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Maysoon Al Zubairy and Nada Moawad conceptualised the study and collection of data; All authors participated in the conduct of experiments. Maysoon Al Zubairy contributed to writing the manuscript. All authors read and approved the final version of the manuscript.

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Quantitative Analysis of Mycoflora and Aflatoxins in Green Coffee Beans from Sana'a, Yemen

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Abstract:

Coffee is one of Yemen's most significant agricultural commodities, renowned since antiquity for its superior quality and distinctive flavor. In light of its economic and cultural importance, this study investigated fungal contamination in green coffee beans and quantified their aflatoxins content. Twenty samples were collected from retail outlets across Sana'a and analyzed for mycoflora using the agar plate method. All samples showed contamination with filamentous fungi. Fungal loads ranged from 20 CFU/40 beans (Sample 10) to 137 CFU/40 beans (Sample 4). The genus *Aspergillus* was predominant (71.84% of isolates), followed by *Rhizopus* (18.59%) and *Penicillium* (9.55%). Among *Aspergillus* species, *A. niger* was most frequent (518 CFU/40 beans; 33.68%; frequency: 95%), followed by *A. melleus* (185 CFU/40 beans; 12.03%; frequency: 80%) and *A. aculeatus* (166 CFU/40 beans; 10.79%; frequency: 65%). Total aflatoxins levels, determined using the AgraQuant Aflatoxins ELISA Test Kit, were detected in all samples, ranging from 17.61 ppb (Sample 19) to 23.34 ppb (Sample 9). These findings highlight the need for monitoring these under-investigated products and the establishment of regulatory oversight in the dietary supplement industry, particularly for botanical-based products such as green coffee.

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INTRODUCTION

Coffee is a globally renowned beverage, characterized by its rich, slightly acidic flavor developed through the roasting of coffee beans. These beans are cultivated in over 70 countries, with the majority of global production concentrated in Brazil, Vietnam, Colombia, Indonesia, and Ethiopia (Shahbandeh, 2020). As of 2019, coffee ranked as one of the most traded commodities worldwide, with an estimated annual output of approximately 171 million 60-kg bags (USDA, 2020).

Yemen was once a prominent global producer and exporter of coffee, with its renowned Mocha variety historically linked to the port of Al Mokha, a major center of the coffee trade. Since its introduction, coffee has been cultivated across Yemen's mountainous and valley regions, where it has adapted to the country's arid climate. Both rainfed and irrigated varieties are grown, primarily on terraced farms, with Arabica being the dominant species. Despite its historical significance, coffee is currently planted on only 2.4% of Yemen's cultivable land, approximately 34,981 hectares. In 2019, the region produced 20,812 tons of coffee, accounting for approximately 6% of national agricultural export revenues in 2020, equivalent to USD 20.2 million out of a total of USD 320 million (FAO, 2023). Green coffee, derived from unroasted beans, is increasingly recognized for its health-promoting properties. Rich in bioactive compounds such as chlorogenic acids, caffeine, diterpenes, and soluble fiber, it is widely incorporated into dietary supplements (Belviso and Barbosa-Pereira, 2019).

The health-promoting properties of green coffee bean extracts are primarily attributed to their antioxidant activity. Additionally, green coffee bean extracts have gained commercial attention for their potential roles in weight management and glycemic control (Bagchi *et al.,* 2017). However, coffee beans are susceptible to contamination by various toxigenic fungi and bacteria throughout multiple stages of production, including harvesting, processing (such as washing, fermentation, and drying), transportation, and storage (Batista *et al.,* 2009; Martins *et al.,* 2003).

The mycocenosis of green coffee beans comprises a diverse fungal community that colonizes coffee throughout its cultivation, processing, and storage, particularly in South and Central America, regions renowned for coffee production. This microbial ecosystem plays a critical role in determining coffee quality and presents potential health risks due to the presence of mycotoxins, harmful secondary metabolites capable of causing serious health issues in humans. Microbiological studies of coffee cherries and beans have identified *Aspergillus* and *Penicillium* as common natural contaminants (Viegas *et al.,* 2017).

Aflatoxins are primarily synthesized by *Aspergillus flavus* and *Aspergillus parasiticus* (Iqbal *et al.,* 2021). High moisture content in coffee beans promotes the growth of *Aspergillus flavus* and the subsequent production of aflatoxins (Paterson *et al.,* 2014). The optimal temperature range for the biosynthesis of aflatoxin B_1 and B_2 is between 16°C and 31°C (Silva *et al.,* 2008). Multiple studies have investigated the fungal contamination and aflatoxins contents in green coffee beans (Nogaim and Gowri, 2013; Al Attiya *et al.,* 2021; Humaid *et al.,* 2019; Lu *et al.,* 2022).

Although fungal contamination and aflatoxins production in green coffee beans have been widely investigated in major producing regions such as South and Central America, systematic data on Yemeni coffee remain scarce. The fungal community associated with Yemeni coffee under its distinctive arid climate, terraced farming systems, and traditional processing practices has not been comprehensively characterized. Moreover, the extent of mycotoxin contamination and its implications for consumer health and export competitiveness are poorly understood. This lack of knowledge limits both scientific understanding and the development of effective strategies to safeguard the quality and health benefits of Yemeni coffee in global markets.

