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Microbiological contamination and mycotoxin detection in guarana integument: addressing safety concerns in guarana agro-industrial co-products

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Abstract

This study characterized the microbiota of guarana integument, a co-product of guarana production chain, and validated an ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS) method for mycotoxin detection. Despite its low water activity, guarana integument harbors diverse microorganisms, including pathogens (*Bacillus cereus*), enteric opportunists (*Enterococcus casseliflavus*, *Citrobacter freundii*, *Kosakonia radicincitans*, *Enterobacter cloacae*, and *Enterobacter bugandensis*), and spoilage organisms (molds, yeasts, and spore-forming bacteria). Mold and yeast counts above the quantification limit were found only in samples from Producers 1 and 3 (3.00 and 4.35 $\log_{10}$ colony-forming unit [CFU]/g, respectively). Aerobic mesophilic bacteria were present in all samples (2.65–4.27 $\log_{10}$ CFU/g), while *Enterobacteriaceae* contamination was detected only in Producer 3 (1.77 $\log_{10}$ CFU/g). *B. cereus* vegetative cells were found in Producers 1 and 3 (2.85 and 2.30 $\log_{10}$ CFU/g), raising safety concerns. No *Salmonella* sp. or *Listeria monocytogenes* were detected. Aerobic mesophilic spore-forming bacteria were present in all samples, with Producer 3 showing the highest count (> 6 $\log_{10}$ spores/g) and being the only thermophilic spores above the limit (2.17 $\log_{10}$ spores/g). Aflatoxins B1 and B2, and ochratoxin A were below detection limits. These findings highlight potential risks to casquilho's microbiological stability.

Keywords: *Paullinia cupana*; seed coat; co-product; microbiological characterization; mycotoxins.

Practical Application: The results of this study reveal that guarana integument, despite its low water activity, can harbor a range of microorganisms, including potential pathogens and spoilage organisms. These findings reinforce the need for guarana producers to implement stricter hygiene and handling protocols during harvest, drying, and storage. The validated ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS) method for mycotoxin detection offers a reliable tool for routine quality control. Ensuring microbiological safety is essential to enable the safe reuse of guarana integument as a functional ingredient or alternative protein source.

1 INTRODUCTION

Guaraná (*Paullinia cupana*) is a woody vine or sprawling shrub native to the central Amazon Basin (Erickson et al., 1984). The seeds are commercially produced on some 10,000 ha in the state of Amazonas (CONAB, 2022). Approximately 70% of the production is utilized by the soft drink and energy drink industries, while the remaining 30% is processed into guaraná powder for direct consumption in capsules or as a water-soluble product. It also serves as a raw material for the pharmaceutical and cosmetics industries (Schimpl et al., 2013).

Microbial colonization by fungi and bacteria in agricultural products, such as edible seeds, is influenced by practices and conditions during pre-harvest, harvest, and post-harvest processing. During the pre-harvest phase, growing plants are vulnerable to various sources of microbial contamination, including soil, animal waste, and irrigation water quality. The harvest process involves several stages, such as collection, sorting, packaging, and transportation, where microbial contaminants can be introduced through cross-contamination of equipment, containers, transport vehicles, and workers. In the post-harvest phase, potential contamination sources include materials, equipment, inadequate hygiene, and even the handlers and environments. Size reduction operations, like milling, also contribute to increased microbial contamination in seed derivatives (Faour-Klingbeil et al., 2016; Murray et al., 2017; Rasines et al., 2023). Although seeds have low water activity (a_w) and are typically considered microbiologically safe, outbreaks of *Salmonella*

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and *Escherichia coli* have been reported in various edible seeds (Silva et al., 2022).

Since seeds are rich in nutrients such as starch, fat, and protein, they are highly susceptible to fungal infections and mycotoxin contamination (Chen et al., 2015). It is important to note that the optimal conditions for fungal growth are not always the same as those for mycotoxin production, which are toxic metabolites produced by certain filamentous fungi (Lima et al., 2021). However, by controlling fungal growth, mycotoxin production can be indirectly managed. Additionally, a synergistic effect can occur when different fungal species contaminate the same food, leading to the production of multiple toxins from the same substrate under favorable conditions. Therefore, it is essential to understand each toxin individually and the specific conditions that promote its production (Oga et al., 2014). Given the high incidence of mycotoxins in such food matrices and the potential for subsequent biosynthesis, studies focused on detecting these toxic metabolites in edible seeds are crucial (Wei et al., 2023).

