hyphae tend to break up into arthric conidia.(Botha and Botes 2014).

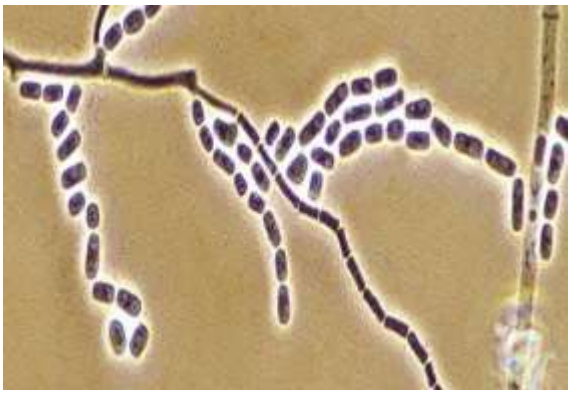


Figure 14 - Geotrichum candidum arthroconidium formation. Hyphal elements are progressively compartmentalised by fragmentation of septa. Conidial secession is by the centripetal separation (schizolysis) of a so called double septum and concomitant rupture of the original outer hyphal wall layer (De Hoog and Smith 2011).

The disarticulation of septa in the lateral hyphae leads to the formation of chains of spores. The state of these spore chains may differ according to culture conditions. They may be aerial and erect, decumbent, or flat on the surface of the medium or under the surface. Cylindrical spores diversify in shape, they can be barrelshaped, ellipsoidal or subglobose, sizes usually span from 2-12 µm and may go up to 50 µm (Carmichael 1957). It seems that a single spore contains several nuclei, usually two and can be up to four units.

The presence of other microorganisms, diffusible fungal metabolites or the absence of an essential somatic phase factor can instantly trigger sporulation, which can also be triggered by inducers, which are either produced by older colonies or by other microorganisms present in the environment. Late self-sporulation induction can occur in case of large *G. candidum*

biomass quantity in an insufficient volume of medium (Park and Robinson 1969). Furthermore, nutrient concentration could influence spore formation. For instance, carbon and nitrogen sources seem positively enhance sporulation and thus, should be maintained in large amount in media. These conditions determine whether *G. candidum* switches from somatic state to arthrospore state or remains in somatic state. Glucose and ammonium nitrate were proven to inhibit *G. candidum* sporulation (Park and Robinson 1970). Overall, nutrients, fungal charge and the presence of other microorganisms are important regulating the sporulation in *G. candidum* (Hudecová et al. 2009b). Optimal *G. candidum* germination was correlated with minimal sporulation, even if the medium lacked nutrients. This means that when germination mechanism is launched, the sporulation mechanism is shut down, and vice-versa. To cancel germination inhibition, nutrient composition should be increased in the medium (reinforcement with glucose and ammonium based elements) (Park and Robinson 1970). Somatic tips of *G. candidum* are characterized by axially placed, mitochondria, while arthrospores are determined by peripherally placed, short, oval mitochondria. Protoplasm of *G. candidum* comprises nuclei, mitochondria, free ribosomes, fragments of endoplasmic reticulum, various small vacuoles and particles of glycogen, which are covered by three or four wall layers in resting arthrospore. A novel layer of wall materials generates between the original spore wall and the cytoplasm, and is restricted to the zone where germtube emergence appears. This new layer is continuous with the germ-tube wall. Vesicles are observed at the apices of germlings after the emanation of germ-tube (Steele and Fraser 1973).

d. *G. candidum* growth conditions

Effect of temperature

The optimal growing temperature varies from one *G. candidum* strain to another. Three main types of strain are described:

- Type I: colonies with creamy color looked like yeast form, and grow optimally at temperature ranging from 22 to 25°C, yet show the weakest growth at 30°C. They are mostly acidifying agents with weak proteolytic activity and produce numerous arthrospores, but little mycelium
- Type II: intermediary between type-one and types-three strains.
- Type III: colonies definitely white, more or less felting. Optimal growing temperature ranges from 25 to 30°C. However, it shows less growth at 22°C. Compared to the first type, it forms much more mycelium, but less arthrospores during the growth phase. It is considered as an alkalinizing agent with remarkable proteolytic activity (Gueguen 1982)

A study on the effect of temperature on germination and growth of *G. candidum* has demonstrated that this imperfect fungus is relatively able to form spores at all temperature range from 4 to 37°C in which the highest germination and growth rate is within 25 to 30°C (Plaza et al. 2003).

Regarding cheese production, *G. candidum* is able to grow on surface at temperatures from 5°C to a highest of 38°C, the optimal condition being around 25°C at pH 5.0-5.5 (R. Boutrou and Guéguen 2005). The doubling time of *G. candidum* is one of the shortest among eukaryotes (1.1 h at 30°C) according to its specific growth rate during exponential phase in broth medium (Trinci 1972). Surprisingly, *G. candidum* was able to grow at 5°C on skimmed milk agar but with low radial growth rate while maximal growth rate was observed at 25°C at which exponential growth phase lasted for 2 weeks (Hudecová, Valík, and Liptáková 2009b, 2009a). In addition to temperature, the difference of strains of *G. candidum* and the designs of experiment (temperature modifications, change from glucose to sorbitol as a sole carbons source and insertion cycloheximide) are factors influencing the growth rate (Caldwell and Trinci 1973; Hudecová, Valík, and Liptáková 2009b). In most surveys, and regardless of the purposes of its application, *G. candidum* was incubated at 25°C on solid or in liquid medium, so it can be assumed that it may be its optimal growing temperature (Dieuleveux, Lemarinier, and Guéguen 1998; Couriol, Amrane, and Prigent 2001; Trinci and Collinge 1975; Helinck et al. 2000).

pH influence

Every type of fungi requires its specific pH range that allows it to optimally grow, sporulate and produce metabolites. For instance, *Chaetomium funicola* and *Pythium acanthicum* show fastest growth in a neutral medium, while *G. candidum* and *Sordaria fimicola* grow optimally in acidic medium (pH 4.0 for *G. candidum*) (Child et al. 1973). In fact, *G. candidum* tends to modify the pH of the medium if it does not meet its need for growth (Assas et al. 2002; Botha and Botes 2014; Assas et al. 2000).

When it comes to colonizing natural substrates, *G. candidum* can grow on cheese surface in a wide pH range from 3 to 11 (R. Boutrou and Guéguen 2005). The reported optimal pH was more or less acidic, located between 5.0 and 6.0, which confirm what was previously found (Child et al. 1973). The study of acidifying and none acidifying races of *G. candidum* isolated from soil showed that this organism could grow in a large range of pH from 2 to 9 in potato dextrose broth medium. All isolates studied grew well at pH 2 to pH 7 with optimal growth at pH 6 (Suprapta et al. 1996). In uncontrolled pH and controlled pH conditions, cells of *G. candidum* grew to the highest level at pH of 5.5, but a controlled pH environment showed better results at the same pH (Bhattacharyya and Banerjee 2007; Bhattacharyya et al. 2008).

Regarding a whole other application, Assas et al. (2002) conducted studies that consisted of decolorizing olive mill water wastes using *G. candidum* (Assas et al. 2002). During experiments on fresh olive mill water wastes, the pH dropped from 5.1 to 4.2 probably due to sugar consumption. In contrast, on old and stored-black olive mill water waste already charged with high quantities of organic acids, the pH rose rapidly from 5.1 to 7.8 which was induced by the consumption of those organic acids by *G. candidum* (Assas et al. 2002; Assas et al. 2000).

Effect of oxygen

For aerobic microorganisms, the presence of oxygen is essential for cellular respiration. On one hand the appropriate amount of dissolved oxygen is generally beneficial for the full growth and high yield of metabolites and on the other hand, lower than critical concentration results in lower cell growth and metabolites production which is strongly caused by a significant change in the physiology of the organism (Bhattacharyya et al. 2008). The role of oxygen is important to ensure the proper growth; once the oxygen supply became inadequate, the decrease of growth rate of *G. candidum* was observed (Kier et al. 1976). However, the way that the oxygen is distributed inside a bioreactor or fermenter tank has a major impact on microorganism growth especially when aeration and mechanical agitation can alter the yeast-like filamentous fungi morphology.

Improvement in the growth and branching of *G. candidum* mycelium was achieved by the application of aeration without agitation (Assas et al. 2000). Bhattacharyya et al. (2007) reported that the cell biomass production was negatively affected by the use of a higher agitation rate (Bhattacharyya and Banerjee 2007). The experiments to find the optimal agitation rate and aeration rate were realized in a stirred tank bioreactor containing synthetic medium: peptone 6 g/L, malt extract 4 g/L, yeast extract 3 g/L, brain heart infusion 1.5 g/L, meat extract 1.5 g/L, and glucose 20 g/L (Bhattacharyya and Banerjee 2007).

As a result, the maximum growth of *G. candidum* was obtained when applying the slowest agitation rate of 200 rpm at high aeration rate of 0.6 vvm, while the best performance of carbonyl reductase enzyme was reached at low aeration rate of 0.4 vvm by agitating at the same rate. Moreover, the study of optimizing lipase production in the peptone and soil oil based medium, reported that the stirred fermenter at 300 rpm and 1 vvm and the airlift bioreactor at 2.5 vvm gave similar maximum lipase activities, but productivity in the airlift was about 60% higher than in the stirred fermenter (Burkert et al. 2005). The use of oxygen in an airlift fermenter improves the productivity of *G. candidum* rather than in a stirred one. This is due to its low shear rate and stress which causes less injury to the filamentous fungi, allowing faster growth with less mycelium damage (Burkert et al. 2005).

e. Benefits and utilities of *Geotrichum candidum*

G. candidum is recognized by its proteolytic capacities and its aromatic characteristics (Boutrou and Guéguen 2005). Given the diversity of its metabolic pathways, *G. candidum* contributes to the maturation of many kinds of soft and semi-hard cheeses and positive flavor and aroma development (Boutrou et al. 2006).

As described above, *G. candidum* is found in raw milk and on the dairy product surfaces. It is involved in cheese ripening given the fact that it produces various volatile compounds and induces typical flavors. This contributes to a wide cheese variety. Investigations show that *G. candidum* generates aromatic volatile molecules belonging to different chemical classes such as alcohol, aldehydes, esters, sulfides, terpens… This filamentous microorganism produces

mainly sulfuric compounds (Spinnler et al. 2001; Martin et al. 2001; Mdaini et al. 2006; Arfi et al. 2002). A study demonstrated that, when cultivated on glucose-enriched medium, *G. candidum* accumulates flavor volatile compounds in the broth that had a fruity flavoring note, usually pineapple-like, such as esters and alcohols. The later included ethyl esters of acetic acid and butyric acid, methyl-3-butan-1-ol, and methyl2-propan-1-ol that were mainly detected during the stationary growth phase of the strain. The main concentrated molecules were 2-hexanoic acid ethyl ester and benzaldehyde detected at 9.5 g/L and 1.6 g/L respectively. It was suggested that ethyl alcohol may be an intermediate metabolite to this pathway since it was found in lower concentrations (Mdaini et al. 2006).

Despite its role in cheese ripening and generating fruity aromatic compounds, investigations showed that *G. candidum* is also known as an effective biological control agent, capable of inhibiting potentially pathogenic Gram-positive bacteria, such as *Listeria monocytogenes, Staphylococcus aureus, Bacillus cereus* and *Enterococcus faecalis.* It also plays a role in inhibiting Gram-negative bacteria such as *Provodencia stuartii*, *Klebsiella oxytoca, Salmonella* and *Escherichia coli* (Dieuleveux et al. 1998a; Dieuleveux et al. 1998b). In 1998, Dieuleveux et al. conducted several studies to evaluate and understand how *G. candidum* stops the growth of *Listeria*. The inhibition of this pathogen is caused by two inhibitors which are D-3- phenyllactic acid (PLA) and D-3-indollactic acid (ILA) (Dieuleveux et al. 1998a; Dieuleveux et al. 1998b).

C. Phenyllactic acid (PLA)

a. Physical and chemical characteristics of PLA

Phenyl lactic acid (PLA) or 3-phenyllactic acid (2- Hydroxy-3-phenyl propionic acid) is under molecular formula $C_9H_{10}O_3$ whose molecular weight is 166 g/mol (Chaudhari 2016). This organic acid has two chiral isomers, L-PLA and D-PLA (figure 15), characterized by an antimicrobial activity (Chaudhari 2016). These none proteic-compounds have a huge stability over a wide pH range and can be resistant to the sterilizing temperature of 120°C for 20 min.

D(+)-phenyl lactic acid L(-)-3-phenyl lactic acid

Figure 15 - Chemical structure of D-(+)-phenyllactic acid and L(-)-3-phenyllactic acid

b. PLA sources

PLA is produced by many microorganisms, including lactic acid bacteria during the fermentation of certain foodstuffs such as dairy products. Following several studies, this metabolite is considered to be an ideal antimicrobial agent with both antibacterial and antifungal activities. Based on this fact, it could be used as a food additive, thus replacing the use of antibiotics in the world of the agro-food industry. PLA is found in very large amount in several types of honey (and more precisely thistle, heather, and manuka honey), in higher proportions than other phenolic acids (Tuberoso et al. 2011; Dimitrova et al. 2007; Tan et al. 1988).

c. PLA producing microorganisms

A wide range of microorganisms have been diagnosed for their ability to produce PLA. Table 7 et 8 present the different PLA-producing strains (Mu et al. 2012) including lactic acid bacteria and other genera.

Table 7 - Lactobacillus PLA-producing strains.

Lactic acid bacteria	
Strain	Reference
Lactobacillus plantarum 21B	(P. Lavermicocca et al. 2000)
Lb. plantarum MiLAB393	(Zhang et al. 2017)
Lb. alimentarius ATCC29643, Lb. mesenteroides ITMY30, Leuconostoc citreum ITM22A, Weissella confusa ITM14A, Enterococcus faecium ATCC882	(Valerio et al. 2004)
Lb. plantarum VLT01	(Armaforte et al. 2006)
Lactobacillus sp SK007	(X. Li, Jiang, and Pan 2007)
Lb. plantarum 1081,778,1073,1063,1501	(Gerez et al. 2010)
Lb. plantarum VE56, Weissella cibaria FMF4B16, Weissella paramesenteroides LC11	(Ndagano et al. 2011)
Lb. plantarum CRL778	(Dallagnol et al. 2011)
Lb. plantarum IMAU10014	(H. K. Wang et al. 2012)
Lb. plantarum CECT-221	(Rodríguez et al. 2012)

Table 8 – Other PLA producing strains.

Other genera	
Strain	References
Brevibacterium lactofermentum	(Mu et al. 2012)
Geotrichum candidum	(Dieuleveux et al. 1998)
Propionibacterium jensenii DSMZ 20535, Propionibacterium thoenii DSMZ 20276, Propionibacterium acidipropionici DSMZ 4900, Propionibacterium freudenreichii DSMZ 20271	(X. Li, Jiang, and Pan 2007)
Bacillus coagulans	(Zheng et al. 2011)

d. PLA antibacterial and antifungal activity

The assay to investigate its inhibitory activity mentioned that D-phenyllactic acid is more effective than its L form in reducing *L. monocytogenes* growth (Dieuleveux et al. 1998; Lind et al. 2007). The ability of commercial 3-phenyllactic acid, both the L and D forms, to inhibit the growth of two fungi, *A. fumigatus* and *P. roqueforti* was tested (Liu, et al. 2009; Zhang et al. 2017). The results showed that for both fungi the Minimal Inhibitory Concentration (MIC) for total growth inhibition was 7.5 g/L at pH 4. The same MIC value of PLA was tested against yeast and was effective enough to inhibit its growth (Schwenninger et al. 2008). The clear mechanism of the inhibitory action is not described yet, but through the use of scanning

electron microscopy (SEM), it was observed that when PLA interacts with bacteria, the cell wall is destroyed and then creates aggregates, leading to the release of polysaccharides and loss of cell wall rigidity, resulting in cell death (Dieuleveux et al. 1998a; Dieuleveux et al. 1998b).

e. PLA biosynthesis pathway

The PLA biosynthesis pathway is well described in Lactic Acid Bacteria (LAB) strains, which can produce large amounts of PLA. The PLA biosynthesis starts from the catabolism of the phenylalanine into phenylpyruvic acid (PPA). Then, PPA is reduced into PLA (figure 16). The transamination of the amino acid takes place in the first step, in which phenylalanine is transaminated to PPA by an aromatic amino acid transferase (AAT) (Chaudhari 2016). This enzyme is pyridoxal 5'- phosphate-dependent enzyme or the other name is cystathionine γ-lyase (EC 4.4.1.1) (Yvon et al. 1997; Gente et al. 2007). This enzyme also transaminates other amino acids including leucine and methionine (Yvon et al. 1997). AAT catalyzes the transfer of the amino group from amino acid to a suitable ∝-keto acid acceptor, mainly ∝-ketoglutarate. The last step is the conversion of PPA to PLA by the action of various dehydrogenases, among them lactate dehydrogenase (L-LDH) is the most important (Mu et al. 2012). The production of PLA by lactic acid bacteria increases dramatically after the addition of PPA to the initial medium, or during fermentation. PPA is an intermediate for the production of phenylalanine. Indeed, once the PPA is added to the culture medium, the production of PLA by *Lactobacillus sp* KS007 was increased by 14 times (Li et al. 2007). Mu et al. (2009) have developed a method for optimizing the production of PLA during fed-batch fermentation by controlling the pH and the amount of PPA added in the process (Liu et al. 2009; Chen et al. 2009).

Until now there is no study proposing a possible biosynthesis pathway for PLA production in *G. candidum*. However, according to Gente et al. (2007), aiming to develop a test based on the use of RT-PCR to detect the expression of the cystathionine gamma lyase (*cgl*) gene of *G. candidum* revealed that *G. candidum* possesses this gene (Gente et al. 2007). Therefore, one could assume that *G. candidum* can synthesize PLA by this same biosynthesis pathway.

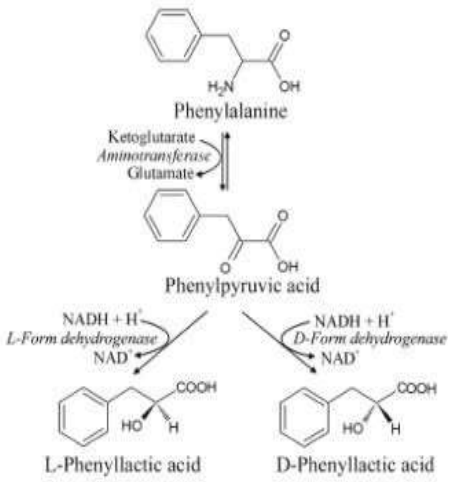


Figure 16 - PLA biosynthesis pathway in the metabolism of lactic acid bacteria (Mu et al. 2012°;

D. Other metabolites

Indole lactic acid (ILA) is an organic acid characterized by its molecular formula $C_{11}H_{11}NO_3$ and its molecular weight 205.2 g/mol. It derives from the metabolism of tryptophan in several microbial strains such as *G. candidum, Candida* spp. and *Bifidobacterium* (Aragozzini et al. 1979; Rao 1976; Naz et al. 2013). This had been reported to inhibit the growth of both Gram negative and Gram positive bacteria such as *Escherichia coli* and *Bacillus cereus*. Figure 1 represents the metabolic pathway of indole lactic acid and shows the implication of several enzymes of which tryptophan aminotransferase, tryptophan decarboxylase and indole-3-lactic acid dehydrogenase (Frankenberger and Poth 1988; Sardar and Kempken 2018). The synthesis of indole-3-pyruvic acid from tryptophan requires some environmental conditions specific to cheese ripening: low temperature at around 13°C, a pH 5, and a minimum of 4% NaCl to activate the aminotransferase (Roudot-Algaron and Yvon 1998). Even though ILA is known for its great antimicrobial potential, its formation is unfavorable, as it may alter the organoleptic criteria of food and feeds (Gummalla and Broadbent 2001; El Soda 1993). This would probably be a restraint to the use of this metabolite in food and feed matrices.

Another aromatic amino acid reported to be produced by *G. candidum* and other microbial strains is Phenylethyl alcohol (PEA) characterized by a rose-like odor. This metabolite, also known as 2-Phenylethanol (2-PE), with the molecular formula $C_6H_5CH_2CH_2OH$ and characterized by low molecular weight 122.16 g/mol. PEA is mainly a bacteriostatic agent, that can reversibly inhibit the synthesis of bacterial deoxyribonucleic acid when administrated in culture media at low concentration (0.3% in culture media) (Lucchini et al. 1993). At higher concentrations, it breaks down the cellular barrier which increases the membrane permeability accelerating passive ions and metabolites diffusion (Silver and Wendt 1967; Ingram and Buttke 1985). Therefore, its antimicrobial effect is revealed at relatively high concentrations (superior to 3 g/L when produced by *Saccharomyces cerevisiae*) (Ingram and Buttke 1985). The effect of PEA produced by *Saccharomyces cerevisiae* was identified against several bacterial strains including *Salmonella, Shigella, Aerobacter, Klebsiella, Escherichia coli* and *Pseudomonas* (Fabre et al. 1998; Etschmann et al. 2002).

The metabolic pathway of PEA is mostly described in yeasts. The whole process is detailed in the study conducted in 2002 by Etschmann et al (2002) (Etschmann et al. 2002). The biosynthesis of phenylethyl alohol, also called 2- phenylethanol (2-PE), was identified by Ehrlich and consequently named after him (Hazelwood et al. 2008). PEA distinguishes by rose-like aroma and is a desirable flavor molecule in various alcoholic beverages. In red wine and beer for example, PEA is one of the major alcohols produced at concentrations ranging from of 13.3 mg/L to 70.6 mg/L (Mallouchos et al. 2002). PEA has also been shown to protect strawberries against fungal growth and thus prolong its shelf life strawberries (Mo and Sung 2007). In fact, during this study, strawberry sample batches were either fumigated with PEA extracted from *Pichia anomala SKM*-T culture broth or were not treated at all (control condition). PEA extraction and purification method was described in Mo et al. study (2003) (Mo et al. 2003). PEA was also extracted from *Galactomyces geotrichum* and used to fumigate the fruit. It is important to note that *Galactomyces geotrichum* is indeed the teleomorphic form of *G. candidum* (Perkins et al. 2020).

Main objectives of this survey

As mentioned in the previous chapter, the establishment and expansion of *Fusarium* species, has become a major health concern for the entire cereal industry, due to the proven health risks of their toxins.

The French Institute of Brewing and Malting is particularly concerned with this issue. As a matter of fact, malting conditions are favorable to the development of *Fusarium* species, brought by contaminated barley grains to the production chain. In September 1999, the Institute selected strains of *Geotrichum candidum,* filamentous yeasts, that are capable to reduce fungal growth during the malting step of the brewing process. Its effectiveness was proven on several microorganisms, such as *Penicillium*, *Aspergillus* and *Fusarium*. The particularity of this innovation relies on the implementation of a biocontrol will be based on a preventive approach. It consists on using *G. candidum,* an ecofriendly and safe yeast to reduce the contamination with T-2 toxin in the malt. This project seeks the development of a safe and efficient brewing process. It targets the comprehension and amelioration of this innovative and environmentally friendly biocontrol used to fight against the occurence of *Fusarium* species, *F. langsethiae* and *F. sporotrichioides* in particular and their T-2 toxin production.

The proposed work will be divided into three main chapters.

The first part will focus on improving knowledge and understanding the interaction mechanisms between *G. candidum* and *Fusarium* species. It will focus on several tasks. Preliminary tests will consist on evaluating direct contact between the two microorganisms through co-culture conditions in order to assess the effect on T-2 toxin concentration. Indirect interactions held during sequential cultures will help clarify the type of interaction that supposedly leads to reduction of the mycotoxin's concentration in order to optimize the implementation of the biocontrol. Another task is to identify the active agent produced by *G. candidum*. Different metabolites inhibiting the growth of microorganisms have been identified as mentioned above. Thus, potential presence of one or more of these metabolites in *G. candidum* cultures will be assessed.

The second part will consist in optimizing the implementation of biocontrol on a micro-malting scale. The efficiency of previous findings will be tested in a simulated malting environment. Indeed, several conditions will be monitored to define better conditions for the biocontrol use.

The third and last part will seek to test several culture conditions of *G. candidum* in order to improve yeast growth as well as the production yield of the active agent. During these trials, several variable will be tested and the development of *G. candidum* as well as the production of the agent will be tracked. The aim is to optimize the implementation of the biocontrol in order to improve its use mainly during the malting step.

Material and Methods

A. Reagents and chemicals

Yeast extract (Y1625), malt extract (70167) and dextrose (D9434) were supplied by Sigma-Aldrich. Peptone (2585K) was supplied by Fisher Scientific. Alpha-D (+)-Glucose, 99% anhydrous (Code: 170080025) was supplied by ACROS ORGANICS Di-Potassium hydrogen orthophosphate anhydrous graded (Code: P/5245/53) as analytical reagent was supplied by Fisher Scientific. L-Phenylalanine (78019) was supplied by Fluka BioChemika. Potatoes Dextrose Agar (PDA) medium (P2182) was supplied by Sigma-Aldrich. Acetonitrile (HPLC grade) and glacial acetic acid were purchased from Fisher Scientific. Water was produced by a Millipore Milli-Q system (Millipore, Bedford, MA, USA). Standards T-2 toxin, HT-2 toxin and Phenyl Lactic Acid (PLA) were purchased from Sigma-Aldrich.

