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MYCOBIOTA AND CO-OCCRURENCE OF MYCOTOXINS IN GREEN AND ROASTED COFFEE BEANS

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ABSTRACT

A total of 20 samples of green and roasted coffee beans (the same varieties as green coffee beans were used) [*Coffea arabica* L. (19 samples) and *Coffea robusta* L. (1 sample)] were collected from the various coffee roasters in Slovakia (2017/2020) and their mycobiota were analyzed. Mycological analysis was carried using standard media with focus on genera *Aspergillus* and *Penicillium*. To determine endogenous and exogenous mycobiota the method of direct placing of surface-sterilized coffee beans (green and roasted) on agar plates and the plate dilution method were used. All obtained pure cultures were classified into the genera and identified to the species according to micro- and macromorphological properties. Next the potentially toxigenic isolates were tested on their ability to produce mycotoxins (cyclopiazonic acid, penitrem A, sterigmatocystin, aflatoxins (AFB_1, AFG_1), ochratoxin A, patulin, roquefortine C, citrinin, and griseofulvin). From green coffee samples with higher isolation frequency (FR%) and relative density (RD%) were the genus *Aspergillus* (FR 100% and RD 67.39%) and the genus *Penicillium* (FR 90% and RD 24.60%) recorded. *Aspergillus* section *Nigri* was the most widespread in green coffee samples (RD 47.7%). The genus *Aspergillus* was the most occurred genus in roasted coffee bean samples, too (RD 36.58%; FR 90%). In green and roasted coffee samples were detected mainly producers of aflatoxins (AFB_1 and AFG_1), cyclopiazonic acid, OA, sterigmatocystin and patulin. Due to the detected presence of mycotoxins in green as well as in roasted coffee bean samples, it is very important to prevent fungal contamination and control of coffee beans before and after roasting process.

Keywords: green coffee beans, roasted coffee beans, *Aspergillus* spp., *Penicillium* spp., mycotoxins, contamination

INTRODUCTION

Coffee is one of the most frequent beverages (75%) consumed globally (**Toci *et al.*, 2016**). The quality of coffee beans at the end of processing affects the price, and therefore shortcomings in the coffee beans reduce its quality. Coffee disorders can occur in the field during collection, processing, transport, and storage (**Illy and Viani, 2005**). These changes also affect the sensory characteristics of the coffee. Mainly the contact of coffee beans with the soil causes contamination by microorganisms. Coffee beans are often contaminated mainly with microscopic fungi because of suitable conditions for growth and reproduction in tropical countries. Contamination causes the formation of acidic, black, and defective coffee beans. Fungal damage reduces the quality and endangers the safety of the final product. Microbiological studies have shown that the primary microscopic fungi that attack coffee beans include the genera *Aspergillus* and *Penicillium*. These genera are present in both field and storage conditions **(Silva *et al.*, 2008a)**. Some can produce mycotoxins in coffee beans (**Toci and Farah, 2008**). The most common mycotoxin in coffee is ochratoxin A (OA). This mycotoxin is nephrotoxic and has teratogenic and mutagenic effects (**Taniwaki *et al.*, 2003**). Based on the risk that OA poses to human health, the European Union has developed rules for maximum levels of OA in coffee. The maximum permitted amounts of OA in coffee are 5 µg/kg in roasted and ground coffee and 10 µg/kg in instant coffee (**EC, 2006; Bessaire *et al.*, 2019**). Major producers of OA in coffee include *Aspergillus ochraceus*, which has been isolated from various coffee beans (**Taniwaki *et al.*, 2003; Batista *et al.*, 2003; Suárez – Quiroz *et al.*, 2004**). In addition, species such as *A. niger*, *A. carbonarius* (**Taniwaki, 2006**), and *A. westerdijkiae* (**Taniwaki *et al.*, 2014**) also can produce ochratoxin A in coffee. **Bokhari and Aly (2009)** found the presence of various mycotoxins in coffee beans, namely ochratoxin A, aflatoxins, patulin, and sterigmatocystin. The occurrence of aflatoxins (AF) in coffee has also been previously reported by several authors (**Soliman, 2002; Batista *et al.*, 2003; Silva *et al.*, 2008b**), but information on their occurrence in green and roasted coffee beans are limited. Also, these mycotoxins are one of the most dangerous mycotoxins with limits at low levels µg/kg for many commodities (**EC, 2006**). Aflatoxins are produced predominantly by species of *A. flavus* and *A. parasiticus*. The higher levels of coffee moisture tend to favor *A. flavus* growth and production of AF (**Paterson *et al.*, 2014**). The optimal temperature for AFB_1 and B_2 production is between 16 and 31 °C (**Silva *et al.*, 2008b**). Some studies have evaluated the risk of mycotoxins in coffee and their impact on human health (**Garcia-Moraleja *et al.* 2015a; Garcia-Moraleja *et al.*, 2015b**). The results showed that contaminated coffee with individual mycotoxins did not pose a risk to consumers but also confirmed that further studies on the co-occurrence of mycotoxins in coffee are still needed.

The objective of our study was to monitor the endogenous and exogenous mycobiota of coffee beans (*Coffea arabica* L., *Coffea robusta* L.) consummated in Slovakia with a focus on potentially toxicogenic species of the genera *Aspergillus* and *Penicillium*.

MATERIAL AND METHODS

Coffee bean samples origin

In total, 20 samples of green and 20 samples of roasted coffee beans (the same varieties as green coffee beans were used) [*Coffea arabica* L. (19 samples) and *Coffea robusta* L. (1 sample)] were analyzed. The coffee bean samples were taken from stored green coffee beans and coffee beans shortly after roasting from various coffee roasters in Slovakia in 2017 and 2020. After sampling, the samples were stored in a cool place in sterile paper bags with vents, and no later than 24 hours, they were transported to the laboratory and analyzed. The water activity (aw) was measured on LabMaster-aw neo (Lachen, Switzerland) for each sample. The list of coffee bean samples used for analyses is summarized in Table 1.

Table 1 Origin, variety, processing, and water activity of coffee bean samples used in this study

Sample	Coffee variety	Origin and region of coffee production	Processing method	Water activity (a_w)
South America				
1.	Cerrado	Brazil, 2000 m.a.s.l	dry road	0.535
2.	Supremo	Columbia, Medellin, 1700–1950 m.a.s.l	washed coffee	0.605
3.	–	Ecuador, Galapagos	hand harvest, washed coffee	0.618
4.	Tabaconas	Peru, Tabaconas		0.679
5.	Excelso (decaf)	Columbia, Medellin, 1700–1950 m.a.s.l		0.656
6.	Excelso	Columbia, Medellin, 1700–1950 m.a.s.l		0.678
7.	Mundo novo	Brazil, Capricornio coffees, 750–1000 m.a.s.l	hand harvest, sun-dried	0.522
8.	Obata	Brazil, California, 1000–1200 m.a.s.l	hand harvest, double fermentation	0.989
9.	Obata	Brazil, Capriocornio	hand harvest, semi-wet road	0.554
10.	Sitio Bom Jesus	Brazil, California,	machine collection, fermentation	0.972
Central America				
11.	Caturra	Honduras, Montecillos, 1200–1600 m.a.s.l	hand harvest, wet road	0.674
12.	Catuai	Nikaragua, Jinotega, 1600–2000 m.a.s.l	dry road, sun-dried	0.431
13.	Bourbon	Salvador, Balsamo, 1100 m.a.s.l	dry road	0.565
North America				
14.	Bourbon	Cuba, Guantanamo, 900 m.a.s.l	dry road	0.489
15.	Bourbon	Dominican Republic, Cibao, 1300–1800 m.a.s.l		0.497
Oceania				
16.	Bourbon	Papua New Guinea, Goroka, 1300–1800 m.a.s.l	dry road	0.507
Indian subcontinent				
17.	Robusta	India, Wayanaad, 500-1000 m.a.s.l	dry road, washed coffee	0.689
South Asia				
18.	Caturra	Nepal, Nuwakot, 700 m.a.s.l	dry road	0.654
East Africa				
19.	Forrest coffee	Ethiopia, Sidamo, 1500 – 2000 m.a.s.l	wet road	0.618
20.	SL 34	Kenya, Kirinyaga, 1800 m.a.s.l		0.625