Considering the growing demand for alternative protein sources, the valorization of agro-industrial co-products, and the enhancement of Brazil's agrobiodiversity, this study is particularly relevant as it provides data on bacterial, fungal, and mycotoxin contamination in guarana integument, the shell that remains attached to the guarana seed. Furthermore, since guarana is a fruit native to the Brazilian Amazon region, it is important to consider the risks to which the products are exposed, due to the climatic conditions of the region, characterized by high humidity and temperature. Such conditions are preponderant factors for the development of microorganisms and their metabolites, especially mycotoxigenic fungi, which may represent a potential risk to the health of the consumer.

Relevance of work

This study underscores the importance of microbiological safety in the processing of guarana integument, a promising by-product with potential as an alternative protein source. By identifying contamination risks, it highlights the need for good agricultural practices, hygienic handling, and regular microbial monitoring. These measures are essential to ensure product safety, protect consumer health, and support the sustainable use of agro-industrial residues.

2 MATERIAL AND METHODS

2.1 Acquisition of material

The guarana integument samples were collected from three producers in the State of Amazonas, Brazil. The first producer (PR1) was located in the municipality of Maués (latitude: -3.38173, longitude: -57.7152; 3°22′54″S, 57°42′55″W), while the other two were from Presidente Figueiredo: one from the Morena Community in PDS 240 (PR2) and the other from the Jardim Floresta Community along Rodovia BR 174 (PR3) (latitude: -2.03435, longitude: -60.0259; 2°2′4″S, 60°1′33″W). The collected material was then packaged and sent to the Faculty of Food Engineering at the Universidade de Campinas, São Paulo.

2.2 Microbiological characterization

All microbiological analyses were performed in technical duplicates, meaning two portions from each batch were collected and analyzed. The data presented in Table 1 represent the average of these duplicates. Samples were placed in sterile glass flasks, mixed with the respective diluents, and homogenized in an orbital shaker (New Brunswick Scientific, PsycroTherm, USA) at 250 rpm for 10 min. The quantification limit for pour plate analyses was 1 $\log_{10}$ colony-forming unit (CFU) or spores/g, while for spread plate analyses, it was 2 $\log_{10}$ CFU/g, unless otherwise specified.

2.2.1 Molds and yeasts

In a sterile bottle, 25 g of each sample was homogenized with 225 mL of 0.1% (w/v) peptone water (Oxoid Ltd., Basingstoke, UK). The count was performed using the serial dilution technique, with surface plating on Dichloran Glycerol Agar (DG-18) (Merck, Darmstadt, Germany). The plates were incubated at 25°C for 5 days (Ryu & Wolf-Hall, 2015). The results were expressed as the total count of fungi and yeasts per gram of sample (CFU/g).

2.2.2 Mesophilic bacteria

In a sterile bottle, 25 g of each sample was homogenized with 225 mL of 0.1% (w/v) peptone water (Oxoid Ltd., Basingstoke, Hampshire, UK). The count was performed using the serial dilution technique, with surface plating on Standard Counting Agar (PCA) (KASVI, Pinhais, Brazil), and the plates were

Table 1. Microbiological characterization of guaraná waste.

Microbial groups	Counts ($\log_{10}$ CFU or spores/g)#		
	PR1	PR2	PR3
Molds and yeasts	3.0 ± 0.06^{a}	$< 2.0^{*c}$	4.3 ± 0.02^{b}
Mesophilic bacteria	3.5 ± 0.05^{a}	2.6 ± 0.07^{c}	4.2 ± 0.05^{b}
Enterobacteriaceae	$< 1.0^{*b}$	$< 1.0^{*b}$	1.7 ± 0.10^{a}
Bacillus cereus	2.8 ± 0.02^{a}	$< 2.0^{*c}$	2.3 ± 0.00^{b}
Aerobic mesophilic spore-forming bacteria	3.8 ± 0.08^{a}	1.4 ± 0.00^{c}	6.1 ± 0.17^{b}
Aerobic thermophilic spore-forming bacteria	$< 1.0^{*b}$	$< 1.0^{*b}$	2.1 ± 0.02^{a}

