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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.

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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

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