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Ochratoxin- and aflatoxin-producing fungi associated with green and roasted coffee samples consumed in Argentina

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Abstract

The aims of this work were to identify the *Aspergillus* sections *Nigri* and *Flavi*, and to evaluate the natural occurrence of ochratoxin A (OTA) and aflatoxins in green and roasted coffee bean samples. The capacity to produce these toxins by *Aspergillus* species was also studied. Fifty samples of Colombian coffee beans (25 green and 25 roasted) were obtained from a processor plant located in the south of Córdoba province (Argentina). OTA and aflatoxin analysis were performed by high performance liquid chromatography (HPLC). OTA production by strains belonging to *Aspergillus niger* aggregate were cultivated using YES medium and detected by HPLC. Aflatoxin production was tested in strains belonging to section *Flavi* on malt extract agar and was detected by thin liquid chromatography (TLC). From green coffee samples, the predominant species isolated belonged to *A. niger* aggregate, 60 and 55%, in dichloran rose bengal chloramphenicol agar (DRBC) and dichloran 18% glycerol agar (DG18) respectively. While *A. flavus* strains were isolated in 14% from DRBC and *A. parasiticus* strains in 12% and 28% in DRBC and DG18, respectively. From roasted coffee samples, *A. flavus* was the most predominant fungi, isolated in similar percentages from both media (28%); followed by *A. niger* aggregate isolated in 28 and 14% in DRBC and DG18, respectively. In green and roasted coffee samples mean colony counts ranged from 2×10^3 to 3.5×10^4 colony forming units per gram of sample (cfu/g). OTA and aflatoxins were not detected in any sample analyses (<1 and 0.5 ng/g for OTA and aflatoxins, respectively). Twenty-five percent of black *Aspergillus* strains were OTA producers. The total of *A. flavus* strains assayed produced aflotoxin B_1 (AFB_1) and 80% of the *A. parasiticus* strains were AFB_1 and aflotoxin G_1 producers. The high percentage of *A. flavus* and *A. parasiticus* aflatoxin-producing strains suggest a potential risk for contamination in coffee with aflatoxins.

Keywords: aflatoxins, *Aspergillus* section *Nigri* and *Flavi*, coffee, ochratoxin A

1. Introduction

Like other crops, coffee cherries and beans are subjected to contamination and colonisation by fungi during different phases of development, harvesting, preparation, transport and storage. Fungal spoilage activity affecting the quality and safety of the final products will depend on environmental conditions as well as crop and product management (Magan and Olsen, 2004).

Studies on the mycoflora of coffee cherries and beans in the world have shown that *Aspergillus*, *Penicillium* and *Fusarium* are the main toxigenic fungal genera isolated (Batista *et al.*, 2003; Martins *et al.*, 2003; Silva *et al.*, 2000; Urbano *et al.*, 2001).

Ochratoxin A (OTA) is receiving worldwide attention because of its nephrotoxic, hepatotoxic, teratogenic, carcinogenic and immunosuppressive properties. Evidence that it may cause cancer in human beings, is being

evaluated (Ehrlich *et al.*, 2002). The occurrence of OTA has been reported in several agricultural crops for human consumption, including stored cereal grains, beer, species, coffee and cocoa beans (Fazekas *et al.*, 2002; Lombaert *et al.*, 2002; Magan and Olsen, 2004; Scudamore and Patel, 2000, Sugita-Konishi *et al.*, 2006).

Aflatoxins are potent carcinogenic and teratogenic metabolites produced in different pre- and post-harvested crops by several species of *Aspergillus* section *Flavi*. *A. flavus* and *A. parasiticus* are aflatoxins producers. Among the main commodities susceptible to contamination are cereals, oilseeds, coffee beans and species (Jelinek *et al.*, 1989; Magan and Olsen, 2004). Aflatoxin B_1 (AFB_1) was evaluated as a class 1 human carcinogen by the International Agency for Research on Cancer (IARC, 1993). In the tropical regions, *A. ochraceus* was found to be a prevalent fungus in the mycobiota of some green coffee bean samples, and was identified as the main source of OTA in this substrate. Recently, several other *Aspergillus* species have been described as OTA producers including black *Aspergillus* (*A. carbonarius* with a minor contribution by *A. niger* aggregate) (Bucheli *et al.*, 2000; JECFA, 2001; Magnoli *et al.*, 2004; 2006a,b).

