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Biodiversity of mycobiota throughout the Brazil nut supply chain: From rainforest to consumer

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ABSTRACT

A total of 172 Brazil nut samples (114 in shell and 58 shelled) from the Amazon rainforest region and São Paulo state, Brazil was collected at different stages of the Brazil nut production chain: rainforest, street markets, processing plants and supermarkets. The mycobiota of the Brazil nut samples were evaluated and also compared in relation to water activity. A huge diversity of *Aspergillus* and *Penicillium* species were found, besides *Eurotium* spp., *Zygomycetes* and dematiaceous fungi. A polyphasic approach using morphological and physiological characteristics, as well as molecular and extrolite profiles, were studied to distinguish species among the more important toxigenic ones in *Aspergillus* section *Flavi* and *A.* section *Nigri.* Several metabolites and toxins were found in these two sections. Ochratoxin A (OTA) was found in 3% of *A. niger* and 100% of *A. carbonarius.* Production of aflatoxins B and G were found in all isolates of *A. arachidicola, A. bombycis, A. nomius, A. pseudocaelatus* and *A. pseudonomius,* while aflatoxin B was found in 38% of *A. flavus* and all isolates of *A. pseudotamarii.* Cyclopiazonic acid (CPA) was found in *A. bertholletius* (94%), *A. tamarii* (100%), *A. caelatus* (54%) and *A. flavus* (41%). Tenuazonic acid, a toxin commonly found in *Alternaria* species was produced by *A. bertholletius* (47%), *A. caelatus* (77%), *A. nomius* (55%), *A. pseudonomius* (75%), *A. arachidicola* (50%) and *A. bombycis* (100%). This work shows the changes of Brazil nut mycobiota and the potential of mycotoxin production from rainforest to consumer, considering the different environments which exist until the nuts are consumed.

1. Introduction

Brazil nuts are one of the most important products extracted from the Amazon rainforest region. Trees of *Bertholletia excelsa* grow wild, reaching up to 60 m, take 12 years to bear fruit and may live up to 500 years. The Amazon rainforest has multiple ecosystems with a huge biodiversity, which plays an important role in the global weather balance. The equatorial climate is hot and humid, with an average temperature of 26 °C and relative humidity of 80–95%. Brazil nut production is considered totally organic and environmentally correct, since no chemical products are used to control pests and weeds and nor is there the need for fertilizers. It also favors a unique biodiversity of fungal species different from those found in cultivated areas.

Studies on the presence of fungi and aflatoxins in Brazil nuts have been investigated elsewhere (Arrus et al., 2005; Baquião et al., 2012, 2013; Calderari et al., 2013; Gonçalves et al., 2012; Iamanaka et al., 2014; Massi et al., 2014). All of these studies have shown the high occurrence of *Aspergillus* section *Flavi* in Brazil nut samples. However, few studies have been carried out on the changes of Brazil nut mycobiota from rainforest to consumer, considering the different environments which exist until the nuts are consumed.

In the studies on Brazil nut mycobiota, the most commonly isolated species were *Aspergillus flavus, A. nomius, A. pseudonomius, A. niger, A. tamarii, Penicillium glabrum, P. citrinum, Penicillium* spp., *Rhizopus* spp., *Fusarium oxysporum, Fusarium* spp., *Phialemonium* spp., *Phaeoacremonium* spp, among others (Calderari et al., 2013; Gonçalves et al., 2012; Olsen et al., 2008; Freire et al., 2000; Bayman et al., 2002; Reis et al., 2012) and *A. bertholletius* a new species described recently, belonging to *A.* section *Flavi* (Taniwaki et al., 2012). More recently a new species of *Penicillium* named

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P. excelsum was also isolated from Brazil nuts and its ecosystem (Taniwaki et al., 2015).

Over the last few decades, molecular studies have proven to be a valuable tool for identification of fungi. The application of molecular techniques has helped to overcome problems of the traditional methods and have revealed the existence of a much larger number of species than was known previously, many of them not yet identified or formally accepted. Besides that, the molecular identification is often faced with the limitation of sequences deposited in databases such as NCBI and MycoBank, which cannot contemplate all Brazilian fungal biodiversity. Therefore, the aim of this research was to evaluate the mycobiota of Brazil nuts from the Amazon rainforest to consumer, using traditional methods for identification and, when available, molecular techniques and production of extrolites from the isolates as a tool for species identification. Additionally, the potential for mycotoxin and other metabolite production was analyzed for species in *A.* section *Flavi* and *A.* section *Nigri*.

2. Materials and methods

Sampling. A total of 172 Brazil nut samples (114 in shell and 58 shelled), each of approximately 2 Kg, was collected at different stages of: rainforest (57 samples in pods), street markets (54 samples, of which 32 were in shell and 22 shelled), processing at the manufacturing plants (40 samples, of which 21 in shell and 19 shelled) and supermarkets (21 samples, of which 4 in shell and 17 shelled). In the Amazon rainforest which corresponds to Amazonas and Pará states, around 5 pods of Brazil nuts were collected from the ground close to *Bertholletia excelsa* trees. Each pod contained 12 to 20 Brazil nuts inside. At the street markets and supermarkets, in the Amazon region and São Paulo state, respectively, Brazil nut samples were purchased in shell and shelled. At the processing plants, Brazil nut samples in shell and shelled were collected at different steps: arrival of Brazil nuts from the rainforest, storage, before drying, after drying, shelling, before sorting, after sorting and at the packaging area. Each sample was placed in a plastic bag, and kept inside an icebox during transport from the collecting place to the laboratory. Samples from rainforest and processing plants were collected from March to May, while from street markets and supermarkets during the whole year.

Fungal isolation from Brazil nut samples. The pods at the rainforest stage were opened using a machete to get the in shell Brazil nuts. From each of the 172 samples (114 in shell and 58 shelled), approximately 100 g were taken randomly and, after all the in shell samples were broken using a manual opener in order to separate the shell and kernel, they were disinfected by immersion in 0.4% sodium hypochlorite solution for 2 min. Then fifty pieces of each kernels and shells were sampled randomly and plated onto Dichloran 18% Glycerol agar (DG18), according to the methodology of Pitt and Hocking (Pitt and Hocking, 2009). Plates were incubated at 25 °C for 5 days. After incubation, the plates were examined and all the fungal species were first isolated in Petri plates containing Czapek Yeast Autolysate (CYA) agar to be later identified by specific protocols for each genus.

