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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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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, *Rhodosporidiobolus 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

1. INTRODUCTION

Mycotoxins are fungal secondary metabolites toxic to human and animal and remain stable during industrial processing (Campos-Avelar et al., 2020). *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, we assessed 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.

2. MATERIALS AND METHODS

Sampling

Two kilograms of coffee cherries from Robusta coffee (*Coffea canephora*) were hand-picked at the mature stage (red cherries) from a farm at 100-200 m above sea level situated in Akoupé, Ivory Coast. The cherries were dried by the natural method; directly after harvest, the cherries were spread on a concrete platform for 12 days until reaching a moisture content of 11-12 %. The environmental temperature was 25-30 °C on average. The dried cherries were then stored at -20 °C and sent to Montpellier, 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.

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) was used for the isolation of lactic acid bacteria. MEA (Malt Extract Agar, Merck) and DG18 agar (Dicloran Glycerol 18 %, Merck) supplemented with 0.1 % chloramphenicol (Merck) was used to isolate both yeasts and fungi.

The isolation of fungi and bacteria was performed by two different techniques. The serial dilution consisted of adding ten grams of fruits to 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 plated in triplicate on MRS for bacterial populations and on DG18 and MEA 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 isolates through this technique were used for enumeration (data not shown) and isolated at -20 °C.

For direct plating, ninety coffee cherries were surface disinfected (+SD) with 2 % sodium hypochlorite for 10 mins while stirring, rinsed in sterile water for 20 minutes and then dried and plated. Surface disinfection was performed to isolate microorganisms present in internal layers of the coffee cherry. Another ninety coffee cherries were plated without surface disinfection (-SD). The coffee cherries were directly plated (45 coffee cherries per medium; 5 and 10 coffee cherries per plate) on 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. All yeast isolates were coded as Y plus a different letter for each and used for confrontations on solid media.

The fungal occurrence of *Aspergillus* sections *Flavi*, *Nigri* and *Circumdati*; *Penicillium* and *Fusarium* was recorded, and representative strains were isolated, purified and stored at -20 °C. These strains were tested for mycotoxin production capacity. To obtain the isolation frequency, all bacterial and fungal colonies growing from directly plated coffee cherries on MRS, MEA and DG18 were recorded and calculated according to the following equation:

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

Screening for mycotoxigenic fungi

Determination of OTA production capacity on Coconut Cream Agar

To identify the isolates producers of OTA or aflatoxins (AFLA) all the isolates identified as *Aspergillus* section *Nigri* or *Penicillium* sp. 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. The excitation and emission wavelengths range of OTA and AFLA are of 301-395 nm and 400-475 nm,

respectively (Cagnasso et al., 2019). Consequently, they were examined under long UV light (366 nm or 312 nm) at days 3, 5 and 7 after inoculation. The colours observed were green fluorescence for OTA, and blue for AFLA.

To confirm that the fluorescence produced on CCA was due to OTA, a standard range by adding OTA to a concentration of 500, 1000 and 3000 $\mu\text{g/kg}$ was created, and poured before the medium solidified. Fifty mm Petri dishes were used with 4 mL of CCA per Petri dish. Fluorescence was visible only above a concentration of 1000 $\mu\text{g/kg}$ of OTA.

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 μL (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. 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 two different Coffee Based Medium (CBM). Whole coffee cherries and grounded dried coffee cherries were used to prepare CBM1 and CBM2, respectively. CBM was prepared similarly to (Akbar & Magan, 2014), using 300 g of coffee cherries 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 µL 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 and detection 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 µL 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 µM PTFE filter for the HPLC analysis. 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 × 4.6 mm. The injection volume was 100 µL, injected during 20 min at a flow rate of 1 mL/min. OTA was detected by absorption at 333 nm excitation and 360 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, red PD 226 R. Biopharm, Glasgow, UK) and the limit of detection and quantification was 0.05 ng/mL and 0.15 ng/mL respectively. Results were expressed in µg 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).

Solid-based confrontations against fungi

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 with 10^6 yeast cells was spread on the PDA plate, then 5 µL with 10^6 fungal spores 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.

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 *Lactobacillus 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 μ L of bacterial suspension (10^7 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 as previously done by Campos-Avelar et al. (2020) to adjust the optimal growth peaks of isolates. Then, 10 μ L of 10^8 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.

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. 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 μ m) to obtain the CFS.