The length of time at a water activity >0.80 at any moment until roasting defines the risk of fungal growth, OTA and aflatoxins production (Viani, 2002). As contamination of coffee beans is especially important, several authors have reported that OTA is not completely degraded during roasting at different temperatures (Blanc *et al.*, 1998; Micco *et al.*, 1989; Tsubouchi *et al.*, 1987; Van der Stegen *et al.*, 2001). Aflatoxin levels were also reduced by approximately 42 to 56% during the roasting process (Soliman, 2002).

In our region, the presence of OTA and its potentially producers has recently been detected in dried vine fruits (Magnoli *et al.*, 2004), corn and corn-based feeds (Dalcero *et al.*, 2002; Magnoli *et al.*, 2005, 2006a), and peanut seeds (Magnoli *et al.*, 2006b). The Argentinean population consumes Brazilian and Colombian coffee, and there is no available information about the mycological and toxicological quality of coffee grains allowed for the consumers. The aims of this work were to isolate and identify the *Aspergillus* and *Penicillium* fungal population and to evaluate the natural occurrence of OTA and AFB_1 in green and roasted coffee bean samples consumed in Argentina. The capacity of *Aspergillus* species to produce these toxins was also studied.

2. Materials and methods

Sampling

Fifty samples of Colombian coffee beans of *Coffea arabica* L. (25 green and 25 roasted) were obtained from a processor plant located in the south of Córdoba province (Argentina). The roasted coffee bean samples were processed at 220 °C in a roasting machine for 45 min. The green coffee beans were collected from 50 kg bags. Whereas roasted coffee beans were collected after seven days of the roasting process directly from the tank where they were stored previously for packaging. The tank with roasted coffee beans had been open since it was roasted (approximately a week) and the green coffee beans were kept in bags placed in the same room. The samples (two kg each) were collected at a single sampling time. In the laboratory, the beans (without disinfection) were finely ground in a Buehler laboratory mill. It was disinfected between each sample with sodium hypochlorite at 10%, and washed with distilled water. The milled samples were immediately analysed for fungal contamination and the rest were kept at 4 °C until the OTA and aflatoxins analysis.

Isolation and identification of mycobiota

Isolation and quantitative enumeration of total fungal propagules was performed on solid media using the surface-spread method by stomaching 10 g portions of each sample with 90 ml of 0.1% peptone water solution by stomacher® 400 circulator (Seward, Warwickshire, United Kingdom) for 2 minutes. Serial dilutions of 10^{-2} to 10^{-4} concentration were made from each material and 0.1 ml aliquots were inoculated in triplicate onto dichloran rose bengal chloramphenicol agar (DRBC) and dichloran 18% glycerol agar (DG18) for general fungal enumeration (Pitt and Hocking, 1997). The plates were incubated at 25 °C for seven days in darkness. On the last day of incubation, plates that only contained between 10-100 cfu were used for counting and the results were expressed as colony forming units per gram of sample (cfu/g). The colonies of filamentous fungi were picked, transferred to malt extract agar (MEA) slants, and allowed to grow at 25 °C for seven days for general identification according to Pitt and Hocking (1997) and Samson *et al.* (2000).

Each strain of *Aspergillus* and *Penicillium* spp. was identified in species according to the method proposed by Klich (2002) and Pitt (1988), respectively.

