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The biodiversity of *Aspergillus* section *Flavi* in brazil nuts: From rainforest to consumer

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ABSTRACT

A total of 288 brazil nut samples (173 kernel and 115 shell) from the Amazon rainforest region and São Paulo State, Brazil were collected at different stages of brazil nut production. Samples were analysed for: percentages of aflatoxigenic fungal species and potential for aflatoxin production and presence of aflatoxins. *Aspergillus nomius* was the most common species found (1235 isolates) which amounted to 30% of the total species with potential to produce aflatoxins. This species is of concern since 100% of all isolates produced aflatoxins B_1, B_2, G_1 and G_2. *Aspergillus flavus* was almost equally common (1212 isolates) although only 46% produced aflatoxins under laboratory conditions, and only aflatoxins B_1 and B_2. Low number of other species with the potential to produce aflatoxins was isolated: *Aspergillus arachidicola* and *Aspergillus bombycis* produced B and G aflatoxins whilst *Aspergillus pseudotamarii* produced only aflatoxin B_1. The total aflatoxin levels found in samples taken from the rainforests was 0.7 μg/kg, from processing plants before and after sorting 8.0 and 0.1 μg/kg respectively, from street markets in the Amazon region 6.3 μg/kg and from supermarkets in São Paulo State 0.2 μg/kg. Processing, which included manual or mechanical sorting and drying at 60 °C for 30 to 36 h, eliminated on average more than 98% of total aflatoxins. These results showed that sorting is a very effective way to decrease aflatoxin content in brazil nuts.

1. Introduction

Brazil nuts are one of the most important products extracted from the Amazon rainforest region. Trees of *Bertholletia excelsa* grow wild, reach up to 60 m in height, take 12 years to bear fruit and may live for up to 500 years. The trees are found in groves of 50–100 individuals and the groves are separated by up to 1 km. Pollination is by wild, large bodied bees, especially *Euglossinae* species (Wadt et al., 2005). The equatorial climate is hot and humid, with an average temperature of 26 °C and relative humidity of 80–95%. Brazil nut production is organic and an environmentally sound system, as no chemical products are used to control pests and weeds. This ecosystem favours a unique biodiversity of fungal species different from those found in cultivated crops. Brazil nuts, encased in hard pods the size of small coconuts, fall from trees during the rainy season, between November and February. Local people collect the pods from the forest but, to avoid serious injury, only once the pods have stopped falling. The pods are opened in the forest or nearby and the brazil nuts are transported to processing plants by boat. Nuts and shells are sorted usually manually, to segregate deteriorated or physically damaged kernels. Kernels or nuts in shell are dried at a temperature of 60 °C for 24 h or until they reach 11 to 15% of moisture content. Dried brazil nuts are packed in plastic bags or boxes to be sold.

Brazil nuts contain a range of fungi. Those from *Aspergillus* section *Flavi* are of major concern because some have the potential for aflatoxin production. A major challenge for brazil nut production is the control of contamination by aflatoxigenic fungi and aflatoxins (Olsen et al., 2008; Gonçalves et al., 2012).

Aflatoxins are of widespread occurrence in nuts and some cereals (Pitt and Hocking, 2009). Aflatoxins are carcinogenic and may cause other human health problems, so many countries have imposed regulations aiming at minimising human exposure to aflatoxins. This has resulted in rejection of products, including brazil nuts, causing great economic losses for producers, processors and marketers. The International Agency for Research on Cancer recognises mixtures of aflatoxins as Group 1 carcinogens (IARC, International Agency for Research on Cancer, 1993). CAC (Codex Alimentarius Commission) (2010a) has recommended a maximum level for aflatoxins in brazil nuts for further processing of 15 μg/kg and for ready to eat of 10 μg/kg.

The occurrence of aflatoxins in brazil nuts has been reported in samples sold in several countries including the United States (Pohland, 1993), Japan (JECFA, Joint WHO/FAO Expert Committee on Food Additives, 1998), the United Kingdom (Food Standards

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Agency, 2004), Sweden (Marklinder et al., 2005) and Brazil (de Mello and Scussel, 2007) with contamination levels ranging up to 620 μg/kg.