The confrontations were run against the highest ochratoxigenic *A. carbonarius*. After a 7 days pre-culture on PDA at 25 °C. Ten μ L of fungal spore suspension (10^7 spores/mL) and 10 μ L of yeast suspension of 10^7 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.

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. One mL volume of final cells, washes, and supernatants were extracted for OTA and OTA quantification by LC-MS/MS according to the extraction and quantification protocol from Gil-Serna et al. (2022) adapted from the methodology developed by Malachová et al. (2014). Analysis was run in five replicates.

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 (OsO₄) at 4 °C overnight.

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 CO₂ 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).

Morphological and molecular identification

The filamentous fungi were initially cultured on PDA medium and observed with an optical microscope for preliminary identification. First, the colony, especially color and shape was analysed using the guide of Samson et al. (2019). And then, the identification of the general *Aspergillus* sp., *Penicillium* sp. and *Fusarium* sp. with microscopic slide examination of spores and conidiophores was carried out. 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 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 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 (1 μ M) 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 1. Primers used for amplification and sequencing.

Locus	Primer	Direction	Primer sequence (5'-3')	Reference
ITS	ITS5	Forward	5'-GGAAGTAAAAGTCGTAACAAGG-3'	(White et al., 1990)
	ITS4	Reverse	5'-TCCTCCGCTTATTGATATGC-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'-ACCCTCAGTGTAGTGACCCTTGGC-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'-GCATATCAATAAGCGGAGGAAAAG-3'	(Taverna et al., 2013)
	NL-4	Reverse	5'-GGTCCGTGTTTCAAGACGG-3'	(Taverna et al., 2013)

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.

3. RESULTS

Occurrence of bacteria and fungi and isolation

Figure 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 Figure S1). 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 on both disinfected and non-disinfected cherries. 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 S1.

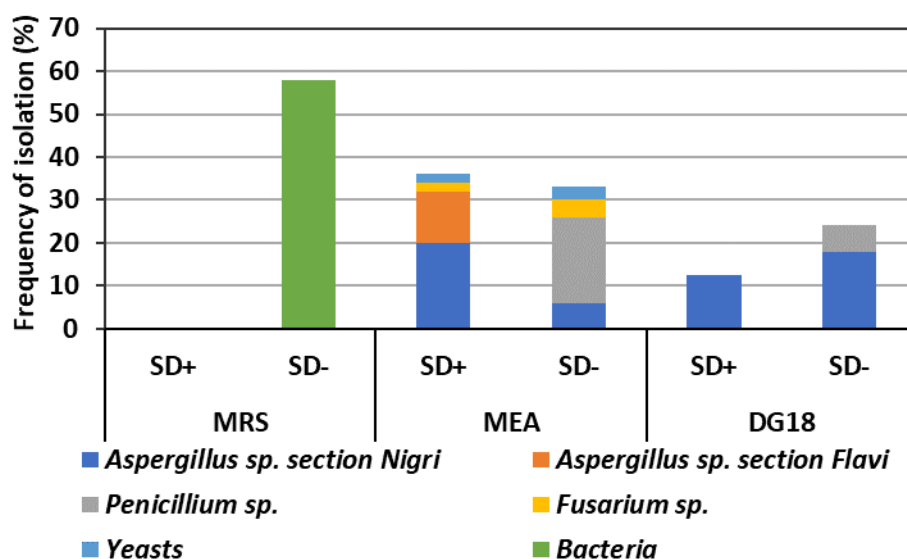


Figure 1. Frequency of bacterial, yeast and fungal isolation (%) in coffee and morphologically identified genus. SD+: surface disinfected/ SD-: non-surface disinfected. MRS: De Man, Rogosa and Sharpe agar; MEA: Malt Extract Agar, DG18: Dichloran-glycerol 18% agar. Data result of direct plating technique.

Regarding the isolation of microorganisms for potential confrontations, 17 yeasts, 59 *Aspergillus* section *Nigri*, 36 *Penicillium* sp., 5 *Aspergillus* section *Flavi* and 4 *Fusarium* sp. isolates were morphologically identified and analysed.

Screening for the ability to produce Ochratoxin A

Forty-seven isolates of *Aspergillus* section *Nigri* were macroscopically and microscopically characterized, purified through monosporic culture (Figure S1), and assessed for OTA production using CCA (Figure S2).