Ochratoxin A analysis

The detection of OTA in the green and roasted coffee samples was performed by HPLC, following the methodology proposed by Diaz *et al.* (2004), with some modifications according to the sample. A 5 gram portion of

mixed ground roasted coffee sample was extracted with a solution of methanol: sodium bicarbonate at 1% (50:50, v/v) and blended at high speed for one minute. The pure extract was filtered to remove particulate matter, and 20 ml of extract was taken and diluted with 20 ml of PBS containing 0.01% Tween 20. The diluted extract was filtered through a microfibre filter. A ten-milliliter portion was taken and added to an immunoaffinity column (OchraTest™, Vicam, Digen Ltd., Oxford, UK), and allowed to elute under gravity. The column was washed with 10 ml PBS containing 0.01% Tween 20 and then with 10 ml double-distilled water. OTA was eluted from the column with methanol (HPLC grade), at a flow rate of one to two drops per second.

A 25 g portion of mixed ground green coffee sample was extracted with a solution of methanol: sodium bicarbonate at 1% (70:30, v/v) and blended at high speed for one min. The pure extract was filtered to remove particulate matter, and a 10 ml of extract was taken and diluted with 40-ml PBS containing 0.01% Tween 20. The diluted extract was filtered through microfibre filter. A ten-milliliter portion was taken and added to an immunoaffinity column (OchraTest™), and allowed to elute under gravity. The column was washed with 10 ml PBS containing 0.01% Tween 20 and then with 10 ml double-distilled water. OTA was eluted from the column with methanol (HPLC grade), again at a flow rate of one to two drops per second.

OTA detection was performed by HPLC, following the methodology proposed by Scudamore and McDonald (1998), with some modifications. The HPLC apparatus used for OTA determination was a Hewlett Packard chromatography with a loop of 100 µl, equipped with a spectrofluorescence detector (excitation, 330 nm; emission, 460 nm) and a C_{18} column (Supelcosil LC-ABZ, Supelco; 150×4.6 mm, 5 µm particle size), connected to a precolumn (Supelguard LC-ABZ, Supelco; 20×4.6 mm, 5 µm particle size). The mobile phase was pumped at one ml/min and consisted of an isocratic system as follows: 57% acetonitrile, 41% water, and 2% acetic acid. OTA was quantified on the basis of HPLC fluorometric response compared with OTA standard (Sigma Aldrich Co., St. Louis, MO, USA, purity >99%). The lowest limit of detection was 1.0 ng/g.

Aflatoxin analysis

The aflatoxins in green and roasted coffee samples were extracted and cleanup was performed according to AOAC (2000). Aflatoxin detection was performed by HPLC according to the methodology proposed by Trucksess *et al.* (1994). Purified extract aliquot (200 µl) was derivatised with 700 µl trifluoroacetic acid: acetic acid: water (20:10:70, v/v). The derivatised aflatoxin was analysed using an HPLC system. Chromatographic separations were performed on stainless steel, C_{18} reversed phase column (VARIAN, 150×4.6 mm id., 5 µm particle size). Water: methanol: acetonitrile (4:1:1, v/v) was used as mobile phase at a flow rate of one ml/min. Fluorescent aflatoxin derivatives was recorded at excitation and emission wavelengths of λ 360 nm and λ 460 nm, respectively. The detection limit of the analytical method was 1.0 ng/g.

Assay of spiking and recovery of Ochratoxin A and aflatoxins

Toxin-free samples (5 g of roasted and 25 g of green coffee for OTA and 25 g of roasted and green coffee for AFB_1) contained in a 250 ml Erlenmeyer flask were spiked with standard solutions of OTA and aflatoxins to reach an equivalent of 1, 5 and 10 ng OTA/g and of 5, 10 and 20 ng AFB_1/g. Spiking and a single analysis of the blank sample were carried out twice. After leaving it for 16 h to evaporate the solvent, extraction solvent was added and toxins concentration were determined using the protocols previously described. Recovery percentage was calculated for each substrate.

Ochratoxin A production

OTA production was tested in 100 strains belonging to *A. niger* aggregate. The toxin was determined following the methodology described by Téren *et al.* (1996). The strains were grown in stationary cultures using 20 ml quantities of YES medium (2% yeast extract, 15% sucrose) in 125 ml Erlenmeyer flasks and were inoculated with 1 ml of dense conidial suspensions and incubated at 30 °C for 10 days in darkness. After incubation, 1 ml portions of these culture media were then mixed with 1 ml of chloroform and centrifuged at 4,000 rpm for 10 min. The chloroform phase was transferred to a clean tube, evaporated to dryness and re-dissolved in methanol. The conditions of OTA detection were previously described.

