



## Research Article

# BIOCONTROL ACTIVITY OF YEAST STRAINS ISOLATED FROM GREEN COFFEE BEANS AGAINST OCHRATOXIN A-PRODUCING *Aspergillus* SPECIES

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**Abstract-** Coffee is one of the most consumed products worldwide, but when not properly processed, it can be contaminated with ochratoxin A (OTA)-producing fungi such as *Aspergillus* species. The yeasts *Wickerhamomyces anomalus*, *Meyerozyma guilliermondii*, *Meyerozyma caribbica*, *Hyphopichia burtonii*, and *Aureobasidium pullulans* were isolated from green coffee beans and co-inoculated with the OTA-producing fungi *Aspergillus ochraceus* and *Aspergillus carbonarius*. Growth inhibition *in vivo* and *in vitro* as well as OTA levels were assessed. *W. anomalus*, *M. caribbica*, and *H. burtonii* had the best results. These yeasts have the potential to inhibit growth and reduce OTA production by fungi naturally occurring in coffee beans.

**Keywords-** *Mycotoxins, Wickerhamomyces anomalus, Hyphopichia burtonii, Coffea arabica, Volatile compounds*

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## Introduction

Coffee is one of the most important commodities in the world market, especially for subtropical countries [1]. Arabica coffee accounts for the majority of the global production, and the dry method is frequently used in coffee processing [1]. Studies comparing different post-harvest practices indicate that 92% of the beans processed using this method were contaminated with fungi [2]. This happens because bark and mucilage are not separated from ripe coffee beans while drying directly in the sun, and thus contamination can occur since coffee mucilage provides a good substrate for many microorganisms [3,4]. Several microorganisms have been isolated from coffee beans in many stages of maturation, such as fungi, yeasts, and bacteria [5-8]. Among them are fungi of the genera *Aspergillus* and *Penicillium*, which can produce ochratoxin A (OTA), a secondary metabolite that naturally occurs in nature and can be produced during storage in response to changes in temperature and moisture or addition of H<sub>2</sub>O<sub>2</sub> [9-11]. OTA is nephrotoxic, hepatotoxic and a potentially carcinogenic, being related to the Balkan endemic nephropathy (irreversible and fatal kidney disease) [12]. OTA is commonly found in foods, such as grapes, cacao and coffee besides it is resistant to high temperatures, making it very difficult to control [13-17]. The presence of OTA in coffee is a result of post-harvest and storage conditions, as well as drying and transportation processes [18]. However, the use of chemicals to inhibit the growth of OTA-producing fungi and OTA contamination in foods has been banned in several countries [19]. In this scenario, yeasts produce secondary metabolites that arise as alternatives to control potentially toxigenic fungi during pre and post-harvest processes [20]. This is mostly due to their ability to grow in harsh conditions such as high and low pH, and long periods under dry and anaerobic conditions [21, 22]. Thus, in this study, the objective was to assess the ability of yeast to inhibit OTA production and growth of *Aspergillus* species. To this end, we isolated yeast strains from green coffee beans and used *in vitro* and *in vivo* inhibition methods.

## Materials and Methods

### Cultures

Yeast strains were isolated from green *Coffea arabica* beans collected in 2011 in Agronomic Institute of Paraná, located in Londrina, PR, Brazil. Green coffee beans were processed by the dry method, collected aseptically, and stored in sterile plastic bags protected from sunlight. The reference strain of OTA-producing *Aspergillus ochraceus*, INCQS 40013 (ATCC 22947), was obtained from the National Institute of Quality Control in Health (INCQS) of the Oswaldo Cruz Foundation, Rio de Janeiro, Brazil and the strain of OTA-producing *Aspergillus carbonarius* 187 UEL was provided by the Laboratory of Molecular Biology of the Londrina State University, Londrina, Brazil. Yeasts and fungi were stored at 4°C.

### Culture media

Four media were used in this study: Sabouraud agar (SB), Czapek Dox agar (CZA), Coffee agar (CA) and YEPD-MB-Phosphate Citrate Buffer agar (YEPD). The former was used for the isolation and inhibition tests and consisted of 40 g/L glucose, 15 g/L peptone, 10 g/L agar. CZA was used in inhibition tests and consisted of 30g/L of sucrose, 2g/L of sodium nitrate, 1g/L of dipotassium phosphate, 0.5g/L of magnesium sulfate, 0.5g/L of potassium chloride, 0.01g/L of iron sulfate and 15g/L of agar.

