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BIOCONTROL OF OCHRATOXIGENIC FUNGI BY ENDOGENOUS
LACTIC ACID BACTERIA AND YEASTS FROM IVORIAN
ROBUSTA COFFEE IN THE CONTEXT OF CLIMATE CHANGE

APPLIED MYCOLOGY GROUP
CENTRE FOR SOIL, AGRIFOOD AND BIOSCIENCES
SCHOOL OF WATER, ENERGY AND ENVIRONMENT

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Supervisor: Angel Medina Vaya and Sabine Schorr-Galindo
Associate Supervisor: Carol Verheecke-Vaessen, Angélique
Fontana & Caroline Strub
September 2023



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Bacteria and Yeasts from Ivorian coffee in the context of climate
change

Supervisor: Angel Medina Vaya & Sabine Schorr-Galindo
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Fontana, Caroline Strub
September 2023

This thesis is submitted in partial fulfilment of the requirements for
the degree of Doctor of Philosophy

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Microorganisms will give you anything you want,
if you know how to ask them.

Kin'ichiro Sakaguchi

Abstract

This doctoral research delves into the innovative domain of biocontrol strategies targeting mycotoxigenic fungi in the context of climate change. Focusing on Ivorian coffee, a vital economic and agricultural commodity, the study explores the potential of indigenous lactic acid bacteria (LAB) and yeasts as biocontrol agents. Mycotoxins, toxic secondary metabolites produced by fungi, pose significant health risks and economic losses. As climate change amplifies the proliferation of mycotoxigenic fungi, the demand for sustainable and eco-friendly interventions intensifies. The research encompasses comprehensive isolation, identification, and characterization of LAB and yeasts from Ivorian coffee, evaluating their antagonistic properties against mycotoxigenic fungi. Furthermore, the study elucidates the mechanisms underlying the biocontrol activity, shedding light on how these microorganisms mitigate mycotoxin contamination. This research is pivotal in the pursuit of climate-resilient strategies for mycotoxin management, contributing to both food safety and agricultural sustainability.

Keywords: biocontrol, mycotoxins, lactic acid bacteria, yeasts, detoxification, metabarcoding

Résumé

Cette recherche doctorale explore le domaine novateur des stratégies de biocontrôle ciblant les champignons mycotoxigènes dans le contexte du changement climatique. En se concentrant sur le café ivoirien, une denrée économique et agricole vitale, l'étude explore le potentiel des bactéries lactiques indigènes (LAB) et des levures en tant qu'agents de biocontrôle. Les mycotoxines, des métabolites secondaires toxiques produits par les champignons, présentent des risques importants pour la santé et des pertes économiques. Alors que le changement climatique amplifie la prolifération des champignons mycotoxigènes, la demande en interventions durables et respectueuses de l'environnement s'intensifie. La recherche englobe l'isolement complet, l'identification et la caractérisation des LAB et des levures à partir du café ivoirien, évaluant leurs propriétés antagonistes contre les champignons mycotoxigènes. De plus, l'étude élucide les mécanismes sous-jacents de l'activité de biocontrôle, éclairant comment ces micro-organismes atténuent la contamination par les mycotoxines. Cette recherche est cruciale dans la poursuite de stratégies résilientes au climat pour la gestion des mycotoxines, contribuant à la fois à la sécurité alimentaire et à la durabilité agricole.

Mots-clés : biocontrôle, mycotoxines, bactéries lactiques, levures, détoxification, métabarcoding

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List of Abbreviations

AF	Aflatoxin
AFB₁	Aflatoxin B ₁
ANOVA	Analysis of Variance
a_w	Water activity
BCA	Biological Control Agent
CC	Climate Change
CFEs	Cell Free Supernatants
CFU	Colony Forming Unit
CO₂	Carbon Dioxide
DG18	Dicloran-18-glycerol agar
DNA	Desoxiribonucleic acid
EC	European Comission
EFSA	European Food Safety Authority
et al.	<i>Et alii</i>
EU	European Union
FAO	Food Agriculture Organization
FDA	Food Drug Administration
FLD	Fluorescence Detector
g	Gram
h	Hour
HPLC	High-Pressure Liquid Cromatography
IARC	International Agency for Research on Cancer
IPCC	Intergovernmental Panel on Climate Change
IT	Information Technology
Kg	Kilogram
LCMSMS	Liquid-Cromatography-Mass Spectroscopy
LOD	Limit of Detection
MC	Moisture Content
MEA	Mal Extract Agar
mg	Miligram
min	Minute
mL	Mililitre

mm	Milimetre
ng	Nanogram
OP-OTA	Open ring ochratoxin A
OTA	Ochratoxin A
OTα	Ochratoxin alpha
°C	Degree Celsius
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
Phe	Phenylalanine
ppm	Parts per million
PTFE	Polytetrafluoroethylene
RNA	Ribonucleic Acid
s	Second
SD	Standard deviation
SD-	No surface disinfection
SD+	Surface disinfection
SEM	Scanning Electron Microscopy
USDA	United States Department of Agriculture
UV	Ultraviolet
VOCs	Volatile Organic Compounds
w/v	Weight per volume
x g	Times gravity
μg	Microgram
μL	Microlitre
μm	Micrometer
v/v	Volume per volume

1 Introduction

Coffee worldwide production in 2021 reached 168 million bags, which means around 10.08 million tonnes (60 kg/bag) and the biggest producing countries were Brazil, Vietnam, and Colombia (ICO, 2021b). The two main varieties of coffee are Arabica and Robusta, which provide different organoleptic qualities, being Arabica considered of better quality. Robusta presents a characteristic bitter flavour, lower acidity, and higher caffeine content (Dos Santos et al., 2021). Ivory Coast is one of the biggest Robusta coffee producers in the world, with 66 million kilograms of coffee in 2022 (USDA, 2022).

Ochratoxin A (OTA) is a mycotoxin produced by some species of fungi belonging to the genera *Aspergillus* sp. and *Penicillium* sp.. This mycotoxin affects many foodstuffs such as coffee, cacao, grapes, spices, and cereals. In coffee, OTA is mainly produced by *Aspergillus westerdijkiae* and *Aspergillus carbonarius* the last being the most occurrent in coffee in Ivory Coast. OTA is a nephrotoxic, immunotoxic, teratogenic and cancerogenic compound (IARC (International Agency for Research on Cancer), 1993). Because of its molecular structure and great thermostability, once OTA is produced, practices like roasting can only marginally reduce OTA content (Castellanos-Onorio et al., 2011). Besides, coffee consumption contributes to the 10% of OTA human ingestion in Europe (Miraglia & Brera, 2002). Due to these facts, the European legislation does not recommend a level above 3 µg/kg for roasted coffee and above 5 µg/kg for soluble coffee (regulation 1831/2003 consolidated in 2023 European-Commission, 2023).

Contamination with OTA happens at the steps of drying and storage of coffee cherries. During these two phases, elevated water activity (a_w) and temperature (28-32 °C) set the optimal conditions for growth and OTA production by *A. carbonarius*. Climate change models project an increase in temperature of between 2-4 °C, associated with drought stress episodes. As well, the atmospheric CO₂ concentration, which at present is about 400 ppm, is expected to increase to 1000 ppm in the next 25 years (IPCC, 2023). Consequently, these ochratoxigenic species could be considered as well adapted to future climate change scenarios, which would increase the risk of OTA contamination of coffee

(Gil-Serna et al., 2014). Additionally, climate change might provoke the emergence of contamination with aflatoxins or fumonisins producing fungi in coffee. Thus, there are three-way interacting factors (temperature x elevated CO₂ x drought stress) which will significantly impact crop quality (Medina et al., 2017).

As good agricultural practices are insufficient to mitigate plant diseases, contamination by fungi and mycotoxin risk, the use of chemical fungicides is nowadays the main tool to fight fungal development. However, their negative impacts on environment and human health, as well as the pathogenic resistance they generate, have urged the scientific community to develop new strategies for crop disease management. For instance, biocontrol by beneficial microorganisms represents an environmentally friendly alternative to chemical treatments. Therefore, the selection of microorganisms resilient to future climatic conditions is key for the success of this strategy in the long term.

Lactic Acid Bacteria (LAB) and yeasts hold a great potential as biocontrol agents due to their capacity to produce a wide variety of secondary metabolites, including antimicrobial agents and enzymes. In addition, they apply a series of antagonistic mechanisms, such as competition for substrates, that confer them the ability to combat pathogenic fungi. Furthermore, they are potential detoxifiers of mycotoxins through adsorption or degradation activities especially for OTA by either the production of enzymes or the polarity towards their cell wall. For these main reasons, the use of LAB and yeasts as biocontrol constitute a promising strategy to handle fungal contamination and OTA risk on coffee.

2 Literature review

2.1 Ochratoxin A occurrence in coffee: how to reduce the risk?

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2.1.1 Abstract

Ochratoxin A (OTA) is a mycotoxin mainly produced by *Aspergillus* section *Circumdati* and section *Nigri* across the coffee chain. Ochratoxin A is nephrotoxic and poses a threat to human and animal health. Non-compliance to food and feed regulation is responsible for great economic losses. This review summarises current knowledge on how to reduce OTA concentration in the coffee from farm to cup. After a brief introduction on the OTA occurrence on coffee, current good management practices are introduced. The core of the review focuses on biocontrol and microbial decontamination by Lactic Acid Bacteria, yeasts and fungi and their associated enzymes currently reported in the literature. A special attention is given to publications closest to *in vivo* applications of Biocontrol Agents and microbial OTA-adsorption or degradation agents. Finally, it provides an opinion on which future techniques to minimize OTA are recommended for the coffee supply chain.

Keywords: ochratoxin A, toxigenic fungi, post-harvest management, lactic acid bacteria, yeasts, biocontrol, biodegradation, detoxification

2.1.2 Introduction

Coffee is the main drink consumed worldwide after water, with a worldwide production increased by 60% in the last 20 years (FAO, 2018; ICO, 2019). In 2020, the main producing countries in order of importance were Brazil, Vietnam, Colombia, Indonesia, Ethiopia, Honduras, India and Uganda (ICO, 2021). There are two main varieties of coffee, Arabica and Robusta, accounting for 60% and 40% of the worldwide production, respectively (Leitão, 2019; ICO, 2021). Arabica and Robusta provide different organoleptic qualities, being Arabica considered of better quality. Robusta presents a characteristic flavour, lower acidity and higher caffeine content (Dos Santos et al., 2021).

International coffee trade is subjected to compliance with regulation regarding mycotoxin concentration, especially ochratoxin A (OTA). Ochratoxin A is a heat-stable mycotoxin with a molecular weight of 0.4 kDa considered mutagenic, nephrotoxic and potentially carcinogenic for humans (group 2B by the International Agency for Research on Cancer (IARC (International Agency for Research on Cancer), 1993). The European Commission holds a maximum threshold of 3 µg/kg for roasted coffee beans and 5 µg/kg for soluble coffee (European-Commission, 2023). The OTA found in coffee beans is generally produced by fungi belonging to the genus *Aspergillus*.

Contamination with *Aspergillus* spp. can begin at the pre-harvest step through the flowers, by carriage of spores on coffee berry borers or in case of overripe cherries. However, it is at post-harvest that most of the fungal contamination occurs (Maman et al., 2021). Good Management Practices (GMP) help reduce fungal growth, OTA production and facilitate the development of competitive microorganisms (FAO, 2006). These practices start just after harvest when coffee should immediately be processed to prevent mould formation.

Just after harvest, coffee can go through two main processes: natural (dry method) and washed coffee (wet method) (C. F. Silva et al., 2008). In the dry method, coffee is first sorted and then sun-dried. During the wet method, coffee is fermented in water and then sun-dried, giving as final product parchment coffee (without mucilage)(Hamdouche et al., 2016). The microorganisms attached to the

equipment used for fermentation and drying influence the contamination with mycotoxigenic fungi, so these should be disinfected (de Melo Pereira et al., 2020; Shetty & Jespersen, 2006).

Microbiologically, the drying step consists of three phases. The first phase lasts between six and eight days. It is dominated by harmless hydrophilic microorganisms, mainly bacteria, thanks to the high-water activity (a_w ; 0.95 a_w). The transition phase (0.90 a_w) lasts around 6 days. This phase facilitates the growth of mesophilic and xerophilic microorganisms, such as yeasts and fungi, and it will be during this phase that ochratoxigenic fungi will produce OTA especially in the dry method in which cherries will be dry with pulp. At the last step, when coffee reached a moisture content of 12% and 0.60 a_w the coffee is suitable for long-term storage in a dry environment and rewetting should be avoided (FAO et al., 2006; C. F. Silva et al., 2008). Controlling parameters such as temperature and a_w through the whole process, helps reducing fungal growth and mycotoxin production.

Once OTA is produced it is very hard to remove due to its high stability. Even during roasting, OTA is not totally removed (Castellanos-Onorio et al., 2011). The reduction of OTA after roasting widely varies between 90% and 12%, depending on the roasting temperature and the initial OTA contamination (Sueck et al., 2019). Climate change, fungicide-resistance, and net zero targeted policies are pushing towards resilient and natural methods to reduce OTA in coffee (Adhikari et al., 2020).

There is evidence of biocontrol microorganisms such as bacteria, yeasts, atoxigenic fungi or their metabolites that prevent and reduce OTA concentration in coffee (Medina, Mohale, et al., 2017). Biocontrol Agents (BCA) have different strategies to prevent filamentous fungi from growing and/or producing mycotoxins. Some of the strategies to prevent growth are production of anti-germinative compounds, chitinase activity or production of Volatile Organic Compounds (VOCs), as well as competition for nutrients and space. To prevent the production of the mycotoxin the BCA blocks the biogenesis of the toxin and/or

gene expression of the gene cluster responsible of the biosynthesis of OTA (Pellan L., 2020).

Another approach is the reduction of mycotoxins once they are formed, known as decontamination (Abrunhosa et al., 2010). Detoxification can be biological, (adsorption or degradation of mycotoxins), physical (radiation or ultrasounds) and chemical (ozone or adsorbents) (Abrunhosa et al., 2014). BCA-based prevention and decontamination pose several advantages in front of the physical and chemical methods since they can be very specific, efficient, environmentally friendly as well as preserving or improving the organoleptic and nutritive quality of coffee (de Bellis et al., 2015). However, it has been shown that these microorganisms must be non-pathogenic, as well as not provoking changes to the organoleptic qualities of the final product. Furthermore, the products result of the mycotoxin degradation must be non-toxic (Fapohunda et al., 2017; Nahle et al., 2022). In the case of Lactic Acid Bacteria (LAB) they are Generally Recognized As Safe (GRAS) which makes them one of the preferred biocontrol agents (Muhialdin et al., 2020).

This review aims to present the occurrence of mycotoxins contamination on coffee, and summarise recent efficient strategies to prevent, control and/or remove OTA contamination in the post-harvest phases of the coffee production chain. Moreover, good management practices and the required future research in OTA biocontrol and detoxification technologies on coffee were also highlighted for future application.

2.1.3 Occurrence and contamination of ochratoxin A in coffee

The main OTA producers in coffee belong to *Aspergillus* sections *Circumdati* and *Nigri* (FAO et al., 2006). In section *Circumdati*, *A. ochraceus*, *A. westerdijkiae* and *A. steynii* are producers; with the biggest OTA producers being *A. westerdijkiae* and *A. steynii*. In section *Nigri*, *A. carbonarius* and *A. nigri*, are producers, with *A. carbonarius* being the biggest producer of both (Bucheli & Taniwaki, 2002). The optimal ranges of a_w and T (°C) for mould growth, sporulation and toxin production for these fungi are detailed on Table 2.1.3-1. These ranges should be considered throughout all the coffee chain, since the optimal conditions may

trigger a low presence of potentially ochratoxigenic fungi to grow and produce a high quantity of OTA (Batista et al., 2003).

A detailed summary of OTA occurrence at different stages of the coffee production chain is listed in Table 2.1.3-2. Between the producing continents, Africa have the highest concentration of OTA in coffee followed by Asia and then South and Central America.

Table 2.1.3-1. Optimal conditions of growth, sporulation and OTA production for the most ochratoxigenic fungi on coffee.

Species	Optimal growth		Optimal spores' germination		Optimal OTA production		Reference
	T (°C)	a _w	T (°C)	a _w	T (°C)	a _w	
<i>A. ochraceus</i>	24-31	0.95-0.99	20-30	0.95-0.99	25-30	0.98	Daou et al. (2021), Pardo et al. (2005)
<i>A. westerdijkiae</i>	20-30	0.95-0.99	20-30	0.95-0.99	28	0.99	Akbar et al. (2020)
<i>A. steynii</i>	28-32	0.95-0.99	20-30	0.95-0.99	28	0.99	Gil-Serna et al. (2014)
<i>A. carbonarius</i>	32-37	0.95-0.99	32-35	0.95	15-25	0.96-0.98	Bucheli & Taniwaki, (2002), Camardo Leggieri et al. (2014)

Pardo et al. (2004), analysed 57 green coffee samples (41 of Arabica Coffee and 16 from Robusta) from America, Asia and Africa. The OTA concentration results ranged between 0.1 and 80 µg/kg, and the average was 6.7 µg/kg, two folds above the limit for roasted coffee beans. From the 57 samples, 12 corresponded to African samples, which results ranged between 2.4 and 23.3 µg/kg, and the average was 10.9 µg/kg, more than 3 times above the current OTA limit for roasted coffee which is 3 µg/kg. A meta-analysis from Khaneghah et al. (2019), based on 36 published articles, studied the prevalence and concentration of OTA in different countries. This article found that higher poverty, lower Human

Development Index (HDI) and increasing annual rainfall significantly increase the prevalence and concentration of OTA in coffee.

In the present review, we reported the median, minimum, and maximum level of OTA on coffee from different origins and markets (Table 2.1.3-2). We found that Asia's OTA level has a higher median than other great coffee-producing continents such as Africa or Central and South America. However, the maximum values of OTA were found in African coffees. Central and South America remained as the coffee-producing area with less OTA contamination, although, the three producing areas showed maximum OTA levels above the European regulation. Thanks to the regulation the OTA levels in the European market were never above the limit decreed by the EC. As well, the number of samples analysed was the highest for Europe and the lowest for Africa, which highlights the need for more OTA quantification on coffee in this continent. Specially, taking into account that the number of positive samples is more than half of the total samples for America and Europe, thus with an increasing number of samples, there is also an increased number of positive samples, improving the representativity of the results.

In Table 2.1.3-2, a slightly higher level of OTA on Robusta coffee was found probably because the dry process is more used for Robusta. Regarding the OTA levels for green, roasted and soluble coffee, Table 2 shows that there are similar levels in green and roasted coffee. For soluble coffee, the concentration of OTA was very low.

Table 2.1.3-2. OTA levels found in different coffees from 1997 to July 2022

Country	Coffee stage	Coffee Species	No Positive/Total	Average (µg/kg)	Range (µg/kg)	Fungi	Year	Reference
Asia's coffee producing countries								
NS ¹ Asiatic countries	Green	NS	NS/14	6	1.6-31.5	NS	NS	Pardo et al. (2004)
India	Green	Robusta dry	2/2	0.12	0.1-0.14	NS	NS	Pérez de Obanos et al. (2005)
India	Roasted	Arabica	1/1	2.3	ND ²	NS	NS	Napolitano et al. (2007)
India	Roasted ground	Robusta	1/1	0.79 ± 0.11	ND	NS	NS	Benites et al. (2017)
Thailand	Roasted	Arabica	1/1	0.39	ND	<i>A. westerdijkiae</i>	February 2018	Maman et al. (2021)
Vietnam	NS	Arabica	1/3	2.3	ND	<i>A. carbonarius</i> , <i>A. westerdijkiae</i> , <i>A. niger</i>	NS	Hashimoto et al. (2015)
Vietnam	NS	Robusta	1/13	6.3	ND	<i>A. carbonarius</i> , <i>A. niger</i>	NS	Hashimoto et al., (2015)

Vietnam	Roasted	Robusta	1/1	1.5	ND	NS	NS	Napolitano et al. (2007)
Vietnam	Green	Robusta dry	9/9	2.54	0.64-8.05	NS	NS	Pérez de Obanos et al. (2005)
Vietnam	Roasted ground	Robusta	1/1	1.56 ± 0.33	ND	NS	NS	Benites et al. (2017)
No positive/Total samples in Asia: 18/46 Median: 2.3 µg/kg Max: 6 µg/kg; Min: 0.12 µg/kg								
Africa's coffee producing countries								
NS African countries ¹	Green	NS	NS/12	10.9	2.4-23.3	NS	NS	Pardo et al. (2004)
Cameroon	Green	Arabica wet	0/2	ND	ND	NS	2017	Nadege & Francky (2021)
Cameroon	Green	Robusta dry	4/4	0.52	0.1-0.7	NS	2017	Nadege & Francky (2021)

Cameroon	Green	Arabica dry	1/2	0.1	ND-0.1	NS	2017	Nadege & Francky (2021)
Cameroon	Roasted	Robusta	1/1	2.3	ND	NS	NS	Napolitano et al. (2007)
Ethiopia	Roasted	Arabica	1/1	2	ND	NS	NS	Napolitano et al. (2007)
Ivory Coast	NS	Robusta	5/5	7.03	+/- 0.726	<i>Aspergillus</i> section <i>Nigri</i>	2008 & 2009	Olga et al. (2015)
Ivory Coast	Roasted	Robusta	1/1	2.3	ND	NS	NS	Napolitano et al. (2007)
Uganda	Roasted ground	Robusta	1/1	1.51 ± 0.10	ND	NS	NS	Benites et al. (2017)
Uganda	Green	Robusta dry	2/2	0.295	0.28-0.31	NS	NS	Pérez de Obanos et al. (2005)
No positive/Total samples in Africa: 16/33				Median: 2 µg/kg;	Max: 10.9 µg/kg; Min: 0.1 µg/kg			
America's producing countries and markets								

NS American countries	Green	NS	NS/31	5.4	1.3-27.7	NS	NS	Pardo et al. (2004)
Argentine market	Green	NS	5/5	5.77	0.20-20.3	NS	NS	Vanessa & Ana, (2013)
Argentine market	Roasted ground	NS	13/24	1	0.11-5.78	NS	NS	Vanessa & Ana, (2013)
Argentine market	Soluble	NS	17/22	1.99	0.22-13.66	NS	NS	Vanessa & Ana, (2013)
Brazil	Roasted	NS	5/16	ND	0.9-9	NS	NS	Bandeira et al. (2012)
Brazil	Roasted ground	Arabica	1/1	ND	ND	NS	NS	Benites et al. (2017)
Brazil	Green	Arabica	9/40	2.45	0.47-4.82	NS	NS	Batista et al. (2003)
Brazil	Roasted	Arabica	1/1	1.4	ND	NS	NS	Napolitano et al. (2007)
Brazil	Roasted ground	NS	23/34	0.9	0.3-6.5	NS	NS	Leoni et al. (2000)
Brazil	Soluble	NS	8/16	2.2	0.5-5.1	NS	NS	Leoni et al. (2000)

Brazil	Green	Arabica dry	5/11	0.2	0.01-1.63	NS	NS	Pérez de Obanos et al. (2005)
Canada market	Roasted ground	NS	38/59	0.4	0.1-2.3	NS	NS	Lombaert et al. (2002)
Canada market	Soluble	NS	15/21	0.8	0.1-3.1	NS	NS	Lombaert et al. (2002)
Chile market	Roasted	NS	24/24	0.47 ± 0.20	0.30-0.84	NS	NS	Galarce-Bustos et al. (2014)
Chile market	Soluble	NS	39/39	1.8 ± 1.81	0.28-5.58	NS	NS	Galarce-Bustos et al. (2014)
Colombia	Roasted ground	Arabica	1/1	ND	ND	NS	NS	Benites et al. (2017)
Colombia	Green	Arabica wet	3/3	0.57	0.08-0.12	NS	NS	Pérez de Obanos et al. (2005)
Costa Rica	Green	Arabica wet	4/9	0.042	0.02-0.12	NS	NS	Pérez de Obanos et al. (2005)
Costa Rica	Roasted	Arabica	1/1	1.5	ND	NS	NS	Napolitano et al. (2007)

Honduras	Roasted ground	Arabica	1/1	0.5 ± 0.04	ND	NS	NS	Benites et al. (2017)
Peru	Roasted ground	Arabica	1/1	0.51 ± 0.07	ND	NS	NS	Benites et al. (2017)
Puerto Rico	Roasted ground	Arabica	1/1	0.86 ± 0.50	ND	NS	NS	Benites et al. (2017)
Sao Tome and Principe	Roasted ground	Arabica	1/1	0.6 ± 0.19	ND	NS	NS	Benites et al. (2017)
Santos	Roasted	Arabica	1/1	1.4	ND	NS	NS	Napolitano et al. (2007)
No positive/Total samples in America: 217/363				Median: 1 µg/kg;	Max: 5.4 µg/kg; Min: 0.042 µg/kg			
European markets								
France	Roasted ground	NS	30/30	1.1	0.025-11.9	NS	NS	Tozlovanu & Pfohl-Leszkowicz (2010)
Germany	Green	NS	22/82	1.29	0.23-24.5	NS	NS	Otteneder & Majerus (2001)

Germany	Roasted	NS	191/419	0.99	12.1-0.21	NS	NS	Otteneder & Majerus (2001)
Germany	Soluble	NS	29/41	1	4.8-0.28	NS	NS	Otteneder & Majerus (2001)
Hungary	NS	NS	33/50	0.57	0.17-1.3	NS	NS	Fazekas et al. (2002)
Italy	Roasted	NS	8/30	1.03 ± 0.17	ND	NS	NS	Prelle et al. (2013)
Portugal	Roasted	NS	2/6	1.84 ± 0.03	10.31-0.71	NS	NS	Benites et al. (2017)
Portugal	Roasted ground	NS	1/5	1.45 ± 0.02	ND	NS	NS	Benites et al. (2017)
Spain	Roasted	NS	43/45	2.07 ± 0.61	1.30 – 5.24	NS	NS	Coronel et al. (2011)
UK	Roasted	NS	17/20	0.6	0.2-2.1	NS	NS	Patel et al. (1997)
UK	Soluble	NS	64/80	1	0.1-8	NS	NS	Patel et al. (1997)

No Positive/Total samples in Europe: 440/808	Median: 1.03 µg/kg;	Max: 2.07 µg/kg; Min: 0.57 µg/kg
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¹NS = Non-Specified; ²ND = Non-Detected

2.1.4 Good practices to reduce mycotoxin occurrence in coffee during post-harvest

Good management practices for OTA prevention at post-harvest

Figure 2.1.4-1 represents the time course of Ochratoxin A formation from pre- to post-harvest stages. Fermentation, drying, or storage are the critical control steps to focus on prevention of mycotoxigenic fungi development. Fermentation is used to limit oxygen availability and encourage harmless competitive microorganisms while degrading the mucilage. It is recommended to limit the fermentation time by immediately drying after fermentation without mucilage removal (FAO et al., 2006). The drying should be done evenly to avoid moisture content (m.c.) or water activity (a_w) above 12% or 0.4 or 0.6, respectively (Anokye-Bempah et al., 2023). As well, techniques such as the Collapsible Dryer Case™ will help to avoid direct contact with the soil, source of contaminants (Velooso et al., 2020). Other scenarios could be concrete ground or bamboo table, which have been proven to prevent fungal contamination (Maman et al., 2021).

During drying and storage moisture content should be monitored according to ISO 6673 method (Codex Alimentarius, 2009). Cervini et al. (2022) suggested the use of DryCard™ developed by the University of California Davis (UC Davis). This tool would help monitoring moisture content (m.c.) during the drying and storing. Before storage, coffee is manually sorted and dehusked in order to remove the most contaminated fractions, reducing OTA (FAO et al., 2006).

After drying, coffee is normally stored in jute sacks, which tend to be reused, representing a critical factor for post-harvest contamination with spoilage and mycotoxigenic fungi (Anokye-Bempah et al., 2023). The Purdue Improved Crop Storage (PICS, 2015) bags are a hermetic storage technology consisting of two inner layers of polyethylene protected by an outer layer of woven polypropylene bag. PICS bags have been previously successfully used in peanuts in order to reduce weight loss and pest contamination (PICS, 2015). As well, other bags like GrainPro SuperGrain bag or GrainPro Safeliner, evaluated for their efficacy

preserving m. c. on coffee (Anokye-Bempah et al., 2023), significantly maintain the m. c. and a_w , preventing OTA contamination during storage.

According to the Codex Alimentarius (2009) the best temperature and m. c. to prevent OTA during transportation and storage are low environmental temperature (3-7°C) and $a_w \leq 0.7$ and an environmental relative humidity (ERH) $\leq 60\%$ to avoid rewetting of the beans, which should be kept at m.c. $<12.5\%$ (Codex Alimentarius, 2009).

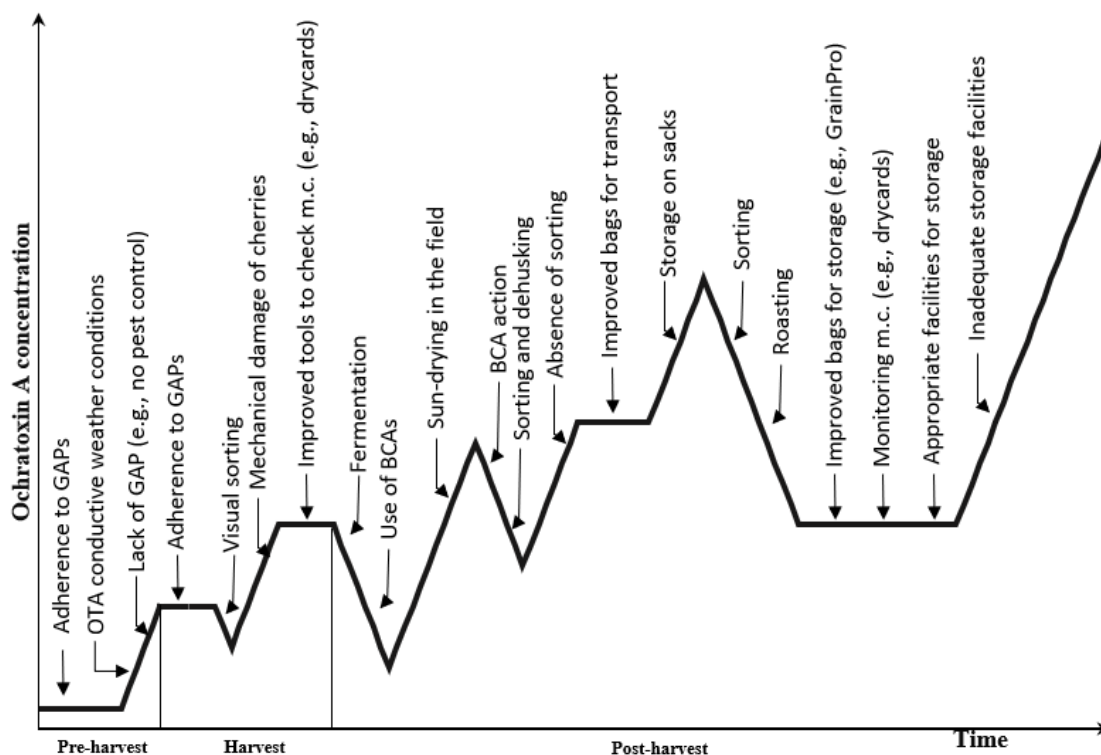


Figure 2.1.4-1. Time course of ochratoxin A formation in relation to proposed mitigation strategies in coffee during pre-harvest, harvest, drying, and post-harvest (Pitt et al., 2013). M.c. means moisture content, GAP means Good Agricultural Practices and BCA means Biocontrol Agent.

Moreover, complementary methods to efficiently reduce contamination in coffee are ongoing. Hereafter, microbiological strategies will be presented, with a special focus on LAB, yeasts and fungi isolated from coffee.

2.1.5 Biological strategies for ochratoxin A prevention in coffee

Biocontrol of ochratoxigenic fungi on coffee

One of the strategies of reduction of mycotoxins in coffee is linked to the capacity of the BCA to reduce the fungal growth, sporulation and germination of spores. One of the side results of these inhibition, is the reduction of OTA occurrence. The different BCA and the conditions of study are summarized in Table 2.1.5-1.

2.1.5.1 By LAB

LAB have been largely used as antifungals for food. The most prevalent group of LAB are *Lactiplantibacillus* spp. From LAB, *L. plantarum* is the most widely studied against different strains of fungi, some of which are potentially mycotoxigenic in coffee (*A. niger*, *A. flavus*)(Sadiq et al., 2019) (Bangar et al., 2021). For instance, Djossou et al. (2011) isolated from coffee two strains of *L. plantarum* with the ability to reduce on dual-culture agar plate *A. carbonarius*'s diameter in between 20- and 30-mm. Some examples of bacteria with activity against the growth, sporulation and spore germination of ochratoxigenic fungi are exposed on Table 2.1.5-1.

The reduction of fungal growth and mycotoxin production can be linked to the production of metabolites such as organic acids, carbon dioxide, hydrogen peroxide, phenyl lactic acid (PLA) and bioactive low molecular weight peptides (Sadiq et al., 2019). The main group of metabolites produced by LAB that reduce fungal growth are organic acids. Thanks to their lipophilic nature organic acids diffuse through the cell wall and are accumulated in the cytoplasm. The organic acid produced by LAB in highest quantity is lactic acid. This acid though has the lowest inhibitory activity. Other acids such as acetic acid or propionic acid with a higher pKa present a significantly lower minimum inhibitory concentration (MIC) than lactic acid (Fidan et al., 2022).

PLA is produced as a by-product of the metabolism of phenylalanine. This acid has been proved to retard the growth of *Aspergillus* and *Penicillium*. However, high concentrations of PLA are needed to inhibit fungal growth, while LAB naturally produce much less quantity. A solution is the addition of phenyl pyruvic

acid (PPA) during LAB growth on MRS in order to enhance the PLA production. The use of PPA remains unclear due to potential safety issues. The replacement of propionate and sorbate by PLA and acetic acid is interesting to produce food products with “clean label” (without chemical preservatives)(Sadiq et al., 2019).

As well, other orders of bacteria have been studied. Shang et al. (2019) isolated from soil the strain *Bacillus megaterium* with the capacity to reduce *A. ochraceus* mycelial growth in a 41.9% on solid media. Also, Campos-Avelar et al. (2020) studied ability of actinobacteria and their metabolites to degrade OTA and/or decrease its production by *Penicillium verrucosum*. In this study, two actinobacteria's cell free supernatant reduced the OTA specific production and fungal growth to 62% and 72% of the control, respectively.

2.1.5.2 By Yeasts

Between yeasts, the most widely studied is *Saccharomyces cerevisiae*. The strain *S. cerevisiae* CCMA 0159 has been notified for its biocontrol properties against *A. carbonarius*, *A. ochraceus* and *A. westerdijkiae* (Neves et al., 2021). During this study confrontations at temperatures between 18 and 38 °C and water activities between 0.91 and 0.99 a_w on Coffee-Based medium were analysed. The results showed that *S. cerevisiae* significantly reduced the risk of contamination with OTA and the growth of the species *A. carbonarius* and *A. westerdijkiae* at 28 °C and 0.99 a_w conditions when the growth of the yeasts was optimal. The possible mechanism used by the yeast to reduce growth and OTA was substrate competition.

According to Lino de Souza et al. (2020) this same strain of *S. cerevisiae* can reduce OTA production through VOCs. However, in the in vivo experiments at a pilot scale (using 10kg of coffee cherries as substrate), inoculation of the *S. cerevisiae* CCMA 0159 and *A. carbonarius* before drying and until coffee reached 11-12% of the m.c., did not reduce the OTA concentration on the final dried coffee. This remarks the importance of testing all these possible BCAs in field conditions. For coffee culture it could be difficult to do but this is used in good condition in vine treatments.

Besides *S. cerevisiae*, there are many other yeasts species with potential BCA properties. For example, Masoud & Kaltoft, (2006) isolated three strains of yeasts, *Pichia anomala*, *P. kluyveri* and *Hanseniaspora uvarum* which had good effect reducing growth and OTA biosynthesis of *A. ochraceus* on MEA. However, on coffee media only *P. anomala* and *P. kluyveri* were able to reduce growth and OTA production in a 50% and 95%, respectively. Also, they run tests on yeast glucose peptone broth and found out that the yeast-free supernatant reduced the germination of the spores by depletion of the amounts of glucose, or production of volatile compounds (VOCs) such as ethyl acetate. This observation underscores the profound influence of incubation media on experimental outcomes, thereby emphasizing the importance of exhaustive parameter assessment. Systematically scrutinizing an array of parameters holds promise in elucidating the mechanisms behind biocontrol properties.

De Melo Pereira et al. (2016) isolated from coffee a yeasts strain identified as *Pichia fermentans* LPBYB13, that reduces considerably growth and OTA production of *A. westerdijkiae* on synthetic media in vitro. Besides, this yeast strain has proved to reduce OTA production by the same fungi on coffee beans in vivo. The suspected mechanisms used by this yeast might be competition for nutrients and space or production of extra-cellular toxic metabolites.