Aflatoxins production

Aflatoxin production was tested in 30 strains belonging to section *Flavi* (*A. flavus* and *A. parasiticus*). The toxins were determined following the methodology described by Geisen (1996). The strains were grown at 25 °C for seven days in MEA medium in darkness, after which mycelium and conidia were collected from the agar surface with a sterile brush and transferred to an Eppendorf tube. Aflatoxins were extracted with 500 µl chloroform and centrifuged at 4,000 rpm for 10 min. The chloroform phase was transferred to a clean tube, evaporated to dryness and re-dissolved in chloroform. The extract was spotted together with standards and screened for aflatoxins by TLC method on silica gel plates without a fluorescent indicator (0.25 mm, G60 Merck); and using chloroform: acetone (9:1, v/v) as developing solvent. Aflatoxin concentration was determined by visual comparison with standards. Chromatograms were dried and observed under 365 nm UV light.

Statistical analysis

The mean colony counts (cfu/g) were analysed by analysis of variance (ANOVA) and means compared by LSD test to determine the significant difference between green and roasted coffee samples. The analysis was conducted using PROC GLM in SAS (SAS Institute, Cary, NC) (Quinn and Keough, 2002).

3. Results

The percentage of green and roasted coffee samples contaminated by fungal genera and *Aspergillus* species was used to characterise the ochratoxigenic mycoflora. Mycological survey of 50 samples of coffee indicated the presence of seven genera of filamentous fungi. Among them, *Aspergillus* spp. was the most frequent fungi of the mycobiota that occurred in 86% and 71% in DRBC and DG18 media of green coffee samples, respectively (Figure 1A). Whereas the percentage of roasted coffee samples contaminated with this genus was 43% in both culture media (Figure 1B). Five and four species of *Aspergillus* from green and roasted coffee samples, respectively, were isolated. Three common contaminating food species of *Penicillium* genus were identified: *P. citrinum*, *P. crustosum* and *P. minioluteum* in percentage ranging from 14% to 28% (Figures 2A and 2B).

From green coffee samples, the predominant species isolated belonged to *A. niger* aggregate, 60 and 55%, in DRBC and DG18 respectively. While *A. flavus* strains were isolated in 14% from DRBC and *A. parasiticus* strains in 12% and 28% in DRBC and DG18, respectively (Figure 2A). From roasted coffee samples, *A. flavus* was the most predominant fungi, isolated in similar percentages of the samples from both media (28%); followed by *A. niger* aggregate isolated in 14 and 28% in DRBC and DG18, respectively. Whereas *A. parasiticus* was isolated in the same percentage (14%) in both culture media (Figure 2B).