#Mean ± SEM of technical duplicates; *Values below the limit of quantification of the technique. For pour plate analyses, the limit of quantification was 1 $\log_{10}$ CFU or spores/g, and for spread plate analyses, it was 2 $\log_{10}$ CFU/g; [a]Within a column, means without a common superscript differ significantly according to Tukey's test ($p \leq 0.05$).

incubated at 35°C for 48 h (Ryser & Schuman, 2014). The results were expressed as the total mesophilic aerobic count per gram of sample (CFU/g).

2.2.3 Enumeration of Enterobacteriaceae

Enterobacteriaceae were enumerated using 25 g of each sample. The count was performed using serial dilution, followed by the pour plating technique using the Violet Red Bile Glucose Agar (VRBG) media (Merck, Darmstadt, Germany). The plates were incubated at 37°C for 24 h (Kornacki et al., 2014). After this period, the colony count (CFU/g) was performed.

2.2.4 Salmonella sp.

A 25-g sample was homogenized in 225 mL of buffered peptone water (BPW, Neogen, Lansing, MI, USA) and incubated at 37 ± 1°C for 18 ± 2 h. Selective enrichment was performed using Rappaport Vassiliadis Soy Peptone Broth (RVS) (KASVI, Pinhais, Brazil) at 41.5 ± 1°C for 24 ± 3 h and Muller–Kauffmann tetrathionate/novobiocin broth (MKTTn) (Merck, Darmstadt, Germany) at 37 ± 1°C for 24±3 h. Both broths were plated onto Xylose-Lysine Deoxycholate Agar (XLD) (Oxoid Ltd., Basingstoke, England) and Brilliant Green (BG) agar (Oxoid Ltd., Basingstoke, England) and incubated at 37 ± 1°C for 24 ± 3 h (ISO, 2017a).

2.2.5 Listeria monocytogenes

A 25-g sample was weighed and homogenized in 225 mL of Half–Fraser broth (Merck, Darmstadt, Germany). After incubating at 37°C for 24 h, 0.1 mL was transferred to 10 mL of Fraser broth (Merck, Darmstadt, Germany) for selective enrichment. Following another 24-h incubation, selectively enriched samples were plated onto Chromogenic Listeria Agar by Ottaviani & Agosti (ALOA, Oxoid Ltd., Basingstoke, England) and Oxford Agar (Oxoid Ltd., Basingstoke, UK) for *Listeria* isolation, and incubated at 37°C for 24 h (ISO, 2017b).

2.2.6 Bacillus cereus

The enumeration of *B. cereus* was carried out by weighing 25 g of the sample, adding it to 225 mL of BPW, and subjecting the mixture to agitation. Then, serial dilutions were made in BPW and plated on Mannitol Egg Yolk Polymyxin Agar (MYP) (Merck, Darmstadt, Germany) using the spread plate technique. The plates were incubated at 30°C for 24 h. Subsequently, up to five characteristic colonies (pink with an opaque halo) were selected, isolated, and subjected to a hemolysis test on blood agar (30°C for 24 h). The occurrence of hemolysis was considered a presumptive indicator for *B. cereus* in the sample (ISO, 2004).

2.2.7 Aerobic mesophilic spore-forming bacteria

The method of Stevenson and Lembke (2014) with minimal modifications was used. Initially, 50 g of sample was added to flasks containing 450 mL of 0.1% (w/v) peptone water and homogenized. Then, they were heated in a water bath at 80°C for 10 min and subsequently cooled in an ice bath. Serial dilutions were made, and 1 mL of each was plated onto Tryptone Glucose Beef Extract Agar (TGEA) (Neogen, Indaiatuba, Brazil) medium using the pour plate method and incubated at 35°C for 48 h.