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 2). 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 (Figure S2) 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. As well, one isolate of *Penicillium* sp. showed fluorescence when inoculated on CCA. However, after quantifying the production of OTA through HPLC, this was not as significant as the amount produced by *Aspergillus* section *Nigri* isolates. Consequently, this isolate was not chosen for the confrontation experiments.

Table 2. 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 direct	30629.33 ± 5781.30	158.00 ± 31.11	3250.22 ± 74.79
M3	MEA direct	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 direct	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
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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 - grounded coffee.

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

A total of 17 yeasts isolates, 7 strains of *Lactobacillus 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, Y16 and Y17 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, Y15, Y16 and Y17 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 2). 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 2).

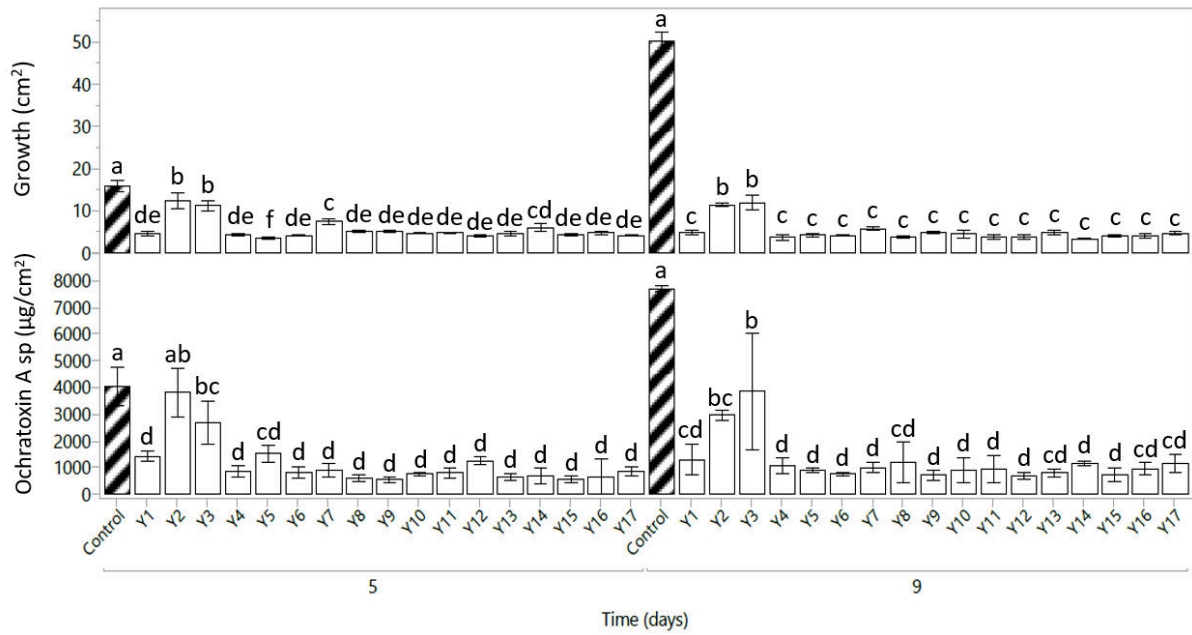


Figure 2. Effect of 17 strains of yeasts in overlay culture assay with *Aspergillus carbonarius* on fungal growth and Ochratoxin A sp (specific production (ng/cm²)) on PDA at days 5 and 9. Different letters express significant differences according to Tukey-Kramer HSD ($p < 0.05$).

During the dual culture assays, different growth inhibition levels were observed, as shown in Figure 3. Y2 showed a lower reduction of growth than Y1 and Y4. As well, the shape of the mycelium varied depending on the confrontation.

