

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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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

1. 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 a various 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, 2006).

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 (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.

2. MATERIALS AND METHODS

2.1 GENERAL OVERVIEW OF THE COFFEE SAMPLING AND HANDLING PROCESSES

Coffee samples were collected from differently processed Robusta coffees. The coffee was cultivated in Akoupe, Ivory Coast (6° 23' north, 3° 54' west) at an altitude of 126 m and the same batch was sampled before and just after drying. The cherries were handpicked and then followed five different dry processes detailed in Figure 1. The traditional, primary post-harvest processing named DP (Dry Process), consisted in directly sun-drying fresh coffee cherries. This process is commonly practiced by Ivorian coffee farmers. The processes named SDP (Soaked Dry Process) and SHDP (Soaked Heaped Dry Process) went through a soaking step. The soaking consists of putting the coffee in a tank with sterile water (sterilized at 121 °C for 15 min) for 24-48 h. These are experimental processes in order to assess the effect of partial depulping of coffee thanks to the soaking step. Processes SHDP, HDP (Heaped Dry Process) and EHDP (Extended Heaped Dry Process) go through a spontaneous fermentation. The spontaneous fermentation happens when coffee is stored in heaps, it can happen after soaking (in case coffee was soaked) or just after harvesting. This is a common malpractice between Ivorian coffee farmers. The length of the spontaneous fermentation can vary between 4 and 7 days, depending for how long they are stored in heaps. After fermentation, they are dried on the floor 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 each differently processed coffee and 19 kilograms of fresh cherries, not processed, were collected by hand using sterilized gloves and placed separately in sterilized plastic bags at 4 °C. At reception, all samples were then kept at -20 °C. Three replicates of each fraction were randomly selected after mixing the 2 kg of each processed coffee. For the obtention of the husk and bean fractions, dried cherries were

manually dehusked, helping to understand the different levels of OTA and changes in the mycobiota across the different layers.

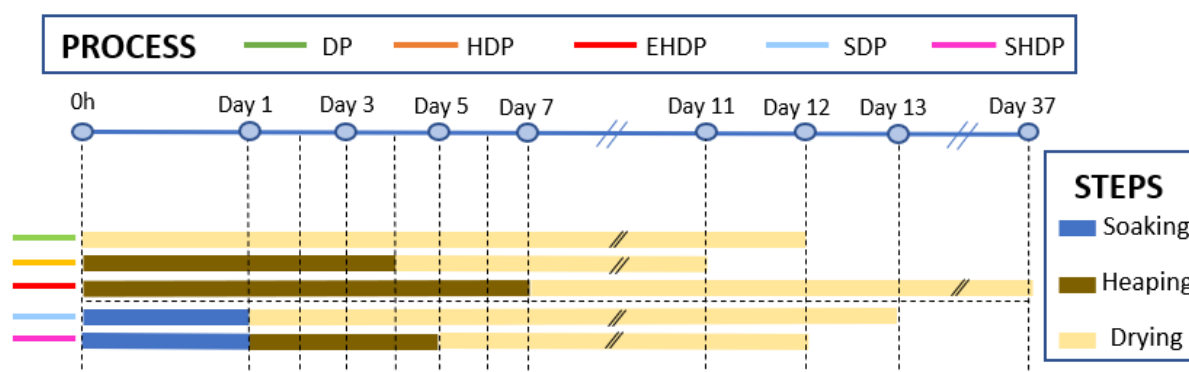


Figure 1. Experimental setup of coffee sampling based on different processing experiments carried out in Akoupe, Ivory Coast. For the processing, the green line depicts the Dry Process (DP); the orange line represents the Heaped Dry Process (HDP); the red line represents the Extended Heaped Dry Process (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.