Morphological examination. The isolated fungi were grown in the CYA and malt extract agar (MEA). The genus *Penicillium* was identified according to Pitt (Pitt, 2000) and Samson et al. (Samson et al., 2010), and the identification of genera *Aspergillus* and *Eurotium* was performed according to Klich (Klich, 2002), Pitt and Hocking (Pitt and Hocking, 2009) and Samson et al. (Samson et al., 2010). The other fungi were identified according to descriptions of Pitt and Hocking (Pitt and Hocking, 2009) and Samson et al. (Samson et al., 2010), supplemented with other sources when necessary.

Isolated *Aspergillus* sp. were inoculated at 3 points in the CYA and MEA plates and incubated for 7 days at 25 °C. Its teleomorphic state *Eurotium* sp. was cultivated in Czapek yeast extract agar with 20% sucrose (CY20S) for 14 days at 25 °C. The *Penicillium* species were grown by following the conditions above, and were also inoculated in CYA at 5 °C and 37 °C. After the cultivation period, the diameters of the colonies were measured and the macro and microscopic features observed in each culture medium were used for species identification.

Extrolite analysis. Extrolite analyses were carried out on representative isolates of *A.* section *Nigri* (79), *A.* section *Flavi* (105) and *Penicillium* (23), using HPLC with diode array detection as reported by Frisvad and Thrane (Frisvad and Thrane, 1987) and modified by Houbraken et al. (Houbraken et al., 2012). The extracts were also analyzed by ultra high performance liquid chromatography (UHPLC) with a maXis 3G Q-TOF orthogonal mass spectrometer (Bruker Daltronics, Bremen, Germany) as described by Klitgaard et al. (Klitgaard et al., 2014), in order to confirm our new reports of production of cyclopiazonic acid, tenuazonic acid, asperfuran, ditryptophenaline, parasiticolide A and miyakamides by some *Aspergillus* species. In addition, the retention time of the compounds was compared to authentic standards (Klitgaard et al., 2014; Kildgaard et al., 2014)]. 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 using 50 min ultrasonication. The solvents were evaporated and the dry extract re-dissolved in 0.4 ml methanol. After filtration the extract was ready for HPLC analysis.

Molecular analysis. In previous studies (Gonçalves et al., 2012; Massi et al., 2014), random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) were used to access the genetic variation among 105 *A.* section *Flavi* isolated from Brazil nut kernels and Brazil nut shells. Representatives of both RAPD and AFLP groups were selected and sequenced by the authors. In the present study, we reanalyzed partial β-tubulin gene sequences to identify or confirm isolate taxonomy taking into account the novelties of the species accepted in *A.* section *Flavi* provided by Samson et al. (Samson et al., 2014).

The sequences of type or neotype strains of all recognized species in *A.* section *Flavi*, were aligned with the published sequences of the β-tubulin gene deposited in the NCBI database (http://www.ncbi.nlm.nih.gov/), using Clustal W (Thompson et al., 1994). The phylogenetic tree was inferred using the neighbour-joining methods (Saitou and Nei, 1987) and the software package MEGA5 (Saitou and Nei, 1987).

Because only molecular data are efficient enough to discriminate between *A. niger* and *A. welwitschiae* (formally *A. awamori*) a total of 20 isolates were randomly selected and subjected to sequencing of a portion of the β-tubulin gene. The methodologies used for DNA extraction, PCR amplification and sequencing, were the same as those described in detail by Gonçalves et al. (Gonçalves et al., 2012). The sequences obtained here were compared, by BLAST and phylogenetic analyses, to those of *A. niger* and *A. welwitschiae* provided by Varga et al. (Varga et al., 2011).

Water activity. Water activity was determined in all kernel and shell samples using Aqualab, Series 3TE equipment (Decagon, USA) at 25 ± 0.1 °C, in triplicate.

3. Results

Mycobiota of Brazil nuts. A huge diversity of fungi was found in several samples throughout the Brazil nut production chain, 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 kernel and shell samples are shown in Tables 1 and 2, respectively. The water activity average in samples of Brazil nut kernels ranged from 0.956 in the rainforest to 0.482 in the supermarkets and a succession of different groups of fungi was observed along the Brazil nut production chain. In the rainforest some kernel samples showed a_W as low as 0.765 indicating that they were on the ground for a long period and became dry. Even being collected in the period of harvest (March to May), some samples in the rainforest had already dried. However, most samples (94%) had a_W higher than 0.90, in which dematiaceus hyphomycetes predominated in kernel and shell samples while *Syncephalastrum racemosum* also had high infection in kernel samples. These groups of fungi prefer substrates with high a_W. In samples with lower a_W *A.* section *Flavi*, *A.* section *Nigri* and species of *Penicillium* especially *P. citrinum* were the most commonly found in kernel and shell samples, showing that there is a succession of diverse fungi that infect Brazil nuts in the rainforests depending on the water availability. In street markets, samples showed variation in a_W from 0.444 to 0.994, with an average of 0.814; 51% with a_W higher than 0.90 and 21% lower than 0.60. Samples from processing plants showed an average of 0.695 a_W which was 0.984 at the beginning of the process which includes samples that had just arrived from the rainforest and samples before drying; and 0.316 at the end of the process, which were ready to eat samples taken from the packaging area. The population of *Eurotium* species increased and took the place of dematiaceus hyphomycetes. *A.* section *Flavi* and *A* section *Nigri* continued appearing in several samples, mainly the ones that had not passed through the sorting process. In the supermarket samples which had a_W lower than 0.597, dematiaceus hyphomycetes decreased greatly although *S. racemosum* still appeared in some samples. *A.* section *Flavi* continued being found in some samples, although at a lower range of infection.