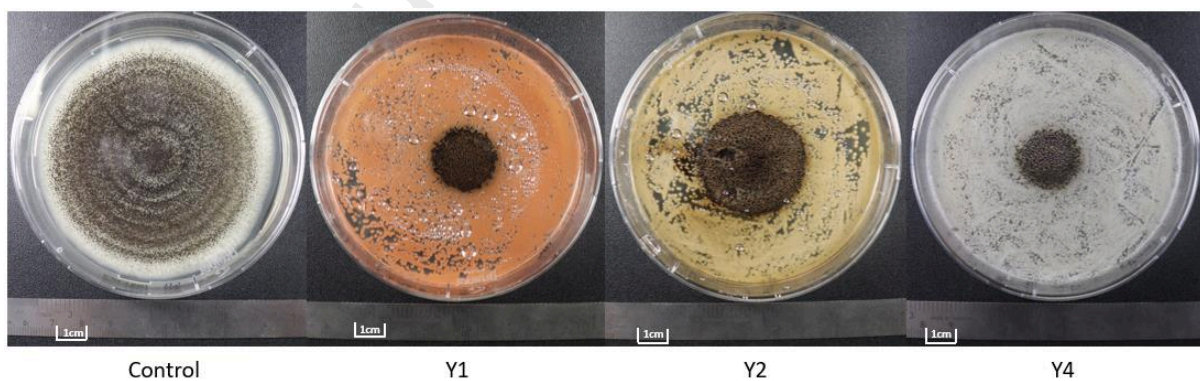


Figure 3. Example of inhibition profiles observed during overlay culture assays of yeasts strains Y1, Y2 and Y4 against *Aspergillus carbonarius* on PDA at 25°C for 9 days. Scale measures 1cm.

Figure 4 depicts the effect of 7 strains of *Lactobacillus 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.