2.2. EXTRACTION AND QUANTIFICATION OF OCHRATOXIN A IN COFFEE

Part of the dried coffee was dehusked, keeping the shell and the bean fractions. Then the husk, the bean and the dried cherry fractions, together with the fresh coffee samples were grounded (Retsch ZM200, Germany), to pass a 0.5 mm sieve, and analysed for OTA content. Three replicates of 10g of every grounded fraction were mixed with 20mL of the extraction solvent (methanol, distilled water 80:20 v/v, and 2g of NaCl) in 50mL 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, extraction was done using immunoaffinity column as per the manufacturer instructions (Ochraprep, R-Biopharm, Glasgow, UK). OTA was eluted with 2mL of HPLC-grade methanol. The elution was evaporated to dryness under a stream of nitrogen at 70°C, and the residue was

redissolved in 1mL of HPLC mobile phase and homogenized in the ultrasounds for 20 mins. The mobile phase consisted of distilled water/acetonitrile/glacial acetic acid (51:48:1). Finally, 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 100µL, injected during 20 min at a flow rate of 1mL/min. OTA was detected by absorption at 333nm excitation and 460nm 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.

For the following tests, the statistical significance of the differences in samples were tested by one-way ANOVA followed by Tukey's honestly significant difference test (Tukey's HSD, $p < 0.05$) using JMP Pro 16.

2.3. DNA EXTRACTION FROM COFFEE

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.

2.4. DNA AMPLIFICATION AND SEQUENCING

2.4.1. Library construction and sequencing

After quantification, the fungal ITS2 region of ribosomal DNA was amplified using the set of primers ITS86 and ITS4 (Vancov and Keen, 2009). The sequences of the set of primers are

shown in Table 1. 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, Proteogene, 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 1. List of Primers used in this study.

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

2.4.2. Data analysis and Statistics

Sequencing data were demultiplexed and trimmed using Cutadapt (Martin, 2011). Forward and reverse paired-end reads were then merged, 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.

3. RESULTS

3.1. OCHRATOXIN A CONCENTRATION IN COFFEE

The samples were analysed for their OTA concentration. Table 2 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. 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 2. 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 range (µg/kg of coffee husk)	OTA mean (µg/kg of coffee bean)	OTA content range (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.

3.2. COFFEE MYCOBIOTA

3.2.1. 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.

3.2.2. 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 2 and Figure 3, respectively. Figure 2 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 2 the fraction corresponding to the coffee beans (red) showed significantly lower richness compared to the dry cherries (green).