A. section *Flavi* and *A.* section *Nigri* were the most common

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

	Processing stage	Rainforest (57)			Street market (54)			Processing (40)			Supermarket (21)		
	Kernel mean a_w (range)	0.956 (0.765–0.997)			0.814 (0.444–0.994)			0.695 (0.316–0.984)			0.482 (0.273–0.597)		
	Fungi	FO(%)	AI (%)	RI (%)	FO(%)	AI (%)	RI (%)	FO(%)	AI (%)	RI (%)	FO(%)	AI (%)	RI (%)
Aspergillus section *Flavi*	*Aspergillus arachidicola*	1.75	0.02	0–2	0	0	0	5	0.05	0–48	0	0	0
	A. bertholletius	0	0	0	11.11	0.61	0–48	5	0.05	0–2	0	0	0
	A. bombycis	0	0	0	1.85	0.02	0–2	0	0	0	0	0	0
	A. caelatus	31.58	2.30	0–98	46.30	4.65	0–100	35	1.70	0–36	23.81	0.29	0–4
	A. flavus	56.14	2.84	0–44	59.26	4.69	0–84	67.5	6.25	0–90	57.14	1.76	0–24
	A. nomius	33.33	3.70	0–96	50.00	2.59	0–90	47.5	3.45	0–48	23.81	0.33	0–6
	A. pseudonomius	0	0	0	1.85	0.07	0–8	0	0	0	0	0	0
	A. pseudotamarii	3.51	0.05	0–4	0	0	0	2.5	0.03	0–2	0	0	0
	A. tamarii	15.79	0.95	0–54	55.56	4.17	0–70	52.5	2.45	0–40	42.86	1.71	0–18
Aspergillus section *Nigri*	*A. carbonarius*	0	0	0	1.85	0.02	0–2	0	0	0	0	0	0
	A. ibericus	8.77	0.16	0–6	1.85	0.02	0–2	15	0.83	0–38	4.76	0.10	0–2
	A. luchuensis	0	0	0	1.85	0.02	0–2	2.5	0.05	0–2	0	0	0
	A. niger	64.91	3.70	0–72	44.44	1.69	0–34	52.5	3.45	0–72	38	1.24	0–20
	A. welwitschiae	0	0	0	7.41	0.09	0–4	3	0.03	0–2	10	0.10	0–2
	Other *A.* section *Nigri*	75.44	6.89	0–96	35.19	0.89	0–20	55	1.90	0–34	28.57	1.38	0–38
Eurotium and others *Aspergillus*	*A. candidus*	0	0	0	1.85	0.02	0–2	0	0	0	0	0	0
	A. terreus	0	0	0	0	0	0	2.5	0.03	0–2	0	0	0
	Aspergillus spp.	0	0	0	1.85	0.02	0–2	0	0	0	0	0	0
	Eurotium amstelodami	1.75	0.12	0–14	1.85	0.02	0–2	22.5	0.35	0–6	4.76	0.05	0–2
	E. chevalieri	8.77	0.98	0–100	11.11	0.15	0–4	30	2.93	0–100	23.81	0.24	0–2
	E. rubrum	0	0	0	1.85	0.02	0–2	0	0	0	0	0	0
	Eurotium spp.	10.53	0.33	0–14	38.89	5.48	0–100	45	8.78	0–98	57.14	3.67	0–42
Penicillium and *Paecilomyces*	*Penicillium brasilianum*	0	0	0	0	0	0	0	0	0	100	1	0–2
	P. chrysogenum	0	0	0	1.85	0.02	0–2	0	0	0	0	0	0
	P. citrinum	63.16	20.49	0–100	48.15	11.81	0–100	42.5	6.45	0–100	9.52	0.71	0–16
	P. excelsum	14.04	1.40	0–100	1.85	0.87	0–94	0	0	0	0	0	0
	P. fellutanum	0	0	0	0	0	0	2.5	0.03	0–2	0	0	0
	P. glabrum	1.75	0.04	0–4	0	0	0	0	0	0	0	0	0
	P. islandicum	1.75	0.04	0–4	0	0	0	7.5	0.20	0–6	0	0	0
	P. paxilii	1.75	0.04	0–4	1.85	0.02	0–2	0	0	0	0	0	0
	P. raistrickii	1.75	0.04	0–4	0	0	0	0	0	0	0	0	0
	P. sclerotiorum	1.75	0.02	0–2	0	0	0	0	0	0	0	0	0
	P. steckii	0	0	0	1.85	0.02	0–2	0	0	0	0	0	0
	P. variabile	0	0	0	3.70	0.78	0–48	0	0	0	0	0	0
	Penicillium spp.	29.82	1.93	0–98	31.48	4.94	0–68	22.5	2.18	0–50	23.81	2.05	0–50
	Paecilomyces spp.	5.26	0.30	0–100	14.81	0.52	0–16	7.5	0.08	0–2	4.76	0.05	0–2
Zygomycetes	*Absidia corymbifera*	3.51	0.33	0–30	1.85	0.04	0–4	2.5	0.08	0–6	0	0	0
	Mucor spp.	1.75	0.81	0–92	7.41	2.43	0–100	0	0	0	0	0	0
	Rhizopus spp.	3.51	0.37	0–60	18.52	0.69	0–22	2.5	0.05	0–4	4.76	0.05	0–2
	Syncephalastrum racemosum	24.56	3.11	0–72	38.89	3.44	0–84	37.5	4.23	0–100	52.38	7.95	0–100
	Other *Zygomycetes*	3.51	0.42	0–36	12.96	2.61	0–78	10	2.20	0–100	4.76	0.10	0–4
Other Fungi	*Cladosporium* spp.	1.75	0.02	0–2	0	0	0	2.5	0.08	0–6	0	0	0
	Trichoderma spp.	3.51	0.05	0–4	1.85	0.02	0–2	2.5	0.05	0–4	0	0	0
	Fusarium sp.	5.26	0.91	0–100	3.70	0.04	0–2	5	0.05	0–2	0	0	0
	Ascomycetes	0	0	0	0	0	0	2.5	0.03	0–2	0	0	0
	Dematiaceus hyphomycetes	42.11	3.11	0–54	37.04	2.57	0–100	22.5	0.70	0–20	14.29	1.90	0–48
	Yeasts	1.75	0.09	0–10	1.85	0.93	0–100	2.5	0.05	0–4	0	0	0

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 beans in a sample).