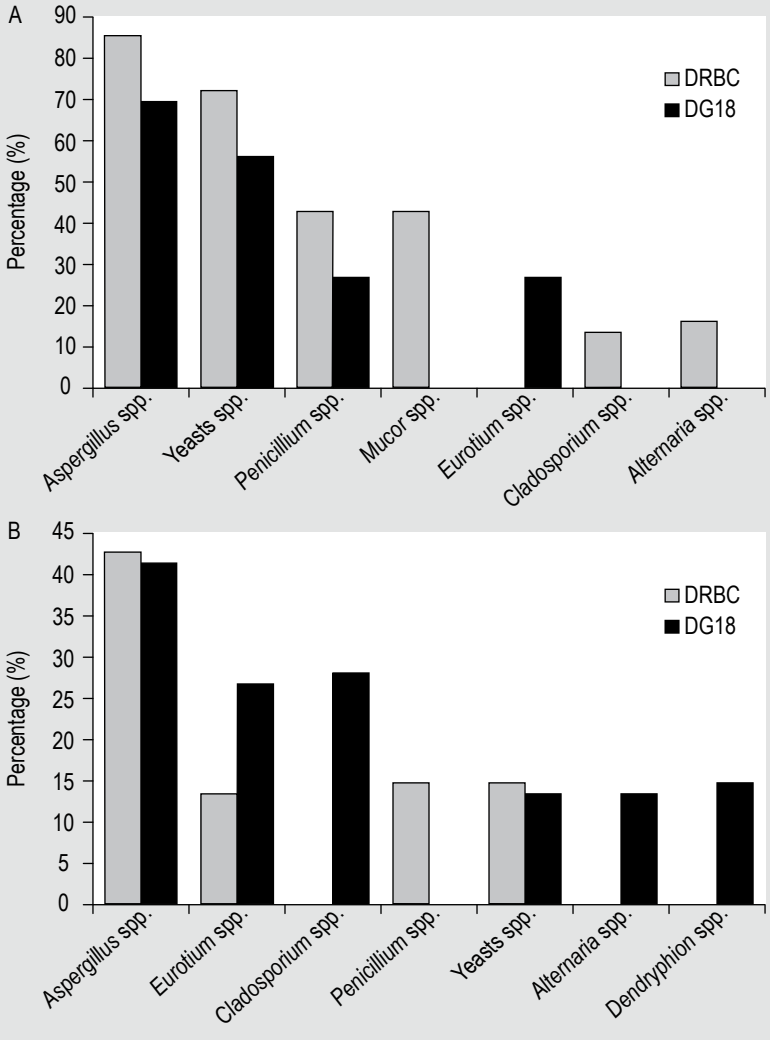

Figure 1. Distribution of fungal genera in (A) green coffee samples (n=25) and (B) roasted coffee samples (n=25).