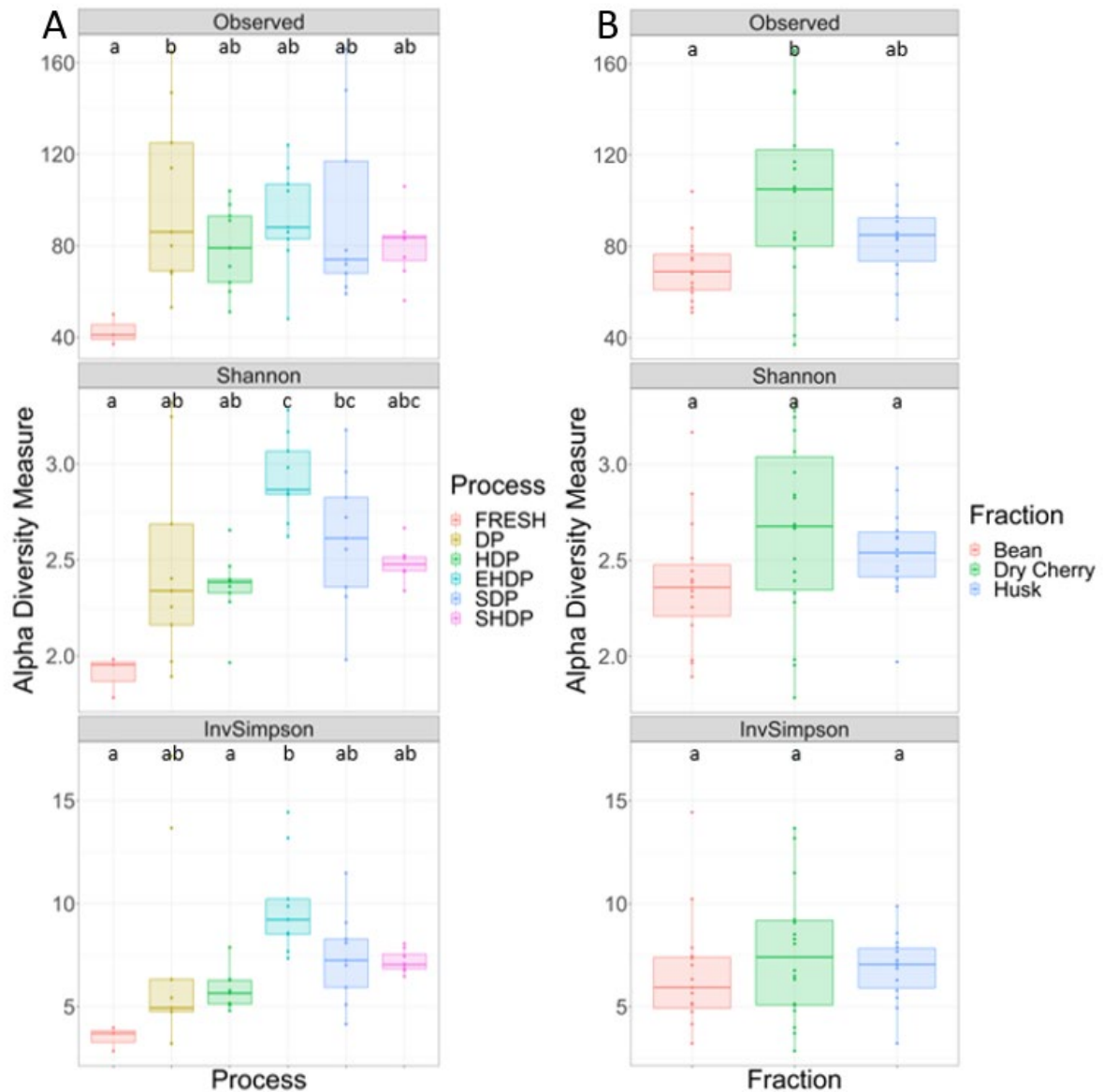


Figure 2. Boxplots illustrating the differences in the observed, InvSimpson and Shannon diversity measures of the fungal communities on the coffee sampled at different post-harvest processes (all fractions included)(A) and on different coffee fractions (B) using the package Phyloseq in the software R 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 3). 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 3) had a different diversity from those dried. Figure 3 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 3 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.