B. Culture media

Ym liquid medium was used during co-cultures and sequential cultures to elucidate the interaction mechanisms between *G. candidum* and *Fusarium* strains.

To study the *G. candidum* growth and PLA production, three semi-synthetic media were prepared, named Ym, Malt Extract Broth (MEB) and Synthetic Medium (SM). Their composition is described in Table 9. The Ym was the reference medium for these experiments. The Synthetic Medium composition was modified from the one described by Mu et al. (2009), in which phenylpyruvic acid was replaced by L-phenylalanine, the precursor of the phenylpyruvic acid. The SM medium contained Glucose, Malt extract, Yeast extract, Peptone, Phenylalanine and K_2HPO_4. The initial pH of these three media was adjusted at seven. All culture media were sterilized at 121°C for 20 minutes.

Table 9- Composition and concentration of different media used during the experiments

MEDIUM	COMPOSITION (G /L)
CARBOXYMETHYLCELLULOSE MEDIUM (CMC)	carboxymethylcellulose 15 g/L; yeast extract 1 g/L; $MgSO_4 7H_2O$ 0.5 g/L; NH_4NO_3 1g/L; KH_2PO_4 1 g/L
MALT EXTRACT BROTH (MEB)	malt extract 20 g/L; peptone 6 g/L; dextrose 20 g/L
BARLEY BASED MEDIUM	Barley kernels submerged in water overnight (1:1 ration of grain:water)
POTATO DEXTROSE AGAR (PDA)	PDA powder 39 g/L
SYNTHETIC MEDIUM (SM)	glucose 30 g/L; malt extract 30 g/L; yeast extract 30 g/L; peptone salt 47 g/L; phenylalanine 5 g/L; K_2HPO_4 0.3 g/L
YEAST AND MALT MEDIUM (YM)	glucose 5 g/L; yeast extract 1.5 g/L; malt extract 1.5 g/L; peptone 2.5 g/L

C. Microorganisms and strains

- *Geotrichum candidum* filamentous malting yeast (IFBM Malting Yeast®, DMS food specialties, La Fertésous Jouarre, France) was purchased from DSM Food Specialties under freeze-dried form. *G. candidum* is already used as a biocontrol agent during the malting process. Figure 17 shows the filamentous state of *G. candidum.*

Figure 17 - G. candidum filamentous form after 3 days of culture on Ym medium at 150 rpm, 22°C. Image taken from an optical microscope 40x magnification.

- *Fusarium* strains: *F. langsethiae* 2297 and *F. sporotrichioides* 186 used to realize experiments, were kindly provided by the French Institute of Brewing and Malting (IFBM. - Nancy, France). There were isolated from barley kernels. These two strains were cultured on PDA at 22°C for 7 days, and then conserved at 4°C. Figure 18 and 19 show *F. langsethiae* 2297 and *F. sporotrichioides* 186 on PDA medium and with round shaped and spindle shaped spores respectively after 7 days of incubation time at 22°C.

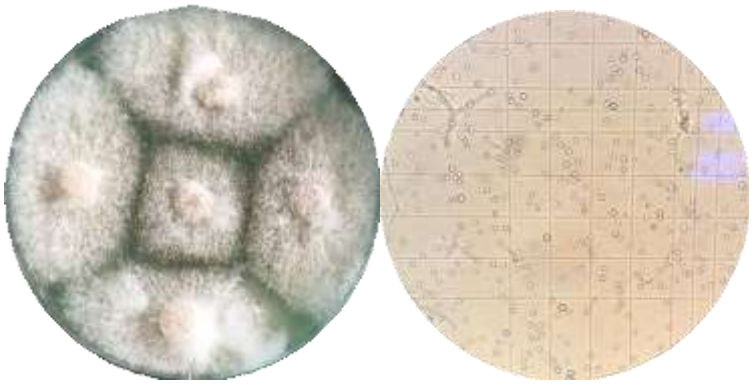


Figure 18 - On the left, F. langsethiae 2297 on PDA after 7 days at 22°C. On the right, F. langsethiae 2297 spores cultured on CMC medium for 15 days at 150 rpm, 22°C (optical microscope 100x)

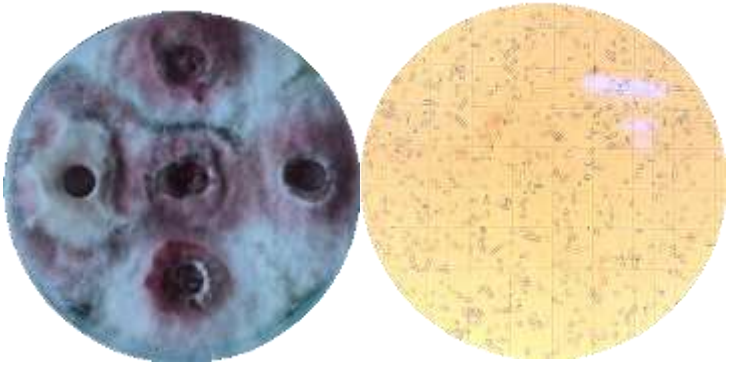


Figure 19 - On the left, F. sporotrichioides 186 on PDA after 7 days at 22°C . On the right, F. sporotrichioides 186 spores cultured on CMC medium for 15 days at 150 rpm, 22°C (optical microscope 100x)

D. Pre-cultures

Plugs of previous *F. langsethiae* 2297 and *F. sporotrichioides* 186 cultures were grown on PDA medium for 7 days at 22°C. The strains are transplanted every 7 days when maintained at 22°C, and every two months when conserved at 4°C.

Fusarium strains sporulation was induced in carboxymethylcellulose (CMC) liquid medium. At least 15 plugs of each *Fusarium* strain from a seven-day-old solid pre-culture were inoculated in 150 mL of CMC medium and incubated in an orbital shaker set at 22◦C at 150 rpm for 15 days in the dark. At the end of the incubation time, the spore suspension was collected and filtrated using sterilized Miracloth. Spores were counted on Thoma cell counting chamber and ultimately used to inoculate culture during further experiments or conserved in 40% glycerol at -80°C.

G. candidum strain was supplied in freeze-dried form. In most experiments, a pre-culture to revivify it before experimental use was carried out. Thus, 24 g/L of freeze-dried powder was added in 250 mL of Yeast and Malt (Ym) liquid medium and incubated in an orbital shaker set at 22◦C at 150 rpm for 24 h. At the end of the incubation time, this pre-culture was used as a starter culture. For other experiments, the freeze-dried powder was added directly to the medium without a preliminary revivification step.

E. Culture conditions

a. Kinetic of PLA production by *G. candidum*

In a 250 ml Erlenmeyer flask, 150 mL of Ym medium was inoculated with *G. candidum* starter culture with a concentration adjusted at 0.2 g/L and then incubated in an orbital shaker set at 22◦C at 150 rpm for different fermentation times ranging from 6 h to 120 h. At the end of the fermentation time, the medium was aseptically divided into two volumes. First, 50 mL were used to determine *G. candidum* growth by measuring the dry weight and PLA concentration by HPLC-DAD at each sampling time. The remaining 100 mL was aseptically filtered to remove *G. candidum* cells and recover the metabolites excreted in the medium. The medium nutrients were then adjusted according to the volume and the pH was adjusted at 7. These media were used in the sequential cultures experiments and are henceforth referred to as pre-fermented media (in section E.c). Experiments were conducted four times in triplicate.

b. Co-cultures of the two *Fusarium* species and *G. candidum*

First, 250 ml Erlenmeyer flasks containing 150 mL of Ym medium were inoculated with *G. candidum* starter culture at the concentration of 0.2 g/L. Then, *F. langsethiae* 2297 or *F. sporotrichioides* 186 was inoculated at a concentration of 10^6 spores/mL in their respective

flasks. For control conditions, each microorganism was inoculated alone at the same concentration. Cultures were incubated in an orbital shaker set at 22◦C at 150 rpm. Several incubation times were tested: 24 h, 48 h, 72 h, 120 h and 168 h.

For some experiments, Erlenmeyer flasks containing 150 mL of Ym medium were inoculated with *G. candidum* starter culture adjusted to 0.4 g/L. The concentration of *Fusarium* spores inoculated was maintained at 10^6 spores/mL in their respective flasks. All culture conditions were also sustained.

At the end of all sampling times for all culture conditions the total dry weight, PLA and T- 2 toxin concentration were measured. All experiments were conducted twice in duplicate.

c. Sequential cultures of the two *Fusarium* species and *G. candidum*

For sequential cultures, 100 mL of pre-fermented Ym medium at different fermentation times ranging from 6 h to 120 h were inoculated with *F. langsethiae* 2297 or *F. sporotrichioides* 186 at the concentration of 10^6 spores/mL in their respective flasks. Cultures were incubated in an orbital shaker set at 22◦C at 150 rpm for 7 days. For the control condition, *F. sporotrichioides* 186 or *F. langsethiae* 2297 were inoculated in a non-fermented Ym liquid medium at the same concentrations. At the end of the incubation time, fungal growth was determined by measuring the dry weight and T-2 toxin concentration was measured by HPLC-DAD. All experiments were conducted twice in duplicate.

d. Phenyllactic Acid Effect on *F. sporotrichioides 186* and *F. langsethiae 2297* growth and T-2 toxin Concentration

To confirm that PLA is the metabolite produced by *G. candidum*, which is involved in *Fusarium* growth reduction and T-2 toxin concentration reduction, fungal cultures were conducted in Ym liquid medium supplemented with PLA. PLA standard stock solution was prepared at 40 g/L in a mixture of acetonitrile/water (30/70, v/v) and appropriate volumes of PLA stock solution were added in order to obtain several different concentrations: 0.05 g/L; 0.1 g/L; 0.2 g/L; 0.3 g/L; 0.4 g/L and 0.5 g/L in Erlenmeyer flasks containing 100 mL of Ym liquid medium. Then, *F. langsethiae* 2297 or *F. sporotrichioides* 186 was inoculated at the concentration of 10^6 spores/mL in their respective flasks. Cultures were incubated in an orbital shaker set at 22◦C at 150 rpm for 7 days. At the end of the incubation time, *Fusarium* growth was evaluated by measuring the dry weight and T-2 toxin concentration by HPLC-DAD. PLA dilutions were prepared to add only 75 µL of acetonitrile in the culture medium, this concentration having been identified as a no-effect dose on both fungal growth and T-2 toxin concentration. Control cultures were performed by adding only 75 µL of acetonitrile in the medium.

e. Micro-malting assays

The purpose of these experiments is to mimic malting conditions and test several parameters to figure out the most effective way to reduce T-2 toxin concentration. According to the IFBM, malting conditions are held in a 1:1 ratio of grain:water. The kernels are left in water for 10- 15 hours, then the filamentous yeast is poured directly in freeze-dried form and set for 3 to 5 days at 22°C. During these experiments, several parameters were tested: the preparation method of the filamentous yeast inoculum, the inoculation rate and the incubation time.

Therefore, 200 g of barley kernels were submerged overnight in 200 mL water in 2L bottles and then sterilized at 121°C for 20 minutes. Some recipients were inoculated with *G. candidum* starter culture at the final concentration of 0.2 g/L, others were inoculated with 0.2 g/L of freeze-dried yeast powder. Then, *F. langsethiae* 2297 or *F. sporotrichioides* 186 was inoculated at a final concentration of 10^6spores/mL in their respective flasks. For control conditions, each microorganism was inoculated alone at the same conditions. Cultures were incubated at 22◦C. Two incubation times were tested: 72h (3 days) and 120h (5 days).

In other experiments, bottles of sterilized barley kernels were inoculated with either *G. candidum* starter culture or freeze-dried yeast powder but with a double final concentration of 0.4 g/L. The final concentration of the two *Fusarium* species spores inoculated was maintained at 10^6 spores/mL in their respective flasks. All culture conditions were also sustained.

At the end of all sampling times for all culture conditions, 400 mL of ethyl acetate were used to extract the whole content bottles. Then, bottles were shaken on Universal Shaker SM 30 B Control Edmund Bühler® set at 150 rpm overnight. PLA and T-2 toxin concentrations were measured. All experiments were conducted twice in duplicate.

The pattern presented in figure 20 explains schematically the different conditions of the micro-malting assays.

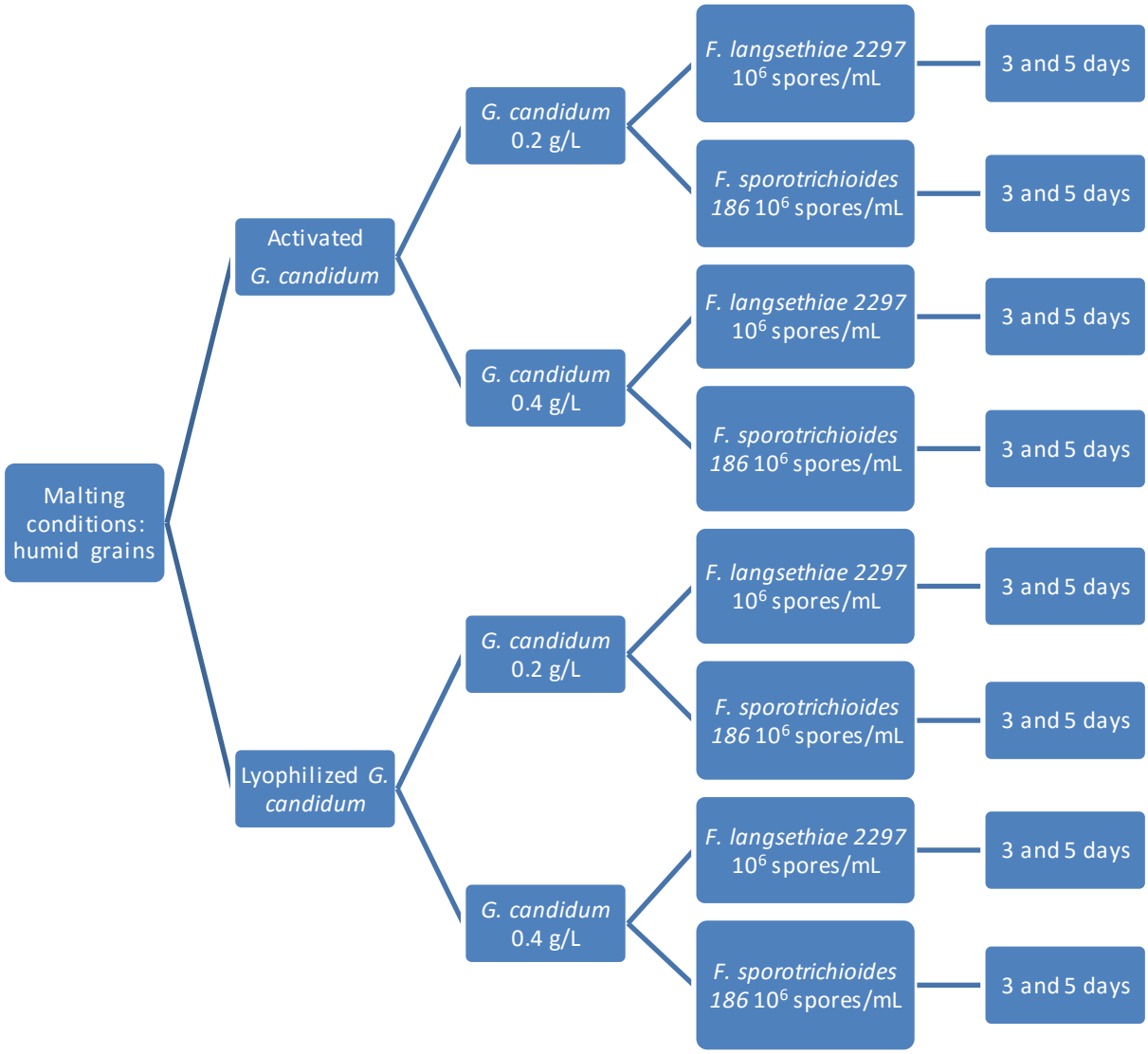


Figure 20 - Schematic representation of different conditions used during micro-malting assays

f. Effect of culture media on *G. candidum* growth and PLA concentration

G. candidum was supplied in freeze-dried form. so it is necessary to be pre-cultured to activate its metabolism and reduce the latent phase. Thus, 2.4 g of freeze-dried *G. candidum* was cultivated in a 250 mL Erlenmeyer flasks containing 100 mL of Ym medium, and incubated in an orbital incubator set at 22°C and 150 rpm for 24h. After incubation, *G. candidum* pre- culture was used as a starter culture.

The objective of inoculating *G. candidum* in three different media (Ym, MEB and SM) is to select the medium that improves the development of this yeast and, importantly, that leads to the optimal PLA production. Therefore, starter culture was used to inoculate those media with a concentration of 0.2 g/L. The inoculum was put into 250 mL Erlenmeyer flasks containing 50 mL of each medium. The incubation conditions were the same as described above. The kinetic of *G. candidum* growth and PLA concentration were studied through different sampling time kinetics including 17 h, 24 h, 41 h, 48 h, 65 h and 72 h.

g. Effect of the preparation of the starter culture on *G. candidum* growth and PLA concentration

The objective of the activation of *G. candidum* in a medium enriched in phenylalanine enriched medium (SM) is to procure the PLA intermediate. Indeed, yeast does not have to synthesize it and therefore takes less time to produce PLA. This could increase the metabolite concentration in subsequent culture.

Therefore, 2.4 g of freeze-dried *G. candidum* was cultivated in a 250 mL Erlenmeyer flask containing 100 mL of SM medium, and incubated in an orbital incubator set at 22°C and 150 rpm for 24 h. Then, *G. candidum* pre-culture was as a seed culture and used to inoculate Ym medium with a final concentration of 0.2 g/L. The inoculum was put into 250 mL Erlenmeyer flask containing 50 mL of medium. The incubation conditions were the same as described above. The kinetics of *G. candidum* growth and PLA concentration were investigated through different sampling time kinetics including 17 h, 24 h, 41 h, 48 h, 65 h and 72 h.

F. *G. candidum, F. sporotrichioides* 186 and *F. langsethiae* 2297 biomass analysis

To evaluate microorganism growth during the incubation period, vacuum filtration was performed to determine the dry weight (g/L). First, cellulose nitrate filters (pore size 0.45µm, Sartorius StedimBiotech, Goettingen, Germany) were left to dry overnight in an oven set at 105◦C. Afterward, 10 mL of culture medium were vacuum-filtered at each sampling time and

filters were then incubated at 105◦C for 24 h. The microorganism dry weight refers to the difference between filters post-filtration and pre-filtration.

G. PLA and T-2 Toxin Quantification by HPLC-DAD

a. PLA quantification

At each sampling time, 1 mL of culture media was withdrawn and filtrated through 0.45 µm PTFE syringe filters to eliminate microorganisms from the supernatant prior to injection into HPLC apparatus. Analysis of PLA were performed using a Luna C18 (2) column (5µm, 250×4.6 mm) and a pre-column with the same characteristics (Phenomenex, Torrance, CA, USA). The detection of PLA was performed using a Dionex Ultimate 3000 UHPLC system coupled with a diode-array detector (DAD) set at 210 nm (Thermo Fisher Scientific, Illkirch,France). The analysis was performed in a gradient mode using acidified water (0.2% of acetic acid glacial) as solvent A and pure HPLC grade acetonitrile as solvent B. Flow rate was set at 1.2 mL/min with A/B ratios of 90:10, 50:50, 50:50, 0:100 and 90:10, with run times of 0.0, 4.0, 9.0, 10.0 and 15.0 min, respectively. Injection volume was set at 50 µL. PLA quantification was determined according to a standard calibration curve with concentrations ranging between 10 and 1000 mg/L.

b. T-2 Toxin extraction and quantification

After the incubation period, cultures were filtrated with Nalgene™Rapid-Flow™Filters of 0.45µm pore size (Thermofischer Scientific, Waltham, MA, USA) to remove microorganisms. Filtrates were then extracted with 70 mL of ethyl acetate and shaken on a Universal Shaker SM 30 B Control EdmundBühler®(Thermofischer Scientific, Waltham, MA, USA) set at 150 rpm overnight. The organic phase was recovered and evaporated until dry under a rotavapor set at 60◦C. Samples were resuspended with 2 mL of acetonitrile/water (30/70, v/v) mixture and filtered through 0.45µm PTFE syringe filters (Sigma Aldrich, St. Quentin Fallavier, France). Samples were conserved at 4◦C until further analysis. T-2 toxin was analyzed by Gemini C18 columns, 150 mm×4.6 mm, 3µm and a pre-column with the same characteristics (Phenomenex). T-2 toxin was detected and quantified using HPLC-DAD (Dionex, Sunnyvale, CA, USA) according to the methodology described by Medina et al. [59]. T-2 toxin quantification was calculated according to a standard calibration curve with concentrations ranging between 0.2 and 50µg/mL. According to the study, LC-DAD limits of detection (LOD) and limits of quantification (LOQ) were 0.1612 and 0.2294 $mgkg^{-1}$ agar for HT-2 and 0.1648 and 0.2541 $mgkg^{-1}$ agar for T-2, respectively.

H. Statistical Analysis

First, the normal distribution of data was tested by the Shapiro-Wilk test. Then, one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test was used to analyze the effect of PLA on *F. langsethiae* 2297 and *F. sporotrichioides* 186 growth and their T-2 production. One-way ANOVA followed by a Tukey's multiple comparisons test was used to analyze the differences between control and co-culture or sequential culture conditions. Differences were considered to be statistically significant when the p-value was lower than 0.05. Graphical values are represented by mean ± standard deviation (SD). In micro-malting experiments, Two-Way ANOVA followed by a Tukey's multiple comparisons post-hoc test was used to analyze all conditions compared to each other. Data with different letters are significantly different p-value < 0.05). The statistical analysis of data was carried out with GraphPad Prism 8 software (GraphPad Software, La Jolla, CA, USA). Differences were considered to be statistically significant when the p-value was lower than 0.05. Graphical values are represented by mean ± standard deviation (SD).

Experimental work

Chapter 1 – Elucidation of the interaction mechanisms between *Geotrichum candidum* and *Fusarium langsethiae* and *F. sporotrichioides*.

As described in the first part of this manuscript, *Fusarium* species are frequent contaminants of barley crops. They are phytopathogen fungi responsible for the Fusarium Head Blight (FHB) disease (Stanca et al. 2016). *Fusarium* species are considered a major concern due to their widespread occurrence and capacity to produce T-2 and HT-2 toxin, well-known mycotoxins belonging to the type A trichothecenes family (Morcia et al. 2016b). T-2 and HT-2 toxins are present in cereal-based food and feed products and have an impact on farm animals and pets consuming contaminated food. Consumption of contaminated food can also have an impact on human health.

F. langsethiae and *F. sporotrichioides* had been observed on barley for a little more than ten years, especially on malting barley. Due to the wide occurrence of these mycotoxinogenic fungi, it is not possible to completely avoid the contamination of malting barley with T-2 and HT-2 toxins. Mycotoxin contaminations are resistant to normal grain processing methods and high temperatures. Therefore, it is important to monitor the development of these contaminant fungi and to establish biological methods to reduce the risk of these mycotoxins to insure the sanitary quality of beer.

The implementation of the filamentous yeast *G. candidum* during the malting step met with great success in breweries. Not only does this microorganism improve the organoleptic quality of the finished product, but it also reduces T-2 toxin concentration. However, interactions between *Fusarium* species and this yeast, leading to this reduction, have not been elucidated up until now.

The aim of this study was mainly to better understand the interaction mechanisms between the filamentous yeast and two strains of *Fusarium*: *F. langsethiae* and *F. sporotrichioides*. According to the literature, we hypothesized that *G. candidum* reduces T-2 toxin concentration through phenyllactic acid (PLA) production.

First, the effect of *G. candidum* on *Fusarium* species was studied by direct interaction in co-culture experiments. For that, the two *Fusarium* species and *G. candidum* were inoculated simultaneously in the same liquid medium Ym and the total microbial biomass, the T-2 toxin and PLA concentrations were monitored during their development. Second, sequential cultures were conducted to evaluate the indirect interaction between *F. langsethiae* and *F. sporotrichioides* and *G. candidum*. At first, *G. candidum* was cultured in Ym liquid medium for several incubation times. Yeast biomass and PLA concentration were analyzed. Then, the Ym fermented medium were filtered aseptically to eliminate *G. candidum.* In the study, these samples were called pre-fermented medium. The latter were then inoculated with *Fusarium* spores and incubated for seven days. At the end of the incubation time, fungal biomass and T-2 toxin concentration were evaluated to determine the effect of *G. candidum* metabolites on the two *Fusarium* species. Third, it was essential to test the effect of PLA pure concentrations to validate our hypothesis.

Overall, results were promising and showed that PLA was produced during the early stages of *G. candidum* growth. The highest PLA concentration was correlated with the lowest T-2 toxin concentration. In addition to that, sequential cultures have demonstrated a tight

interdependence between *Fusarium* growth and T-2 toxin concentration. Thus, the reduction of the T-2 toxin concentration was likely due to the fungal biomass reduction. The metered addition method proved that the addition of pure PLA doses at the concentrations measured during *G. candidum* kinetics had same reduction effects on both fungal growth and T-2 concentration.