Qualitative and quantitative mycological analysis of coffee bean samples

Endogenous mycobiota of coffee beans

The method of direct placing of surface-sterilized coffee beans (green and roasted) on agar plates according to **Samson *et al*. (2002)** was used to determine endogenous mycobiota. More than 200 coffee beans (100 beans of green coffee and 100 beans of roasted coffee) were superficially sterilized with a 0.4% chloramine solution, for 2 minutes with constant stirring. Subsequently, the coffee beans were washed three times with distilled water and dried on filter paper. Next the coffee beans in 7-8 pieces (Figure 1), using a total of 100 beans from each coffee sample were placed on DRBC (agar with dichloran, rose bengal and chloramphenicol) (HiMedia, India) and DG18 (agar with dichloran-glycerol and chloramphenicol) (HiMedia, India). Prepared Petri dish were cultivated for 5-7 days at 25±1 °C in the dark**.**

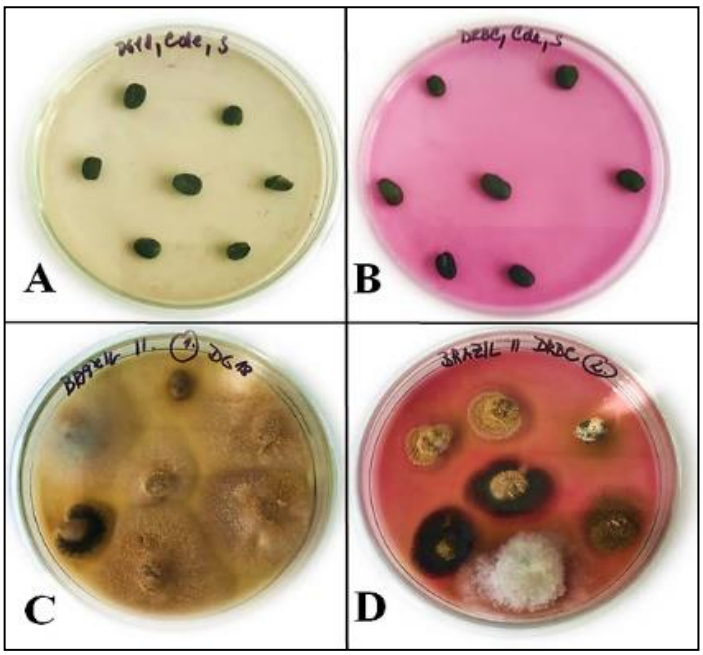

Figure 1 Coffee bean samples prepared for cultivation (sample no. 2): A) on DG18 plates, B) on DRBC plates; samples after cultivation (5-7 days at a temperature of 25±1 °C in the dark) (sample no. 7): C) on DG18 plates, D) on DRBC plates

Exogenous mycobiota and mycobiota of crushed coffee beans

The plate dilution method according to **Samson *et al*. (2002)** were used. The coffee bean samples were homogenized by crushing in an electric grinder. Subsequently, 20 g of crushed coffee beans with 180 ml of saline and 20 µl of Tween 80 were added into polyethylene resealable bags and mixed the contents in the bag for 2 minutes. The procedure was the same for whole coffee beans. Amount of 20 g (exactly) whole coffee beans were transferred to the Ehrlenmayer banks. Subsequently, 180 ml of saline and 20 µl of Tween 80 were added to the Ehrlenmayer banks. The mixture was left on a horizontal shaker for 30 minutes. From this suspension, dilutions up to 10^{-3} were prepared. From each dilution, 100 µl of the solution was applied to a Petri dish with nutrient medium (DRBC and DG18) in triplicate (Figure 2). Prepared Petri dich were cultivated in the same was as before (5-7 days at 25±1 °C in the dark).

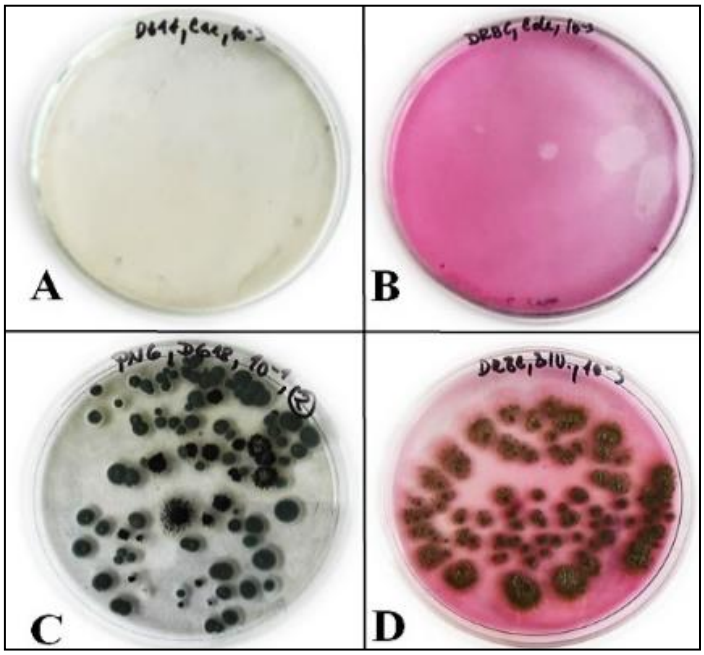

Figure 2 Prepared dilutions from coffee bean samples for cultivation (sample no. 5 – dilution 10^{-3}): A) on DG18 plates, B) on DRBC plates; samples after cultivation (5-7 days at 25±1 °C in the dark): C) on DG18 plates (sample no. 16 – dilution 10^{-1}), D) on DRBC plates (sample no. 9 – dilution 10^{-3})

Results evaluation of endogenous and exogenous coffee beans mycobiota

The presence of mycobiota in/on coffee beans were expressed as Isolation frequency (FR) and relative density (RD) of genera and species. Obtained values were calculated according to following formula:

$$FR\ (\%) = \left(\frac{ns}{N}\right) * 100$$

$$RD\ (\%) = \left(\frac{ni}{Ni}\right) * 100$$

Where ns = number of samples with species or genus; N = total number of samples; ni = number of isolates of a species or genus; Ni = total number of isolated fungi **(Gonzáles *et al*. 1996)**.

Fungal isolation and identification

To obtain pure cultures, the grown microscopic fungi on coffee beans or from individual dilutions were re-inoculated on nutrient media. Next obtained cultures were classified into the genera and next were re-inoculated on standard identification media (Table 2) and cultivated for next 5-7 days at different temperatures (suitable for a particular genus), at 25±1 °C in the dark. Only fungal isolates belonging to the genera *Aspergillus* spp. and *Penicillium* spp. were identified to the species according to micro- and macromorphological properties used the following identification keys: **Samson *et al*., (2002)**; **Varga *et al*. (2004)**; **Samson *et al*. (2004a)**; **Samson *et al*. (2004b)**; **Frisvad *et al*. (2005)**; **Varga *et al*. (2007)**; **Samson *et al*. (2007)**; **Houbraken *et al*. (2007)**; **Pitt and Hocking, (2009)**; **Houbraken *et al*., (2010)**; **Varga *et al*. (2011)**; **Samson *et al*. (2011a)**; **Samson *et al*. (2011b)**; **Jurjevic *et al*. (2012)**; **Samson *et al*. (2014)**; **Gautier *et al*. (2016)**; **Chen *et al*. (2016a)**; **Chen *et al*. (2016b)**; **Chen *et al*. (2017)**; **Yamairi *et al*. (2019)**; **Iamanaka *et al*. (2019)**; **Frisvad *et al*. (2019)**.