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

	Processing stage	Rainforest (57)			Street market (32)			Processing (21)			Supermarket (4)		
	Shell mean a_w (range)	0.956 (0.717–0.997)			0.814 (0.444–0.994)			0.695 (0.316–0.984)			0.482 (0.273–0.597)		
	Fungi	FO(%)	AI (%)	RI (%)	FO(%)	AI (%)	RI (%)	FO(%)	AI (%)	RI (%)	FO(%)	AI (%)	RI (%)
Aspergillus section *Flavi*	*Aspergillus arachidicola*	5.26	0.05	0–2	6.25	0.06	0–2	0	0	0	0	0	0
	A. bertholletius	1.75	0.02	0–2	6.25	0.63	0–36	14.29	0.19	0–4	25	0.5	0–4
	A. bombycis	0	0	0	3.13	0.03	0–2	4.76	1.57	0–66	0	0	0
	A. caelatus	24.56	1.65	0–88	65.63	4.81	0–100	76.19	7.29	0–58	50	1.25	0–8
	A. flavus	47.37	2.19	0–40	81.25	4.53	0–48	90.48	7.81	0–64	14.29	5.75	0–26
	A. nomius	40.35	3.30	0–92	78.13	6.44	0–52	80.95	10.71	0–82	75	4.75	0–18
	A. pseudocaelatus	0	0	0	0	0	0	4.76	0.05	0–2	0	0	0
	A. pseudotamarii	0	0	0	3.125	0.06	0–4	4.76	0.05	0–2	0	0	0
	A. tamarii	17.54	0.54	0–14	68.75	4.84	0–46	61.90	5.33	0–42	100	3.75	2–14
Aspergillus section *Nigri*	*A. carbonarius*	0	0	0	3.13	0.03	0–2	0	0	0	0	0	0
	A. ibericus	5.26	0.05	0–4	3.13	0.03	0–2	33.33	0.95	0–14	0	0	0
	A. luchuensis	0	0	0	0	0	0	4.76	0.05	0–2	0	0	0
	A. neoniger	0	0	0	0	0	0	4.76	0.05	0–2	0	0	0
	A. tubingensis	0	0	0	6.25	0.25	0–12	0	0	0	0	0	0
	A. welwitschiae	0	0	0	3.13	0.03	0–2	0	0	0	0	0	0
	A. niger	61	3.44	0–46	40.63	1.81	0–24	61.90	4.95	0–40	100	3	2–18
	Other *A.* section *Nigri*	45.61	3.16	0–66	37.50	0.88	0–10	76.19	4.29	0–26	75	5.75	0–30
Eurotium and other *Aspergillus*	*A. candidus*	0	0	0	0	0	0	0	0	0	0	0	0
	A. terreus	0	0	0	0	0	0	0	0	0	0	0	0
	A. wentii	0	0	0	3.12	0.03	0–2	0	0	0	0	0	0
	A. westerdijkiae	1.75	0.02	0–2	0	0	0	0	0	0	0	0	0
	Aspergillus spp.	1.75	0.16	0–18	0	0	0	0	0	0	0	0	0
	Eurotium amstelodami	1.75	0.11	0–12	0	0	0	14.29	0.43	0–14	0	0	0
	E. chevalieri	8.77	0.32	0–26	0	0	0	47.62	1	0–14	0	0	0
	E. rubrum	0	0	0	0	0	0	4.76	0.10	0–4	0	0	0
	Eurotium spp.	7.02	0.18	0–8	12.50	2.06	0–100	42.86	4.90	0–96	75	2.50	0–14
Penicillium. Paecilomyces and Talaromyces	*Penicillium bilaiae*	0	0	0	3.13	0.03	0–2	0	0	0	0	0	0
	P. citrinum	73.68	17.47	0–100	56.25	7.31	0–100	38.10	4.38	0–48	0	0	0
	P. excelsum	5.26	0.77	0–54	6.25	2.34	0–100	0	0	0	0	0	0
	P expansum	1.75	0.16	0–18	0	0	0	0	0	0	0	0	0
	P. paxilii	1.75	0.02	0–2	0	0	0	0	0	0	0	0	0
	P. raistrickii	3.51	0.11	0–10	0	0	0	0	0	0	0	0	0
	P. sclerotiorum	5.26	0.05	0–2	0	0	0	4.76	0.38	0–16	0	0	0
	P. steckii	0	0	0	0	0	0	4.76	0.05	0–2	0	0	0
	P. variabile	0	0	0	0	0	0	4.76	0.05	0–2	0	0	0
	Penicillium spp.	33.33	2.16	0–100	31.25	2.69	0–52	19.05	1.76	0–28	25	2.75	0–22
	Paecilomyces variotii	1.75	0.04	0–2	0	0	0	0	0	0	0	0	0
	Paecilomyces spp.	7.02	0.21	0–12	18.75	1.09	0–44	4.76	0.10	0–4	0	0	0
	Talaromyces wortmannii	1.75	0.02	0–2	0	0	0	0	0	0	0	0	0
	Talaromyces spp	0	0	0	3.13	0.03	0–2	0	0	0	0	0	0
Zygomycetes	*Absidia corymbifera*	10.53	0.98	0–32	9.38	1.75	0–56	0	0	0	0	0	0
	Mucor sp.	1.75	0.09	0–10	9.38	0.66	0–24	0	0	0	0	0	0
	Rhizopus spp.	7.02	0.18	0–8	9.38	0.38	0–12	23.81	0.76	0–14	0	0	0
	Syncephalastrum racemosum	21.05	1.84	0–86	40.63	1.72	0–30	38.10	2.81	0–46	25	2.75	0–22
	Other Zygomycetes	7.02	1.95	0–100	6.25	0.59	0–32	4.76	0.76	0–32	0	0	0
Others Fungi	*Trichoderma* spp.	10.53	0.51	0–38	46.88	3.94	0–100	28.57	0.62	0–8	0	0	0
	Fusarium sp.	8.77	1.49	0–100	3.13	0.03	0–2	9.52	0.10	0–2	0	0	0
	Ascomycetes	1.75	0.02	0–2	0	0	0	4.76	0.05	0–2	0	0	0
	Dematiaceus hyphomycetes	63.16	9.81	0–100	81.25	15.69	0–100	42.86	7.05	0–100	25	1.50	0–12
	Yeasts	0	0	0	0	0	0	4.76	0.10	0–4	0	0	0

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 beans in a sample).

groups appearing at all stages of the Brazil nut production chain. Ten species of *A.* section *Flavi* could be distinguished from the kernels and shells using the polyphasic identification approach. Fig. 1 shows an overview of genetic diversity of *A.* section *Flavi* isolates from Brazil nuts based on partial β-tubulin gene sequences. *A. flavus, A. nomius, A. caelatus* and *A. tamari* were the most common species found and were present at all stages, while few samples showed the presence of *A. arachidicola, A. bombycis, A. pseudocaelatus, A. pseudonomius* and *A. pseudotamarii*. In kernel samples there was a slight increase in the infection by the main *A.* section *Flavi* species (*A. flavus, A. tamarii, A. caelatus*) indicating the low quality and poor condition of these samples. The new species *A. bertholletius* was found in samples from all stages mainly in the shells. A. *pseudonomius* was only found in kernel samples from street markets.

Seven species of *A.* section *Nigri* were distinguished using molecular or extrolite profiles: *A. carbonarius, A. ibericus, A. luchuensis, A. niger, A. neoniger, A. tubingensis* and *A. welwitschiae*. The most common species found in the collected samples were *A. niger/A. welwitschiae*, which are indistinguishable by morphological and extrolite profiles. As shown in Fig. 2, the β-tubulin gene sequence analysis revealed that both species, *A. niger* and *A. welwitschiae*, are well adapted in Brazil nuts. More studies on *A.* section *Nigri* isolated from Brazil nuts are being carried out.