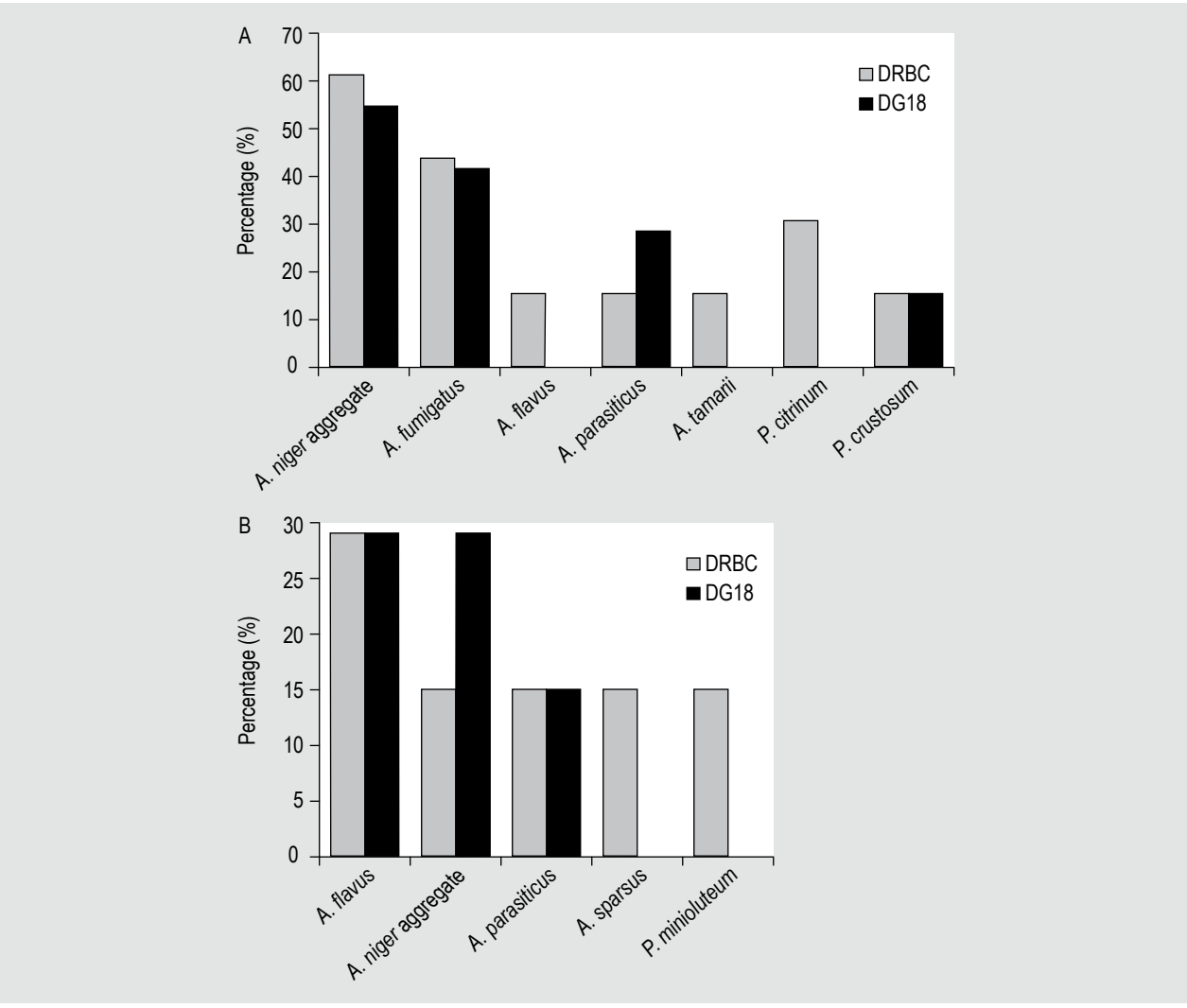

Figure 2. Distribution of *Aspergillus* and *Penicillium* species in (A) green coffee samples (n=25) and (B) roasted coffee samples (n=25).

Table 1 shows the total fungal colony counts (cfu/g) in green and roasted coffee samples from DRBC and DG18 media. Mean colony counts ranged from 2×10^3 to 3.5×10^4 cfu/g. The highest counts were observed in roasted coffee from DRBC media ($P<0.0001$).

Mean colony counts of *Aspergillus* section *Nigri* and *Flavi* were 10^2 cfu/g from green coffee samples, whereas from roasted coffee these counts ranged from 10^2 to 10^4 cfu/g. The counts obtained for *A. niger* aggregate species in roasted coffee samples from DG18 media were significantly

Table 1. Total fungal counts from green and roasted coffee beans.

Samples	DRBC		DG18	
	Range[1]	Mean counts[2] ± SD	Range[1]	Mean counts[2] ± SD
Green coffee	$8x10^2$-$3.45x10^3$	$1.9x10^3$±1.13 $x10^3$ [b]	$5.5x10^2$-$4.75x10^3$	$2.0x10^3$±$1.7x10^3$ [b]
Roasted coffee	$2.5x10^2$-$8.5x10^4$	$3.5x10^4$±4.4 $x10^4$ [a]	$2.2x10^3$-$2.2x10^4$	$9.4x10^3$±1.0 $x10^4$ [b]

[1] Minor and major fungal count (cfu/g).

[2] Cfu/g. SD: standard deviation.

[a,b] Values with no common superscripts are significantly different ($P<0.0001$) according to LSD test.

higher than those obtained in green coffee. The highest counts of *A. flavus* and *A. parasiticus* were obtained from roasted coffee (P<0.0001) (Table 2).

OTA and aflatoxins were not detected in any sample analyses at detection limits of techniques applied (<1 and 0.5 ng/g for OTA and aflatoxins, respectively). Twenty-five percent of 100 black *Aspergillus* strains assayed were OTA producers. The toxin levels produced ranged from 2.7 to 14 ng/ml.

Table 3 shows the potential for OTA production by *Aspergillus* section *Nigri* isolated from green and roasted coffee samples. The total of *A. flavus* assayed strains produced AFB_1 with mean levels of 9.5 to 17.5 ng/g of mycelium. Regarding *A. parasiticus*, 80% of the strains were AFB_1 and AFG_1 producers, with mean levels ranging from 11.5 to 48 ng/g and 6 to 28 ng/g of mycelium, respectively.