Table 2 Identification media and cultivation condition used in this study

Type of identification media	Cultivation conditions	Genera
Czapek Yeast Autolyzate agar (CYA)	25±1 °C and 37±1 °C for 5-7 days in the dark	*Aspergillus* spp., *Penicillium* spp.
Malt Extract agar (MEA)	25±1 °C for 5-7 days in the dark	*Aspergillus* spp., *Penicillium* spp.
Creatine Sucrose Agar (CREA)	25±1 °C for 7-14 days in the dark	*Penicillium* spp.
Yeast Extract Agar (YES)	25±1 °C for 7-14 days in the dark	*Aspergillus* spp., *Penicillium* spp.

Mycotoxins screening by TLC method in *in vitro* conditions

For mycotoxins screening the method of thin layer chromatography (TLC method) according to **Samson *et al*. (2002)**, modified by **Labuda and Tančinová (2006)** was used. For the determination of intracellular mycotoxins: cyclopiazonic acid (CPA), penitrem A (PA) and sterigmatocystin (SC), the identified species of potential toxigenic fungi were cultivated on CYA plates (25±1 °C, 14 days in the dark). For the screening of extracellular mycotoxins aflatoxins (AFB_1, AFG_1), ochratoxin A (OA), patulin (PAT), roquefortine C (RC), citrinin (C) and griseofulvin (G), the fungi were cultivated on YES plates (25±1 °C, 14 days in the dark). The method and visualization of mycotoxins are described in **Císarová *et al*. (2015a), Císarová *et al*. (2015b), Císarová *et al*., (2015c) and Císarová *et al*. (2020).** The identification of the screened mycotoxins was obtained by recalculating their retention factors according to **Samson *et al*. (2002)** for AFB_1 (R_f 0.56), AFG_1 (R_f 0.39), OA (R_f 1.39), C (R_f 0-1.49), S (R_f 1.75), PAT (R_f 0.98), PA (R_f 0.7), CPA (R_f 0-1.37), G (R_f 0.53) and by retention standards (Sigma-Aldrich, Germany).

RESULTS AND DISCUSSION

Mycobiota of green coffee samples

Coffee is one of the most popular drinks consumed by millions of people every day. However, coffee beans are often contaminated with microorganisms, mainly microscopic fibrous fungi. This damage can occur at various stages of the development and growth of coffee, the collection, transport, and storage of coffee beans. Microbiological studies have shown that coffee berries are often contaminated with microscopic fungi belonging to the genera *Aspergillus*, *Penicillium*, and *Fusarium* (**Iamanaka *et al*., 2014**). Also, in this work, representatives isolate of these genera in the analyzed coffee beans samples were isolated and identified. Results are summarized in Table 3. A total of 5308 isolates of microscopic filamentous fungi were isolated from the analyzed green coffee samples. The most common fungi belonged to the genera *Aspergillus* and *Penicillium*. The relative density (RD) of genus *Aspergillus* in analyzed green coffee samples is represented by RD 49.77% (2642 isolates) for endogenous and RD 17.61% (935 isolates) for exogenous determination of mycobiota. The genus *Penicillium* was represented by RD 22.76% (1208 isolates) for endogenous and RD 1.85% (98 isolates) for exogenous determination of mycobiota in green coffee samples. These two genera in green coffee have been demonstrated in several studies (**Iamanaka *et al*., 2014; Rehmat *et al*., 2019; Sousa *et al*., 2019**). The next most frequently isolated genus in green coffee samples were *Mucor* spp. with RD 1.22% (65 isolates – end.) and RD 0.92% (46 isolates – ex.). This genus was followed by *Rhizopus* with RD of 1.22% (65 isolates) in endogenous and with RD 0.79% (42 isolates) in exogenous mycobiota. *Rhizopus* spp. has been isolated by several authors in different types of green coffee (**Pardo *et al*., 2004; Pitt and Hocking, 2009**). Some species of the genus *Rhizopus* are used in the biotechnology industry because they cause changes in the aromatic precursors in green coffee, thus achieving a complete change in its aroma. For example, *Rhizopus oligosporus* degrades the non-volatile components of green coffee, degrading ferulic acid and increasing the level of volatile phenols in coffee berries (**Lee *et al*., 2016**).

Table 3 The overall presence of individual isolated fungal genera in green coffee bean samples (n=20)

Identified fungal genera	Mycobiota of green coffee beans						
	Exogenous		**Endogenous**		**Overall amount**		
	Numbers of micromycetes	**RD (%)**	**Numbers of micromycetes**	**RD (%)**	**Numbers of micromycetes**	**RD (%)**	**FR (%)**
Penicillium spp.	98	1.85	1208	22.76	1306	24.60	90
Aspergillus spp.	935	17.61	2642	49.77	3577	67.39	100
Cladosporium spp.	5	0.09	20	0.38	25	0.47	55
Mucor spp.	49	0.92	65	1.22	114	2.15	55
Mycelium sterillium	24	0.45	95	1.79	119	2.24	85
Absidia spp.	2	0.04	5	0.09	7	0.13	15
Rhizopus spp.	42	0.79	65	1.22	107	2.02	85
Alternaria spp.	6	0.11	1	0.02	7	0.13	15
Eurotium spp.	36	0.68	10	0.19	46	0.87	65
Σ	1197		4111		5308		

Legend: RD (%) – relative density, FR (%) – isolation frequency

The genus Rhizopus was followed by the genus *Eurotium* with a relative density of RD 0.19% (10 isolates – end.) and RD 0.68% (36 isolates -ex.) > and *Cladosporium* spp. with RD 0.38% (20 isolates – end.) and RD 0.09% (5 isolates – ex.). The least common genera in the green coffee samples were *Alternaria* (1 isolate from endogenous and six isolates from endogenous mycobiota) with RD 0.02% and RD 0.11% and *Absidia* (2 isolates from exogenous mycobiota and five isolates from endogenous mycobiota) with RD 0.09% and RD 0.04% respectively. Amount of 119 isolates with RD 0.45% (exogenous) and 1.79% (endogenous) were classified as *Mycelium sterillium.* These isolates did not form the necessary fructification structures during the seven days of cultivation period; therefore, their identification was not possible. The results showed a high isolation frequency (FR) of *Aspergillus* isolates in green coffee samples. The genus *Aspergillus* was found in all samples (20) of green coffee. The incidence of this genus was 100%, followed by *Penicillium* spp. with FR 90% and *Mycelium sterillium* and *Rhizopus* spp. with FR 85% for both, and *Mucor* spp. and *Cladosporium* spp. with FR 55%, respectively. Almost the genus *Penicillium* was found in all samples except two (samples no. 2 and 6). *Absidia* spp. (samples no. 8,12, and 15) and *Alternaria* spp. (samples no. 6,17, and 19), with FR 15%, were the least occurring genera in the green coffee samples. Our results agree with the results of other authors. Various microbiological studies have shown that the genera *Aspergillus* and *Penicillium* are the most common contaminants of coffee beans**. Viegas *et al*. (2017)** examined the mycobiota of green coffee beans before. Coffee samples came from different countries of the world (Brazil, Timor, Honduras, Angola, Vietnam, Costa Rica, Colombia, Uganda, Guatemala, Nicaragua, India, and Uganda). In their study, the

fungal contamination showed 67% of the tested coffee beans samples. The most common isolated genus in these coffee samples was *Aspergillus*, found in 96% of contaminated samples. The identified species of the genus *Aspergillus* mostly belonged to the sections *Nigri*, *Flavi*, and *Circumdati*. Also, **Vega *et al.* (2010)** investigated fungal contamination of 5 coffee bean samples from different countries (Colombia, Hawaii, Mexico, Puerto Rico). They obtained a total of 843 fungal isolates, and the most numerous group was the genus *Penicillium* (220 isolates).

According to our results, the most contaminated green coffee sample was no. 8 from Brazil and 11 from Honduras. From these samples of green coffee, a total of 1960 different genera of fungal isolates were isolated with the genus *Penicillium* and *Aspergillus* as the most frequent genera (Figure 3). The cleanest samples of green coffee included samples no. 1 from Brazil and no. 2 from Columbia. However, only in sample no. 1, the lowest occurrence of *Aspergillus* (28 isolates) and *Penicillium* (1 isolate) isolates was recorded.