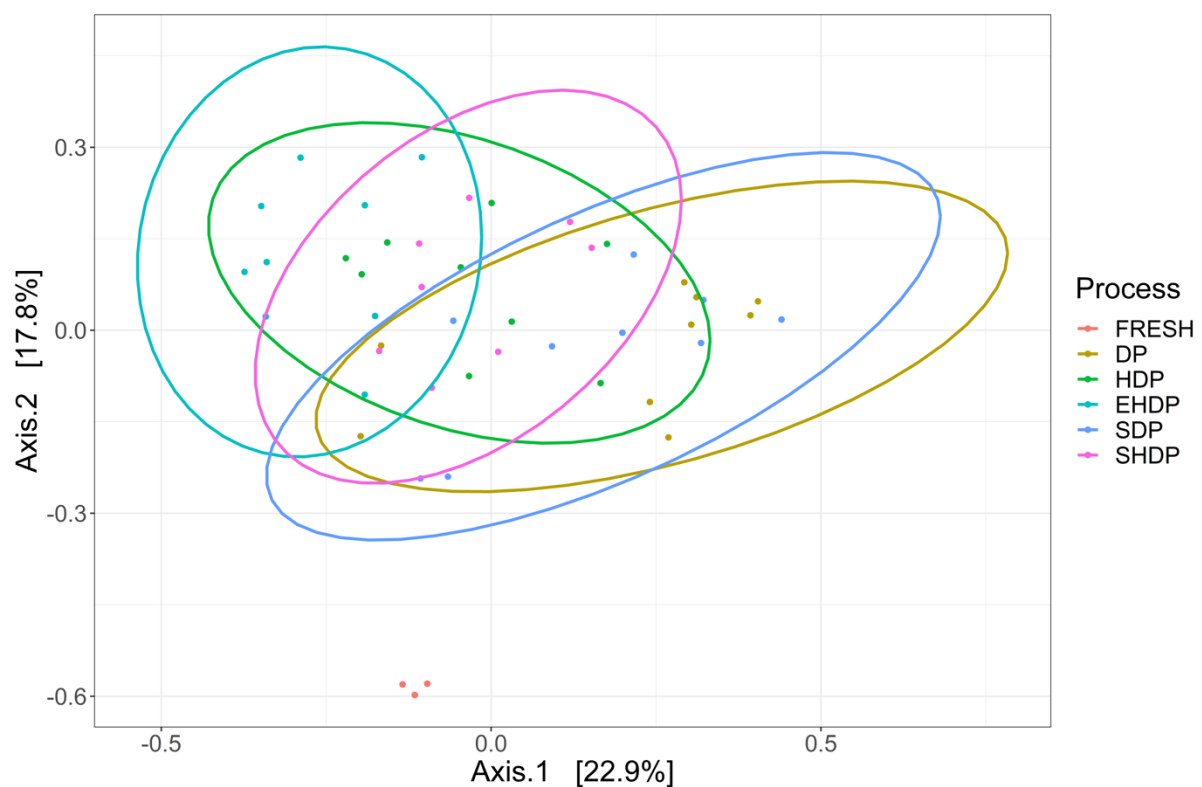


Figure 3. Principal coordinate analysis (PCoA) of fungal populations associated to Robusta coffee samples at the end of five post-harvest processes based on the beta diversity metric Bray

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%), *Chytridiomycota* (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. 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).

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.