The present work was carried out to elucidate the source of aflatoxins in brazil nuts at different stages of the production chain from the Amazonian rainforest, to Amazon street markets, to samples for drying and processing, and packaged for sale in supermarkets in São Paulo State.

2. Materials and methods

2.1. Fungal isolation and identification from brazil nut samples

A total of 288 brazil nut samples (173 kernel and 115 shell) each of approximately 2 kg were collected in the Amazon region and São Paulo State, Brazil. Samples of shells were also analysed for fungi because they are porous allowing penetration into the kernel. From each sample, approximately 100 g of shelled nuts and 100 g of shells were disinfected separately by immersion in 0.4% sodium hypochlorite solution for 1 min. Fifty pieces of nuts or shells were plated onto Dichloran 18% Glycerol agar (DG18), according to the methodology of Pitt and Hocking (2009). Plates were incubated for 5 days at 25 °C.

Isolates that had the appearance of belonging to *Aspergillus* section *Flavi* were grown on Czapek yeast autolysate agar (CYA) and incubated at 25 °C for 7 days (Pitt and Hocking, 2009). Isolates were then examined on standard identification media for *Aspergillus* species, Czapek yeast autolysate agar, at 25 °C, 37 °C and 42 °C and on *Aspergillus flavus* and parasiticus agar (AFPA) at 25 °C (Pitt and Hocking, 2009). The incubation time for all media and conditions was 7 days. Although Pitt and Hocking (2009) recommended incubation of AFPA at 30 °C for 48 h, in the present study AFPA was incubated like the other media, as AFPA was used for identification, not isolation. Representatives of each species, distinguished by morphological and physiological characteristics, were analysed by molecular methods and for the production of extrolites. Isolates were finally identified using polyphasic approaches following Pitt and Hocking (2009), Samson et al. (2010) and Varga et al., 2011.

2.2. Potential for aflatoxin production by Aspergillus section Flavi isolates

Fungi identified as potential producers of aflatoxins were inoculated onto yeast extract sucrose agar for 7 days at 25 °C and then the agar plug technique (Filtenborg et al., 1983) was used to evaluate the capability of isolates to produce aflatoxins. Fungal extracts taken as plugs with a cork borer were placed on TLC plates, developed in a toluene: ethyl acetate: formic acid 90%: chloroform (7:5:2:5, v/v/v/v) mobile phase, and visualized under UV light at 365 nm. A mixture of standard preparations of aflatoxins B_1, B_2, G_1 and G_2 (Sigma Aldrich, St. Louis, MO, USA) was used for comparison.

2.3. Water activity

The water activity of kernel and shells was determined using an Aqualab Series 3TE instrument (Decagon, Pullman, WA, USA) at 25 ± 0.1 °C, in triplicate. The kernels were ground using a laboratory mill (IKA A 11 basic, USA) and the shells were broken manually.

2.4. Molecular analysis

Sixty two isolates were cultivated on yeast extract and lactose (YEL) solid medium (Price et al., 2005) at 28 °C for 7 days. From each culture, a suspension of approximately 10^7 conidia suspended in Tween 80 (2.5 ml) was inoculated into bottles containing YEL liquid (50 ml), and incubated in a shaker (180 rpm) at 28 °C for 16 to 24 h. After incubation, mycelia were collected on sterile filter paper by vacuum filtration and washed in sterile water. Nucleic acids were extracted according to Azevedo et al. (2000), and treated with ribonuclease A (20 μg/ml). Partial amplification of the ß-tubulin gene was performed using standard amplification reactions and the following primer pair: Bt2a (5′ GGT AAC CAA ATC GGT GCT TTC 3′) and Bt2b (5′ ACC CTC AGT GTA GTG ACC CTT GGC 3′), as described by Glass and Donaldson (1995). Fragments generated by PCR were purified with Wizard® SV Gel and PCR Clean-Up System (Promega, USA). The amplicons were submitted to direct sequencing in both directions with a Big Dye Terminator Cycle Sequencing Standard kit Version 3.1 (Applied Biosystems, USA) under the following conditions: denaturation at 95 °C for 60 s, followed by 30 cycles of denaturation at 95 °C for 20 s, annealing at 50 °C for 15 s, extension at 60 °C for 1.5 min, and a final extension at 60 °C for 3 min. A volume of HiDi formamide (10 μl) was added to the sequencing products, which were processed in an ABI 3500XL Genetic Analyser (Applied Biosystems, USA). One sequence from each species was deposited into GenBank with the following accession number: *Aspergillus tamarii* (JX157590), *Aspergillus flavus* (JX157586), *Aspergillus caelatus* (JX157591), *Aspergillus nomius* (JX157589) *Aspergillus arachidicola* (JX157587), *Aspergillus bombycis* (JX157588) and *Aspergillus pseudotamarii* (JX157592).