CA was used in inhibition tests and was prepared by boiling 20g of green coffee beans in 1 liter of water, which was then filtrated with a 0.45mm filter. The water collected was mixed with 15g/L of agar. The YEPD was used in metabolite production detection and was prepared using 10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose, 0.01g/L methylene blue, 0.1M Phosphate Citrate Buffer until pH 4 and 20 g/L agar.

## Yeast isolation

Microorganisms from green coffee bean samples were isolated by direct plating of four beans on SB and incubated at 28°C for 72 h. Yeast colonies were collected and purified for further identification and stored at 4°C [23]. Isolates were then deposited in the Coleções de Culturas Microbiológicas da Rede Paranaense – CMRP rede TAXonline (Federal University of Paraná, Brazil).

## Yeast identification

DNA extraction from yeast cells was performed from pure cultures incubated at 28°C for 48 h, samples were diluted to 20ng/μL [24]. For identification of yeasts, the ITS1-5.8S-ITS2 region of ribosomal DNA was amplified using the following primers: ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3'). DNA amplification was carried out in a 25 μL volume, and each reaction contained 1x buffer, 2.5 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 0.1 μM each primer (ITS1 and ITS4) and 0.1 μM Taq DNA polymerase (Applied Biosystems, USA) [25]. The PCR reaction was performed in an automatic thermal cycler (2720, Applied Biosystems) under the following conditions: initial denaturation at 94°C for 2 min; 30 cycles at 94°C for 35 sec, 52°C for 30 sec and 72°C for 1 min; and final extension at 72°C for 7 min. The PCR fragments were sequenced with an ABI 3500 automated sequencer (Applied Biosystems).

## Growth inhibition tests of *Aspergillus* species

### Inhibitory effect of yeasts in SB, CZA and CA

Yeasts were inoculated in the three cultures media for 48 hours at 28°C and then transferred to tubes containing 0.85% saline solution at a cell concentration of 1x10<sup>6</sup> cells/mL (estimated using a counting chamber). *A. ochraceus* and *A. carbonarius* were inoculated for 7 days at 28°C. One milliliter of yeast suspension was added to 5 mL of the melted culture medium where the yeast was inoculated, and the suspension was spread over a sterile Petri dish containing 15 mL of the same culture medium. A 4-mm diameter disc was removed from the center of each plate and 10 μL of a suspension of *A. ochraceus* or *A. carbonarius* (1x10<sup>6</sup> spores/mL) was added. The tests plates were incubated for 7 days at 28°C, and the growth of *Aspergillus* sp. was evaluated. The assay was carried out with three repetitions and the control group consisted of plates containing only the standards of *A. ochraceus* and *A. carbonarius* [20]. The diameter of the *Aspergillus* sp. colonies was measured to determine growth inhibition using the formula

$$(PI\%) = ((Dc-Dt) / Dc) \times 100$$

Where, Dt as the diameter of the colony of *Aspergillus* sp. in the inhibition test and Dc the diameter of the control group of *Aspergillus* sp. [26].

### Reduction of OTA production by *Aspergillus* species

Yeasts were inoculated for 48 hours at 28°C then transferred to tubes containing 0.85% saline solution (1.5 x 10<sup>8</sup> cells/mL) and spread on SB plates [27]. A 7 mm disc was removed from the center of each plate, and a SB disc containing *Aspergillus* mycelia incubated for 7 days at 28 °C was inserted. The experiments were carried out with three repetitions for 7 days at 28°C, and plates containing only *Aspergillus* mycelia were used as control. Three small aliquots (7mm plugs) of fungi were collected and transferred to tubes containing 1.5 mL of methanol. The suspensions were placed for one hour in the dark and then centrifuged at 4000 rpm. The toxin was filtered through glass wool and evaporated at 50°C and added 1 mL of the mobile phase of the HPLC (SYS-LC-240-E, Varian, USA), acetonitrile: acidified water (2%) (50:50 (v:v)) filtered through a 0.22-μm pore filter [28, 29]. The samples were analyzed by HPLC with fluorescence detection (330 nm excitation and 470 nm emission) (Fluorescence detector model 363, Varian, USA), using a C18 column (5 μm, 25 x 4.6mm, Varian, USA) a full loop was injected though a ProStar 410 Auto-Sampler at a flow rate of 1 mL/min. The concentrations were expressed in ng/L, and the detection limit of this method was 0.5 ng/L, the default OTA values for *A. carbonarius* and *A. ochraceus* were established by extracting the toxin of standard strains. The experimental data were expressed as percentages of inhibition, according to the following formula:

$$\% \text{ Reduction} = 100 - [(C_{OTA \text{ sample}} / C_{OTA \text{ control}}) \times 100] [30].$$