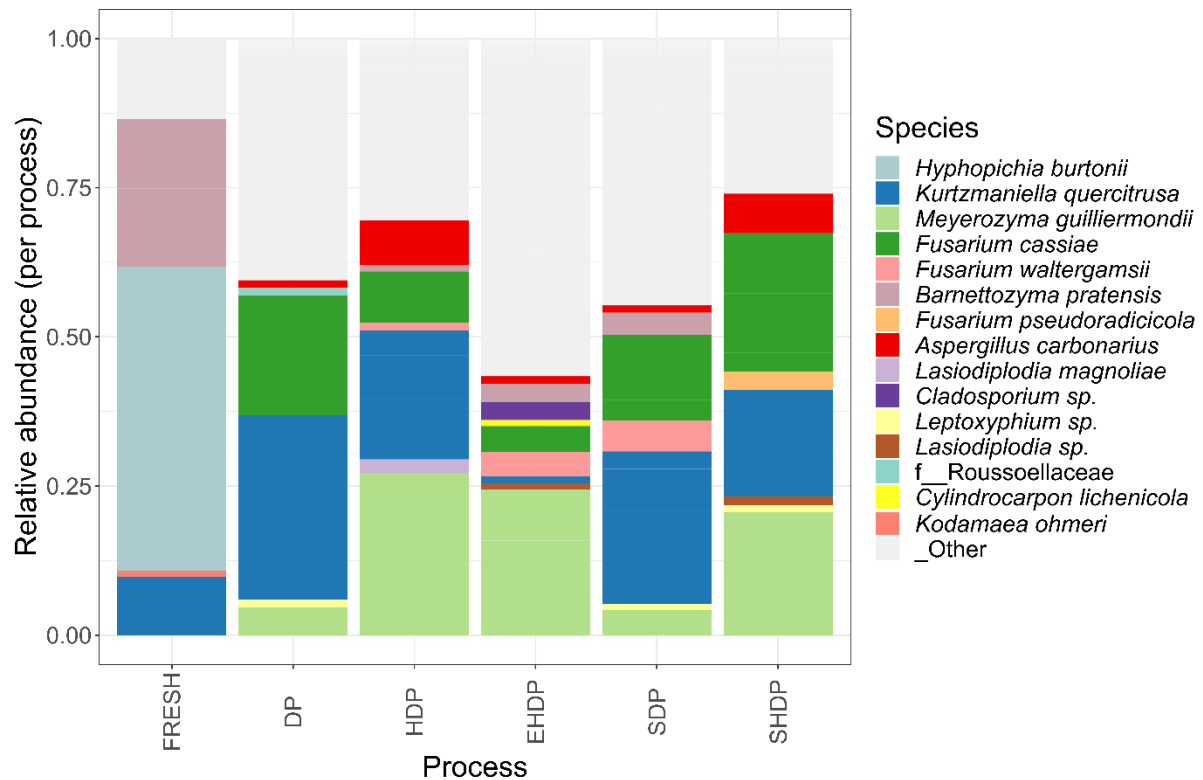


Figure 4. Relative abundance of fungal top 10 species detected in differently processed Robusta coffee. Fungal species with less than 1% are reported as “other”. When the taxonomic identification wasn’t possible to the species level, the ASV was identified by the lowest possible level of the phylogenetic tree. DP = dry processed, HDP = Heaped Dry Process; EHDP = Extended Heaped Dry Process, SDP = Soaked Dry Process; SHDP = Soaked Heaped Dry Process.

Figure 5 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.

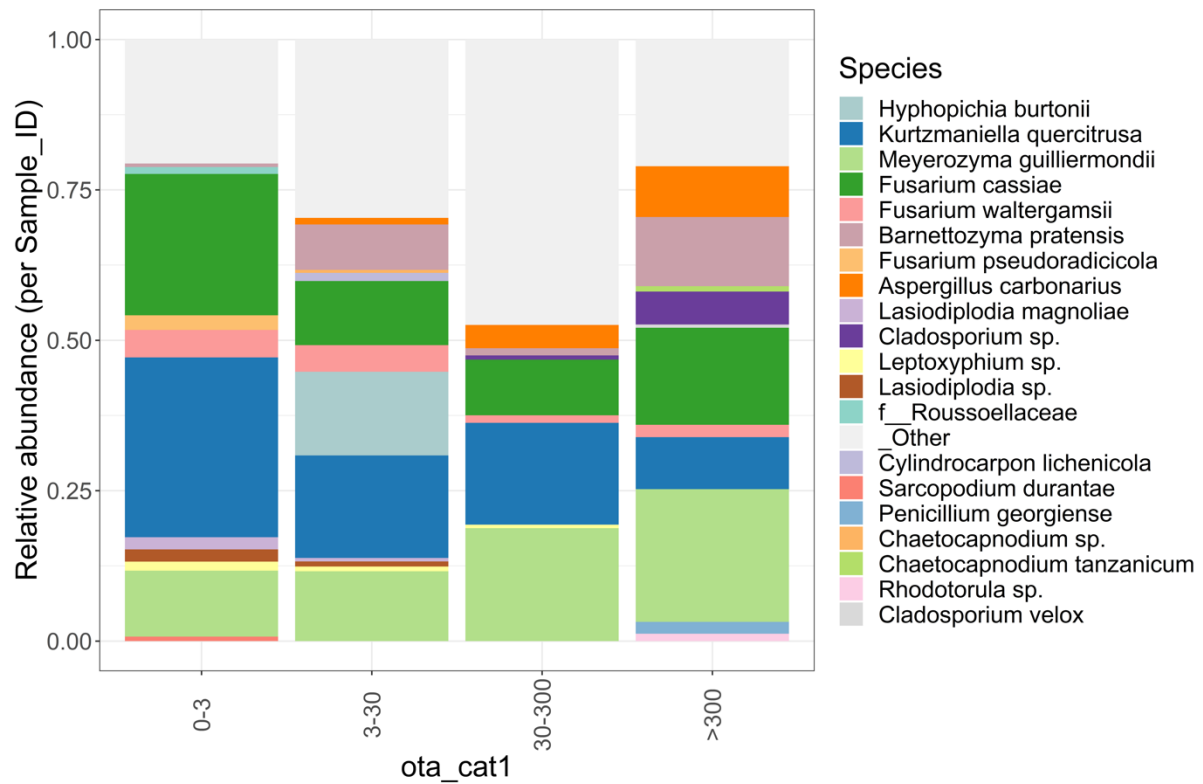


Figure 5. Relative abundance of fungal top 10 species detected in Robusta coffee samples grouped in function of their OTA levels expressed in μg of OTA per kg of coffee. Fungal species with less than 1% are reported as “other”. When the taxonomic identification wasn’t possible to the species level, the ASV was identified by the lowest possible level of the phylogenetic tree.