2.5. Extrolite analysis

Extrolite analyses were carried out on 176 isolates using HPLC with diode array detection as reported by Frisvad and Thrane (1987) and modified by Houbraken et al. (2012). Isolates were grown on both CYA and YES. Five plugs taken from each agar medium were pooled into the same vial and extracted with 0.75 ml of a mixture of ethyl acetate/dichloromethane/methanol (3:2:1) (v/v/v) with 1% (v/v) formic acid.

2.6. Aflatoxin analysis of samples

Aflatoxin analysis was carried out on kernels, based on the method of Stroka et al. (2000) as follows.

2.6.1. Clean-up

Samples (25 g) of finely ground kernels were added to NaCl (2 g) and extracted with methanol: water solution (100 ml; 8:2, v/v) for 3 min at high speed (10,000 rpm) using an Ultra-Turrax homogenizer (Polytron, Switzerland). The homogenized solution was filtered through Whatman No. 2 filter paper and Whatman A-H glass microfiber filter (Whatman, UK). The filtrate (10 ml) was diluted in phosphate buffered saline (60 ml) and applied to an Aflatest WB immunoafinity column (Vicam, USA) at a flow rate of 2–3 ml/min. The column was then washed with distilled water (30 ml) and aflatoxins eluted with methanol (1250 μl) and diluted with Milli Q water up to 3 ml.

2.6.2. Chromatographic conditions

A Shimadzu LC-10VP HPLC system (Shimadzu, Japan) was used with a fluorescence detector set at 362 nm excitation and 455 nm emission for aflatoxins G_1 and G_2 and 425 nm emission for aflatoxins B_1 and B_2. A Shimadzu CLC G-ODS (4×10 mm) guard column and Shimadzu Shimpack (4.6×250 mm) column were employed. The mobile phase used was water: acetonitrile: methanol (6:2:3, v/v/v) containing KBr (119 mg/l) and nitric acid (4 M, 350 μl/l). The flow rate was 1 ml/min. A post-column derivatization of aflatoxins B_1 and G_1 was performed with bromine using a KobraCell (R-Biopharm Rhône Ltd, UK). The injection volume was 100 μl.

2.6.3. Methodology validation

Three different levels of contamination were tested to determine the recovery of aflatoxins on brazil nuts, and tests carried out in triplicate. Brazil nuts kernels were spiked with total aflatoxin at levels of

Table 1
Frequency of occurrence, mean and variation in the level of infection of brazil nut kernel by fungi in rainforests, processing plants, street markets and supermarkets.

Processing stage õ(no of samples examined)	Rainforests (59)			Processing plants (40)			Street markets (53)			Supermarkets (21)		
Mean a_w (range)	0.950 (0.576–0.997)			0.695 (0.316–0.984)			0.814 (0.444–0.994)			0.482 (0.273–0.597)		
Fungi	FO (%)	AI (%)	RI (%)	FO (%)	AI (%)	RI (%)	FO (%)	AI (%)	RI (%)	FO (%)	AI (%)	RI (%)
Aspergillus arachidicola	0	ND	ND	2.5	0.05	ND-2	0	ND	ND	0	ND	ND
A. bombycis	0	ND	ND	0	ND	ND	1.9	0.04	ND-2	0	ND	ND
A. caelatus	32.2	4.5	ND-98	35.0	3.4	ND-36	45.3	9.3	ND-100	23.8	0.6	ND-4
A. flavus	57.6	6.1	ND-60	67.5	12.5	ND-90	58.5	9.5	ND-84	66.6	3.7	ND-24
A. nomius	35.6	7.2	ND-96	47.5	6.8	ND-48	49.0	5.4	ND-96	23.8	0.7	ND-6
A. pseudotamarii	3.4	0.07	ND-2	5	0.1	ND-2	0	ND	ND	0	ND	ND
A. tamarii	16.9	2.5	ND-54	50	5.1	ND-40	56.6	8.7	ND-70	42.8	2.7	ND-18
Other *Aspergillus* section *Flavi* species	5.1	0.1	ND-4	10	0.2	ND-2	19	0.4	ND-4	9.5	0.2	ND-2