2.2.8 Aerobic thermophilic spore-forming bacteria

The quantification followed the methodology of Olson and Sorrells (2014). Briefly, 20 g of casquilho was added to 100 mL of sterile distilled water and homogenized. Then, 10 mL of the dilution was added to a flask with 100 mL of Dextrose Tryptone Agar (DTA) medium (Neogen, Indaiatuba, Brazil), boiled in a water bath for 30 min, followed by cooling in an ice bath, and then the medium was poured into five Petri dishes. The plates were incubated at 55°C for 72 h.

2.2.9 Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis

If present, presumptive colonies from the analyses of *Salmonella* sp., *L. monocytogenes*, *Enterobacteriaceae*, and *B. cereus* were isolated and purified. Subsequently, these cultures were identified using the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) technique. The procedure followed the manufacturer's direct smear extraction protocol, which is also described by Kumar et al. (2024). Briefly, a single colony was transferred to a MALDI-TOF MS target plate (MSP 96 polished steel BC, Bruker Daltonics, Germany) using a sterile toothpick. Protein extraction was achieved by adding 1 µL of 70% formic acid to each sample spot, followed by air drying at room temperature. Subsequently, 1 µL of α-cyano-4-hydroxycinnamic acid (CHCA) matrix solution (Bruker Daltonics) was applied to the spot. Mass spectra were acquired in positive linear mode using a Microflex LT mass spectrometer (Bruker Daltonics, Bremen, Germany). Instrument parameters included ion source voltages of 2.0 kV and 1.8 kV, a lens voltage of 6.0 kV, and a laser frequency of 60 Hz, with a mass range of 2,000–20,000 Da. The spectra were analyzed using Biotyper software (version 3.1, Bruker Daltonics), comparing them with the integrated database. Log score values ≥ 2.0 were deemed reliable for species-level identification, according to the manufacturer's criteria. Equipment calibration was conducted using the Bacterial Test Standard (Bruker Daltonics, Bremen, Germany).

2.2.10 Analysis of mycotoxin presence

2.2.10.1 Mycotoxin extraction

The extraction of aflatoxins (AFs) B1 (AFB1) and B2 (AFB2), and ochratoxin A (OTA) was conducted following the modified AOAC 991.31 method (AOAC, 2002) and Shen and Singh (2022). Initially, 25 g of ground sample was mixed with 125 mL of a methanol and deionized water solution (70:30 v/v) and 5 g of NaCl. The mixture was homogenized in a high-speed blender for 2 min and then filtered using Whatman No. 4 filter paper. From the filtrate, 12 mL was diluted with 20 mL of deionized water, and the resulting solution was filtered again through

a 1.5-µm glass microfiber filter (Vicam, Milford, MA, USA). Next, a 20-mL aliquot was purified using an AflaOchraTest® immunoaffinity column (Vicam, Milford, MA, USA), following the manufacturer's instructions. Finally, AFB1, AFB2, and OTA were eluted with 1 mL of methanol.

2.2.10.2 UPLC-MS/MS conditions

The chromatographic analysis was conducted using a Waters Acquity ultra-performance liquid chromatography (UPLC) system coupled with an Xevo TQD Triple Quadrupole mass spectrometer (Waters, Milford, MA, USA). The equipment used an electrospray ionization (ESI) source in positive mode, operating under multiple reaction monitoring (MRM). The separation of AFB1, AFB2, and OTA was performed on a BEH C18 analytical column (2.1×100 mm, 1.7 µm, Waters) maintained at 40°C. The mobile phase consisted of a mixture of ultrapure water and methanol containing 0.1% formic acid, at a 30:70 (v/v) ratio. The system operated in isocratic mode, with a flow rate of 0.3 mL/min and an injection volume of 5 µL. The ionization source conditions were as follows: a desolvation temperature of 300°C, a desolvation gas flow of 800 L/h, a capillary voltage of 2.5 kV, and a source temperature of 150°C. Mass spectrometer parameters were optimized by injecting a standard solution of AFs and OTA using MassHunter Optimizer software. Data acquisition and processing were carried out in MassLynx software (Waters). Detailed optimization parameters are presented in Table 2.