All the details of this study published in *Toxins Journal* on March 2020 are presented in publication form.

toxins

MDPI

Article

Phenyllactic Acid Produced by *Geotrichum candidum* Reduces *Fusarium sporotrichioides* and *F. langsethiae* Growth and T-2 Toxin Concentration

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Abstract: *Fusarium sporotrichioides* and *F. langsethiae* are present in barley crops. Their toxic metabolites, mainly T-2 toxin, affect the quality and safety of raw material and final products such as beer. Therefore, it is crucial to reduce *Fusarium spp.* proliferation and T-2 toxin contamination during the brewing process. The addition of *Geotrichum candidum* has been previously demonstrated to reduce the proliferation of *Fusarium spp.* and the production of toxic metabolites, but the mechanism of action is still not known. Thus, this study focuses on the elucidation of the interaction mechanism between *G. candidum* and *Fusarium spp.* in order to improve this bioprocess. First, over a period of 168 h, the co-culture kinetics showed an almost 90% reduction in T-2 toxin concentration, starting at 24 h. Second, sequential cultures lead to a reduction in *Fusarium* growth and T-2 toxin concentration. Simultaneously, it was demonstrated that *G. candidum* produces phenyllactic acid (PLA) at the early stages of growth, which could potentially be responsible for the reduction in *Fusarium* growth and T-2 toxin concentration. To prove the PLA effect, *F. sporotrichioides* and *F. langsethiae* were cultivated in PLA supplemented medium. The expected results were achieved with 0.3 g/L of PLA. These promising results contribute to a better understanding of the bioprocess, allowing its optimization at an up-scaled industrial level.

Keywords: phenyllactic acid; biocontrol agent; T-2 toxin; *F. langsethiae*; *F. sporotrichioides*; G. candidum; mycotoxin.

Key Contribution: Phenyllactic acid production by *G. candidum* reduces T-2 toxin concentration by reducing *F. langsethiae and F. sporotrichioides* growth.

1. Introduction

Beer is the most consumed alcoholic beverage worldwide and the third most popular drink overall after water and tea. In 2018, beer production in the European Union was estimated to be nearly 406,050 10^8 L and its consumption was calculated to be around 370,092 10^8 L [1]. Barley is the main ingredient in the brewing process and its quality directly influences the characteristics of the final product. However, barley crops can be contaminated by several fungal species belonging to *Aspergillus, Penicillium* and *Fusarium* genera [2]. The latter is the most prevalent genus all over the world and the main genus in Europe [3]. *Fusarium* species are responsible for the production of toxic metabolites called mycotoxins, which are of increasing concern at both health and economic levels [4]. Indeed, recent surveys carried out in Europe have demonstrated that barley crops are frequently contaminated

by *Fusarium* species and their associated mycotoxins [5–8]. The use of such contaminated raw materials in the brewing process impacts the quality of the produced beer [9]. *Fusarium* species can produce several kinds of toxins belonging to the trichothecenes family, of which types A and B are commonly found in food and feed. The most important of them are deoxynivalenol (DON), nivalenol (NIV), T-2 and their derivatives: the 15-acetyldeoxynivalenol (15-ADON), 3-acetyldeoxynivalenol (3-ADON) and HT-2 toxin [10].

The T-2 toxin belonging to the type A family was first isolated in *F. tricinctum* cultures, now called *F. sporotrichioides* and then detected in several cereal grains such as wheat, oats, barley and their derivatives. T-2 toxin is mainly produced by *F. sporotrichioides* and *F. langsethiae* [11,12].

T-2 toxin is known to be the most cytotoxic of the type A trichothecenes and has adverse effects on cellular metabolism [13]. It is 1.5–1.7 times more toxic than its deacetylate form HT-2 toxin. Even though its carcinogenicity was proven in certain affected animals, no evidence of such effect was detected in humans. Therefore, the IARC classified the T2-toxin in group 3 as not classifiable with regard to its carcinogenicity for humans [14], thus leading the European Union (EU) to propose recommendations on the presence of T-2 toxin in cereals and cereal products. Thus, the maximum limits in unprocessed cereals are 100 µg/kg for wheat, rye and other cereals, 200 µg/kg for barley (including malting barley) and corn and 1000 µg/kg for oats. [15].

In order to limit mycotoxin contamination, several pre-harvest and/or post-harvest methods can be adopted [16–18]. These techniques either directly target fungal development or limit mycotoxin levels. Pre-harvest methods include good agricultural practices (GAPs) and good manufacturing practices. Crop rotation, tillage and fungicide treatment are mainly implemented to control fungal infection [19,20]. Fungicides are commonly used during agricultural practices but have numerous disadvantages such as detrimental effects on human and animal health, environmental contamination and subsequently, they have a strong impact on microbial biodiversity [21,22]. Indeed, fungicides of the azole family are used in small grain cereals to control *Fusarium spp.* They target the CYP51 (sterol 14α-demethylase) an important enzyme involved in ergosterol biosynthesis, which is essential to maintain fungal membrane fluidity and permeability [23]. By reducing fungal growth, they disturb the natural microbial ecosystem, causing the potential emergence of new microorganisms that may be even more dangerous [24]. Moreover, fungal resistance to these compounds has developed in recent years, thus reducing their effectiveness [25]. In an attempt to limit the proliferation of these toxinogenic and phytopathogens fungal species, biocontrol approaches are starting to be published. Recently, Rahman et al. (2018) proposed the concept of the "plant holobiont". They demonstrated that barley is consistently associated with beneficial bacteria inside their seeds and that this type of association should be encouraged to help the plant react to fungal attack. This could open up new possibilities for applying seeds formulated with endophytic bacteria as bioinoculants for sustainable agriculture [26]. Post-harvest methods include physical treatments such as high temperature treatment exposure and chemical agents. However, these procedures can lead to the deterioration of nutritional quality and alteration of the organoleptic properties of the food matrix [27–29]. Therefore, it is important to conceive a bioprocess to minimize these side effects. This implies the use of natural and environmentally friendly ways to maintain the safety and the quality of the final product. The brewing process comprises several stages and among them, the malting step provides the best conditions (22 °C and high humidity) for *Fusarium* development and T-2 toxin production [30,31]. To reduce mycotoxin concentration during the malting process, several studies have reported the use of lactic acid bacteria (LAB), which are characterized by their antifungal and anti-mycotoxigenic properties [32,33]. However, LAB are fermenting bacteria and can spoil beer, leading to acidification, turbidity, off-flavors and ropiness, depending on the bacterial strain [34,35].

The French Institute for Brewing and Malting (IFBM) filed a patent in September 1999 entitled "The inoculation by *Geotrichum candidum* during malting of cereals or other plants" [36]. The invention consists of using *G. candidum* strain, a filamentous yeast, to inhibit the development of undesirable microorganisms such as *Fusarium spp.* during the malting process to avoid the contamination of beer

products by T-2 toxin. Antibacterial activity was previously attributed to this microorganism as it can inhibit the growth of several bacteria such as *Listeria monocytogenes* [37]. *G. candidum* was also found to inhibit other Gram-positive bacteria, such as *Staphylococcus aureus* and *Enterococcus faecalis*, and Gram-negative bacteria, such as *Providencia stuartii* and *Klebsiella oxytoca* [38]. As a matter of fact, three metabolites produced by *G. candidum* have been reported as antimicrobial compounds. Phenyllactic acid (PLA) and indoleacetic acid (ILA) induce behavioral and structural alterations to *L. monocytogenes*, which completely inhibit its growth [37]. The third metabolite, phenylethyl alcohol (PEA), is responsible for the "aromatic rose" character of soft cheese, and promotes membrane damage and inhibition of RNA and protein synthesis of Gram-positive and Gram-negative bacteria, such as *S. aureus* and *Escherichia coli* [39]. Among these three metabolites, PLA is the most effective against bacteria growth [37].

However, the *G. candidum* mechanism against *Fusarium spp.* and T-2 toxin production during the malting process is still unidentified. Given the data in the literature considering PLA as a powerful antimicrobial, the production of PLA by *G. candidum* now needs to be monitored and its effect on *Fusarium spp.* growth as well as on T-2 toxin concentration needs to be quantified.

Thus, this study aims to decipher the interaction mechanisms between *G. candidum* and two *Fusarium* strains: *F. langsethiae* 2297 and *F. sporotrichioides* 186, determine on which level these interactions occur and identify the metabolite responsible for the T-2 toxin concentration reduction.

2. Results

2.1. Effect of Co-Culture between G. candidum and Fusarium Strains on Fungal Growth and T-2 Toxin Concentration

The co-culture experiment consisted of simultaneously inoculating *G. candidum* and *Fusarium* strains into Ym medium for different incubation times (ranging from 24 to 168 h) at 22 °C, 150 rpm. For each incubation time, microbial dry weight, T-2 toxin and PLA concentrations were analyzed in control cultures (*G. candidum*, *F. langsethiae* 2297 *and F. sporotrichioides* 186 alone) and in co-cultures. Two co-culture experiments were conducted: *G. candidum* with *F. langsethiae* 2297 (Gc/Fl) and *G. candidum* with *F. sporotrichioides* 186 (Gc/Fs).

In control cultures, *G. candidum* dry weight increased during the first 3 days of incubation reaching 3.9 g/L and then slightly decreased to stagnate at 2.3 g/L during the last hours of the experiments. For *Fusarium* control cultures, fungal biomass increased throughout the whole experimental duration; *F. langsethiae* 2297 attained a maximum of 3.8 g/L whereas *F. sporotrichioides* 186 almost reached 3 g/L. In co-culture conditions, where microorganisms were simultaneously inoculated, for the two co-culture experiments (Gc/Fl and Gc/Fs), the total biomass increased during the first 3 days of incubation and then stabilized until the end of the experiment. However, in both co-culture experiments, for each incubation time, the total microbial dry weight was not the sum of dry weights obtained separately in control culture. Thus, co-culture leads to microbial growth reduction without distinguishing the growth of *G. candidum* from *Fusarium* species (Figure 1).

Figure 2, Panel A, shows that in control culture (*F. langsethiae* 2297 alone), T-2 toxin was detected from 48 h and the concentration was 99.65 µg/L (±7.28), and reached 332.7 µg/L (±29.42) after an incubation time of 168 h. In co-culture (*G. candidum* with *F. langsethiae* 2297), T-2 toxin was detected from 72 h (20.22 µg/L ± 4.32) and attained 116.44 µg/L (±10.89) after incubation for 168 h. The percentage of T-2 toxin reduction was 100%, 94%, 84% and 65% at 48 h, 72 h, 120 h and 168 h, respectively. These results were similar to those previously obtained for the *F. langsethiae 033* strain [40]. The same phenomenon was observed in the second co-culture experiment using *F. sporotrichioides* 186 strain with slightly different degrees of reduction (Figure 2, Panel B). In control culture (*F. sporotrichioides* 186 alone), T-2 toxin was detected from 48 h and the concentration was 82.3 µg/L (±6.1), reaching 294.65 µg/L (±4.74) after incubation for 168 h. As for the first co-culture experiment, in the co-culture *G. candidum* with *F. sporotrichioides* 186, T-2 toxin was detected from 72 h (18.8 µg/L ± 6.12) and reached 106.25 µg/L (±3.04) after incubation for 168 h. The percentage of T-2 toxin reduction was 100%, 92%,

74% and 64% at 48 h, 72 h, 120 h and 168 h, respectively. To ensure that T-2 toxin was not degraded, HT-2 toxin was also monitored and was not detected.

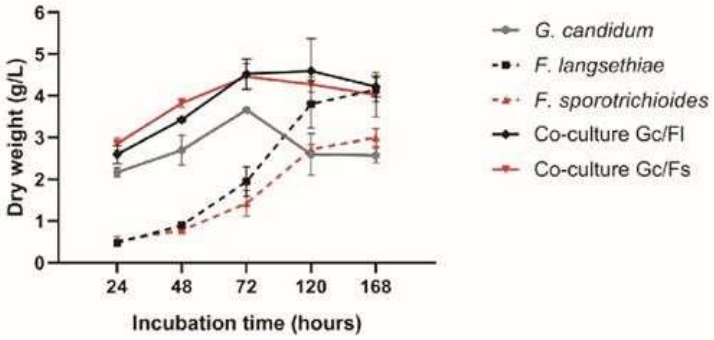

Figure 1. Microbial dry weight analysis in control cultures (*G. candidum*, *F. langsethiae* 2297 and *F. sporotrichioides* 186 alone) and in co-culture experiments (*G. candidum* with *F. langsethiae* 2297 and *G. candidum* with *F. sporotrichioides* 186).

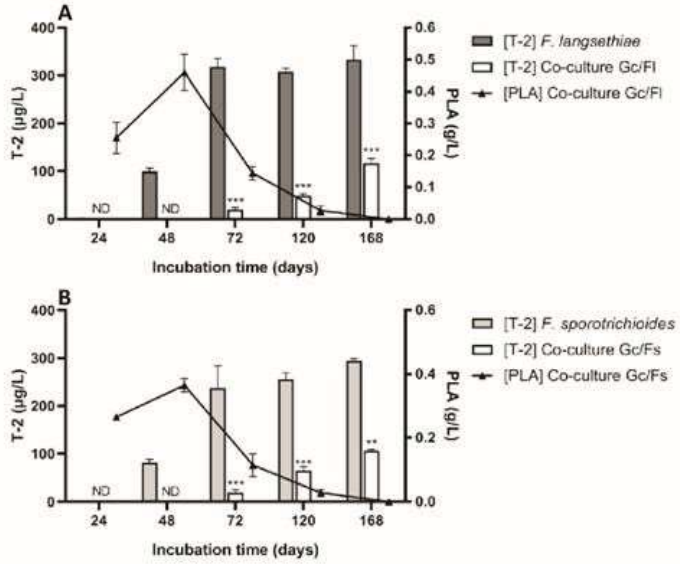

Figure 2. T-2 concentration (µg/L) and phenyllactic acid (PLA) concentration (g/L) in co-culture experiments. Panel **A**: Co-culture experiment of *G. candidum* and *F. langsethiae* 2297. Panel **B**: Co-culture experiment of *G. candidum* and *F. sporotrichioides* 186 (One-way ANOVA, Tukey's multiple comparisons post-hoc test, ** p-value < 0.01; *** p-value < 0.001) ND = not detectable.

In both co-culture experiments, the PLA concentration increased rapidly during the first two days of incubation. In the co-culture with *F. langsethiae* 2297, PLA concentration attained 0.25 g/L (±0.05) at 24 h and 0.46 g/L (±0.06) at 48 h. Afterward, it radically decreased starting at 72 h (0.14 g/L± 0.02) to reach a null value at the end of the incubation time. The same profile was observed in the co-culture with *F. sporotrichioides* 186: PLA concentration attained 0.26 g/L (±0.02) at 24 h and 0.36 g/L (±0.04) at 48 h. PLA concentration in co-culture conditions was inversely proportionate to the T-2 toxin concentration. Indeed, the increase in T-2 toxin concentration was correlated with the reduction of PLA concentration in the medium. When PLA was at its highest level (0.46 g/L in Gc/Fl and 0.36 g/L in Gc/Fs), T-2 toxin was not detected.

2.2. *G. candidum* Growth and PLA Production Kinetics

The *G. candidum* strain selected by the IFBM and used in this study produces PLA during the brewing process. To study the growth of this filamentous yeast, Ym medium was initially inoculated with 0.2 g/L of a *G. candidum* starter culture and incubated at 22 °C, 200 rpm for 5 days. Samples were withdrawn at the starting point and after 6 h, 12 h 24 h, 48 h, 72 h, 96 h, and 120 h of fermentation time and the PLA concentrations were measured.

After 48 h of incubation, the concentration of PLA reached a maximal concentration of 0.41 g/L for 2.25 g/L of yeast dry weight. After 72 h of culture, both *G. candidum* dry weight and PLA concentration started decreasing, growth went from a maximum of 3.43 g/L (±0.51) to 2.46 g/L (±0.46) and PLA concentration drastically decreased from a maximum of 0.41 g/L (±0.03) to 0.03 g/L (±0.01) (almost 17 times less) (Figure 3, Panel A). Figure 3, Panel B demonstrates the specific production of PLA relative to *G. candidum* biomass through the fermentation time. It clearly shows that the PLA is highly accumulated in the medium at the early stages of *G. candidum* growth between 12 and 48 h and then drastically disappears.

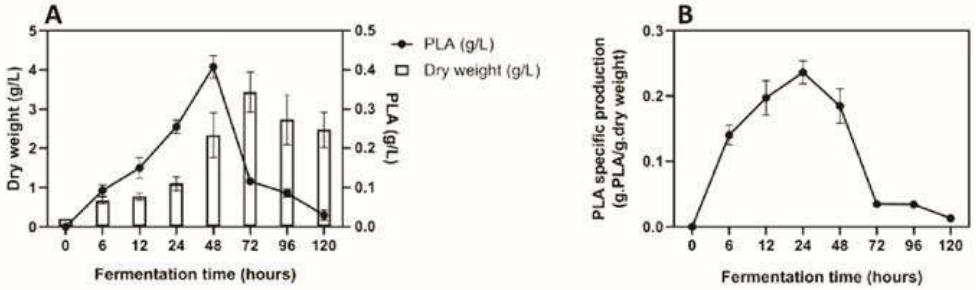

Figure 3. PLA concentration (g/L) and *G. candidum* biomass (g/L) in Ym medium (Panel **A**) and PLA specific production (g PLA/g dry weight) in Ym medium (Panel **B**).

2.3. Sequential Cultures

This experiment studied the indirect interactions between *G. candidum* and the two *Fusarium* strains. Therefore, the same Ym medium used in Section 2.2 to grow *G. candidum* was filtrated after 6 h, 12 h, 24 h, 48 h, 72 h, 96 h and 120 h of fermentation time into sterilized Erlenmeyer flasks. Henceforth, the obtained filtrate will be called the "pre-fermented medium" which contains all the metabolites secreted by *G. candidum*.

In the sequential culture experiment with *F. langsethiae* 2297, the dry fungal weight was gradually reduced on pre-fermented media up to 48 h (Figure 4, Panel A). The most significant reduction in the *F. langsethiae* 2297 dry weight occurred in the flasks pre-fermented for 12 h, 24 h and 48 h with a 62%, 72% and 66% reduction percentage, respectively. Beyond 24 h of pre-fermentation, it appeared that *F. langsethiae* 2297 growth increased slowly. In Ym medium pre-fermented for 120 h, the fungal strain was able to proliferate naturally (3.1 g/L of fungal biomass compared to 3.4 g/L in a non-fermented Ym medium). These results demonstrated that the fungal growth inhibition was more efficient in Ym medium pre-fermented for two days by *G. candidum*. Previous results showed that the PLA was produced during the early growth phase of the yeast reaching its peak (between 0.25 and 0.41 g/L of PLA) at around 24–48 h of fermentation time. This suggests that the PLA was involved in the reduction of fungal biomass at a rate of 72% (going from 3.4 g/L in a non-fermented medium to 0.95 g/L in 24 h pre-fermented medium). A significant reduction in T-2 toxin concentration was observed for fungal cultures performed in Ym medium pre-fermented from 6 h to 72 h (Figure 4-Panel B). To ensure that T-2 toxin was not degraded, HT-2 toxin was also monitored and was not detected.

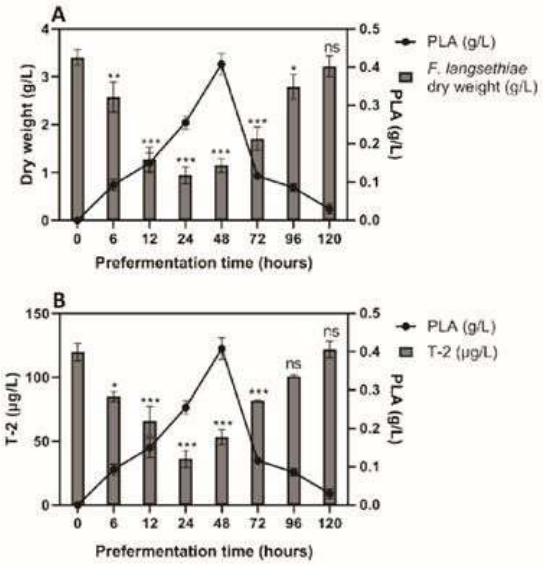

Figure 4. Sequential culture of *F. langsethiae* 2297 inoculated in pre-fermented medium by *G. candidum* and incubated 7 days at 22 °C. Panel **A**: Dry weight of *F. langsethiae* 2297 (g/L) in comparison with PLA concentration (g/L). Panel **B**: T-2 concentration (µg/L) in comparison with PLA concentration (g/L). One-way ANOVA, Dunnett multiple comparisons post-hoc test, * p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001; ns = not significant).

The most significant reduction in T-2 toxin concentration occurred in the flasks pre-fermented for 24 h and 48 h, with a 70% and 56% reduction, respectively. These percentages correlated perfectly with the biomass reduction rate (72% and 66%, respectively). This suggested that the reduction in fungal biomass in the medium is responsible for the reduction in T-2 toxin concentrations. Indeed, specific productions were calculated and demonstrated that the T-2 toxin reduction is correlated to fungal biomass reduction (data not shown).

The same experiment was conducted using *F. sporotrichioides* 186 (Figure 5). Fungal growth was drastically reduced in medium pre-fermented for 6 h, 12 h, 24 h and 48 h at almost the same rate of 70% in correlation with the increase of PLA in the medium. As expected, the Ym medium pre-fermented for 6 h, 12 h, 24 h and 48 h showed an important reduction in T-2 toxin of up to 78%. The equivalence between the growth reduction and the toxin reduction percentages also suggests that it is due to the cessation of fungal growth.

These experiments demonstrated that the interaction between *G. candidum* and *Fusarium* strains occurs through a compound released by *G. candidum* in the medium. As previous results showed, it is highly probable that the PLA, present in *G. candidum* filtrate is the metabolite responsible for the reduction of fungal dry weight and the subsequent reduction in T-2 toxin concentration. To validate this hypothesis, further experiments using pure PLA compound were required.

2.4. Effect of Pure PLA on Fungal Growth and T-2 Toxin Concentration

D-(+)-3Phenyllactic acid was purchased as a pure compound and several concentrations were tested. To validate the results presented in previous sections, PLA solution was prepared at concentrations found at different fermentation times: 0.5 g/L, 0.4 g/L, 0.3 g/L and 0.2 g/L. Lower concentrations of PLA were also tested to determine the minimal inhibitory concentration (MIC): 0.05 g/L and 0.1 g/L. The effect of this pure compound on *Fusarium* strains growth and its ability to produce T-2 toxin was evaluated.

Both *F. langsethiae* 2297 growth and T-2 toxin production were highly affected by the addition of D-PLA in Ym medium (Figure 6). As the concentration of D-PLA increased in the medium, lower fungal mass and lower toxin concentration were quantified. In the control condition (without PLA) *F. langsethiae* 2297 dry weight was 3.2 g/L (±0.26) and the T-2 toxin concentration was 148 µg/L (±7.8), whereas in the presence of 0.3 g/L of PLA, both dry weight and T-2 concentration were reduced to a rate of 71%, reaching 0.75 g/L (±0.7) and 43.4 µg/L (±1.2), respectively.

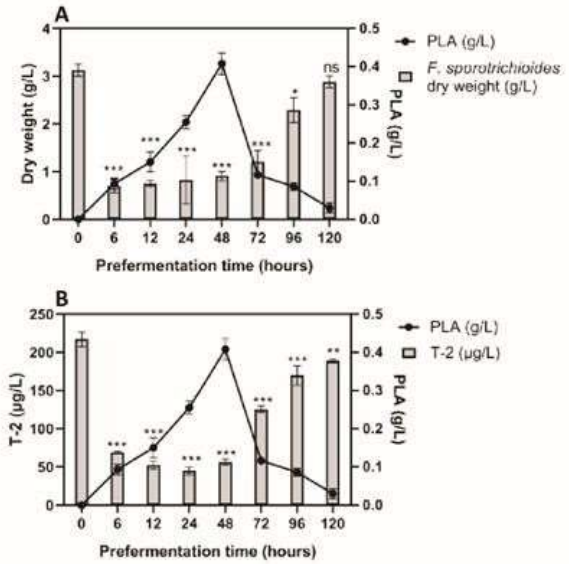

Figure 5. Sequential culture of *F. sporotrichioides* 186 inoculated in pre-fermented medium by *G. candidum* and incubated for 7 days at 22 °C. Panel **A**: Dry weight of *F. sporotrichioides* 186 (g/L) in comparison with PLA concentration (g/L). Panel **B**: T-2 concentration (µg/L) in comparison with PLA concentration (g/L). One-way ANOVA, Dunnett multiple comparisons post-hoc test, * p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001; ns = not significant).

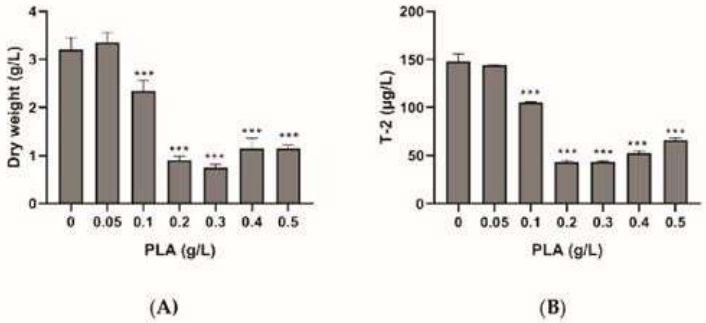

Figure 6. Effect of PLA on the dry weight of *F. langsethiae* 2297 (**A**) and T-2 toxin concentration (**B**) (One-way ANOVA, Dunnett multiple comparisons post-hoc test, *** p-value < 0.001).