FO = frequency of occurrence % (number of samples that contained a fungal species/total of samples evaluated); AI = average of infection (sum of infection level/total number of samples); RI = range of infection % (range of infected kernels in a sample); and ND = not detected.

0.50, 5.0 and 14.6 μg/kg. Detection and quantification limits were estimated according to Eurachem Guides (1998).

3. Results

3.1. Fungal infection in brazil nuts and shells

Fungi belonging to *Aspergillus* section *Flavi* were found in many samples of brazil nuts and shells collected in the rainforest, processing plants, street markets and supermarkets. The frequency of occurrence, the average infection rate, the range of infection and the water activity of the samples are given in Table 1 (kernels) and Table 2 (shells). Seven species were identified using morphological and physiological characteristics, extrolite production and β-tubulin sequences (Table 3). A few other *Aspergillus* section *Flavi* strains were isolated but were not identified to species. Samples of brazil nut kernels from the rainforest were mostly microbially unstable, averaging 0.95 water activity (a_w), whilst those from supermarkets were stable, averaging 0.48 a_w. A few samples from the rainforest were much drier, as low as 0.58 a_w, indicating that they had been on the ground for long periods, but most were higher than 0.90 a_w. Some samples taken from factories before being processed were higher than 0.90 a_w, and may have been stored in warehouses for some days before being dried. After processing, all samples analysed were below 0.70 a_w. Samples from street markets in the Amazon region varied from 0.44 to 0.99 a_w, with an average of 0.81 a_w. All shelled samples from supermarkets in São Paulo State were lower than 0.60 a_w whilst unshelled samples were lower than 0.70 a_w. Most shelled samples were packaged in moisture proof packages.

From all samples, 4172 isolates of *Aspergillus* section *Flavi* were obtained (Table 3). Although in some samples, the frequency of occurrence of *A. flavus* was higher than *A. nomius*, the latter was the most common species found (1235 isolates) which amounted to 30% of the total potentially aflatoxigenic fungi. This species is of concern since 100% of all isolates produced aflatoxins B_1, B_2, G_1 and G_2. *A. flavus* was almost equally common (1212 isolates) but 46% of isolates produced aflatoxins (B_1 and B_2) and 31% produced cyclopiazonic acid. Flavimin was found in all 45 isolates of *A. flavus* tested. Aflavinin was found in 25 out of 59, 17 out of 45 and 2 out of 5 isolates of *A. nomius*, *A. flavus* and *A. pseudotamarii*, respectively. This metabolite was not found in *A. caelatus* and *A. tamarii*.

A. arachidicola was isolated from two samples of nuts before processing and one of shell from a street market whilst *A. bombycis* was found in both kernel and shell of one sample from a street market. Both species produced B and G aflatoxins. *A. pseudotamarii*, which produced only aflatoxin B_1, was found in three samples of nuts from the rainforests, one sample of shell from a street market and one sample of shell from a processing plant. *A. tamarii* was isolated from most samples. Although this species does not produce aflatoxins, all isolates tested produced cyclopiazonic acid.

The average percentages of infection by *A. flavus* and *A. nomius* in samples of brazil nuts from the rainforests was 6.1 and 7.2%, from processing plants, 12.5 and 6.8%, from street markets, 9.5 and 5.4% and from supermarkets 3.7 and 0.75, respectively. These averages are not high, however the highest levels of infection for these two species in samples from the rainforest were 60 and 96%, from processing plants 90 and 48%; from street markets 84 and 96% and from supermarkets 24 and 6%, respectively.