A huge variety of *Penicillium* species was found, with *P. citrinum* being the most common throughout the Brazil nut production chain. A new species *Penicillium excelsum* described recently (Taniwaki et al., 2015) was found mainly in the samples from the rainforest and street markets. More than ten different species of *Penicillium* could be recognized morphologically and by the

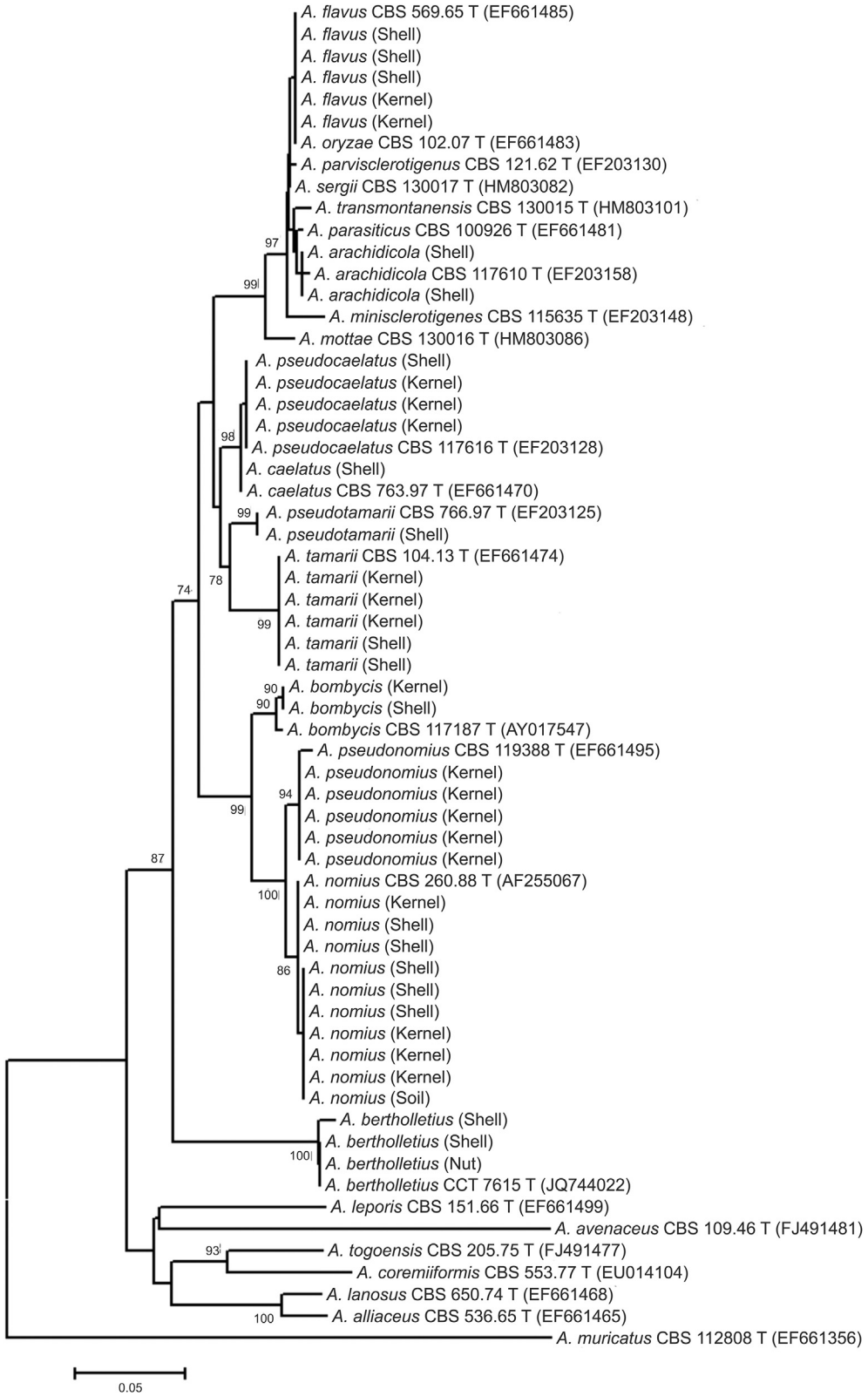

Fig. 1. An overview of genetic diversity of *Aspergillus* section *Flavi* isolates from Brazil nuts based on partial β-tubulin gene sequences. Neighbour joining tree reconstructed from the β-tubulin partial gene sequences aligned with those published sequences of β-tubulin gene of type or neotype strains of all recognized species in *Aspergillus* section *Flavi* deposited in public databases. Numbers at branch nodes refer to bootstrap values (1000 replicates), only values of 70% are shown.

production of their extrolites.

Syncephalastrum racemosum was the most common species among the *Zygomycetes*, being found especially in samples of high a_W. The other isolated fungi were *Absidia corymbifera*, *Nigrospora* sp, *Cladosporium* spp., *Trichoderma* spp, *Fusarium* spp, dematiaceous hyphomycetes and yeasts.