Farbo et al. (2018) isolated from grapes four strains of yeasts, identified as *Cyberlindnera jadinii* 273, *Candida friedrichii* 778, *Candida intermedia* 235 and *Lachancea thermotolerans* 751 with the ability to reduce OTA production and growth of *A. carbonarius* and *A. ochraceus*. The VOCs produced by these yeasts reduced growth of both fungi up to a 74% and OTA production by at least a 95%.

As described in this section, and accordingly, to Choudhary (1992), reduction of toxins levels can be attributed to different strategies. These strategies encompass a gamut of mechanisms ranging from physical competition for space and nutrition, to adsorption or degradation of OTA, strategies that are also considered in this review.

Table 2.1.51. Cultures of yeasts and LAB tested in various conditions for their efficacy to reduce growth, spore germination or OTA production against different OTA producing fungi isolated from coffee. CFS = Cell Free Supernatant, TLC=thin layer chromatography; GCM=green coffee media.

Name	Media used	Inoculum (CFU/mL)	[Fungal] (sp/mL)	OTA Reduction efficacy (%)	Incubation period	Environmental conditions	Incubation condition	References
<i>C. friedrichii</i> 778	Sealed plates YPD /PDA	10 ⁸	10 ⁷	99.9%	7 days	25°C	Against <i>A. carbonarius</i>	Farbo et al., 2018
<i>C. friedrichii</i> 778	Sealed plates YPD /PDA	10 ⁸	10 ⁷	99.9%	7 days	25°C	Against <i>A. ochraceus</i>	Farbo et al., 2018
<i>Candida intermedia</i> 235	Sealed plates YPD /PDA	10 ⁸	10 ⁷	99.9%	7 days	25°C	Against <i>A. carbonarius</i>	Farbo et al., 2018
<i>C. intermedia</i> 235	Sealed plates YPD /PDA	10 ⁸	10 ⁷	99.9%	7 days	25°C	Against <i>A. ochraceus</i>	Farbo et al., 2018
<i>Cyberlindnera jadinii</i> 273	Sealed plates YPD /PDA	10 ⁸	10 ⁷	99.9%	7 days	25°C	Against <i>A. carbonarius</i>	Farbo et al., 2018
<i>C. jadinii</i> 273	Sealed plates YPD /PDA	10 ⁸	10 ⁷	99.9%	7 days	25°C	Against <i>A. ochraceus</i>	Farbo et al., 2018
<i>Hanseniaspora uvarum</i>	MEA/CA	10 ⁶	10 ⁶	OTA not found by TLC	7 days	30°C	Against <i>A. ochraceus</i>	Masoud & Kaltoft, 2006
<i>Lachancea thermotolerans</i> 751	Sealed plates YPD/PDA	10 ⁸	10 ⁷	99.9%	7 days	25°C	Against <i>A. carbonarius</i>	Farbo et al., 2018
<i>L. thermotolerans</i> 751	Sealed plates YPD /PDA	10 ⁸	10 ⁷	99.9%	7 days	25°C	Against <i>A. ochraceus</i>	Farbo et al., 2018

<i>Pichia anomala</i> S12	MYGP broth	10 ⁶	10 ⁶	Spore germination inhibition	72 h	30°C	Against <i>A. ochraceus</i>	Masoud & Kaltoft, 2006
<i>P. anomala</i> S12	MYGP broth	CFS 10 ⁶ cells/mL	10 ⁶	Spore germination inhibition	72 h	30°C	Against <i>A. ochraceus</i>	Masoud & Kaltoft, 2006
<i>P. anomala</i> S12	MEA/CA	10 ⁶	10 ⁶	50% growth inhibition	7 days	30°C	Against <i>A. ochraceus</i>	Masoud & Kaltoft, 2006
<i>P. anomala</i> S12	MEA/CA	10 ⁶	10 ⁶	TLC, OTA not found	7 days	30°C	Against <i>A. ochraceus</i>	Masoud & Kaltoft, 2006
<i>Pichia kluyveri</i>	MEA/CA	10 ⁶	10 ⁶	TLC, OTA not found	7 days	30°C	Against <i>A. ochraceus</i>	Masoud & Kaltoft, 2006
<i>Pichia fermentans</i> LPBYB13	YM	10 ⁶	10 ⁶	50% growth inhibition	5 days	28°C	Against <i>A. westerdijkiae</i>	De Melo Pereira et al. 2016
<i>P. fermentans</i> LPBYB13	Coffee beans	10 ⁶ CFU/g	10 ⁶ sp/g	88%	10 days	28°C	Against <i>A. westerdijkiae</i>	De Melo Pereira et al. 2016
<i>Saccharomyces cerevisiae</i> CCMA 0159	GCM	NS ¹	10 ⁵	97%	10 days	0.99 a _w , 28°C	Against <i>A. carbonarius</i>	Neves et al., 2021
<i>S. cerevisiae</i> CCMA 0159	GCM	NS	10 ⁵	76%	10 days	0.99 a _w 28°C	Against <i>A. westerdijkiae</i>	Neves et al., 2021
<i>S. cerevisiae</i> CCMA 0159	GCM	NS	10 ⁵	73%	10 days	0.99 a _w , 28°C	Against <i>A. ochraceus</i>	Neves et al., 2021
<i>S. cerevisiae</i>	Arabica wet-processed coffee	10 ⁸	NS	100%	10 mins	NS	NS	Velmourougane et al., 2011

<i>S. cerevisiae</i>	Arabica wet-processed coffee	10 ⁸	10 ⁷	96%	10 mins	NS	Against <i>A. ochraceus</i>	Velmourougane et al., 2011
<i>S. cerevisiae</i>	Arabica dried-processed coffee	10 ⁸	NS	78.2%	10 mins	NS	NS	Velmourougane et al., 2011
<i>S. cerevisiae</i>	Arabica dried-processed coffee	10 ⁸	10 ⁷	92.8%	10 mins	NS	Against <i>A. ochraceus</i>	Velmourougane et al., 2011
<i>S. cerevisiae</i>	Robusta wet-processed coffee	10 ⁸	NS	100%	10 mins	NS	NS	Velmourougane et al., 2011
<i>S. cerevisiae</i>	Robusta wet-processed coffee	10 ⁸	10 ⁷	66.7%	10 mins	NS	Against <i>A. ochraceus</i>	Velmourougane et al., 2011
<i>Saccharomyces cerevisiae</i>	Robusta dried-processed coffee	10 ⁸	NS	75%	10 mins	NS	NS	Velmourougane et al., 2011
<i>Saccharomyces cerevisiae</i>	Robusta dried-processed coffee	10 ⁸	10 ⁷	71.4%	10 mins	NS	Against <i>A. ochraceus</i>	Velmourougane et al., 2011
<i>Bacillus amyloliquefaciens</i> RP103	GCM	10 ⁹	5mm agar plug	96.1%	10 days	25°C	Against <i>A. westerdijkiae</i>	Einloft et al., 2017
<i>Bacillus subtilis</i> RP242	GCM	10 ⁹	5mm agar plug	94.9%	10 days	25°C	Against <i>A. westerdijkiae</i>	Einloft et al., 2017

<i>Bacillus safensis</i> RF69	GCM	10 ⁹	5mm agar plug	62.1%	10 days	25°C	Against <i>A. westerdijkiae</i>	Einloft et al., 2017
<i>Lactiplantibacillus plantarum</i>	MRS/CYA double layer	NS	10 ⁴	2cm inhibition fungal growth	72 h	25°C	Against <i>A. carbonarius</i>	Djossou et al., 2011
<i>Lactiplantibacillus brevis</i> 8-2B isolated from grapes	PDA	10 ⁷	10 ⁷	91%	11 days	28°C	Against <i>A. carbonarius</i> from grapes	Li et al., 2021
<i>L. brevis</i> 8-2B	PDB/PDA	CFS of 10 ⁵	10 ⁵	Inhibition of spore germination	24 h	28°C	Against <i>A. carbonarius</i>	Li et al., 2021
<i>L. brevis</i> 8-2B	PDB	10 ⁷	10 ⁷	Inhibition of spore germination	5 h	180 rpm, 28°C	Against <i>A. carbonarius</i>	Li et al., 2021
<i>L. brevis</i> LPBB03	MRS	10 ⁶	10 ⁶	50% growth inhibition	5 days	28°C	Against <i>A. westerdijkiae</i>	De Melo Pereira et al. (2016)

¹NS: Not specified

2.1.5.3 By Non-mycotoxigenic fungi

De Almeida et al., 2019 isolated from coffee 8 non-mycotoxigenic strains: *Rhizopus* spp. (2 isolates), *Lichtheimia* sp. (1 isolated), and *Aspergillus* spp. (5 isolates). All of them have the in vitro capacity to reduce the growth and OTA production of the ochratoxigenic strains *A. ochraceus*, *A. westerdijkiae*, *A. carbonarius*, and *A. niger* on Sabouraud medium. Other studies have isolated non-mycotoxigenic fungi from coffee too (Chaves et al., 2012), but there is a lack of research in the use of these as biocontrol agents.

2.1.5.4 Combination of various biocontrol agents, synergism, or antagonism?

Until now, there are only a few examples of combination of different BCAs for the reduction of fungal growth and mycotoxin production. Da Silva et al. (2015) showed a reduction of 66% of AF on peanuts through the application of *Saccharomyces boulardii*. They also tested the combination of this agent and *Lactiplantibacillus delbrueckii*, a bacterium without significant reduction capacity alone. The synergism of these two biocontrol agents gave place to a reduction of 95% of AF.

De Melo Pereira et al. (2020) in their review also pointed out that the combined use of LAB and yeasts in the coffee chain holds a promising research line since both microorganisms are part of the innate coffee microbiota and their role in coffee fermentation is known to improve organoleptic quality and control mycotoxigenic fungi. However, up until now there is no published data about combination of various biocontrol agents isolated from coffee.

2.1.5.5 Commercialization of BCAs

In the coffee sector, biocontrol is yet a concept not very well known. Instead, they are more familiarised with the concept of starter culture. A starter culture is a set of microorganisms added before fermentation (for wet-processed coffee) or before drying (dry-processed coffee). For coffee, the only commercially available starter culture with antifungal properties is provided by Lallemand Inc. in the shape of a dried yeast powder (LALCafe Oro™, Intenso™, Briosca™, Cima™,

BSC™). It must also be noted that Velmourougane et al. (2011) used for the 1st time a commercial baker's yeast procured from a local bakery (*Saccharomyces cerevisiae*) during the fermentation of parchment and cherries of Arabica and Robusta coffee. They confirmed in vivo the capacity of the yeast to reduce the incidence of ochratoxigenic fungi and the OTA contamination in both types of coffee and processing. The option of a starter culture made of LAB for coffee has not yet been commercialised. Thus, there is plenty of room for development of this solution in the coffee industry.

However, it is also important to check the capacity of the BCAs to detoxify the mycotoxins once these are produced. Thus, we will summarize and develop the OTA adsorption and degradation mechanisms of different microorganisms on synthetic or coffee-based matrices.

2.1.6 Biological strategies for ochratoxin A detoxification in coffee

2.1.6.1 Biological adsorption of OTA

2.1.6.1.1 By LAB

Mycotoxin adsorption by the cell wall of LAB is the most common mechanism of mycotoxin removal in media or food. It is a direct and quick pathway compared to biodegradation (Nahle et al., 2022). The cell wall of LAB is composed of a cytoplasmic membrane with polysaccharides, proteins, lipoteichoic acids and teichoic acids are attached. The adsorption of mycotoxins by the cell wall of LAB is mainly linked to the presence of polysaccharides and peptidoglycans. Differences in the mycotoxin binding capacity are specially influenced by the structure of the peptidoglycans and the number of available binding sites. Peptidoglycans are composed of linear glycan strands of N-acetyl glucosamine (GlcNAc) and N-acetyl muramic acid (MurNAc) which are interconnected by short peptides. The variable length of these peptides modulates the structure of peptidoglycans among different LAB strains (Sadiq et al., 2019). As well, some mycotoxins bind to bacteria by non-covalent interactions such as hydrophobic or ionic interactions and hydrogen bonds (Nahle et al., 2022; Chiocchetti et al., 2019). Mycotoxin binding capacity is a phenomenon not yet well understood, which depends on different factors such as initial concentration of mycotoxins,

bacterial state, LAB cell number, LAB strain, growth media, the complexity and pH of the food, the incubation temperature and the hydrophobicity of the bacterial cell wall (Nahle et al., 2022; Muhialdin et al., 2020; Sadiq et al., 2019).

Acid and heat treatment of the LAB cell walls are strategies to increase their binding capacity which could be linked to an extension of the peptidoglycan net. A proof of this was recently reported by Zhang et al. (2022). This study analysed the ability to remove OTA on PDB by *Bacillus velezensis* E2 cell pellets. Their results suggested that the mechanisms responsible for 68.4% of the OTA might be adsorption. This adsorption mechanism was boosted in the heat-inactivating treated cell pellets, which show a higher OTA reduction capacity, while heat-inactivated cell-free supernatant did not show any difference.

Another example was reported by Shang et al. (2019), where heat inactivation increased the binding of OTA to the wall of *Bacillus megaterium*. The pH has an important role modifying hydrophobic and electrostatic interactions between mycotoxins and the LAB cell wall. It has been reported a pH value of 5 for optimal removal of OTA from MRS. However, other studies have shown that a lower pH between 3.5 and 4.1 increases OTA binding to the LAB cell wall. The medium of growth also plays an important role. It has been seen that OTA binding capacity of *L. plantarum*, *L. sanfranciscensis* and *Pediococcus pentosaceus* is higher in MRS than in phosphate buffer (Taroub et al., 2019). As well, Ben Taheur et al. (2021) reported the culture medium dependant ability of two bacterial strains (*Lactiplantibacillus kefir* KFLM3 and *Acetobacter syzygii* KFGM1) to bind AFB1. These two strains reduced 80% of AFB1 in milk and 0% in MRS medium. This might be explained by a faster growth or biosynthesis of specific cell wall components in milk.

There are other factors such as binding time which is inversely correlated to the mycotoxin binding capacity. Or number of cells, for which there is correlation of initial bacterial count and mycotoxin binding. The stability of the mycotoxin-LAB complex is reversible and depends on the strain, pH, environmental conditions, and type of treatment to which it is subjected (Sadiq et al., 2019). It is important to mention the role of the hydrophobicity of the bacterial cell wall, since *E. coli*, a

Gram-negative bacterium is not capable of removing mycotoxins due to its moderately hydrophilic nature (Nahle et al., 2022).

Not only the cell itself, but also the metabolites produced by LAB can bind to the mycotoxins reducing their toxicity. These metabolites can be acids, phenolic compounds, fatty acids, reuterin and low molecular weight bioactive (Muhialdin et al., 2020).

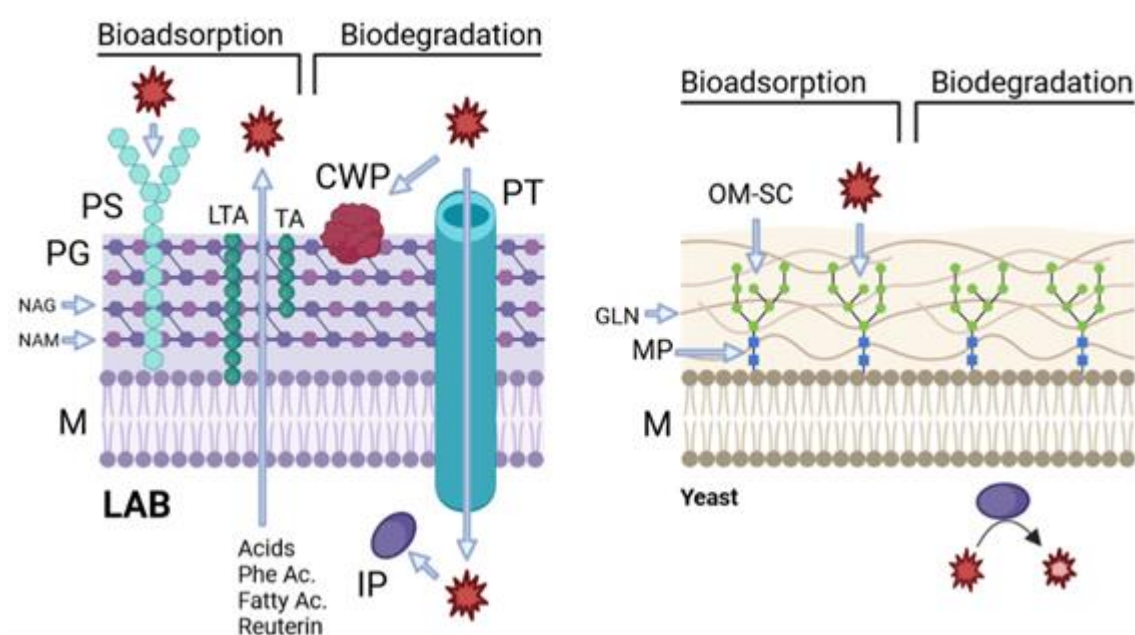


Figure 2.1.6-1. OTA adsorption and degradation mechanisms of LAB and yeasts found in coffee. Created in Biorender.com.

2.1.6.1.2 By Yeasts

Part of the mycotoxins adsorption abilities of the yeasts relies on the structure of their wall. The cell wall represents about 30% of the total weight of the cell and is a bi-layered structure, mainly made up of two polysaccharides, names β - (1, 3)-D-glucan and β - (1, 6)-D -glucan. The distribution between these two glucans plays an important role in the efficacy of the adsorption capacity (Chiocchetti et al., 2019). Linked to these glucans are the cell wall proteins, called mannoproteins. Mannoproteins are composed mainly of carbohydrates, from which mannan oligosaccharides play an important role in mycotoxin adsorption to the cell surface (Shetty & Jespersen, 2006). Both β -glucans and mannan

oligosaccharides provide the cell wall with weak hydrogen and van der Waals non-covalent bonds to a wide range of mycotoxins (OTA, T-2 and HT-2 toxins, fumonisin, aflatoxin B1, deoxynivalenol, zearalenone and Alternaria toxins) (Piotrowska, 2021; Vila-Donat et al., 2018).

Ul Hassan et al. (2021) analysed the ability to bind OTA of the cell walls of 4 yeasts *Cyberlindnera jadinii* 273, *Candida friedrichii* 778, *Candida intermedia* 235 and *Lachancea thermotolerans* 751, previously mentioned for their biocontrol properties, and *S. cerevisiae*. The strains presented a reduction efficacy of OTA of between a 59 and a 70% after incubation at 37 °C for 60 min in a buffer solution of pH 5. This mechanism is known to be fast and reversible.

2.1.6.2 Biodegradation of OTA

2.1.6.2.1 By LAB

Biodegradation is the second most widely known process to reduce OTA by LAB after adsorption. However, it poses two disadvantages compared to adsorption, it is more time-consuming and might produce harmful metabolites. Besides, for OTA reduction, degradation is the least common mechanism, and most of the studies contemplate adsorption or do not give an explicit explanation (Sadiq et al., 2019). LAB produce several enzymes that can degrade mycotoxins to other compounds. These enzymes can be proteolytic enzymes, such as cell-wall bound proteinase, that hydrolyze proteins into polypeptides, peptide transporters, that transfer the peptides into the cells and intracellular peptidases that degrade the transferred peptides into amino acids (Muhialdin et al., 2020). There is very little information about purified and characterized enzymes involved in OTA degradation. The first reported one was carboxypeptidase A (CPA), isolated from bovine pancreas. From porcine pancreas, the enzyme pancreatin was isolated. *A. niger* has been a source of many enzymes such as a lipase, a protease A, an enzyme called prolyve PAC and a cell-free crude enzyme preparation. From *S. cerevisiae* the enzyme carboxypeptidase Y (CPY) has been notified (Abrunhosa et al., 2010). Generally, there are two types of reactions of OTA with these enzymes: hydrolysis of the amide bond, or hydrolysis of the lactone ring (Wang et al., 2022).

As reported by various studies degradation is a process that normally occurs together with other detoxification mechanisms such as adsorption. Du et al. (2021) studied the potential of Tibetan Kefir Grains (TKG), composed mainly of *Lactiplantibacillus kefiranofaciens* and *Kazachstania unisporus* AC-2, to detoxify dairy products. In their experiments on liquid media, they discovered that 75% of the OTA was binded to the cell wall and only 25% of the OTA was degraded, giving as main degradation products ochratoxin alpha (OT α) and phenylalanine (Phe). Similarly, Zhang et al. (2022) analysed the ability to remove OTA of *Bacillus velezensis* E2 on *A. westerdijkiae* fc-1 on liquid medium. Their results suggested that the mechanisms responsible for 98.4% of the OTA by the cell-free supernatant might be of enzymatic origin. The second reduction mechanisms analysed in this study was the alkaline hydrolysis. Which consists in the opening of the lactone ring of OTA under alkaline conditions (pH 8-12), converting the OTA into open ring OTA (OP-OTA). Both the enzymatic and the alkaline hydrolysis theories were supported by the identification of degradation products OT α and OP-OT α .

Einloft et al., 2017 isolated two strains of *Bacillus* genus, *B. amyloliquefaciens* RP103 and *B. subtilis* RP242. These strains showed high OTA inhibition when confronted to *A. westerdijkiae* on coffee-based medium. Specially, *B. amylolicheniformis* showed an OTA reduction of 92% which according to this article might be linked to a degradation strategy by cells and/or cell-free metabolites. Besides, *B. subtilis*'s cell-free supernatant has been related in this article to important OTA degradation during confrontation with *A. ochraceus*. If we look at other food commodities with a similar problematic, for example, *Pediococcus parvulus*, isolated from Douro wines, degraded 90% OTA into OT α (Abrunhosa et al., 2014). Two bacteria isolated from vineyard soil of the genus *Acinetobacter* sp. reduced OTA in more than an 80%.

2.1.6.2.2 By Yeasts

Until the date there are no publications of yeasts isolated from coffee or used on coffee media or coffee beans with OTA degradation ability. Most of the enzymes discovered capable of degrading OTA are isolated from soil samples. For

example, the enzyme carboxypeptidase Y (CPY) isolated from *S. cerevisiae* has been notified (Abrunhosa et al., 2010). However, there are some successful yeasts isolated from grapes. For example, *Yarrowia lipolytica* Y-2 isolated from the surface of grapes, had the ability to degrade OTA into OTα through intracellular enzymes (X. Zhang et al., 2018).

Table 2.1.61. Culture or enzymes of bacteria or yeasts with the ability of OTA degradation on food commodities similar to coffee.

Microorganism or enzymes	OTA concentration	Removal (%)	Incubation time/condition	Degradation products	Reference
<i>Acinetobacter calcoaceticus</i> 396.1	1 µg/mL	82	6 d in liquid medium	OTα	de Bellis et al. (2015)
<i>Acinetobacter</i> sp.	1 µg/mL	91	6 d in liquid medium	OTα	de Bellis et al. (2015)
<i>Pediococcus parvulus</i>	1 µg/mL	90	25 h in liquid medium	OTα	Abrunhosa et al. (2014)
<i>Yarrowia lipolytica</i> intracellular enzymes	1 µg/mL	97.5	1 d in liquid medium	OTα	X. Zhang et al. (2018)

2.1.6.2.3 By Non-mycotoxigenic fungi

An article from Zou et al. (2022) analysed the detoxification properties of a UV-mutated strain of *A. niger*. This strain on optimal conditions of pH 8 and 30°C temperature can degrade 89.4% of OTA to non-toxic derivatives such as ochratoxin α. It has also been tested on contaminated wheat bran where it degraded 59.8% of OTA. Besides, Zou et al. (2022) reported the identification of a new metabolite product of the demethylation and oxidation of OTA by their UV-mutated strain.

There is very little information about purified and characterized enzymes involved in OTA degradation. The first reported one was carboxypeptidase A (CPA), isolated from bovine pancreas. From porcine pancreas, the enzyme pancreatin was isolated. *A. niger* has been a source of many enzymes such as a lipase, a protease A, an enzyme called prolyse PAC and a cell-free crude enzyme preparation. Generally, there are two types of reactions of OTA with these enzymes: hydrolysis of the amide bond, or hydrolysis of the lactone ring (L. Wang et al., 2022).

2.1.7 Physical strategies for reduction of mycotoxins on coffee

There are various physical strategies such as extrusion, ultraviolet (UV) and gamma radiation, high-pressure, ultrasound, and cold plasma that can detoxify mycotoxins in coffee (L. Wang et al., 2022). These strategies can be applied at different stages of the coffee chain, after harvest, before fermentation or after roasting. Until now, there is report of using cold plasma on roasted coffee. When used in coffee, cold plasma has been able to inhibit the growth of OTA-producing fungi and reduced 50% OTA content. This technique does not affect nutritional and sensory properties of coffee, but it can generate reactive species such as O₂, O₃, OH, NO and NO₂ (Casas-Junco et al., 2019).

The daily process of coffee torrefaction has as well an impact reducing OTA on coffee although the reduction is normally heterogeneous. Depending on the desired characteristics of the final product, conditions such as time or temperature account for the variation in the reduction percentage (Pérez de Obanos et al., 2005). Torrefaction process has been reported to degrade OTA into less cytotoxic products, 14-decarboxy-ochratoxin A and 14-(R)-ochratoxin A, through decarboxylation and isomerization reactions, respectively (L. Wang et al., 2022)

Ultrasounds reduce the OTA in coffee through inactivation of filamentous fungi and through a direct degradation of the OTA. This method has been used before fermentation without affecting the quality of coffee. Gamma irradiation and high-pressure processing (HPP) are proposed as well because they would not have any change on the organoleptic properties of coffee. Gamma irradiation efficacy

reducing OTA concentration is directly correlated to a_w . A a_w of 0.82, 0.65 and 0.59 reduce 90%, 9% and 5% OTA. HPP at 400 MPa or higher inhibits fungal growth but does not reduce OTA concentration. Besides, HPP increases the content of beta-aminobutyric acid after 50 days of storage, which improves the nutritional value of coffee (Leitão, 2019).

Byun et al. (2020) analysed the effect of UV-C irradiation on *A. flavus* and *A. parasiticus* spores' survival on roasted coffee beans. The results confirmed that UV-C was effective preventing the contamination of roasted coffee beans by *A. flavus*, but not sufficiently effective with *A. parasiticus*. This might be linked to the thickness of the spore cell wall, and to photoprotective pigments as melatonin. As well, this method directly reduces the content in OTA on coffee through its degradation. Regarding the effect on roasted coffee beans quality, UV-C irradiation modified the Hunter colour, lowered the pH, and decreased the hedonic points by enhancing sourness.

2.1.8 Chemical strategies for reduction of mycotoxins on coffee

Between the chemical methods for OTA detoxification, we can find bases, oxidizing agents, organic and inorganic adsorbents, and ozone. Potassium carbonate solution and sodium hydrosulphite have been notified as well, together with acids such as lactic acid, citric acid and acetic acid. However, the use of these chemicals in food is regulated due to safety and nutritional concerns (L. Wang et al., 2022). The treatment with gaseous ozone on coffee was investigated by Akbar et al. (2020a). In their study, gaseous ozone was evaluated for its efficacy in curbing ochratoxigenic fungi and OTA presence on Arabica green coffee beans contaminated with *A. carbonarius*, *A. westerdijkiae*, and *A. ochraceus* under experimental conditions mimicking storage environments. The conclusions drawn around ozone, indicated a reduction of a 26% in some cases. However, its potential application is only relevant for stored coffee after fermentation, and during medium term storage and transport.

2.1.9 Conclusions

There are many approaches to reduce OTA occurrence in coffee. If prevention techniques such as biocontrol do not fully avoid mycotoxin contamination, decontamination techniques such as microbiological, physical, or chemical treatments can help to remove a part of the remaining OTA content. Of these alternatives, biocontrol emerges as the most promising approach, effectively reducing coffee losses by proactively preventing the apparition of OTA. This review has highlighted the diversity of protocols used and microorganisms tested until now. Some were tested as whole cultures, while other were tested as supernatants. Those promising results were most often limited to *in vitro* conditions.

The results summarized in the review highlight the link between biocontrol of toxigenic fungi and adsorption/degradation of mycotoxin strategies exposing the need for further tests to recognize the mechanisms of OTA reduction. The review of biocontrol strategies reveals a lack of *in vivo* tests and proposes the *P. fermentans* LPBYB13 test as the most advanced study towards potential application during coffee fermentation.

2.1.10 Future research needs

Biocontrol on coffee is a promising field to be developed soon. However, the heterogeneity of the tested conditions and the microorganisms studied has led to difficulties in comparing results. Consequently, we suggest insights for future studies on coffee biocontrol.

To improve the reliability on the information of future articles, it must be considered to include more details, such as coffee species (Robusta or Arabica), process (wet or dry), fungi related to this contamination or harvesting year. As well, there is a very low number of studies on soluble coffee which is alarming since the OTA threshold for this type of coffee is higher than for roasted coffee.

For potential biocontrol agents, there is a need for taking a step further using coffee based medium as a step before *in vivo* tests and avoiding synthetic media that can give biased results. Besides, for all BCA and mycotoxin

adsorbents/degraders the protocols should include inoculum concentration (in CFU/mL) and analysis of OTA degradation products.

The candidates validated through all the previous steps should be analyzed in vivo on coffee beans. The protocol established by De Melo Pereira et al. (2016), which evaluates the biocontrol efficacy of diverse microorganisms on coffee beans, serves as a valuable benchmark. A marginal enhancement could involve the incorporation of multiple ochratoxigenic species to ascertain the biocontrol agent's potential in diminishing OTA.

By applying these recommendations, we could have comparable data between the different BCA, or adsorption/degradation agents. The future of those methods is the development and formulation of inoculums of OTA BCA or adsorption/degradation agents to optimise efficacy and provide solutions to the coffee chain. The demand for this kind of biological agents will further increase in the future.

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3 Aim and Objectives

The overall aim of this study was to investigate and understand the effect of climate change on mycotoxin production and to create a resilient inoculum to reduce the presence of ochratoxin A (OTA) on Ivorian Robusta Coffee. This requires a good understanding of the microbial composition of coffee at the postharvest stage and the ecophysiology of *Aspergillus carbonarius* which might impact the control strategies.

The present work was divided into three sequential chapters/articles, each with its own aims and objectives:

The **first chapter** was created to analyse the microbial ecology and the OTA quantity of fresh and differently dried coffees harvested in Ivory Coast. The microbial ecology was evaluated through the metabarcoding technique which allowed to assess the bacterial and fungal parameters such as alpha and beta diversity, relative abundance and composition, and the co-occurrence networks.

The **second chapter** was to establish the fungal diversity on dried Robusta coffee in terms of isolation frequency, and relative fungal populations for *A. carbonarius* and other species. The ability to produce OTA by *Aspergillus* section *Nigri* strains isolated was examined. As well, this chapter was designed to isolate and identify ochratoxigenic fungi and possible candidates of antagonistic microorganisms from Ivorian dried coffee (bacteria and yeasts) which were antagonistic to the pathogen *A. carbonarius*. These studies were done in dual-culture assays on solid media. Antagonism was assessed through the quantification of the inhibition of *A. carbonarius* growth rate and OTA production during the confrontations against the BCA. This enabled the identification of some potential candidates for further studies.

Finally, in this chapter, we targeted the analysis of the mechanisms of growth and OTA reduction of the BCAs. For this, the effect of the BCAs on fungal growth and OTA production by *A. carbonarius* was measured *in vitro* on liquid media. Liquid media confrontations allowed to assess the capability of extracellular metabolites to inhibit the growth and OTA production of *A. carbonarius*. The interaction

between the BCA and *A. carbonarius* was physically assessed through Scanning Electron Microscopy to confirm if any physical mechanism was taking place. Finally, the ability of the BCAs to adsorb OTA was measured through a confrontation on liquid media of the BCAs and the OTA standards alone.

The **third chapter** was planned to study the effect of different interacting environmental conditions on growth and OTA production *in vivo* using fresh Robusta coffee. This phase was designed to understand the patterns and optimal conditions for growth and OTA production of the *A. carbonarius* strain, and the resilience of the BCA isolated from Ivorian Coffee.

Each chapter and the specific aims are described below:

Chapter 1. Metabarcoding and OTA quantification profiles of fresh and differently dried Robusta coffee from Ivory Coast

- a) To study the microbial ecology during post-harvest by applying the metabarcoding technique, which allows to assess diversity, composition, and co-occurrence of species.
- b) To assess the differences in microbial ecology between different coffee fractions.
- c) To calculate the level of OTA on fresh and dried Robusta coffee and to analyse the distribution of OTA in the cherry.
- a) To examine the *A. carbonarius* isolated for their ability to produce Ochratoxin A using HPLC-FLD.

Chapter 2. Screening for potential biocontrol agents (BCAs) for control of *Aspergillus carbonarius* and ochratoxin A production *in vitro* and Identification of the mechanisms of inhibition and detoxification of the selected biocontrol agents

- a) To isolate ochratoxigenic fungi and their ability to produce Ochratoxin A using HPLC-FLD.
- b) To determine the growth and OTA production inhibition of the isolated yeasts (n = 17) and bacteria (n = 5) on *A. carbonarius* isolate using solid media.
- c) To select the best potential BCAs to control OTA production *in vitro*.

- d) To find out the mechanism or mechanisms involved in the inhibition through the confrontation of cells and cell free supernatant of selected BCAs against *A. carbonarius*.
- e) To examine the detoxification capacity of the selected BCAs in presence of OTA, and to assess the adsorption or degradation mechanism through the analysis of the cells and the supernatant every 1, 4, 8 and 24h.

Chapter 3. Biocontrol resilience under simulated climate change scenarios in fresh Robusta coffee from Ivory Coast

- a) To simulate the climate change scenarios in UV-irradiated fresh coffee cherries at 0.95 and 0.90 a_w , 27, 30 and 33 °C and 400 and 1000 ppm CO₂ to examine the effects on ochratoxin A (OTA) production and fungal colonization 7 days after the inoculation with the BCAs and *A. carbonarius*.
- b) To examine the resilience of the BCAs under CC scenarios against *A. carbonarius*.

4 Effect of post-harvest management practices on the mycobiome and ochratoxin A contamination of differently processed Robusta coffees from Ivory Coast

4.1 Article presentation

During post-harvest treatment of vegetal productions, temperature and humidity conditions should be controlled carefully, otherwise fungi can develop and lead to a potential risk of mycotoxin contamination. Specially, for the case of coffee, drying is the main step where fungi can develop and produce harmful mycotoxins. The mycobiota and its relationship to different post-harvest management strategies can help understanding the interactions between the different fungi and their impact on dried coffee safety.

This article presents the analysis of the mycobiota and the Ochratoxin A contamination of fresh coffee compared to dried coffee processed using different post-harvest practices such as soaking, heaping or extended drying. As well, there is a screening specific to the fungus *A carbonarius*, a ubiquitous fungus found regularly as a contaminant in coffee, cocoa and grapes, and the main producer of OTA among the *Aspergillus* section *Nigri*. It was found that a higher contamination with OTA was accompanied by a higher relative abundance of *A. carbonarius* studied through Metabarcoding. Furthermore, all samples were positively contaminated with OTA and certain process appear riskier for this contamination. An interesting observation in this work was the impact of drying on the diversity of the mycobiota. Indeed, dried coffee presented a more diverse mycobiota, with a high relative abundance of yeast species such as *Kurtzmaniella* sp., and *Meyerozyma* sp., and some other yeasts with negative co-occurrence with *A. carbonarius* could be good candidates for biological control.

This study represents the first metabarcoding analysis of Robusta coffee available in the literature. For the aim of the present work this first chapter frames the results presented in the next chapters.

This article was published in the journal Post-harvest Biology and Technology.