Samples collected from the ground in the rainforest were already infected with aflatoxigenic fungi. Infection levels were similar in samples from Amazon street markets and processing plants. Samples from supermarkets in São Paulo State showed lower infection rates. The fungal species found in kernels were also found in shells although not at the same frequency.

Table 2
Frequency of occurrence, mean and variation in the level of infection of brazil nut shell by fungi in rainforests, processing plants, street markets and supermarkets.

Stage of brazil nut chain (no of samples examined)	Rainforests (59)			Processing plants (21)			Street markets (31)			Supermarkets (4)		
Mean a_w (range)	0.943 (0.556–0.999)			0.745 (0.474–0.999)			0.862 (0.552–0.999)			0.518 (0.430–0.649)		
Fungi	FO (%)	AI (%)	RI (%)	FO (%)	AI (%)	RI (%)	FO (%)	AI (%)	RI (%)	FO (%)	AI (%)	RI (%)
Aspergillus arachidicola	0	ND	ND	0	ND	ND	3.2	0.06	ND-2	0	ND	ND
A. bombycis	0	ND	ND	0	ND	ND	3.2	0.06	ND-2	0	ND	ND
A. caelatus	27.1	3.2	ND-86	66.6	7.3	ND-36	67.7	9.7	ND-100	75	3	ND-8
A. flavus	45.8	4.2	ND-40	85.7	14.8	ND-62	80.6	9.6	ND-48	75.0	10.5	ND-26
A. nomius	38.9	6.5	ND-92	95.2	20.8	ND-82	77.4	12.6	ND-58	75.0	8.5	ND-16
A. pseudotamarii	3.4	0.1	ND-4	0	ND	ND	0	ND	ND	0	ND	ND
A. tamarii	22.0	1.3	ND-14	66.6	16.2	ND-58	64.5	8.8	ND-46	100	10	ND-14
Other *Aspergillus* section *Flavi* species	6.7	0.2	ND-4	9.5	0.3	ND-6	12	0.4	ND-6	7.5	3	ND-8

FO = frequency of occurrence % (number of samples that contained a fungal species/total of samples evaluated); AI = average of infection (sum of infection level/total number of samples); RI = range of infection % (range of infected shells in a sample); and ND = not detected.

Table 3
Percentage of isolates of *Aspergillus* section *Flavi* producing aflatoxins and other polyphasic characteristics.

Species	No isolates	% Aflatoxin producers (type of aflatoxins produced)	Extrolites other than aflatoxins	GenBank no	Conidium colour on CYA 25 °C/7 d	Reverse on AFPA 25 °C/7 d
A. arachidicola	5	100 (B_1 B_2 G_1 and G_2)	Aflavinin, kojic acid, miyakamides, parasiticolide, tenuazonic acid	JX157587	Olive to olive brown	Bright orange
A. bombycis	2	100 (B_1 B_2 G_1 and G_2)	Not analysed	JX157588	Yellow green	Bright orange
A. caelatus	810	0	Kojic acid, tenuazonic acid, specific alkaloids (same as in *A. pseudotamarii*)	JX157591	Yellow green	Yellow/orange
A. flavus	1212	46 (B_1 and B_2)	Flavimin[a], cyclopiazonic acid (CPA), kojic acid, paspaline, a versicolorin, O-methylsterigmatocystin	JX157586	Yellow green, some isolates with large, black sclerotia	Bright orange
A. nomius	1235	100 (B_1 B_2 G_1 and G_2)	Aflavinin, kojic acid	JX157589	Yellow green, bullet shape sclerotia. Colonies very floccose.	Yellow/orange
A. pseudotamarii	10	100 (B_1)	Aflavinin, kojic acid, specific alkaloids (same as in *A. caelatus*)	JX157592	Brown	Red dark brown
A. tamarii	847	0	CPA, ditryptophenaline, kojic acid	JX157590	Yellow brown to dark green	Red dark brown
Other *Aspergillus* section *Flavi* species	51	45 (B and G); 6 (B); 20 (none); 29 (not tested)	Not analysed	Not analysed		
Total of isolates	4172					

[a] A diketopiparazine that is unique to *Aspergillus flavus*. The exact structure of this alkaloid is not yet known.