2.2.10.3 Method validation

The linearity and calibration parameters were evaluated using an analytical curve with concentrations ranging from 1 to 20 µg/kg for AFB1, AFB2, and OTA. The method's precision was analyzed through recovery tests, in which a standard mycotoxin solution was added to blank samples at three concentration levels (5, 10, and 20 µg/kg). The limits of detection (LOD) and quantification (LOQ) were calculated based on the ratio between the standard deviation of the lowest point of the analytical curve and the slope coefficient, with the result multiplied by 3.3 for the LOD and 10 for the LOQ (Inmetro, 2020).

2.2.11 Statistical analysis

Data were analyzed using R software (v. 4.3.2, R core team). Differences between the mean values were assessed with Tukey's test at a 5% significance level ($p < 0.05$).

3 RESULTS AND DISCUSSION

In Table 1, the results of the microbiological characteristics in *casquilho* samples from the three producers in the state of Amazonas are presented.

The microbiological characterization of the guarana integument samples revealed that this co-product of the guaraná production chain, despite its low water activity, harbors a diverse range of microorganisms, including pathogens (*B. cereus*) (Logan & Vos, 2015), enteric opportunists (*Enterococcus casseliflavus*, *Citrobacter freundii*, *Kosakonia radicincitans*, *Enterobacter cloacae*, and *Enterobacter bugandensis*) (Coutinho et al., 2024; Hathcock et al., 2023; Yoshino, 2023; Zhang et al., 2024), and spoilage organisms, such as molds, yeasts, and spore-forming bacteria, which warrant attention (Pitt & Hocking, 2009; Snyder et al., 2024).

It was initially observed that only samples Producer 1 and Producer 3 presented values above the quantification limit for molds and yeasts, registering 3.00 and 4.35 $\log_{10}$ CFU/g, respectively. All samples showed mesophilic bacterial counts, ranging from 2.65 $\log_{10}$ CFU/g (Producer 2) to 4.27 $\log_{10}$ CFU/g (Producer 3). Only sample Producer 3 showed contamination by *Enterobacteriaceae* above the quantification limit, with 1.77 $\log_{10}$ CFU/g. Additionally, two samples, Producer 1 and Producer 3, were contaminated with vegetative cells of *B. cereus* (confirmed by MALDI-TOF MS), with values of 2.85 and 2.30 $\log_{10}$ CFU/g, respectively.

The absence of *Salmonella* sp. and *L. monocytogenes* was confirmed in all samples. Presumptive colonies of these pathogens and/or *Enterobacteriaceae* were subsequently identified using MALDI-TOF MS, revealing the species *Lysinibacillus xylanilyticus*, *E. casseliflavus*, *C. freundii*, *K. radicincitans*, *E. cloacae*, and *E. bugandensis*.

Notably, the presence of vegetative cells of *B. cereus* was worrying since its spore form is highly resistant to osmotic (den Besten et al., 2006) and thermal stress (Desai & Varadaraj, 2010; Samapundo et al., 2014). This resistance, whether in its spore form or, as evidenced here, in its vegetative state, enables this bacterium to survive in this residue from guarana production, increasing the risk of its presence in the final product and, consequently, its exposure to consumers.

All samples were contaminated with aerobic mesophilic spore-forming bacteria, with sample Producer 3 reaching the highest value, exceeding 6 $\log_{10}$ spores/g. Regarding aerobic thermophilic spore-forming bacteria, only sample Producer 3

Table 2. Optimized UPLC-MS/MS conditions for the analysis of the studied mycotoxins.

Mycotoxin	Molecular formula	RT (min)	Precursor ions (m/z)	Product ions (m/z)	Collision energy (V)	Cone voltage (V)
AFB1	$C_{17}H_{12}O_6$	1.35	313.1	285.0	23	50
				241.2	33	50
AFB2	$C_{17}H_{14}O_6$	1.30	315.2	259.2	31	50
				287.1	25	50
OTA	$C_{20}H_{18}ClNO_6$	2.73	404.2	239.0	22	32
				358.0	15	32

RT: retention time; AFB1: aflatoxin B1; AFB2: aflatoxin B2; OTA: ochratoxin A.