Table 2. Counts of *Aspergillus* and *Penicillium* species from green and roasted coffee samples.

Species	Mean fungal counts[1] ± SD			
	Green coffee		Roasted coffee	
	DRBC	DG18	DRBC	DG18
A. niger aggregate	$5.75x10^2$±5.4 [g]	$4.0x10^2$±0.0 [g]	$2.0x10^2$±0.0 [g]	$6.5x10^3$±900 [d]
A. tamarii	$2.0x10^2$±0.0 [g]	ND[2]	ND	ND
A. fumigatus	$1.13x10^3$±189 [ef]	$5.2x10^2$±500 [fg]	ND	ND
A. parasiticus	$1.0x10^2$±0.0 [g]	$1.5x10^2$±71 [g]	$1.5x10^4$±0.0 [c]	$2x10^3$±0.0 [e]
A. flavus	$3.5x10^2$±0.0 [g]	ND	$2.0x10^4$±0.0 [b]	$1.07x10^3$±700 [e]
A. sparsus	ND	ND	$2.5x10^4$±0.0 [a]	ND
P. citrinum	$1.0x10^2$±0.0	ND	ND	ND
P. minioluteum	ND	ND	$1.0x10^2$±0.0	ND
P. crustosum	$1.0x10^2$±0.0	$3.5x10^2$±0.0	ND	ND

[1] Cfu/g; SD: standard deviation.
[2] ND: not detected.
[a-g] Values with no common superscripts are significantly different (p< 0.0001) according to LSD test.

Table 3. Aflatoxins production by *Aspergillus* section *Flavi* strains from coffee samples.

Species	Percentage of producer strains (%)	Mean levels (ng/ml)[1] ± SD				
		AFB_1	AFB_2	AFG_1	AFG_2	Total aflatoxins
A. flavus	20	9.5±3.0	ND2	ND	ND	9.5
	20	12.5±1.5	ND	ND	ND	12.5
	30	15.5±3.5	ND	ND	ND	15.5
	30	17.5±5.5	ND	ND	ND	17.5
A. parasiticus	10	11.5±3.0	ND	6	ND	11.5
	1	15±2.5	ND	7	ND	22
	15	17±5.5	ND	9	ND	26
	15	19±5.5	ND	10	ND	29
	5	20±0.0	ND	11	ND	31
	10	25±4.0	ND	13	ND	38
	20	47±9.5	ND	26	ND	73
	15	48±8.5	ND	28	ND	76

[1] Aflatoxins were detected by TLC method.
[2] ND: not detected.

4. Discussion

In general, fungi genera identified have been previously isolated in coffee beans (Martins *et al.*, 2003). Batista *et al.* (2003) observed that the percentage of contaminated samples by *Aspergillus* spp. was higher than that found in our study; and the percentage of samples containing *Penicillium* spp was similar to green coffee in DRBC medium. Another study from Belgium, Sibanda *et al.* (2001), has reported 100% and 14% of green coffee samples contaminated by *Aspergillus* and *Penicillium* spp., respectively. The results observed in our study indicated that the fungal total counts from roasted coffee showed a higher value than those from green coffee whereas the percentage of *Aspergillus* spp. was lower in roasted coffee. The high diversity of *Aspergillus* and *Penicillium* spp. isolated from roasted coffee reveals poor hygienic conditions in the environment when it is stored and packet before commercialisation. In the seven-day period between the roasting and packaging of coffee beans, the environmental conditions, such as temperature, humidity and environment mycobiota, allowed contamination and development of different fungal species. The sampling was done in September, when the temperature and humidity ranged between 15-25 °C and 50-60%, respectively. The high *A. flavus* and *A. parasiticus* counts in roasted coffee beans can be attributed to the presence of these species in green coffee beans and in the environment where they are stored. This fact establishes the importance of controlling the microbiological quality of the environment where these types of products are processed and/or packaged. These results indicate, in addition, the need to apply good manufacturing practices (Moss, 2002).