The same PLA concentrations were tested on *F. sporotrichioides* 186 and similar results were obtained (Figure 7). The most important reduction occurred in Ym medium supplemented with 0.3 g/L of D-PLA.

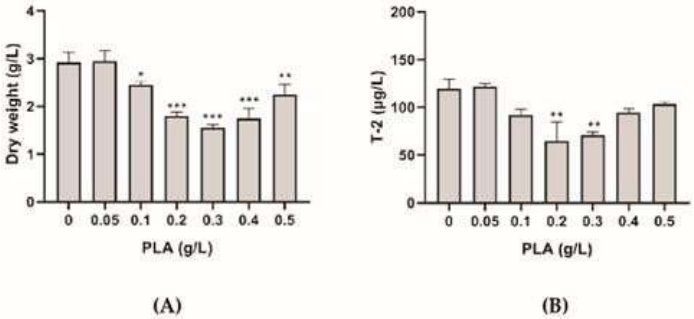

Figure 7. Effect of phenyllactic acid (PLA) on dry weight of *F. sporotrichioides* 186 (**A**) and T-2 toxin concentration (**B**) (One-way ANOVA, Dunnett multiple comparisons post-hoc test, * p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001).

In both cases, it seems clear that the reduction of T-2 toxin concentration in the medium is directly related to the reduction in fungal growth. Specific production was calculated for each PLA concentration and demonstrated that the T-2 toxin reduction is correlated to the fungal biomass reduction (Figure 8).

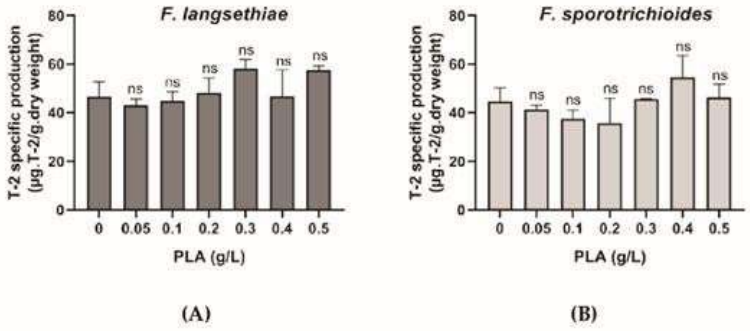

Figure 8. Specific production of T-2 toxin by *F. langsethiae* 2297 (**A**) and *F. sporotrichioides* 186 in Ym medium supplemented with pure phenyllactic acid (PLA) (**B**) and incubated 7 days at 22 °C (One-way ANOVA, Dunnett multiple comparisons post-hoc test, ns = not significant).

3. Discussion

The contamination of food raw material by fungal species has many consequences. In addition to the alteration of commodities, the loss of nutritional qualities, and the strong reduction in yield, fungal development can lead to the accumulation of toxic compounds such as mycotoxins. In France, the occurrence of several *Fusarium* species in barley crops intended for brewing has become a source of concern over the past ten years. In barley crops, the introduction of *F. sporotrichioides* and *F. langsethiae* has been recently observed, progressively replacing *F. poae* [41–43]. The risk associated with these *Fusarium* species is the production of T-2 toxin, the most toxic compound in the type-A trichothecenes family. During the brewing process, the malting step provides the best conditions (22 °C and high humidity) for *Fusarium* development and T-2 toxin production [30]. Currently, *G. candidum* is used during the brewing process to reduce T-2 toxin contamination. However, its efficiency is variable and the mechanisms of interaction between *G. candidum* and *Fusarium* species are still unknown. Previously, Gastélum-Martinez et al. (2012) used the co-culture method between those two microorganisms and demonstrated that the direct interaction between *G. candidum* and *F. langsethiae* 033 led to a drastic T-2 toxin concentration reduction (93% in comparison to the control culture) [40]. To decipher the mechanism of interaction that lead to this reduction, in the presented study, the two

microorganisms were also cultivated sequentially. First, *G. candidum* was cultivated, and removed from the medium before *Fusarium* strain inoculation. Several incubation times for *G. candidum* culture were tested (0 to 146 h) and *Fusarium* incubation was always 7 days. Results obtained in these sequential cultures show a reduction in the T-2 toxin concentration linked to a reduction in fungal growth. In addition, the reduction in T-2 toxin concentration varies according to the medium pre-fermentation time by *G. candidum*. In sequential cultures, T-2 toxin concentrations are inversely correlated with the production of PLA by *G. candidum*, demonstrating that the mechanism leading to T-2 toxin reduction, was linked directly to the PLA concentrations in the medium. Indeed, while the PLA concentration was at its highest level after 48 h of pre-fermentation time, the T-2 toxin concentration was at its lowest. The correlation between *G. candidum* growth evolution and PLA concentration in the medium suggests that PLA is a primary metabolite as it is secreted during the growth phase (from 0 h to 48 h, the PLA concentration varied from 0 to 0.41 g/L) and then gradually disappeared from the culture media. PLA biosynthesis is not yet described in *G. candidum* but well described in lactic acid bacteria (LAB) strains, which can produce large amounts of PLA. In fact, in lactic acid bacteria, the PLA results from amino acid metabolism of phenylalanine and α-ketoglutarate. In a glucose, citric acid or fructose enriched medium, the phenylalanine amino acid group is transferred to α-ketoglutarate under the action of aromatic amino acid transferase (AAT), leading to the formation of phenylpyruvic acid (PPA), an intermediate to PLA. Depending on the type of lactate dehydrogenases (L-LDH or D-LDH) present in lactic acid bacteria, PPA is converted to either L-PLA or D-PLA [44–46]. A potential PLA synthesis pathway is explicitly demonstrated by Chaudhari and Gokhale (2016) and simplified in Figure 9 [47]. Studies have shown that the D form of PLA is more effective as an antimicrobial compound than the L form [37].

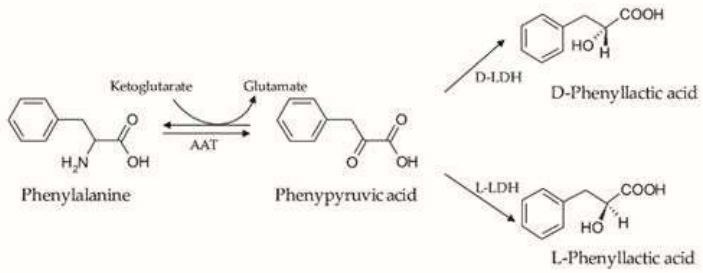

Figure 9. Hypothetical phenyllactic acid biosynthesis pathway. Adapted from Chaudhari and Gokhale (2016) [47]. AAT: amino acid transferase; D-LDH: D-lactate dehydrogenase; L-LDH: L-lactate dehydrogenase.

Several studies have been conducted on LAB and more precisely, on the *Lactobacillus* genus, which is frequently involved in their antifungal activity [48–50]. *Lactobacillus* strains and *L. plantarum* in particular, have been found to produce PLA in sourdough bread. The use of these strains is a means of natural food preservation. Indeed, studies have shown that they improve the shelf life of bread and bakery products by decreasing and/or inhibiting fungal activities. PLA is considered one of the responsible inhibitory compounds along with lactic acid and acetic acid [51]. To our knowledge, no studies have been carried out on PLA metabolism and its toxicity effect in the human body. In 2002, Lavermicocca et al. (2003) studied the fungicidal activity of PLA on 23 fungal strains belonging to *Aspergillus*, *Penicillium* and *Fusarium* genera. Among these strains, 90% showed at least a 50% growth inhibition at PLA concentrations lower than 7.5 g/L. Other strains presented a growth delay of at least three days [52]. Dieuleveux et al. have proved that PLA produced by *G. candidum* strains at 20 g/L also has antibacterial activity against *L. monocytogenes*, *S. aureus*, *E. coli* and *A. hydrophila* [37,38,53].

Fusarium strains used in this study were more susceptible to PLA that those tested by Lavermicocca et al. (2003). Indeed, *F. langsethiae* 2297 growth was drastically reduced (72%) when it was exposed to 0.2 g/L of PLA, whereas *F. sporotrichioides* 186 growth was slightly reduced (47%) when it was

exposed to 0.3 g/L of PLA. Thus, there is relevant variability in susceptibility among fungal species. Although the antimicrobial action mechanism is still not elucidated, some suggest that the PLA causes the bacteria to form aggregates with the secretion of polysaccharides described as a "response to the attack". Indeed, as the concentration of PLA increased, a larger amount of polysaccharides were found in the medium and alteration in cell wall rigidity after only 27 h of incubation was observed leading to cell death [38,47]. In this study, as indicated by the specific production of T-2 toxin obtained for each fungal strain, the reduction in the concentration of T-2 toxin is correlated with the reduction in fungal growth. However, in some cases, the inhibition of fungal growth by sub-lethal concentrations of fungicide or some natural products enhances mycotoxin production [54–56]. This must be taken into account in the development of biocontrol strategies.

In this study, to provide an explanation for the phenomenon of T-2 toxin concentration control during the malting process previously observed by the IFBM, in vitro experiments were carried out under environmental conditions close to those of the brewing process. Currently, the filamentous yeast is added in a freeze-dried form (100 g per 25 tons of barley) directly into the barley steeping water for at least 10 h. Then, the water is discarded and the steeped barley remains at rest for 3 to 5 days at 16–20 °C. This stage is the most critical step in the brewing process because the operating conditions favor *Fusarium* growth and T-2 toxin production. Results demonstrate that the reduction in *Fusarium* contamination and T-2 toxin during the malting process is due to the PLA produced by *G. candidum*. Based on the results of this study, in order to develop an effective biocontrol method to use *G. candidum*, preparation of the strain seems essential to activate the PLA production metabolism. Mu et al. developed a medium favorable to PLA production by *Lactobacillus sp.* strains, highly enriched with glucose, phenylpyruvic acid (phenylalanine intermediate in the PLA biosynthesis pathway) and yeast extract [46]. This medium significantly enhanced *Lactobacillus sp.* proliferation, and thus PLA yield. However, the use of such broth on an industrial level does not seem to be applicable for several reasons. On one hand, it may alter the organoleptic characteristics of the final product. On the other, using these components in large amounts would have a considerable economic impact on the industry. Consequently, it seems important to combine optimized growth factors (*G. candidum* activation medium and initial concentration, fermentation duration, temperature, water activity, rotation speed, oxygenation levels, etc.) to enhance PLA production naturally, and to develop an ecofriendly, toxin-free beer product. Moreover, the presence of PLA during the malting step not only helps to reduce *Fusarium* flora and consequently, to reduce T-2 toxin concentration, but it also improves the organoleptic properties of the final beer product [36,57,58]. This study demonstrates for the first time, the role of PLA as a biocontrol agent in reducing T-2 toxin concentration.

4. Materials and Methods

4.1. Reagents and Chemicals

T-2 toxin and phenyllactic acid (PLA) were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Stock solutions were prepared in dimethylsulfoxyd (DMSO) and acetonitrile–water (30:70 v/v) mixture, respectively, and stored at −18 °C until use. Solvents used for T-2 toxin extraction and high-performance liquid chromatography (HPLC) were analytical grade quality and purchased from Thermo-Fisher Scientific (Illkirch, France). Ultrapure water used for HPLC was purified at 0.22 µm by an ELGA purification system (ELGA LabWater, High Wycombe, United Kingdom).

4.2. Strains, Media and Culture Conditions

In this study, two *Fusarium* strains were used: *F. sporotrichioides* 186 and *F. langsethiae* 2297. Both strains were previously isolated from contaminated barley kernels and were kindly provided by the French Institute of Brewing and Malting (IFBM). The filamentous yeast *Geotrichum candidum* is already used as a biocontrol agent during the malting process (IFBM Malting Yeast®, DMS food specialties, La Ferté sous Jouarre, France) and was purchased from DSM Food Specialties.

Fusarium pre-cultures were performed on potato dextrose agar medium (PDA 39 g/L) and incubated at 22 °C for 7 days. Cultures were then used to induce sporulation or conserved at 4 °C. *Fusarium* strains sporulation was induced in carboxymethylcellulose (CMC) liquid medium (CMC: carboxymethylcellulose 15 g/L; yeast extract 1 g/L; $MgSO_4$ $7H_2O$ 0.5 g/L; NH_4NO_3 1g/L; KH_2PO_4 1 g/L). Briefly, at least 15 plugs of each *Fusarium* strain from a seven-day-old solid pre-culture were inoculated in 150 mL of CMC medium and incubated in an orbital shaker set at 22 °C at 150 rpm for 15 days in the dark. At the end of the incubation time, the solution was filtrated using sterilized Mira cloth. Spores were counted on Thoma cell counting chamber and ultimately used to inoculate culture during further experiments or conserved in 40% glycerol at −80 °C.

G. candidum strain was supplied in freeze-dried form, thus a pre-culture was essential to revivify it prior to experimental use. A 24 g/L culture was prepared in 250 mL of yeast and malt (Ym) liquid medium (Ym: glucose 5 g/L; yeast extract 1.5 g/L; malt extract 1.5 g/L; peptone salt 2.5 g/L pH 7) and incubated in an orbital shaker set at 22 °C at 150 rpm for 24 h. At the end of the incubation time, this culture was used as a starter culture.

Ym liquid medium was used during all experiments (co-cultures and sequential cultures) to elucidate the interaction mechanisms between *G. candidum* and *Fusarium* strains.

4.3. Kinetic of PLA Production by G. candidum

In an Erlenmeyer flask, 150 mL of Ym medium was inoculated with *G. candidum* starter culture with a final concentration adjusted at 0.2 g/L and then incubated in an orbital shaker set at 22 °C at 150 rpm for different fermentation times ranging from 6 h to 120 h. At the end of the fermentation time, the medium was aseptically divided into two volumes. First, 50 mL were used to evaluate *G. candidum* growth by measuring the dry weight and PLA concentration by HPLC-DAD at each sampling time. The remaining 100 mL was aseptically filtered to eliminate *G. candidum* cells, leaving only its excreted metabolites in the medium. The medium nutrients were then adjusted according to the volume and the pH was adjusted at 7. These volumes were used during the sequential cultures experiments and are henceforth referred to as pre-fermented medium. Experiments were conducted four times in triplicate.

4.4. Co-Culture of Fusarium Strains and G.candidum

Erlenmeyer flasks containing 150 mL of Ym medium were inoculated with *G. candidum* starter culture at the final concentration of 0.2 g/L. Then, *F. langsethiae* 2297 or *F. sporotrichioides* 186 was inoculated at a final concentration of 10^6 spores/mL in their respective flasks. For control conditions, each microorganism was inoculated alone at the same concentrations. Cultures were incubated in an orbital shaker set at 22 °C at 150 rpm. Several incubation times were tested: 24 h, 48 h, 72 h, 120 h and 168 h. At the end of all sampling times for all culture conditions the total dry weight, PLA and T-2 toxin concentration were evaluated. All experiments were conducted twice in duplicate.

4.5. Sequential Cultures of Fusarium Strains and G.candidum

For sequential cultures, 100 mL of pre-fermented Ym medium at different fermentation times ranging from 6 h to 120 h (used in Section 4.3) were inoculated with *F. langsethiae* 2297 or *F. sporotrichioides* 186 at the final concentration of 10^6 spores/mL in their respective flasks. Cultures were incubated in an orbital shaker set at 22 °C at 150 rpm for 7 days. For the control condition, *F. sporotrichioides* 186 or *F. langsethiae* 2297 were inoculated in a non-fermented Ym liquid medium at the same concentrations. At the end of the incubation time, fungal growth was evaluated by measuring the dry weight and T-2 toxin concentration by HPLC-DAD. All experiments were conducted twice in duplicates.

4.6. Phenyllactic Acid Effect on F. sporotrichioides 186 and F. langsethiae 2297 Growth and T-2 Toxin Concentration

To confirm that PLA is the metabolite produced by *G. candidum*, which is involved in *Fusarium* growth reduction and T-2 toxin concentration reduction, fungal cultures were conducted in Ym liquid

medium supplemented with PLA. PLA standard stock solution was prepared at 40 mg/mL in a mixture of acetonitrile/water (30/70, v/v) and appropriate volumes of PLA stock solution were added in order to obtain several different concentrations: 0.05 g/L; 0.1 g/L; 0.2 g/L; 0.3 g/L; 0.4 g/L and 0.5 g/L in Erlenmeyer flasks containing 100 mL of Ym liquid medium. Then, *F. langsethiae* 2297 or *F. sporotrichioides* 186 was inoculated at the final concentration of 10^6 spores/mL in their respective flasks. Cultures were incubated in an orbital shaker set at 22 °C at 150 rpm for 7 days. At the end of the incubation time, *Fusarium* strains' growth was evaluated by measuring the dry weight and T-2 toxin concentration by HPLC-DAD. PLA dilutions were prepared to add only 75 µL of acetonitrile in the culture medium, this concentration having been identified as a no-effect dose on both fungal growth and T-2 toxin concentration. Control cultures were performed by adding only 75 µL of acetonitrile to the medium.

4.7. G. candidum, F. sporotrichioides 186 and F. langsethiae 2297 Biomass Evaluation

To estimate microorganism growth during the incubation period, vacuum filtration was performed to determine the dry weight (g/L). First, cellulose nitrate filters (pore size 0.45 µm, Sartorius Stedim Biotech, Goettingen, Germany) were left to dry overnight in an oven set at 105 °C. Afterward, 10 mL of culture medium were vacuum-filtered at each sampling time and filters were then incubated at 105 °C for 24 h. The microorganism dry weight refers to the difference between filters post-filtration and pre-filtration.

4.8. PLA and T-2 Toxin Quantification by HPLC-DAD

4.8.1. PLA Quantification

At each sampling time, 1 mL of culture media was withdrawn and filtrated through 0.45 µm PTFE syringe filters (Thermo Scientific Fisher, Villebon-Sur-Yvette, France) to eliminate microorganisms from the supernatant prior to injection into HPLC apparatus. Analyses of PLA were performed using a Luna C18(2) column (5 µm, 250 × 4.6 mm) and a pre-column with the same characteristics (Phenomenex, Torrance, CA, USA). The detection of PLA was performed using a Dionex Ultimate 3000 UHPLC system coupled with a diode-array detector (DAD) set at 210 nm (Thermo Fisher Scientific, Illkirch, France). The analysis was performed in a gradient mode using acidified water (0.2% of acetic acid glacial) as solvent A and pure HPLC grade acetonitrile as solvent B. Flow was set at 1.2 mL/min with A/B ratios of 90:10, 50:50, 50:50, 0:100 and 90:10, with run times of 0.0, 4.0, 9.0, 10.0 and 15.0 min, respectively. Injection volume was set at 50 µL. PLA quantification was calculated according to a standard calibration curve with concentrations ranging between 10 and 1000 mg/L.

4.8.2. T-2 Toxin Extraction and Quantification

After the incubation period, cultures were filtrated with Nalgene™ Rapid-Flow™ Filters of 0.45 µm pore size (Thermofischer Scientific, Waltham, MA, USA) to remove microorganisms. Filtrates were then extracted with 70 mL of ethyl acetate and shaken on a Universal Shaker SM 30 B Control Edmund Bühler® (Thermofischer Scientific, Waltham, MA, USA) set at 150 rpm overnight. The organic phase was recovered and evaporated until dry under a rotavapor set at 60 °C. Samples were resuspended with 2 mL of acetonitrile/water (30/70, v/v) mixture and filtered through 0.45 µm PTFE syringe filters (Sigma Aldrich, St. Quentin Fallavier, France). Samples were conserved at 4 °C until further analysis. T-2 toxin was analyzed by Gemini C18 columns, 150 mm × 4.6 mm, 3 µm and a pre-column with the same characteristics (Phenomenex). As for PLA, T-2 toxin was detected and quantified using HPLC-DAD (Dionex, Sunnyvale, CA, USA) according to the methodology described by Medina et al. [59]. T-2 toxin quantification was calculated according to a standard calibration curve with concentrations ranging between 0.2 and 50 µg/mL.

4.9. Statistical Analysis

First, the normal distribution of data was tested by the Shapiro-Wilk test. Then, one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test was used to analyze the effect of PLA on *F. langsethiae 2297* and *F. sporotrichioides 186* growth and their T-2 production. One-way ANOVA followed by a Tukey's multiple comparisons test was used to analyze the differences between control and co-culture or sequential culture conditions. The statistical analysis of data was carried out with GraphPad Prism 8 software (GraphPad Software, La Jolla, CA, USA). Differences were considered to be statistically significant when the *p*-value was lower than 0.05. Graphical values are represented by mean ± standard deviation (SD).

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Chapter 2 - Impact of *Geotrichum candidum* inoculation method, used as biocontrol agent, on T-2 toxin produced by *Fusarium* species during the malting process

As mentioned before, beer is the most consumed alcoholic beverage in the world. The contamination of malting barley by *Fusarium* species and their mycotoxins, T-2 and HT-2 toxins in particular, is a source of public health concern, especially for heavy drinkers.

The beer production process involves various operations that can have an impact on the concentration of these mycotoxins. Among them, the malting step consists of three main steps:

- Steeping: barley kernels are soaked in water for 36 – 52 hours at 12 – 22°C
- Germination: enzymes are activated to produce malt for 4 – 6 days at 15 – 22°C
- Kilning: malt is dried and heated to allow the development of flavors and beer color for 24 – 48 hours at 50 – 85 °C

Steeping and germination conditions (temperature, water activity, humidity and incubation time) are propitious for *Fusarium* species growth and hence T-2 toxin production. Kilning temperatures are high and can eliminate fungal biomass in malt. However, T-2 toxin, as all the other mycotoxins, is thermostable and cannot be degraded even under high heat.

Hence, it seems necessary to reduce *Fusarium* contamination at the beginning of the malting step. The core of this thesis is to optimize the use of *Geotrichum candidum* as a biocontrol to reduce the concentration of T-2 toxin in the brewing process. In the previous study, *G. candidum* was proved to be efficient against *Fusarium sporotrichioides* and *F. langsethiae* growth and T-2 toxin concentration through the production of phenyllactic acid.

This second part of the thesis focused on conducting co-culture experiments on synthetic medium to evaluate the effectiveness of *G. candidum* inoculation form. In fact, in most breweries, the yeast is poured under its freeze-dried form over malting barley and left to react. That being the case, *G. candidum* was inoculated simultaneously either under its freeze dry form or after activation under sourdough form. At the end of incubation time, PLA and T-2 concentrations were evaluated. Moreover, the effect of *G. candidum* initial inoculum concentration on PLA and T-2 toxin concentrations were also monitored. For that, an increase in *G. candidum* inoculum was tested and compared to standard inoculation conditions.

Subsequently, the same parameters were conducted on barley kernels in micro-malting essays miming malting conditions in order to validate previous findings on an up-scaled level.

Results showed that inoculating *G. candidum* under freeze-dried form at both concentrations delayed phenyllactic acid production by one day and lead to lower concentrations over time. Second, same experiments were conducted on artificially infected barley kernels with the two *Fusarium* species in malting miming conditions in micro-malting. Similarly, results showed that activating *G. candidum* allowed earlier production of phenyllactic acid therefore reducing T-2 toxin concentration. Moreover, doubling *G. candidum* inoculum to 0.4 g/L enhanced phenyllactic acid production leading to better efficiency against T-2 toxin concentration in

both co-culture experiments and micro-malting assays. Indeed, T-2 toxin reduction percentages ranged from 87 to 100% in co-culture experiments and from 76 to 99% in micro-malting assays depending on the *Fusarium* species and incubation time. Based on these findings, the activation and a double concentration for the inoculation of *G. candidum* allow a better reduction of T-2 toxin concentration during the first three days of malting.

All the details of this study have been submitted for publication in International Journal of Food Microbiology, and are presented hereinafter in the form of an article.

Impact of *Geotrichum candidum* inoculation method, used as biocontrol agent, on T-2 toxin produced by *Fusariul langsethiae* and *F. sporotrichioides* during the malting process

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Abstract

Barley, the main ingredient in beer production, is often contaminated by several *Fusarium* species, T-2 toxin producers. Their occurrence on malting barley can decrease the processing potency, quality and sanitary safety of beer. In previous work, a correlation between the phenyllactic acid production by *G. candidum* and the reduction of *Fusarium* growth and T-2 toxin concentration was established. In the present study, the effect of *G. candidum* initial inoculum form on phenyllactic acid and T-2 toxin concentrations were evaluated. For that, first, co-culture experiments with two *Fusarium* species (*F. sporotrichioides* 2297 and *F. langsethiae* 186) and *G. candidum* were conducted in a synthetic medium. *G. candidum* was inoculated either under freeze-dried form or under activated form at 0.2 or 0.4 g/L. Results showed that inoculating *G. candidum* under the freeze-dried form at both concentrations delayed the production of phenyllactic acid by one day and lead to the concentration reduction over time. Second, the same experiments were conducted on artificially infected barley kernels with the two *Fusarium* species in malting miming conditions in a micro-malting. Similarly, results showed that activating *G. candidum* allowed earlier production of phenyllactic acid therefore reducing T-2 toxin concentration. Moreover, doubling *G. candidum* inoculum to 0.4 g/L enhanced phenyllactic acid concentration leading to better efficiency against T-2 toxin concentration in co-culture experiments and micro- malting assays. Indeed, T-2 toxin reduction percentages ranged from 87 to 100% in co-culture experiments and from 76 to 99% in micro-malting assays depending on the *Fusarium* species and incubation time. Based on these findings, the activation and a double concentration for the inoculation of *G. candidum* were established as the best conditions for T-2 toxin reduction during the first three days of malting.