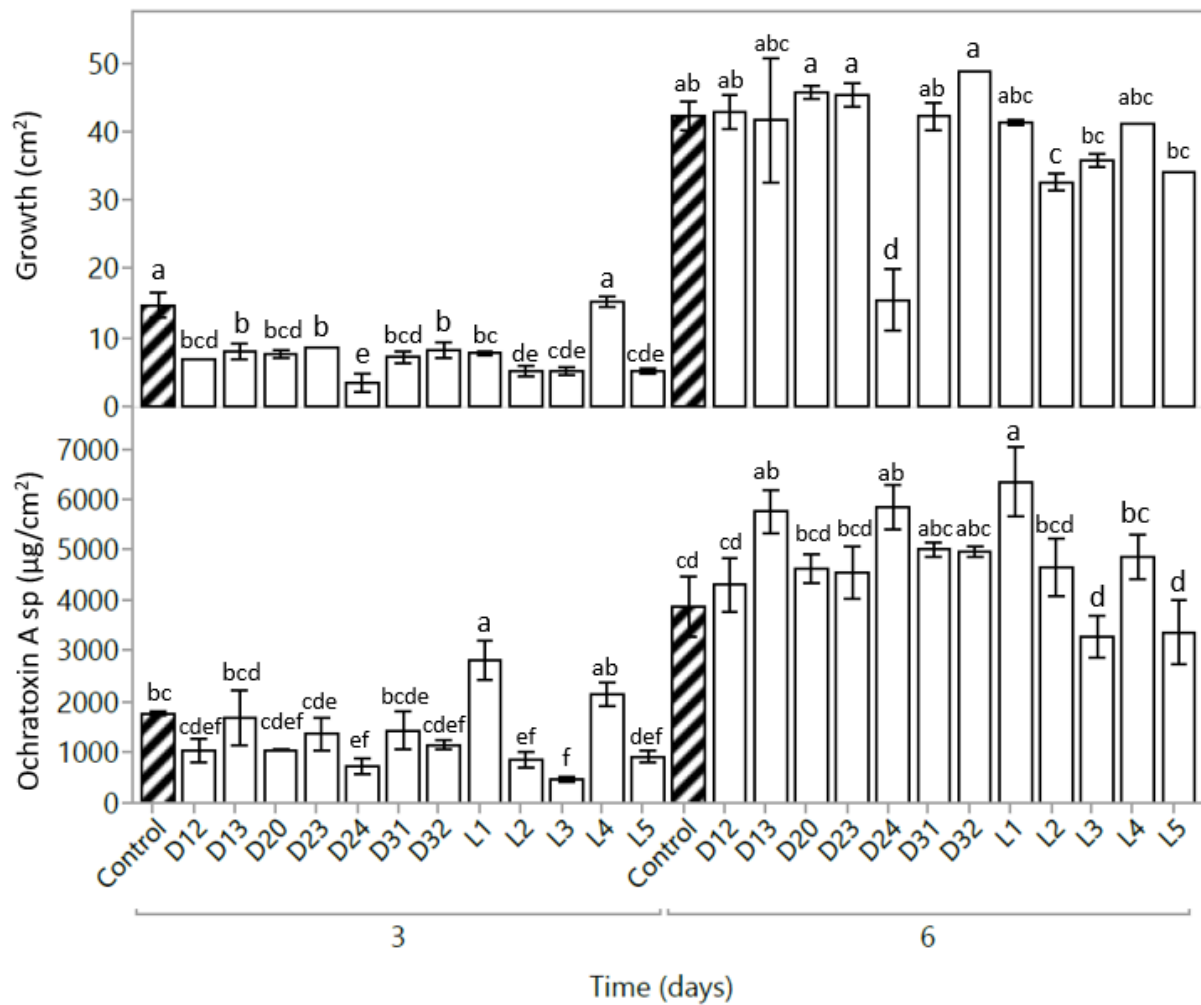


Figure 4. Effect of 7 strains of *Lactobacillus plantarum* (D12, D13, D20, D23, D24, D31, D32) and 5 strains of bacteria (L1, L2, L3, L4, L5) in overlay culture assay with *Aspergillus carbonarius* on fungal growth and ochratoxin A (OTA) specific production (ng/cm²). Each confrontation represents the 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 3).

Table 3. 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>

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 4). The cells showed a significant reduction of the growth and OTA production ($p < 0.05$). Y1 reduced by 2-fold the growth and totally inhibited the OTA production. Y4 reduced by 1-fold the growth and 13-fold the OTA production. CFSs added in the liquid growth medium of *A. carbonarius* did not show significant inhibition of fungal growth and OTA production ($p > 0.05$). The results for OTA concentration reported in Table 4 are ± 5 -100% of the average value, which must be taken into consideration for the interpretation of the results in the discussion section.

Table 4. Effect of yeast cells 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 ± 0.02^a	0.12 ± 0.02	2306.01 ± 2839.19^a	2306.01 ± 2839.19^a
<i>Rhodosporidiobolus ruineniae</i> (Y1)	0.05 ± 0.01^{bc}	0.12 ± 0.01^a	$<\text{LOD}^{1b}$	1149.41 ± 2189.62^a
<i>Meyerozyma caribbica</i> (Y4)	0.07 ± 0.02^b	0.12 ± 0.01^a	167.21 ± 169.03^b	1434.80 ± 812.00^a

¹LOD (Limit of Detection): 0.5 ng/mL

Adsorption test

The two yeast strains (Y1, Y4) were further tested for OTA adsorption capacity (Table 5). As shown in Table 5, *Rhodospiridiobolus 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) 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 %. The degradation metabolite OTα was not detected in these samples (data not shown).

Table 5. 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>Rhodospiridiobolus 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

Microscopy

The interaction between *Rhodosporidiobolus ruineniae* (Y1) and *A. carbonarius* on solid media was further studied under Scanning Electron Microscope (SEM; Figure 5). Figure 5.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 5b.1 and c.1 the blue arrows indicate yeast cells, and the orange arrows fungal mycelium.