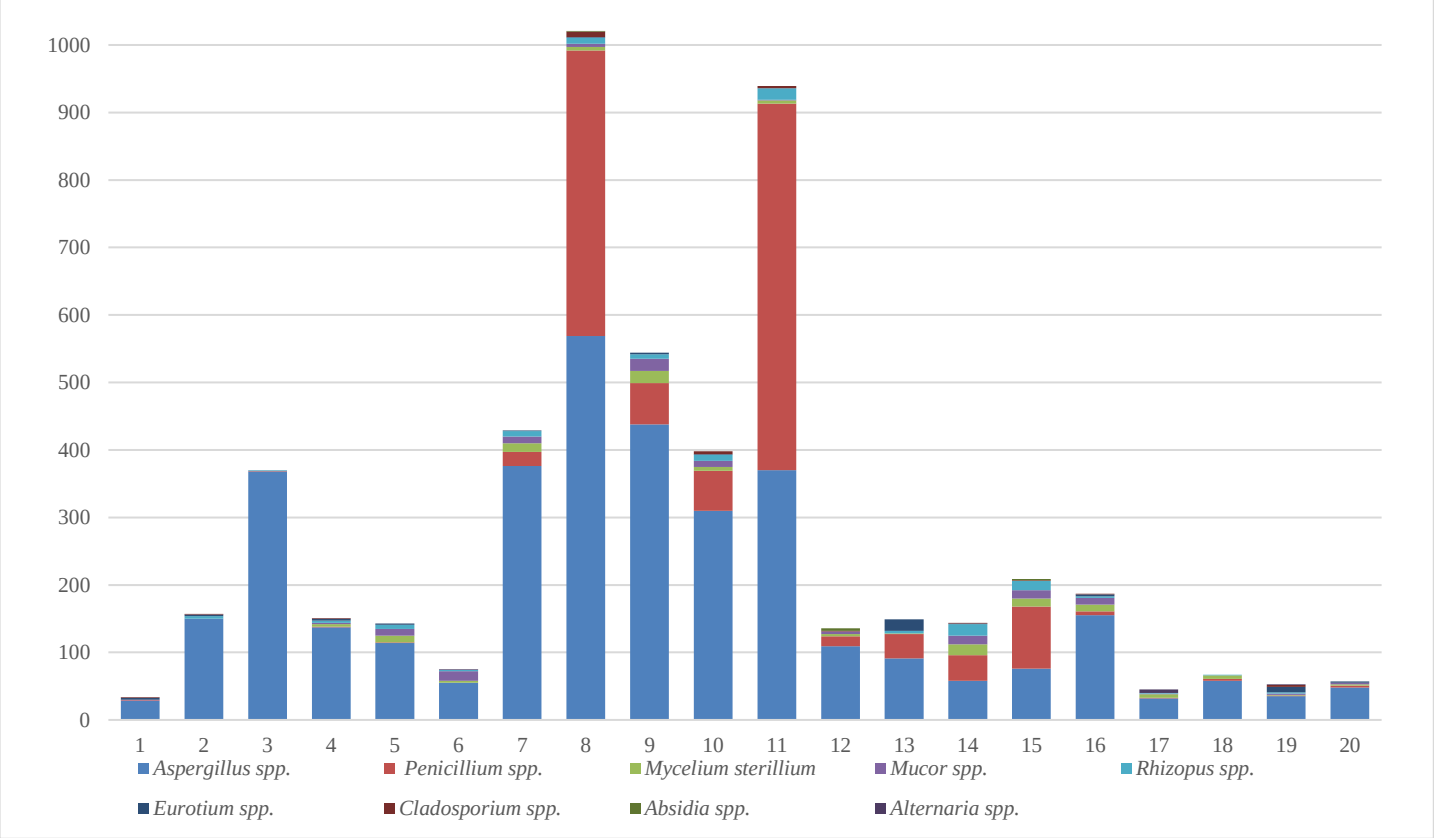

Figure 3 Presence of identified genera of micromycetes in individual green coffee samples (n=20)

Relative density (RD) of micromycetes species of the genus *Aspergillus* obtained from green coffee samples is shown in Figure 4. In presence, studies have described those species capable of producing mycotoxins, especially. Some isolates of the genus *Aspergillus* could not be taxonomically identified because they co-existed with *Rhizopus* in the samples. A total of 857obtainded isolates of the genus *Aspergillus* were classified into ten species (including non-toxinogenic ones) from different sections, and 1706 isolates were classified as black *Aspergillus* belongs to the section *Nigri* (RD 47.7%). *Aspergillus* section *Nigri* was also the most numerous section isolated from green coffee samples. They have been isolated from various foods such as grapes, cashews, peanuts, but often from coffee (**Pitt and Hocking, 2009; Lamboni *et al.*, 2016**). The second most frequently isolated species of the genus *Aspergillus* belongs to section *Flavi*: *A. flavus* (145 isolates), *A. oryzae* (142 isolates), and *A. parasiticus* (48 isolates). *A. flavus* is an opportunistic pathogen that can produce several toxic secondary metabolites. It attacks the most common crops such as corn, peanuts, and cotton (**Fakruddin *et al.*, 2015**). This species has also been isolated from several types of green coffee beans (**Batista *et al.*, 2003; Medina *et al.*, 2014**). *A. oryzae* is often used in fermentation processes to produce food such as soy juice (**Sucu *et al.*, 2018**). However, also *A. oryzae* can produce toxic secondary metabolites (**Blumenthal, 2004**), such as cyclopiazonic acid. A total of 102 isolates were included in the *Nidulans* section and identified as *A. versicolor* (RD 2.9%). **Vilela *et al.* (2010)** studied green coffee from Minas Gerais, Brazil, and among the main species they isolated was *A. versicolor*. Among the species of microscopic fungi that have been often isolated from green coffee samples were species of *A. terreus* with RD 2.2% and *A. clavatus* with RD 2.9%. **Kouadio *et al.* (2012)** also isolated *A. clavatus* from green coffee with a relative density of 4%, and **Kouadio *et al.* (2014)** isolated this genus with a relative density of 3.6%.

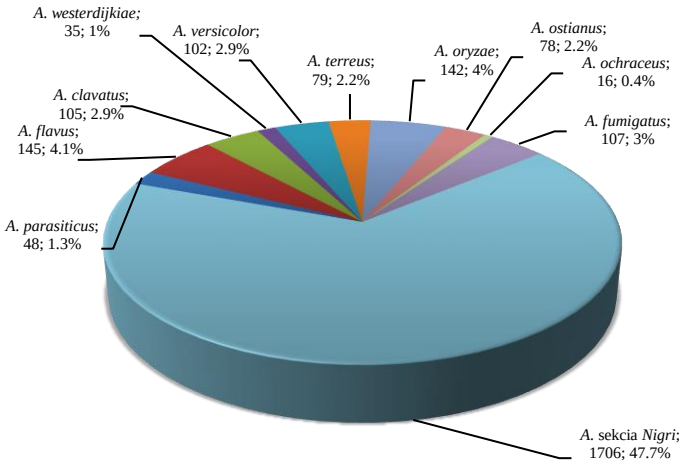

Figure 4 Relative density (RD in%) of micromycetes species of the genus *Aspergillus* obtained from green coffee samples (n=20)

The genus *Penicillium* has also been isolated from several types of green coffee, namely *Penicillium verrucosum* and *Penicillium nordicum*, *P. islandicum*, *P. corylophilum*, *P. citrinum* **(Kusumaningrum and Rasyidah, 2019)**, *P. brevicompactum*, *P. crustosum* **(Vega *et al.*, 2006)**. In our study the genus *Penicillium* was the most represented by species *P. crustosum* (19 isolates: RD 1.5%). *P. crustosum* is a microscopic fungus that often causes food spoilage especially coffee beans. Authors **Peterson *et al.* (2003**) also isolated *P. crustosum* from green coffee from Mexico. In addition, it is often found in meat, grains, fruits, and nuts. This species can produce the mycotoxin penitrem A **(Rundberget *et al.*, 2004)**. Another frequently occurring species of the genus *Penicillium* in the green coffee samples was *P. chgrysogenum P. citrinum* and *P. expansum* (Figure 5). *P.*

chrysogenum isolated **Bokhari (2007)** from green coffee samples grown in Saudi Arabia. **Alvindia and Guzman (2016)** tested green coffee from five Philippine provinces. The total number of microscopic fungi of the genus *Penicillium*, 4 isolates were identified as *P. citrinum*. **Houbraken *et al.* (2010)** isolated *P. citrinum* from green coffee grown in Thailand. *P. expansum* is often isolated from fruit, especially apples and pears, where it is one of the major producers of patulin. This species was isolated from coffee by **Batista *et al*. (2003)**. Other *Penicillium* species were found in green coffee samples with low relative density.