Keywords: *F. sporotrichioides*, *F. langsethiae*, phenyllactic acid, mycotoxin, barley, micro-malting

1. Introduction

Cereal crops are highly susceptible to fungal contamination and barley harvests are no exception. Considered as one of the main ingredients in beer production along with water, hops and yeast, the quality of barley kernels is decisive for the sanitary quality and market acceptance of the beer (Piacentini et al., 2017). Moreover, beer is the most consumed alcoholic beverage in the world, thus its contamination can be a source of public health concern, especially for heavy consumers. Fungal infection of barley grains generally occurs in the field and is most often associated with *Fusarium* species, which are phytopathogenic fungi. This genus is held responsible for the *Fusarium* Head blight disease (Nielsen et al., 2014), resulting in quality deterioration of grain but also in harmful secondary metabolites production, called mycotoxins. According to Magan et al., applying fungicides to crops can indeed limit *Fusarium* species proliferation. However, mycotoxin contamination remains intact (Magan et al., 2002).

In beer production, the malting step offers favorable conditions (22°C and high aw) for fungal proliferation from barley contaminated by spores. Indeed, nutrients abundance, grain moisture, temperature and process duration can induce growth and mycotoxin production. Fungal proliferation is responsible for several issues such as starch degradation, lower grain germination, variations of soluble protein and nitrogen content, worth and final beer color alteration, beer aroma alteration and gushing (uncontrolled, eruptive foaming of beer, due to the presence of hydrophobins, small fungal proteins) (Sarlin et al., 2005; Schwarz et al., 2002; Vaughan et al., 2005). At the end of the malting step, during the kilning, temperature increases progressively, which reduces *Fusarium* biomass. However, mycotoxins persist even at the end of the production process, as they are thermostable (Piacentini et al., 2019). Inoue et al., conducted a survey in 2013 to evaluate the fate of mycotoxins during beer brewing and fermentation (Inoue et al., 2013). They clearly stated that trichothecenes should be carefully controlled due to their toxicity and its outspread such as deoxynivalenol (DON), nivalenol (NIV) and T-2/HT-2 toxins (T-2/HT-2) as their residual content is at least 30% higher than other mycotoxins. Other studies have analyzed european beer products leading to the same conclusion to establish new european regulations that limit the maximum level of these frequently occurring mycotoxins in commercialized beer products to ensure consumer safety (Grajewski et al., 2019; Peters et al., 2017; Varga et al., 2013). Among these mycotoxins, T-2 toxin is a type A trichothecenes, produced in warm and moist conditions mainly by *Fusarium langsethiae, F. poae* and *F. sporotrichioides* (Kalantari and Moosavi, 2010). It is the most cytotoxic of type A trichothecenes and has adverse effects on cellular metabolism (van der Fels-Klerx and Stratakou, 2010). Even though carcinogenicity was proven on certain affected animals, no evidence of such effects was detected on humans. Therefore, the IARC classified the molecule as group 3 (not classifiable as to its carcinogenicity to humans) (IARC, 1993).

Several biological, chemical and physical strategies applied before and/or after harvest are proposed to reduce the waste of food and agricultural commodities associated with fungal spoilage and/or mycotoxins accumulation. Among these strategies, biocontrol methods may

be of interest, especially those that involve the use of microorganisms to reduce these contaminations. Thus, the French Institute of Brewing and Malting (IFBM) filed a patent that proposes the use of a Geotrichum candidum strain, a filamentous yeast during the malting step to reduce the development of undesirable flora such as *Fusarium* species to avoid the contamination of beer product (Boivin and Malanda, 1999). Nowadays, brewers mix the *G. candidum* strain under the freeze-dried form with humid barley kernels and leave it for three to five days in proper malting conditions.

Previous work shed the light on the interaction mechanisms between *G. candidum* and two Fusarium species to concretize the use of this yeast as a biocontrol (Kawtharani et al., 2020). This study has demonstrated that *G. candidum* produces phenyllactic acid (PLA) at the early stages of growth and that PLA is responsible for the reduction of both Fusarium growth leading to the reduction of T-2 toxin concentration. Indeed, T-2 toxin concentration and fungal growth were reduced by almost 90% when PLA reaches its highest value after 24 hours of incubation.

The objective of the present study is to evaluate the impact of the method of preparation (i.e. concentration and activation or not) of *G. candidum* strain on T-2 toxin concentration reduction. Experiments were conducted using *G. candidum* under freeze-dried or activated forms at two concentrations (0.2 and 0.4 g/L). At first, experiments were conducted in a synthetic medium. Then, results were validated at the micro-malting scale.

2. Material and methods

2.1 Reagents and chemicals

T-2 toxin and phenyllactic acid (PLA) were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Stock solutions were respectively prepared in dimethylsulfoxyd (DMSO) and in acetonitrile–water (30:70 v/v) mixture and stored at -18°C until use. Solvents used for T-2 toxin extraction and High-Performance Liquid Chromatography (HPLC) were analytical grade quality and purchased from Thermo-Fisher Scientific (Illkirch, France). Ultrapure water used for HPLC was purified at 0.22 µm by an ELGA purification system (ELGA LabWater, High Wycombe, United Kingdom).

2.2 Strains, media and culture conditions

Two *Fusarium* strains were used: *F. sporotrichioides* 186 and *F. langsethiae* 2297. Both strains were isolated from contaminated barley kernels and were kindly provided by the French Institute of Brewing and Malting (IFBM). *Fusarium* spore solutions and pre-cultures were performed as described by Kawtharani et al. (2020). The filamentous yeast *Geotrichum candidum* (IFBM Malting Yeast®) was purchased from DSM Food Specialties (La Ferté-sous-Jouarre, France) and supplied under the freeze-dried form. In this study, *G. candidum* was used either under the freeze-dried form or under the activated form. For the latter, *G. candidum* was revivified prior experimental use. For that, a 24g/L culture was prepared in 250 mL of Yeast and Malt liquid medium (Ym - glucose 5 g/L; yeast extract 1.5 g/L; malt extract 1.5

g/L; peptone salt 2.5 g/L pH 7) and incubated in an orbital shaker set at 22°C at 150 rpm for 24 hours. At the end of the incubation time, this culture was used as a starter culture set at 0.2 or 0.4 g/L.

Ym liquid medium was used during co-culture experiments. To mimic the malting conditions, micro-malting assays were performed. For that, microbial cultures were performed in 2L bottles containing 200 g hulless barley kernels (JMT, Labège, France) submerged overnight in 200 mL distilled water (1:1 grain:water ratio) and sterilized 20 min at 121°C prior inoculation.

2.3 Co-culture of Fusarium strains and G. candidum in Ym synthetic medium

For co-culture experiments, Erlenmeyer flasks containing 150 mL of Ym medium were inoculated with *G. candidum* either under the freeze-dried form or under activated form at the final concentration of 0.2 g/L or 0.4 g/L. Then, *F. langsethaie* 2297 or *F. sporotrichioides* 186 was inoculated at the final concentration of 10^6 spores/mL in their respective flasks. Cultures were incubated in an orbital shaker set at 22°C at 150 rpm. Several incubation times were tested on day 1, day 2, day 3, day 4, day 5 and day 7. At the end of all sampling times for all culture conditions the total dry weight, PLA and T-2 toxin concentrations were measured. For control conditions, each microorganism was inoculated alone at the same concentrations. All experiments were conducted in duplicates.

2.4 Micro-malting assays

Two-liter bottles containing 200 g of barley kernels were submerged with 200 mL of distilled water and left overnight to allow grains to soak in water. The next day, bottles were sterilized at 121°C for 20 minutes. After complete cooling, *G. candidum* was inoculated using either under the freeze-dried form or under activated form final concentration of 0.2 g/L or 0.4 g/L. Then, *F. langsethiae* 2297 or *F. sporotrichioides* 186 was inoculated at the final concentration of 10^6 spores/g in their respective flasks. Cultures were incubated at 22°C. Two incubation times were tested: day three and day five. At the end of all sampling times for all culture conditions, PLA and T-2 toxin concentrations were measured. For control conditions, each microorganism was inoculated alone at the same concentrations. All experiments were conducted in duplicates.

2.5 G. candidum, F. sporotrichioides 186 and F. langsethiae 2297 biomass evaluation in co-cultures

To evaluate microorganism growth during the incubation period, vacuum filtration was performed to determine the total dry weight (g/L) as previously described by Kawtharani et al. (2020). Briefly, cellulose nitrate filters (pore size 0.45 µm, Sartorius Stedim biotech) were left to dry overnight in an oven set at 105°C. Afterward, 10 mL of culture medium were vacuum-filtered at each sampling time and filters were then incubated at 105°C for 24h. The microorganism dry weight refers to the difference between filters post-filtration and pre-filtration.

2.6 T-2 toxin and PLA quantification by HPLC-DAD

2.6.1 T-2 toxin quantification

For co-culture experiments in Ym synthetic medium, at the end of the incubation period, cultures were filtrated with Nalgene™ Rapid-Flow™ Filters of 0.45 µm pore size to remove microorganisms. Then, filtrates were extracted with 70 mL of ethyl acetate and shaken overnight at 150 rpm. Concerning micro-malting experiments, 400 mL of ethyl acetate were used to extract the whole content bottles. Then, bottles were shaken on Universal Shaker SM 30 B Control Edmund Bühler® set at 150 rpm overnight.

In both cases, the organic phase was recovered and evaporated until dryness under a rotavapor set at 60°C. Samples were resuspended with 2 mL of acetonitrile/water (30/70, v/v) mixture and filtered through 0.45 µm PTFE syringe filters. Samples were conserved at 4°C until further analysis. T-2 toxin was analyzed by C18 Gemini column C18, 150 mm × 4.6 mm, 3 µm and a pre-column with the same characteristics (Phenomenex, Torrance, CA, USA). T-2 toxin was detected and quantified using HPLC-DAD according to the methodology previously described by Kawtharani et al. (2020). T-2 toxin quantification was calculated according to a standard calibration curve with concentrations ranging between 0.2 and 500 µg/mL.

2.6.2 PLA quantification

In the case of co-culture experiments in Ym synthetic medium, 1 mL of culture media was withdrawn at each sampling time and filtrated through 0.45 µm PTFE syringe filters (Thermo Scientific Fisher, Villebon-Sur-Yvette, France) to eliminate microorganisms from the supernatant prior to injection to HPLC apparatus. Regarding micro-malting assays, PLA was quantified in the same extracts as for the T-2 toxin quantification. Analyses of PLA were performed using a Luna C18(2) column (5µm, 250×4.6mm) and a pre-column with the same characteristics (Phenomenex, Torrance, CA, USA). The PLA was detected and quantified using HPLC-DAD according to the methodology previously described by Kawtharani et al. (2020). PLA quantification was calculated according to a standard calibration curve with concentrations ranging between 0.01 to 1 g/L.

2.7 Statistical analysis

One-Way ANOVA followed by a Tukey's multiple comparisons test was used to analyze the differences between control and co-culture conditions. Differences were considered to be statistically significant when the p-value was lower than 0.05. Graphical values are represented by mean ± standard deviation (SD). Two-Way ANOVA followed by a Tukey's multiple comparisons post-hoc test was used to analyze all conditions compared to each other. Data with different letters are significantly different p-value < 0.05). The statistical analysis of data was carried out with GraphPad Prism 8 software (GraphPad Software, La Jolla, USA).

3. Results

3.1 Co-culture between G. candidum and Fusarium strains in Ym synthetic medium

Co-culture experiments were conducted by using the *G. candidum* strain either under the freeze-dried form or under activated form inoculated at 0.2 or 0.4 g/L with *F. langsethiae* 2297 (Gc/Fl) or with *F. sporotrichioides* 186 (Gc/Fs). When *G. candidum* was inoculated under the freeze-dried form in both co-culture experiments, T-2 toxin was detected from the second day of the experiment. Besides, T-2 toxin concentration increased over time to reach a value, which was not significantly different after seven days of incubation time from that observed in *Fusarium* pure cultures (Figure 1, Panel A to D). When *G. candidum* was inoculated under activated form at 0.4 g/L, in both co-culture experiments, T-2 toxin was detected after three days of incubation at very low concentrations (11.8 µg/L (±2.6) in Gc/Fl and 2.7 µg/L (±1.1) in Gc/Fs). Moreover, values remained low after seven days of incubation time (15.8 µg/L (±1.2) in Gc/Fl and 28.4 µg/L (±2.2) in Gc/Fs) (Figure 1, Panel E and F).

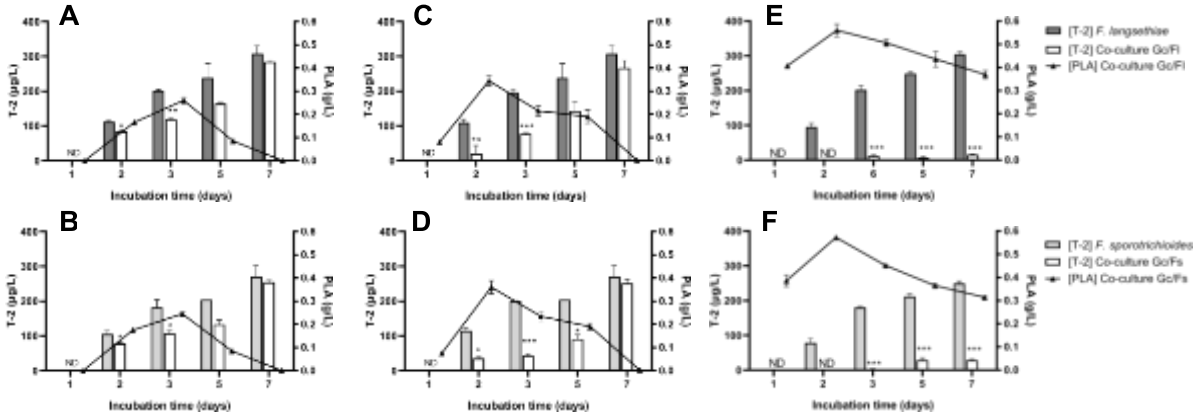

*Figure 1 - T-2 toxin concentration (µg/L) and PLA concentration (g/L) in co-culture experiments of G. candidum with F. langsethiae 2297 (Gc/Fl) or F. sporotrichioides 186 (Gc/Fs). G. candidum was inoculated under freeze-dried form at 0.2 g/L (Panels A and B), under freeze-dried form at 0.4 g/L (Panels C and D) and under activated form at 0.4 g/L (Panels E and F). One-way ANOVA, Tukey's multiple comparisons post-hoc test, * p-value < 0.05; ** p- value < 0.01 *** p-value < 0.01 ND = not detected.*

Based on T-2 toxin concentrations measured in the co-culture experiments, the T-2 toxin reduction percentages were calculated for each culture condition and are presented in Table 1. When *G. candidum* was inoculated under freeze-dried form at 0.2 or 0.4 g/L, the percentage of T-2 toxin reduction decreases rapidly over time. However, when it was inoculated under activated form at 0.4 g/L, the reduction percentage of T-2 toxin remained high throughout the experiment (95% in Gc/Fl and 89% in Gc/Fs after seven days of incubation).

Table 1 - T-2 toxin reduction percentage in co-culture experiments

	F. langsethiae 2297				*F. sporotrichioides* 186			
	Incubation time (days)				Incubation time (days)			
G. candidum **inoculation condition**	**2**	**3**	**5**	**7**	**2**	**3**	**5**	**7**
0.2 g/L freeze-dried	26%	41%	31%	7%	27%	41%	35%	6%
0.4 g/L freeze-dried	82%	61%	39%	13%	69%	78%	54%	7%
0.4 g/L activated	100%	94%	96%	95%	100%	98%	87%	89%

Regarding PLA concentrations in co-culture experiments where *G. candidum* was inoculated under freeze-dried form at 0.2 g/L, the PLA concentration highly increased during the first three days to reach 0.26 g/L (± 0.01) in Gc/Fl and 0.24 g/L (±0.01) in Gc/Fs (Figure 1, Panel A and B). When the concentration of *G. candidum* was increased to 0.4 g/L, the highest concentration of PLA was attained on the second day of incubation and reached 0.35 g/L (± 0.01) in Gc/Fl and 0.36 g/L (± 0.01) in Gc/Fs (Figure 1, Panel C and D). Afterward, in both co- culture conditions, PLA concentration decreased until reaching the null value after 7 days of incubation. On the contrary, when *G. candidum* was inoculated under activated form at 0.4 g/L, in both co-culture experiments, PLA concentrations were 0.56 g/L (± 0.02) in Gc/Fl and 0.57 g/L (± 0.03) in Gc/Fs at 48 hours and remained high until the end of the experiment (Figure 1, Panel E and F). In control conditions, where *G. candidum* was inoculated alone, PLA concentrations measured were equivalent to those measured in the corresponding co-cultures (Figure 2).

Regarding *G. candidum* growth in pure culture, inoculated under freeze-dried form at 0.2 g/L and 0.4 g/L, dry weight gradually increased to reach a maximum value of 3.5 g/L (± 0.1) at day five and 4.5 g/L (± 0.1) at day five respectively (Figure 2, Panels A and B). However, when *G. candidum* was inoculated under activated form at 0.4 g/L, the dry weight increased abruptly within 24 hours to reach a value of 3.2 g/L (± 0.01) and then, increased slowly to attain a maximum value of 4.8 g/L (± 0.1) after five days of incubation (Figure 2, Panel C).

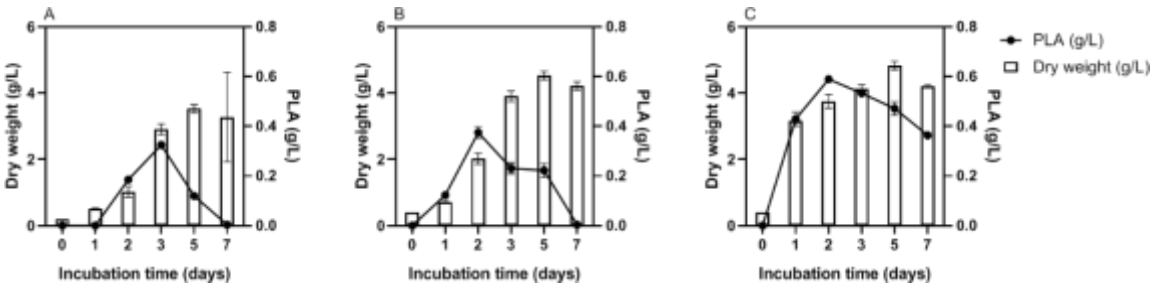

Figure 2 - PLA concentration (g/L) and G. candidum biomass (g/L) in control conditions. G. candidum was inoculated under freeze-dried form at 0.2 g/L (Panel A), under freeze-dried form at 0.4 g/L (Panel B) and under activated form at 0.4 g/L (Panel C).

Since growth values are different from one experiment condition to another, *G. candidum* PLA specific productions were calculated and presented in Table 2. PLA was highly accumulated in the medium between the first and second days in all culture conditions. When *G. candidum*

was inoculated under the freeze-dried form, PLA specific production was drastically reduced at three days of incubation. However, when *G. candidum* was inoculated under the activated form, the PLA specific production was slightly reduced after three days of incubation and remained stable until the end of the incubation time.

Table 2 – PLA specific production (g PLA/g dry weight) in Ym medium inoculated in different conditions (ND=not detected)

Inoculation condition	INCUBATION TIME (DAYS)				
	1	2	3	5	7
0.2 g/L freeze-dried	ND	0.181 ± 0.038	0.112 ± 0.004	0.034 ± 0.001	0.001 ± 0.0002
0.4 g/L freeze-dried	0.175 ± 0.011	0.186 ± 0.004	0.059 ± 0.003	0.049 ± 0.008	0.001 ± 0.0002
0.4 g/L activated	0.136 ± 0.006	0.157 ± 0.01	0.129 ± 0.002	0.098 ± 0.008	0.086 ± 0.001

In co-culture conditions (Gc/Fl and Gc/Fs) the mass of either microorganism could not be distinguished because microorganisms were simultaneously inoculated. Thus, the total microbial dry weight was measured. For the three co-culture experiments, the total microbial dry weight was lower than the sum of dry weights obtained separately in the control culture (data not shown).

When *G. candidum* was inoculated under the freeze-dried form, the highest concentration of PLA was obtained on the third day of incubation and reached 0.325 g/L (± 0.007) at 0.2 g/L and on the second day at 0.37 g/L (± 0.02) when inoculated at 0.4 g/L (Figure 3). In addition to that, PLA disappeared from the medium after five days of incubation time in both conditions. However, inoculating *G. candidum* under activated form at 0.4 g/L helped to maintain PLA values high during the whole incubation time which lead to a more important reduction of the T-2 toxin concentration as seen in Figure 1 Panels E and F. High PLA concentration was detected even after only one day of culture time and reached its maximum value after 2 days. Moreover, the maximum value reached was higher than when *G. candidum* was inoculated under the freeze-dried form.

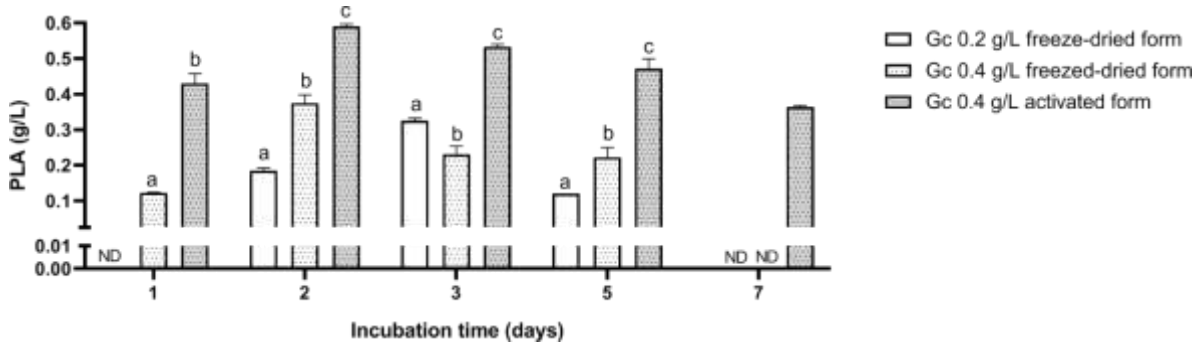

Figure 3 - Comparison of PLA concentration (g/L) in Ym medium inoculated with G. candidum under freeze-dried or activated form (Two-way ANOVA, Tukey's multiple comparisons post-hoc test, data with different letters are significantly different p-value < 0.05). ND = not detected

Experiments conducted in synthetic Ym medium showed that activating *G. candidum* before inoculation helps to launch its metabolism and allowed the production of PLA during the early stages of growth. The highest T-2 toxin reduction levels corresponded to the highest values of PLA and this occurred between the second and third day of incubation time. Based on the results obtained in this study, the optimal culture conditions of *G. candidum* during the malting process were activating the yeast and doubling its inoculum concentration for a better T-2 toxin reduction.

3.2 Micro-malting assays

Micro-malting assays were conducted with *F. langsethiae* 2297 or with *F. sporotrichioides* 186 to mimic the malting process conditions and held between three and five days, according to the duration of the malting step in beer industries. The aim was to upscale the findings in Ym synthetic medium and validate them on an intermediate level before assessing them on an industrial level. In the case of *F. langsethiae* 2297, T-2 toxin concentration results are presented in Figure 4. The inoculation of *G. candidum* under the freeze-dried form or activated form either inoculated at 0.2 or 0.4 g/L significantly reduced the T-2 toxin final concentration at the two incubation times. T-2 toxin final concentration did not depend on the *G. candidum* inoculation way. T-2 toxin reduction percentage for each *G. candidum* inoculation condition for the two incubation times was calculated and is presented in Table 3. For the micro-malting experiment with *F. sporotrichioides* 186, results are presented in Figure 5. After three incubation days, only the activated forms of *G. candidum* could reduce significantly the T-2 toxin final concentration in comparison with the control condition. After five days of incubation time, T-2 toxin concentrations significantly decreased in all experimental conditions. However, the most efficient results were obtained when *G. candidum* was inoculated at 0.4 g/L. In the latter conditions, the highest reduction of the toxin was obtained under its activated form. T-2 toxin reduction percentage for each *G. candidum* inoculation condition for the two incubation times was calculated and is presented in Table 3. Concerning *F. langsethiae* 2297, the best reduction values were obtained at three days of incubation (between 87% and 99%). Even though the reduction lowered at five days of incubation, it ranges between 76% and 79% when inoculating *G. candidum* at 0.4 g/L. In the case of *F. sporotrichioides* 186, high reduction values between 75 and 87% were only obtained with activated forms of *G. candidum* at three days.