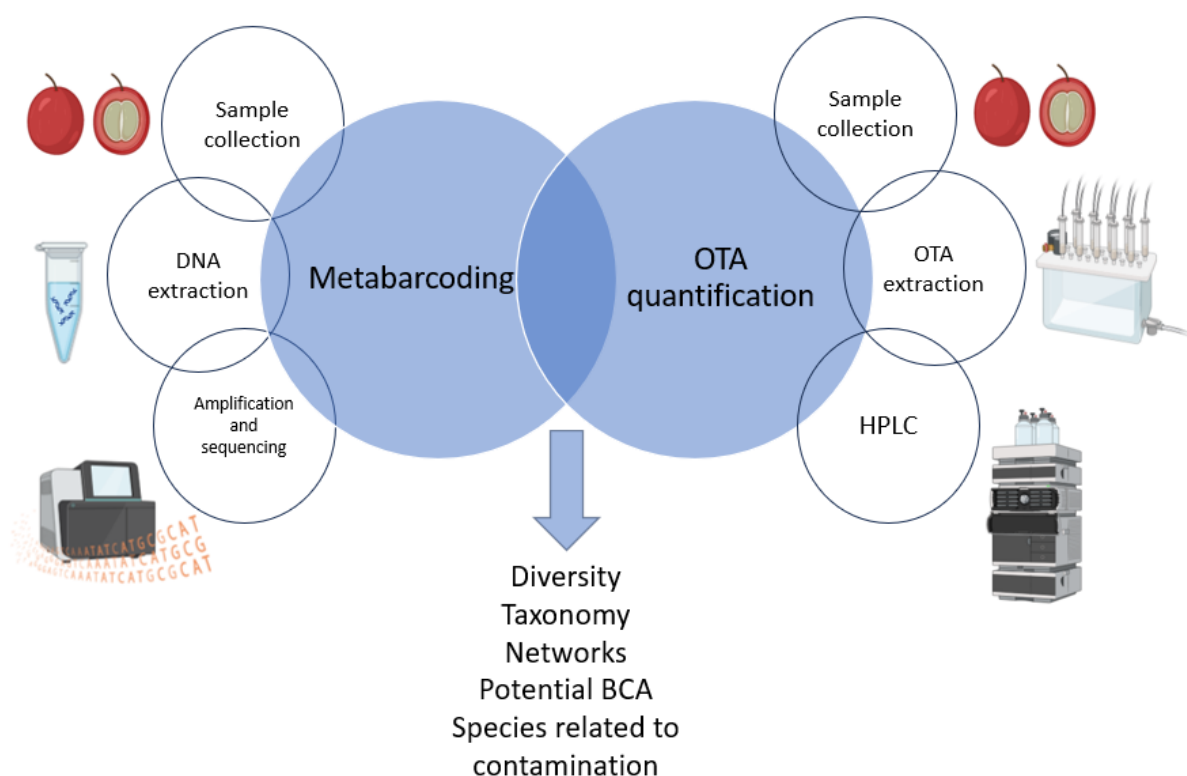


Figure 2.1.11 Graphical abstract. Created with biorender.com

4.1.1 Preliminary work

Metabarcoding is a technique very dependent on the initial quantity and quality of DNA. This is why it is important to choose a method to extract the DNA that is adapted to the type of DNA targeted, and that reduces the presence of inhibitors and coffee DNA. With this aim, the comparison of the five different DNA extraction kits listed in Table 4.1.1-1 was performed.

Table 4.1.1-1. List of different kits used for DNA extraction from coffee.

Number	Kit ID
1	FastDNA SPIN kit (MPBio)
2	FastDNA SPIN Kit for Soil (MPBio)
3	NucleoSpin Kit Soil (MN)
4	NucleoSpin Kit for Food (MN)
5	DNAEasy Plant Pro (Qiagen)

The protocols included in every kit were followed. The results of the DNA analysis performed with Nanodrop showed that the kit with the higher DNA concentration was NucleoSpin kit For Food. The $A_{260/280}$ ratio was used for the analysis of the DNA purity. The NucleoSpin kit for Soil showed the best purity, considering the $A_{260/280}$ ratio value 1.8 as the best DNA purity.

Table 4.1.1-2. Results of the Nanodrop analysis of the extracted DNA by five different kits (listed in Table 4.1.1-1).

KIT	1	2	3	4	5
DNA concentration (ng/ μ L)	62.86	17.16	14.32	88.93	7.174
$A_{260/280}$ ratio	2.02	3.32	1.75	1.88	1.89

The DNA from the extraction with kits 1 and 4 was chosen to perform the PCR, thanks to the high DNA concentration obtained. For the PCR the fungal ITS2 region of ribosomal DNA was amplified using the set of primers ITS86 and ITS4. The ITS2 PCR was performed in a total volume of 25 μ L containing 12.5 μ L of AmpliTaq Gold™ 360 Master Mix (Thermo Fisher Scientific), 1 μ L of each primer (100 μ M), PCR-grade water and 1 μ L of DNA. Then, the reactions were incubated in a Mastercycler X50 (Eppendorf France SAS; Montesson, France) under the following cycling conditions: 94 °C for 10 min, 30 cycles of 95 °C for 30 s, 55 °C for 45 min, 72 °C for 30 s, 72 °C. Finally, we run our samples in a 2% agarose gels for 45 min at 75v.

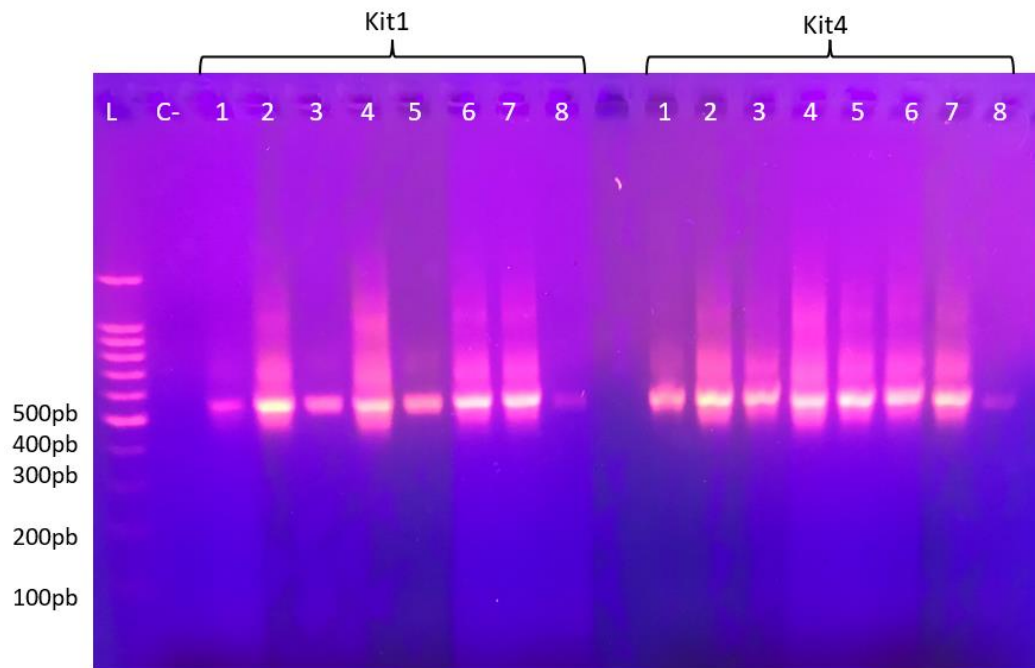


Figure 4.1.1-1. Bands of the fragments result of the ITS PCR performed for FastDNA SPIN kit (kit 1) and NucleoSpin Kit for Food (kit 4). L designates Ladder, and C- for negative control.

The results of the electrophoresis are shown in Figure 4.1.1-1. All the samples tested present a good band intensity, but kit 4 shows a higher intensity than kit 1 for all samples. The results showed that even though kit 3 presents the $A_{260/280}$ ratio closest to 1.8, the concentration of DNA was too low to consider it in front of kits 1 and 4. For kits 1 and 4, the fragment bands in the electrophoresis experiment were stronger for kit 4. Consequently, NucleoSpin Kit for Food was chosen to perform the Metabarcoding analysis.

EFFECT OF POST-HARVEST MANAGEMENT PRACTICES ON THE MYCOBIOME AND OCHRATOXIN A CONTAMINATION OF DIFFERENTLY PROCESSED ROBUSTA COFFEES FROM IVORY COAST

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Highlights

- Ochratoxin A (OTA) was detected in fresh cherries and in all treatments.
- Extended drying increase OTA contamination.
- Ninety-five percent of the OTA is detected in the husk.
- Microbial communities' composition of fresh and dried Robusta coffee is different.
- A higher *A. carbonarius* presence after drying was detected through Metabarcoding.
- A higher *A. carbonarius* abundance was concomitant with higher OTA levels.

Abstract: Ochratoxin A (OTA) is a secondary metabolite produced primarily by the genus *Aspergillus* sp. sections *Circumdati* and *Nigri*. It can accumulate in coffee at post-harvest stage. In the present study five different dry processed coffees were sampled from Ivory Coast at harvesting and after drying. The OTA was detected in all the samples, from 3.62 µg/kg of coffee just after harvest to a higher-level of contamination in the dried coffee, between 11.04 and 760.24 µg/kg of coffee. Metabarcoding was used to further study the changes in the mycobiota. The alpha and beta diversity analysis revealed the presence of unique and more diverse fungal communities on the coffee after drying compared to fresh harvested coffee. Specially, coffee dried for a longer period showed a higher diversity. Fungal species such as *Hypopichia* sp. or *Bernettozyma* sp., were replaced in the top ten abundant species after drying by species such as *Kurtzmaniella* sp., *Meyerozyma* sp. and *Fusarium* sp. The increasing abundance of the ochratoxigenic fungi, *A. carbonarius* was correlated to the increased concentration of OTA on coffee. The construction of the negative correlation network shows that some yeasts could be good candidates for the biocontrol of *A. carbonarius*. The combination of DNA metabarcoding and OTA quantification was effective at deciphering the post-harvest origin of the OTA contamination. Overall, this study highlights the changes in the mycobiota under different drying conditions. Future perspectives include developing actions of prevention and control of the contamination with OTA during the post-harvest stages.

Keywords: metagenomics, ochratoxin A, Robusta coffee, toxigenic fungi, post-harvest management, coffee safety

4.2 Introduction

Ivory Coast is the 7th producer of Robusta Coffee in the world, and the 2nd in Africa with a production of 66 million kilograms of coffee in 2022 (USDA, 2022). Climate is divided between equatorial with higher precipitations in the south, and tropical in the north. These conditions, place Ivory Coast amongst the largest African producers of cocoa and coffee and favour the production of several food commodities cultures such as corn, banana, and palm oil between others (FAO et al., 2018). Coffee is native to Africa; Arabica has been supposedly originated in Ethiopia and Robusta in the Atlantic Coast and Great Lakes. However, coffee was introduced in Ivory Coast after the French colonisation (Bunn et al., 2015; Kufa, 2010).

Coffee can contain ochratoxin A (OTA), a toxic fungal secondary metabolite or mycotoxin produced by several fungi of the *Aspergillus* genera (Houbraken et al., 2020). Among this genera, *Aspergillus carbonarius* and *A. westerdijkiae* are the main producers of OTA and are considered important post-harvest fungal species of coffee (Taniwaki, 2005). OTA presents a major safety concern for coffee because of its severe acute and chronic effects on human. Chronic health risks of OTA ingestion may include mutagenicity, possible carcinogenicity (group 2B by the International Agency for Research on Cancer (IARC)), hepatotoxicity and nephrotoxicity effects (International Agency for Research on Cancer, 1993). Maximum regulated levels were established by the European Union (EU) ranging between 3 and 5 µg/kg, respectively for roasted coffee beans and soluble coffee (European-Commission, 2023).

Post-harvest handling of coffee can follow three different ways, wet, semi-dry and dry. The wet process consists of coffee depulping, then fermentation in water for between 12 and 36 hours, and finally sun-drying for approximately 12 days, or until coffee reaches a 12% humidity. The dry processed coffee is sun-dried right after harvest (L. R. Batista et al., 2009). Lastly, the semi-dry process is a variation of wet process, in which coffee is depulped, but the fermentation occurs spontaneously while coffee is sun-dried (Evangelista et al., 2014). Generally, it is during drying that growth of fungi occurs, and consequently there is a higher risk

of OTA contamination. Once OTA is produced, practices like roasting can only marginally reduce OTA content (Castellanos-Onorio et al., 2011).

During postharvest, yeasts (Lino de Souza et al., 2021), and fungi (de Almeida et al., 2019) are implicated on the degradation of the pulp and mucilage and the reduction of the risk of contamination with mycotoxigenic fungi. Many microbial species have been isolated from coffee through culture-dependant methods (Feng et al., 2016, Silva et al., 2008). However, in the last years these techniques are used complementarily to culture-independent methods such as metabarcoding (de Oliveira Junqueira et al., 2019; Madrid Restrepo et al., 2022). Metabarcoding is used for rapidly and accurately identify the composition, distribution, and dynamics of microbial communities. Previous work of metabarcoding on coffee has analysed Arabica coffee's fungal species composition and diversity during fermentation (De Bruyn et al., 2017). However, for Robusta coffee little is known about the diversity of the mycobiome. In fact, most research related to Robusta mycobiome has concentrated on microbial diversity of plant pathogens and the rhizome (Duong et al., 2021; Tran, 2022). Therefore, the study of Robusta coffee mycobiome at different key processing stages of coffee supply chain is a promising tool to better understand the contamination with mycotoxigenic fungi and the OTA production.

In this study, metabarcoding was used to determine the composition of fungal communities on coffee cherries and compare the effect of different post-harvest practices on its diversity. In parallel, OTA concentration was quantified using HPLC-FLD to identify critical post-harvest steps for contamination. These data will allow us to analyse the relation between OTA and fungal occurrence at key processing stages.

4.3 Materials and methods

4.3.1 General overview of the coffee sampling and handling processes

Coffee samples were collected from differently processed Robusta coffees during the harvest season corresponding to the year 2020. The coffee was cultivated in

Akoupé, (6° 23' north, 3° 54' west) in a highly producing area in the southeast of Ivory Coast (Figure 4.3.1-1) at an altitude of 126 m. The same batch was sampled before and just after drying.

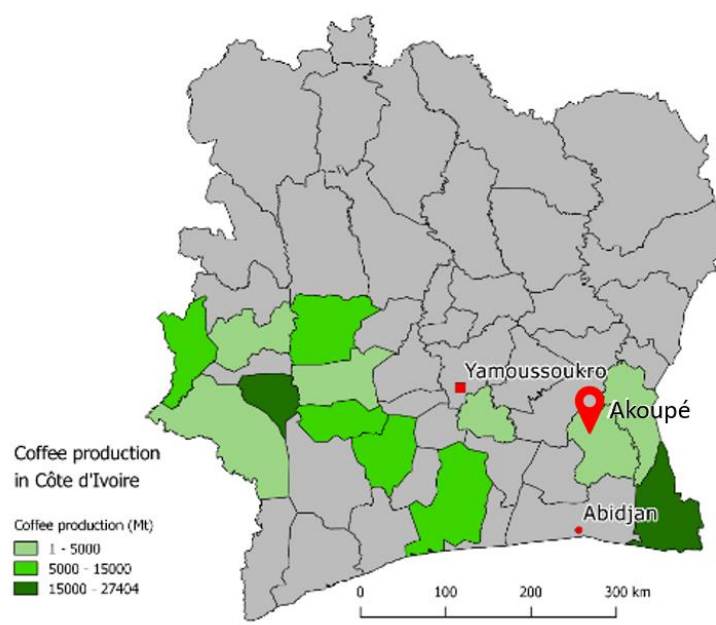


Figure 4.3.1-1. Coffee production by districts and supply levels (2016). Source: Global Coffee platform.

The cherries were handpicked and then followed five different dry processes detailed in Figure 4.3.1-3. The traditional, primary post-harvest processing named DP (Dry Process), consists of directly sun-drying fresh coffee cherries by laying them on the ground, usually in layers of 5cm thickness (Figure 4.3.1-2). This process is commonly practiced by small-scale and low-income coffee farmers, which represent most of the coffee farmers in Ivory Coast due to the low performance of this crop in this country (Musebe, et al., 2007; Binam et al., 2003). The processes named SDP (Soaked Dry Process) and SHDP (Soaked Heaped Dry Process) went through a soaking step before the drying process. The soaking consists of putting the coffee in a tank with sterile water (sterilized at 121 °C for 15 min) for 24-48 h (Firdissa et al., 2022). Soaking helps in softening the outer skin and slows down the rate of fermentation, consequently, it is used during peak season in farms with limited capacity to process the coffee cherries immediately (Ikumi et al., 2017). The heaping process consists of distributing the

coffee cherries in piles of various volumes, to hasten the drying process in areas of limited available space. These are experimental processes designed to assess the effect of the soaking, heaping and drying steps. Processes SHDP, HDP (Heaped Dry Process) and EHDP (Extended Heaped Dry Process) go through a spontaneous fermentation. The spontaneous fermentation happens when coffee is piled in heaps outdoors and uncovered, after soaking (in case coffee was soaked) or just after harvesting. This is a common malpractice between coffee farmers. The length of the spontaneous fermentation can vary between 4 and 7 days, depending for how long they are stored in heaps (Velmourougane, et al., 2006). After fermentation, they are dried on the ground in layers of a thickness of 5 cm. They can be dried for 7, 12 or 30 days. In the case of EHDP, this process was an experiment to evaluate the effect of prolonged fermentation on the mycobiota and OTA content. Two kilograms of dried coffee cherries from each of the 5 different processes and 19 kilograms of fresh cherries, not processed, were collected by hand using sterilised gloves and stored separately in sterilised plastic bags within a 4 °C portable fridge for a period of 2 weeks, before sending to the laboratory in France. At reception, all samples were then kept within a refrigerator unit at -20 °C until the experiments began.



Figure 4.3.1-2. Image of the drying technique carried out in Ivory Coast. Coffee drying usually takes place on bamboo tables, or on the ground. Photo borrowed from CIRAD's private repository.

The experimental process started by gathering the appropriate coffee replicate samples and separating them in 3 different fractions: 1) the complete cherry, 2) the husk, and 3) the bean. Three replicates were randomly selected from each one of the 2 kg processed coffee bags, after carefully mixing their content for uniformity. For the obtention of the husk and bean fractions, dried cherries were manually dehusked. This helped to understand the different levels of OTA and changes in the mycobiota across the different layers of the coffee cherries before and after drying (Bucheli & Taniwaki, et al., 2002).

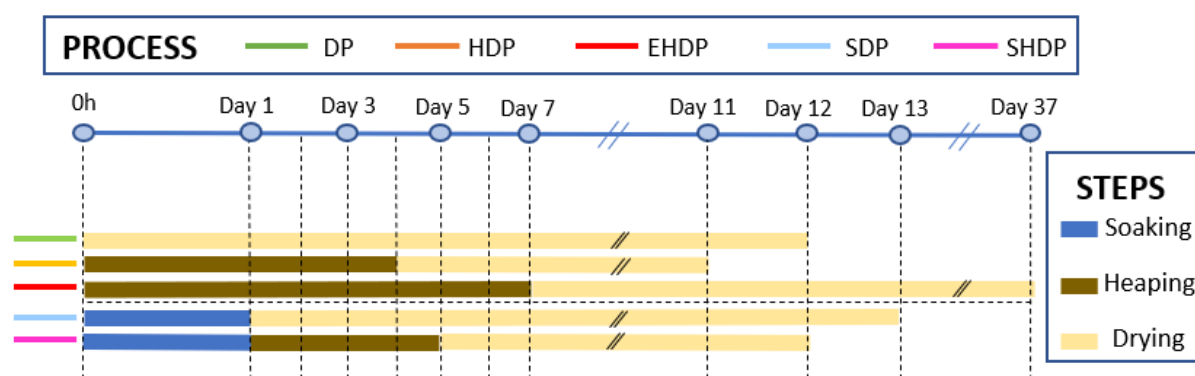


Figure 4.3.1-3. Experimental setup of coffee sampling based on different processing experiments carried out in Akoupé, Ivory Coast. For the processing, the green line depicts the Dry Process (DP); the orange line represents the Heaped Dry Process (HDP); the red line represents the Extended Heaped Dry Process (EHDP); the blue line corresponds to the Soaked Dry Process (SDP); the pink line corresponds to Soaked and Heaped Dry Process (SHDP). The samples used correspond only to the final product, dried cherries.

4.3.2 Extraction and quantification of ochratoxin a in coffee

I husk, the bean and the cherry fractions, and the fresh coffee samples were ground at a speed of 10,000 rpm (Retsch ZM200, Germany), to pass a 0.5 mm sieve, and analysed for OTA content. Three replicates of 10g of every ground fraction were mixed with 20 mL of the extraction solvent (methanol, distilled water 80:20 v/v, and 2 g of NaCl) in 50 mL Falcon flasks. The mixture was shaken gently in a vertical tube rotator at 50 rpm for 30 minutes, and then centrifuged at 6000 rpm for 10 min. Two millilitres of the supernatant were pipetted into 18mL

of PBS buffer. Then, the extraction of OTA was done using the immunoaffinity column as per the manufacturer instructions (Ochraprep, R-Biopharm, Glasgow, UK). The OTA was eluted with 2 mL of HPLC-grade methanol. The elution was evaporated to dryness under a stream of nitrogen at 70 °C, and the residue was redissolved in 1 mL of HPLC mobile phase and homogenised in ultrasounds for 20 mins. The mobile phase consisted of distilled water/acetonitrile/glacial acetic acid (51:48:1, v/v/v), that injected at a flow rate of 1 mL/min (Castellanos-Onorio et al., 2011). Finally, the OTA was quantified by HPLC-FLD (Shimadzu, UFCL-XR, LC 20AD, Kyoto, Japan), using a C18 reverse-phase HPLC column, Uptisphere type 5 ODB, ODS, 5 µm particle size, 250 x 4.6 mm. The injection volume was 100 µL, injected during 20 min (Campos-Avelar et al., 2020). OTA was detected by absorption at 333 nm excitation and 460 nm emission at a retention time of 13.3-13.5 min. A standard curve of OTA was established from an ochratoxin A standard (1000 ng/mL, ref PD 226 R. Biopharm, Glasgow, UK) and the limit of detection and quantification was 0.05 ng/mL and 0.15 ng/mL respectively.

The normality and homoscedasticity of the samples was validated through the Shapiro-Wilk and Levene's tests, respectively. The statistical significance of the differences in the samples was assessed by one-way ANOVA followed by Tukey's Honestly Significant Difference test (Tukey's HSD, $p < 0.05$) using The statistical package JMP® Pro 16 (SAS Institute Inc., Cary NC, USA).

4.3.3 DNA extraction from coffee

The DNA extraction was performed on a total of 48 samples, corresponding to three replicates of every postharvest process. The DNA was obtained from the resulting washing water of mixing 10g of coffee with 40 mL of Mili-Q® water and 0.1% Tween 80, previously filtered through a 0.2µm filter chamber. The DNA of the washing water was extracted with the NucleoSpin Food kit (Macherey-Nagel, Germany). The washing water was centrifuged at 4500 *g* for 10 mins, the pellet weighted and then the rest of the extraction was made as specified by the manufacturer's instructions. The DNA purity ratio and concentration were then measured using a Nanodrop ND 8000 spectrophotometer (Thermo Fisher

Scientific, Waltham, MA, USA). A DNA purity ratio $A_{260/280}$ of 1.8-2 were accepted.

4.3.3.1 DNA amplification and sequencing Library construction and sequencing

After quantification, the fungal ITS2 region of ribosomal DNA was amplified using the set of primers ITS86 and ITS4 (Vancov & Keen, 2009). The sequences of the set of primers are shown in Table 4.3.41. Those primers were modified to include Illumina adapters subsequent multiplexing. The ITS2 PCR was performed in a total volume of 25 μ L with each primer (0.25 μ M), PCR-grade water and 5 μ L template DNA. The reactions were incubated in a Mastercycler X50 (Eppendorf France SAS; Montesson, France) under the following cycling conditions for ITS: 94 °C for 10 min, 30 cycles of 95 °C for 30 s, 55 °C for 45 min, 72 °C for 30 s, a final elongation at 72 °C for 10 mins.

After a magnetic bead purification (Clean PCR, Proteigene, Saint-Marcel, France), the indexing PCR was performed in a total volume of 18 μ L (5 μ L of first round PCR products, 9 μ L Phusion® High-Fidelity PCR Master Mix (NEB, Evry, France), 2 μ L I5 index-adapter, 2 μ L I7 index-adapter). Cycling conditions: 95 °C for 3 min then 10 cycles of 95 °C 30 s, 55 °C 30 s, 72 °C 30 s then final elongation of 5 min at 72 °C. A set of 96 in-house index pairs was used to be able of multiplexing the whole samples on a single MiSeq run. After purification with magnetic beads, these final PCR products were pooled and paired-end sequenced on a MiSeq Illumina sequencer using MiSeq Reagent Kit v3 (600-cycle; Illumina, San Diego, CA, USA).

Table 4.3.3-1. List of Primers used in this study.

Primer	Sequence (5'-3')
ITS86F	GTGAATCATCGAATCTTTGAA
ITS4R	TCCTCCGCTTATTGATATGC

4.3.4 Data analysis and Statistics

Sequencing data were demultiplexed and trimmed using Cutadapt functions filterAndTrim (maxN = 0, maxEE = 2, truncQ = 2, truncLen = 250 forward/210 reverse, trimLeft = 20, minLen = 20, rm.phix = TRUE)(Martin, 2011). Forward and reverse paired-end reads were then merged (function mergePairs, overlap = 12, maxmismatch = 0), cleaned from chimeras, filtered, and assigned a taxonomy using Dada2 based workflow (Callahan et al., 2016). The taxonomy assignment database used was UNITE 8.2.

Alpha and Beta diversity population structure and composition were analysed using the phyloseq package (version 1.32.0) in R (version 4.2.1) after rarefaction to an even number of reads of 35133. For alpha diversity, the indexes of Shannon (Shannon, 1948) and Inverse Simpson (Simpson, 1949) were used to take species abundance into account (evenness). Shannon index reflects species richness of a sample, while the inverse Simpson index also places a greater weight on dominant species. For beta diversity, Bray-Curtis distances (Beals, 1984) was used to investigate the variability in community composition. Statistical analyses were carried out in R. Alpha diversity was compared between sampling steps using Tukey's HSD pairwise comparison ($\alpha < 0.05$). Beta diversity differences between sampling steps was tested using permutational ANOVA (Permanova, adonis function) with 99999 permutations.

The co-occurrence network diagram was generated using the functions spiec.easi() and sparcc() of the Sparse InverseE Covariance estimation for Ecological Association Inference (SPIEC-EASI) package (Kurtz et al., 2015). The spiec.easi function was executed with the following specifications to construct the networks: method="mb" from Meinshausen & Bühlmann (2006) and rep.num = 99. All the count tables were filtered prior to running SpiecEasi to include only genera found in at least 3 samples.

4.4 Results

4.4.1 Ochratoxin A concentration in coffee

The samples were analysed for their OTA concentration. Table 4.4.1-1 represents the mean of quantified OTA ($\mu\text{g/kg}$ of coffee) and the content range. All the coffee samples were positive for OTA. At a coffee process level, there was a difference between the fresh coffee (even expressed in dry matter) and the dry cherries of the Extended Heaped Dry Process (EHDP), which showed the highest OTA content of the dry cherries fraction. However, this difference is not significant due to the high standard deviation of the samples. In the husk fraction, OTA quantity was significantly lower in Dry Process (DP) than in EHDP or Soaked Heaped Dry Process (SHDP) (Tukey-Kramer HSD, $p < 0.05$). Compared to the husk, the bean was less contaminated. For the bean fraction, there were not significant differences between processes.

Table 4.4.1-1. OTA content on coffee samples collected from differently process coffee after and before drying. DP = dry processed, HDP = Heaped Dry Process; EHDP = Extended Heaped Dry Process, SDP = Soaked Dry Process; SHDP = Soaked Heaped Dry Process. NA states for Not Applicable.

Fraction	Dry cherry		Husk		Bean	
Process	OTA mean ¹ (µg/kg of coffee)	OTA content range ² (µg/kg of coffee)	OTA mean (µg/kg of coffee husk)	OTA content (µg/kg of coffee husk)	OTA mean (µg/kg of coffee bean)	OTA content (µg/kg of coffee bean)
FRESH	6.66 ^a ± 3.94	3.62- 12.23	NA	NA	NA	NA
DP	83.75 ^a ± 70.01	11.04- 178.31	53.30 ^b ± 29.64	28.73- 95.00	4.93 ^a ± 5.90	0.67- 13.27
HDP	74.17 ^a ± 22.45	45.85- 100.77	111.42 ^{a,b} ± 45.39	61.69- 171.43	2.02 ^a ± 1.56	0.77- 4.21
EHDP	296.72 ^a ± 328.26	42.65- 760.24	408.06 ^a ± 122.29	292.16- 577.17	6.91 ^a ± 1.33	5.04- 8.05
SDP	39.81 ^a ± 7.31	33.75- 50.09	183.41 ^{a,b} ± 99.19	88.53- 320.33	1.07 ^a ± 0.61	0.55- 1.93
SHDP	54.42 ^a ± 37.16	22.10- 106.47	260.03 ^a ± 77.98	195.38- 369.72	1.96 ^a ± 0.71	1.36- 2.96

¹Means with different letters are significantly different between processes according to ANOVA followed by post-hoc Tukey's HSD test, $p < 0.05$. ²The lowest and highest ochratoxin A concentration reported across all samples. The LOD and LOQ of the method were calculated and the values were determined as follows: LOQ: 0.15 ng/mL and LOD: 0.5 ng/mL.

4.4.2 Coffee mycobiota

4.4.2.1 Sequencing results

Sequences were paired end aligned, quality filtered, chimeras, singletons, mitochondrial and chloroplast sequences were deleted, and rarefied to an even depth of 35,133 reads per sample, i.e., samples with less than 35,133 reads were removed. Finally, a total of 1,651,251 fungal internal transcribed spacer (ITS) reads were recovered and assigned to 1,640 fungal amplicon sequence variants (ASVs) in a total of 47 samples composed by the triplicates of every processed and fraction combination. The rarefaction curves tended towards saturation, illustrating that the current sequencing depth was sufficient for the fungal diversity analysis. The number of ASV reflects the communities present around the dry cherry, in the husk, and in the outer bean, but not the entire host mycobiome.

4.4.2.2 Fungal mycobiota of the differently processed coffees

Alpha and beta diversities were analysed to understand the differences inside a single community and between communities in Figure 4.4.2-1 and Figure 4.4.2-2, respectively. Figure 4.4.2-1 shows the results of alpha-diversity indexes comparing samples from different dry processes. The ANOVA performed on the fungal alpha diversity data shows a significant difference across samples for the process variable ($p < 0.01$). The indexes used are observed index, which measures richness (number of species), Inversed Simpson index, measuring dominance, and Shannon index, which assess evenness between the abundance of the species. Considering the three diversity indexes analysed, fungal communities from pure DP were the richest, and the EHDP presented the highest diversity and species evenness. The high values of observed diversity are related to rich fungal communities, as noticed in coffee samples belonging to the DP. The high values of the inverse Simpson and Shannon indexes indicate that there is no species dominance in the EHDP.

All alpha-diversity indexes were significantly lower in the case of fresh samples in comparison with the rest of the dried coffee samples. There are no differences in species richness regarding the samples that underwent either heaping or soaking steps, although the samples that experienced extended heaping

presented a significantly higher diversity and species evenness. Moreover, a clear species dominance was found in the case of dry processed coffee with low values of the inverse Simpson index, lower than in the rest of processes. As well, in Figure 4.4.2-1 the fraction corresponding to the coffee beans (red) showed significantly lower richness compared to the dry cherries (green).

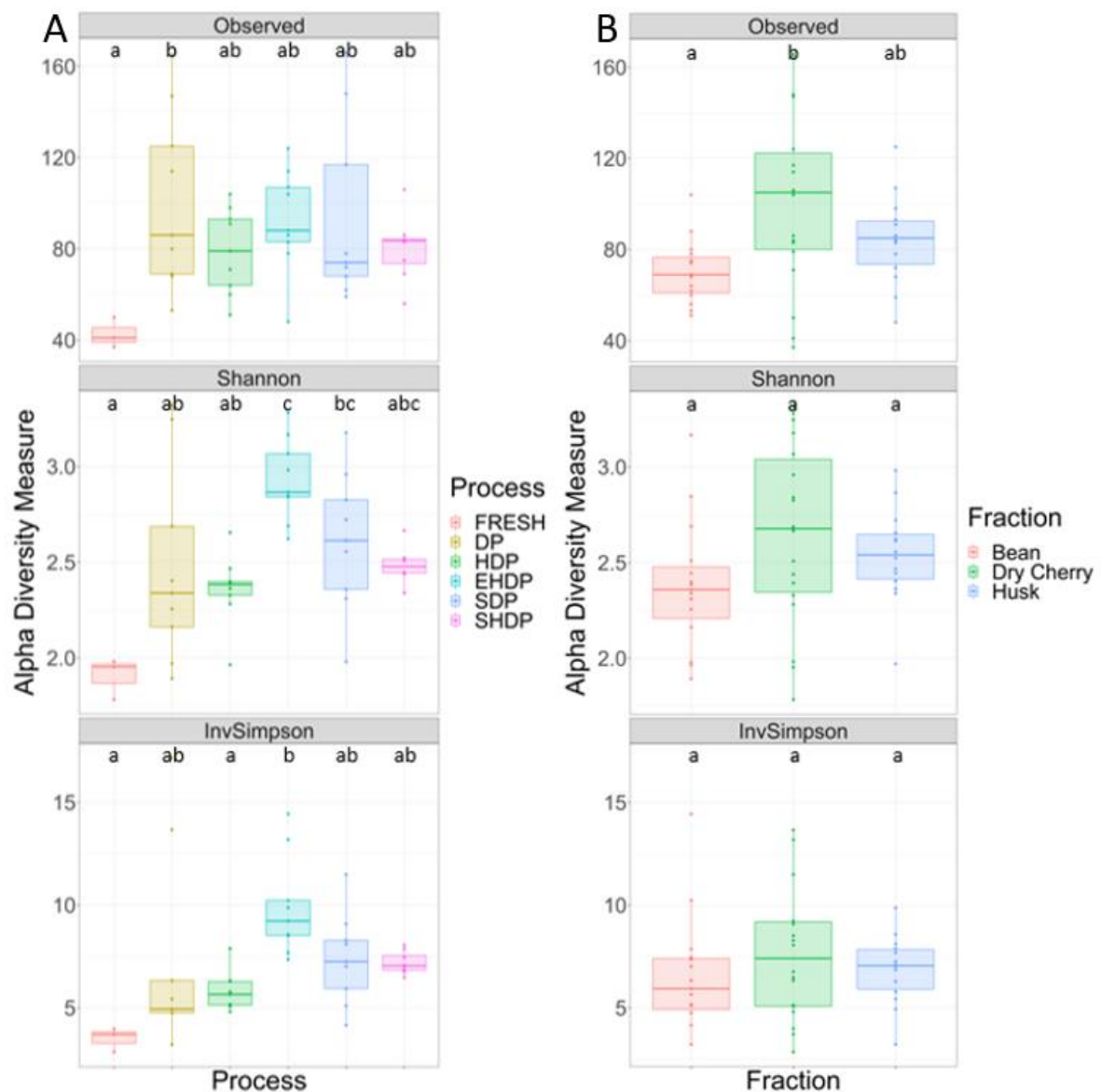


Figure 4.4.2-1. Boxplots illustrating the differences in the observed, InvSimpson and Shannon diversity measures of the fungal communities on the coffee sampled at different post-harvest processes (all fractions included)(A) and on different coffee fractions (B) using the package Phyloseq in the software R Studio

(version 4.2.1.). DP = dry processed, HDP = Heaped Dry Process; EHDP = Extended Heaped Dry Process, SDP = Soaked Dry Process; SHDP = Soaked Heaped Dry Process.

Beta diversity of the communities was also studied using a Principal coordinate analysis (PcoA) of the Bray-Curtis distances, also called Bray-Curtis PcoA (Figure 4.4.2-2). The application of the beta diversity analysis to the whole dataset helped to confirm the significant differences between the different mycobiomes. The PcoA showed that fresh samples (three red dots on the bottom of Figure 4.4.2-2) had a different diversity from those dried. Figure 4.4.2-2 exposes as well that samples that underwent heaping exhibited differences in fungal communities compared to the standard DP or the Soaked Dry Process (SDP) samples, meaning that the step where coffee is put in heaps after harvesting or soaking had a significant effect on the microflora of dried coffee (PERMANOVA, $p < 0.001$). Figure 4.4.2-2 displayed a distinct clustering of the samples based on the sampling steps, with an important cluster separating the extended heaped samples from the rest of the post-harvest processes. This revealed the presence of unique fungal communities on dried coffee from the EHDP. For the fresh coffee samples, a markedly different beta diversity was demonstrated in comparison to the post-harvest processed samples.