To detect potential interactions between the different species found in coffee, we illustrated fungal co-occurrence networks among species that appear within the threshold of 3 samples using the SPIEC-EASI pipeline (Kurtz et al., 2015). These association networks can be indicative of cooperative or competitive interactions. Figure 6 presents fungal species exhibiting positive interactions on coffee, and Figure 7 the taxa exhibiting negative interactions on coffee. An antagonist relationship was observed between *A. carbonarius* and the yeast genus *Zygorhynchus* sp. The latter was positively correlated to *Saccharomyces* sp. positively correlated to other *Saccharomyces* sp. At the same time, *Sacharomyces* sp. showed a negative correlation with *Cladosporium* sp. In Figure 6 *A. carbonarius* doesn’t show any positive

interaction, which indicates that this fungus does not appear at the same time than other species in at least 3 samples.

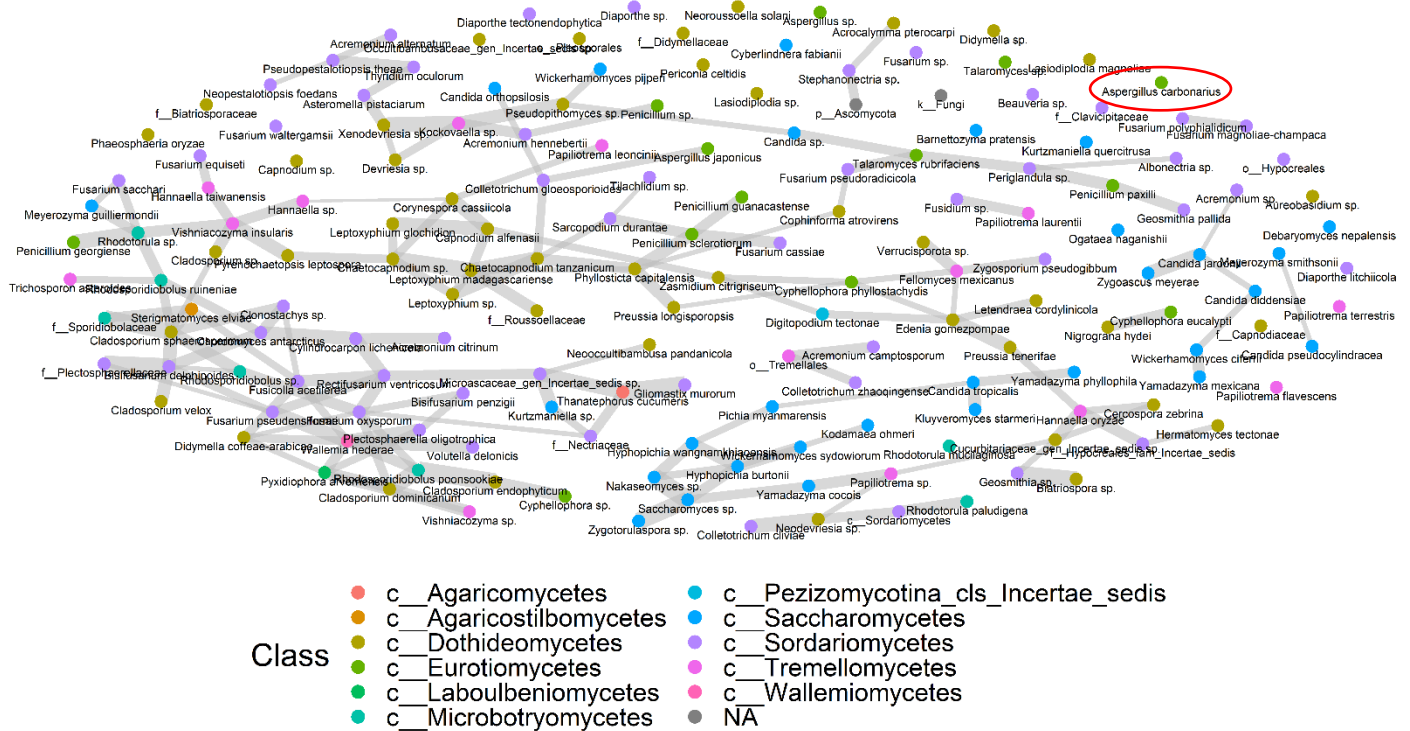


Figure 6. Co-occurrence network diagram (based on SPIEC-EASI) of the fungal species present in at least 3 samples showing cooperative associations (positive interactions). Each node represents a microbe from the mycobiome, and each grey link represents a pairwise co-occurrence. The thickness of the line 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*.