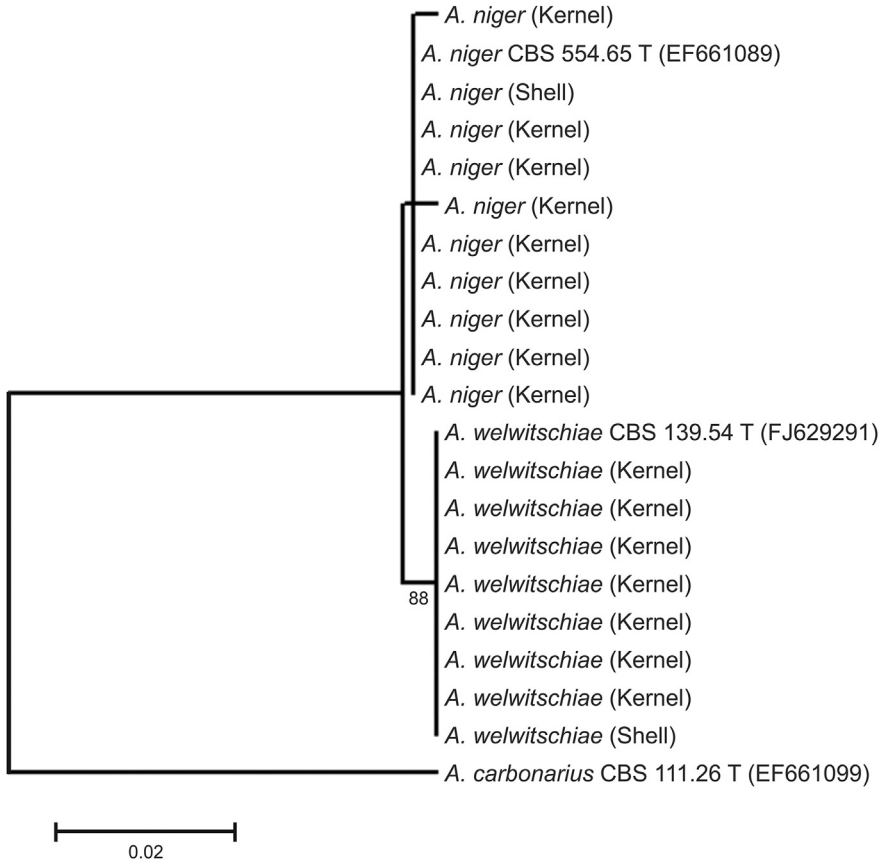

Fig. 2. Neighbour joining tree reconstructed from the β-tubulin gene sequences aligned with sequences of *Aspergillus niger* and *Aspergillus welwitschiae* type strains deposited in public databases. Numbers at branch nodes refer to bootstrap values (1000 replicates).

E. chevalieri (*Aspergillus chevalieri*), *E. rubrum* (*Aspergillus ruber*), *E. amstelodami* (*Aspergillus montevidensis*) and other *Eurotium* species were found in most dried samples and could be distinguished using microscopic characters such as ascopore size and ornamentation. One kernel sample directly obtained from the rainforest had an a_W of 0.942. Another from a processing plant had a reduced a_W of 0.316 but, even so, both were infected with 100% of *E. chevalieri* (*A. chevalieri*). As the latter was a ready to eat sample, it can be seen that this species still survives industrial processing.

Extrolites of the isolates. The extrolite profiles showed themselves to be a very useful tool to distinguish species of *A.* section *Flavi* and *A.* section *Nigri* from each other as presented in Tables 3 and 4, respectively. As can be seen in Table 3, production of aflatoxins B and G were found in all representative isolates of *A. arachidicola, A. bombycis, A. nomius, A. pseudocaelatus* and *A. pseudonomius,* while aflatoxin B was found in 38% of *A. flavus* isolates and all isolates of *A. pseudotamarii.* Cyclopiazonic acid (CPA) was found in most of *A. bertholletius* (94%) and *A. tamarii* (100%) isolates and some of *A. caelatus* (54%) and *A. flavus* (41%). Tenuazonic acid was produced by some isolates of *A. bertholletius* (47%), *A. caelatus* (77%), *A. nomius* (55%), *A. pseudonomius* (75%), *A. arachidicola* (50%) and one isolate of *A. bombycis.* Flavimin and ditryptophenalin were only produced by some isolates of *A. flavus*. Fig. 3 shows the LC-DAD-MS peaks of *Aspergillus bertholletius* extract which confirms the production of CPA and tenuazonic acid by this species. Other species also confirmed the production of these toxins as shown in Table 5.

Ochratoxin A was found in 3% and 100% of isolates of *A. niger* and *A. carbonarius*, respectively but other analyzed species of *A.* section *Nigri* did not produce this toxin. The most common metabolites found in *A. niger* were atromentin, cycloleucomelon, funalenone, kotanin, naphtho-γ-pyrones, pyranonigrin A and tensidol B, while most *A. luchuensis* isolates produced cycloleucomelon, fonsecin, funalenone, naphtho-γ-pyrones, pyranonigrin A and tensidol B. Asperazine was only produced by *A. tubingensis*. *A. welwitschiae* was discriminated from *A. niger* based on molecular identification; the metabolites did not distinguish between these two species. The isolates of *A. ibericus* only produced naphtho-γ-pyrones and pyranonigrin A (Table 4).

The most common extrolites found in *Penicillium citrinum* were: citrinin, citrinadin, perinadine and quinolactacin. *P. sclerotiorum* produced penicillic acid, rotiorin and sclerotiorin while *P. brocae* produced pyranonigrins, phenopyrrozin and spinulosin. In *Talaromyces wortmannii,* rugulovasine and rugulosin were found.

4. Discussion

Because Brazil nuts come from the Amazon rainforest, most common good agricultural practices do not apply during harvesting when pods are falling down from the 30–60 m high trees. The pods can remain in contact with soil for long periods in the humid tropical forest until they are collected. During this period microorganism proliferation will take place and fungal infection may occur because of the porosity of the shells and the pods, or through the action of insects, birds and other physical damage. These factors, in association with the climatic conditions of the region, may favor the infection and propagation of fungi from fruit pod to shells and from shells to kernels (Arrus et al., 2005; Reis et al., 2012; Castrillón and Purchio, 1988).

The infection of Brazil nuts by *A.* section *Flavi* reached 100% in

Table 3
Extrolites produced by the *Aspergillus* section *Flavi* isolated from Brazil nuts (Number of positive strains/Total number of tested strains).

Extrolites	*Aspergillus arachidicola*	*A. bertholletius*	*A bombycis*	*A. caelatus*	*A. flavus*	*A. nomius*	*A. pseudocaelatus*	*A. pseudonomius*	*A. pseudotamarii*	*A. tamarii*
Aflatoxin type B	2/2	0/17	1/1	0/13	13/34	27/27	1/1	4/4	2/2	0/4
Aflatoxin type G	2/2	0/17	1/1	0/13	0/34	27/27	1/1	4/4	0/2	0/4
Aflavinin	1/2	7/17	0/1	0/13	29/34	25/27	1/1	0/4	2/2	0/4
Alcaloid (alkca)	0/2	0/17	0/1	13/13	0/34	0/27	0/1	0/4	2/2	0/4
Aspirochlorine	0/2	0/17	0/1	4/13	1/34	0/27	0/1	0/4	0/2	0/4
Cyclopiazonic acid	0/2	16/17	0/1	7/13	14/34	0/27	0/1	0/4	0/2	4/4
Ditryptophenalin	0/2	0/17	0/1	0/13	21/34	0/27	0/1	0/4	0/2	2/4
Flavimin	0/2	0/17	0/1	0/13	24/34	0/27	0/1	0/4	0/2	0/4
Kojic acid	1/2	17/17	1/1	13/13	14/34	27/27	1/1	4/4	2/2	3/4
Mellamide	1/2	0/17	0/1	0/13	6/34	9/27	0/1	0/4	0/2	0/4
Miyakamide	1/2	0/17	0/1	0/13	7/34	26/27	1/1	0/4	0/2	0/4
Paspalinin	0/2	0/17	0/1	0/13	15/34	4/27	0/1	0/4	0/2	0/4
Scytalone	0/2	0/17	0/1	1/13	0/34	0/27	0/1	0/4	2/2	0/4
Tenuazonic acid	1/2	8/17	1/1	10/13	0/34	15/27	0/1	3/4	0/2	0/4
Ustilaginoidin C	0/2	9/17	0/1	0/13	1/34	0/27	0/1	0/4	0/2	0/4
Versicolorin	1/2	0/17	0/1	0/13	2/34	8/27	0/1	3/4	0/2	0/4

Table 4
Extrolites produced by the *Aspergillus* section *Nigri* isolated from Brazil nuts (Number of positive strains/Total number of tested strains).