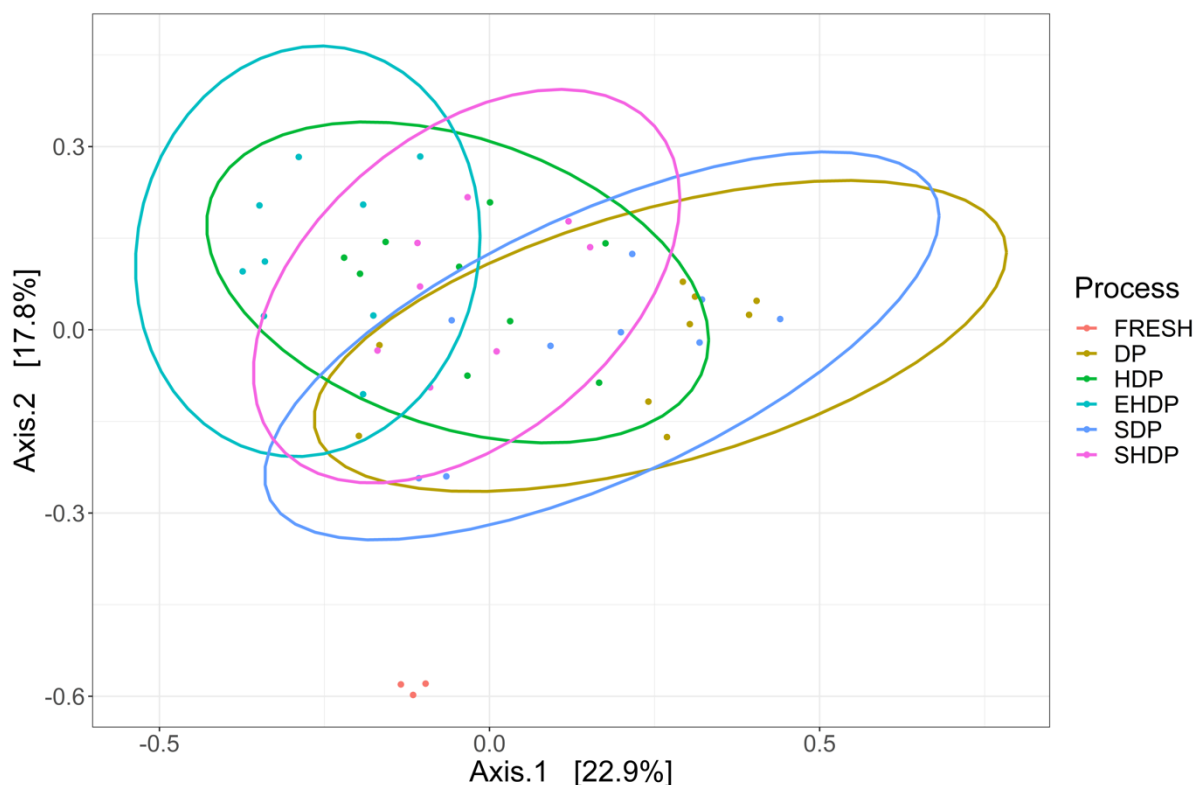


Figure 4.4.2-2. Principal coordinate analysis (PcoA) of fungal populations associated to Robusta coffee samples at the end of five post-harvest processes based on the beta diversity metric Bray Curtis. DP = dry processed, HDP = Heaped Dry Process; EHDP = Extended Heaped Dry Process, SDP = Soaked Dry Process; SHDP = Soaked Heaped Dry Process.

A total of 180 identified fungal genera belonging to 3 phyla, 18 classes, 43 orders, and 107 families were detected in the total of samples. Fungal ASVs, across all samples were assigned to *Ascomycota* (81.86%), *Basidiomycota* (17.64%), *Chytriodomycota* (0.49%). The *Ascomycota* were mainly represented by the Class *Dothideomycetes* (38.55%), *Sordariomycetes* (34.94%) and *Saccharomycetes* (14.45%) and *Basidiomycota* were represented mainly by *Agaricomycetes* (30.55%) and *Tremellomycetes* (30.55%). The class *Dothideomycetes* were largely represented by the families *Capnodiaceae* and *Mycosphaerellaceae*, the class *Sordariomycetes* by the family *Nectriaceae* and *Plectosphaerellaceae*, and the class *Saccharomycetes* contained mostly the *Debaryomycetaceae* and *Saccharomycetaceae* families.

The differences, between the five post-harvest management strategies, in the composition and the relative abundance (RA) of the top 10 abundant fungal species were shown in Figure 4.4.2-3. When the taxonomic identification wasn't possible at the species level, the ASVs were identified using the lowest level possible in the phylogenetic tree. The species represented with less than 1% of the total number of reads were clustered as "other". The composition and RA of the fungal ASVs was different between fresh samples and the post-harvest processes. The latter shared important similarities in the taxa (Figure 4.4.2-3).

Fresh coffee appears to feature different mycobiota than coffee after drying, with dominance of *Hypopichia burtonii* and *Barnettozyma pratensis* only in fresh coffee. In dried coffee, the most abundance genus were *Kurtzmaniella*, *Meyerozyma*, and *Fusarium* spp. Moreover, OTA producing species *A. carbonarius* appeared in all the drying processes studied with more abundance in HDP and SHDP processes.

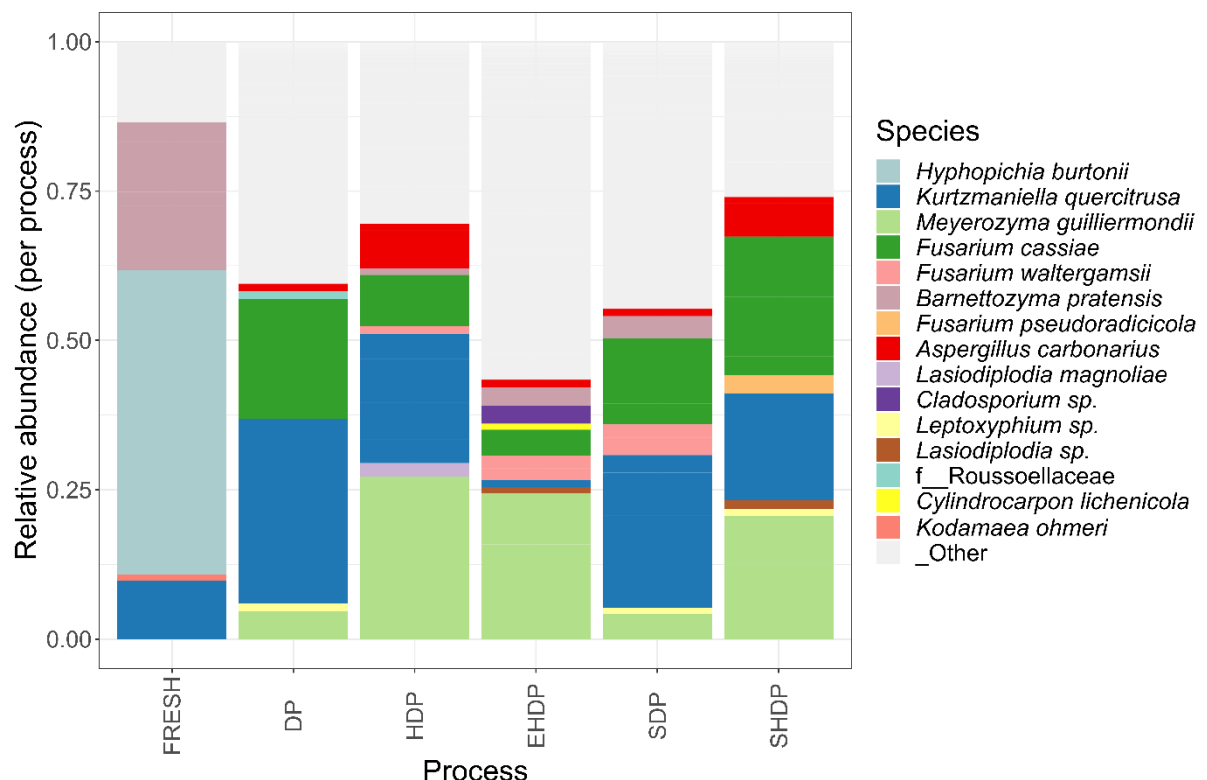


Figure 4.4.2-3. Relative abundance of fungal top 10 species detected in differently processed Robusta coffee. Fungal species with less than 1% are

reported as “other”. When the taxonomic identification wasn’t possible to the species level, the ASV was identified by the lowest possible level of the phylogenetic tree. DP = dry processed, HDP = Heaped Dry Process; EHDP = Extended Heaped Dry Process, SDP = Soaked Dry Process; SHDP = Soaked Heaped Dry Process.

Figure 4.4.2-4 shows the relative abundance of the top 10 most abundant species in relation to the concentration of OTA. *A. carbonarius* and *Meyerozyma guilliermondii* relative abundances increases with a higher OTA. At the same time, other fungi such as *Kurtzmaniella quercitrusa* or *Fusarium waltergamsii* present lower relative abundance with increasing OTA concentration. There is no clear co-occurrence between *A. carbonarius* and any other species in this top 10 most abundant.

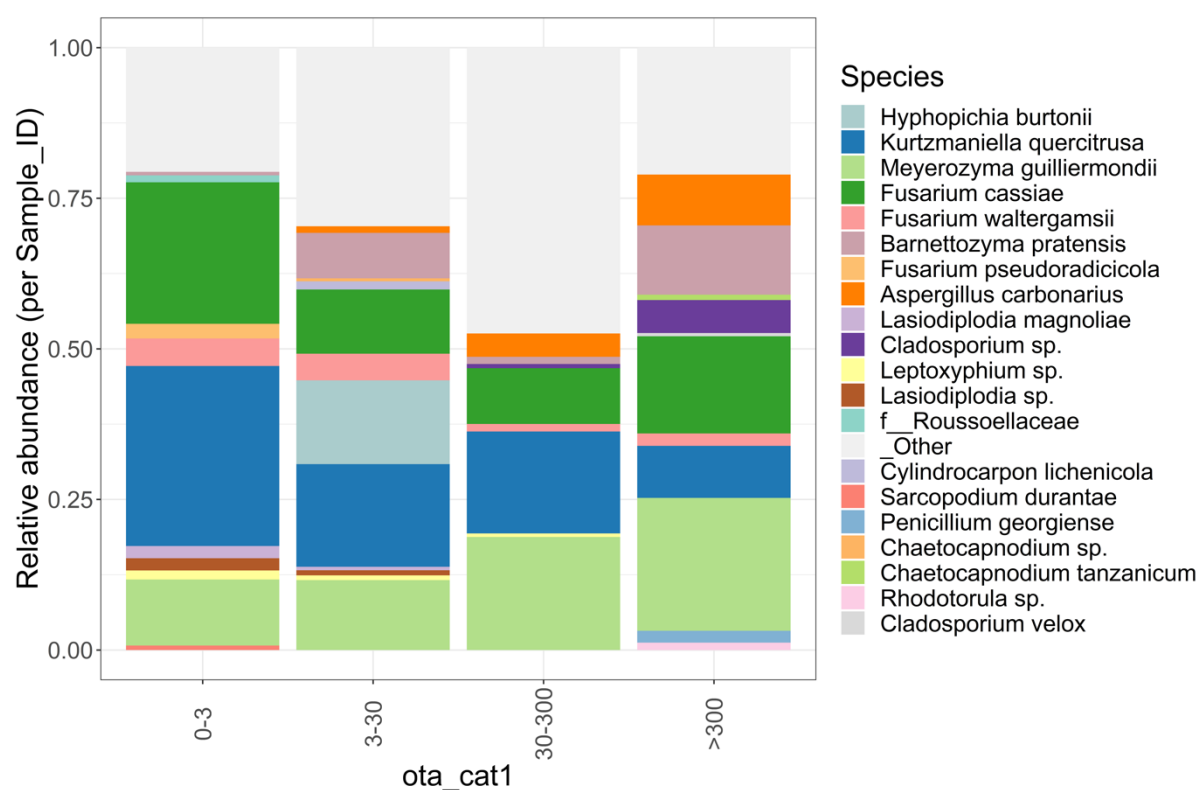


Figure 4.4.2-4. Relative abundance of fungal top 10 species detected in Robusta coffee samples grouped in function of their OTA levels expressed in µg of OTA per kg of coffee. Fungal species with less than 1% are reported as “other”. When

the taxonomic identification wasn't possible to the species level, the ASV was identified by the lowest possible level of the phylogenetic tree.

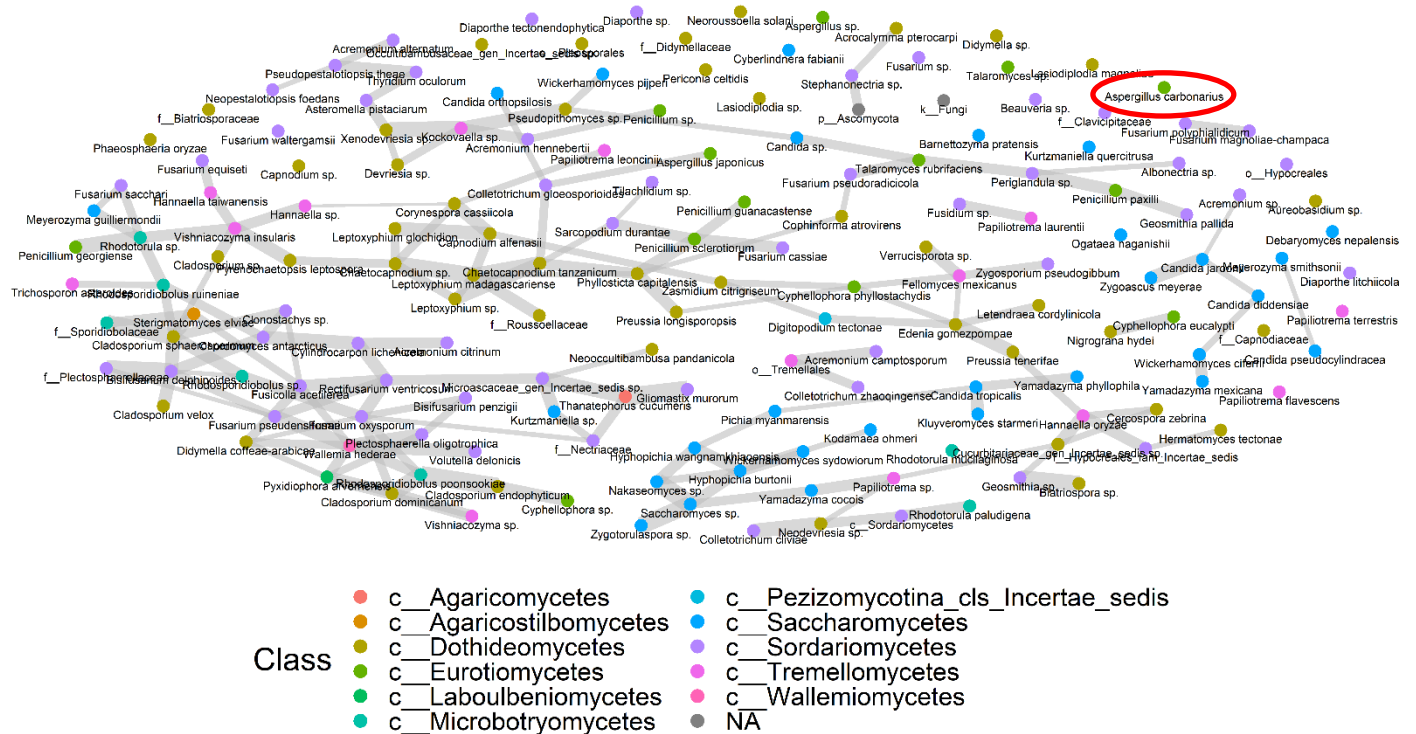


Figure 4.4.2-5. Co-occurrence network diagram (based on SPIEC-EASI) of the fungal species present in at least 3 samples showing cooperative associations (positive interactions). Each node represents a microbe from the mycobiome, and each grey link represents a pairwise co-occurrence. The thickness of the line highlights the degree of interaction between two species, the thickest lines presenting stronger interactions. When the taxonomic identification wasn't possible to the species level, the ASV was identified by the lowest level of the

phylogenetic tree. Species in the red oval corresponds to *Aspergillus carbonarius*.

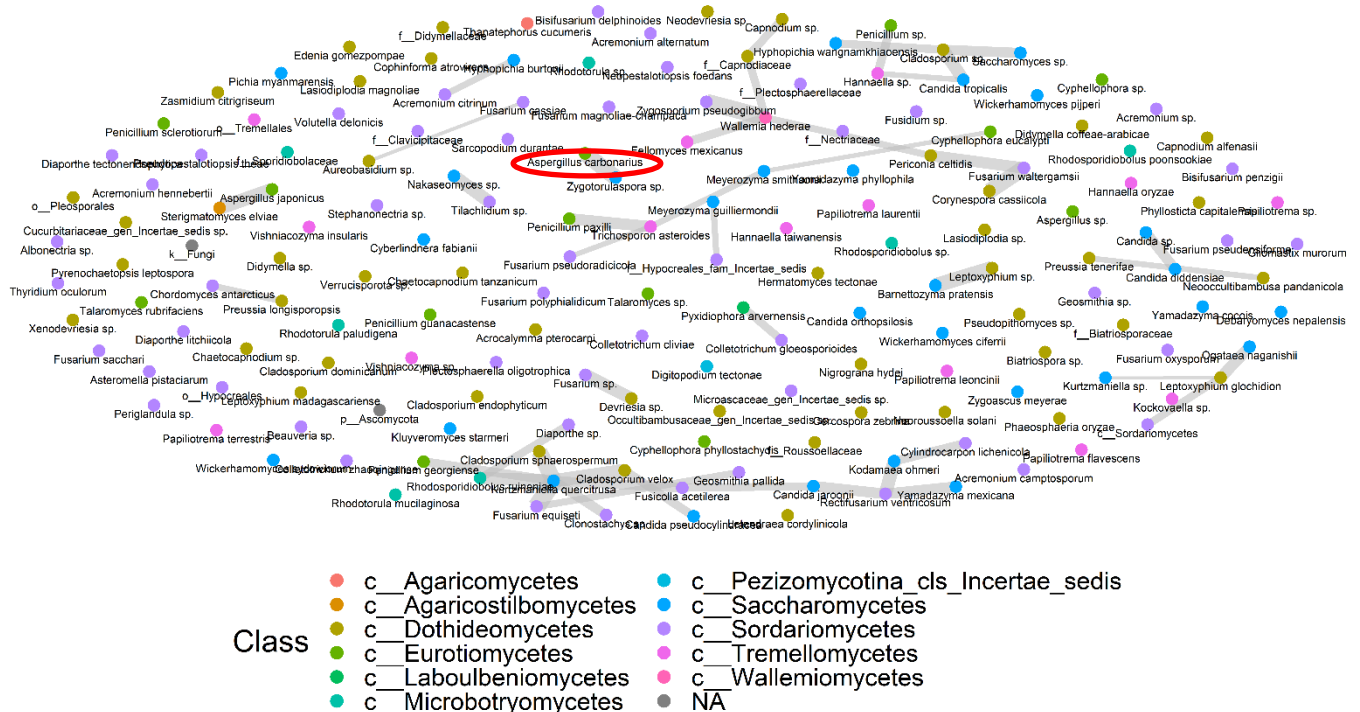


Figure 4.4.2-6. Co-exclusion network diagram (based on SPIEC-EASI) of the fungal species present in at least 3 samples showing opposite associations (negative interactions). Each node represents a microbe from the mycobiome and each gray link represents a pairwise co-occurrence. The thickness of the line highlights the degree of interaction between two species, the thickest lines presenting stronger interactions. When the taxonomic identification wasn't possible to the species level, the ASV was identified by the lowest level of the phylogenetic tree. Species in the red oval corresponds to *Aspergillus carbonarius*.

4.5 Discussion

The present study compared coffee processing practices and associated OTA concentration and fungal community associated with coffee cherry fractions (i.e., husk and bean) and dry coffee cherries from different dry process practices.

The main OTA producers in coffee belong to *Aspergillus* sections *Circumdati* and *Nigri* (FAO et al., 2006). In section *Circumdati*, *A. ochraceus*, *A. westerdijkiae* and *A. steynii* are producers; with the main OTA producers being *A. westerdijkiae* and *A. steynii*. In section *Nigri*, *A. carbonarius* and *A. niger*, are producers, with *A. carbonarius* being the main producer of both (Bucheli & Taniwaki, 2002). *A. carbonarius* has been previously reported as the main OTA producer in Robusta coffee in the South of Thailand, since this variety grows in lower altitudes where temperatures are higher (Paterson et al., 2014). The Robusta coffee used in this experiment was sampled from the South of Ivory Coast at an altitude of 126m, the hotter weather of this region might explain the high relative abundance of *A. carbonarius*. Moreover, *A. westerdijkiae* has been principally isolated in Arabica coffee from Central and South America, and Asia (Maman et al., 2021; Suárez-Quiroz et al., 2004; Taniwaki et al., 2003). Regarding the processing methods, dry method, during which there is a longer exposure to sun, is linked to higher presence of black *Aspergilli* probably due to the high melanin content of these fungi (Pitt & Hocking, 2009).

As it has been previously described (Pardo et al., 2004), the presence of *A. carbonarius* does not guarantee a higher OTA in the coffee, even though this is an important OTA producer. However, the general indigenous postharvest mycobiome could influence the level of OTA, due to competitive effect of some microbial species, especially other yeasts that show inhibition of mycotoxigenic fungi such as *Saccharomyces cerevisiae* (Lino de Souza et al., 2021; Neves et al., 2021; Velmourougane et al., 2011b), *Pichia fermentans* (Pregolini et al., 2021) or *Hanseniaspora uvarum* (Masoud & Kaltoft, 2006a) and therefore improve prevention practices against OTA. Although in this work we did not detect these genera, we still detected an important presence of *Meyerozyma* sp. which belongs to the same order as the previously mentioned yeasts and could potentially have some biocontrol properties against *Aspergillus* sp. as shown by Qiu et al. (2022) against *Penicillium expansum* growth and spore germination.

The results of OTA concentration of the fresh coffee and dry cherry confirmed increase of OTA concentration in processed samples. Previous studies have already identified that poor practices such as slow drying can increase the OTA, especially when coffee stays over 0.80 a_w for four consecutive days (Paterson et al., 2014). In our study this was observed with OTA content increasing in longer drying periods, such as DP and EHDP coffees, contributing to a higher OTA content. Studying the different coffee fractions, allows us to calculate the % of OTA that remains in each coffee fraction. Ninety-five percent of OTA contamination was detected in the husk. This confirms the effectivity of dehusking by reducing the OTA content on coffee beans to a 1-5% of the total OTA level in the dry cherry, considering that the total weight of the dry cherry is composed by 45% husk and 55% bean. However, in view of the heterogeneous reduction of OTA after roasting (Attiya et al., 2021) and the limits set for OTA on roasted coffee (3 μg of OTA per kg of coffee), DP and EHDP beans of our study would not be allowed to produce coffee for human consumption.

This study provides the first data of the effect of heaping on OTA content in Ivory Coast coffee. EHDP samples had the higher amount of OTA content in dried cherries. This suggests that the combination of a longer heaping and drying periods could contribute to a higher OTA content. Additionally, the soaking step didn't significantly increase the OTA content, on the contrary, lower levels of OTA were found in dry cherry and bean fractions of SDP and SHDP. Similarly, the study carried out by Batista et al. (2009) concludes that fermentation happening during wet processing gave lower levels of OTA compared to dry process. This might indicate that steps that enhance controlled fermentations in coffee contribute to a lower OTA contamination.

So far, most mycobiome studies associated with coffee have mainly focused on the evaluation of wet processed Arabica (Silva et al., 2008; Zhang et al., 2019). The only study focusing on fungal communities of dried processed Robusta until today was carried out by Hamdouche et al. (2016). This article uses the Denaturing Gradient Gel Electrophoresis (DGGE) technique for the characterization of the microbial communities and its association to specific post-

harvest processes. They showed that mycobiome structure differs depending on the method applied (wet or dry), geographical origin and coffee species. As well, they identified *Wallemia* sp. as a discriminant fungus in Robusta coffee.

In the present study, the richness, diversity, evenness, and composition of the mycobiome of different coffee fractions at different dry processes was investigated and discussed. Fresh coffee showed a lower diversity compared to the processed coffees, which was also noticed by De Bruyn et al. (2017). Moreover, the beta diversity plot for the fungal diversity analysis demonstrated a clear clustering within all samples based on the type of process, with two different clusters separating the fresh coffee samples and the rest of the processed samples. Those results revealed that there was a higher similarity between the fungal communities after drying, and that fresh coffee samples share a less diverse fungal community.

The results of the fungal composition showed that *Ascomycota* and *Basidiomycota* were the predominantly detected phyla across all samples coinciding with the results on coffee of Vaughan et al. (2015). Freshly harvested coffee cherries show a fungal community structure composed mainly by the genera *Hyphopichia* sp., and *Barnettozyma* sp. (Figure 4.4.23). *Hyphopichia* sp. was previously isolated from coffee waste in Ethiopia (Birhanu et al., 2016). *Barnettozyma* sp. has been previously isolated from coffee husk in the study performed by Cerda et al. (2017). In this work, thanks to their lignocellulose degrader properties, *Barnettozyma* sp. strains were used for the production of cellulases during Solid State Fermentation (SFF).

Once the coffee cherries underwent drying, these microorganisms were succeeded by the genera *Meyerozyma* sp., yeast genus widely detected in dry processed coffee (Hamdouche et al., 2016; Martins et al., 2020; Pereira et al., 2022). As well, filamentous fungi such as the genera *Fusarium* sp. and *Aspergillus* sp. were isolated. The pathogenic genus *Fusarium* sp. is associated to coffee corky-disease (Gamboa-Becerra et al., 2021) and the ochratoxigenic species *A. carbonarius* previously isolated in Robusta coffee (Djossou et al., 2011). Other genus in the top 10 more abundant is *Cladosporium* sp. previously

isolated in West African coffee by Hamdouche et al. (2016) and in Australian (Elhalis et al., 2020) and Brazilian coffee (Silva et al., 2008). This fungus is related to good quality (Silva et al., 2000) and its potential use as Biocontrol Agent against the ochratoxigenic fungi *Aspergillus ochraceus* has been studied by Lima Angelico et al. (2017). Furthermore, *Cylindrocarpon lichenicola* was detected, in a lower relative abundance. This fungus was previously isolated by Alvindia and Acda (2010) in coffee beans from the Philippines.

Moreover, our analysis detected the genera *Lasiodiplodia* sp. and *Leptoxylum* sp. on fresh and dried coffee (data not shown). *Lasiodiplodia* sp. was reported for the first time on Arabica coffee by Freitas-Lopes et al. (2020) and on Robusta coffee by Oliveira Ramos et al. (2023) as the pathogen responsible of dieback disease on coffee plants. However, due to its recent discovery, its effect on coffee losses is not yet well understood. *Leptoxylum* sp., a sooty mould which reduces the photosynthesis of the plant, was previously isolated from Arabica coffee in Thailand (Maharachchikumbura, 2018). Thus, metabarcoding helped into the detection of potential causal agents of diseases in the coffee plants not previously detected in Ivory Coast.

Our study provides a new insight into the relationship between the OTA concentration and the fungal composition and abundance in coffee. It has been observed that at higher OTA concentration, there is also an increased abundance of the ochratoxigenic fungi *A. carbonarius*. This raise in the OTA level might be linked to the conditions undergone during coffee postharvest processing which might increase the growth and OTA production of *A. carbonarius* as shown in other papers (Leong et al., 2007; Taniwaki et al., 2003).

Co-presence/co-exclusion data of fungal genera could help us identify potential useful relationships between the different fungal species within the microbial community, including with *A. carbonarius*. The competitive interactions can enable the identification of microorganisms that could be tested in vitro for their potential biocontrol effect against mycotoxigenic fungi (Zheng et al., 2021). For instance, *A. carbonarius* shows a negative correlation with *Zygorhiza* sp. This genus is closely phylogenetically related to *Torulaspora* sp., genus

containing recently discovered biocontrol strains (Abu-Mejdad et al., 2020; Bosqueiro et al., 2023). Besides, *Zygorhiza* sp. was positively correlated to other yeast such as species of *Saccharomyces* sp. genus which could also be good candidates to control ochratoxigenic fungi.

4.6 Conclusions

This study showed elevated occurrence of OTA on Ivorian coffee samples correlated with high abundance of *A. carbonarius* in the mycobiota of coffee. The coffee processing impacted the OTA concentration and the diversity and species composition of the fungal ecosystem. Strategies such as soaking, or heaping, did not have a significant effect on OTA accumulation in post-harvest treatments. However, extended heaped drying seemed to be the most favourable condition for OTA production by ochratoxigenic fungi such as *A. carbonarius* which was found in the more abundant taxa of the mycobiota. This study is the first time that novel metabarcoding approach were used to establish the composition of the Robusta dry processed coffee and revealed the presence of species that had not been detected before. The results obtained in this work confirm different species composition depending on the process used. Considering all the aspects discussed in this paper, further applied research is needed to find an effective and sustainable post-harvest management strategy to further reduce OTA concentration.

CrediT authorship contribution statements

Claudia Lopez Rodriguez: conceptualization, formal analysis, investigation, writing – original draft, project management; **Caroline Strub:** conceptualization, Writing – Review & Editing, supervision; **Vincent Chochois:** software, formal analysis, data curation, visualization ; **Noël Durand:** validation, investigation; **Carol Verheecke-Vaessen:** Writing -Review and Editing, supervision; **Christophe Jourdan:** investigation, resources; **Angélique Fontana:** validation, supervision; **Tagro Guehi:** resources, supervision; **Angel Medina Vaya:** supervision, funding acquisition; **Sabine Schorr-Galindo:** Writing – Review & Editing, supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data Availability

No data was used for the research described in the article.

Abbreviations

<i>A. carbonarius</i>	<i>Aspergillus carbonarius</i>
IARC	International Agency for Research on Cancer
EU	European Union
ITS	Internal transcribed variant
ASV	Amplicon sequence variant
RA	Relative abundance

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5 Biocontrol activities of yeasts or lactic acid bacteria isolated from Robusta coffee against *Aspergillus carbonarius* growth and ochratoxin A production *in vitro*

5.1 Article presentation

Biocontrol agents have gained popularity recently due to the search of alternatives to chemical products. These biocontrol agents can help reducing fungal development and mycotoxin decontamination of food matrices. The addition of starting cultures before drying is a promising alternative to reduce OTA contamination in coffee.

This article presents the screening of yeasts and bacteria for their antifungal and antimycotoxinogenic capacities against *Aspergillus carbonarius*, one of the main fungus responsible of OTA contamination in coffee. It was found that most of the tested yeast isolates were capable to inhibit fungal growth and OTA production. The evaluation of the yeasts' cell free supernatants and cells against the fungi and the OTA produced alone, allowed to determine that they may use their cell walls or some intracellular metabolite to reduce OTA levels. Interestingly, the results of the Scanning Electron Microscopy confirmed that the yeast *Rhodosporidiobolus ruineniae* adheres to the wall of *A. carbonarius* reducing the turgency of the mycelium and potentially reducing fungal growth. As well, the tests against OTA confirmed that the mechanism to reduce OTA might be adsorption of the mycotoxin to the cell wall of the yeast. Complementarily, another yeast, whose genus was previously identified through Metabarcoding, *Meyerozyma caribbica* proved to reduce OTA through a mechanism of degradation after 24h.

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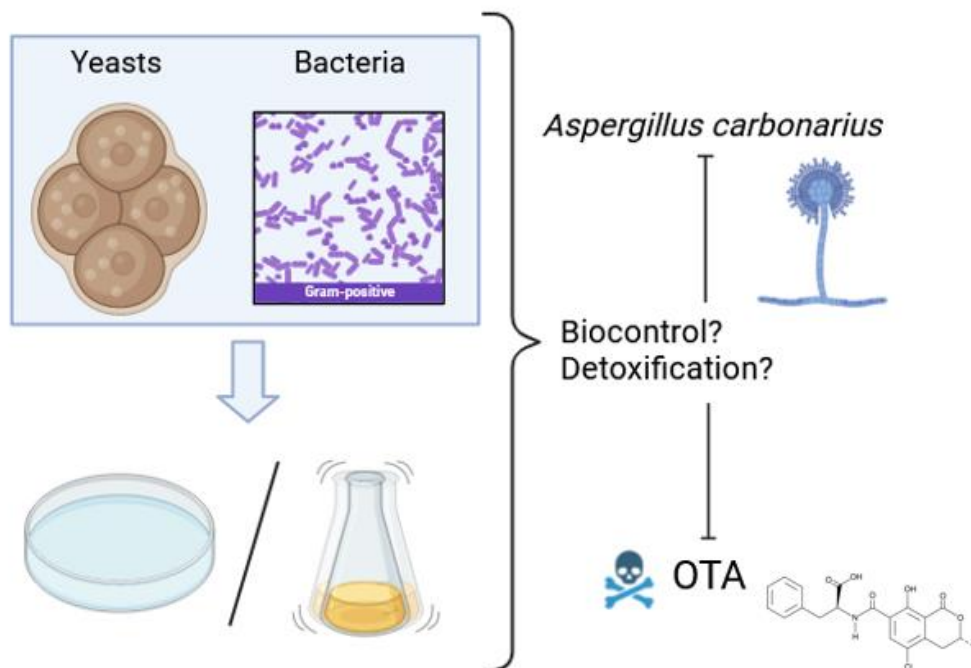


Figure 4.4.2-1. Graphical abstract. Created in Biorender.com

BIOCONTROL ACTIVITIES OF YEASTS OR LACTIC ACID BACTERIA ISOLATED FROM ROBUSTA COFFEE AGAINST *ASPERGILLUS CARBONARIUS* GROWTH AND OCHRATOXIN A PRODUCTION *IN VITRO*

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Highlights

- *Aspergillus carbonarius* is the main ochratoxin A producer isolated in Ivorian Robusta coffee.
- Seventeen yeast isolates reduced *A. carbonarius* growth and Ochratoxin A specific production.
- *Rhodospiridiobolus ruineniae* showed adhesion to the hyphae and Ochratoxin A adsorption.
- *Meyerozyma caribbica* degrades Ochratoxin A *in vitro*.

Abstract: Biocontrol Agents (BCAs) can be an eco-friendly alternative to fungicides to reduce the contamination with mycotoxigenic fungi on coffee. In the present study, different strains of bacteria and yeasts were isolated from Ivorian Robusta coffee. Their ability to reduce fungal growth and Ochratoxin A (OTA) production during their confrontation against *Aspergillus carbonarius* was screened on solid media. Some strains were able to reduce growth and OTA production by 85 % and 90 % and were molecularly identified as two yeasts, *Rhodospiridiobolus ruineniae* and *Meyerozyma caribbica*. Subsequent tests on liquid media with *A. carbonarius* or solely with OTA revealed adhesion of *R. ruineniae* to the mycelium of *A. carbonarius* through Scanning Electron Microscopy, and an OTA adsorption efficiency of 50 %. For *M. caribbica* potential degradation of OTA after 24h incubation was observed. Both yeasts could be potential BCAs good candidates for Ivorian Robusta coffee protection against *A. carbonarius* and OTA contamination.

Key Words: fungi, ochratoxin A, post-harvest, coffee cherries, detoxification, microscopy

5.2 Introduction

Mycotoxins are fungal secondary metabolites toxic to human and animal and remain stable during industrial processing (Campos-Avelar et al., 2020b). *Aspergillus carbonarius* is a mycotoxigenic fungus contaminating food commodities such as cocoa, coffee and grapes and can produce ochratoxin A (OTA) (Cervini et al., 2019; Crespo-Sempere et al., 2010). OTA is a heat-stable mycotoxin with a molecular weight of 400 Da considered mutagenic, nephrotoxic and potentially carcinogenic for humans (group 2B by the International Agency for Research on Cancer (IARC, 1993)). The European Commission have set maximum limit of 3 µg/kg for roasted coffee beans and 5 µg/kg for soluble coffee (European-Commission, 2023).

Biocontrol Agents (BCAs) are becoming a high priority for post-harvest disease control management programmes to reduce the use of chemicals (Taye et al., 2023). BCAs such as yeasts or lactic acid bacteria, have been widely used to reduce contamination by mycotoxins since they are generally recognized as safe for animal and human consumption (GRAS)(Nguyen et al., 2017; Panebianco & Caridi, 2021; Rahayu et al., 2021).

Applying BCAs directly onto the food matrix has been widely used as a strategy for the control of OTA contamination (Álvarez et al., 2021; de Melo Pereira et al., 2016; Di Canito et al., 2021). In coffee, it is during the post-harvest stages that OTA contamination takes place (De Bruyn et al., 2017; Maman et al., 2021). Specific temperature (30 °C) and water activity (a_w ; $a_w > 0.85$) (equivalent to 60 % moisture content) during coffee cherries drying, represent good conditions for *A. carbonarius* growth and OTA production (Joosten et al., 2001). As well, other microorganisms such as Lactic Acid Bacteria and yeasts have been described as dominant microbiota during drying (Silva et al., 2008). Before drying, the application of BCAs could prevent OTA accumulation on coffee (Ben Taheur et al., 2019; Djossou et al., 2011). BCAs can inhibit mycotoxin biosynthesis, and/or detoxify mycotoxins once they are produced, either by binding them to their cell wall, or by degrading them into less toxic compounds (Chiocchetti et al., 2019).

The study of these mechanisms will help to ensure the success of the BCAs once applied on the coffee cherries (Wang et al., 2016; Zhang et al., 2018).

The aim of this work was to isolate from coffee and identify potential BCAs and to study the mechanism behind the OTA reduction. Firstly, the investigation focused on assessing the population of yeasts, bacteria and *Aspergillus* sp. on dried coffee and isolated potential BCAs (bacteria and yeasts) and ochratoxigenic fungi. Then, yeasts and bacteria strains were confronted with *A. carbonarius* to evaluate their inhibitory effect on fungal growth and OTA specific production (OTAsp). Secondly, the interactions between *A. carbonarius* and the yeasts strains were characterised during dual culture assays on a liquid medium. The Cell Free Supernatant (CFS) was added to the growth media of *A. carbonarius* to understand the role of constitutive yeasts metabolites on inhibiting fungal growth and/or OTA biosynthetic pathway. Interactions with OTA were studied by adding the toxin to the media during yeasts growth. Finally, yeasts interactions with the fungus were studied through Scanning Electron Microscopy (SEM), to draw conclusions about potential cell adsorption mechanisms of the BCA.