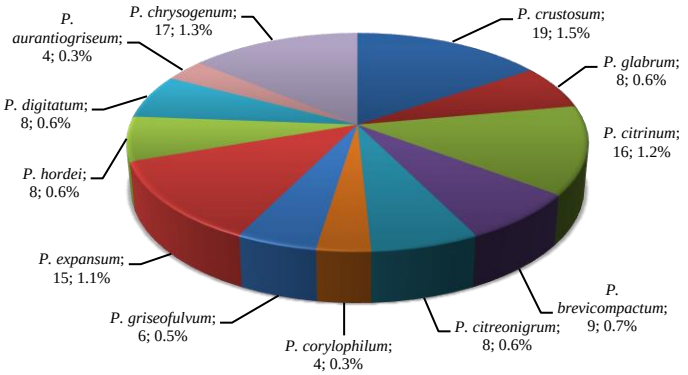

Figure 5 Relative density (RD in%) of micromycetes species of the genus *Penicillium* obtained from green coffee samples (n=20)

Toxigenity of the fungal isolates obtained from green coffee bean samples

Coffee is often contaminated with different species of microscopic fungi which can produce mycotoxins. The most studied mycotoxin in coffee is OA and it is also one of the mycotoxins covered by legislation **(RASFF, 2017)**. Nevertheless, available studies showed the presence of OA in both green and roasted coffee. **Nielsen *et al*. (2015)** studied and demonstrated the presence of OA and fumonisins B in green, roasted, and soluble coffee. **Bokhari and Aly (2009)** detected OA, aflatoxins, patulin and sterigmatocystin in green coffee beans. The results are summarized in Table 4 and Table 5. Coffee samples that did not contain *Aspergillus* and *Penicillium* species and were not tested for mycotoxin production are not included in Table 4 and 5. In our study, all tested isolates of *A. flavus* (n=145) showed the ability to produce cyclopiazonic acid and 81 isolates were able to produce aflatoxin B_1. The ability to produce aflatoxin B_1 and G_1 was demonstrated by all tested isolates (n=48) of *A. parasiticus*. **Soliman (2002)** tested 30 samples of green coffee for aflatoxin production. *A. flavus* species was isolated from up to 17 of 30 coffee bean samples, which means that 56% of green coffee beans were contaminated. He proved the production of aflatoxins by *A. flavus* in all (n=17) samples. **Humaid *et al*. (2019)** demonstrated the presence of aflatoxins B_1, B_2 and G_1 in 50 samples of green coffee beans from Yemen. **Jeszka-Skowron *et al*. (2017)** tested Arabica green coffee for the presence of aflatoxins B_1, G_1 and G_2. Among these mycotoxins, AFB_1 was dominant with the highest measured level of 17.45 ng/g^{-1}. They identified *A. flavus* and *A. tamarii* species as the main producers of this mycotoxin. From the section *Flavi*, also 142 isolates of *A. oryzae* species were tested for the potential ability to produce CPA in this study. None of the tested isolates showed this ability. *A. oryzae* is very similar to *A. flavus* and is considered a domesticated species. Some strains are able to produce CPA, but this ability may not always manifest **(Chang *et al*., 2009)**. Ochratoxin A production was confirmed by TLC in all tested isolates of *A. westerdijkiae* and *A. ochraceus* (Table 4). Out of the 281 isolates obtained from green coffee samples belonging to section *Nigri*, only 26 isolates showed the ability to produce ochratoxin A. The presence and production of OA by this section and mentioned species in green coffee beans were demonstrated by many authors **(Napolitano *et al*., 2007; Barcelo *et al*., 2017)**. A total of 102 isolates of *A. versicolor* were screened for sterigmtocystin production by TLC. Totally 45 isolates of this species were able to produce this mycotoxin. **Culliao and Barcelo (2015)** tested coffee beans from Philippines for the presence of mycotoxins. From 885 fungal isolates the most frequent was the genus *Aspergillus*. The authors noted a high content of sterigmatocystin (193.7 µg/kg) in dried coffee fruits. **Nasser (2008)** states that STER can be present in green coffee beans in quantities of 60-600 µg/kg. The last mycotoxin tested in this study was patulin. For production of this mycotoxin were screened 105 isolates of *A. clavatus*. According to our results, up to 60 isolates of *A. clavatus* were able to produce patulin in *in vitro* condition. The occurrence of patulin in coffee beans is not often reported in the literature and is not monitored. Nevertheless, **Bokhari and Aly (2009)** report that up to 16.6% of all mycotoxins detected in 30 coffee bean samples was patulin. The occurrence of this mycotoxin in coffee should not be underestimated, because it is one of the legally monitored mycotoxins, especially in apple products in Slovakia.

The genus *Penicillium* is reported to be the second most common fungal contaminant occurred in coffee beans **(Geremew *et al*., 2016; Casas – Junco *et al*. 2018)**. The most important mycotoxins that *Penicillium* spp. can produce in coffee beans include patulin, citrinin, cyclopiazonic acid, and ochratoxin A **(Prencipe *et al*., 2018)**. In this study citrinin was produced by all tested *P. citrinum* (16 isolates) and by 10 of 15 tested isolates of *P. expansum*. **Bessaire *et al.* (2019)** tested green coffee for the presence of 31 mycotoxins. They detected the presence of many mycotoxins, including citrinin produced by *P. citrinum*. *P. expansum* (5 isolates), 2 isolates of *P. griseofulvum* and 3 isolates of *P. chrysogenum* showed the ability to produce RC. The largest producer of patulin from the genus *Penicillium* was *P. crustosum* (11 isolates) and patulin were able to produce 5 isolates of *P. crustosum* and one isolate of *P. chrysogenum*, too. **Batista *et al.* (2003)** tested contamination of green coffee beans obtained caused by microscopic filamentous fungi. They obtained 25 isolates of *P. expansum* and proved their ability to produce patulin in all cases. Also, the ability of tested *Penicillium* spp. produced CPA was confirmed in 11 cases, of which the largest producer was again *P. crustosum* (12 isolates), followed by *P. griseofulvum* (5 isolates) and *P. chrysogenum* (1 isolate). The potentially production of PR toxin and griseofulvin by the TLC method did not been confirmed in any of tested isolates.

Mycobiota of roasted coffee samples

The type of coffee processing modifies its composition and bioactivity (**Bauer *et al*., 2018)**. Roasting is the primary processing of raw coffee. During this process, essential taste properties develop in the coffee beans. Roasting also affects the composition of coffee beans and their potential biological activity due to the interactions of the compounds found in coffee (**Choi *et al*., 2018**).