Extrolites	*Aspergillus carbonarius*	*A. ibericus*	*A. luchuensis*	*A. niger*	*A. neoniger*	*A. tubingensis*	*A. welwitschiae*
Aflavinin	0/2	0/10	2/5	1/58	1/1	0/1	0/2
Asperazine	0/2	0/10	0/5	0/58	0/1	1/1	0/2
Atrometin	2/2	0/10	0/5	46/58	0/1	1/1	2/2
Atrovenetin	0/2	0/10	0/5	2/58	1/1	0/1	0/2
Cycloleucomelon	2/2	0/10	3/5	46/58	0/1	1/1	0/2
Fonsecin	0/2	0/10	3/5	12/58	0/1	0/1	2/2
Funalenone	0/2	0/10	5/5	58/58	1/1	1/1	2/2
Kotanin	0/2	0/10	0/5	45/58	1/1	0/1	0/2
Naphtho-γ-pyrones	2/2	10/10	5/5	58/58	0/1	1/1	2/2
Nigragilin	0/2	0/10	1/5	0/58	0/1	1/1	0/2
Ochratoxin A	2/2	0/10	0/5	2/58	0/1	0/1	0/2
Cf. Pyranonigrin	0/2	0/10	1/5	11/58	0/1	1/1	2/2
Pyranonigrin A	2/2	10/10	5/5	58/58	1/1	1/1	2/2
Tensidol B	0/2	0/10	5/5	58/58	1/1	0/1	2/2

some kernel and shell samples and these species were found all along the Brazil nut production chain, mainly in the rainforest, street markets and processing places. The samples showing high a_W content (>0.99) were more favorable for dematiaceous fungi and *Zygomycetes* such as *Syncephalastrum racemosum* and *Rhizopus* species than for *Aspergillus* and *Penicillium* species. Brazil nut pods fall to the ground from January to April when the Amazon rainforest environment is very humid due to constant rain. The pods stay in contact with soil and the entry of water into the pods can easily occur from the operculum. This may be the reason why the a_W of some samples was so high. Pods recently fallen down from the trees also have a high a_W. However, some pods can remain on the ground for a long period of time and lose their moisture content, especially during the dry season from May to October when rainfall is reduced. Under warm, humid subtropical or tropical climates in which the growing season is unusually hot and dry, species of *Aspergillus* such as *A. flavus* and *A. nomius* can infect seeds early in the field (Calderari et al., 2013; Marin et al., 1998). Many species of *Fusarium*, as well as some species of *Penicillium*, also infect grain in the field as well as in storage. As a consequence of the different ecophysiological adaptations of fungi, the naturally contaminating mycobiota of food commodities follow a typical succession from the early days of development in the field until the end of storage. *Fusarium*, *Cladosporium* and dematiaceous fungi as found in the present work are typically the predominant field contaminants which are established before harvest, while *Penicillium* and *Aspergillus* tend to predominate during storage. At a_W lower than 0.75 xerophilic fungi like *Eurotium* species are able to grow (Pitt and Hocking, 2009). Fungal growth can be prevented by drying the nuts to a_W below 0.65 and keeping it at this level. However, if the moisture of the stored product increases due to microbial or insect activity, moisture migration or during an increase in environmental relative humidity, other fungal species besides xerophiles begin to grow. For example, the *Eurotium* species have more advantage at a_W 0.80 to 0.85, and many *Penicillium* and *Aspergillus* species begin to grow at a_W above 0.85. *A. flavus* grows well at a_W of 0.94, and *A. niger* is dominant at a_W of 0.98 (Pitt and Hocking, 2009; Marin et al., 1998). Freire et al (Freire et al., 2000) detected *Aspergillus* spp. as the predominant species in Brazil nut shells, while other isolated genera were: *Penicillium, Cunninghamella, Rhizopus, Fusarium* and *Acremonium*.

In the present study, over 50 species of fungi could be identified using polyphasic approaches with morphological examination, extrolite analysis and DNA sequence data. Ten species of *A.* section *Flavi* could be distinguished following the taxonomy for *Aspergillus* section *Flavi* (Varga et al., 2011; Pildain et al., 2008). *A. parasiticus* was not found at any stage of Brazil nut production, as has been shown in other reports (Baquião et al., 2013; Reis et al., 2012; Castrillón and Purchio, 1988). There might be problems in distinguishing between *A. parasiticus* and *A. arachidicola* because these two species are very close molecularly and morphologically and both produce aflatoxins B and G with a lack of CPA production.

Mycotoxins, other than aflatoxins such as CPA were found in *A. bertholletius* (94%), *A. tamarii* (100%), *A. caelatus* (54%) and *A. flavus* (41%). These data differ from Reis et al. (Reis et al., 2012) which did not find CPA production in *A. caelatus* and *A. tamarii*