5.3 Materials and methods

5.3.1 Sampling

Two kilograms of coffee cherries from Robusta coffee (*Coffea canephora*) were sampled as described in section 4.3.1. through Dry Process at an environmental temperature of 25-30 °C on average. The dried cherries were then stored at 4 °C and sent to France in a portable fridge and kept at -20 °C until further analysis. To ensure representativity, before taking any replicate, the 2 kg of coffee were thoroughly mixed (Cheli et al., 2009).

5.3.2 Occurrence and isolation of different groups of microorganisms

Occurrence and isolation of microorganisms was carried out using 3 different culture media. MRS agar and broth (De Man Rogosa Sharpe, Merck) containing 0.1 % cycloheximide (Merck) were used for the isolation of lactic acid bacteria (Junqueira et al., 2022). MEA (Malt Extract Agar, Merck) was used for the

isolation of yeasts and fungi, and DG18 agar (Dicloran Glycerol 18 %, Merck) for the isolation of xerophilic fungi. Both MEA and DG18 media were supplemented with 0.1 % chloramphenicol (Merck) to avoid the growth of gram-positive, gram-negative and *Rickettsia* bacteria (Suárez-Quiroz et al., 2004).

The occurrence of fungi and bacteria was assessed by two different techniques, serial dilution, and direct plating (Czaban & Wroblewska, 2006). The serial dilution consisted of adding 10 g of dried cherries through Dry Process in a bottle containing 90 mL of saline-peptone water 0.1 % (1:10 w/v) mixing for 5 min at 200 rpm, in an orbital shaker, and preparing ten-fold dilutions. 100 µL from each dilution were spread and plated in three plates of MRS media for bacterial populations and on three plates of DG18 and MEA media for fungal populations. After inoculation, plates were incubated at 30 °C for 48 h in anaerobic conditions for bacteria, and 25 °C for 5 days for filamentous fungi and yeasts. The isolated microorganisms were counted (data not shown) and stored at -20 °C in triplicates. As well, the isolated fungi were morphologically characterised.

For direct plating, 90 coffee cherries were surface disinfected (+SD) with 2 % sodium hypochlorite for 10 mins while stirring, then rinsed in sterile water for 20 minutes and then dried and plated. Another 90 coffee cherries were plated without surface disinfection (-SD). Five and ten coffee cherries were directly plated in triplicates on plates of MRS, MEA and DG18 media. After inoculation, plates were incubated at 30 °C for 48 h in anaerobic conditions for bacteria, and 25 °C for 5 days for filamentous fungi and yeasts. Subsequently, the bacterial and yeast isolates were purified and stored at -20 °C in triplicates. All isolates were coded as L for bacteria and Y for yeasts, plus a different number for each isolate. All isolates were used for confrontations on solid media (section 5.3.4.).

To assess the fungal occurrence of *Aspergillus* sections *Flavi*, *Nigri* and *Circumdati*; *Penicillium* and *Fusarium*, these genera were morphologically characterised based on Samson et al. 2019. Then, the number of species was quantified and the number of times the same species was isolated was recorded. Representative strains were isolated, purified and stored at -20 °C. These strains were tested for mycotoxin production capacity as explained in section 5.3.3. To

obtain the isolation frequency, all bacterial and fungal colonies growing from directly plated coffee cherries on MEA and DG18 were recorded and calculated according to the following equation (Urbano et al., 2001):

$$\% \text{ isolation frequency} = \frac{\text{number of coffee cherries colonized by a species}}{\text{total number of coffee cherries per plate}} \times 100$$

5.3.3 Screening for mycotoxigenic fungi

5.3.3.1 Determination of OTA production capacity on Coconut Cream Agar

To identify the isolates producers of OTA all the isolates identified as *Aspergillus* section *Nigri* or *Penicillium* were grown on Coconut Cream Agar (CCA). CCA was prepared from commercial canned coconut cream (Kara) according to Dyer & McCammon (1994) using 400 g of coconut cream, 400 mL of distilled water and 12 g of agar. Fungal isolates were central point inoculated into CCA in duplicate and incubated at 25 °C. They were examined under long UV light (366 nm or 312 nm) at days 3, 5 and 7 after inoculation. The excitation and emission wavelengths range of OTA are 301-395 nm and 400-475 nm, respectively (Cagnasso et al., 2019).

To confirm that the fluorescence produced on CCA was due to OTA, a standard range was created by adding OTA to a concentration of 500, 1000 and 3000 µg/kg, and poured on the 50mm Petri dishes containing 4 mL of CCA before the medium solidified. Fluorescence was visible only above a concentration of 1000 µg/kg of OTA.

5.3.3.2 Quantification of OTA on PDA and Coffee-Based Medium

To select the most ochratoxigenic fungal isolates, all the OTA-producing isolates were tested to confirm OTA production and the mycotoxin was quantified by HPLC-FLD. OTA-producing fungi were grown on PDA in Petri dishes for 7 days at 25 °C. The inoculum was collected by flooding the surface of the plates with sterile peptone water with Tween 80 (0.05 % v/v) and scraping the surface with a spatula. A 10 mL sample of the fungal culture suspension was filtered through sterile cotton to remove mycelium and transferred to a sterile tube and shaken to obtain a homogeneous suspension. The spore concentration was determined

using a Thomas cell counting chamber. Spores' suspension was diluted to a concentration of 10^6 spores/mL. A volume of 5 μ L (10^6 spores/mL) was centrally inoculated and plates were incubated during 9 days at 25 °C, growth measurements and OTA sampling for OTA quantification were made on days 5, 7 and 9 (Gomez-Lopez, et al., 2005). Tests were carried out in triplicate.

To choose the ochratoxigenic fungi with highest potential OTA production on coffee, the 6 highest OTA-producing isolates on PDA were further tested on Coffee Based Medium (CBM). CBM was prepared according to Akbar & Magan (2014), milling 300 g of coffee beans, and then boiling them in 1 L of water for 30 minutes. A double layer of muslin was used to filter the resulting concentrates. These were used at 6 % concentration and 17 g/L of agar was added prior to autoclaving at 121 °C for 15 min. The plates were central point inoculated with 5 μ L of fungal spore suspension (10^6 spores/mL) and incubated for 9 days at 25 °C, growth measurements and sampling for OTA quantification were made on days 5, 7 and 9. Tests were carried out in triplicate.

OTA extraction was made following the protocol described by Campos-Avelar et al. (2020). Half of the content of the Petri dish was transferred into a plastic container. Acidified methanol (3.85 % formic acid) was added to the sample with a ratio of 1.5/5, followed by 20 min of agitation. A total of 500 μ L of the mixture were evaporated, then 2 mL of the mobile phase composed of methanol/water/acetic acid, 69:30:1 was added. Finally, samples were filtered through a 0.45 μ M PTFE filter for the HPLC analysis. Results were expressed in μ g of mycotoxin/kg. The diameter of the fungal colonies was measured using ImageJ (1.52a, Wayne Rasband National Institute of Health, Bethesda, MD, USA, 2018).

5.3.4 Solid-based confrontations against fungi

5.3.4.1 Yeasts

Seventeen yeasts isolated from the dried-processed coffee beans were grown in 10 mL of Yeasts Extract and Glucose liquid medium (YEG) (9 % yeast extract (9 g/L) and 20 % glucose (20 g/L) (D(+)) Glucose monohydrate, Merck, Germany))

at 25 °C during 24 h in agitation. A volume of 100 µL of 10⁶ yeast cells/mL of suspension was spread on the PDA plate, then 5 µL of fungal spores' suspension (10⁶ spores/mL) were centrally inoculated. Plates were incubated for 9 days at 25 °C, growth measurements and sampling for OTA quantification were made on days 5 and 9. Samples were done in triplicate (Gomez-Lopez, et al., 2005).

5.3.4.2 Bacteria

Five bacteria were isolated from the dried-processed coffee beans. In addition to the bacteria isolated in this study, we used 7 strains of *Lactiplantibacillus plantarum* previously isolated from Ivorian Robusta coffee, and kindly provided from a project between the University of Nangui Abrogoua (Ivory Coast) and the French Agricultural Research Centre for International Development (CIRAD) (Beugre et al., 2023). An amount of 100 µL of bacterial suspension (10⁷ CFU/mL) were spread on the surface of MRS plates. After bacterial inoculation, plates were incubated for 24 h to allow the bacteria to grow. Then, 10 µL of 10⁷ spores/mL of fungi was central point inoculated. Plates were incubated for 6 days at 28 °C, growth measurements and sampling for OTA quantification were made on days 3 and 6. Samples were done in triplicate.

5.3.5 Liquid-based confrontations

Two yeast strains identified as *Rhodosporidiobolus ruineniae* (Y1) and *Meyerozyma caribbica* (Y4) were selected because of their strong ability to inhibit fungal growth and OTA production. The yeasts were cultivated in 10 mL of YEG liquid medium in agitation at 25 °C for 24 h. To determine the mechanism of OTA inhibition, yeast Cell Free Supernatant (CFS) and cultured cells were analysed. To obtain the CFS, four mL of cultured cells were centrifuged at 10000 g/5 min/4 °C and supernatants were collected and filtered through Sartorius PES membrane (0.22 µm). The cultured cells were yeast cells growing in the YEG media, the cells were not extracted from the media (Muleta et al., 2007).

The confrontations were run against the highest ochratoxigenic *A. carbonarius*. After a 7 days pre-culture on PDA at 25 °C, 10 µL of fungal spore suspension (10⁷ spores/mL) and 10 µL of yeast suspension of 10⁷ spores/mL or 1 mL of CFS

were inoculated on 15 mL of YEG broth. Flasks were incubated in agitation 170 rpm at 25 °C. OTA and growth inhibition were measured at day 6. OTA was extracted as done on solid media, and growth was measured by weighing the dried biomass of the cells and the mycelium. One negative control inoculated only with the fungal spores, and 2 positive controls, with cells or supernatant were prepared. Everything was run in triplicate.

5.3.6 Adsorption tests

The same two yeasts were tested for their OTA adsorption capacity. Ten mL of PDB at a concentration of 10 µg of OTA/mL were inoculated with 10 µL of yeast cell suspension (10^7 cells/mL) and incubated in Erlenmeyer at 25 °C in agitation for 1, 4, 8 and 24 h. In each sampling point, the cells of the Erlenmeyer's were centrifuged and washed twice with PBS (Armando et al., 2012). One mL volume of final cells, washes, and supernatants were extracted for OTA quantification by LC-MS/MS according to the extraction and quantification protocol developed by Malachová et al. (2014). Analysis was run in five replicates.

5.3.7 Microscopy

Scanning Electron Microscopy (SEM) was performed as follows. The control and treated mycelia of *A. carbonarius* was collected, and the mycelia was fixed with 2.5 % of glutaraldehyde at 4 °C for 24 h, washed with PBS buffer for 3 times for 20 min each time, and post-fixed with 1 % osmic acid (OsO_4) at 4 °C overnight (Yang et al., 2021).

Then dehydrated with distilled water, 30 %, 50 %, 70 %, 85 % and 90 % of ethanol solution for 15 min each time, finally dehydrated twice with 100 % of ethanol for 20 min each time. After dehydration, the mycelia were placed on the slide and dried in CO_2 critical point drying (CPD) instrument (Baltec CPD 030). Subsequently, the mycelia were fixed into stubs and placed on a gold coater holder. Finally, the sample was coated with a 1.4 nm gold layer and examined by SEM (Hitachi S-2600N), visualized, identified, and photographed at different magnifications using the data acquisition software Aztec (Oxford Instruments).

5.3.8 Morphological and molecular identification

The filamentous fungi were initially cultured on PDA medium and observed with an optical microscope for preliminary identification. First, we analysed the colony, especially colour and shape, using the guide of Samson et al. (2019). And then, we proceed to identification of the general *Aspergillus* sp., *Penicillium* sp. and *Fusarium* sp. with microscopic slide examination of spores and conidiophores. For yeasts, all yeasts isolates were characterized by determining their morphology and identified through biochemical test API 20C AUX (BioMerieux).

The most ochratoxigenic fungi, *A. carbonarius* (D3) was identified by sequencing analysis of ITS1 and ITS2 regions, and the partial regions of the β -tubulin and Calmodulin (CaM) genes (primers specified in Table 5.3.8-1). Yeasts strains showing antimycotoxigenic activity, were identified by sequencing analysis of ITS1 and ITS2 regions, and the D1/D2 domains of the 26S rRNA gene (Table 5.3.8-1). DNA was extracted from culture in YEG broth using DNAEasy Plant Pro (Qiagen). The PCR of each primer was performed in a total volume of 25 μ L PCR mixture containing 12.5 μ L Thermo Fisher 2X AmpliTaq Gold™ 360 Master Mix, 10.5 μ L of biomolecular water, 1 μ L of each primer and 1 μ L of DNA (100 ng/ μ L). The reactions were incubated in a Mastercycler X50 (Eppendorf France SAS, Montesson, France) under specific cycling conditions. For the fungi initial denaturation at 94 °C for 5 min, 35 cycles of 94 °C for 50 sec, 55 °C for 50 sec, 72 °C for 1 min, and a final extension for 7 min at 72 °C. For yeasts, initial denaturation at 95 °C for 5 min, 30 cycles of 95 °C for 40 sec, 58 °C for 40 sec, 72 °C for 40 sec, and a final extension for 7 min at 72 °C. PCR products were analyzed on 1.5 % agarose gel electrophoresis and sequenced in both directions in a Seq ABI 3500xL (Sequencing Platform, University of Montpellier). Strain identification was done by MEGA11 for alignment of the sequences. Species names were assigned considering the NCBI database results after blasting the previously aligned nucleotides.

Table 5.3.8-1. Primers used for amplification and sequencing.

Locus	Primer	Direction	Primer sequence (5'-3')	Reference
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ITS	ITS5	Forward	5'-GGA-AGT-AAA-AGT-CGT-AAC-AAGG-3'	(White et al., 1990)
	ITS4	Reverse	5'-TCC-TCC-GCT-TAT-TGA-TATGC-3'	(White et al., 1990)
β -tubulin	Bt2a	Forward	5'-GGT-AAC-CAA-ATC-GGT-GCT-GCT-TTC-3'	(Glass & Donaldson, 1995)
	Bt2b	Reverse	5'-ACC-CTC-AGT-GTA-GTG-ACC-CTT-GGC-3'	(Glass & Donaldson, 1995)
Calmodulin	Cmd5	Forward	5'-CCG-AGT-ACA-AGG-AGG-CCT-TC-3'	(Hong et al., 2005)
	Cmd6	Reverse	5'-CCG-ATA-GAG-GTC-ATA-ACG-TGG-3'	(Hong et al., 2005)
26S rRNA	NL-1	Forward	5'-GCA-TAT-CAA-TAA-GCG-GAG-GAA-AAG-3'	(Taverna et al., 2013)
	NL-4	Reverse	5'-GGT-CCG-TGT-TTC-AAG-ACGG-3'	(Taverna et al., 2013)

5.3.9 Statistics

Statistical analysis was performed using the statistical package JMP® Pro 16 (SAS Institute Inc., Cary NC, USA). The normality and homoscedasticity of the samples set was validated through the Shapiro-Wilk and Levene's tests, respectively. In the case of the screening for the most ochratoxigenic fungi, the results failed the requirements for analysis of variance. Therefore, nonparametric Wilcoxon/Kruskal-Wallis ($p=0.05$) was used for these comparisons. For the rest of results, one-way ANOVA followed by Tukey's Honestly Significant Difference test (Tukey's HSD, $p<0.05$) was performed. The figures were generated using the JMP software.

5.4 Results

5.4.1 Frequency of isolation of bacteria and fungi and identification

Figure 5.4.1-1 shows the relative frequency of isolation of bacteria, yeast, and fungi from dried Robusta coffee cherries when plated with or without surface disinfection. There was a lower frequency of isolation in the disinfected plated coffee cherries than in those which were not disinfected (see Table 5.4.6-1). The fungal isolation also showed that 50% of the isolates belonging to *Aspergillus* and 30% to *Penicillium* were the principal fungal genera contaminating dried coffee cherries. *Aspergillus* sp. section *Nigri* was isolated through all the methods. We also isolated *Aspergillus* section *Flavi* from surface disinfected cherries. None produced aflatoxin when tested for fluorescence on CCA (data not shown). The total number of isolated bacteria, yeasts and *Aspergillus* sp. and *Penicillium* sp. isolates is available in Table 5.4.6-1.

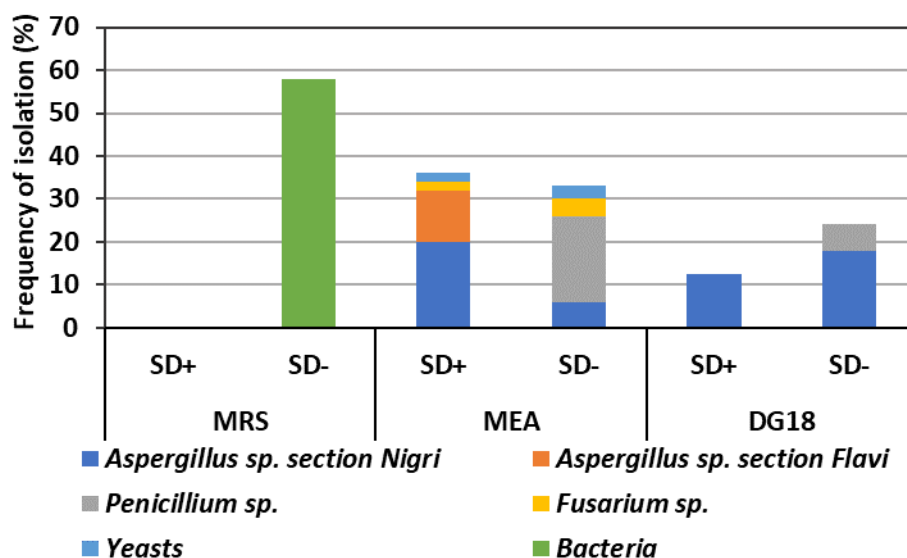


Figure 5.4.1-1. Frequency of bacterial, yeast and fungal isolation (%) in coffee and identified genus. SD+: surface disinfected/ SD-: non-surface disinfected. MRS: De Man, Rogosa and Sharpe agar; MEA: Malt Extract Agar, DG18: Dichloran-glycerol 18% agar.

5.4.2 Screening for the ability to produce Ochratoxin A

Forty-seven isolates of *Aspergillus* section *Nigri* were macroscopically and microscopically characterized, purified through monosporic culture, and assessed for OTA production using CCA (Table 5.4.6-1).

A total of forty *Aspergillus* section *Nigri* isolates with the highest fluorescence on CCA were analysed for OTA production on PDA by HPLC-FLD quantification. Among these, 6 were the highest producers of OTA, and were selected for further tests on coffee media (Table 5.4.2-1). The production of OTA was media-dependant, with PDA after 9 days being the most conducive for OTA production for all the isolates. For the toxigenic isolate D3 (Table 5.4.2-1) the amount of OTA produced was higher than in for the rest of isolates in all the media analysed and was chosen for further confrontations against BCAs.

Table 5.4.2-1. OTA production by the *Aspergillus* section *Nigri* strains isolated from dried coffee cherries.

Strain	Isolation strategy	OTA concentration (µg/kg)		
		PDA/day 9	CBM1/day 12	CBM2/day 12
M1	MEA dilution	32988.89 ± 4098.00	103.32 ± 0.95	2915.56 ± 97.42
M2	MEA disinfection	30629.33 ± 5781.30	158.00 ± 31.11	3250.22 ± 74.79
M3	MEA disinfection	25304.44 ± 1552.86	161.36 ± 32.23	4436.89 ± 4.39
D1	DG18 dilution	29948.44 ± 32501.20	184.22 ± 14.26	2951.11 ± 398.49
D2	DG18 disinfection	20355.56 ± 3037.70	96.62 ± 9.86	2741.78 ± 241.98
D3	DG18 Direct	36095.56 ± 4101.32	409.33 ± 130.10	4554.67 ± 148.96

OTA + producer/ - non producer. OTA – Ochratoxin A; coffee sample used only for isolation; values correspond to average ±SD (n=3). CBM1 – whole dry cherry; CBM2 – ground coffee.

5.4.3 Screening for potential biocontrol agents (BCAs) against *A. carbonarius* on solid media

A total of 17 yeasts isolates, 7 strains of *L. plantarum*, and 5 bacteria isolated from Ivorian coffee were confronted against *A. carbonarius* to test their capability to inhibit the growth and OTA production of the fungus. Most of the yeast isolates showed antagonistic activity on mycelial growth and OTA production of the toxigenic fungus, from which two isolates were further studied due to their specific ability as BCAs and belonging to different genera.

All 17 yeast isolates from the dried coffee were tested by in vitro dual culture assays against *A. carbonarius* (D3) on PDA medium at 25 °C for 5 and 9 days. OTA-specific production (sp) was calculated according to Campos-Avelar et al. (2020). Our results show that, after 5 days of incubation, all the strains significantly reduced *A. carbonarius* growth. From the 17 isolates Y1, Y4, Y5, Y6, Y7, Y8, Y9, Y10, Y11, Y12, Y13, Y14, Y15 and Y16 showed a growth inhibition of at least 70%. With regards to the OTA-sp, Y1, Y4, Y6, Y7, Y8, Y9, Y10, Y11, Y12, Y13, Y14 and Y15 were the most efficient at reducing it in at least a 70%. Only the yeast Y2 did not significantly reduce the OTA-sp. As well, Y3 exhibited a lower OTA-sp during the confrontation, even though the OTA sp was significantly lower. At day 9, most of the yeasts significantly reduced the growth and the OTA-sp by at least 90% and 85%, respectively (Figure 5.4.31). Except for strains Y2 and Y3, which showed an inhibition of *A. carbonarius* growth of an 80% and OTA-sp reduction in a 50% (Figure 5.4.3-1).

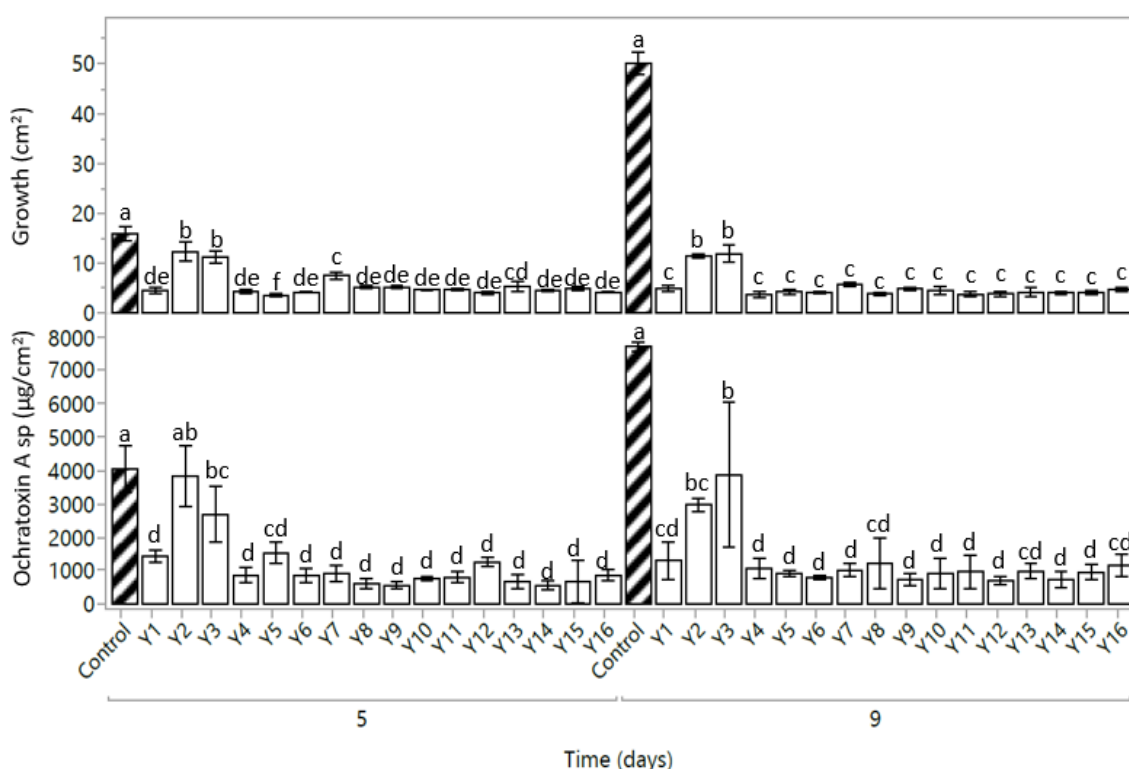


Figure 5.4.3-1. Effect of 17 strains of yeasts in overlay culture assay with *Aspergillus carbonarius* on fungal growth and Ochratoxin A sp (specific

production (ng/cm²) on PDA at days 5 and 9. Different letters express significant differences according to Tukey-Kramer HSD ($p < 0.05$).

During the dual culture assays, different growth inhibition levels were observed, as shown in Figure 5.4.3-2. Y2 showed a lower reduction of growth than Y1 and Y4. As well, the shape of the mycelium varied depending on the confrontation.

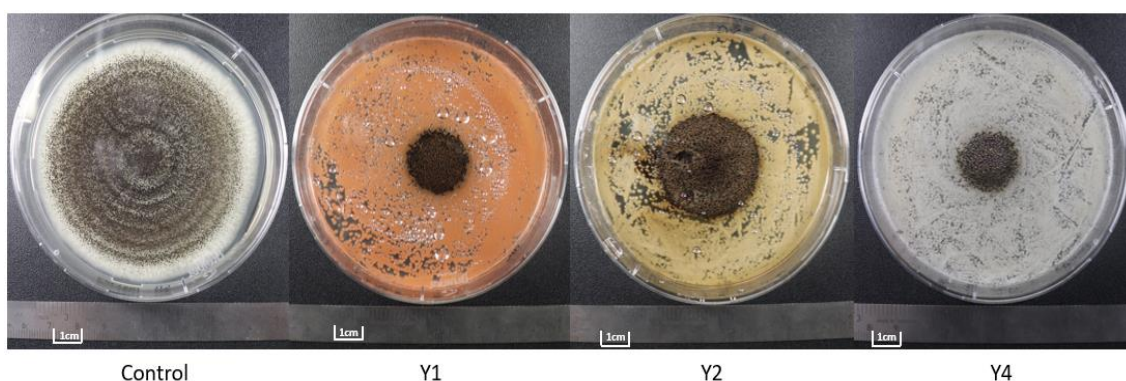


Figure 5.4.3-2. Example of inhibition profiles observed during overlay culture assays of yeasts strains Y1, Y2 and Y4 against *Aspergillus carbonarius* on PDA at 25°C for 9 days. Scale measures 1cm.

Figure 5.4.3-3 depicts the effect of 7 strains of *L. plantarum* (D12, D13, D20, D23, D24, D31, D32) and 5 isolates of bacteria (L1, L2, L3, L4, L5) on confrontation against *A. carbonarius* at days 3 and 6. At day 3, all isolates except L4 showed a significant growth inhibition. Interestingly, the isolate L1 showed a significant growth inhibition of a 50% while at the same time a 1.5 times significantly higher OTA-sp. Strain D24 and isolates L2 and L3 were the only ones showing between two- and three-times lower OTA-sp at day 3. At day 6, only the strain D24 and isolate L2 showed a significant growth inhibition, decreasing between 3 and 1 folds the fungal growth, respectively. For OTA-sp none of the bacterial strains or isolates demonstrated significant reduction at day 6.

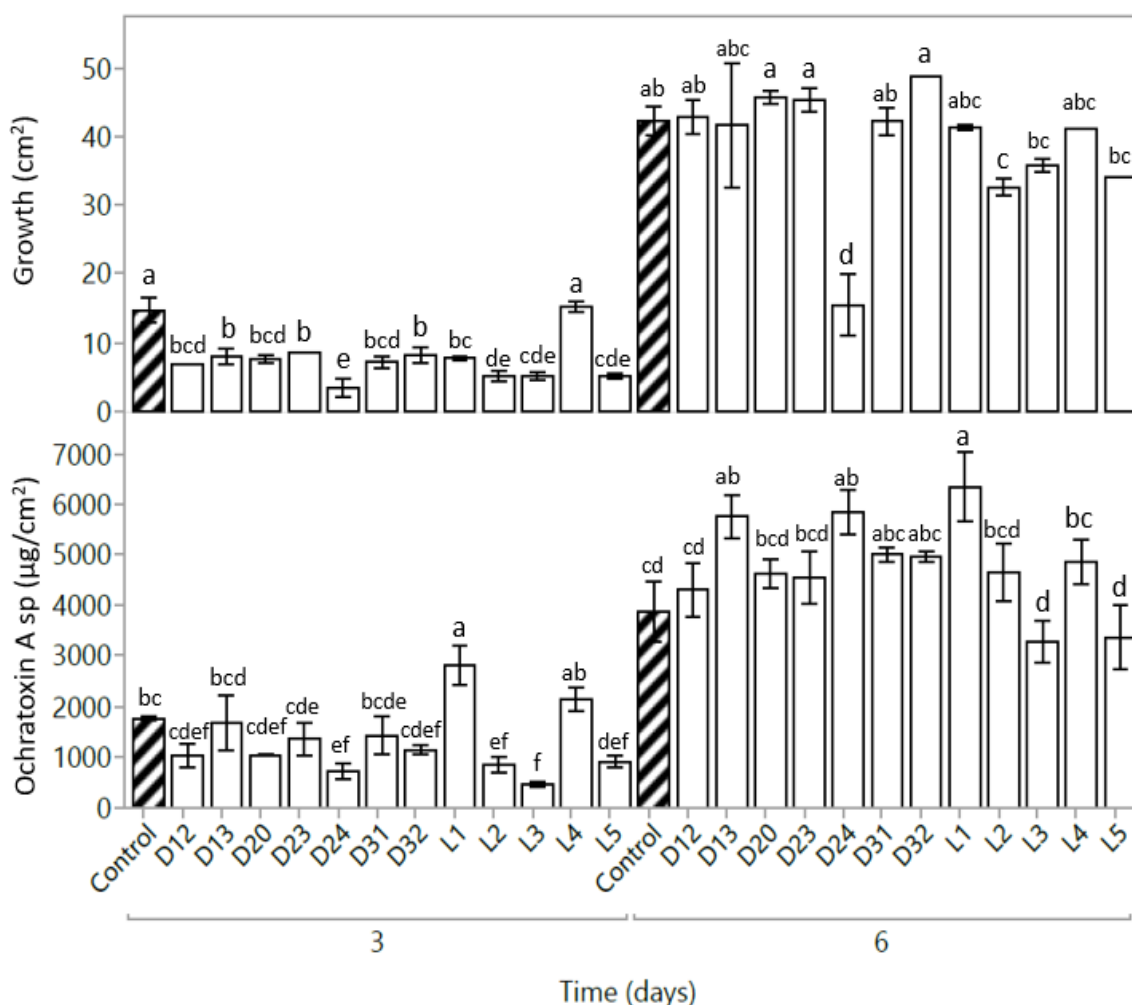


Figure 5.4.3-3. Effect of 7 strains of *L. plantarum* (D12, D13, D20, D23, D24, D31, D32) and 5 strains of bacteria (L1, L2, L3, L4, L5) in overlay culture assay with *Aspergillus carbonarius* on fungal growth and ochratoxin A (OTA) specific production (ng/cm²). Each confrontation represents the average of three replicates.

Since the screened yeasts reduced the concentration of OTA more efficiently than bacteria, further identification was performed, and the yeasts were tested by API. The identification showed Y1 as *Rhodotorula mucilaginosa*, Y2 as *Cryptococcus laurentii* and Y3 to Y15 as *Candida guilliermondii*. Y1 and Y4 (Table 3) were selected for further experiments as these had the most inhibitory capacity for OTA sp and belong to different genera. The selected ochratoxigenic fungus and the two yeast strains was validated by molecular identification (Table 5.4.3-1).

Table 5.4.3-1. Yeasts, bacteria and fungi used for confrontation experiments.

	Strains	Origin	API Identification	Molecular identification
Biocontrol agents	Y1	Coffee	<i>Rhodotorula mucilaginosa</i> (98%)	<i>Rhodosporidiobolus ruineniae</i>
	Y4	Coffee	<i>Candida guilliermondii</i> (73%)	<i>Meyerozyma caribbica</i>
Pathogen	D3	Coffee		<i>Aspergillus carbonarius</i>

5.4.4 Screening for potential biocontrol agents (BCAs) against *A. carbonarius* in liquid media

The chosen yeast strains cells and their Cell Free Supernatant (CFS) were tested against *A. carbonarius* in liquid media (Table 5.4.4-1). The cells showed a significant reduction of the growth and OTA production ($p < 0.05$). Y1 reduced by 2-folds the growth and totally inhibited the OTA production. Y4 reduced by 1-fold the growth and 13-folds the OTA production. CFSs added in the liquid growth medium of *A. carbonarius* did not show significant inhibition of fungal growth and OTA production ($p > 0.05$).

Table 5.4.4-1. Effect of yeast cells' culture and Cell Free Supernatants (CFS) against *Aspergillus carbonarius* growth and OTA production in liquid media after 6 days. Values with different letters are statistically different ($p < 0.05$). Data are means $\pm$ standard deviations (SDs).

Selected strains	Dry weight (g)		Ochratoxin A concentration ($\mu\text{g/kg}$)	
	Cells	CFS	Cells	CFS
Control	0.12 $\pm 0.02^a$	0.12 $\pm 0.02^a$	2306.01 $\pm 2839.19^a$	2306.01 $\pm 2839.19^a$
<i>Rhodosporidiobolus ruineniae</i> (Y1)	0.05 $\pm 0.01^{bc}$	0.12 $\pm 0.01^a$	<LOD ^{1b}	1149.41 $\pm 2189.62^a$
<i>Meyerozyma caribbica</i> (Y4)	0.07 $\pm 0.02^b$	0.12 $\pm 0.01^a$	167.21 $\pm 169.03^b$	1434.80 $\pm 812.00^a$

¹LOD (Limit of Detection): 0.5 ng/mL

5.4.5 Adsorption test

The two yeast strains (Y1, Y4) were further tested for OTA adsorption capacity (Table 5.4.5-1). As shown in Table 5.4.5-1, *Rhodosporidiobolus ruineniae* (Y1) reduced by 50% the amount of OTA in the culture medium after 1h and 24h incubation. For Y1, a 20% of OTA was recovered from the 1st wash and the cell pellet after 24h, indicating a weaker binding of the OTA to the yeast's cell wall. *Meyerozyma caribbica* (Y4) reduced by 80% the amount of OTA in the culture medium after 24h (Table 5.4.5-1) and did not impact OTA quantity at 1h and 8h (data not shown). The removed OTA quantity was only partially recovered from the media, washing solutions and cell pellets at a percentage of recovery between 45 and 100 %.

Table 5.4.5-1. Ochratoxin A (OTA) recovery from adsorption tests. Cells were suspended in PDB in the presence of OTA (10 µg/mL) and incubated at 25°C for 1h and 24h. Data are means ± standard deviations (SDs); NA means non applicable.

Process	Control		<i>Rhodosporidiobolus ruineniae</i> (Y1)		<i>Meyerozyma caribbica</i> (Y4)	
	1h	24h	1h	24h	1h	24h
OTA in culture medium (µg)	9.87 ±0.43	9.903 ^a ±0.37	4.52 ±0.64	5.46 ^c ±0.33	10.05 ±1.63	1.96 ^b ±1.16
OTA recovered from 1 st washing solution of yeast pellet (µg)	NA	NA	<LOD ¹	0.98 ±0.06	<LOD	2.79 ±1.04
OTA recovered from 2 nd washing solution of yeast pellet (µg)	NA	NA	<LOD	<LOD	<LOD	0.91 ±0.01
OTA recovered from cell pellets	NA	NA	<LOD	0.87	<LOD	0.98 ±0.01
Total recovery (%)	98.74	99.03	45.15	73.10	100.00	66.40

¹LOD (Limit of Detection): 0.91 ng/mL

5.4.6 Microscopy

The interaction between *Rhodosporidiobolus ruineniae* (Y1) and *A. carbonarius* on solid media was further studied under Scanning Electron Microscope (SEM; Figure 5.4.6-1). Figure 5.4.6-1.a shows *A. carbonarius* mycelium in absence of Y1. Figure 5b, 5c and 5b.1 and 5.c.1. shows *R. ruineniae* attached to the

mycelium and internal wall or septum of the hyphae. As well, Fig. 5b.1 and 5c.1 show from up close the less turgent state of the mycelium during the confrontation against *R. ruineniae*. In Figure 5.4.6-1b.1 and c.1 the blue arrows indicate yeast cells, and the orange arrows fungal mycelium.