3.2. Aflatoxin assays

Using the methodology described above, recovery of total aflatoxins were 83.9, 85.3 and 85.0% for spiking levels of 0.50, 5.0 and 14.6 μg/kg, respectively. The detection limit for total aflatoxins was 0.05 μg/kg and the quantification limit was 0.25 μg/kg.

The incidence of aflatoxins in brazil nuts at different processing stages is shown in Table 4. The average level of total aflatoxins in samples from the rainforests was 0.7 μg/kg, from Amazon street markets 6.3 μg/kg, from processing plants before and after sorting 8.0 and 0.1 μg/kg; and from supermarkets 0.24 μg/kg. However, much higher levels of total aflatoxins were seen in some samples: from the rainforests, 18.1 μg/kg, from street markets 151 μg/kg, from samples before and after processing, 80.63 and 0.91 μg/kg and from supermarkets 0.98 μg/kg. Although some nuts arrived at processing plants with high aflatoxin levels, manual or mechanical sorting of bad nuts produced final products with less than 1 μg/kg total aflatoxins. Drying at 60 °C for 30 to 36 h followed by sorting eliminated 98% of total aflatoxin on average.

Table 5 shows fungal infection and water activities of samples with high aflatoxin. The highest level of aflatoxins in brazil nuts was from samples sold in the street markets in the Amazon. The aflatoxin content of one unshelled sample was 151 μg/kg, whilst one shelled sample contained 140 μg/kg. These samples were ready to be eaten and posed a risk to consumers. Often the nuts sold in such markets are of low quality and price, and may have been rejected by processors. The shelled sample had low fungal infection (4% *A. tamarii*) and no aflatoxigenic fungi could be recovered; the reduced a_w (0.569) indicated that the sample had already dried, reducing the levels of viable fungi. Fungi with potential to produce aflatoxin rarely grow below 0.80 a_w.

Table 4
Aflatoxins (μg/kg) incidence in brazil nut kernels in rainforests, processing plants, street markets and supermarkets.

Stage of brazil nut chain	Aflatoxin B_1	Aflatoxin B_2	Aflatoxin G_1	Aflatoxin G_2	Total aflatoxins
Amazon rainforests (56 samples)					
Mean	0.31	<LOD	0.39	<LOD	0.70
Median	<LOD	<LOD	<LOD	<LOD	<LOD
Range	<LOD–9.20	<LOD–0.19	<LOD–8.60	<LOD–0.24	<LOD–18.10
No of positive samples[a]	8 (14%)	2 (4%)	8 (14%)	3 (5%)	8 (14%)
Processing plants before sorting (21)					
Mean	6.08	0.33	1.60	0.03	8.04
Median	0.05	<LOD	<LOD	<LOD	0.05
Range	<LOD–45.44	<LOD–5.61	<LOD–33.34	<LOD–0.63	<LOD–80.63
No of positive samples[a]	8 (38%)	3 (14%)	6 (29%)	1 (5%)	8 (38%)
Processing plants after sorting (19)					
Mean	0.10	<LOD	0.04	<LOD	0.14
Median	0.06	<LOD	<LOD	<LOD	0.07
Range	<LOD-0.31	<LOD	<LOD–0.28	<LOD	<LOD–0.91
No of positive samples[a]	8 (42%)	0	7 (37%)	0	8 (42%)
Street markets (53)					
Mean	3.72	0.06	2.34	0.04	6.26
Median	0.03	0	0	0	0
Range	<LOD–111.52	<LOD–1.84	<LOD–75.00	<LOD–0.73	<LOD–151.14
No of positive samples[a]	11 (21%)	4 (7%)	15 (28%)	6 (11%)	15 (28%)
Supermarkets (21)					
Mean	0.13	0.01	0.11	0.01	0.24
Median	0.05	<LOD	0.01	<LOD	0.11
Range	<LOD–0.58	<LOD–0.04	<LOD–0.35	<LOD–0.04	<LOD–0.98
No of positive samples[a]	7 (33%)	0	9 (43%)	1 (5%)	9 (43%)
Limit of detection (LOD)	0.02	0.01	0.01	0.01	0.05
Limit of quantification (LOQ)	0.1	0.05	0.05	0.05	0.25

[a] Higher than LOQ.