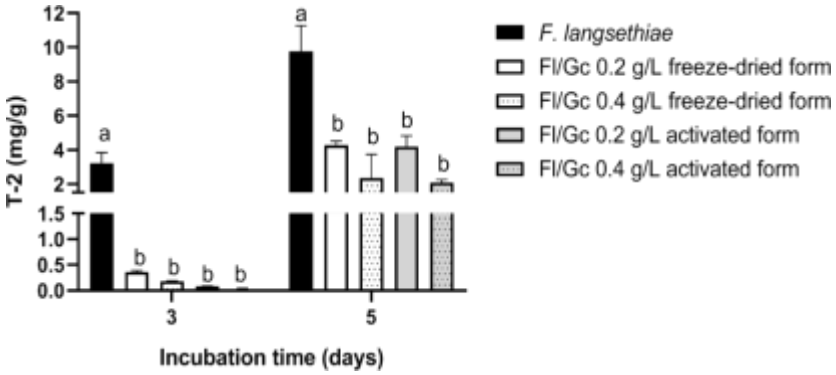

Figure 4 - T-2 toxin concentration (mg/L) in micro-malting experiment with F. langsethiae 2297. G. candidum was inoculated at 0.2 or 0.4 g/L under freeze-dried or activated form (Two-way ANOVA, Tukey's multiple comparisons post-hoc test, data with different letters are significantly different p-value < 0.05).

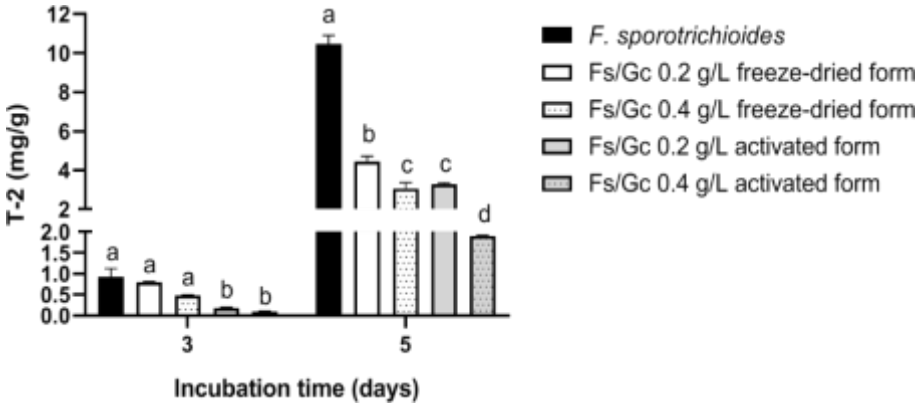

Figure 5 - T-2 toxin concentration (mg/L) in micro-malting experiment with F. sporotrichioides 186. G. candidum was inoculated at 0.2 or 0.4 g/L under freeze-dried or activated form (Two-way ANOVA, Tukey's multiple comparisons post-hoc test, data with different letters are significantly different p-value < 0.05).

Table 3 – T-2 toxin reduction percentages in micro-malting experiments

	***F. langsethiae* 2297**		***F. sporotrichioides* 186**	
	Incubation time (days)		**Incubation time (days)**	
***G. candidum* inoculation condition**	**3**	**5**	**3**	**5**
0.2 g/L freeze-dried	87%	61%	70%	60%
0.4 g/L freeze-dried	94%	79%	77%	73%
0.2 g/L activated	98%	51%	75%	68%
0.4 g/L activated	99%	76%	87%	82%

PLA concentrations were also monitored and are detailed in Table 4. In micro-malting experiments where *G. candidum* was inoculated under the freeze-dried form, the PLA production was lower than in micro-malting experiments where it was inoculated under activated form at three days of incubation time. At five days of incubation time, a reduction of PLA concentrations was observed except when *G. candidum* was inoculated at 0.4 g/L under its activated form. Values of PLA concentrations in the control condition (*G. candidum*

cultivated alone) were also monitored and showed similar values to the ones obtained in micro-malting experiments with both *Fusarium* species (data not shown).

Table 4 - PLA concentration (g/g) in micro-malting experiments

G. candidum inoculation condition	*F. langsethiae* 2297		*F. sporotrichioides* 186	
	Incubation time (days)		Incubation time (days)	
	3	5	3	5
0.2 g/L freeze-dried	0.29 (± 0.00)	0.13 (± 0.02)	0.30 (± 0.01)	0.14 (± 0.01)
0.4 g/L freeze-dried	0.49 (± 0.03)	0.25 (± 0.01)	0.43 (± 0.01)	0.23 (± 0.01)
0.2 g/L activated	0.49 (± 0.01)	0.19 (± 0.01)	0.50 (± 0.01)	0.22 (± 0.01)
0.4 g/L activated	0.73 (± 0.02)	0.39 (± 0.02)	0.72 (± 0.02)	0.77 (± 0.01)

The effectiveness of *G. candidum* in micro-malting assays is different depending on the *Fusarium* strain but it is at least 50%. Indeed, The T-2 toxin reduction is less effective with *F. sporotrichioides* 186. For both *Fusarium* strains best T-2 toxin reduction percentages are obtained at three incubation days, except for *F. sporotrichioides* 186 when *G. candidum* was inoculated at 0.2 g/L under the freeze-dried form. Moreover, T-2 toxin reduction percentages are correlated with PLA concentrations.

The statistical analysis demonstrates that *G. candidum* significantly reduces the T-2 toxin concentration regardless of the form and quantity of the inoculum in the micro-malting experiment with *F. langsethiae* 2297. On the contrary, in the micro-malting experiment with *F. sporotrichioides* 186, at three incubation days, only *G. candidum* under activated form significantly reduces the T-2 toxin concentration. For both *Fusarium* species, depending on *G. candidum* inoculation form, T-2 toxin was reduced during the first three days of incubation time, then slightly increasing over time but stayed relatively lower in comparison with control conditions. However, the best results were obtained when *F. langsethiae* 2297 and *F. sporotrichioides* 186 were co-cultured with activated forms of *G. candidum* at 0.4 g/L specifically after three days of culture.

4. Discussion

In France, the occurrence of *Fusarium* species in barley crops brewing is a source of concern for several years. The risk associated with these fungal species is the production of T-2 toxin, the most toxic compound in the type-A trichothecenes family. During the brewing process, the malting step provides the best conditions (22°C and high humidity) for Fusarium development and T-2 toxin production (Strub et al., 2010). To reduce the development of undesirable microflora and more particularly *Fusarium* species, The French Institute of Brewing and Malting filed a patent in September 1999 that recommends the use of *G. candidum* species obtained by selection to substantially inhibit the development of the

undesirable flora during the malting step (Boivin and Malanda, 1999). Moreover, studies showed that inoculating barley kernels with *G. candidum* starter culture significantly reduced the presence of filamentous fungi belonging to *Fusarium, Aspergillus* and *Rhizopus* genera (Bielecki and Tramper, 2000). Better yet, its use lowered worth viscosity, and thus improved the physical, chemical and organoleptic characteristics of the beer. Using starter cultures as a mean to limit the development of undesirable microflora and to eliminate the potential presence of unfavorable fungi metabolite is considered as a replacement of chemical agents used for grain disinfection during the brewing process (Wolf-Hall, 2007). Nevertheless, the extent of the information ends at this point and no further explanation was given on this matter.

In our previous study, the interaction mechanisms between two *Fusarium* species and *G. candidum* were deciphered in Ym synthetic medium at 22°C (Kawtharani et al., 2020). The reduction of fungal biomass and the T-2 toxin concentration were correlated to the production of PLA by *G. candidum*. In this study, *G. candidum* was inoculated under activated form at 0.2 g/L and the highest PLA concentration was obtained on the third day of incubation and reached 0.41 g/L (± 0.03). Results showed that the reduction of the T-2 toxin concentration was due to fungal growth inhibition. However, nowadays, brewers inoculate *G. candidum* under the freeze-dried form with humid barley kernels and leave it for three to five days in proper malting conditions. Thus, the present study aimed to evaluate the influence of the *G. candidum* inoculum in terms of form (i.e. freeze-dried or activated form) and amount in in vitro experiments in Ym synthetic medium and at the micro-malting scale.

Experimentations conducted in Ym synthetic medium at 22°C showed that PLA production was highly dependent on the inoculation method of *G. candidum*. Indeed, when added in the freeze-dried form at 0.2 or 0.4 g/L, PLA production was delayed and it was only detected after 48 hours instead of 24 hours of incubation time. Besides, regardless of the inoculation concentration of *G. candidum,* the PLA concentration reached lower values when it was inoculated in freeze-dried form. However, the final dry weight of *G. candidum* was the same as when it was inoculated under activated form demonstrating a reduced specific production when it was inoculated under the freeze-dried form. As expected, PLA values increased and maintained longer when *G. candidum* inoculum concentration under activated form was doubled. Experimentations conducted in both Ym synthetic medium and micro-malting assays showed that activating *G. candidum* before inoculation helps to launch its metabolism and permitted the production of PLA during early stages of growth leading to a higher reduction of T-2 toxin concentration. The highest T-2 toxin reduction levels corresponded to the highest values of PLA and this occurred between the second and third day of incubation time. These findings support the hypothesis that the PLA is a primary metabolite produced during the early stages of growth (Kawtharani et al., 2020). Based on the results obtained in the present study, the optimal culture conditions of *G. candidum* during the malting process were activating the yeast and doubling its inoculum concentration for better T-2 toxin concentration reduction. If the brewer wishes to maintain the inoculum at 0.2 g/L, the malting step should be reduced to

a maximum of three-day period otherwise PLA values would decrease leading to an increase in T-2 toxin levels. Nonetheless, if the brewer can not afford to activate the yeast, the alternative is to double *G. candidum* inoculum concentration to guarantee the safety of the beer product. In this case, the malting step should also be limited to a three-day extent to avoid high T-2 toxin concentration.

Freeze-drying of biocontrol agents allows their stabilization and their conservation. Moreover, compared to liquid forms, dried products are easier to use in industrial processes. Specific procedures are required to guarantee long-term viability and metabolism stability. However, it may be considered as a source of stress for the microorganism itself (Thammavongs et al., 2008).

Regarding freeze-drying methods, a study was conducted to test the effect of freezing, freeze-drying process and the use of protectants on the viability of the biocontrol yeast Candida sake. Results showed that liquid nitrogen freezing caused high deterioration levels to the cells with viability < 10% (Abadias et al., 2001a). However, freezing cells at -20°C could be a better alternative to the previous method as it registered around 30% of cell viability. Adding exogenous substances as protective agents such as lactose, glucose or fructose increased survival chances by 35% when added at 10% in appropriate media (Berner and Viernstein, 2006). A hypothesis suggests that liquid nitrogen quick-freezes cells immediately to a point where internal water freezes inside causing membrane damage (Usall et al., 2000). Therefore, it is advised to adopt rehydration and growth media, enriched with these protective agents, to increase yeast viability after freeze-drying (Abadias et al., 2001b). Moreover, the efficiency of C. sake, used as a biocontrol agent against Penicillium expansum on Golden Delicious apples was tested. It appears that, even after adding different protective agents during freeze-drying process (the best one being a combination of 10% lactose with 1% peptone) and using a rehydration media rather than plain water, the efficacy of freeze-dried cells was reduced compared to fresh ones. When C. sake cells were freeze-dried, the biocontrol against P. expansum was effective at 65%. Storage temperatures also influenced the stability of lyophilized yeast. (Abadias et al., 2001b).

In the use of *G. candidum* during the malting step, it is the production of PLA that is important to reduce T-2 toxin concentration. Thus, in the freeze-drying process, it is necessary to ensure that the capacity to produce PLA is maintained at the same level as the strain under the fresh form. In the present study, the difference in efficacy between the freeze-dried and the activated form may be due to the freeze-drying process. Indeed, this latter can reduce microorganism viability and finally the activation step allows to overcome this reduction of viability by inoculating with a culture in an exponential growth phase. Temperature and duration storage were also proven to influence cell viability yield (Sullivan and Bradford, 2011). For instance, storage at temperatures ranging from ambient temperature to 40°C was found to be harmful for dried yeast cells, which caused 90% loss of viability per month. Thus, conservation of active dry yeast should be done at cool temperature and used as soon as possible.

G. candidum was chosen among several species as a fermenting agent during the brewing process for many reasons: the production of thermostable hydrolytic enzymes (cellulase, β-glucanases, pentosanase) that facilitate the fermentation, flavoring of the malt due to the synthesis of amino acids, lowering of the pH and inactivation of the development of undesirable microflora. While it has many advantages, it is regrettable not to optimize its use to maximize the benefits and obtain a safe end product (Arfi et al., 2002; Boutrou and Guéguen, 2005; Kure et al., 2001; Martin et al., 2001, 1999; Naz et al., 2013).

Furthermore, other factors can influence yeast growth and have an impact on the brewing process. As a matter of fact, magnesium and zinc are key factors to improve the brewing process. They act as modulators of yeast stress as they maintain the stability and dynamics of the cell membrane. Studies on Saccharomyces cerevisiae showed that these metal ions are linked to better viability of yeast during the brewing process (Walker et al., 2006). They enhance signaling systems that allow a better response to environmental stress and decrease the permeability of the cell membrane (Walker, 2004). Thus, a zinc and magnesium-enriched rehydration medium might be one solution among others to increase the chances of active dry yeast viability.

In conclusion, important factors can influence the effectiveness of *G. candidum* against *F. langsethiae* and *F. sporotrichioides* and of course T-2 toxin, such as dehydration methodology, growth media and conditions, the addition of protective agents, rehydration conditions, stress induction and storage conditions to standardize and attain high levels of cell viability. All these elements should be taken into account during the brewing process to optimize the efficiency of the biocontrol against these mycotoxigenic fungi and thus ensure a safe product.

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Chapter 3 - Influence of several culture parameters on *Geotrichum candidum* growth and phenyllactic acid production

Because of its organoleptic benefits and effectiveness against *F. langsethiae* and *F. sporotrichioides* growth during the malting step, *G. candidum* is considered as an efficient preventive approach proposing sober and salubrious beer products. In fact, the correlation between phenyllactic acid (PLA), *G. candidum* metabolite and fungal growth was obvious: *F. langsethiae* and *F. sporotrichioides* proliferation and thus T- 2 toxin concentration were significantly reduced when PLA reached its highest values. In order to optimize the implementation of this innovative and environmentally friendly biocontrol later on, the third part of this work consisted on determining preliminarily the influence of certain parameters on *G. candidum* development and PLA production.

As previous results showed, PLA is produced during early stages of *G. candidum* growth. Providing adequate nutrients to increase *G. candidum* growth may contribute to higher PLA production. Therefore, three media have been proposed to enhance the growth of *G. candidum*: Synthetic medium (SM), Malt Extract Broth (MEB) and Yeast and Malt medium (Ym). Fermentations carried out on SM medium formulated with high doses of glucose, yeast extract and supplemented with phenylalanine showed increase biomass and higher PLA productivity.

However, the large-scale production of PLA on complex media commonly used in the laboratory for *G. candidum* growth such as SM is not economically viable due to its large amount of expensive nutrients such as yeast extract, phenylalanine, peptones and salts. Therefore, activating *G. candidum* in SM medium prior to its actual inoculation in malting conditions seemed more reasonable. As a result, the presence of PLA precursor, phenylalanine, boosted PLA production without influencing *G. candidum* growth.

Influence of several culture parameters on *Geotrichum candidum* growth and phenyllactic acid production

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Abstract

Geotrichum candidum, through the production of PLA, was previously demonstrated to reduce *Fusarium sporotrichioides* and *F. langsethiae* growth during malting process, leading to the reduction of T-2 toxin concentration. Since culture conditions influence microorganism metabolism and cause regulatory changes that might alter metabolite synthesis, the aim of this study is to determine the effect of several culture parameters on *G. candidum* growth and PLA production. First, the kinetics of *G. candidum* growth and PLA concentration were monitored in Yeast and Malt medium (Ym) which is the reference medium from 0h to 72h. Then, the analysis of *G. candidum* growth and PLA production was conducted in two other liquid media: Malt Extract Broth (MEB) and Synthetic Medium (SM). The use of SM medium for the starter culture instead of the Ym medium was also tested in order to try to enhance *G. candidum* growth and PLA production. Finally, the influence of phenylalanine (Phe), a PLA precursor, on *G. candidum* growth and PLA production was also analyzed. Results showed that the SM medium is the medium turning out maximum PLA yield of 2.00 g/L, which was in between two and ten times higher than in other used media. Moreover, PLA production was significantly improved (almost doubled) for a longer period of time when phenylalanine was added into the medium used for the started culture.

Key words *Geotrichum candidum*, Phenyllactic acid, Yeast and Malt medium, Malt Extract Broth, Synthetic Medium, Rotational Speed, Phenylalanine.

1. Introduction

Previously known as *Oidium lactis, Geotrichum candidum* is often described as halfway between yeast and mold. It is a widespread filamentous yeast, often found in various habitat such as soil, air, water, milk, silage, plant tissues and even in human and mammals digestive tracts. This microorganism is commonly used as a ripening agent during cheese maturation (Perkins et al. 2020; Dugat-Bony et al. 2015; Boutrou and Guéguen 2005; Boutrou et al. 2006). Given the fact that it can be found in raw milk, *G. candidum* can also be detected in low levels in raw cheese (Cosentino et al. 2001). Its development contributes to the appearance of the crust and the flavor of the products (Mdaini et al. 2006). Known for its quality enhancement during the malting process, inoculating *G. candidum* at this step results in a higher extract yield of the malt, a lower viscosity of the worth, a stronger activity of β-glucanase and α- amylase and a higher diastatic power (Dziuba et al. 2000; Piegza et al. 2014).

Since it's rather acidophilic, *G. candidum* helps to neutralize the curd and fights undesirable molds (Somerville and Proctor 2013). Indeed, *G. candidum* had been credited for inhibiting *Listeria monocytogenes* on cheeses, responsible for human and animal listeriosis, through competition for nutrients (Farber and Peterkin 1991; Goerges et al. 2006). Noticeably, the most considerable feature of *G. candidum* strains is a substantially complete inhibition of undesirable flora developing during malting, a lack of mutagenic activity and a reduction of the final quantity of T-2 toxin in beer.

Phenyllactic acid (PLA), which is an organic acid widely existing in honey and lactic acid bacteria fermented food, can be produced by many microorganisms, *G. candidum* in particular (Rodríguez et al. 2012; Dieuleveux et al. 1998). It was proved to be an ideal antimicrobial compound with broad and effective antimicrobial activity against both bacteria and fungi (Mu et al. 2012; Ohhira et al. 2012). In a previous study, it was proven that *G. candidum* strain produced PLA and reduced *Fusarium langsethiae* 2297 and *F. sporotrichioides* 186 growth by nearly 90% after 48 hours in co-culture. This growth inhibition resulted in the reduction of T- 2 toxin concentration, a common mycotoxin produced by these fungal species that are susceptible to contaminate several food matrices, of which malting barley (Kawtharani et al. 2020; Morcia et al. 2016b). Thus, it is seems important to evaluate certain cultivation parameters to enhance *G. candidum* growth and consequently its production of this antimicrobial agent.

Microorganism cultivation requires consideration of three things: the strain, cultivation medium, and environmental culture conditions. Therefore, the aim of this study is to determine the impact of several parameters such as culture media and yeast activation medium of *G. candidum* on its growth and its PLA production.

2. Materials and Methods

2.1. Reagents and chemicals

Yeast extract (Y1625), malt extract (70167) and dextrose (D9434) were supplied by Sigma-Aldrich (Saint-Quentin-Fallavier, France). Peptone (2585K) was supplied by Fisher Scientific.

Alpha-D (+)-Glucose, 99% anhydrous (Code: 170080025) was supplied by ACROS ORGANICS Di-Potassium hydrogen orthophosphate anhydrous graded (Code: P/5245/53) as analytical reagent was supplied by Fisher Scientific. L-Phenylalanine (78019) was supplied by Fluka BioChemika. Acetonitrile (HPLC grade) and glacial acetic acid were purchased from Fisher Scientific (Illkirch, France). Ultrapure water used for HPLC was purified at 0.22 µm by an ELGA purification system (ELGA LabWater, High Wycombe, United Kingdom). Phenyllactic acid (PLA) standard was purchased from Sigma-Aldrich.

2.2. Strains, Media and culture conditions

Geotrichum candidum filamentous malting yeast (IFBM Malting Yeast®) was purchased from DSM Food Specialties (DMS food specialties, La Fertésous Jouarre, France). It was supplied under freeze-dried form. *G. candidum* is currently used as a biocontrol agent during the malting process.

To study *G. candidum* growth and PLA production, three semi-synthetic media were prepared, namely Yeast and Malt (Ym), Malt Extract Broth (MEB) and Synthetic Medium (SM). The Ym medium (glucose 5 g/L; yeast extract 1.5 g/L; malt extract 1.5 g/L; peptone salt 2.5 g/L) was the reference medium. MEB composition was as follows: malt extract 20 g/L; peptone salt 6 g/L; dextrose 20 g/L. SM composition was modified from the one used in Mu et al. (2009), in which phenylpyruvic acid was replaced by phenylalanine (glucose 30 g/L; malt extract 30 g/L; yeast extract 30 g/L; peptone salt 47 g/L; phenylalanine 5 g/L; K_2HPO_4 0.3 g/L). For all culture media, the pH was adjusted to pH 7 prior sterilization.

2.3. Culture conditions

G. candidum was purchased in under freeze-dried form, thus it was decided to be pre-cultured in order to activate its metabolism and reduce the latent phase before each experiment (Gastélum-Martínez et al. 2012). For that, 2.4 g of freeze-dried *G. candidum* was inoculate in a 250 mL Erlenmeyer flasks containing 100 mL of Ym medium, and incubated in an orbital incubator set at 22°C and 150 rpm during 24h. After incubation, this *G. candidum* pre-culture was used as a starter culture in the following experiments.

2.3.1. Kinetic of PLA production by *G. candidum* in Ym medium

In a 250 mL Erlenmeyer flask, 100 mL of Ym medium was inoculated with *G. candidum* starter culture at a final concentration of 0.2 g/L and then incubated in an orbital shaker set at 22◦C at 150 rpm for several fermentation times ranging from 6 h to 120 h. At each sampling time, *G. candidum* growth was determined by measuring the dry weight and PLA concentration by HPLC-DAD.

2.3.2. Influence of culture media on *G. candidum* growth and PLA concentration

The objective of inoculating *G. candidum* in three different media (Ym, MEB and SM) is to select the medium that improves the development of *G. candidum* and, importantly, that leads to the best PLA concentration. Ym medium has similar properties as oat-based worth

prepared for brewing in term of carbon and nitrogen sources. The MEB medium was generally used for isolation, cultivation and enumeration of yeast and filamentous fungi (Moreira et al. 2001; Black 2020) . SM medium is noticeably composed of phenylalanine, a precursor of PLA biosynthesis in *G. candidum* (Naz et al. 2013). First, the starter culture used in this experiment was prepared in Ym medium as previously described and then, 250 mL Erlenmeyer flasks containing 100 mL of each medium were inoculated with *G. candidum* starter culture at a final concentration of 0.2 g/L. The incubation conditions were the same as described above. The kinetic of *G. candidum* growth and PLA concentration were studied through different sampling time kinetics including 0h, 3h, 6h, 12h, 17 h, 24 h, 41 h, 48 h, 65 h and 72 h.

2.3.3. Effect of the preparation of the starter culture on *G. candidum* growth and PLA concentration

The objective of the activation of *G. candidum* in a medium enriched in phenylalanine (SM) is to procure the PLA precursor. Indeed, *G. candidum* does not have to synthesize it and therefore takes less time to produce PLA. This could increase the PLA concentration in subsequent culture. Therefore, 2.4 g of freeze-dried *G. candidum* was cultivated in a 250 mL Erlenmeyer flask containing 100 mL of SM medium, and incubated in an orbital incubator set at 22°C and 150 rpm for 24 h. Then, *G. candidum* pre-culture was used to inoculate Ym medium at a final concentration of 0.2 g/L in a 250 mL Erlenmeyer flask filled with 100 mL of medium. The incubation conditions were the same as described above. The kinetics of *G. candidum* growth and PLA concentration were investigated through different sampling time including 0h, 3h, 6h, 12h, 17 h, 24 h, 41 h, 48 h, 65 h and 72 h.

2.4. Biomass quantification

Vacuum filtration was used in order to estimate the development of *G. candidum*. Dry mass of biomass is the difference between dry mass of a membrane filter (cellulose nitrate, pore size 0.45 µm, Sartorius stedim biotech) after and before filtration and drying 100 mL of *G. candidum* culture at each sampling time. To obtain the dry mass filters were dried in an oven for 24h at 105°C and then weighed after cooling down in a desiccator. The dry biomass was expressed as grams per liter (g/L).

2.5. Detection and quantification of PLA

At each sampling time, 1 mL of culture media was withdrawn and filtrated through 0.45 µm PTFE syringe filters to eliminate microorganisms from the supernatant prior to injection into HPLC apparatus. Analysis of PLA were performed using a Luna C18 (2) column (5µm, 250×4.6 mm) and a pre-column with the same characteristics (Phenomenex, Torrance, CA, USA). The detection of PLA was performed using a Dionex Ultimate 3000 UHPLC system coupled with a diode-array detector (DAD) set at 210 nm (Thermo Fisher Scientific). The analysis was performed in a gradient mode using acidified water (0.2% of acetic acid glacial) as solvent A and pure HPLC grade acetonitrile as solvent B. Flow rate was set at 1.2 mL/min with A/B ratios of 90:10, 50:50, 50:50, 0:100 and 90:10, with run times of 0.0, 4.0, 9.0, 10.0 and 15.0 min,

respectively. Injection volume was set at 50 µL. PLA quantification was determined according to a standard calibration curve with concentrations ranging between 10 and 1000 mg/L.