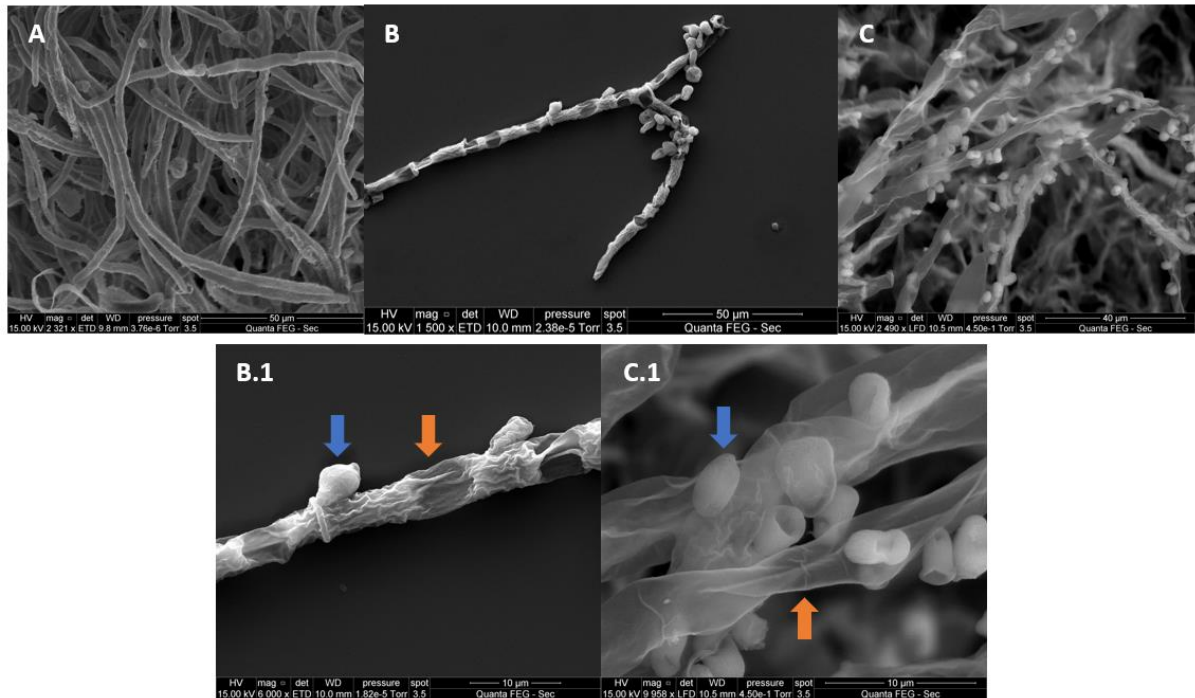


Figure 5.4.6-1. SEM analysis of antagonistic effect of *Rhodosporidiobolus ruineniae* Y1 yeast against *A. carbonarius*. (A) Hyphae of *A. carbonarius* with increase of 2321 X, (B) Hyphae of *A. carbonarius* co-cultured with Y1 yeast at day 3 with increase of 1500 X, (C) Hyphae of *A. carbonarius* co-cultured with Y1 at day 6 with increase of 2480 X. B.1 and C.1 are a zoom of B and C, with 6000 X and 9958 X increase, respectively. Blue arrow indicates yeast cells, orange arrow points *A. carbonarius* hyphae.

5.5 Discussion

This is the first study assessing in vitro biocontrol activities of bacteria and yeasts against ochratoxigenic fungus isolated from Ivorian coffee. This is particularly important as Ivory Coast is considered an important Robusta coffee producing country (Bellachew, 2009). Along the coffee postharvest it is during drying when the undesired mycotoxigenic fungi proliferate due to high humidity (Bucheli et al., 2000). Good Management Practices (GMP) such as sorting of cherries or drying in homogeneous and thin layers, can partially improve the mycotoxigenic level of the final dried coffee beans (Codex Alimentarius, 2009). However, complementary techniques are needed such as the application of BCAs (Habschied et al., 2021; Medina et al., 2017).

In the present study on Robusta Ivorian coffee, we found that *Aspergillus* section *Nigri* isolates represent more than 50% of the total amount of fungal isolates. Species belonging to *Aspergillus* section *Nigri* have been previously isolated on Arabica coffee in Brazil (Taniwaki et al., 2003) and Mexico (Suárez-Quiroz et al., 2004); and on Robusta coffee in Vietnam (Hashimoto et al., 2015) and in Ivory Coast (Olga et al., 2015). Olga et al., (2015) identified that 52% of the isolates from Robusta Ivorian coffee beans belonged to section *Nigri*. *A. carbonarius* was previously reported as the species with the most ochratoxigenic capacity (Leong et al., 2007). Our result confirms the high prevalence of *Aspergillus* section *Nigri* and particularly *A. carbonarius* in Robusta Ivorian coffee. This suggests that section *Nigri* might be predominant in Robusta coffee from Ivory Coast. However, this might not be linked to the type of coffee or the location, but to the drying method, since dry-method is related to more black *Aspergilli*, due to the melanin content they have more chances to survive, and wet-method to section *Circumdati*, during which drying time above 0.80 aw is critical for OTA production (Paterson et al., 2014).

In this work, three of the thirty-three isolates of *Penicillium* sp. showed fluorescence on CCA, and from these one of them showed production of OTA after incubation on PDA for 5 days. Vega et al. (2006) found strains of *Penicillium* sp. with ochratoxigenic activity isolated from coffee plants. However, this genus

is not commonly found on coffee beans during postharvest, due to the low water activities that are reached at the end of drying and kept during storage. Nevertheless, it is interesting to analyse the presence of this genus and its ochratoxigenic activity on coffee beans in future studies, in order to reach a global perspective of the changes in the ecotoxicology of coffee beans.

In this study, we also isolated twelve isolates belonging to *Aspergillus* section *Flavi* from surface disinfected cherries. None produced aflatoxin when tested for fluorescence on CCA (data not shown). In a study carried out by Batista (2003) eighteen strains of *Aspergillus flavus* were isolated from Brazilian Arabica coffee, and one-third of the strains produced aflatoxins B1 and B2. In a recent study, Pandit et al. (2015) isolated strains of *Aspergillus* section *Flavi* but did not analyse the production of aflatoxins. According to Paterson et al., 2014, the presence of *Aspergillus* section *Flavi* strains will become more regular due to higher temperatures associated with climate change. This will lead to the potential emergence of aflatoxins, the most poisonous mycotoxin (Frisvad et al., 2019). Besides, the impact of this fungus in the coffee mycobiota is uncertain and might give place to competition with the proposed BCAs (Duong et al., 2020). To predict the effect of this fungus, it is fundamental to analyse the aflatoxin production of potentially aflatoxigenic isolates from coffee, alone and in competition with the BCAs and other fungi found in coffee.

The use of bacteria as BCAs to reduce OTA in coffee has been widely studied (de Melo Pereira et al., 2016, 2020; Junqueira et al., 2022; Pereira et al., 2016). Lactic Acid Bacteria (LAB), including *L. plantarum*, isolated from coffee have been identified as the BCAs with the best results inhibiting *A. carbonarius* growth on synthetic media (Djossou et al., 2011). In our study, 7 isolates of *L. plantarum* were tested but none showed efficient fungal growth and OTA production. This might be due to a reduced number of bacteria cells in the inoculum or the use of different synthetic media for the confrontations (PDA in our experiment, CYA in the study mentioned). As well, due to the low resistance of bacteria to lower water activities, the efficiency of this kingdom to the coffee drying step might reduce its efficiency as BCA *in situ*.

In the present work, *Rhodospiridiobolus ruineniae* (Y1) and *Meyerozyma caribbica* (Y4) mechanisms involved in the reduction of the fungal growth and the OTA production was further investigated. Competition for nutrients and space is a shared mechanism that they use in combination with other mechanisms as shown in the results on solid media. Similar results have been obtained in previous studies by using other yeasts as BCAs against ochratoxigenic fungi. Armando et al. (2013) analysed the capacity of *Saccharomyces cerevisiae* reducing the growth and OTA production during the confrontation against *A. carbonarius*. *S. cereveisiae* significantly reduced the growth and the OTA production of *A. carbonarius*, suggesting as inhibitory mechanism competition for nutrients and space. As well, de Souza et al. (2021) studied the ability of different yeast strains such as *Candida* sp., *Meyerozyma* sp., *Pichia* sp., *Rhodotorula* sp. or *Saccharomyces* sp. between others to reduce growth and OTA production of *A. ochraceus* and *A. carbonarius*. These authors found that *S. cerevisiae* strains used volatile organic compounds and competition for nutrients and space that hampered spore production, mycelial growth and OTA production, being the only efficient strains both in vitro and in vivo. Similarly, in the present study the reduction of growth and OTA is due to a combination of mechanisms.

Besides the competition for nutrients and space, Y1 uses adsorption by the yeast wall, as shown by the 50% reduction of the OTA in the media after 1h incubation. *S. cerevisiae* OTA adsorption capacity have previously been studied, with an OTA reduction of up to 91 % in 15 min (Ul Hassan et al., 2021). It is interesting to notice the low OTA recovery during the incubation with Y1, this could be linked with the nature of the bond between the yeast and OTA as discussed by Mateo et al. (2010) during their work on detoxification by bacteria isolated from wine.

In our study *Meyerozyma caribbica* (Y4) isolated from Robusta coffee was identified as a potential BCA candidate. The species *M. caribbica* has been previously isolated in Arabica coffee (Martins et al., 2020; Pregolini et al., 2021) and has been widely used as starter culture for enhancing coffee's organoleptic quality (Bressani et al., 2020). Y4 would use competition for nutrients and space in combination with degradation, since Y4 reduced the concentration of OTA in

the culture medium in an 80% after 24h incubation. During the degradation process, OTA could possibly degrade into less toxic molecules such as O α and L- β -phenylalanine (Campos-Avelar et al., 2020b; Shetty & Jespersen, 2006). However, in our study no O α was found during the incubation of Y4 with OTA. This suggests that either a subsequent degradation step into unknown products or a different mechanism of detoxification happened which needs to be investigated.

This study highlights for the first time the potential of *M. caribbica* and *R. ruineniae* to also improve coffee safety towards OTA. *M. caribbica* and *R. ruineniae* strains should be tested in vivo on coffee beans, as de Melo Pereira et al. (2016) did with *Pichia fermentans* against *A. westerdijkiae* on coffee beans. This would help to select the BCA that adapts better to the coffee substrate biochemical and microbial peculiarities while assessing its effectiveness and resilience under different climate conditions.

5.6 Conclusions

This study points at *Aspergillus* section *Nigri* fungi as the main OTA contaminant on Ivorian coffee, from which a strain of *A. carbonarius* was selected as the highest OTA producer. Different strains of bacteria and yeasts were isolated to test their ability as BCAs in confrontation against *A. carbonarius*. From 12 strains of bacteria and 17 of yeasts, two yeasts, *R. ruineniae* (Y1) and *M. caribbica* (Y4), were selected for their remarkable capacity of reducing fungal growth and OTA production and were further studied to determine the mechanisms involved in the inhibition. Confrontations in liquid media of the cell-free supernatant demonstrated that the mechanism is not related to extracellular enzymes produced by the yeast cells. Instead, the tests in liquid media inoculated with OTA showed that Y1's inhibition might be related to an adsorption of the mycotoxin to the cell wall, and Y4's inhibition due to a degradation of the mycotoxin. Considering all aspect discussed in this paper, these promising BCAs provide a solid base to verify their effectiveness on coffee cherries *in vivo*, their resilience under future climate change conditions and better know and understand their modes of action.

CrediT authorship contribution statements

Claudia Lopez Rodriguez: conceptualization, formal analysis, data curation, visualization, investigation, writing – original draft, project management; **Caroline Strub:** validation, supervision; **Angélique Fontana:** Writing – Review & Editing, validation, supervision; **Carol Verheecke-Vaessen:** Writing -Review and Editing, validation, supervision; **Noël Durand:** validation, investigation; **Corinne Beugré:** resources; **Tagro Guehi:** Writing – Review & Editing, resources; **Angel Medina Vaya:** Writing – Review & Editing, supervision, funding acquisition; **Sabine Schorr-Galindo:** Writing – Review & Editing, supervision, funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data Availability

No data was used for the research described in the article.

Abbreviations

<i>A. carbonarius</i>	<i>Aspergillus carbonarius</i>
IARC	International Agency for Research on Cancer
EU	European Union

OTA	Ochratoxin A
ITS	Internal Transcribed Spacer
CCA	Coconut Cream Agar

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5.8 Supplementary materials

Table 5.4.6-1 shows the number of isolates obtained when doing serial dilution, direct plating of disinfected and non-disinfected beans. The results show bacteria and *Aspergillus* section *Nigri* got the higher number of isolates.

Table 5.4.6-1. Summary of the number of bacteria, yeasts and filamentous fungi isolated from coffee.

Type of isolation / Media		Bacteria		Yeasts	Filamentous fungi		Total
					<i>Aspergillus</i> section <i>Nigri</i>	<i>Penicillium</i>	
Serial dilution		MEA	--	11	13	15	39
		DG18	--	1	7	5	13
		MRS	28	--	--	--	28
Direct plating	Non-disinfected beans	MEA	--	3	3	10	16
		DG18	--	0	9	3	12
		MRS	58	--		--	58
	Disinfected beans	MEA	--	2	10	--	12
		DG18	--	--	5	--	5
		MRS	--	--	--	--	
Total		86	17	47	33		

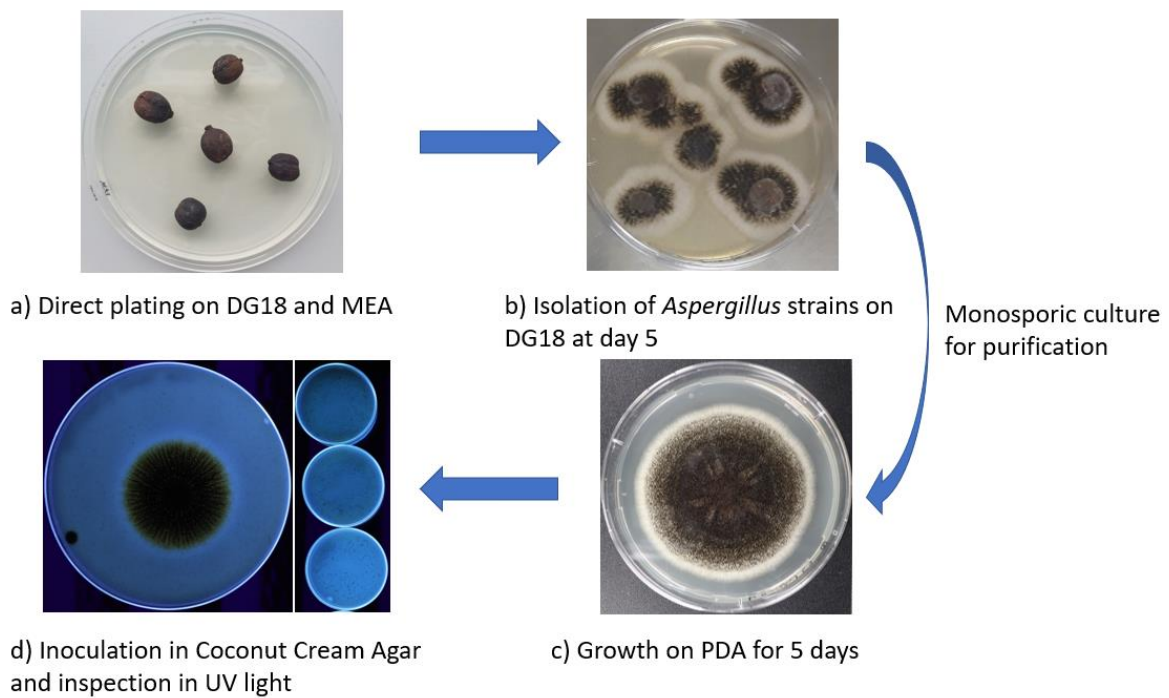


Figure 5.4.6-1. Process of (a) direct plating, isolation of *A. carbonarius* (b), purification through monosporic culture (c) and detection of fluorescence under UV light (d).

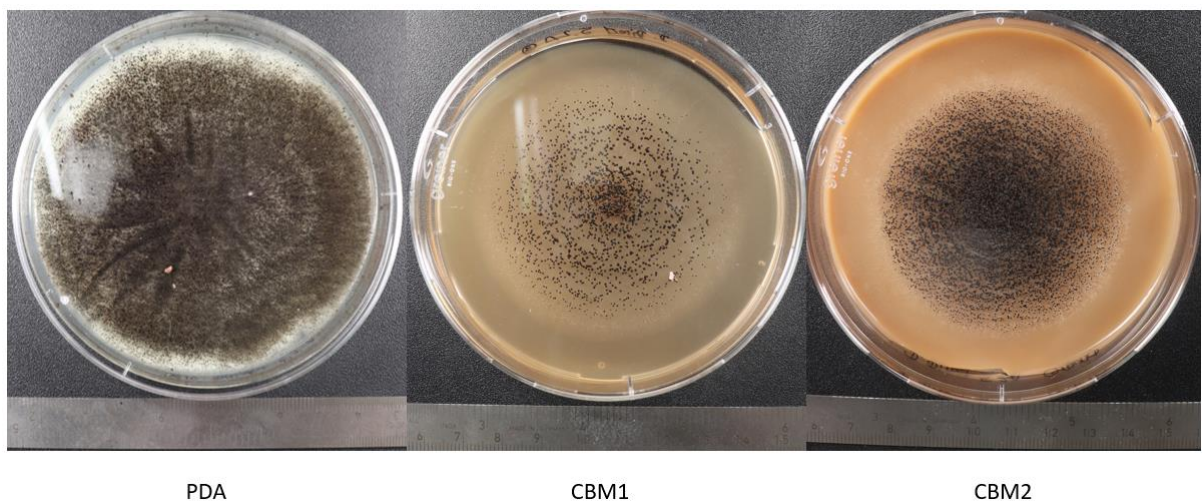


Figure 5.4.6-2. Plates from the studies of *A. carbonarius* when using PDA at day 9, and CBM1 – whole dry cherry and CBM2 - ground coffee, as substrate on day 12 at 25°C for the toxigenic strain D3.

6 Biocontrol resilience in confrontation with *Aspergillus carbonarius* under simulated climate change scenarios in Ivorian Robusta coffee *in vivo*

6.1 Article presentation

By 2050, global warming might be responsible of an increase of between 2 to 4 degrees, more frequent periods of drought and the CO₂ levels are expected to reach 1000 ppm. The impact of climate change on coffee contamination with mycotoxins is a significant worry. Projections indicate that coffee might experience an increase in ochratoxin A and also aflatoxin contamination as a consequence of these changing climatic conditions.

In this study, two strains, *Rhodosporidiobolus ruineniae* and *Meyerozyma caribbica*, isolated from coffee showed the best results inhibiting *A. carbonarius* growth and OTA production in previous chapters. This is why, they were chosen for the confrontation against *A. carbonarius in vivo* on coffee beans. This experiment was run under current and future conditions of water activity, temperature, and CO₂ to assess the resilience of the strains in different scenarios. The results showed that *M. caribbica* was the better adapted to different combinations and thus, the most resilient one.

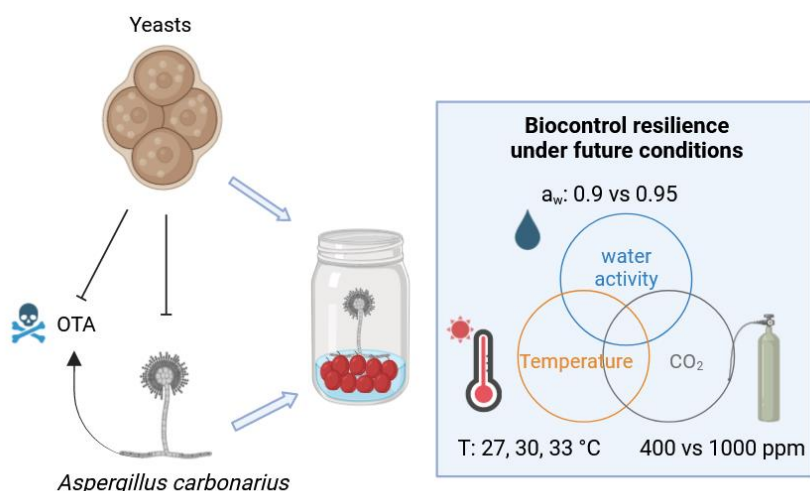


Figure 5.4.6-1. Graphical abstract. Created in Biorender.com. Conditions of incubation were temperature (T), water activity (a_w), and CO₂.

6.2 Preliminary work

To carry out an experiment *in vivo*, it is important to make sure that the results of the inoculation of the coffee cherries will be reliable. For this, we checked that the surface sterilization technique we use is efficient at eliminating the microorganisms that can bias our results. We then performed a pre-experiment where fresh cherries were exposed to different times of UV light. Then, the cherries were directly plated on three different media and incubated for 72h to observe the growth of microorganisms.

In the results in Supplementary materials 2, it is shown the control plates (cherries without UV exposure) and the plates with cherries exposed to UV light for 1h15mins. The plates exposed to UV light presented at least 90% less contamination than the control. This means that the UV light is an effective tool reducing the microbial contamination of coffee cherries.

Another preliminary experiment was the creation of an adsorption curve adapted to our coffee cherries (Supplementary material 3). This curve helps predicting the amount of water needed to be added to 5 g of coffee in order to reach a specific water activity a_w . For its creation, we dried 100g of fresh coffee cherries until they reached $<0.7 a_w$. Then, different amount of water from 1 to 6 mL were added to each 5 g of coffee. The coffee was sealed in a universal glass vial and left overnight at 4 °C so coffee adsorbs the water and equilibrates. Next day, the coffee was left on the bench for 2 h and then the final a_w was measured.

BIOCONTROL RESILIENCE IN CONFRONTATION WITH *ASPERGILLUS CARBONARIUS* UNDER SIMULATED CLIMATE CHANGE SCENARIOS IN IVORIAN ROBUSTA COFFEE *IN VIVO*

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Abstract: *Aspergillus carbonarius* is the main producer of Ochratoxin A (OTA) in coffee from Ivory Coast. To control the presence of this mycotoxin in coffee, there has been an increasing interest to develop formulations of yeast strains in the last years. Climate change abiotic conditions of increased temperature (+2-4 °C), elevated CO₂ (existing levels of 400 vs. 1000 ppm), and drought stress will impact on the control of pests and diseases. This study has examined the effect of three-way interaction of temperature (27, 30 and 33 °C) x water activity (a_w) (0.90 and 0.95 a_w) x CO₂ (400 vs. 1000 ppm) on *A. carbonarius* growth and OTA contamination and the resilience of three yeast strains biocontrol capacity on fresh coffee cherries. Low a_w (0.90) and higher CO₂ and temperature levels boosted the production of OTA by *A. carbonarius*. All the strains tested significantly reduced the fungal growth by at least 20% and OTA production up to 85% *in vivo*. From the three strains used, strain *Meyerozyma caribbica* (YE) showed the best resilience to climate change scenarios since it reduced both growth (20-50%) and OTA contamination (60-85%) and should be selected for scale-up in farm experiments.

Keywords: biological control, fungi, mycotoxin, *Coffea canephora*, safety, post-harvest control, abiotic factors.

6.3 Introduction

In 2021/2022, 10.1 million tonnes of coffee were produced worldwide (ICO, 2023). The main food safety concern linked with coffee is the contamination with Ochratoxin A (OTA). This mycotoxin, produced by *Aspergillus* spp. in coffee and *Penicillium* sp. in another foods, represents a major safety concern due to its acute and chronic effects on humans. Health impact of OTA ingestion may include mutagenicity, possible carcinogenicity (group 2B by the International Agency for Research on Cancer (IARC)), hepatotoxicity and nephrotoxicity (IARC, 1993). Maximum regulated levels established by the European Union (EU) range between 3 and 5 µg/kg, respectively for roasted coffee beans and soluble coffee (European-Commission, 2023).

The main sources of OTA contamination in Ivorian Coast coffee are *A. carbonarius* (Lopez-Rodriguez et al., 2023). The growth and OTA production of these species can be influenced by variables such as temperature, water activity (a_w) and the ambient concentration of CO₂ (Medina et al., 2017). By the year 2100, global temperatures are predicted to be 2-4°C warmer than today, the frequency of droughts is expected to rise and CO₂ levels would reach 1000 ppm (IPCC, 2023). The three-way interacting climate-related abiotic factors have been previously studied to evaluate their influence on the growth and production of OTA by *Aspergillus* sections *Circumdati* and *Nigri* on stored coffee beans (Akbar et al., 2016, 2020b). This study found a stimulation of the OTA production by *A. westerdijkiae* under elevated CO₂ and low water activity (0.90, a_w) at 35°C, seven times higher than under 400ppm CO₂ levels.

The use of biocontrol microorganisms during coffee postharvest have shown the capacity to modulate the final OTA level (Medina et al., 2017). Microorganisms such as yeast, have shown promising results during coffee fermentation thanks to their resistance to variable environmental conditions and their absence of spores or toxic metabolites during its inhibitory action (Lino de Souza et al., 2021; Masoud & Kaltoft, 2006b). Yeasts are part of coffee's endogenous mycobiota and play a crucial role in breaking down coffee mucilage while regulating the presence of detrimental microorganisms (Lopez-Rodriguez et al., 2023). Some of them are

considered Biocontrol Agents (BCA) thanks to their ability to reduce the growth and OTA production of ochratoxigenic fungi (Velmourougane et al., 2011b).

The use of BCA to reduce OTA production by *Aspergillus* section *Circumdati* and *Nigri* under the double effect of temperature and a_w has been previously explored on coffee-based media (Neves et al., 2021). In this study, they found that the strain *S. cerevisiae* CCMA0159 reduced growth and OTA production by 80 and 70%, respectively for all toxigenic strains. As well, this strain showed good resilience under temperatures and a_w between 18-28 °C and 0.95 and 0.99 a_w . However, nothing is known of the resilience of these BCA under the triple effect of temperature, a_w and elevated CO₂ on fresh coffee cherries.

The aim of this study was to identify a potential resilient BCA against *A. carbonarius* and OTA production on coffee cherries. We studied the effect of the three-way interacting climate-related abiotic factors (temperature (27-33 °C) x drought stress (0.90-0.95 a_w) x existing (400 ppm) and elevated (1000 ppm) CO₂) in fresh coffee cherries on i) *A. carbonarius* growth and OTA production, and ii) BCA efficiency of three yeast strains to reduce growth and OTA production of *A. carbonarius*.

6.4 Materials and methods

6.4.1 Coffee sampling

Following the same process as described in section 4.3.1, 19 kg of fresh Robusta coffee cherries, not processed, were collected and stored and transported at 4 °C. Upon reception, all samples were then kept at -20 °C. The coffee was cultivated. The coffee was irradiated to eliminate the microbiota while conserving the biochemical composition. This process started by placing the coffee in a Petri dish without cover, inside a Biosafety cabinet equipped with UV-C (254.7 nm) lamps. The coffee was then irradiated with UV-C for 1 hour and 15 minutes and turned every 15 minutes (Byun et al., 2020).

6.4.2 Yeasts and fungal strains

Three yeasts were isolated from Ivorian Robusta coffee cherries. Based on phenotypic characteristics, biochemical tests and sequence analyses of rRNA genes, the following species were identified: *Rhodospordiobolus ruineniae* (Y1), *Vishniacozyma* sp. (Y2), and *Meyerozyma caribbica* (Y4). They were selected as BCA based on their capacity to reduce fungal growth and OTA production in previous in vitro studies (Lopez-Rodriguez et al., 2024). The native toxigenic strain of *A. carbonarius* was isolated from Ivorian Robusta coffee cherries and characterised as high OTA production capacity was used as the ochratoxigenic fungus (Lopez-Rodriguez et al., 2024).

6.4.3 Inoculation of coffee beans

The yeast strains were sub-cultured on PDB (Potato Dextrose Broth) and incubated in agitation for 48 h at 25 °C. Then, the cells were counted using a counting chamber (Neubauer improved, Paul Marienfeld, GE) and suspended in a 1 % (w/v) Tween®80 solution. From this solution, a specific volume was added to reach a final concentration of 10^6 cells/g of fresh coffee. The ochratoxigenic strain was sub-cultured on PDA (Potato Dextrose Agar). After 7 days at 25 °C, the spores were removed from the colony using a sterile loop, and transferred to a 1 % (w/v) Tween®80. Similarly, from this solution, a specific volume was added to reach a final concentration of 10^3 spores/g of fresh coffee (Gomez-Lopez, et al., 2005). The final stock solution was used to modify the a_w of the cherries. The control and confrontations were run in five replicates.

6.4.4 Incubation conditions and elevated CO₂ treatment

Fifteen grams of fresh coffee cherries were aseptically distributed into sterile glass culture vessels of a 100 mL capacity (Magenta™, Sigma, USA) with a lid vented (10 mm with polypropylene membrane 0.22 µm pore size) to allow gases exchange but keep the environment inside the vessel sterile. Then, 2 or 12 ml of sterile water was added to reach 0.90 or 0.95 a_w respectively based on the moisture adsorption curve (see Appendix B.2). After adding the water, the jars were manually shaken twice to ensure good rehydration and were kept at 4 °C

for 24 h for full adsorption and equilibration (Gasperini et al., 2019). Next day, the jars were inoculated with 1 ml of inoculum, either yeast and fungus or only fungus, depending on the treatment, and equally shaken to ensure the inoculum soaked all the cherries. All treatments were run in five replicates. The jars were placed in 12-L air-tight containers and incubated in a dynamic climatic chamber (SANYO Electric Co., Ltd., MLR-350H) at three different temperatures (27, 30, 33°C) corresponding to the current (27 °C) and future temperatures of the southeast region of Ivory Coast. The temperature inside the incubators was controlled during the whole length of the experiment with a thermometer. The containers included a beaker (300 mL) of water with glycerol at 0.90 and 0.95 a_w to minimise moisture loss (Medina et al., 2015). They were flushed twice a day either with current (400 ppm) or increased (1000 ppm) CO₂ concentrations by using specialty grade mixtures gas cylinders (British Oxygen Company, Guildford, Surrey, UK), with a flow rate of 3 L·min⁻¹ to renew 3× the air volume of the incubation containers (Verheecke-Vaessen et al., 2019)(Figure 6.4.4-1). After 7 days, the samples were collected, and oven dried at 65 °C for 48 h. The samples were visually assessed for fungal growth and a scoring was assigned according to figure S1 prior to storage at 4 °C.

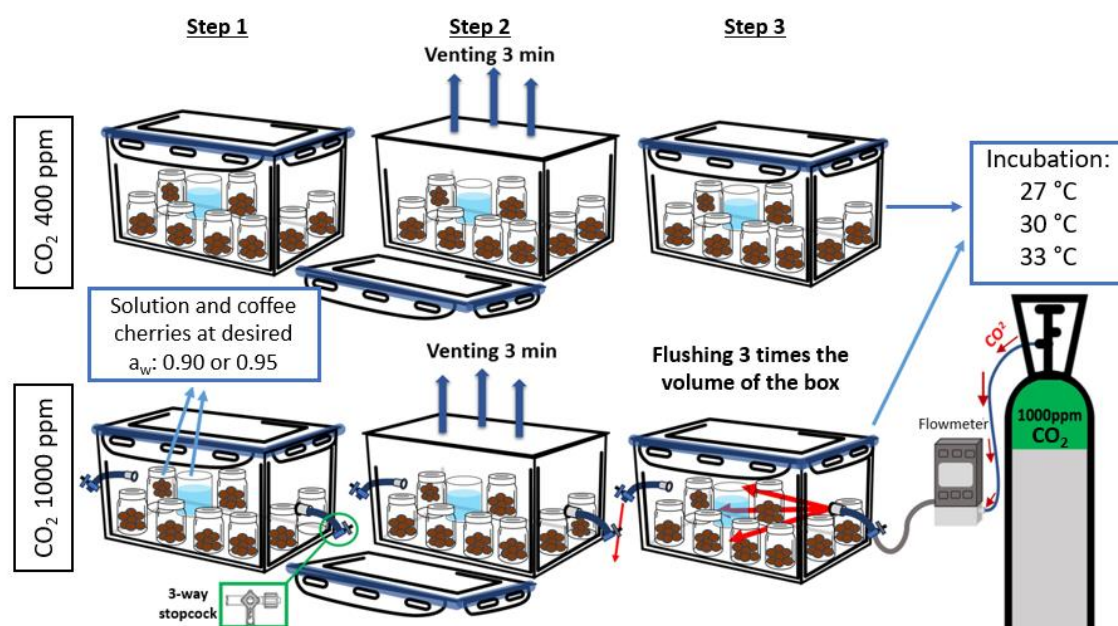


Figure 6.4.4-1. Representation of the incubation setting and conditions for the confrontation experiments under different climatic conditions *in vitro* on fresh coffee cherries. Figure modified from Dr Alessandra Gasperini's Thesis Manuscript.

6.4.5 Ochratoxin A quantification

The samples were processed according to Malachova et al., (2018). Briefly, the sample were ground using the laboratory blender with stainless steel blade (Waring, Stamford, USA). Five grams of sample were extracted with 20 mL of extraction solvent (acetonitrile/water/acetic acid 79:20:1, v/v/v). The samples were extracted for 90 min using a VWR VX-2500 Multitube Vortexer (VWR, Radnor, PA, USA) at 1800 rpm speed, and subsequently centrifuged for 5 min at 3000 rpm on a centrifuge (Thermo Scientific Sorvall ST40 Centrifuge, Loughborough, UK). The extracts were transferred into glass vials and diluted with the same volume of dilution solvent (acetonitrile/water/acetic acid 20:79:1, v/v/v).

Quantification was performed using a qTRAP-LC-MS/MS 6500+ system (Exion Series) without further pre-treatment. An ACE 3-C₁₈ column (2.1 × 100 mm, 3 μm particle size; Hichrom, Berkshire, UK) equipped with a C₁₈ security guard cartridge (4 × 3 mm, Gemini Agilent, Oxford, UK) kept at 60°C was used for

chromatographic separation with the following gradients: Solvent A: water:methanol:acetic acid (89:10:1, v:v:v) and Solvent B: methanol:water:acetic acid (97:2:1, v:v:v), both supplemented with 5 mM ammonium acetate, which was applied for 20 min. The flow rate of the mobile phase was 0.6 mL/min, and the injection volume was set to 1 μ L. MS/MS was performed in a scheduled multiple reaction monitoring (MRM) with a 60 s window. Analyst version 1.6.3 (AB SCIEX™, Framingham, MA, USA) and MultiQuant version 3.0.3 (AB SCIEX™, Framingham, MA, USA) were used for data acquisition and analysis, respectively, following the method described in Malachova et al., (2018).

6.4.6 Data analysis

Statistical analysis was performed using the statistical package JMP® Pro 16 (SAS Institute Inc., Cary NC, USA). Shapiro-Wilk tests was used to determine normality and Levene's test to assess the equality of variance. The OTA production data failed normality therefore on Fit Least Squares tests ($P=95\%$) were used for analysis, followed by LS Means Tukey HSD test to assess potential differences across treatments.

6.5 Results

6.5.1 Effect of climate change on *A. carbonarius* growth

The growth score was assessed on a scale of 1 to 5, where a rating of 1 denoted minimal growth, and 5 corresponded to the highest level of growth observed, equivalent to the growth of the control group, which consisted solely of fungus (Figure 6.5.4). The control exhibited consistent growth across varying temperature, water activities and CO₂ levels tested.

6.5.2 Effect of BCA and abiotic factors on fungal growth

Upon co-inoculation of *A. carbonarius* with BCA, notable alterations in fungal growth became evident. As outlined in Table 6.5.2-1, the yeast YE consistently decreased the growth of *A. carbonarius* by at least one level. Conversely, Y1 and Y2 demonstrated inhibitory effects on fungal, primarily observed at lower a_w (0.90 a_w). Regarding the combined effect of the BCA and the exposure to different

climate conditions, the growth score changed according to the type of strain and the a_w . Factors such as CO₂ or temperature did not impact the growth scoring. Strains Y1 and Y2 demonstrated comparable outcomes, diminishing the growth to a level 3 at 0.90 a_w , with no reduction in growth observed at 0.95 a_w . Strain Y4 showed a growth of level 2 at 0.90 a_w and level 4 at 0.95 a_w .

Table 6.5.2-1. Growth level of *A. carbonarius* during confrontation with Y1, Y2 and Y4 at different CO₂, temperature, and water activities levels. Scoring set according to Figure 6.5.4, where 5 represents same growth as control, and 1 no growth.

Strains	CO ₂	Temperature	Water activity	Growth
Y1	400/1000ppm	27/30/33	0.90	3
	400/1000ppm	27/30/33	0.95	5
Y2	400/1000ppm	27/30/33	0.90	3
	400/1000ppm	27/30/33	0.95	5
Y4	400/1000ppm	27/30/33	0.90	2
	400/1000ppm	27/30/33	0.95	4

6.5.3 Effect of climate change on *A. carbonarius* ochratoxin A production

As presented in Table 6.5.4-1, all factors significantly impact the production of OTA by *A. carbonarius*. Under current CO₂ conditions, there was a lower OTA production at higher temperatures (Figure 6.5.3-1). This reduction was significant at 30 and 33 °C and 0.90 a_w , and at 0.95 a_w the reduction was significant only at 33 °C. While at current CO₂ levels the OTA was significantly reduced at higher temperatures, under future CO₂ levels (1000 ppm) the OTA production did not show significant differences between temperatures. Exposure to elevated CO₂ levels led to a significant increase of OTA production at 33 °C at both a_w .