Table 5
Brazil nut samples with highest aflatoxin (μg/kg) and aflatoxigenic fungi incidence at each stage of brazil nut production.

Stage of brazil nut chain	Aflatoxins (μg/kg)					% infection of fungi	Water activity
	B_1	B_2	G_1	G_2	Total		
Rainforests (in-shell sample no 117)	9.20	0.19	8.60	0.12	18.10	2% *A. flavus* 96% *A. nomius* 2% *A. tamarii*	0.992
Processing plants before sorting (in-shell sample no 52)	66.30	5.61	<LOD	<LOD	71.91	90% *A. flavus* 8% *A. nomius* 2% *A. caelatus* 6% *A. tamarii*	0.667
Processing plants before sorting (in-shell sample no 171)	45.44	1.22	33.34	0.63	80.63	4% *A. flavus* 8% *A. nomius* 2% *A. arachidicola* 4% *A. tamarii* 2% other aspergilli producer of B and G aflatoxins	0.929
Street markets (shelled sample no 17)	111.52	1.04	26.27	0.73	139.56	4% *A tamarii*	0.569
Street markets (in-shell sample no 259)	73.80	1.84	75.00	0.50	151.14	8% *A. flavus* 10% *A. nomius* 28% *A. tamarii*	0.980
Supermarkets (shelled sample no 197)	0.58	0.03	0.34	0.03	0.98	14% *A. flavus* 18% *A. tamarii*	0.586

4. Discussion

Species of *Aspergillus* isolated from brazil nuts and capable of producing aflatoxins were *A. flavus*, *A. nomius*, *A. bombycis* and *A. arachidicola*. These species have been reported from unshelled and shelled brazil nuts previously (Olsen et al., 2008; De Mello and Scussel, 2007; Gonçalves et al., 2012). *A. pseudotamarii*, a producer of aflatoxin B_1, was also isolated in the present study and is reported for the first time from this substrate. Lopez Castrillon and Purchio (1988) reported *A. parasiticus* from brazil nuts, but that report likely referred to *A. nomius*, described in the year before (Kurtzman et al., 1987). In agreement with more recent reports (Olsen et al., 2008; Gonçalves et al., 2012), *A. parasiticus* was not found in the present study.

Among the five aflatoxin producing species identified in this study, *A. nomius* and *A. flavus* cause the greatest concern because of their high level of occurrence in brazil nuts: 30% and 29%, respectively. All isolates of *A. nomius* produced aflatoxins B_1 and G_1 and 46% of *A. flavus* isolates produced aflatoxins B_1 and B_2. Although Olsen et al. (2008) reported *A. nomius* as a source of aflatoxins in brazil nuts, only three strains of *A. nomius* were isolated (among 22 strains of *Aspergillus* section *Flavi*). In the present study 1235 strains of *A. nomius* were isolated among 4172 strains of *Aspergillus* section *Flavi*, confirming that *A. nomius* is indeed a major source of aflatoxins in brazil nuts.

Aflatoxigenic fungi were found throughout the brazil nut chain. According to Johnsson et al. (2008), the aflatoxigenic fungal growth and aflatoxin production increases rapidly between 40 to 90 days after collection of nuts and arrival at the processing plant for final drying.

Brazil nuts are an extractivist product, so good agricultural practices for cultivated crops do not apply. Pods can remain in contact with moist soil for long periods before being collected. As seen in Tables 1 and 2, water activities of brazil nuts both kernel and shell were often very high and infections by fungi with the potential to produce aflatoxin was over 90%. Sorting in processing plants after shelling was shown here to be a very effective way to decrease aflatoxin content in brazil nuts.

The Code of Practice for handling brazil nuts published by the CAC (Codex Alimentarius Commission) (2010b) recommends that brazil nuts should be dried to a safe moisture level (less than 0.7 a_w) within 10 days after collection. Brazil nuts must be dried in the extractivist communities or transported and dried in processing plants. In practice, rapid drying is not always possible because collection of brazil nuts in the Amazon region depends on climatic and logistic conditions. However, we have shown that a safe product can be produced by an effective sorting system in processing plants. On the other hand, a better surveillance system is needed for brazil nuts sold in street markets in the Amazon region.

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