## Investigation of the mechanism inhibition

### Inhibitory effect of volatile compounds produced by yeasts

Yeasts and fungi were inoculated in SB, incubated for 48 hours (yeasts) and 7 days (fungi) at 28°C, then transferred to tubes containing 0.85% saline solution to obtain suspensions at the concentrations of 1x10<sup>6</sup> cells/mL and 1x10<sup>6</sup> spores/mL. Yeast suspensions (100 μL) were spread on SB plates, while in another SB plate a 4 mm disc was removed from the center and 10 μL of the suspension of *A. ochraceus* or *A. carbonarius* was added. Plates were fitted to each other and sealed to prevent the leakage of volatile compounds produced by yeasts. Plates were incubated at 28°C for 14 days, and the growth of *Aspergillus* sp. was measured at 7 and 14 days of testing [20]. The assay was carried out with three repetitions and control plates containing only *Aspergillus* sp. were used. The diameter of *Aspergillus* sp. colonies was measured to determinate growth inhibition with the formula [item 2.5.1].

### Detection of production of metabolites by yeasts

The yeasts were inoculated in the media YEPD-MB-Phosphate Citrate Buffer-Agar with conditions that enable the metabolite production to assay the capability of those to produce metabolites [31]. An 6mm agar plug with a 2 days colony were placed in a plate containing the YEPD media. After 2 days the color of the media was analyzed and clear halos were indicative of metabolite production.

## Analysis of the inhibitory potential of selected yeast *in vivo* in green coffee beans against *Aspergillus* sp.

### Inoculum preparation

The yeast with the highest *in vitro* inhibition rates, *W. anomalus* - CAT 1 L1 inoculated for 48 hours and *Aspergillus* sp. for 7 days on SB plates were selected for the *in vivo* tests and transferred to tubes containing 0.05% of Tween 80. The concentrations were standardized in 1x10<sup>5</sup> and 1x10<sup>7</sup> cells/mL for yeast, and 1x10<sup>2</sup> spores/mL for *Aspergillus* sp. [32].

### Coffee beans preparation

The grains must undergo a superficial disinfection to avoid contamination. Three hundred grams of green coffee beans were disinfected with autoclaved water, alcohol (70%), and hypochlorite (3.5%) in a laminar flow hood, where they remained drying on a sterile paper filter for 1 hour [32].

### Inoculation in green coffee beans

Beans were immersed in 500 mL of yeast suspension for 2 minutes. For the negative control, beans were immersed for 2 minutes in 500 mL of 0.05% tween 80. After drying in a laminar flow hood, 5 mL of *Aspergillus* sp. suspension was sprayed on coffee beans that were then dried on a sterile filter paper. Tests were carried out with three repetitions, beans were placed in jute bags and incubated in the dark at 26°C and 75% humidity for 24 days [32]. The growth of *Aspergillus* sp. was evaluated every 2 days by collecting 10 grams of coffee beans and examining under stereoscope (Zeiss, Germany). *Aspergillus* sp. growth was expressed as percentages. The temperature was standardized at 26°C (temperature at which the grains are normally stored in the field). The following moisture conditions were tested in the incubation of the grains, 0% (no microorganism proliferation) and 90%.

### Statistical analysis

Analysis of Variance (ANOVA) was performed using a completely randomized design (DIC). When significant differences were detected by the F test, the analyzes were followed by the Tukey test (p <0.01) using the statistical software ASSISTAT version 7.7 [33].