Optimum conditions for OTA production vary between CO₂ levels, being 27 °C the optimum at 400 ppm CO₂, and between 27 and 33 °C at 1000 ppm CO₂. Regarding a_w, at lower CO₂ and lower a_w OTA production was optimal, while at higher CO₂, it was a high a_w that OTA production was higher. So, at higher CO₂, the optimal conditions for OTA production are between 30 and 33 °C and 0.95 a_w, while at lower CO₂ levels, it is at 27 °C and 0.95 a_w that present optimal OTA production.

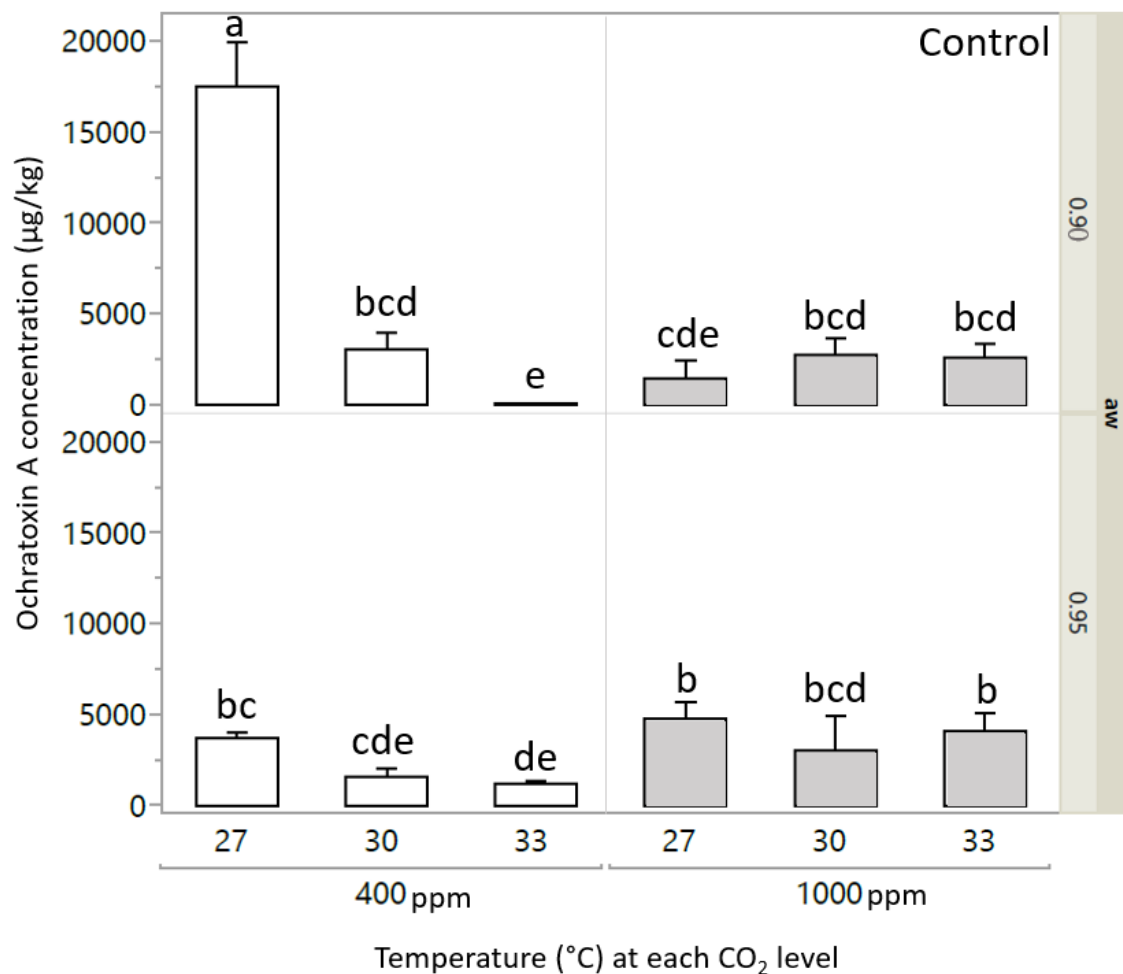


Figure 6.5.3-1. Effect of CO₂ and water activity at 27, 30 and 33 °C on the Ochratoxin A concentration on fresh coffee inoculated with *A. carbonarius*. Different letters indicate significant differences ($p > 0.05$) between different temperatures at a specific a_w and CO₂ using Fit Least Squares test and LSMeans differences Tukey HSD. Bars represent average (AV) ± standard deviation (SD).

6.5.4 Effect of BCA and abiotic factors on ochratoxin A production

The impact of BCA strains on OTA reduction was further investigated jointly with the three-way interacting CC factors. All single factors and possible interacting factors have a significant effect ($p < 0.05$) on the % of reduction of OTA (Table 6.5.4-1).

Under current CO₂ conditions and 0.90 a_w , there is a strong reduction of OTA at 27 and 30 °C (Figure 6.5.4-1). At 0.95 a_w , the reduction is higher at 33 °C, and significantly higher by the strain Y4. Under future CO₂ conditions and 0.90 a_w there is a strong reduction of OTA at 30 and 33 °C. The OTA reduction by the three strains is higher at 27 °C and 0.95 a_w than at 0.90 a_w . Higher CO₂ and temperature induce an increased reduction of OTA by the three strains at 0.90 a_w . At 0.90 a_w , there is a difference in OTA reduction between current and future CO₂ levels. Under current CO₂ levels, OTA reduction is higher at lower temperatures, while at future CO₂ levels, OTA is reduced more at higher temperatures. Under both present and future CO₂ conditions strains Y1 and Y4 significantly reduce the production of OTA under more scenarios.

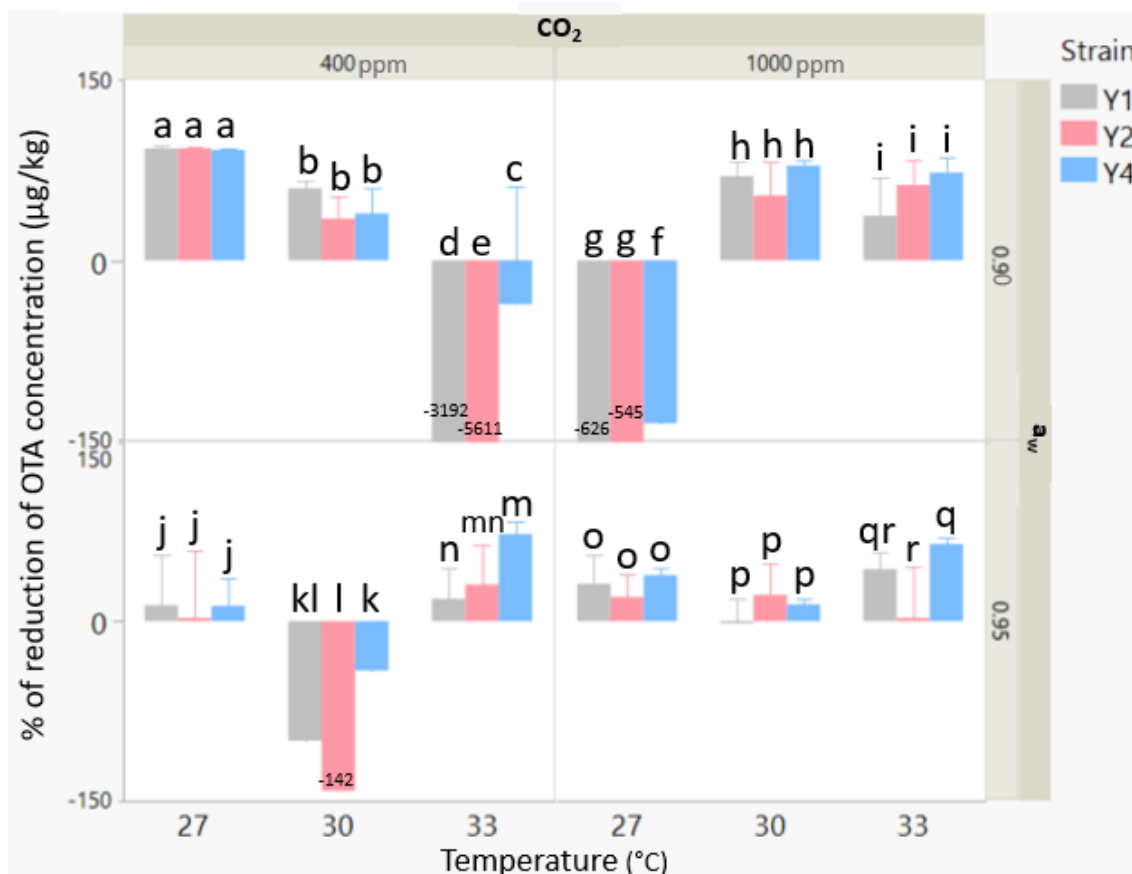


Figure 6.5.4-1. Effect of CO₂, temperature, and water activity on the % of reduction of ochratoxin A concentration in fresh coffee cherries. Different letters indicate significant differences ($p>0.05$) between strains at the same CO₂, temperature and a_w , using Fit Least Squares method and Tukey HSD test. Bars represent $AV\pm SD$. Values in negative correspond to an increase in the production of OTA.

In Table 6.5.4-1, the significance of the effect of abiotic factors on each strain's capacity of OTA's reduction is analysed. For strains Y1 and Y2 all factors have a significant effect on their capacity of OTA reduction. For strain Y4, factors CO₂ and a_w do not affect the reduction of OTA.

Table 6.5.4-1. Summary of the statistical analysis for the effect of interacting climate-related abiotic factors on % of reduction of ochratoxin A by each BCA after 7 days. The probability values in bold were significant ($p<0.05$). Results

based on Fit Least Squares Test for the effects of water activity (a_w), temperature (T), CO₂ level and BCA.

Factors	DF	<i>p</i> -value (Prob > F)	Factors	DF	<i>p</i> -value (Prob > F)
		OTA (μg/kg)			OTA (μg/kg)
BCA: Y1					
a_w	1	<0.0001	T* CO ₂	2	<0.0001
T	2	<0.0001	T* a_w	2	<0.0001
CO ₂	1	0.0001	CO ₂ * a_w *T	2	<0.0001
a_w *CO ₂	1	0.0005			
BCA: Y2					
a_w	1	<0.0001	T* CO ₂	2	<0.0001
T	2	<0.0001	T* a_w	2	<0.0001
CO ₂	1	<0.0001	CO ₂ * a_w *T	2	<0.0001
a_w *CO ₂	1	<0.0001			
BCA: Y4					
a_w	1	0.3581	T* CO ₂	2	<0.0001
T	2	0.0027	T* a_w	2	<0.0001
CO ₂	1	0.9192	CO ₂ * a_w *T	2	<0.0001
a_w *CO ₂	1	0.0089			

As shown in Figure 6.5.4-2, Strains Y1 and Y2 significantly increased the production of OTA at 400 ppm of CO₂, 33 °C and 0.90 a_w . The statistics show that there is a significant difference for Y1 and Y2 at 400 ppm CO₂, 33 °C and

0.90 a_w versus the remaining conditions tested. This might be due to the high variance result of the variability of the replicates. Consequently, although not significantly, both strains also increased the production of OTA at 400 ppm, 30 °C and 0.95 and at 1000 ppm, 27 °C and 0.90 a_w . Overall, Y4 increased OTA production less than the other two strains. However, under 1000 ppm, 27 °C and 0.90 a_w Y4 significantly increased OTA production.

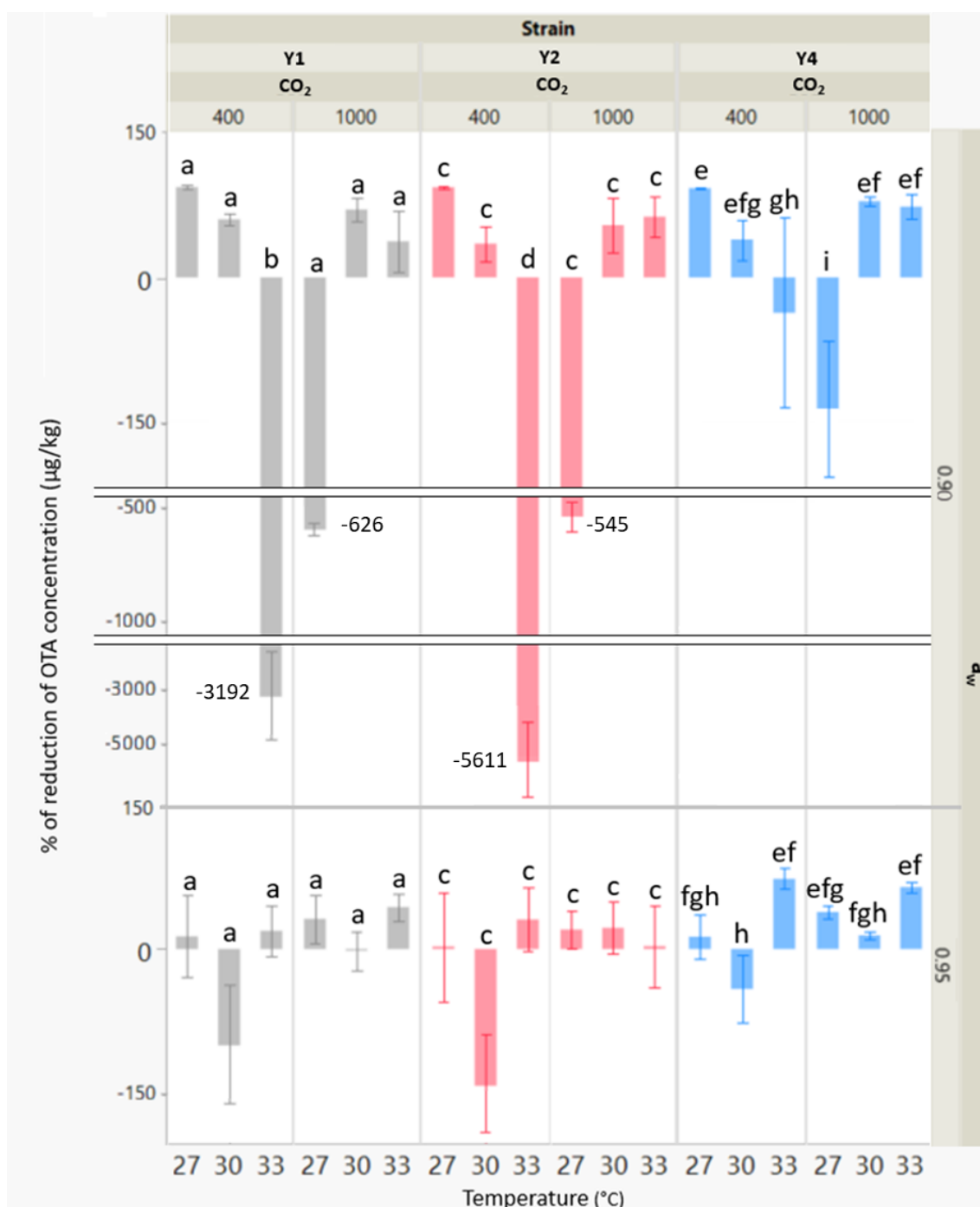


Figure 6.5.4-2. Effect of CO₂, temperature, and water activity on the % of reduction of ochratoxin A concentration in fresh coffee cherries. Different letters indicate significant differences ($p < 0.05$) in the % of reduction of OTA of each strain, using Fit Least Squares method and Tukey HSD test. Bars represent $AV \pm SD$. Values in negative correspond to an increase in the production of OTA.

In Figure 6.5.4-3, for Y1 and Y4, elevated CO₂ increases the reduction of OTA in most scenarios. While for Y2, elevated CO₂ influences it under less scenarios.

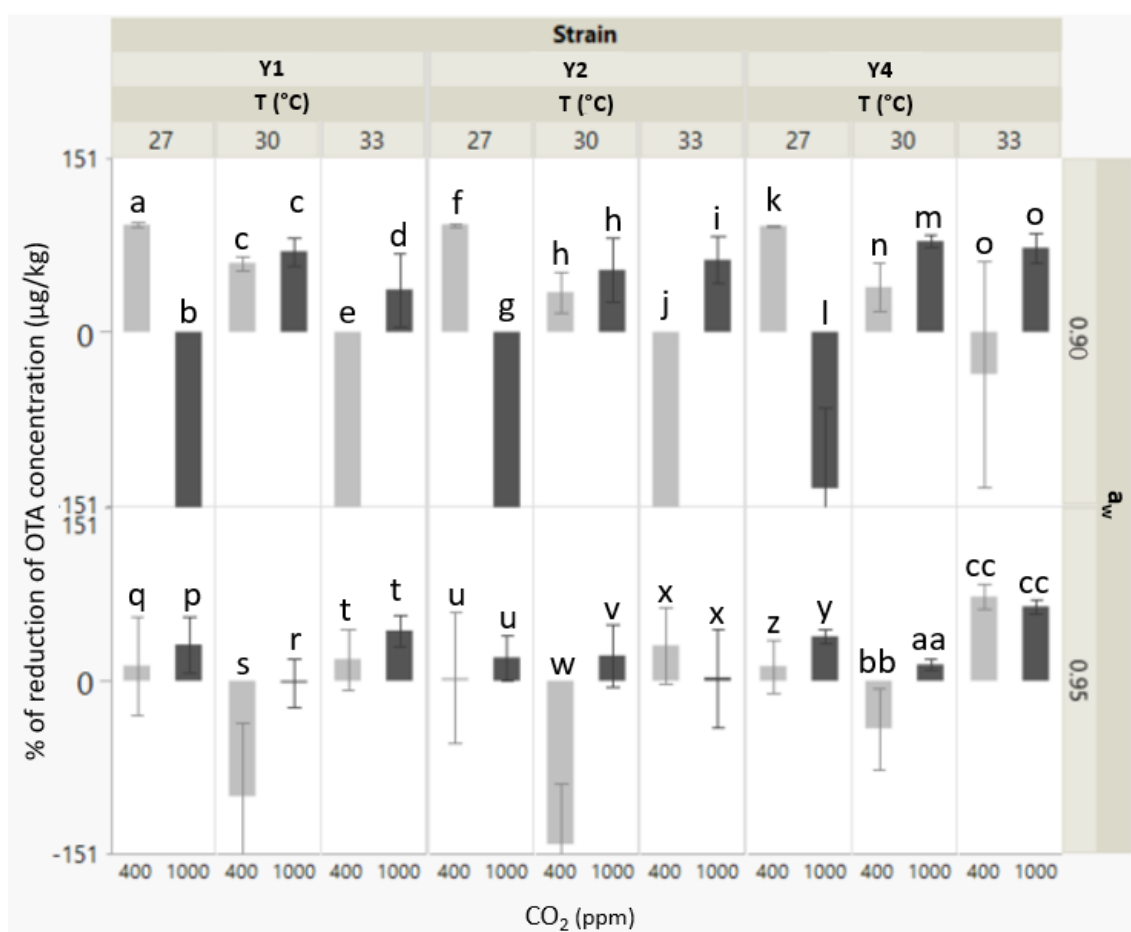


Figure 6.5.4-3. Effect of CO₂, temperature, and water activity on the % of reduction of ochratoxin A concentration in fresh coffee cherries. Different letters indicate significant differences ($p>0.05$) in the % of reduction of OTA of each strain at a specific temperature and a_w , using Fit Least Squares method and Tukey HSD test. Bars represent $AV \pm SD$. Values in negative correspond to an increase in the production of OTA.

6.6 Discussion

This study has addressed for the first time how the triple effect of temperature, the a_w and CO₂ levels impact the efficiency of different yeasts as potential BCA against the production of OTA by *A. carbonarius* on fresh coffee beans. Previous studies have only analysed the effect of a_w and temperature alone, on coffee-

based media, using mostly *Aspergillus* section *Circumdati* strains, and without the inoculation of potential BCAs (Estrada-Bahena et al., 2022; Gil-Serna et al., 2014; Oliveira et al., 2019).

In this work, it was found that the most important abiotic factor increasing *A. carbonarius*' fungal growth was a_w . Previous studies have described the optimal growth of *A. carbonarius* to be between 0.93 and 0.96 a_w on coffee-based medium (Neves et al., 2021), which aligns with our results, in which at 0.95 a_w the growth is elevated. Surprisingly, temperature did not influence fungal growth, while in other studies it has been positively correlated with a higher *A. carbonarius* growth (Cervini et al., 2019). This might be due to the influence of a_w in combination with CO_2 and T.

To our knowledge the present study would be the first that analyses the effect of yeasts on fungal growth using coffee beans as substrate. The strain Y4 reduced growth more than Y1 and Y2, at both 0.90 and 0.95 a_w . As well, the fungal growth was lower at low a_w , which might confer the BCAs an advantage in the fight for space and nutrients under such conditions. There are many studies that show the effect of yeasts on *A. carbonarius* fungal growth. Neves et al. (2021) isolated the strain *Saccharomyces cerevisiae* CCMA 0159 from coffee beans and analysed its growth inhibitory effect on *A. carbonarius* in coffee-based media. The results of this study showed that 28 °C and 0.99 a_w were the optimum conditions for fungal growth inhibition by *S. cerevisiae* CCMA 0159, while in our experiment a higher a_w did not give any advantage to the yeasts' capacity to reduce fungal growth.

Regarding the OTA production, the present work shows that under future CO_2 conditions there is an increase on the OTA levels. Similar results were obtained by Akbar et al. (2016) that analysed the OTA production of different *Aspergillus* section *Circumdati* strains under different conditions of CO_2 , temperature and a_w on coffee-based medium. This study concluded that elevated CO_2 and temperatures increased their OTA production. In our study we could observe that under elevated CO_2 OTA was produced under a wider range of temperatures.

This suggests that future temperatures which are expected to raise towards 30 °C in coffee producing areas, will increase the OTA concentration in coffee (Adhikari et al., 2020). With respect to a_w , higher a_w showed a correlation with slightly higher OTA production by *A. carbonarius*. Previous studies have also shown that water activities above 0.95 a_w show the highest OTA production levels by *A. carbonarius* (Oliveira et al., 2019).

The present study has shown that, in confrontation with the selected yeast strains, there is a significant reduction of OTA production under some of the conditions. Under current conditions of CO₂ and low a_w all the strains showed a significant reduction of OTA production at 27°C, however, high CO₂ concentration cancelled the ability of the yeasts to reduce OTA and even elevated the OTA production with respect to control. For higher temperatures of 30 and 33°C, elevated CO₂ and 0.95 a_w , strains Y2 and Y4 kept a significant inhibition of the OTA production. However, it is the strain Y4 the one with a better resilience, since it reduced the OTA production under more scenarios, both current and future, than the rest of strains. As well, Y4 significantly reduced OTA concentration in current conditions under both 0.90 and 0.95 a_w . On the other hand, Y1 would be the second most suitable strain for current conditions but does not show a good adaptation to future conditions with elevated CO₂ and temperature.

Previous studies have shown that yeasts species such as *S. cerevisiae* are able to reduce the production of OTA during the confrontation against *A. carbonarius* on different food matrixes and under different climatic conditions. For example, Neves et al. (2021) confronted the strain *S. cerevisiae* CCMA 0159 against *A. carbonarius* on coffee-based medium, showing a significant reduction of the OTA production on both low and high temperatures and a_w . This reduction might be related to substrate competition as strategies, acting downregulating OTA's biosynthetic gene expression. Another example, it's shown in the work by Cubaiu et al. (2012), where *S. cerevisiae* strains isolated from wine were tested *in vitro* and *in vivo* against *A. carbonarius*. This study demonstrated that *S. cerevisiae* DISAABA1182 strain was capable of inhibiting OTA production by at least a 65% and diminish the transcription of the polyketide synthase (pks) gene as well.

Complementary studies of the effect of the proposed BCAs on the downregulation of OTA's biosynthetic gene expression of the *A. carbonarius* strain would be recommended. Other studies such as the one performed by de Melo Pereira et al. (2016) analysed not only yeasts such as *Pichia fermentans* but also the Lactic Acid Bacteria strain *L. brevis* against *Aspergillus westerdijkiae* on coffee beans. However, the results showed that the OTA production was reduced in an 88% by *P. fermentans* and stimulated during the confrontation with *L. brevis*.

It should be noted that the two studies previously mentioned didn't monitored the confrontations under controlled temperature, a_w nor CO_2 conditions. Also, it is remarkable the low number of publications using BCAs such as bacteria or filamentous fungi on coffee-based medium or directly on coffee cherries. There are mainly studies of BCAs on coffee such as *Pseudomonas* sp. and *Trichoderma* sp. used for the treatment of coffee plant infections such as coffee rust or coffee wilt disease (de Sousa et al., 2022; Mulatu et al., 2022).

Finally, we recommend the study of the proposed BCAs in confrontations against other strongly ochratoxigenic strains such as *A. westerdijkiae* or *A. ochraceus*. This would help to find out the spectrum of OTA inhibition of these BCAs to tackle OTA contamination in other coffee cultivations (de Melo Pereira et al., 2016; Durand et al., 2019).

6.7 Conclusions

This multifactorial study has shown that the use BCAs such as *Rhodospiridiobolus ruineniae* (Y1), *Vishniacozyma* sp. (Y2), and *Meyerozyma caribbica* (Y4) in co-culture with a high OTA producing strain of *A. carbonarius* on coffee cherries, could result in considerable reduction of fungal growth and OTA production. Thus, these BCAs represent an alternative to create a more sustainable and safe coffee post-harvest chain. Besides, the efficiency of the BCAs was affected by temperature, a_w and CO_2 , resulting in the *Meyerozyma caribbica* (Y4) strain being the most resilient BCA since growth and OTA levels were reduced more efficiently under future conditions of elevated CO_2 and temperature. The application of *Meyerozyma caribbica* (Y4) as a BCA on fresh coffee cherries could reduce the contamination with OTA in at least 12 folds,

representing an adaptable BCA whose correct formulation could give place to a competitive agribusiness product.

CRedit authorship contribution statements

Claudia Lopez Rodriguez: conceptualization, formal analysis, data curation, visualization, investigation, writing – original draft, project management; **Carol Verheecke-Vaessen:** Writing -Review and Editing, conceptualization, data curation, validation, supervision; **Caroline Strub:** supervision; **Angélique Fontana:** supervision; **Tagro Guehi:** resources; **Sabine Schorr-Galindo:** supervision, Funding acquisition; **Angel Medina Vaya:** Writing -Review and Editing, conceptualization, data curation, supervision, funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data Availability

No data was used for the research described in the article.

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6.9 Supplementary materials

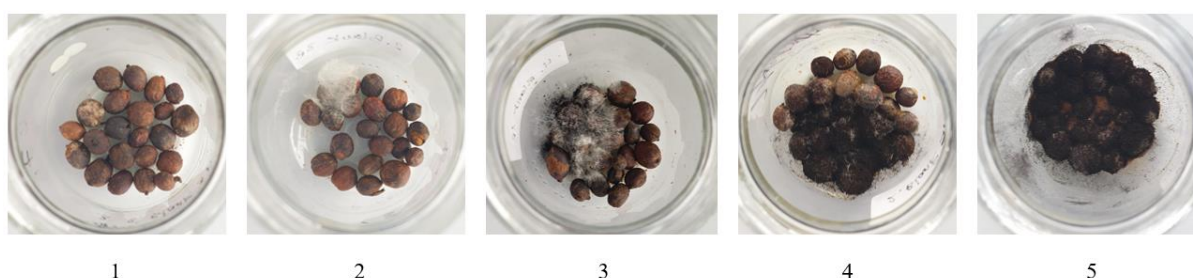


Figure 6.5.4. Examples of the five different level of growth used to assess the growth level of *A. carbonarius* on the coffee cherries during the climate change

experiment. Increasing growth from level 1 to 5. Dimensions of the glass vessel are 77 x 77 x 97 mm.

Table 6.5.4-1 Summary of the statistical analysis for the effect of interacting climate-related abiotic factors on ochratoxin A production by *A. carbonarius* after 7 days. The probability values in bold were significant ($p < 0.05$). Results based on Fit Least Squares Test for the effects of water activity (a_w), temperature (T) and CO₂ level.

Factors	DF	p -value (Prob > F)	Factors	DF	p -value (Prob > F)
		OTA ($\mu\text{g/kg}$)			OTA ($\mu\text{g/kg}$)
a_w	1	<0.0001	T* CO ₂	2	<0.0001
T	2	<0.0001	T* a_w	2	<0.0001
CO ₂	1	<0.0001	CO ₂ * a_w *T	2	<0.0001
a_w *CO ₂	1	<0.0001			

Table 6.5.4. Summary of the statistical analysis for the effect of interacting climate-related abiotic factors and BCA on ochratoxin A % of reduction by *A. carbonarius* after 7 days. The probability values in bold were significant ($p < 0.05$). Results based on Fit Least Squares Test for the effects of water activity (a_w), temperature (T), CO₂ level and BCA.

Factors	DF	p -value (Prob > F)	Factors	DF	p -value (Prob > F)
		OTA ($\mu\text{g/kg}$)			OTA ($\mu\text{g/kg}$)
BCA	2	<0.0001	BCA* CO ₂	2	<0.0001
a_w	1	<0.0001	BCA *T	4	<0.0001
T	2	<0.0001	BCA * a_w	2	<0.0001

CO ₂	1	<0.0001	BCA * CO ₂ *a _w	2	<0.0001
a _w *CO ₂	1	<0.0001	BCA *T*a _w	4	<0.0001
T* CO ₂	2	<0.0001	BCA*CO ₂ *T*a _w	4	<0.0001
T*a _w	2	<0.0001	CO ₂ *a _w *T	2	<0.0001

The *p*-value (Prob > F) was calculated from the values of F ratio and DF (degrees of freedom) of each effect.

7 General discussion

Ochratoxin A contamination poses a significant challenge to the coffee industry worldwide, including in Ivory Coast (Chilaka et al., 2022). Coffee production and safety is of paramount importance in Ivory Coast, not only for protecting the health of consumers but also for contributing to both rural livelihoods and export revenue (Due, 1969). The country has a long history of coffee cultivation, but like many coffee-producing nations, Ivory Coast faces a range of challenges that impact its coffee industry. Ivory Coast is the 2nd largest Robusta coffee producer in Africa, with coffee farming often practiced by smallholder farmers in various regions of the country. Coffee Arabica and Robusta are the two main coffee varieties grown, with Robusta being the dominant type due to its adaptability to the local climate (Olga et al., 2015). This discussion explores according to the results obtained during our study, the problematic around Ochratoxin A contamination of Robusta coffee in Ivory Coast, including quality control, and sustainable practices to mitigate mycotoxin risk, with a focus on biocontrol.

The coffee chain industry in this country is very delicate due to price fluctuation, insufficient infrastructure, climate change and poor farming practices, which lead to post-harvest losses due to safety and quality deterioration (Adeyeye et al., 2021). Besides, changing weather patterns and increased occurrence of pests and diseases due to climate change threaten coffee contamination with mycotoxins (Medina et al., 2017). As well, lack of access to training and awareness about mycotoxin risks and prevention measures to the farmers can result in poor farming practices that affect productivity and sustainability (Cervini et al., 2022).

The development of post-harvest management strategies can enhance the competitiveness of Ivorian coffee in global markets (FAO, 2006). For instance, wet-processed coffee requires substantial water usage, and implementing efficient dry-processed practices while keeping the safety and quality can reduce the environmental footprint of coffee production (Adnan & Lestari, 2021).

Proper drying, dehusking, and storage are crucial to prevent the growth of fungi and the production of mycotoxins (Samuel et al., 2021). Reducing the reliance on synthetic agrochemicals, such as pesticides, that leave harmful residues on coffee beans, and adopting sustainable farming practices can improve coffee safety (Kufa, 2010). One of the alternative sustainable farming practices is the application of microorganisms to manage mycotoxin contamination which can improve the coffee safety and quality and enhance the overall resilience of the sector (Habschied et al., 2021; Nguyen et al., 2017).

Ochratoxin A contamination presents a significant concern in the coffee industry, potentially compromising quality and safety standards and impacting trade relations (European Coffee Co-operation, 1983). The European Union (EU) establishes stringent thresholds to safeguard consumer health and regulate the coffee trade (European-Commission, 2023). In our study, we analysed a comprehensive set of Robusta coffee samples, revealing widespread mycotoxin presence; however, none exceeded the limits stipulated by the European Commission. This study underscores the importance of routine mycotoxin testing as an essential measure to ensure the integrity of the coffee supply chain and international market access.

Normally, in Ivory Coast, and other developing countries, the contamination with mycotoxin in the food chain is frequent due to relatively slow or poor drying conditions (Bucheli et al., 2000; Hamdouche et al., 2015; Templalaxis et al., 2021). Therefore, the initial studies were focused on deciphering the effect of different post-harvest management strategies on the composition of the mycobiota and its relation to OTA content on fresh and dried coffee cherries. Different drying methods were analysed in the present study, from which longer drying was pointed out for involving a higher risk of OTA contamination due to a higher time for the growth of ochratoxigenic fungi. The drying length can be shortened by reducing layer thickness, covering the coffee at night and during the hours of the day with higher temperatures, turning coffee every hour or using bamboo tables to dry coffee (Enyan, 2011; FAO et al., 2006). A positive correlation between the increasing abundance of *A. carbonarius* and the

concentration of OTA on coffee was found. Another factor studied was the effect of heaping the coffee before drying. This step is a common practice, and in the present study we noticed a correlation with a higher OTA contamination. Although delays before processing may lead to quality deterioration (FAO et al., 2006), there is no evidence enough to reject this practice. These results highlight the important role of adequate ventilation and spreading in layers on reducing the risk of mycotoxin development in the drying yard.

Afterwards, we focused on the diversity shifts. The alpha and beta diversity analysis revealed the presence of unique and more diverse fungal communities on the coffee after drying compared to fresh harvested coffee. Specially, coffee dried for a longer period showed a higher diversity. Fungal species such as *Hypopichia* sp. or *Bernettozyma* sp., were replaced in the top ten abundant species after drying by species such as *Kurtzmaniella* sp., *Meyerozyma* sp. and *Fusarium* sp. As well, some yeasts genus such as *Torulaspora* sp. are suggested as possible BCAs native to Ivorian Robusta coffee. Understanding the potential implications of the diversity shifts requires further investigation. Many studies have focused on studying the effect of wet-processing on the microbiota (Elhalis et al., 2020; Ribeiro et al., 2018), but very few focus dry-processed coffee. A general comprehensive analysis of the different processes and coffee species could be the key to the determination of the microbial fingerprint. The microbial fingerprint would help to better understand and predict the effect of different process on the microbiota and therefore on the final safety and quality of the coffee (Hassoun et al., 2023). Consequently, the possibility of a more sustainable coffee produced reducing the water usage but keeping its safety and quality properties could be considered.

The biochemical composition of dried coffee has been hypothesized as different from wet coffee (L. R. Batista et al., 2009). Until today, there are studies that analyse the biochemical changes that different processing does on coffee biochemical composition. However, they tend to be correlated to the organoleptic qualities, and not to safety attributes. A study done by Eshetu et al. (2022) analysed the effect of artificial drying (90 °C), solar tunnel (35-40 °C) and sun

drying (21-23 °C) on coffee biochemical composition, mainly for caffeine, chlorogenic acid, trigonelline and sucrose. The results showed that artificial drying reduces the loss of biochemical compounds from the bean. As sun drying involves a lower temperature, probably the drying time was longer, which has been linked in other studies to an acidification and lower content of chlorogenic acids, and to higher OTA levels (Kouadio et al., 2012). As well, some studies have determined that phenolic compounds, such as chlorogenic acids, are able to inhibit the production of some mycotoxins, for instance aflatoxin B1 (Telles et al., 2017). Although this study does not explore the biochemical composition of the coffee, it reveals the importance of understanding how the different compounds can impact mycotoxin content, especially during drying.

Once the microbial ecology of the coffee was assessed, it was important to be able to correlate it with microbial activity by the study in vitro of the dried coffee to evaluate the fungal and bacterial diversity and isolate potential BCAs and mycotoxigenic fungi. The first experiments revealed the most prevalent fungal groups in order from most frequently isolated to the less: *Aspergillus* section *Nigri*, *Aspergillus* section *Flavi*, *Penicillium* sp., and *Fusarium* sp. which have been previously isolated in other studies (Kouadio et al., 2012; Olga et al., 2015). Then, the isolated fungi potential producers of ochratoxins and/or aflatoxins were screened for their capacity to produce these toxins. From these fungi, 37 belonging to *Aspergillus* section *Nigri* and 3 to *Penicillium* sp., showed fluorescence and thus were chosen for further tests on PDA to quantify their OTA production. Once the 6 most ochratoxigenic strains were selected on PDA, their OTA production capacity was also checked on coffee-based media. Finally, the most ochratoxigenic strain on both media, which belonged to the species *Aspergillus carbonarius*, was chosen for the confrontations against the potential BCAs. As seen in previous studies on coffee from Ivory Coast, the most ochratoxigenic species are normally *Aspergillus carbonarius* and *Aspergillus ochraceus* (Kouadio et al., 2012; Olga et al., 2015).