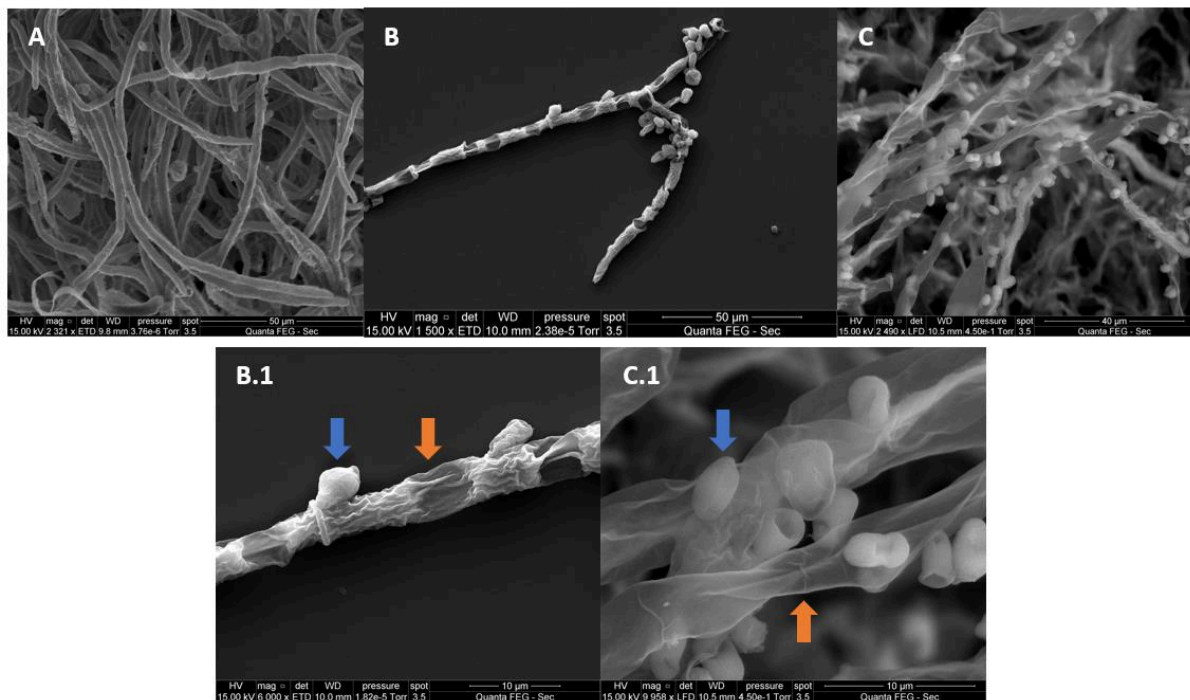


Figure 5. SEM analysis of antagonistic effect of Rhodosporidiobolus ruineniae Y1 yeast against A. carbonarius. (A) Hyphae of A. carbonarius with increase of 2321 X, (B) Hyphae of A. carbonarius co-cultured with Y1 yeast at day 3 with increase of 1500 X, (C) Hyphae of A. carbonarius co-cultured with Y1 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.

4. 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 a_w is critical for OTA production (Paterson et al., 2014).

In this work, three of the thirty-six 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 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 (MRS 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 as displayed in the results on solid media. On liquid media, due to the wide standard deviations on the results for OTA concentration, we could not determine that the mechanism of inhibition is not related to the effect of extracellular metabolites produced by the BCA. The wide standard deviations in the OTA concentrations might be related to a matrix effect produced by the irregular growth of fungi on liquid media, either growing spherically or flat. At the same time, this brings up an added variation to the sampling step.

Previous studies using other yeasts as BCAs against ochratoxigenic fungi have found competition for nutrients and space as the main mechanism of inhibition. Armando et al. (2013) analysed the capacity of *Saccharomyces cerevisiae* reducing the growth and OTA production during the confrontation against *A. carbonarius*. *S. cerevisiae* 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. With respect to the safety of Y1, some strains from the genus *Sporidiobolus* sp., that belongs to the same family as *Rhodosporiobolus* sp., have several patents for their biotechnological use in China and the United States.

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 OT α and L- β -phenylalanine (Campos-Avelar et al., 2020; Shetty & Jespersen, 2006). However, in our study no OT 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. Regarding its safety, *M. caribbica* presents a broad spectrum of antifungal activity in fruits at postharvest, however, some strains can cause diseases, and toxicity studies are lacking (Herrera-Balandrano et al., 2023).

None of the potential BCAs isolated in this study hold the Generally Recognized as Safe (GRAS) status. Nevertheless, there are different applications that have been already studied. Some strains of *Sporidiobolus* sp. are capable of growth inhibition of *A. niger* in grapes and spore germination of *P. expansum* in apples (Kot et al., 2021). Furthermore, in the work done by Aguirre-Güitrón et al. (2018), *M. caribbica* was formulated for its application as a biocontrol using the spray drying process. For the case of the proposed BCAs they would be applied as an spray or by soaking the coffee cherries after harvest and before drying. This way, the growth of *A. carbonarius* would be prevented, and potentially the production of OTA. Further studies on the effect of the application of these BCAs on the safety and organoleptic quality of the final coffee would be needed.

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. 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

A. carbonarius *Aspergillus carbonarius*

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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Supplementary materials

Table S1 **Error! Reference source not found.** 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 S1. 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	