2.6. Statistical analysis

Differences between PLA production and *G. candidum* biomass were analyzed using a One-Way Analysis of Variance (ANOVA) followed by Dunnett Post Test. Data analysis was carried out with GraphPad Prism 4 software (GraphPad Software, La Jolla, CA, USA). Differences were considered to be statistically significant when the p-value was lower than 0.05.

3. Results and Discussion

3.1. *G. candidum* growth and PLA production in Ym medium

To study the growth of *G. candidum*, Ym medium was initially inoculated with 0.2 g/L of *G. candidum* starter culture and incubated at 22°C, 150 rpm for 3 days. Samples were withdrawn at the starting point, after 3h, 6 h, 12 h, 18h, 42h, 48h, 65h and 72 h of fermentation time. For each sampling time, the dry weight and PLA concentration were analyzed (Figure 1). Panel A shows that *G. candidum* grew linearly during the entire incubation time, from 0.2 g/L till 3.66 g/L (± 0.04). Panel B shows the evolution of PLA concentration throughout the incubation time. In fact, PLA values were detected after 6 hours of incubation (0.04 ± 0.01 g/L) and reach its maximum value after 48 hours of incubation time (0.42 ± 0.01 g/L). Between the second (48h) and third day (72h) of incubation time, PLA values was highly reduced to reach 0.23 g/L (± 0.01), almost half the concentration within only 24 hours. Specific production referred to quantity of PLA produced by *G. candidum* comparing to quantity of biomass at each corresponding time. It shows that the yeast is highly active in accumulating PLA in the medium during the first day of incubation, then this activity gradually decreased.

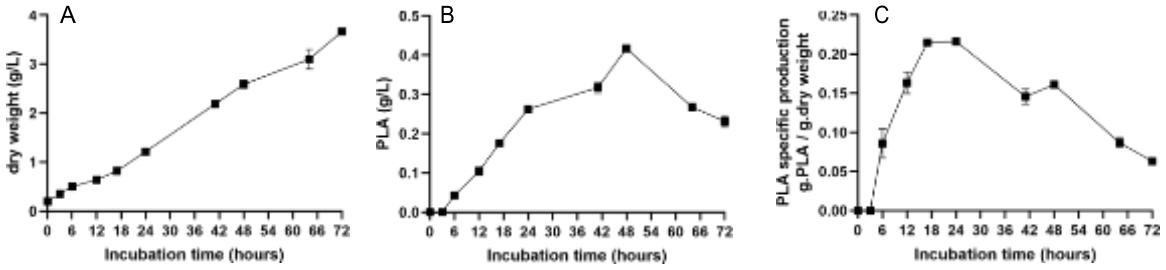

Figure 1 – PLA concentration (g/L) (Panel A) and G. candidum biomass (g/L) (Panel B) in Ym medium and PLA specific production (g PLA/g dry weight) (Panel C).

3.2. Influence of the culture media on kinetics of *G. candidum* growth and PLA production

In this experiment, *G. candidum* strain was activated in Ym medium and then, inoculated in Ym, SM or MEB medium. *G. candidum* biomass and PLA production were analyzed and results are presented in Figure 2. In Ym medium, which is the reference medium, *G. candidum* reached the highest biomass at 24h (3.70 ± 0.13 g/L Figure 2 Panel A) then remained stable until the end of the experiment. In MEB medium, the same profile was observed. Indeed, the

highest biomass was reached at 65h (7.03 ± 0.13 g/L) of incubation time and remain also stable until the end of the experiment. Differently, in SM medium, *G. candidum* biomass increased throughout the experiment to reach a value of 13.91 ± 0.20 g/L at 72h.

For the three culture media, the PLA production has the same profile as the growth kinetics of *G. candidum* (Figure 2 Panel B). Indeed, in SM medium PLA production reached the highest value in the experiment (2.0 ± 0.1 g/L). The PLA specific production kinetics were calculated for each culture medium and for all incubation times, the SM medium support the best PLA production. The specific production on SM medium rose sharply between 0h to 17h and jumped to the top at 17h, resulting in ten-fold and three-fold increase in comparison to that of MEB and Ym media, respectively. Beyond that time, specific production of SM declined steadily because *G. candidum* didn't produce as much amount of PLA as the early stage of fermentation. There was a gradual increase of the specific production from 0h to 48h and from 0h to 24h in Ym and MEB, respectively, after that it fell down until the end of experiment. Moreover, our results show that the PLA concentration increased with an increase in the amounts of nitrogen sources. Ym medium was made from 4 g/L, MEB medium 6 g/L and SM medium 77 g/L of nitrogen substrates yield the maximum product of PLA 0.41 g/L, 0.63 ± 0.2 g/L and 2.00 ± 0.11 g/L respectively (Figure 2 Panel A). From the starting point of fermentation PLA concentration in SM medium was always significantly higher than that in other media reaching to the highest production at 65h while Ym medium at 48h, beyond which there was a decrease, but MEB medium produced the maximum PLA at 41h and remained constant until the end.

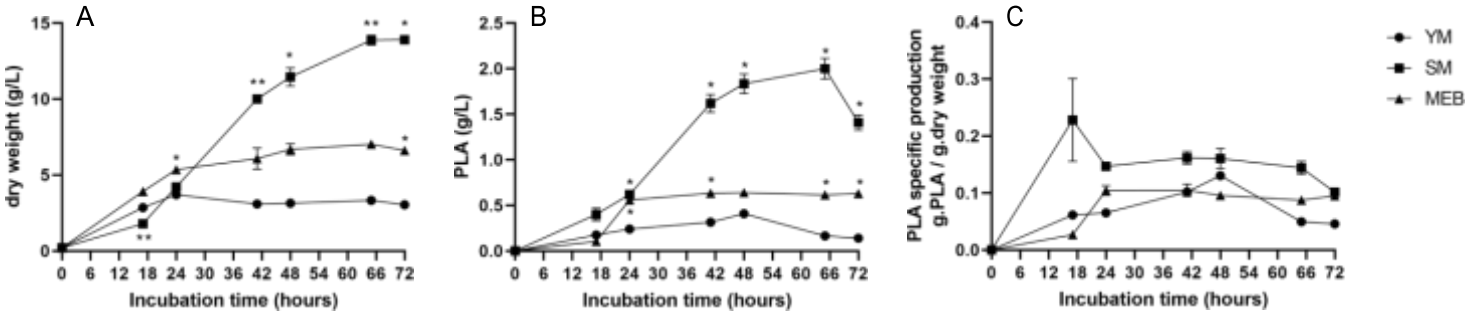

*Figure 2 - PLA concentration (g/L) (Panel A), G. candidum biomass (g/L) (Panel B) and PLA specific production (g PLA/g dry weight) (Panel C) in Ym, SM and MEB media when G. candidum was activated Ym medium - One-way ANOVA, Dunett's multiple comparisons post-hoc test, * p-value < 0,05; ** p-value < 0.01*

The SM medium is a rich medium encompassing large amount of glucose and malt extract (carbon and energy sources) and yeast extract and peptone salt (nitrogen sources) whereas MEB and Ym media have low concentration of those substrates. Therefore, this phenomenon is strongly connected to the availability of carbon and nitrogen sources in each culture medium, which were required for cell biomass and metabolite synthesis. The nitrogen and carbon substrates play important roles in energy generation, cell maintenance and biosynthesis in microorganism (Costa et al. 2002). PLA is a primary metabolite which was detected from cultivation of *L. plantarum* and yeast strains like *G. candidum* (Naz et al. 2013).

The production of primary metabolites in filamentous yeast-fungi were strongly influenced by carbon and nitrogen sources (Wattanachaisaereekul et al. 2014). Nitrogen sources, particularly aromatic amino acids functioned as precursors of corresponding aromatic acids having low molecular weight (<1kDa) including hydroxyl acids such as PLA, hydroxyphenyllactic acid, indolelactic acid, alcohols such as phenylethyl alcohol and others (Prasuna et al. 2012;Naz et al. 2013). Therefore, the release of PLA would be expected simultaneously with consumptions of at least one nitrogen substrate which can be yeast extract, peptone and especially phenylalanine.

3.3. Influence of *G. candidum* activation in SM medium, enriched in phenylalanine (Phe), the PLA precursor, on *G. candidum* growth and PLA production

In this experiment, *G. candidum* starter culture was prepared in SM medium enriched with 5 g/L of phenylalanine (Phe) and then used to inoculate Ym medium at the final concentration of 0.2 g/L. The control condition consisted to use a *G. candidum* starter culture prepared in Ym medium and then to inoculate Ym medium at the final concentration of 0.2 g/L. For both conditions, *G. candidum* growth and PLA production kinetics were evaluated at several sampling times after inoculation in Ym medium, ranging from 0h to 72h.

In both conditions, as shown in Figure 3 Panel A, *G. candidum* grown similarly in Ym medium, going from 0.2 g/L (± 0.00) to nearly 3.65 g/L (± 0.05). Glucose is one of the main substrate in Ym medium which was proved to be the best assimilated carbon source to promote *G. candidum* growth (Arfi et al. 2003). The similarity of *G. candidum* growth behavior in both conditions is justified by the identical composition of Ym medium in which it was inoculated. Nevertheless, the kinetic of PLA production is very different between the two activation conditions (Figure 3 Panel B). When *G. candidum* was activated in SM medium, PLA was detected at low values even at the beginning of the experiment (at 0h at 0.001 g/L (± 0.00) and then highly increased in the medium to reach its peak after 72h to incubation time to attain 0.75 g/L (± 0.01). This suggested that PLA biosynthesis was launched during *G. candidum* activation step. On the contrary, when *G. candidum* was activated in Ym medium, PLA was detected after 6h of incubation time at a value of 0.07 g/L (± 0.01). PLA concentrations slowly increased to reach its highest after 48h at 0.42 g/L (± 0.01) then was reduced at the end of the experiment to reach 0.25 g/L (± 0.01). The reduction of PLA concentration at the end of incubation time is probably due to the hypothesis that PLA might be a primary metabolite and may be involved in other metabolic pathways or used by the *G. candidum* itself (Kawtharani et al. 2020). Activating *G. candidum* in SM medium where the PLA precursor, phenylalanine, was incorporated into the medium clearly enhance PLA production since it was detected at the beginning. Moreover, PLA concentration remains high over a longer period of time. A possible explanation could suggest that higher Phe concentrations were present in the medium and lead to continuous PLA production. Regarding PLA specific production, when *G. candidum* was activated in SM medium, it was significantly higher from 17h of incubation time until the end of the experiment (Figure 3 Panel C). The PLA specific production was twice as

higher for incubation times 12, 17 and 24 hours and three times higher at 72h when *G. candidum* was activated in SM medium

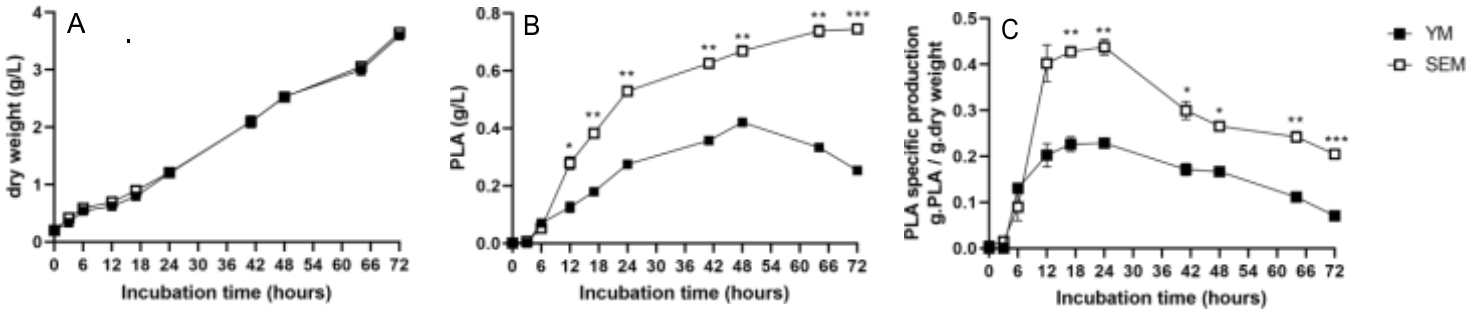

Figure 3 - PLA concentration (g/L) (Panel A), G. candidum biomass (g/L) (Panel B) and PLA specific production (g PLA/g dry weight) (Panel C) in Ym medium, when G. candidum was activated in SM medium or in Ym medium – t-test, * p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001

In a previous study, Naz et al. have demonstrated that *G. candidum* produced five more times PLA when Phe was added to the medium. After only 72 hours of incubation time, more than 75% of Phe was converted into PLA (Naz et al. 2013). They have also demonstrated that adding PPA into the medium lead to an earlier production of larger quantities of PLA since it is the direct intermediate between Phe and PLA in the metabolic pathway (Li et al. 2007; Naz et al. 2013). Moreover, others studies showed similar findings related to PLA produced by *Saccharomyces cerevisiae* and Lactic Acid Bacteria (LAB) (Dickinson et al. 2003; Yvon and Rijnen 2001). All of this reinforces the results obtained during this study. Since Phe is already found as a diet supplement and no data on adverse health effects after chronic ingestion of supplemental phenylalanine in apparently healthy subjects are available, it may be a solution to reduce certain food matrix microbial contamination (Frøyland et al. 2020). However, there is no available data on the effect of PPA incorporation as a supplement on human health.

4. Conclusion

In this study, preliminary results were provided to enhance PLA production by *G. candidum* in an enriched synthetic medium. In fact, the addition of phenylalanine to the culture broth could be efficient; as it demonstrated precocious production of PLA without affecting *G. candidum* growth. The purpose behind this study is to optimize the use of *G. candidum* and PLA production as a biocontrol agent against several foodborne contaminants. Indeed, as previously demonstrated PLA is responsible for the reduction *Fusarium* species growth leading to the reduction of T-2 toxin concentration. The use of *G. candidum* during malting process can reduce T-2 toxin concentration through the production of PLA. Thus, the optimization of PLA production by *G. candidum* during the malting process may be a solution to reduce T-2 toxin concentration in beer product. However, the use of pure amino acid, such as phenylalanine, could not be economically viable. Design of experiments varying nutrient supply, oxygen supply and rotational speeds, temperature and pH values in bioreactors could be the solution to find optimal conditions for *G. candidum* growth and PLA production.

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General Discussion and Perspectives

In the framework of this project, we investigated the issue of T-2 toxin produced by two *Fusarium* species, *Fusarium langsethiae* and *F. sporotrichioides* on malting barley. This doctoral research focuses on three main aspects. The first part of the work consisted on elucidating the mechanisms of interaction between *G candidum* and *Fusarium* species in order to better understand the mode of action leading to T-2 toxin concentration reduction. The identification of the phenyllactic acid, produced by *G. candidum* as the biocontrol agent was at the heart of this section. The second part of this survey consisted on conducting experiments miming malting conditions *in vitro* and then, on a micro-malting level to evaluate the effect of *G. candidum* inoculation methods on T-2 toxin concentration reduction. Finally, in order to find better conditions for the production of PLA by *G. candidum*, several culture parameters were tested to see their effect on *G. candidum* growth and its PLA production. This work is more of an applied research project; the effort was focused on understanding a method capable of limiting the presence of the T-2 toxin produced by the two *Fusarium* species during the malting step.

First of all, the discussion will focus on the experimental continuum anticipated within the framework of this study.

Henceforth, it will primarily go through hypotheses that may be proposed preceding the work presented in this manuscript. In the course of this survey, it was found that *G. candidum* is capable of producing several aromatic compounds, reported to have antimicrobial activities. They can also be produced by several microbial strains in different food and feed matrices. All of these elements, along with the metabolic pathways of these compounds will be discussed.

It is important to note that some of the discussion points already addressed in the articles will not be included in this section.

Continuum of the experimental work

The second part of this work consisted on conducting experiments in malting miming conditions. The objective was to evaluate the impact of the method of preparation (i.e. concentration and activation or not) of *G. candidum* strain on T-2 toxin concentration reduction. Experiments were conducted using *G. candidum* under freeze-dried or activated forms at two concentrations (0.2 and 0.4 g/L) and simultaneously inoculated with either *Fusarium langsethiae* 2297 or *F. sporotrichioides* 186. At first, experiments were conducted in synthetic medium. Then, results were validated at the micro-malting scale.

The logical course of events would be to extrapolate these experiments in an actual brewing industry. Indeed, the French Institute of Brewing and Malting, the subcontractor of this project, will be carrying out a few experiments to validate the results obtained during the thesis on an industrial level.

For starters, a reference experiment similar to the one used during the thesis should be conducted to ensure the repeatability of experimental conditions: *Fusarium* spores (10^6 spores / kg of barley) will artificially contaminate sterilized barley. Simultaneously, freeze-dried *G. candidum* will be added and brought to 0.2 g/kg of barley.

Ideally, several parameters will be tested:

- Decreasing the inoculum concentration of *G. candidum* in order to get as close as possible to the actual malting conditions adopted by the brewers (100g of *G. candidum* for one ton of barley grains)
- Activating *G. candidum* in synthetic medium prior to the malting step and before inoculating it with *Fusarium* spores on sterilized barley
- Inoculating *G. candidum* under its activated form and its freeze-dried form at a concentration of 0.2 g/ kg of naturally contaminated barley grains by the two *Fusarium* species.

In all experimental conditions, after 3 days and 5 days of incubation time, both T-2 toxin and PLA concentrations will be measured. Quantitative polymerase chain reaction (qPCR) will then be carried out in order to quantify the genomes of the two microorganisms and evaluate the effect of co-culture on their development in natural malting conditions. This method will help to confirm whether the reduction of T-2 toxin concentration observed in previous findings was due to the reduction in *Fusarium* growth or not.

On another note, optimizing *G. candidum* culture conditions is essential to properly use the biocontrol agent during the brewing process and maybe other food and feed (temperature, pH, agitation, culture time, batch culture, fed-batch, continuous...) to increase the concentration of the PLA produced by *G. candidum*. An experimental design that varies these culture parameters must be studied and implemented in order to optimize *G. candidum* fermentation conditions and PLA production.

Major antimicrobial metabolites produced from aromatic amino acid metabolism in fermented products

Classical preservation methods have always been at the heart of interest of food industries to insure salubrious products to the consumer. Advent technologies consisted on freezing food matrices, pressure heating, drying or even chemical preservation against spoilage. Although microorganisms are considered as the main reason behind food and feed spoilage, newer alternatives to classical preservation means involve the implementation of certain microorganisms as a biocontrol agent to insure healthier and safer product for the consumer (Naz et al. 2013). In fact, they are able to produce several antimicrobial compounds, aromatic amino acids of various low molecular weight (< 1kDa), such as phenyllactic acid, indolelactic acid, alcohols such as phenylethyl alcohol for example. These metabolites are known to play an important role in biopreservation of fermented food products as beer, wine, sourdough and cheese by inhibiting the growth of pathogens such as *Listeria monocytogenes, Staphylococcus aureus* and *Enterococcuss faecalis* (Lan et al. 2012). Moreover, these aromatic metabolites can also influence the organoleptic characteristics, flavor and aroma profiles of fermented food products (Naz et al. 2013). The microorganisms in fermented products can either be present as wild indigenous flora carrying out spontaneous food fermentation, or can be incorporated as starter culture (Leroy and De Vuyst 2004). It is important to properly select the biocontrol agent that maintains the sensory quality of fermented products on one hand, and to comprehend the interaction mechanisms that occur between the undesirable flora and the microorganism of concern in order to limit spoilage on the other hand (Lan et al. 2012).

According to Naz et al (2013), the knowledge of compounds produced by the biocontrol agent, their production pathways, genes and enzymes involved help decipher the allelopathic phenomenon between microbial strains. The following table enlists few fermented products, the leaven used and its reaction products. As shown, lactic acids (such as phenyllactic acid and indole lactic acid) and alcohols are the main compounds produced by several fermenting microbial strains. Their benefits will be discussed later on. *Geotrichum candidum,* the filamentous yeast of interest in this study, used the malting step in beer production and cheese maturation is a major producer of lactic acids.

Table 10 – Example of fermented products, corresponding microorganisms and their produced compounds

Fermented product	Fermenting microorganism	Produced compounds	References
Beer	*Saccharomyces cerevisiae, S. bayanus, S. pastorianus, S. paradoxus, Candida tropicalis,* ***Geotrichum candidum***	Carbon dioxide, ethanol, dextrin, **lactic acid**	(Evans 2006; Victor R. Preedy 2009; Bokulich and Bamforth 2013; Alloue-Boraud et al. 2015)

Wine	*Oenococcus oeni , Lactobacillus plantarum*	Ethanol, malic acid	(Pozo-bayón et al. 2005)
Sourdough	*Bifidobacterium pseudocatenulatum, Lactobacillus sanfrancisco, Saccharomyces cerevisiae, Lactobacillus pontis, Lactobacillus brevis, Lactobacillus plantarum, Lactobacillus paralimentarius, Lactobacillus rossiae, Lactobacillus sanfranciscensis, Lactobacillus amylovorus, , Lactobacillus fermentum, Lactobacillus reuteri*	Ethanol and ethyl acetate, 2-methylpropanol and 2/3- methyl-1-butanol, d/l-lactic and acetic acids	(Sanz-Penella et al. 2011; Annan 2013; Dallagnol et al. 2011; Lavermicocca and Visconti 2003)
Cheese	*Lactococcus lactis, Streptococcus thermophilus, Debaryomyces hansenii,* ***Geotrichum candidum*** *Penicillium camemberti, Arthrobacter arilaitensis, Brevibacterium aurantiacum, Brevibacterium linen, Corynebacterium casei Halomonas* spp.*, Hafnia alvei, Leuconostoc sp., Psychrobacter sp.,Kluyveromyces marxianus*	**Lactic acid**, propionic acid, acetic acid,	(Pottier et al. 2008; Botha and Botes 2014; Martin et al. 1999; Yvon and Rijnen 2001; Larpin et al. 2006; Larpin-Laborde et al. 2011)
Kefir	*Lactobacillus kefiranofaciens ssp. kefirgranum, Lactobacillus parakefiri, Lactobacillus kefiri, Kluyveromyces marxianus, Kazachstania exigua, Rhodosporidium kratochvilovae, Streptococcaceae*	Alcohol, lactic acid	(Vardjan et al. 2013)

	(primarily Lactococcus spp.*), Gluconobacter japonicus and Lactobacillus uvarum, Lactobacillus helveticus, Acetobacter syzygii, Lactobacillus satsumensis*		

The biocontrol agents use carbohydrates and sugars to trigger its primary metabolism. Consequently, this enables the proteolysis due to launching the enzyme activity. A large and varies spectrum of different amino acids can be produced depending on the fermentation potential of the microbial species. According to Petkova et al. (2013), amino acids are an important group of organic molecules responsible for the product flavor, characterized by the presence of amine and carboxylic acid functional groups, along with side-chain specific to each amino acid (Petkova 2013). The side chain found in the three proteinogenic aromatic amino acids (AAA) namely phenylalanine (phe), tryptophan (trp), and tyrosine (tyr) consists of a common aromatic ring structure (Ji et al. 2018). These aromatic amino acids are detected in many foods such as wine, cheese, barley; wheat and other plant raw materials. Table 2 reports concentrations of free aromatic amino acids in some fermented food products.

Table 11 – Concentrations of phenylalanine, tryptophan and tyrosine in some fermented food matrices.

Fermented product	Amino acid concentration (mg/g)			References
	Phenylalanine (Phe)	Tryptophan (Trp)	Tyrosine (Tyr)	
Beer	0.1612	0.1307	-	(Hellwig et al. 2018; Fumi et al. 2011)
Wine	0.0028 – 0.138	0.0955	0.0173	(Pozo-bayón et al. 2005; Izquierdo-Cañas et al. 2020)
Cheese	1.421	0.14	0.171	(Valerio et al. 2004; Gummalla and Broadbent 2001)
Kefir	0.762	0.0337	-	(Irigoyen et al. 2012)

Phenylalanine is the most abundant among these aromatic compounds. In some cases, it is specifically added to enhance the production of aromatic compounds, such as longan wine (Trinh et al. 2010). A study conducted in 2010 showed that the presence of phenylalanine and

tyrosine in worth improves nutritional values, quality and safety of beer products (Zhu et al. 2010). However, during beer fermentation, only phenylalanine and tryptophan were detected.

Microorganisms use these aromatic amino acid, phenylalanine, tryptophan and tyrosine to produce compounds listed above in table 1 of which lactic acids. *G. candidum* had been reported to produce phenyllactic acids and indole lactic acid, two major antimicrobial metabolites (Naz et al. 2013). Hydroxyphenyl lactic acid, that comes from tyrosine metabolism, was also produced by *G. candidum* but showed very weak antimicrobial potential (Naz et al. 2013). Scientists showed very little interest in this metabolite and thus was not well described in literature. However, a possible hypothesis suggests that tyrosine metabolites might be structurally related to phenylalanine metabolites since tyrosine is the hydroxylated form of phenylalanine.