Seventeen yeast and five bacteria were isolated from the dried Robusta coffee from Ivory Coast. A preliminary API identification allowed to determine that the

isolates belonged to *Rhodotorula mucilaginosa*, *Cryptococcus laurentii*, *Candida guilliermondii*, *L. delbrueckii* and *Leuconostoc mesenteroides*. A molecular identification was performed for those isolates with better biocontrol activity. Complementary, seven *L. plantarum* strains isolated from Robusta coffee in a previous project developed between the Qualisud research unit and the University Nangui Abroguá were studied for their antifungal and antimycotoxigenic capacities. The studies using Lactic Acid Bacteria as BCAs in Ivory Coast foodstuffs mainly focus on cocoa, and very rarely on coffee (Beugre et al., 2023; Papalexandratou et al., 2011). There are many studies using *Saccharomyces* sp., *Candida* sp. or *Lactiplantibacillus* sp. genera for the biocontrol of coffee, but very few or none used the other genera previously mention to improve coffee safety or quality (Bressani et al., 2020; de Melo Pereira et al., 2016; Evangelista et al., 2014; de Melo et al., 2016). This would be then the first study contemplating these strains.

The first approach to evaluate fungi-yeast or fungi-bacteria interactions was performed in vitro double layer assays of the seventeen yeasts against the ochratoxigenic fungi *A. carbonarius*. This general assay allowed at first to identify their fungal growth and OTA production inhibition capacities. For yeasts, results indicated that fungal development and OTA production's inhibition varied between species, suggesting that different mechanisms could be at play. On the opposite, bacteria did not show any relevant ability reducing growth and OTA on solid media, and in some cases, they even enhanced it. Maybe further tests are needed to evaluate these bacteria under different concentrations and media, since there is evidence of the ability of bacteria, and especially Lactic Acid Bacteria to control the growth and OTA production of mycotoxigenic fungi (Bangar et al., 2022; Khalisanni, 2011; Nasrollahzadeh et al., 2022).

The evaluation of the two yeast strains *Rhodosporidiobolus ruineniae* and *Meyerozyma caribbica*'s ability to detoxify ochratoxin A was conducted against the OTA producing *A. carbonarius*. In vitro assays on liquid media were pursued by evaluating the impact of yeast's CFSs on fungal development and mycotoxin production to find potential sources of detoxifying metabolites. However, the use

of CFSs was not efficient, which may indicate that the mechanism used by the BCAs is not related to metabolites. Further studies on the impact of different concentrations of CFSs on fungal growth are strongly recommended. Additionally, mycotoxin adsorption assay revealed that OTA could be adsorbed by the yeast strain *Rhodosporidiobolus ruineniae* and degraded by *M. caribbica*. Adsorption mechanisms of OTA by yeast strains have been previously contemplated by (Caridi et al. (2022) and Pourmohammadi et al. (2022) and might be linked to the adsorption to the cell wall. Regarding the degradation of OTA, some studies have found a degradation of OTA by strains of yeasts (Wei et al., 2022), and the formation of degradation by-products of OTA such as OTB which is considered less toxic than OTA. In our study, no OTA's degradation products, such as OT α were found, maybe due to a degradation into other by-products.

In vivo biocontrol resilience was assessed by inoculation of fresh coffee with the yeast isolates and *A. carbonarius* under different climatic conditions of water activity, temperature and CO₂. The results showed that the strain *M. caribbica* was the most resilient since it had an impact reducing fungal growth and OTA production under more both current and future conditions of temperature, water activity and CO₂ than the other strains tested. Previous studies have focused on analysing the effect of temperature and aw on different ochratoxigenic fungi in coffee-based media (Akbar et al., 2016; Pardo et al., 2005), some including CO₂ (Akbar et al., 2020b) or dry cherries or green coffee beans as substrate (Estrada-Bahena et al., 2022). Some studies have even analysed the impact of environmental factors on the inoculation of yeasts to prevent contamination with *Aspergillus* sp. (Neves et al., 2021). However, the present work is the first study where the triple effect of temperature, a_w and CO₂ and the inoculation with a BCA are analysed on fresh coffee cherries.

8 General conclusions

Overall, the present project has largely been able to address the original questions posed at the beginning of the research. The fungal biodiversity of fresh and differently processed coffees from Ivory Coast was studied, followed by their relative mycotoxin contamination profiles for the first time. Several native strains of yeasts were isolated and used for biocontrol of toxigenic *A. carbonarius* strains and OTA control. The ecology of strains of *A. carbonarius* in terms of growth and toxin production were shown to be different between PDA and coffee-based medium. The efficacy of one chosen native BCA yeast candidate (*Meyerozyma caribbica*) was shown to significantly inhibit OTA production *in vitro* and *in situ* in fresh coffee cherries. In addition, the resilience of the BCA under climate change scenarios was conserved, regardless of storage conditions.

Ensuring coffee safety in Ivory Coast is a multifaceted endeavour that requires the collective efforts of government, industry stakeholders, and academic experts. By raising awareness, implementing best practices, and investing in research and testing, Ivory Coast can mitigate the risk of mycotoxin contamination, ensure the safety of its coffee products, and maintain its reputation in the global coffee market. Collaborating with experts in mycotoxin analysis and mitigation strategies can help developing tailored solutions for Ivory Coast's coffee industry.

9 Future perspectives and steps

Based on the findings of this work, future research should focus on:

- a) Explore native atoxigenic strains with potential as biocontrol agents to overcome the toxigenic strains in the coffee agroecosystem.
- b) Better decipher the modes of action of BCAs especially for degradation.
- c) Investigate the impact on the key structural and regulatory genes involved in the biosynthesis of OTA.
- d) Testing other ochratoxigenic strains of *A. carbonarius* and ochratoxigenic species such as *A. westerdijkiae* or *A. ochraceus* as well as aflatoxigenic strains to find out the spectrum of inhibition of the proposed BCAs.
- e) Increase the pool of samples of coffee from different geographic regions in Ivory Coast.
- f) Apply the BCA *in situ* using formulations, either as a spray or soaking to control *A. carbonarius* in the Ivorian coffee post-harvest chain.
- g) Potential relationship between CC scenarios in different coffee growing countries and potential for increased contamination with aflatoxins and ways to minimise the impact on toxin contamination.

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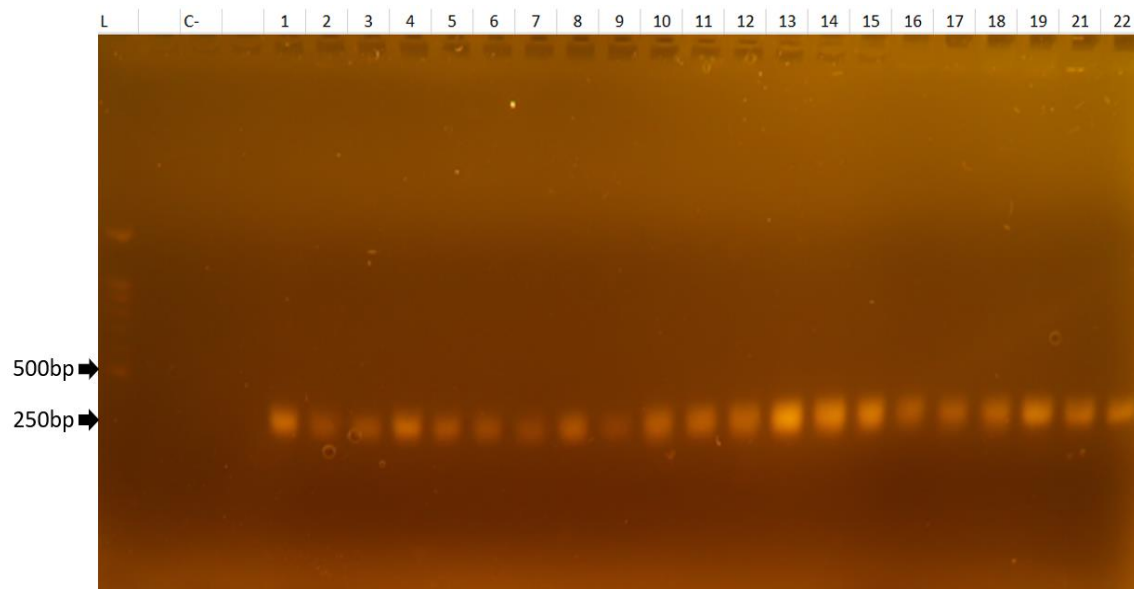
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11 APPENDICES

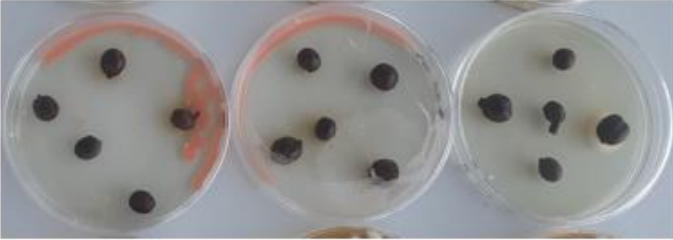
Appendix A



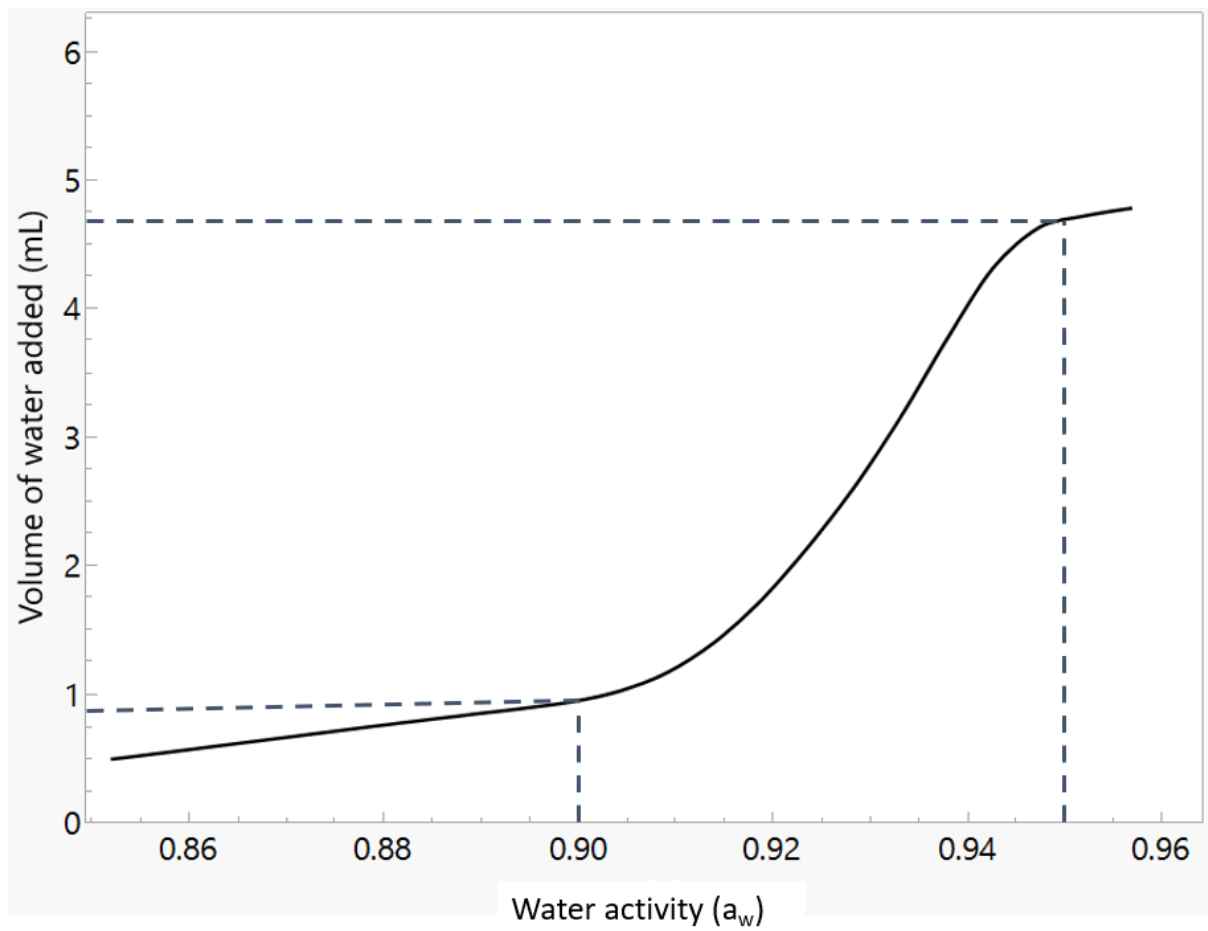
A.1 Example of gel of ITS region DNA quality extract from coffee samples used in the PCR. The first lane (L) is the ladder. The third lane (C-) is the negative control. Lanes 1 to 22 refer to samples. Samples show the targeted ITS fragment with a size of 250bp.

Appendix B

B.1 Effect of UV light for 1h15mins on microbial load on fresh coffee plated on three different media.

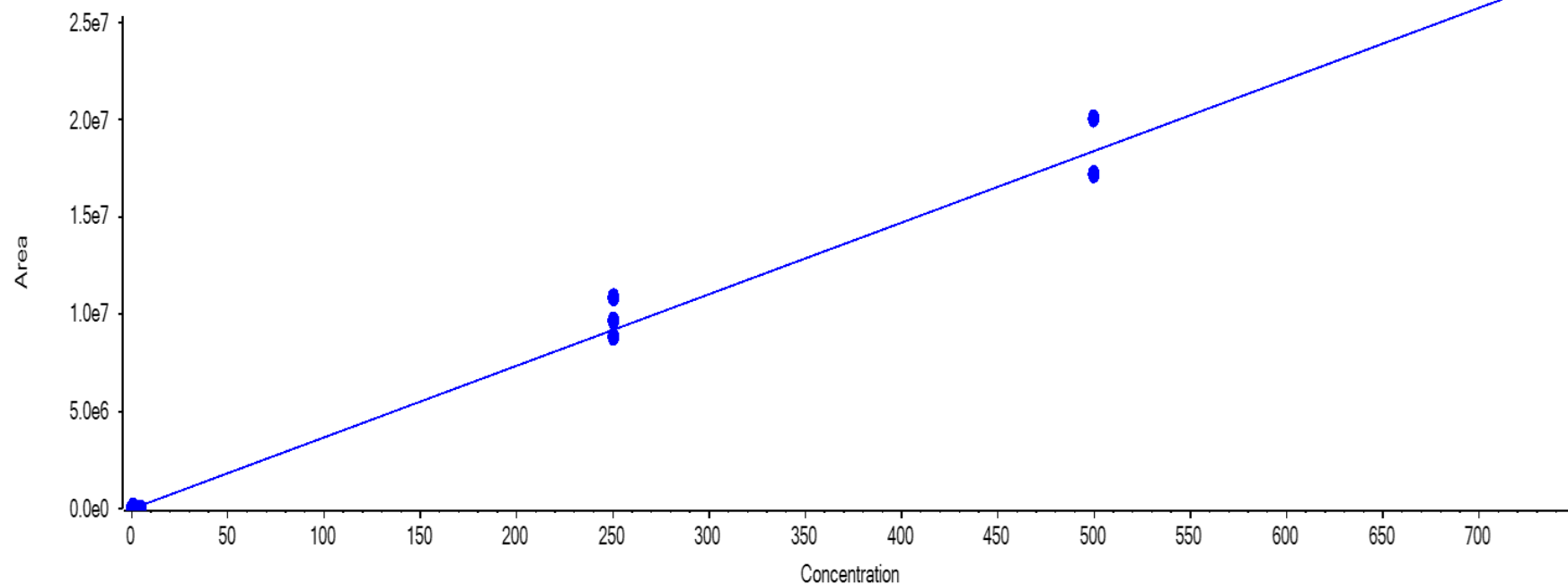
72h	Control	MRS	
		DG18	
		MEA	
72h	UV	MRS	
		DG18	
		MEA	

B.2 Adsorption curve of fresh coffee cherries used in this work.
Sample size of 5g of fresh coffee.



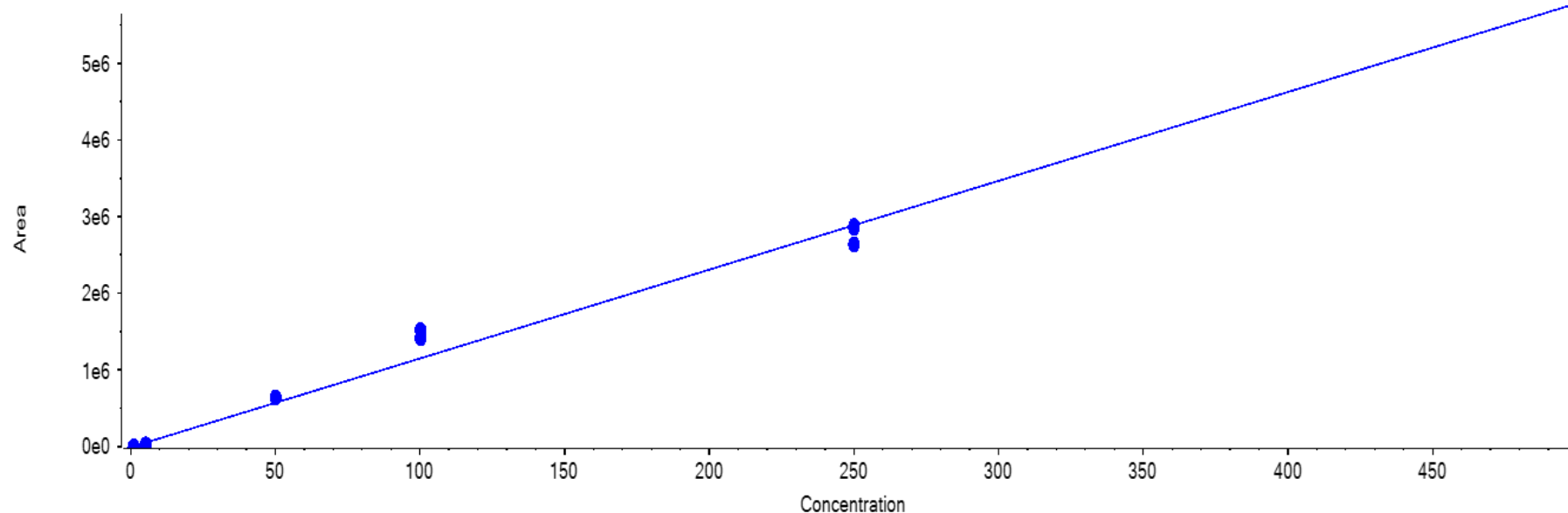
B.3 Calibration curves for OTA quantification for adsorption experiments, and for CC experiments

Calibration for Ochratoxin A a 1: $y = 3.67938e4 x + -10462.45213$ ($r = 0.99406$) (weighting: $1/x$)



B.3. Example of calibration curve for Ochratoxin A using the column ACE 3-C18/2.1 × 100 mm, 3 µm particle size; Hichrom, Reading, UK) equipped with a C18 security guard cartridge (4 × 3 mm, Gemini Agilent) and kept at 40 °C. Obtained from injections of samples belonging to the OTA adsorption experiments.

Calibration for Ochratoxin A a 1: $y = 11590.80397 x + -10400.39665$ ($r = 0.99471$) (weighting: $1 / x$)



B.4 Example of calibration curve for Ochratoxin A using the column ACE 3-C18/2.1 × 100 mm, 3 μm particle size; Hichrom, Reading, UK) equipped with a C18 security guard cartridge (4 × 3 mm, Gemini Agilent) and kept at 40 °C. Obtained from injections of samples belonging to the experiments on Climate Change.

Appendix C

C.1 Publications and communications

Research articles published

1. Effect of post-harvest management practices on the mycobiome and ochratoxin a contamination of differently processed Robusta coffees from Ivory Coast.

Claudia López Rodríguez, Caroline Strub, Vincent Chochois, Carol Verheecke-Vaessen, Noël Durand, Christophe Jourdan, Angélique Fontana, Tagro Guehi, Angel Medina, Sabine Schorr-Galindo

Published in Postharvest Biology and Technology Journal.

Research articles under revision

1. In vitro biocontrol of *Aspergillus carbonarius* growth and ochratoxin a production by yeasts or lactic acid bacteria isolated from robusta coffee.

Claudia López Rodríguez, Caroline Strub, Angélique Fontana, Carol Verheecke-Vaessen, Noël Durand, Corinne Beugré, Tagro Guehi, Angel Medina, Sabine Schorr-Galindo.

Submitted to Food Control journal

2. Assessing the resilience of a yeast inoculum in confrontation with ochratoxigenic fungi under future climate change conditions on coffee *in vivo*.

Claudia López Rodríguez, Carol Verheecke-Vaessen, Caroline Strub, Angélique Fontana, Tagro Guehi, Sabine Schorr-Galindo, Angel Medina

Under revision for submission to International Journal of Food Microbiology

3. Ochratoxin A occurrence in coffee: how to reduce the risk?

Claudia López Rodríguez, Carol Verheecke-Vaessen, Caroline Strub, Angélique Fontana, Sabine Schorr-Galindo, Angel Medina

Under revision for submission to Food Control journal

Scientific congress (Oral communications and posters)

1. International Conference on Engineering and Food, 2023, Nantes, France. Oral communication.

“Biocontrol of mycotoxigenic fungi by Lactic Acid Bacteria and yeasts isolated from Robusta Ivorian coffee”.

Claudia López Rodríguez, Caroline Strub, Vincent Chochois, Noël Durand, Carol Verheecke-Vaessen, Angélique Fontana, Tagro Guehi, Angel Medina, Sabine Schorr-Galindo.

2. Journées Mycotoxines, 2022, Marseille, France. Poster.

“Biocontrol of mycotoxigenic fungi by Lactic Acid Bacteria and yeasts isolated from Robusta Ivorian coffee”.

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3. Mycotoxin Workshop, 2022, Toulouse, France. Poster.

“Biocontrol of mycotoxigenic fungi by Lactic Acid Bacteria and yeasts isolated from Robusta Ivorian coffee”.

Claudia López Rodríguez, Caroline Strub, Vincent Chochois, Noël Durand, Carol Verheecke-Vaessen, Angélique Fontana, Tagro Guehi, Angel Medina, Sabine Schorr-Galindo.

4. 125th BMS Annual International Symposium, 2022, Cranfield, United Kingdom. Poster.

“Biocontrol of mycotoxigenic fungi by Lactic Acid Bacteria and yeasts isolated from Robusta Ivorian coffee”.

Claudia López Rodríguez, Caroline Strub, Noël Durand, Carol Verheecke-Vaessen, Angélique Fontana, Tagro Guehi, Angel Medina, Sabine Schorr-Galindo.

C.2 Poster presented at Journées Mycotoxins, Marseille, France, September 2022



Biocontrol of mycotoxigenic fungi by lactic acid bacteria and yeasts in coffee



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Background



- Ivory Coast - 3rd African producer, more than 135 tn/year (1)
- Ochratoxin A (OTA) appears after harvest and is not totally removed during roasting (2)
- The main factors that affect OTA contamination are water activity, environment and processing method (dry/fermentation stages)(3)
- Lactic Acid Bacteria (LAB) or Yeasts can act as safe and natural biocontrol agents reducing fungal growth and OTA production (4)

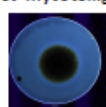
Aim & Objectives

Materials & Methods

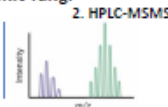
1. Isolation and identification

- 102 fungi: *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp.
- 17 yeasts: *Candida* sp., *Cryptococcus* sp. and *Rhodotorula* sp.
- 3 LAB: *Lactobacillus plantarum*

2. Characterisation and selection of the most mycotoxigenic fungi



1. OTA Fluorescence on Coconut Cream Agar

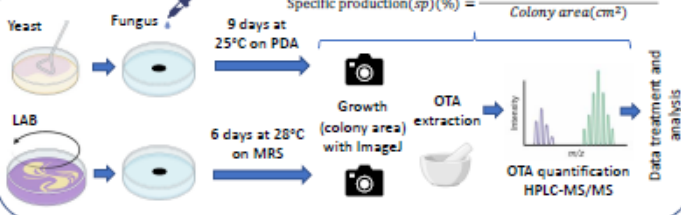


2. HPLC-MS/MS

Aim: Evaluate the effect of Climate Change on mycotoxin production and develop a resilient inoculum to reduce presence of OTA on dried processed coffee from Ivory Coast

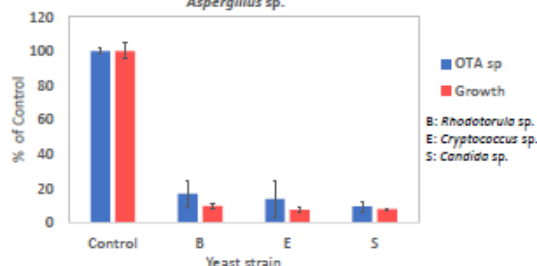
First objectives: (1) Isolate microorganisms from shell coffee (2) Characterize and select the most mycotoxigenic fungi, (3) Identify and select LAB and yeasts that reduce OTA production and growth of *Aspergillus* sp.

3. Confrontation



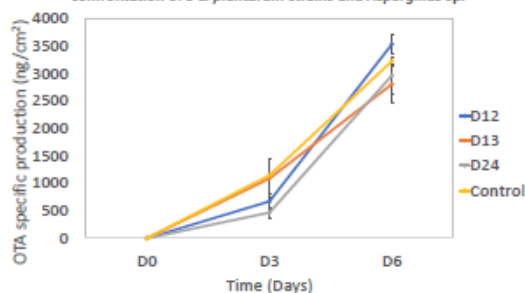
Results & Conclusions

In vitro interactions observed between yeasts and *Aspergillus* sp.



✓ OTA sp and growth were strongly reduced (>75%) for the 17 yeast strains (especially for the 3 presented)

Evolution of the OTA specific production during the confrontation of 3 *L. plantarum* strains and *Aspergillus* sp.



✓ OTA was strongly reduced (>50%) by D12 and D24 at day 3

- The study of these yeasts and bacteria showed that they can have a potential use as biocontrol agents, reducing fungal growth and OTA production.
- The mechanisms behind these inhibitions or removal are yet to be deciphered through confrontations on liquid medium between the biocontrol agents and the fungi or the OTA molecule.
- The transcriptomic analysis of these interactions will allow us to identify the genes of the fungus whose expression is modulated during the inhibition.

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- (3) Leitão, A. L. (2019). Occurrence of ochratoxin A in coffee: Threats and solutions—A mini-review. *Beverages*, 5(2), 36.
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12 Summary in French

La contamination par les mycotoxines pose un défi majeur à l'industrie du café dans le monde entier, y compris en Côte d'Ivoire. La production et la sécurité du café revêtent une importance primordiale en Côte d'Ivoire, non seulement pour protéger la santé des consommateurs, mais aussi pour contribuer aux moyens de subsistance des producteurs en milieu rural ainsi qu'aux recettes d'exportation. Le pays possède une longue histoire de culture du café, mais comme de nombreux pays producteurs de café, la Côte d'Ivoire doit relever une série de défis qui affectent son industrie du café.

La Côte d'Ivoire est le deuxième plus grand producteur de café Robusta en Afrique, la culture du café étant souvent pratiquée par des petits exploitants dans différentes régions du pays. Les variétés de café Arabica et Robusta sont les deux principales variétés de café cultivées, le Robusta étant le type dominant en raison de sa meilleure adaptabilité au climat local. Notre projet explore la problématique de la contamination par l'Ochratoxine A (OTA) du café Robusta en Côte d'Ivoire, y compris le contrôle de la qualité et les pratiques durables visant à atténuer les risques liés aux mycotoxines.

L'industrie de la chaîne du café dans ce pays est très délicate en raison de la fluctuation des prix, de l'insuffisance des infrastructures des traitements post-récolte adaptées, du changement climatique et des mauvaises pratiques agricoles, ce qui entraîne des pertes après récolte en raison de la détérioration de la qualité sanitaire et organoleptique. De plus, les changements des schémas météorologiques et l'augmentation des occurrences de ravageurs et de maladies fongiques liés au changement climatique menacent la contamination du café par les mycotoxines. Enfin, le manque d'accès à la formation et à la sensibilisation aux risques et aux mesures de prévention des mycotoxines pour les agriculteurs peut entraîner de mauvaises pratiques agricoles qui affectent la productivité et la durabilité.

Le développement de stratégies de gestion après récolte peut renforcer la compétitivité du café ivoirien sur les marchés mondiaux. Par exemple, le café

traité par voie humide nécessite une utilisation substantielle d'eau, et la mise en œuvre de pratiques de traitement par voie sèche efficaces tout en maintenant la sécurité sanitaire et la qualité générale peut réduire l'empreinte environnementale de la production de café. Le séchage, le décortilage et le stockage appropriés sont essentiels pour prévenir la croissance des moisissures et la production des mycotoxines. La réduction de la dépendance aux produits agrochimiques synthétiques, tels que les pesticides, qui laissent des résidus nocifs sur les grains de café, et l'adoption de pratiques agricoles durables peuvent améliorer la sécurité du café.

L'une des pratiques agricoles durables prometteuses est l'application de micro-organismes pour gérer la contamination par les mycotoxines, ce qui peut améliorer la sécurité sanitaire et la qualité générale du café et renforcer la résilience globale du secteur. La contamination par les mycotoxines constitue une préoccupation majeure dans l'industrie du café, compromettant potentiellement les normes de qualité et de sécurité et impactant les relations commerciales. L'Union européenne (UE) établit des seuils stricts pour protéger la santé des consommateurs et réguler le commerce du café (Reglement 1881-2006 consolidé en 2023).

Dans notre étude, nous avons analysé un ensemble complet d'échantillons de café Robusta, révélant une présence de mycotoxines dans tous les échantillons, cependant, aucun n'a dépassé les limites fixées par la Commission européenne (Reglement 1881-2006 consolidé en 2023). Ces résultats soulignent l'importance des tests de routine des mycotoxines en tant que mesure essentielle pour garantir l'intégrité de la chaîne d'approvisionnement en café et l'accès au marché international.

Les études antérieures de nos équipes de recherche étaient axées sur le decryptage de l'effet de différentes stratégies de gestion après récolte sur la composition du mycobiote des cerises de café et sa relation avec la teneur en OTA de cerises fraîches et séchées (ref de thèse ou article). Différentes méthodes de séchage ont été analysées dans la présente étude. En effet un séchage prolongé a été signalé comme risque plus élevé de contamination par

l'OTA en raison d'un temps prolongé aux températures et activité de l'eau optimales pour la croissance des champignons ochratoxinogènes. Une corrélation positive entre l'augmentation de l'abondance d'*Aspergillus carbonarius* et la concentration en OTA dans le café a été trouvée. Un autre facteur étudié était l'effet de l'amoncellement du café avant le séchage. Cette étape est une pratique courante mais à risque pour la contamination du café et dans la présente étude, nous avons constaté une corrélation de la mise en tas avec une contamination par l'OTA plus élevée. Cela souligne qu'une ventilation adéquate et un étalage en couches peuvent réduire le risque de développement de mycotoxines dans la cour de séchage.

Ensuite, nous nous sommes concentrés sur les changements de diversité microbienne des échantillons de cerises et de grains de café provenant des différents traitements post-récolte. L'analyse de la diversité alpha et bêta a révélé la présence de communautés fongiques uniques et plus diversifiées sur le café après le séchage par rapport au café fraîchement récolté. En particulier, le café séché pendant une période plus longue a montré une plus grande diversité. Des espèces fongiques telles que *Hypopichia* sp. ou *Bernettozyma* sp., ont été remplacées dans les dix espèces les plus abondantes après le séchage par des espèces telles que *Kurtzmaniella* sp., *Meyerozyma* sp. et *Fusarium* sp. De même, certains genres de levures trouvés par cette technique tels que *Torulaspora* sp. ou *Meyerozyma* sp. sont suggérées comme des BCAs possibles propres au café Robusta ivoirien.

Une fois l'écologie microbienne du café évaluée, nous nous sommes tournés vers l'étude *in vitro* du café séché pour évaluer la diversité fongique et bactérienne des souches cultivables et isoler des champignons mycotoxinogènes et de potentiels agents de biocontrôle (BCAs) parmi les levures et les bactéries lactiques (LAB). Les premières expériences ont révélé les groupes fongiques les plus prévalents dans l'ordre, des plus fréquemment isolés aux moins fréquents : *Aspergillus* section *Nigri*, *Aspergillus* section *flavi*, *Penicillium* sp., et *Fusarium* sp.

Ensuite, parmi les champignons isolés producteurs potentiels d'ochratoxines et/ou d'aflatoxines, un test de screening sur milieu Coconut Agar a été réalisé pour évaluer leur capacité à produire ces toxines. Parmi ces champignons, 40, dont 37 appartenant à *Aspergillus* section *Nigri* et 3 à *Penicillium* sp., ont montré une fluorescence et ont donc été choisis pour des tests ultérieurs sur PDA pour quantifier leur production d'OTA. Une fois les six souches les plus ochratoxinogènes sélectionnées sur PDA, leur capacité de production d'OTA a également été vérifiée sur un milieu à base de café. Enfin, la souche la plus ochratoxinogène sur les deux milieux a été choisie pour les confrontations contre les BCAs potentiels.

Dix-sept levures et cinq bactéries lactiques ont été isolées du café Robusta séché de Côte d'Ivoire. Une identification API préliminaire a permis de déterminer que les isolats correspondaient à *Rhodotorula mucilaginosa*, *Cryptococcus laurentii*, *Candida guilliermondii*, *L. delbrueckii* et *Leuconostoc mesenteroides*. Une identification moléculaire a été réalisée pour les isolats présentant une meilleure activité de biocontrôle. Sept souches de *L. plantarum* isolées du café Robusta dans un projet précédent mené entre l'UMR Qualisud et l'Université Nangui Abroguia ont été ajoutées en raison de leurs capacités antifongiques et antimycotoxinogènes.

La première approche pour évaluer les interactions champignons-levures ou champignons-bactéries a été réalisée *in vitro* sous forme de doubles couches de dix-sept levures contre le champignon ochratoxinogène *A. carbonarius*. Cet essai général a d'abord permis d'identifier leur croissance fongique et leurs capacités d'inhibition de la production d'OTA. Pour les levures, les résultats ont indiqué que l'inhibition du développement fongique et de la production d'OTA variaient entre les espèces, suggérant que différents mécanismes pourraient être en jeu. En revanche, les bactéries n'ont montré aucune capacité pertinente à réduire la croissance et la production d'OTA sur les milieux solides, et dans certains cas, elles l'ont même améliorée du fait d'exigence différente vis-à-vis de l'oxygène. Il est peut-être nécessaire de réaliser d'autres tests pour évaluer les bactéries sous différentes concentrations et milieux.

L'évaluation de la capacité des deux souches de levures, *Rhodosporidiobolus ruineniae* et *Meyerozyma caribbica*, à détoxifier l'ochratoxine A a été réalisée sur le champignon producteur d'OTA *A. carbonarius* isolé de nos échantillons. Des essais *in vitro* sur milieu liquide ont été poursuivis en évaluant l'impact des CFSs des levures sur le développement fongique et la production de mycotoxines pour trouver des sources potentielles de métabolites détoxifiants. Cependant, l'utilisation de CFSs n'était pas efficace, ce qui peut indiquer que le mécanisme utilisé par les BCAs n'est pas lié aux métabolites. Des études ultérieures sur l'impact de différentes concentrations de surnageants sans cellules (CFSs) sur la croissance fongique sont fortement recommandées. De plus, l'essai d'adsorption des mycotoxines a révélé que l'OTA pouvait être adsorbée par la souche de levure *R. ruineniae* et dégradée par *M. caribbica*.

La résilience du biocontrôle *in vivo* a été évaluée par l'inoculation de café frais avec les isolats de levure et *A. carbonarius* dans différentes conditions climatiques. Les résultats ont montré que la souche *M. caribbica* était la plus résiliente, car elle avait un impact réduisant la croissance fongique et la production d'OTA sous des conditions de température, d'activité de l'eau et de CO₂ actuelles et futures plus élevées que les autres souches testées.

Dans l'ensemble, le présent projet a largement réussi à répondre aux questions initiales posées au début de la recherche. La biodiversité fongique des cafés frais et différemment transformés de Côte d'Ivoire a été étudiée, suivie de leurs profils de contamination relative par mycotoxines pour la première fois. Plusieurs souches indigènes de levures ont été isolées et utilisées pour la lutte biologique contre les souches toxigènes d'*A. carbonarius* et pour le biocontrôle de l'OTA. L'efficacité d'un candidat levure BCA indigène choisi (*Meyerozyma caribbica*) a été démontrée pour inhiber de manière significative la production d'OTA *in vitro* et *in situ*. De plus, la résilience du BCA face aux scénarios de changement climatique a été préservée.

Sur la base des résultats de ce travail, les futures recherches devraient se concentrer sur examiner la cinétique des impacts sur les gènes structurels et régulateurs clés impliqués dans la biosynthèse de l'OTA ; tester d'autres souches

ochratoxinogènes telles que *A. westerdijkiae* ou *A. ochraceus* pour découvrir le spectre d'inhibition des BCA proposés ; et finalement appliquer le BCA *in situ* en utilisant des formulations, que ce soit sous forme de pulvérisation ou de trempage, pour contrôler *A. carbonarius* dans la chaîne post-récolte du café ivoirien.