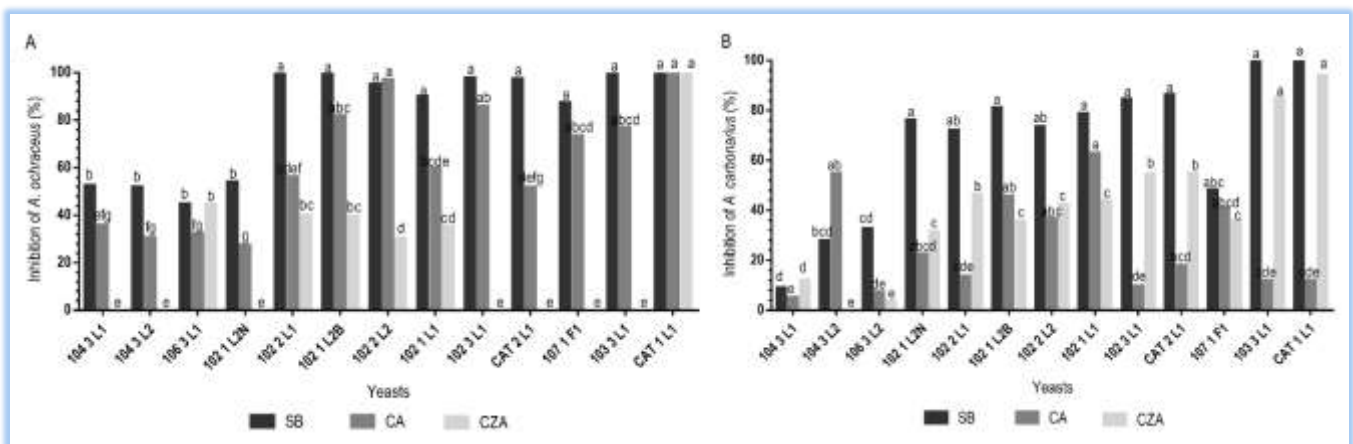
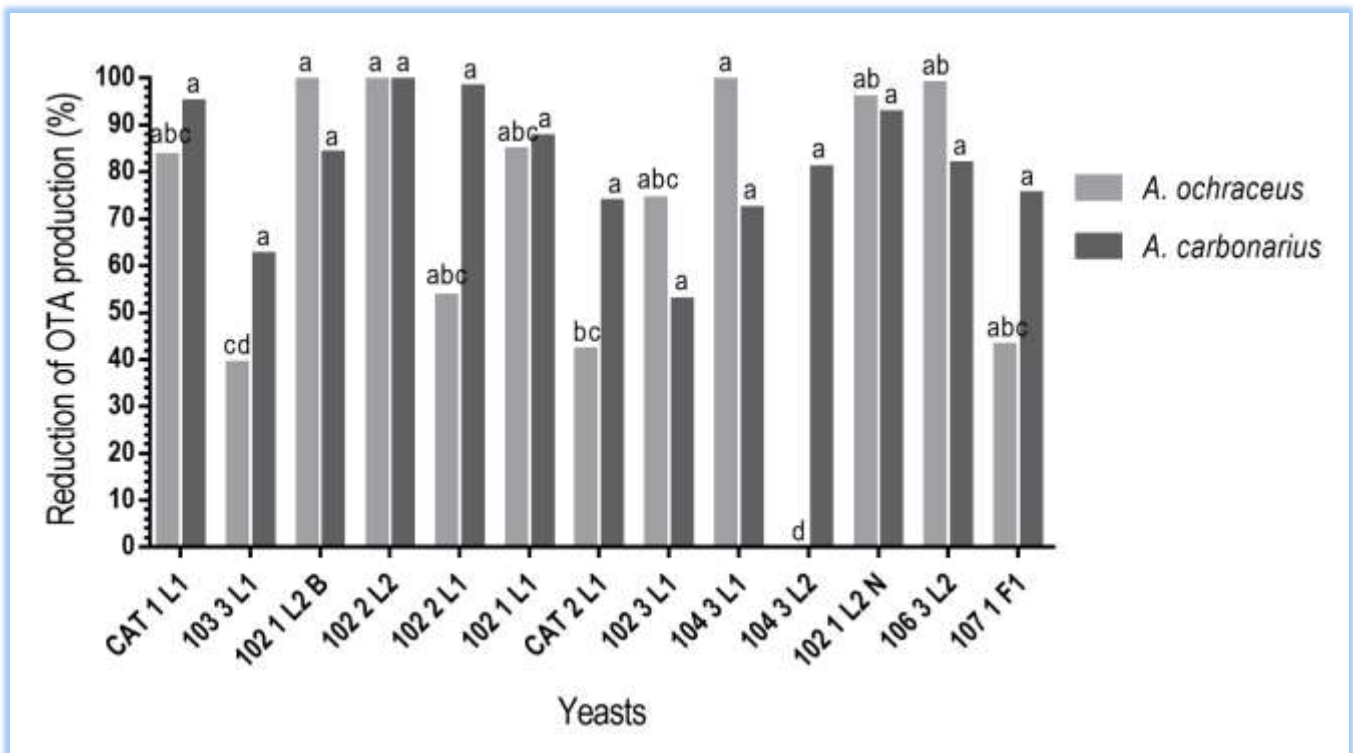
## Results and Discussion

### Yeast isolation and identification

Thirteen yeast isolates were obtained from green coffee bean samples and identified as *Wickerhamomyces anomalus*, *Hyphopichia burtonii*, *Meyerozyma caribbica*, *Aureobasidium pullulans*, and *Meyerozyma guilliermondii* [Table-1]. This is the first report on the isolation of *A. pullulans* on green coffee beans.