The most common genus in roasted coffee samples was *Aspergillus* (158 isolates) with RD 34.20% for exogenous and RD 2.38% (11 isolates) for exogenous mycobiota. The genus *Rhizopus* was the second most abundant genus in roasted coffee samples (63 isolates with RD 13.64% for exogenous mycobiota and nine isolates with RD 1.95% for endogenous mycobiota) (Table 6). **Oliveira *et al.* (2006)** analyzed the presence of mycotoxins in raw and roasted coffee (*Coffea arabica* L.). They also proved the presence of microscopic fungi of the genus *Aspergillus* before and after roasting coffee beans. **Alvindia and Acda (2010)** isolated several species of microscopic fungi, including the genus *Rhizopus.* The least common genera in the roasted coffee samples were *Cladosporium* (RD 6.08% for exogenous and RD 0.7.58% for endogenous mycobiota), *Mucor* (RD 5.41% for exogenous and RD 7.58% for endogenous mycobiota), and *Eurotium* (RD 3.25% for exogenous and RD 2.16% for endogenous mycobiota). Also, **Silva *et al.* (2008b)** tested roasted coffee from Brazil and obtained up to 26 isolates of the genus *Cladosporium.* **Geremew *et al*. (2016)** tested roasted coffee from Ethiopia, and they found that genera such as *Mucor*, *Trichoderma*, and *Rhizopus* were present in less than 8% of the samples. In this study, *Penicillium* spp. was the least represented genus in roasted coffee (RD 3.46% for exogenous and RD 0.87% for endogenous mycobiota). Similarly, **Perez *et al*. (2003)** tested roasted coffee and isolated only four species of the genus *Penicillium* from it.

Aspergillus spp. was the predominated genus in the roasted coffee, with isolation frequency (FR) of 90% (169 isolates) and was found in 18 analyzed samples (except two samples no. 5 and 6). **Noonim *et al*. (2008)** tested roasted coffee from Thailand and found that the genus *Aspergillus* was present in all tested samples (100% frequency). **Grigoryan *et al*. (2005)** performed a fungal analysis of 80 roasted coffee species (*Coffea arabica* L., *Coffea robusta* L.). They identified up to 33 species in the samples. The most numerous was the genus *Aspergillus* (22 identified species). According to our results, the most contaminated coffee samples were with origin in Brazil: no. 7 (65 isolates) and no. 8 from (58 isolates) (Figure 6). In sample no. 8 (42 isolates) and no. 9 (36 isolates) the most isolates of the genus *Aspergillus* are isolated. Both samples came from Brazil. The least contaminated and thus the purest samples of roasted coffee beans included sample no. 18 from Nepal, with only 1 isolates of the genus *Aspergillus*. In total, only 6 isolates were obtained from this sample (no. 18).

Table 4 Production of mycotoxins by *Aspergillus* spp. obtained from green coffee samples after 14 days of cultivation in the dark at 25±1 °C

Tested species	MT	Tested coffee samples																			
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
A. flavus	AFB_1	1[a]/1[b]		10/10	6/6	4/2	1/1	23/5	19/4	25/6	6/6	9/6	7/6	5/5	2/2	2/2	14/8	4/4	3/3		4/4
	CPA	1/1		10/10	6/6	4/4	1/1	23/23	19/19	25/25	6/6	9/9	7/7	5/5	2/2	2/2	14/14	4/4	3/3		4/4
A. parasiticus	AFB_1			18/18	11/11			2/2			2/2	6/6	1/1	1/1			5/5	1/1			1/1
	AFG_1			18/18	11/11			2/2			2/2	6/6	1/1	1/1			5/5	1/1			1/1
A. oryzae	CPA	3/0	3/0	9/0	6/0	4/0	6/0	15/0	4/0	15/0	8/0	14/0	20/0	4/0			16/0	4/0		3/0	2/0
A. clavatus	PAT	3/0	9/9	8/4	4/2	5/5		8/8	10/7	6/2	2/2	3/3		10/5	8/4	5/0	6/6	8/3		10/0	
A. versicolor	STER	4/4	9/4	11/5			5/4	8/7	10/6	6/4	2/1	3/3	4/0	11/1	8/1	8/0	7/2		2/2	3/0	1/1
A. westerdijkiae							12/12	18/0						1/1				1/1	2/2	1/1	
A. section *Nigri*	OA	13/3	42/4	28/6	18/6	23/0	7/0	8/0	23/1	16/0	8/0	18/6	30/0	2/0	4/0	2/0	13/0	3/0	10/0	5/0	8/0
A. ochraceus						1/1	14/14				1/1										

Legend: MT-mycotoxins, [a]Number of tested isolates/[b]number of positive isolates, AFB_1/AFG_1 – aflatoxins, CPA – cyclopiazonic acid, PAT – patulin, STER – sterigmatocystin, OA – ochratoxin A

Table 5 Production of mycotoxins by *Penicillium* spp. obtained from green coffee samples after 14 days of cultivation in the dark at 25±1 °C

Tested species	MT	Tested coffee samples																
		1	**3**	**4**	**5**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**20**
P. expansum	RC					2/1	3/0		2/0	4/4	2/0	1/0		1/0				
	PAT					2/1	3/3		2/0	4/0	2/2	1/0		1/0				
	C					2/1	3/3		2/0	4/4	2/2	1/0		1/1				
P. crustosum	CPA	1/0					3/3	1/0	2/0	2/0	3/3	4/4			1/0			2/1
	RC	1/0					3/0	1/0	2/0	2/0	3/0	4/0			1/0			2/0
	PR toxin	1/0					3/0	1/0	2/0	2/0	3/0	4/0			1/0			2/0
	PAT	1/0					3/3	1/0	2/0	2/0	3/3	4/4			1/0			2/1
P. griseofulvum	RC									2/2		2/0	1/0					1/0
	G									2/0		2/0	1/0					1/0
	PAT									2/0		2/0	1/0					1/0
	CPA									2/2		2/2	1/1					1/0
P. chrysogenum	RC			1/1			5/2	5/0	3/0	1/0		1/0						1/0
	PR toxin			1/0			5/0	5/0	3/0	1/0		1/0						1/0
	CPA			1/1			5/0	5/0	3/0	1/0		1/0						1/0
	PAT			1/1			5/0	5/0	3/0	1/0		1/0						1/0
P. hordei	RC						2/0		2/0	1/0	2/0			1/0				
	G						2/0		2/0	1/0	2/0			1/0				
P. citrinum	C		1/1		1/1		5/5	2/2		2/2	2/2		1/1			1/1	1/1	

Legend: MT – mycotoxins**,** [a]Number of tested isolates/[b]number of positive isolates, RC – roquefortine C, PAT – patulin, C – citrinin, CPA – cyclopiazonic acid, G - griseofulvin

Table 6 The overall presence of individual isolated fungal genera in roasted coffee bean samples (n=20)

Identified fungal genera	**Mycobiota of green coffee beans**						**FR (%)**
	Exogenous		**Endogenous**		**Overall amount**		
	Numbers of micromycetes	**RD (%)**	**Numbers of micromycetes**	**RD (%)**	**Numbers of micromycetes**	**RD (%)**	
Penicillium spp.	16	3.46	4	0.87	20	4.33	30
Aspergillus spp.	158	34.20	11	2.38	169	36.58	90
Cladosporium spp.	28	6.06	35	7.58	63	13.64	80
Mucor spp.	25	5.41	35	7.58	60	12.99	50
Mycelium sterillium	42	9.09	1	0.22	43	9.31	65
Absidia spp.	1	0.22	0	0.00	1	0.22	5
Rhizopus spp.	63	13.64	9	1.95	72	15.58	70
Alternaria spp.	9	1.95	0	0.00	9	1.95	30
Eurotium spp.	15	3.25	10	2.16	25	5.41	50
Σ	357		105		462		