The species *A. sparsus*, *A. minioluteum* and *P. crustosum* have not been previously identified from green coffee beans (Batista *et al.*, 2003). *A. carbonarius* and *A. ochraceus*, the main OTA producers species, have been previously isolated from coffee beans (Joosten *et al.*, 2001; Martins *et al.*, 2003; Urbano *et al.*, 2001) indicating that these species are also potential sources of OTA in coffee. In the present study, these species were not isolated; *A. carbonarius* has been previously isolated from other substrates in Argentina such as dried vine fruits, peanut seeds and wine grapes, but *A. ochraceus* has not been detected in these commodities (Magnoli *et al.,* 2004, 2006b; Ponsone *et al.,* 2007). The percentage of samples infected by *A. niger* aggregate was similar to the one found by Urbano *et al.* (2001); Joosten *et al.* (2001) and Taniwaki *et al.* (2003), but it was lower than the percentage of green coffee samples contaminated with *A. niger* observed by Martins *et al.* (2003). The total fungal counts observed in this study were higher than one found from Indian coffee beans in one of the media used (DRBC), while the counts of *A. flavus* / *A. parasiticus* strains were lower than those detected in green coffee beans (Somashekar *et al.*, 2004). Soliman (2002) has reported 80% and 71% of the green and ground roasted coffee beans contaminated by *A. flavus*. In the same study, aflatoxins were detected in 76.5 and 54.6% of the *A. flavus* infected samples, respectively. These percentages of *A. flavus* are higher than the ones observed in this study. The Argentinean Alimentary Codex and Mercosur regulations have not established limits for total fungal counts in green and roasted coffee beans (CAA, 2007).

In the present work, the percentage of black *Aspergillus* strains producing OTA (25%) is higher than those reported from coffee cherries by Heenan *et al.* (1998), Taniwaki *et al.* (2003) and Urbano *et al.* (2001). However, these percentages correspond with those previously seen in *Aspergillus* section *Nigri* strains isolated from dried vine fruits, corn kernels and peanut seeds from Argentina (Magnoli *et al.*, 2004, 2006a,b). The absence of the main ochratoxigenic species, *A. carbonarius* and *A. ochraceus*, could explain the none detection of OTA contamination in green and roasted coffee samples.

In previous works, there is limited available information on aflatoxigenic capacity in *A. flavus* and *A. parasiticus* isolated from coffee. A low percentage of *A. flavus* aflatoxin B_1 and B_2 producers and contaminated samples have been found in Brazilian green coffee beans (Batista *et al.*, 2003). The high percentage of *A. flavus* (100%) and *A. parasiticus* (80%) aflatoxin- producing strains isolated in this work, suggests a possible aflatoxins coffee contamination. However, these mycotoxins have not been detected in green and roasted coffee samples. Therefore, the industry should work to not only reduce the growth of these fungi, but also prevent flavour alteration, spoilage and aflatoxin formation in green and roasted coffee.

From the first analysis of OTA in green coffee beans reported by Levi *et al.* (1974), the presence of OTA in green and roasted coffee beans has been reported worldwide in variable concentrations (Bucheli *et al.*, 2000, Bucheli and Taniwaki, 2002, Fazekas *et al.*, 2002, Fuji *et al.*, 2006, Lombaert *et al.*, 2002, Martins *et al.*, 2003, Romani *et al.*, 2000, Sibanda *et al.*, 2001, Studer-Rohr *et al.*, 1995, Sugita-Konishi *et al.* 2006, Taniwaki *et al.*, 2003, Trucksess *et al.*, 1999, Urbano *et al.*, 2001). But aflatoxin reports in these substrates are limited (Soliman 2002, Somashekar *et al.*, 2004). The results obtained in this work indicate that any of the coffee samples contained OTA and aflatoxins at detectable levels. Further studies with a significant number of samples at industry and market origin, would be necessary to confirm that coffee in Argentina is not an important source of OTA and aflatoxins for the human population.

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