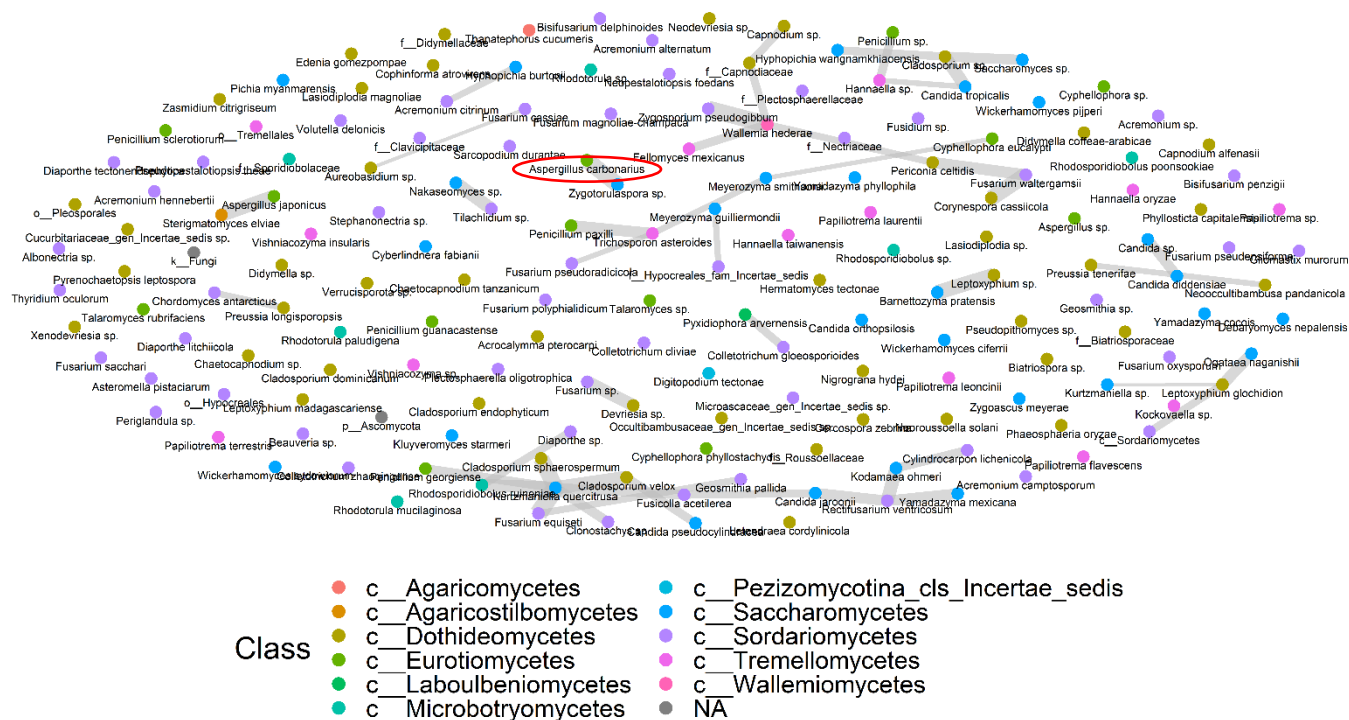


Figure 7. Co-exclusion network diagram (based on SPIEC-EASI) of the fungal species present in at least 3 samples showing opposite associations (negative interactions). Each node represents a microbe from the mycobiome and each gray link represents a pairwise co-occurrence. The thickness of the line highlights 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. 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 and 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., 2011), *Pichia fermentans* (Pregolini et al., 2021) or *Hanseniaspora uvarum* (Masoud and Kaltoft, 2006) 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). *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; M.H. 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 *Zygorhynchus* 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). We don't see another direct negative interaction but as *Zygorhynchus* was positively correlated to other yeast such as species of *Saccharomyces* genus which could also be good candidates to control ochratoxigenic fungi.

5. 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

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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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