Legend: RD (%) – relative density, FR (%) – isolation frequency

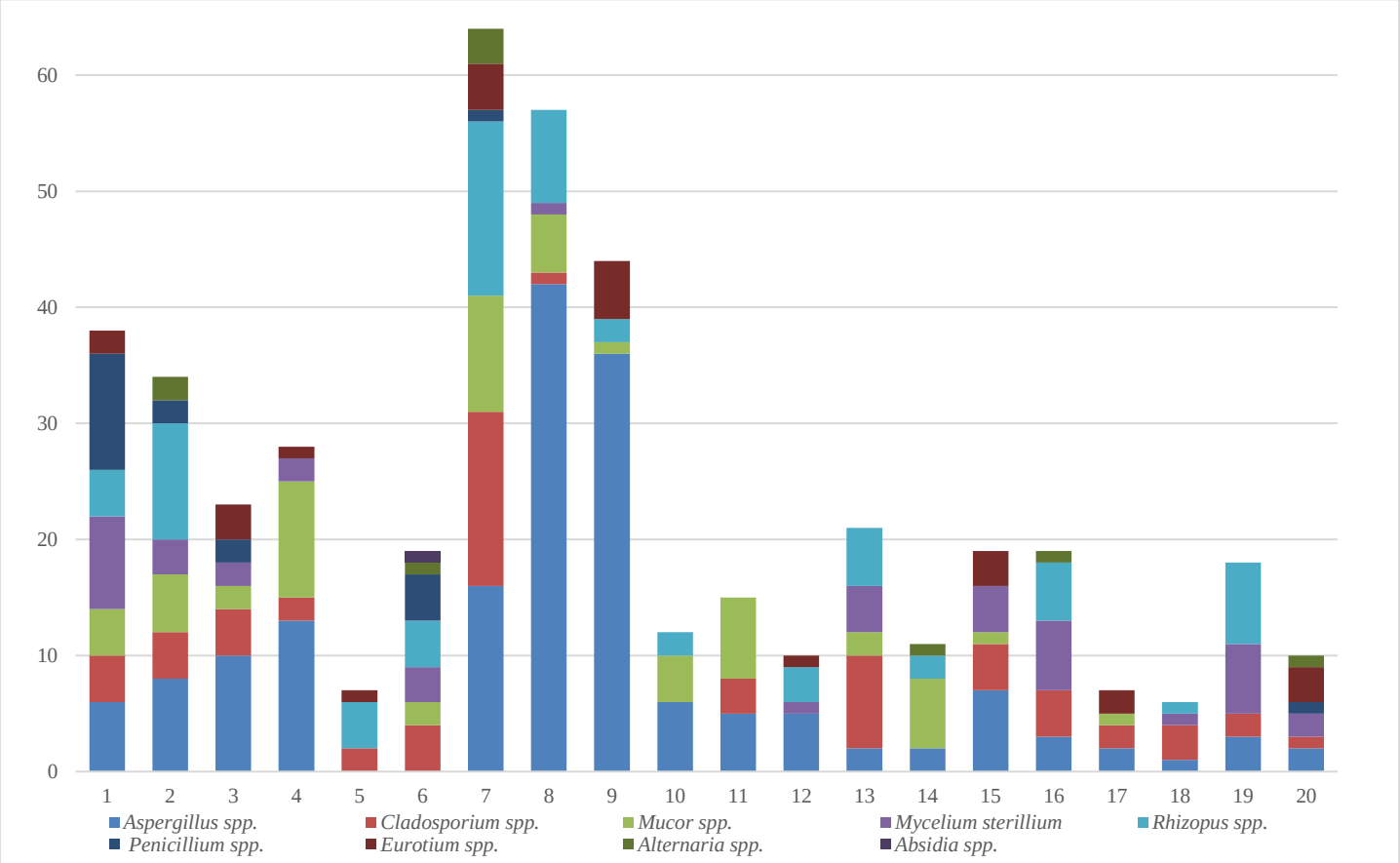

Figure 6 Presence of identified genera of micromycetes in individual roasted coffee samples (n=20)

A total of 158 isolates of the genus *Aspergillus* were isolated from roasted coffee bean samples and then identified (Figure 7). Isolates were classified into 5 species from different sections and 45 isolates were classified as black aspergillus belongs to the section *Nigri* (RD 11.1%). The second most frequently species were *A. parasiticus*. In total, there were 30 isolates, representing a relative species density of RD 7.4%. From section *Flavi* were identified to the species also *A. flavus* (DR 6.4%) and *A. oryzae* (RD 5.2%). **Fungaro *et al*. (2004)** studied the occurrence of *A. carbonarius* in coffee beans. They detected several species of the genus *Aspergillus*, including *A. flavus*. **Magnani *et al*. (2005)** focused on the molecular identification of *Aspergillus* spp. isolated from *Coffea arabica* L. They identified several species of the genus *Aspergillus*, but the most common were *A. flavus* and *A. oryzae* species.

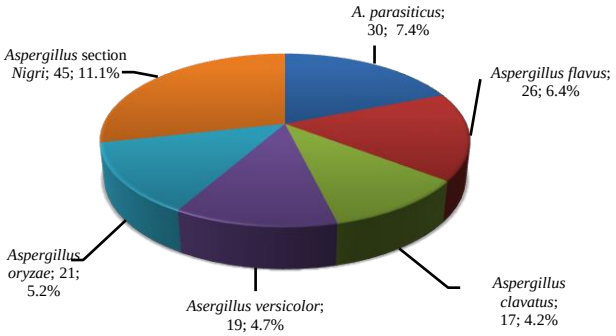

Figure 7 Relative density (RD in%) of micromycetes species of the genus *Aspergillus* obtained from roasted coffee samples (n=20)

Penicillium sp. is one of the largest and most important genera of microscopic fungi. This genus causes economic losses and, in addition, its ability to produce mycotoxins endangers human health **(Yin *et al*., 2017)**. The isolates of the genus *Penicillium* was present in a much smaller number from roasted coffee samples in comparison with green coffee samples (Figure 8). A total of 20 isolates from this genus were identified. *P. citrinum* (7 isolates) was one of the most numerous species with RD 1.7%, followed by *P. crustosum* (4 tisolates RD 1.0%) and *P. chrysogenum* with RD 0.7% (3 isolates). **Silva *et al*. (2000)** studied microbial diversity in roasted coffee from Brazil. The authors noted the presence of several species of the genus *Penicillium*, including *P. citrinum* and *P. crustosum*.

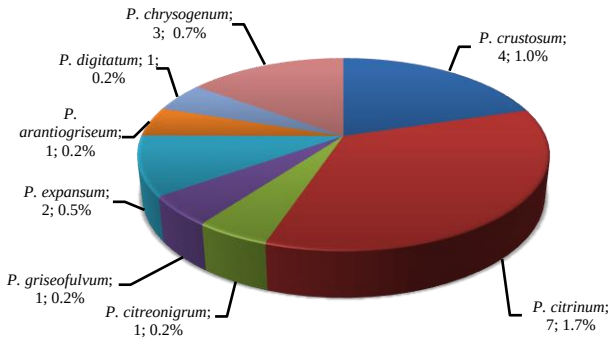

Figure 8 Relative density (RD in%) of micromycetes species of the genus *Penicillum* obtained from roasted coffee samples (n=20)

Toxigenity of the fungal isolates obtained from roasted coffee bean samples

The genus *Aspergillus* consists of various species of micromycetes occurring in many climatic conditions. Contamination of food and feed by the genus *Aspergillus* has become a global problem with a significant global economic impact. The problem with this genus of filamentous fungi occurs mainly due to the production of mycotoxins (**Sheikh-Ali *et al*., 2014**). The most common are aflatoxins, ochratoxin A and fumonisins **(Perrone and Gallo, 2017**). Similar results were obtained in this study for *A. parasiticus* tested for the production of AFB_1 and AFG_1 in roasted coffee samples. The results are summarized in Table 7 and Table 8. Coffee samples that did not contain *Aspergillus* and *Penicillium* species and were not tested for mycotoxin production are not included in Tables 7 and 8. All tested isolates (30 isolates) of this species showed the ability to produce aflatoxin B_1 and G_1 (Table 7). In addition, the ability of all *A. flavus* isolates (26 isolates) to produce aflatoxin B_1 and CPA was also confirmed by the TLC method. None of the *A. oryzae* isolates (21 isolates) were able to produce CPA. The production of PAT was confirmed for nine isolates of *A. clavatus* (17 tested isolates), and STER was produced by four isolates of *A. versicolor* (19 tested isolates). Also, **Rahmani *et al*. (2009)** analyzed samples of raw and roasted coffee

for the presence of mycotoxins. In both cases, the authors found the presence of AFB_1, AFB_2, AFG_1, and STER. In our study, the production of ochratoxin A by isolates of black aspergilli was not confirmed (45 isolates).