Indole lactic acid (ILA) is an organic acid characterized by its molecular formula $C_{11}H_{11}NO_3$ and its molecular weight 205.2 g/mol. It derives from the metabolism of tryptophan in several microbial strains such as *G. candidum, Candida* spp. and *Bifidobacterium* (Aragozzini et al. 1979; Rao 1976; Naz et al. 2013). This had been reported to inhibit the growth of both Gram negative and Gram positive bacteria such as *Escherichia coli* and *Bacillus cereus*. Figure 1 represents the metabolic pathway of indole lactic acid and shows the implication of several enzymes of which tryptophan aminotransferase, tryptophan decarboxylase and indole-3-lactic acid dehydrogenase (Frankenberger and Poth 1988; Sardar and Kempken 2018).

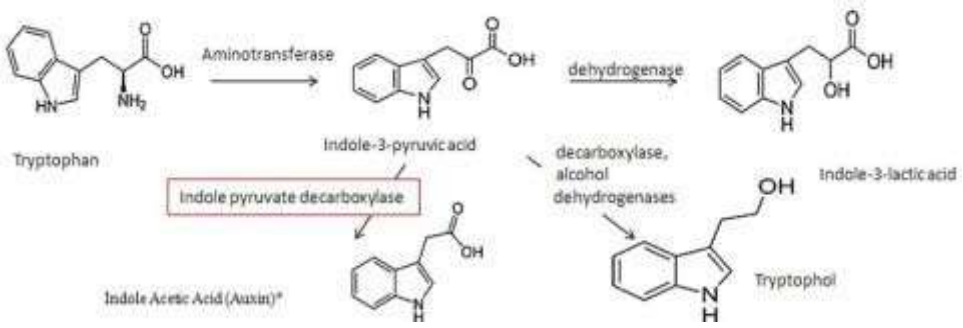

Figure 21 – Proposed indole lactic acid biosynthesis pathway adapted from Naz et al (2013) and Sardar & Kempken (2018)

The synthesis of indole-3-pyruvic acid from tryptophan requires some environmental conditions specific to cheese ripening: low temperature at around 13°C, a pH 5, and a minimum of 4% NaCl to activate the aminotransferase (Roudot-Algaron and Yvon 1998). Even though ILA is known for its great antimicrobial potential, its formation is unfavorable, as it may alter the organoleptic criteria of food and feeds (Gummalla and Broadbent 2001; El Soda 1993). This would probably be a restraint to the use of this metabolite in food and feed matrices.

Henceforth, phenyllactic acid (PLA) pathway was discussed and detailed in previous sections of this manuscript. Briefly, phenylalanine is transaminated to phenylpyruvic acid (PPA) and PPA is further reduced to PLA (McSweeney and Sousa 2000; Vermeulen et al. 2006). The transamination reaction is initiated by an aromatic aminotransferase (AAT) (Yvon et al. 1997). In Lactic acid bacteria, different types of dehydrogenases have been reported to convert PPA to PLA, and lactate dehydrogenase (LDH) is the main type (Mu et al. 2012). Depending on the type of lactate dehydrogenases (L-LDH or D-LDH) present in lactic acid bacteria, PPA is converted to either L-PLA or D-PLA.

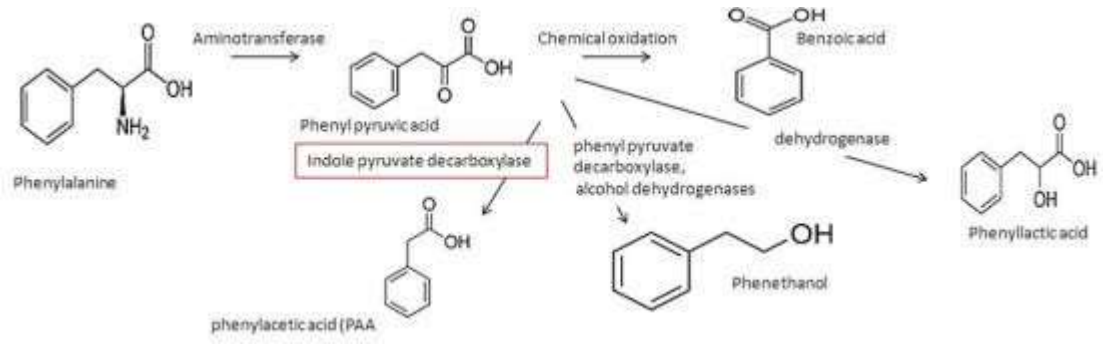

Figure 22 - Proposed phenyllactic acid biosynthesis pathway adapted from Naz et al (2013)

Since PLA had less odor than other organic acids, such as acetic acid, it may be an alternative to antibiotics when synthetically administrated to foodborne. However, few studies have been conducted to evaluate the application of PLA or its salts to diets. A study was conducted to evaluate the potential of PLA addition to the diets of egg laying poultry to function as an alternative to antibiotic growth promoters. Results showed that the addition of PLA can improve the production performance and egg quality (Wang et al. 2009). Additionally, when used as a feed additive in the diet of chicks, PLA exhibits antipathogenic activity in the large intestine, and enhances the meat quality (Wang et al. 2010). It may be beneficial to supplement the diets of laying hens with PLA in the absence of antibiotics. However, more research regarding the effects of PLA is still necessary to approve its use as food and feed additive.

Another aromatic amino acid reported to be produced by *G. candidum* and other microbial strains is Phenylethyl alcohol (PEA) characterized by a rose-like odor. This metabolite, also known as 2-Phenylethanol (2-PE), with the molecular formula $C_6H_5CH_2CH_2OH$ and characterized by low molecular weight 122.16 g/mol. PEA is mainly a bacteriostatic agent, that can reversibly inhibit the synthesis of bacterial deoxyribonucleic acid when administrated in culture media at low concentration (0.3% in culture media) (Lucchini et al. 1993). At higher concentrations, it breaks down the cellular barrier which increases the membrane permeability accelerating passive ions and metabolites diffusion (Silver and Wendt 1967; Ingram and Buttke 1985). Therefore, its antimicrobial effect is revealed at relatively high concentrations (superior to 3 g/L when produced by *Saccharomyces cerevisiae*) (Ingram and Buttke 1985). The effect of PEA produced by *Saccharomyces cerevisiae* was identified against

several bacterial strains including *Salmonella, Shigella, Aerobacter, Klebsiella, Escherichia coli* and *Pseudomonas* (Fabre et al. 1998; Etschmann et al. 2002).

The metabolic pathway of PEA is mostly described in yeasts. The whole process is detailed in the study conducted in 2002 by Etschmann et al (2002) (Etschmann et al. 2002). The biosynthesis of phenylethyl alohol, also called 2- phenylethanol (2-PE), was identified by Ehrlich and consequently named after him (Hazelwood et al. 2008). Figure 23 shows the Ehrlich biosynthesis pathway for PEA. Indeed, Phe undergoes a transamination to form phenylpyruvate (or phenylpyryvic acid), decarboxylation to phenylacetaldehyde caused by a decarboxylase and then reduction to PEA under the action of a dehydrogenase.

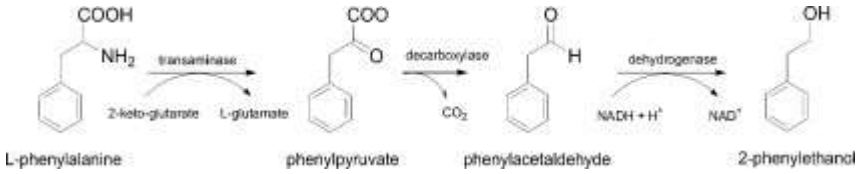

Figure 23 - Ehrlich pathway for 2-PE synthesis

PEA distinguishes by rose-like aroma and is a desirable flavor molecule in various alcoholic beverages. In red wine and beer for example, PEA is one of the major alcohols produced at concentrations ranging from of 13.3 mg/L to 70.6 mg/L (Mallouchos et al. 2002). PEA has also been shown to protect strawberries against fungal growth and thus prolong its shelf life strawberries (Mo and Sung 2007). In fact, during this study, strawberry sample batches were either fumigated with PEA extracted from *Pichia anomala SKM*-T culture broth or were not treated at all (control condition). PEA extraction and purification method was described in Mo et al. study (2003) (Mo et al. 2003). PEA was also extracted from *Galactomyces geotrichum* and used to fumigate the fruit. It is important to note that *Galactomyces geotrichum* is indeed the teleomorphic form of *G. candidum* (Perkins et al. 2020).

Thus, it would be interesting to investigate if *G. candidum* strain provided by the French Institute of Brewing and Malting is a producer of this aromatic alcohol. If that is the case, and since PEA has already been administrated to foodborne as an antifungal product, fumigating barley grains before the brewing process would be a way to inhibit *Fusarium* growth during the malting step. However, this method would not insure the total absence of T-2/HT- 2 toxins in grains as *Fusarium* species are phytopathogen and can grow directly in the field. Therefore, an alternative would be to experience fumigating barley plants on a pre-harvest level as a preventive approach to reduce *Fusarium* growth right at the beginning of the barley food chain.

On another note, PLA and PEA pathways are both related to the presence of phenylalanine as a substrate. They also have the same intermediate, phenylpyruvic acid. In *Saccharomyces*

cerevisiae, decarboxylation of 3-phenylpyruvate can proceed if any of the four genes encoding for decarboxylase enzymes, *PDC1, PDC5, PDC6, or YDR380w*, is functional (Dickinson et al. 2003).

Another metabolite originating from phenylalanine metabolism is benzoic acid. It is found in the volatile fractions of several cheeses and may contribute to the flavor of these products. On an industrial level, this metabolite is widely administrated as a natural food preservative, known as the additive E210. Originally, *Lactobacillus plantarum* strains isolated from beer, yoghurt and orange juice were reported to produce benzoic acid as a defense mechanism against the spoilage yeast *Rhodotorula mucilaginosa* and inhibited fungal growth of *Fusarium avenaceum* (Crowley et al. 2012; Niku-Paavola et al. 1999). Other studies proved the effectiveness of the antimicrobial activity of this organic acid against a variety of food spoilage and pathogenic microorganisms such as *E. coli, L. monocytogenes, Aspergillus sp.* and *Penicillium* spp. (Davidson et al. 2005). *Lactobacillus* strains including *Lactobacillus kefiri*, *L. plantarum* and others (enumerated in table 10) have also been reported to produce benzoic acid in fermented foods such as kefir and sourdough breads (Nambou et al. 2014; Wu et al. 2012). Figure 24 shows a proposed mechanism for benzaldehyde formation from phenylalanine by both enzymatic and chemical steps in *L. plantarum* (Nierop Groot and De Bont 1998). In fact, the metabolite is produced from phenylalanine in cell extracts through the phenylpyruvic acid pathway. Phenylpyruvic acid is initially converted to phenylpyruvic acid by an aminotransferase and the keto acid is further transformed to benzaldehyde and benzoic acid through a chemical oxidation step (Nierop Groot and De Bont 1998).

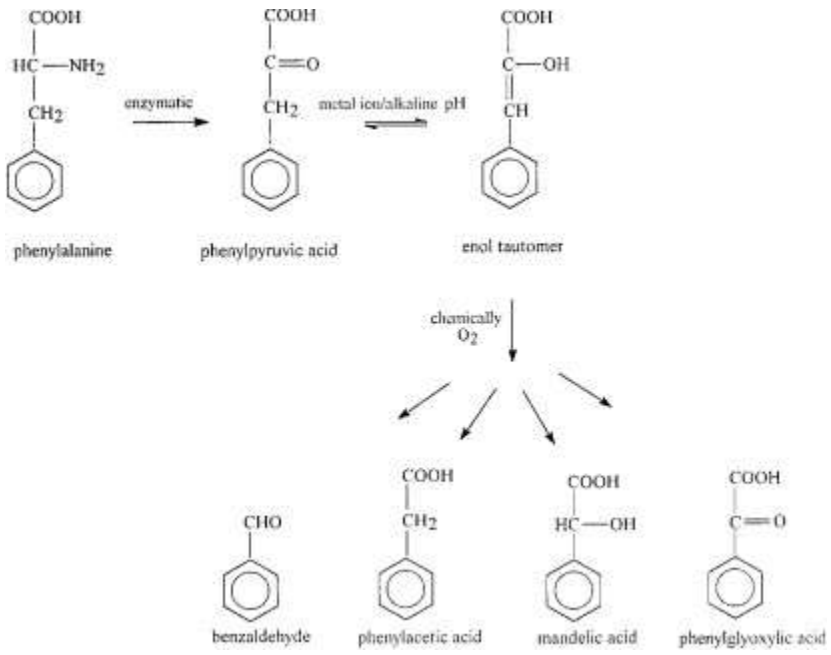

Figure 24 - Proposed mechanism for benzaldehyde formation from phenylalanine by both enzymatic and chemical steps in L. plantarum (Nierop Groot and De Bont 1998).

Amino acid degradation is believed to be important for flavor development in cheese. Straight-out chemical reactions are not believed to play a major role in the production of cheese flavor but rather seem to be enhanced by enzymes (Urbach 1995). In this study, benzaldehyde formation was initiated by an aminotransferase present in the cell extract of *L. plantarum* (Yvon et al. 1997). Aminotransferases from lactic acid bacteria have shown activity under cheese-ripening conditions, but the low oxygen concentration, low ripening temperature, and low pH in cheese do not favor the chemical conversion of phenylpyruvic acid to benzaldehyde (Engels 1997). However, considering the long time involved in cheese ripening, this mechanism may still make a significant contribution.

In conclusion, the production of organic, low molecular weight acidic and alcoholic metabolites as a result of aromatic amino acids in fermented foods can enhance the antimicrobial potential of the microbial flora. In fermented products especially cheese, their applicability can be beneficial as they possess appreciable lipid solubility, which allows them to diffuse more freely through the matrices (Adams and Nicolaides 1997). In other acidic food/feed products as well, the liposolubility of these acids allows them to diffuse across the bacterial plasma membrane into the cytoplasm which makes these metabolites very effective against pathogenic bacteria (Adams and Nicolaides 1997). However, certain food borne pathogens like *L. monocytogenes* have shown enhanced acid adaptation and survival potential in dairy products, including cottage cheese, yogurt, and whole-fat cheddar cheese. Therefore, the use of bioprotective cultures as an adjunct in fermented food products is necessary (Cataldo et al. 2007).

Given all these data and the findings of this work, further studies are required and open up to new perspectives:

- Toxicological studies should be conducted to evaluate the effect of PLA addition on malting barley or even other food matrices.
- Evaluate the ability of *Fusarium* strains to develop resistance against the use of *G. candidum* as a biocontrol.
- Determine whether *G. candidum* produced any of the metabolites sited above (ILA, PEA or benzoic acid).
- Determine whether the administration of phenylalanine in *G. candidum* fermentation media targeted the production of other antimicrobial metabolites.
- In case *G. candidum* produces PEA, purification and administration of this alcohol on barley grains in order to study its effect on *Fusarium langsethiae* 2297 and *F. sporotrichioides* 186 growth and T-2 toxin concentration would be a preventive approach worth being taken into account.
- Determine the effect of *G. candidum* against other fungal species such as *Aspergillus* spp., *Penicillium* spp…
- Conduct same experiment with a *G. candidum* strain that, instead of producing PLA, produces PEA and compare the results in order to optimize the use of the biocontrol and obtain salubrious beer products.

Conclusion

Filamentous fungi have a remarkable ability to grow on varied and relatively simple substrates whose components can be used as nutrients. In France, the establishment of the species *Fusarium* spp., especially *F. langsethiae* and *F. sporotrichioides* on the malting barley has become a major concern. During their development, they also produce secondary metabolites. These molecules are synthesized in response to different types of environmental signals and can be endowed with a toxicogenic potency. In this case, they are called mycotoxins. Known for producing T-2 and HT-2 toxins, *Fusarium* fungi raise the problem of the possible occurence of these toxins in the beer. Findings have shown that the use of the filamentous yeast *G. candidum* as a possible biocontrol agent against several microorganisms, can reduce contamination levels during the malting process, especially *Fusarium* spp. Nevertheless, the mechanism of action was still unknown. This yeast had been found to produced antimicrobial compounds such as Phenyllactic acid (PLA) that has been reported to induce behavioral and structural alterations to bacteria such as *L. monocytogenes*, which completely inhibit their growth.

The primary objective of the work carried out during this thesis was to comprehend the functioning mechanism of a biocontrol, implemented by the French Institute of Brewing and Malting to reduce T-2 toxin produced by *Fusarium* species, a major malting barley contaminant. *G. candidum* was already being used during the malting step as an antifungal agent without understanding the interaction mechanism. Therefore, it was important to clarify this mycotoxin reduction phenomenon, in order to allow the optimization of this biocontrol use on an industrial level.

Three main aspects were treated in this survey:

1) Deciphering the interaction mechanisms between the two microorganisms, *G. candidum* and the two *Fusarium* spp. *in vitro* for starters.
2) Testing findings were then test on an up scaled level, in micro-malting conditions using barley grains; during which *G. candidum* was inoculated in either its activated form or its freeze-dried form at two concentrations.
3) Determining the influence of several culture parameters on *G. candidum* development and PLA production.

On the first hand, the agent produced by *G. candidum,* the PLA, was identified, and its effect on both fungal growth and T-2 toxin concentration was assessed. To begin with, co-culture conditions were monitored to evaluate the direct interaction. Highest T-2 toxin concentration reductions were obtained after 24h, 48h and 72 hours of incubation time at values ranging from 94% to 65% for both *Fusarium* spp. Kinetics of *G. candidum* and its PLA production through fermentation time were essential in order to comprehend its behavior, and determine the best fermentation time and metabolite production ratio. It seemed like PLA was produced during the early yeast growth phase and reached its peak after 24h and 48h of incubation time reaching 0.24g/L and 0.41 g/L respectively. Later, indirect interactions were

assessed through sequential cultures where *G. candidum* was grown at different fermentation time. The pre-fermented media was then filtered and *Fusairum* spp. spores were inoculated. Results showed that the most T-2 toxin concentration reduction were detected when *G. candidum* was fermented during 24h and 48h, where PLA values were at their highest. Toxin concentration reduction was correlated to the observed fungal growth inhibition. Finally, the effect of PLA pure compound on *Fusarium* strains growth T-2 toxin concentration was evaluated. This experiment validated previously found results.

On the second hand, micro-malting experiments were conducted miming actual industrial malting conditions. The purpose of this work section was to determine the best conditions for brewers to limit T-2 toxin presence during the malting process and ensure salubrious beer products. The obtained resultas have demonstrated that activating *G. candidum* before the process allows earlier PLA production and thus T-2 concentration lower by almost 90% after 3 days of malting. However, extending the malting process to a 5-day duration might result in higher T-2 toxin concentartion. A possible solution to that is to double the inoculation concentration of *G. candidum* right from the beginning of the malting step.

Finally, in the perspective of optimizing *G. candidum* use at an industrial level later on, the influence of several culture parameters on yeast growth and PLA production was tested *in vitro*. The composition of culture media played an important role in enhancing both growth and PLA production. Indeed, *G. candidum* grew abundantly when cultivated in Synthetic Medium (SM) that was enriched in yeast extract, phenylalanine and glucose, the best carbon source assimilated by the microorganism. Consequently, PLA values reached its greatest peak value at 2.1 g/L for nearly 15 g/L of biomass. Another explanation is that PLA production is improved by high concentration of phenylalanine in culture. Therefore, activating *G. candidum* in SM medium seemed to boost PLA production rather than its growth. In fact, phenylalanine is an intermediate that contributes to the metabolite production at early yeast growth phase.

Overall, reduction in *Fusarium* spp. growth is correlated to high PLA production by *G. candidum* which consequently reduced T-2 toxin concentration. During the malting step, PLA seemed to reach higher values when the yeast was activated prior to use. This part of the brewing process should ideally be limited to three days in order to insure the safety of the beer product. In addition, T-2 toxin seemed to be significantly reduced when *G. candidum* was previously activated in an enriched medium to allow earlier PLA production.

In summary, the work presented in this thesis opens up a wide field of research in terms of understanding biocontrol use against T-2 toxin production by *Fusarium* spp. in the brewing process in particular and mycotoxins in food matrices in general. The health and economic consequences related to foodborne contamination by these secondary metabolites have motivated numerous studies aimed at finding environmental friendly means to limit their presence and develop relevant control strategies against these contaminants.

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A

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Abstract

The establishment of *Fusarium* species, especially *F. langsethiae* and *F. sporotrichioides* known for producing T-2 toxin on the malting barley has become a major concern during malting process. Studies have shown that the use of the filamentous yeast *Geotrichum candidum* known as biocontrol agent against several microorganisms, can reduce T-2 contamination during the malting process. Nevertheless, the mechanism of action is still unknown. *G. candidum* produces antimicrobial compounds such as Phenyllactic acid (PLA) that have been reported to induce behavioral and structural alterations to bacteria such as *Listeria monocytogenes*, and to completely inhibit their growth.

The first objective was to decipher *in vitro* the interaction mechanisms between *G. candidum* and two *Fusarium species* *F. langsethiae* and *F. sporotrichioides*. For that, the PLA produced by *G. candidum* was identified as responsible for the reduction of fungal growth and T-2 toxin concentration. The implementation of growth kinetics of *G. candidum* is essential to understand its behavior, to determine the optimal fermentation time and the rate of PLA production. It seemed like PLA was produced during the early yeast growth phase, reached its peak after 24h and 48h of incubation time. This was correlated with the highest T-2 toxin concentration reductions when cultivated with *Fusarium* strains. Finally, the effect of pure PLA compound on *Fusarium* growth and their ability to produce T-2 toxin was evaluated. This experiment validated previously found results. The second objective was to reproduce these results on a micro-malting scale by miming industrial conditions and to determine the ideal conditions to limit the presence of the toxin during malting. Results demonstrated that activating *G. candidum* before the process allowed earlier PLA production and thus T-2 concentration lower by almost 90% after 3 days of malting. However, extending the malting process to a 5-day duration might result in higher T-2 toxin concentration. A possible solution to that is to double the inoculation concentration of *G. candidum* right from the beginning of the malting step. Finally, to optimize *G. candidum* use at an industrial level later on, the influence of several parameters on yeast growth and PLA production was tested *in vitro.* The composition of culture media played an important role in enhancing both growth and PLA production. Indeed, *G. candidum* grew abundantly and produced larger amounts of PLA when phenylalanine enriched medium.

Keywords: *Fusarium langsethiae* 2297*, F. sporotrichioides* 186*,* T-2 toxin, *Geotrichum candidum,* biocontrol agent, Phenyllactic acid, micro-malting

Résumé

L'implémentation des espèces *Fusarium* sur l'orge*,* notamment *F. langsethiae et F. sporotrichioides,* producteurs de la toxine T-2, est devenu une préoccupation majeure pendant le maltage. Des études ont montré que l'utilisation de la levure filamenteuse *Geotrichum candidum*, connue comme agent de biocontrôle contre plusieurs micro-organismes, peut réduire la contamination de la toxine T-2 pendant le maltage. Néanmoins, le mécanisme d'action est encore inconnu. *G. candidum* produit des composés antimicrobiens tels que l'acide phényllactique (PLA) qui, selon la littérature, induit des modifications comportementales et structurelles chez des bactéries telles que *L. monocytogenes* et inhibe leur croissance.

Le premier objectif était d'élucider *in vitro* les mécanismes d'interaction entre *G. candidum* et les deux espèces *Fusarium*. Le PLA produit par *G. candidum* a été identifié comme responsable de la réduction de la croissance fongique et de la concentration en toxine T-2. L'étude de la cinétique de croissance de *G. candidum* était essentielle pour comprendre son comportement, déterminer le temps de fermentation optimal et le taux de production de PLA. Ce dernier a été produit au début de la phase de croissance de la levure, et a atteint son pic après 24h et 48h d'incubation. Ceci a été corrélé avec les réductions de concentration de la toxine T- 2 les plus élevées dans les conditions de culture avec les souches fongiques. Enfin, tester l'effet du PLA pur sur la croissance fongique et la concentration de la toxine T-2 a permis de valider les résultats précédents. Le second objectif était de reproduire ces résultats à l'échelle du micro-maltage en mimant les conditions industrielles et de déterminer les conditions idéales pour limiter la présence de la toxine. Les résultats ont démontré que l'activation de *G. candidum* avant le processus permettait une production plus précoce de PLA et donc une réduction de 90% de la concentration de toxine T-2 après 3 jours de maltage. Cependant, l'extension du processus à une durée de 5 jours pourrait entraîner une concentration plus élevée de toxine. Une solution possible à cela est de doubler la concentration d'inoculation de *G. candidum* dès le début de l'étape de maltage. Enfin, pour optimiser ultérieurement l'utilisation de *G. candidum* à un niveau industriel, l'influence de plusieurs paramètres sur la croissance de la levure et la production de PLA a été testée *in vitro*. La composition des milieux de culture a joué un rôle important dans l'amélioration de la croissance et de la production de PLA. En effet, *G. candidum* s'est développée abondamment et a produit de plus grandes quantités de PLA lorsque la phénylalanine était ajoutée au milieu.

Mots clés: *Fusarium langsethiae* 2297*, F. sporotrichioides* 186*,* Toxine T-2, *Geotrichum candidum,* agent de biocontrol, Acide phényllactique, micro-maltage