Table-1 Yeast strains isolated from green coffee beans and identified by sequencing the ITS1-5.8S rDNA-ITS2 region amplified with primers ITS1 and ITS4, the GenBank accession number and Coleção de Culturas Microbiológica da Rede Paranaense -CMRP accession number.

Strain ID	Species	Amplicon size (bp)	Identity <sup>1</sup> (%)	GenBank Accession No.	CMRP Accession No.
CAT 1 L1	<i>Wickerhamomyces anomalus</i>	593	99	KP638728	CMRP3421
103 3 L1	<i>Wickerhamomyces anomalus</i>	570	99	KP638741	CMRP3422
102 1 L2B	<i>Hyphopichia burtonii</i>	443	99	KP638740	CMRP3423
102 2 L2	<i>Hyphopichia burtonii</i>	432	97	KP638737	CMRP3424
102 2 L1	<i>Hyphopichia burtonii</i>	443	99	KP638729	CMRP3425
102 1 L1	<i>Hyphopichia burtonii</i>	403	99	KP638735	CMRP3426
CAT 2 L1	<i>Meyerozyma caribbica</i>	607	99	KP638738	CMRP3427
102 3 L1	<i>Meyerozyma caribbica</i>	607	100	KP638733	CMRP3428
104 3 L1	<i>Aureobasidium pullulans</i>	582	98	KP638730	CMRP3429
106 3 L2	<i>Aureobasidium pullulans</i>	582	99	KP638731	CMRP3430
102 1 L2N	<i>Aureobasidium pullulans</i>	535	99	KP638732	CMRP3431
104 3 L2	<i>Aureobasidium pullulans</i>	584	99	KP638734	CMRP3432
107 1 F1	<i>Meyerozyma guilliermondii</i>	616	99	KP638727	CMRP3433

<sup>1</sup>Percentage of identical nucleotides found in GenBank sequencesFig-1 Percentages of inhibition of *A. ochraceus* and (A) *A. carbonarius* (B) in SB, CA, and CZA. \*Different letters in the same medium indicate significant differences detected by the Tukey test (P < 0.01).Fig-2 Reduction of *Aspergillus*-produced OTA by yeast isolated from green coffee beans. Reduction of OTA was expressed in percentage compared to the control. \*Different letters indicate significant differences, according to the Tukey test for each *Aspergillus* species (P < 0.01).