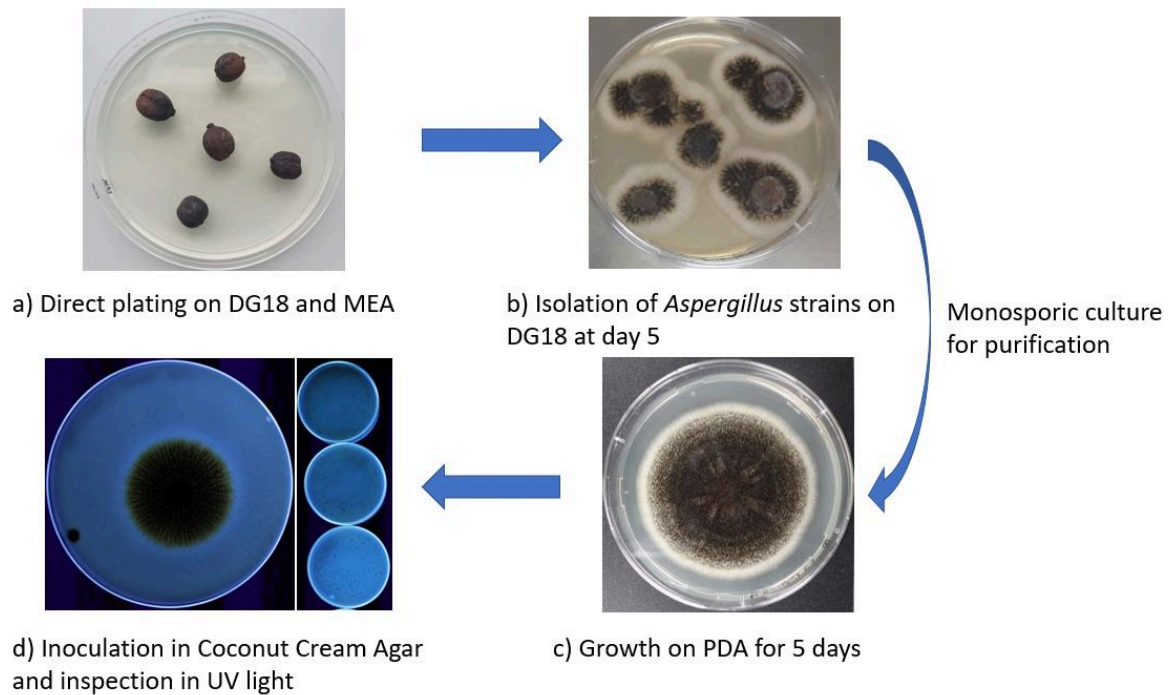


Figure S1. Process of (a) direct plating, isolation of *A. carbonarius* (b), purification through monosporic culture (c) and detection of fluorescence under UV light (d).

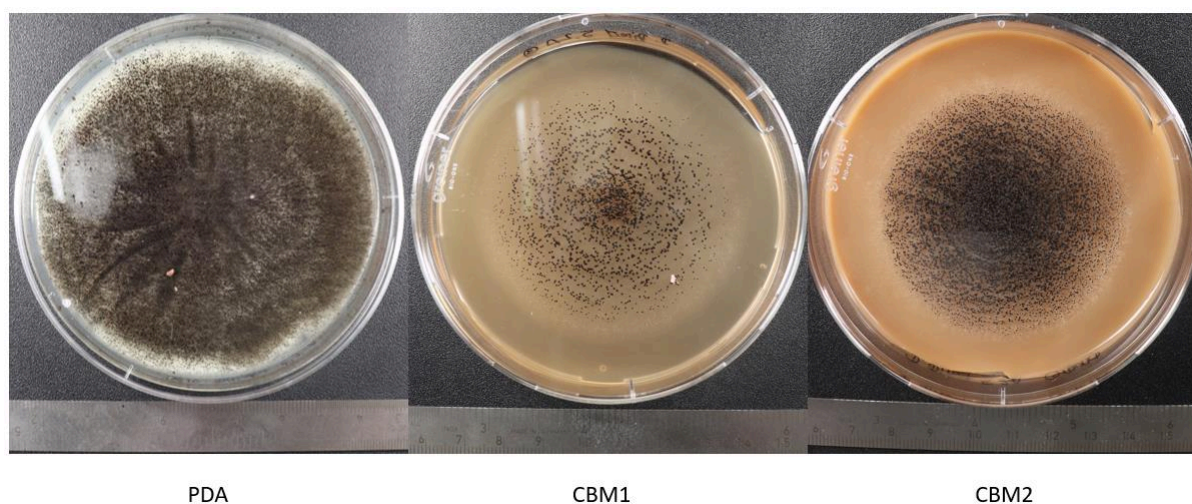


Figure S2. Plates from the studies of *A. carbonarius* when using PDA at day 9, and CBM1 – whole dry cherry and CBM2 - grounded coffee, as substrate on day 12 at 25°C for the toxigenic strain D3.

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.
- *Rhodosporidiobolus ruineniae* showed adhesion to the hyphae and Ochratoxin A adsorption.
- *Meyerozyma caribbica* degrades Ochratoxin A *in vitro*.



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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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