Table 3. Data obtained from UPLC-MS/MS method validation.

Mycotoxin	Equation of the line (R^2)	LOD (μg/kg)	LOQ (μg/kg)	Recovery (%)		
				5 μg/kg	10 μg/kg	20 μg/kg
AFB1	y = 1,105.9x+7,077.1 R^2 = 0.9996	0.12	0.50	97.11	102.25	99.62
AFB2	y = 823.71x+4,432.1 R^2 = 0.9984	0.44	0.50	91.68	103.65	99.57
OTA	y = 3,374.2x-1,617.1 R^2 = 0.9965	0.67	1.00	86.31	102.66	100.09

AFB1: aflatoxin B1; AFB2: aflatoxin B2; OTA: ochratoxin A; R^2: coefficient of determination; LOD: limit of detection; LOQ: limit of quantification.

showed counts above the quantification limit, registering 2.17 $\log_{10}$ spores/g.

Moreover, the presence of mesophilic aerobic spore-forming bacteria (detected in all samples) and thermophilic aerobic spore-forming bacteria poses a significant risk to the microbiological stability (Snyder et al., 2024) of the integument and other products within the guaraná production chain.

The results of the analytical method validation were deemed satisfactory, with determination coefficients (R^2) greater than 0.99 in the calibration curves obtained in the matrix, demonstrating high linearity of the applied method. Furthermore, the recovery assays fully met the guidelines of SANTE/11312/2021 (EC, 2021), ensuring the precision and reliability of the data, as shown in Table 3.

The analyses conducted for the detection of AFB1, AFB2, and OTA in integument samples from different Amazonian producers indicated concentrations below the LOD. These results reinforce the quality and safety of the evaluated samples, demonstrating the absence of contamination by these mycotoxins.

The production of mycotoxins in guaraná is influenced by factors such as the presence of toxigenic fungi, the chemical properties of the product, and the processing methods adopted (Martins et al., 2014a). Although studies indicate that strains of *Aspergillus* sp. and *Penicillium* sp., producers of AFs (aflatoxins) and OTA, were identified in only 2% of the analyzed samples, the OTA levels detected remained below the LOD, indicating low contamination in the evaluated guaraná (Martins et al., 2014a). According to Copetti et al. (2013), the peeling stage is primarily responsible for reducing OTA levels, as the concentration of this mycotoxin in cocoa shells was approximately 10 times higher than in the internal parts (nibs).

Furthermore, studies indicate that bioactive compounds present in guarana, such as caffeine, tannins, and phenolic compounds, especially catechins, have antimicrobial and antioxygenic properties, playing an important role in reducing the biosynthesis of FAs and OTA (Majhenič et al., 2007; Martins et al., 2014b). In particular, caffeine can inhibit the production of these toxins by interfering with fungal metabolism (Akbar et al., 2016; Martins et al., 2014b).

Although guaraná is widely used and recognized for its functional properties, the study of mycotoxin contamination in this product has not been extensively investigated. Therefore, further research is needed to deepen the understanding of the risks and dynamics of contamination throughout the production chain. However, implementing good agricultural and post-harvest practices is crucial to mitigating mycotoxin contamination. This includes harvesting fruits in good condition, promptly removing the husk, efficiently drying seeds to safe moisture levels, and storing them under controlled temperature and humidity conditions. Regular monitoring throughout the production chain is also essential to ensure the safety of guaraná, aligning with quality and public health regulations (Costa et al., 2022; Sousa et al., 2018).

4 CONCLUSION

This is the first study to conduct a microbiological characterization of guarana integument samples, revealing the presence of pathogens, enteric opportunists, and spoilage organisms. A chromatographic method for mycotoxin detection was validated. The results of the analyses carried out on the guarana integuments of the three main producers in Amazonas have shown that the quantity of pathogenic microorganisms found, as well as the fungal metabolites (mycotoxins), was below the LOD, not representing an effective risk to health. However, due to the climatic conditions of the Amazon region, it is important to enhance Brazilian agrobiodiversity and ensure the microbiological safety of integuments, by guarana producers through good agricultural and handling practices to avoid the development of microorganisms and their metabolites.

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