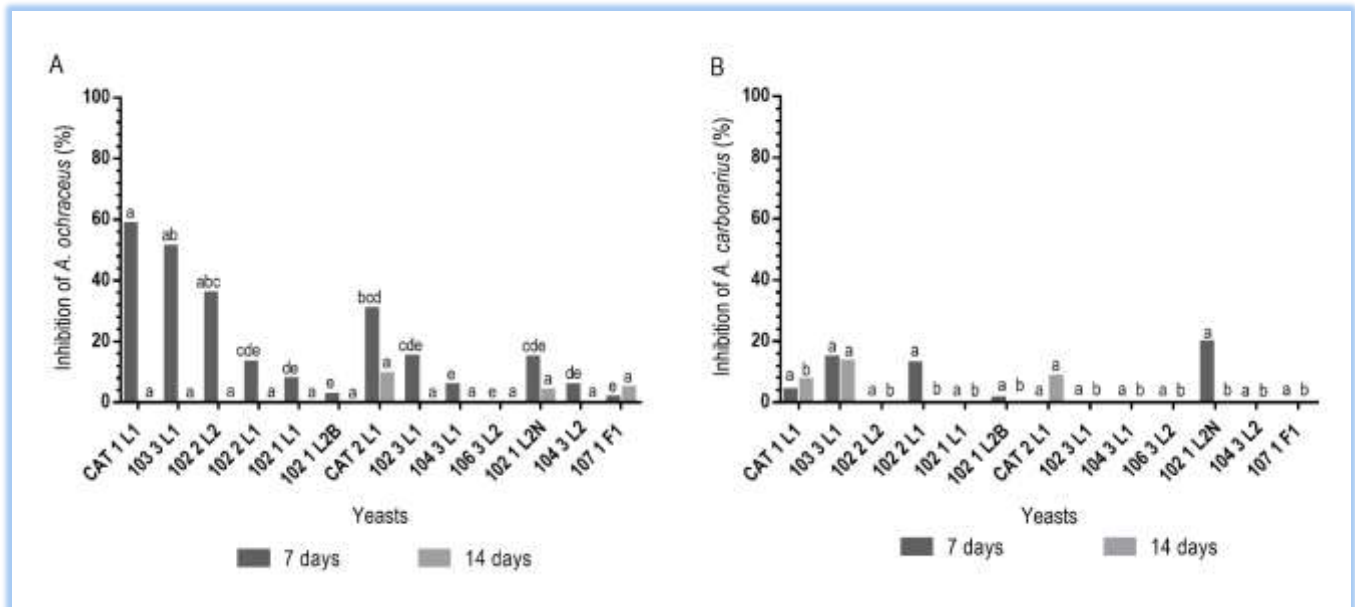


Fig-3 Percentages of inhibition of *A. ochraceus* (A) and *A. carbonarius* (B) by volatile compounds produced by yeast isolates. \*Different letters indicate significant differences of each period by the Tukey test ( $P < 0.01$ ).

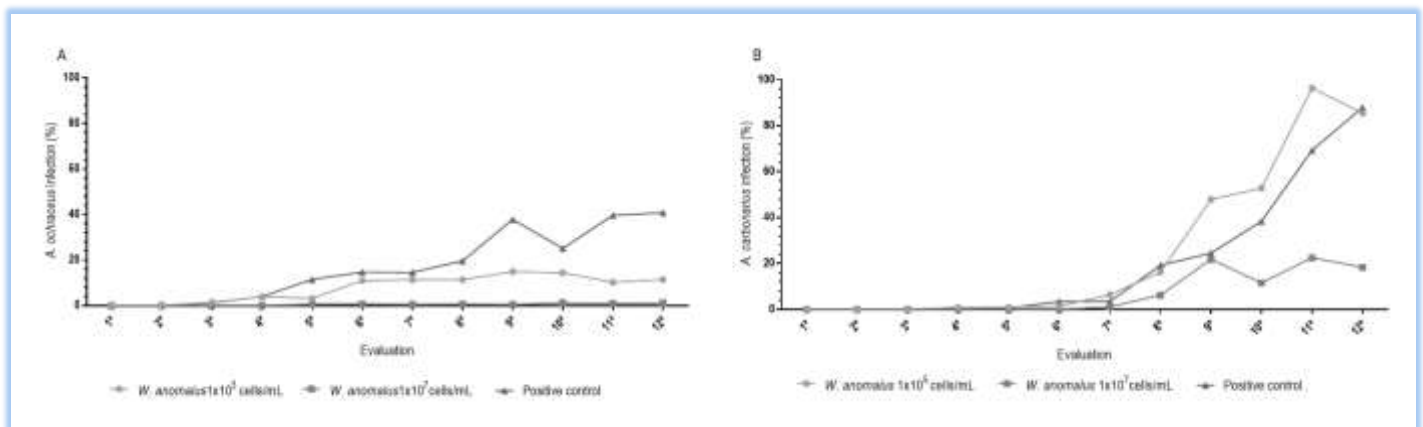


Fig-4 Infection percentage of *A. carbonarius* (A) and *A. ochraceus* (B) in coffee beans inoculated with *W. anomalus*.

The yeasts *M. guilliermondii*, *W. anomalus* and *H. burtonii* have been previously isolated from Brazilian coffee beans in different stages of processing [34]. *M. caribbica* was found in small amounts in coffee beans processed by a semi-dry method [35]. Hamadi *et al.* [36] isolated yeasts from Tanzanian coffee beans processed by the wet method that were capable of fermenting ethanol, among them *M. guilliermondii* and *W. anomalus*, indicating their potential in bioethanol production.