On the contrary, the authors **Vacvalik *et al*. (2013)** detected the presence of ochratoxin A and aflatoxin B_1 and B_2 in green and roasted coffee. The maximum permitted concentration of aflatoxins (AFB_1 + AFB_2 + AFG_1 + AFG_2) in roasted coffee in the European Union is 5 µg /kg and 20 µg/kg in the United States (**Ismail *et al*., 2018; Pankaj *et al*., 2018**). **Benites *et al*. (2017)** tested OA levels in coffee of various origins. They also found the presence of this mycotoxin in all coffee samples except one. Nevertheless, OA levels were lower than 1 g/kg in roasted coffee samples.

Similarly, authors **Galarce-Bustos *et al*. (2014)** monitored the presence of OA in roasted coffee and found that the levels of OA in coffee were 20-40 times lower than the European maximum limit. OA was present in all 63 samples. **Vanesa and Ana (2013**) analyzed 51 coffee samples for the presence of OA. Up to 69% of the coffee samples examined were found to be contaminated with mycotoxins. They determined the amount of 0.24 g/kg OA in roasted coffee. **Barcelo *et al*. (2017)** demonstrated the presence of ochratoxin A in green coffee beans. They also found that roasting coffee beans significantly reduced OA levels. **Napolitano *et al*. (2007)** also tested green coffee and changes in its composition during the collection, processing, storage, and preparation of the coffee beverage. They noted the presence of microscopic filamentous fungi such as *A. ochraceus* and *A. carbonarius*. The most contaminated coffee with ochratoxin A was from Costa Rica (13 µg/kg). However, the authors also noted a reduction in the level of OA after roasting for all types of green coffee to 4 g/kg.

From the genus *Penicillium*, overall, 17 isolates were tested for the ability to produce mycotoxins (Table 8). All isolates of *P. expansum* (2 isolates) produced patulin and citrinin, *P. crustosum* (4 isolates), *P. chrysogenum* (3 isolates), and *P. griseofulvum* (1 isolate) were able to produce CPA. *P. citrinum* (7 isolates) produced citrinin in all tested isolates.

The reason for the high levels of mycotoxins is the favorable environmental conditions in countries which incorrect storage conditions and the low standard of living. Large levels of mycotoxins have not been measured in more developed European countries, which may be due to the tightening of mycotoxin limits, the application of rules and regulations, and advances in post-harvest commodity technologies (**Ismail *et al*., 2018**).

Table 7 Production of mycotoxins by *Aspergillus* spp. obtained from green coffee samples after 14 days of cultivation in the dark at 25±1 °C

Tested species	MT	Tested coffee samples																	
		1	**2**	**3**	**4**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
A. flavus	AFB_1	1[a]/1[b]	4/4	1/1	2/2		3/3	2/2	2/2	1/1	2/2	1/1	1/1		2/2		1/1	1/1	2/2
	CPA	1/1	4/4	1/1	2/2		3/3	2/2	2/2	1/1	2/2	1/1	1/1		2/2		1/1	1/1	2/2
A. parasiticus	AFB_1		2/2	1/1		1/1	19/19	1/1	1/1	1/1		1/1	1/1	2/2					
	AFG_1		2/2	1/1		1/1	19/19	1/1	1/1	1/1		1/1	1/1	2/2					
A. clavatus	PAT				3/2	3/1	8/4			1/0					1/1	1/1			
A. versicolor	STER	2/0	1/0	1/1		4/0	6/3			2/0					2/0				
A. section *Nigri*	OA	5/0	2/0			9/0	12/0	15/0			1/0					1/0			

Legend: MT-mycotoxins, [a]Number of tested isolates/[b]number of positive isolates, AFB_1/AFG_1 – aflatoxins, CPA – cyclopiazonic acid, PAT – patulin, STER – sterigmatocystin, OA – ochratoxin A

Table 8 Production of mycotoxins by *Penicillium* spp. obtained from green coffee samples after 14 days of cultivation in the dark at 25±1 °C

Tested species	Mycotoxins	Tested coffee samples					
		1	**2**	**6**	**7**	**14**	**20**
P. expansum	RC	2/0					
	PAT	2/2					
	C	2/2					
P. crustosum	CPA	1/1		2/2			1/1
	RC	1/0		2/0			1/0
	PR toxin	1/0		2/0			1/0
	PAT	1/0		2/0			1/0
P. griseofulvum	RC					1/0	
	G					1/0	
	PAT					1/0	
	CPA					1/1	
P. chrysogenum	RC	2/0	1/0				
	PR toxin	2/0	1/0				
	CPA	2/2	1/1				
	PAT	2/0	1/0				
P. citrinum	C	4/4	1/1	1/1	1/1		

Legend: [a]Number of tested isolates/[b]number of positive isolates, RC – roquefortine C, PAT – patulin, C – citrinin, CPA – cyclopiazonic acid

CONCLUSION

Plant products, including coffee beans, are susceptible to fungal contamination. Therefore, the study of coffee beans' mycobiota is significant. In this work, the endogenous and exogenous mycobiota of 20 samples of green and roasted coffee beans were analyzed, focusing on toxicogenic species of the genus *Aspergillus* and *Penicillium*. The presence of 5308 micromycetes was detected in green coffee beans (3577 micromycetes were classified into the genera *Aspergillus* with overall RD 67.39% and FR 100% and 1306 isolates to the *Penicillium* genera with RD 24.60% and FR 90%). Some of these isolates were not identified to the species because they co-existed with the genus *Rhizopus* in the samples. From the genus *Aspergillus*, the most isolates from the *Aspergillus* section *Nigri* (1706 isolates) with RD 47.7% were detected. A total of 122 isolates from the genus *Penicillium* were classified and identified to the species. The most common species in green coffee samples were *P. crustosum* (19 isolates) with RD 1.5%. All toxicogenic isolates were screened for their potential to produce mycotoxins. From the genus *Aspergillus*, all tested isolates were able to produce aflatoxins B_1 (100%), a total of 66.84% isolates were able to produce aflatoxins G_1. Also, the presence of other mycotoxins of this genus was detected in green coffee samples as follows: 57.14% isolates showed the ability to produce patulin > 55.86% produced cyclopiazonic acid > 44.12% isolates produced sterigmatocystin > and 23.19% produced OA. The citrinin production by the genus *Penicillium* was confirmed in 83.87% isolates, followed by > 42.85% of CPA production >, and 21.43% demonstrated the ability to produce patulin in green coffee samples. There were fewer isolates from both genera capable of producing mycotoxins detected in roasted coffee. However, all tested isolates of the genus *Aspergillus* were capable of producing AFB_1, AFG_1, and CPA (100% for all mycotoxins), 52.94% of isolates showed the ability to produce PAT and 21.05% production of sterigmatocystin. The production of ochratoxin A by black aspergilli was not confirmed. Also, all tested isolates of the genus *Penicillium* (15) showed the ability to produce at least one tested mycotoxin. 100% of citrinin and cyclopiazonic acid production and 20% for patulin production by tested strains were observed. The genus *Aspergillus* was the most frequent genus with FR 90% (169 isolates) and RD 36.58% from roasted coffee beans samples. The genus *Penicillium* was isolated with lower isolation frequency (FR 30%), and relative density for this genus was RD 4.33% (20 isolates).

The obtained results showed that it is necessary to monitor the occurrence of microscopic fungi in green and roasted coffee beans. These microorganisms are capable of producing secondary metabolites that adversely affect human health. Some of these mycotoxins are classified as human carcinogens (AFBs or OA) and could also be found in roasted coffee because of their thermostability during the roasting process. In total, the presence of microscopic fungi capable of producing aflatoxins and ochratoxin A was found in all green coffee bean samples, and in 18 out of 20 roasted coffee bean samples has detected the production of aflatoxins. Due to the possible presence of mycotoxins, it is imperative to prevent fungal contamination of coffee beans and further analysis and control of coffee beans before and after the roasting process.

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