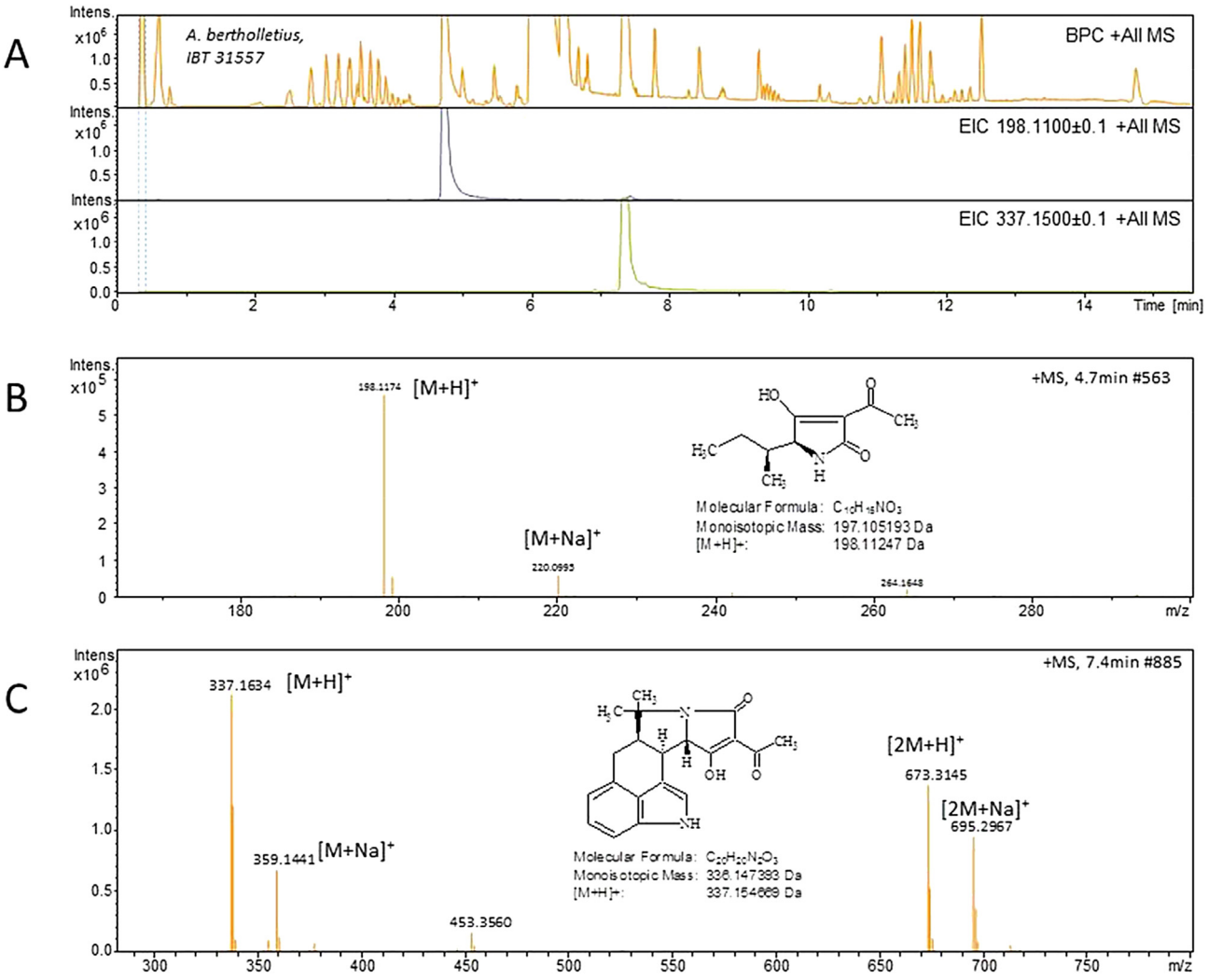

Fig. 3. Chemical analysis of an *Aspergillus bertholletius* extract from cultivation on YES using LC-DAD-MS. **Part A**. Base peak chromatogram (top) of the raw extract scanning m/z in the range from 75 to 1250 Da. Extracted ion chromatogram (middle) targeting $[M+H]^+$ 198.11 Da of tenuazonic acid showing a single major peak. Extracted ion chromatogram (middle) targeting $[M+H]^+$ 337.15 Da of cyclopiazonic acid showing a single major peak. **Part B**. Mass spectrum matching the expected adduct pattern for tenuazonic acid. **Part C**. Mass spectrum matching the expected adduct pattern for cyclopiazonic acid.

Table 5
Compounds that have been positively identified with peaks (such as $[M+H]^+$) in mass spectra generated from LC-DAD-MS analysis of extracts of *A. bertholletius*, *A. bombycis*, *A. caelatus*, *A. flavus*, *A. pseudonomius*, *A. tamarii*, and *A. nomius*.

Compound	Formula	Mass	$[M+H]^+$	*A. bertholletius*	*A. bombycis*	*A. caelatus*	*A. flavus*	*A. pseudonomius*	*A. tamarii*	*A. nomius*
tenuazonic acid	$C_{10}H_{15}NO_3$	197.1052	198.113	Positive	Positive	Positive		Positive		Positive
cyclopiazonic acid	$C_{20}H_{20}N_2O_3$	336.1474	337.1552	Positive		Positive	Positive		Positive	
kojic acid	$C_6H_6O_4$	142.0266	143.0344			Positive	Positive		Positive	
asperfuran	$C_{13}H_{14}O_3$	218.0943	219.1021				Positive			
ditryptophenaline	$C_{42}H_{40}N_6O_4$	692.3111	693.3189				Positive			
parasiticolide A	$C_{26}H_{30}O_8$	470.1941	471.2019					Positive		
miyakamide A	$C_{31}H_{32}N_4O_3$	508.2474	509.2547					Positive		Positive

isolated from Brazil nuts but agree with Peterson et al (Peterson et al., 2000) and Horn et al (Horn et al., 1996) who found CPA production in these two species. Tenuazonic acid, a toxin commonly found in *Alternaria* species was produced by some isolates of *A. bertholletius* (47%), *A. caelatus* (77%), *A. nomius* (55%), *A. pseudonomius* (75%), *A. arachidicola* (50%) and one isolate of *A. bombycis*. Tenuazonic acid is toxic to several animal species, a wide range of plants and microorganisms (Logrieco et al., 2003). The significance of tenuazonic acid in several species of *A.* section *Flavi* deserve further investigation.

The concept of associated mycobiota (Filtenborg et al., 1996) is that every food has its own specific fungal species composition and normally few species are present in a given food commodity. In the case of Brazil nuts more than 50 species were identified using the polyphasic taxonomic approach along the chain. At different stages of Brazil nut production some species or groups of species appeared more frequently. The dominant groups or fungal species were *A.* section *Flavi* (10 species), *A.* section *Nigri* (7 species), *P. citrinum*,

S. racemosum, Rhizopus spp. and dematiaceous hyphomycetes. It can be observed that each fungal species occupies a particular set of niches specified by its ability to adapt to Brazil nut intrinsic parameters associated with extrinsic factors and these are responsible for the profile of the contaminating and dominating mycobiota. This is mostly related to the physiology of fungi to overcome defense systems, to produce enzymes and secondary metabolites and their adaptation to the different matrices and environmental conditions (Filtenborg et al., 1996; Frisvad et al., 2007; Rodrigues et al., 2012).

The prevention of fungal spoilage and mycotoxin production in Brazil nuts can be carried out successfully if the species, which are actually spoiling it, are known. The availability of these data provide an optimum basis of research to study the selection principles of different foods which in turn can lead to the development of new methods to assess and control fungal spoilage including mycotoxin contamination.

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