### Growth inhibition tests of *Aspergillus* species

#### Inhibitory effect of yeasts in SB, CZA and CA medium.

All thirteen yeast isolates were able to inhibit *Aspergillus* species in at least one culture medium [Fig-1]. Our results showed that the inhibition percentage of *A. ochraceus* (mean of 82.29% for SB, 62.15% for CA, and 22.23% for CZA) was higher than the inhibition of *A. carbonarius* (mean 66.89% for SB, 26.29% for CA and 41.43% for CZA), in agreement with the findings by Zhu *et al.* [20]. The best results were obtained using SB, with inhibition varying between 44.65 and 100% for *A. ochraceus* and 8.95 to 100% for *A. carbonarius*. Regarding CZA, inhibition was highest for *A. carbonarius* (ranging from 3.45 to 93.84%), with the best results were obtained using *W. anomalus* (93.84% for CAT 1 L1 and 85.13% for 103 3 L1). For CA, *A. carbonarius* inhibition was highest with *H. burtonii* (102 1 L1), while for *A. ochraceus*, inhibition was highest with *W. anomalus* (CAT 1 L1). Culture media with high carbon concentration (SB) induce yeast growth, consequently inhibiting *A. ochraceus* and *A. carbonarius* [28]. Comparing our results on CA and CZA, *A. ochraceus* inhibition was highest using the former, suggesting that yeasts grown better in an environment similar to the one they were isolated from. Studies

show that the caffeine can control the grow of yeasts, this can be confirmed by the inhibition results when CA produce less inhibition comparing with SB [37]. SB had the highest carbon concentration compared to those of other media used and had the best results. A high nutrient level promotes yeasts grow and consequently *Aspergillus* inhibition, thus SB was used in the tests.

#### Reduction of OTA production by the *Aspergillus* species

The yeast isolates evaluated decreased OTA levels produced by *A. ochraceus* and *A. carbonarius*. The reduction of OTA levels produced by *A. ochraceus* ranged from 42.06 to 100% [Fig-2]. The highest percentages of OTA reduction were obtained for the yeasts *H. burtonii* (102 2 L2 and 102 1 L2B) and *A. pullulans* (104 3 L1). OTA reduction was highest for *A. carbonarius*, with a mean of 76.15%, while lower reduction rates were observed for *A. ochraceus* (mean of 69.01%). Studies evaluating OTA degradation by yeasts have found that *A. pullulans* degrades OTA into OT $\alpha$ , a less toxic compound and phenylalanine [38]. *Candida intermedia*, *Candida friedrichii*, and *Lachancea thermotolerans* degrade OTA in grape juice up to 75%, while *M. guilliermondii* degraded 65.3% of OTA added to a liquid media [39, 40]. Other yeasts can decrease OTA production by inhibiting fungal growth, such as *W. anomalus* and *P. kluyveri* against *A. ochraceus* [28]. Other hand, some yeasts, such as *Candida zemplinina* and *Saccharomyces cerevisiae*, could not degrade OTA after it was produced [20]. The yeast isolates tested were able to reduce OTA production, possibly by inhibiting fungal growth, absorbing the toxin, or producing substances diffusible in the media that can degrade OTA, possibly into OT $\alpha$  and phenylalanine [41, 42]. Further studies should be conducted to investigate the mechanisms associated to OTA reduction.



No studies have reported the reduction of OTA production by *H. burtonii* and *M. caribbica*. The yeast *W. anomalus* (CAT 1 L1) showed significant inhibitory potential in *in vitro* tests and was selected for *in vivo* testing.

### Investigation of the mechanism inhibition

#### Inhibitory effect of volatile compounds produced by yeasts

Yeast isolates produced volatile compounds that inhibited the growth of *A. ochraceus* and *A. carbonarius*. Inhibition rates were highest for *W. anomalus* (CAT 1 L1) against *A. ochraceus* at 7 days (58.2%). No statistically significant differences between the inhibition of *A. carbonarius* were found at 7 days. Two yeasts were statistically superior to the other yeasts [Fig-3] at 14 days testing, *W. anomalus* (8.38%) (CAT 2 L1) and *M. caribbica* (11.33%) (103 3 L1). Volatile compounds produced by yeasts have low-molecular weight and thus can easily evaporate at normal temperature and pressure conditions [43]. In our study, volatile compounds of yeasts inhibited *Aspergillus* species, and according to Hua *et al.* (2014) [44], they can affect spore germination, mycelial growth, toxin production and gene expression. Studies have shown that *A. ochraceus* is inhibited by volatile yeast compound especially those produced by *W. anomalus*, which induced the highest inhibition rates, compared to *P. kluyveri* and *Hanseniaspora uvarum* [45]. Parafati, *et al.* (2017) [46] used volatile compounds of *W. anomalus* and *A. pullulans* against *B. cinerea*, *P. digitatum*, and *P. italicum*. After direct contact between a suspension of yeast and fungus, conidial germination was evaluated, revealing that *W. anomalus* strongly inhibited spore germination of the 3 fungi tested (percentage of germination below 7%), *A. pullulans* also inhibited fungi growth, but with higher percentages of germination.

#### Detection of production of metabolites by yeasts

Six yeasts (107 1 F1, 103 3 L1, CAT 1 L1, 106 3 L2, 104 3 L2, 102 2 L1) shown to be able to produce metabolite. *M. guilliermondii*, *W. anomalus*, *A. pullulans*, *H. burtonii*. Studies indicate that *M. guilliermondii* presents metabolite extracted with ethyl acetate with inhibitory activity against bacteria [47]. *W. anomalus* has the ability to produce volatile secondary metabolites such as ethyl acetate, isoamyl acetate and ethyl propionate [21, 45]. *A. pullulans* produces in its metabolism the pullulan substance that has enormous application in the alimentary area, such as stimulatory effect on insulinotropic activity of pancreatic islets [48]. No data are available on the production of metabolites by *H. burtonii*. These results indicate that yeasts have the ability to produce metabolites of interest, further tests should be performed to assess whether they have antimicrobial capacity.

#### Analysis of the inhibitory potential of selected yeast *in vivo* in green coffee beans against *Aspergillus* sp.

The infection percentage of *A. ochraceus* or *A. carbonarius* in stored coffee beans was calculated by evaluating fungal growth [Fig-4]. The results indicated that *W. anomalus* (CAT 1 L1) in both concentrations used was effective in inhibiting *A. ochraceus* during the 24 days tested, when compared to the positive control. The growth rate of *A. ochraceus* was 40% for the positive control and 11% in the test with yeast at the concentration of  $10^5$  cells/mL and 1.26% for the test using the yeast at the concentration of  $10^7$  cells/mL. Yeast isolates inoculated at the concentration of  $1 \times 10^7$  cells/mL induced the highest reduction in *A. ochraceus* growth. Regarding *A. carbonarius* infection, fungal growth was 25% when yeast was inoculated the concentration of  $1 \times 10^5$  cells/mL, and at a higher concentration ( $1 \times 10^7$  cells/mL) *A. carbonarius* was inhibited with growth rate at 6%. Comparing the growth rate of *A. carbonarius* in the positive control (20%) and in the test with yeast at the concentration of  $10^5$  cells/mL (25%), it is observed that the yeast concentration was not sufficient to inhibit the fungus, this concentration may still have generated a stress condition for the fungus, which may have led to an induction of OTA production. Studies indicate that the *otapksPV* gene (related to OTA production) is regulated by environmental stimulation, suggesting that the inhibition did not occur at this concentration because the fungus may have produced OTA, causing damage to yeast [49, 50]. Yeast isolates from green coffee beans are promising alternatives to inhibit mycotoxin-producing fungi *in*

*vivo*, as they can prevent OTA production and are already used in coffee fermentation [6, 51]. The inoculation of these yeasts in stored coffee beans does not alter the quality of coffee and can prevent the proliferation of fungi under storage conditions [52]. Also, they easily grow in liquid media, making large-scale production viable. Further studies are needed to identify the active metabolites of yeast isolates and apply them to inhibit *Aspergillus* spp. in stored coffee beans.

### Conclusion

Our results indicate that yeast isolates from green coffee beans clearly inhibited *A. ochraceus* and *A. carbonarius* growth (*in vivo* and *in vitro*) and OTA production. A high yeast concentration is required to inhibit fungal growth in coffee beans. We demonstrated that the yeasts, especially *W. anomalus*, have the potential to be used as biocontrol agents.

**Application of research:** These results indicate that yeasts isolated from coffee beans and their products (volatile and non-volatile) can be used to control fungi OTA-producers in stored coffee bean. Those results can be an alternative for the using of chemical products.

**Research Category:** Applied and Environmental Microbiology; Food Microbiology

**Abbreviations:** Ochratoxin A (OTA), Sabouraud agar (SB), Czapek Dox agar (CZA), Coffee agar (CA), YEPD-MB-Phosphate Citrate Buffer agar (YEPD), Analysis of Variance (ANOVA).

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**Author Contributions:** All author equally contributed

**Author statement:** All authors read, reviewed, agree and approved the final manuscript

**Conflict of Interest:** None declared

**Ethical approval:** This article does not contain any studies with human participants or animals performed by any of the authors.

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