This study investigated fungal contamination in Yemeni green coffee beans, identifying key fungal genera, including *Aspergillus* species, and quantified total aflatoxins levels using Agar Quant and ROMER ELISA methods.

MATERIALS AND METHODS

Collection of samples

A total of 20 stored Yemeni green coffee bean samples were randomly collected from various markets and retail shops across Sana'a City, Republic of Yemen, during 2024. Each sample weighed 60 grams and was placed in a sterile polyethylene bag, sealed, and enclosed within a secondary protective bag. Samples were then transported to the Microbiology Laboratory, Department of Biological Sciences, Faculty of Science, Sana'a University, where they were stored at 4 °C until analysis for filamentous fungi and total aflatoxins content.

Isolation of fungi:

Agar plate method:

Forty green coffee beans were individually plated on Potato Dextrose Agar (PDA), prepared using 200 g of potato infusion, 20 g of dextrose, 15 g of agar, and 1000 mL of distilled water, adjusted to pH 5.5. The beans were placed in 9 cm Petri dishes, five beans per dish, and incubated at 25–28 °C for 3 to 7 days. Emerging fungal colonies were counted, subcultured onto fresh PDA for purification, and stored on PDA slants at 4 °C for subsequent identification (Mathur and Kongsdal, 2003).

Count of fungi isolated from the green coffee beans

Recovered fungal colonies were quantified and reported as colony-forming units per 40 beans (CFU/40 beans). The isolation frequency of fungal genera and species was determined following the method described by Marasas et al. (1988) as follows

$$\text{Frequency} = \frac{\text{No .from samples with occurrence of genus / species}}{\text{Total Of samples}} \times 100$$

Identification of fungi isolates:

Purified fungal isolates were identified based on their macroscopic and microscopic characteristics. For morphological identification, isolates were sub-cultured on Czapek Dox Agar medium composed of sucrose (30 g), $NaNO_3$ (3 g), KH_2PO_4 (1 g), $MgSO_4.7H_2O$ (0.5 g), KCl (0.5 g), $FeSO_4$ (0.01 g), agar (20 g), and 1000 mL of distilled water. Identification was carried out to the genus and species level using both colony morphology and microscopic features. Taxonomic classification was conducted using established references (Raper and Fennell, 1977; Pitt, 1979; Moubasher, 1993; Samson *et al.,* 1995).

Aflatoxins analysis in green coffee bean:

Total aflatoxins levels in 20 green coffee bean samples were quantified using the AgraQuant Aflatoxins ELISA Test Kit (1–20 ppb, 96 wells).

Extraction procedure:

The extraction solvent (70% methanol) was prepared by combining 70 mL of reagent-grade methanol with 30 mL of deionized water for each sample. Representative coffee bean samples were ground to a fine powder, comparable in texture to instant coffee; a 20 g portion of the ground sample was weighed and transferred into a screw-cap glass vial. One hundred milliliters of the prepared 70% methanol extraction solvent was added to each vial, maintaining a sample-to-solvent ratio of 1:5 (w/v). The mixture was agitated on a shaker at room temperature for 5-10 minutes; after allowing the particulate matter to settle, 5-10 mL of the supernatant was filtered through Whatman No. 1 filter paper, and the resulting filtrate was collected for aflatoxins analysis.

Assay procedure:

All reagents were equilibrated to room temperature before use. For each standard and sample, one dilution well was placed into a microwell holder, and an equal number of antibody-coated microtiter wells were arranged in a separate holder. Two hundred microliters of conjugate solution were dispensed into each dilution well. Using fresh pipette tips, 100 µL of each standard or sample was added to the corresponding conjugate-containing dilution well and mixed thoroughly by aspirating and dispensing three times. Subsequently, 100 µL from each dilution well was transferred into the corresponding antibody-coated microtiter well. The plates were incubated at room temperature for 15 minutes. After incubation, the contents were discarded into a designated waste container, and each well was washed five times with distilled water. Plates were inverted and tapped gently onto absorbent towels to remove residual moisture. Substrate reagent (120 µL per well) was prepared in a separate container, dispensed into each microwell, and incubated for 5 minutes at room temperature, protected from light. Stop solution (120 µL per well) was then added in the same sequence as the substrate. Aflatoxins concentrations were measured using a Linear Microplate Reader (GEA, Montgat, Spain) set to 450 nm.

RESULTS

Fungal isolates recovered from green coffee bean samples

Twenty green coffee bean samples were collected from local shops across Sana'a City, Yemen, and analyzed for their mycoflora using Potato Dextrose Agar (PDA). All samples showed contamination with filamentous fungi, ranging from 20 CFU/40 beans in Sample 10 to 137 CFU/40 beans in Sample 4 (Figure 1, Plate 1).

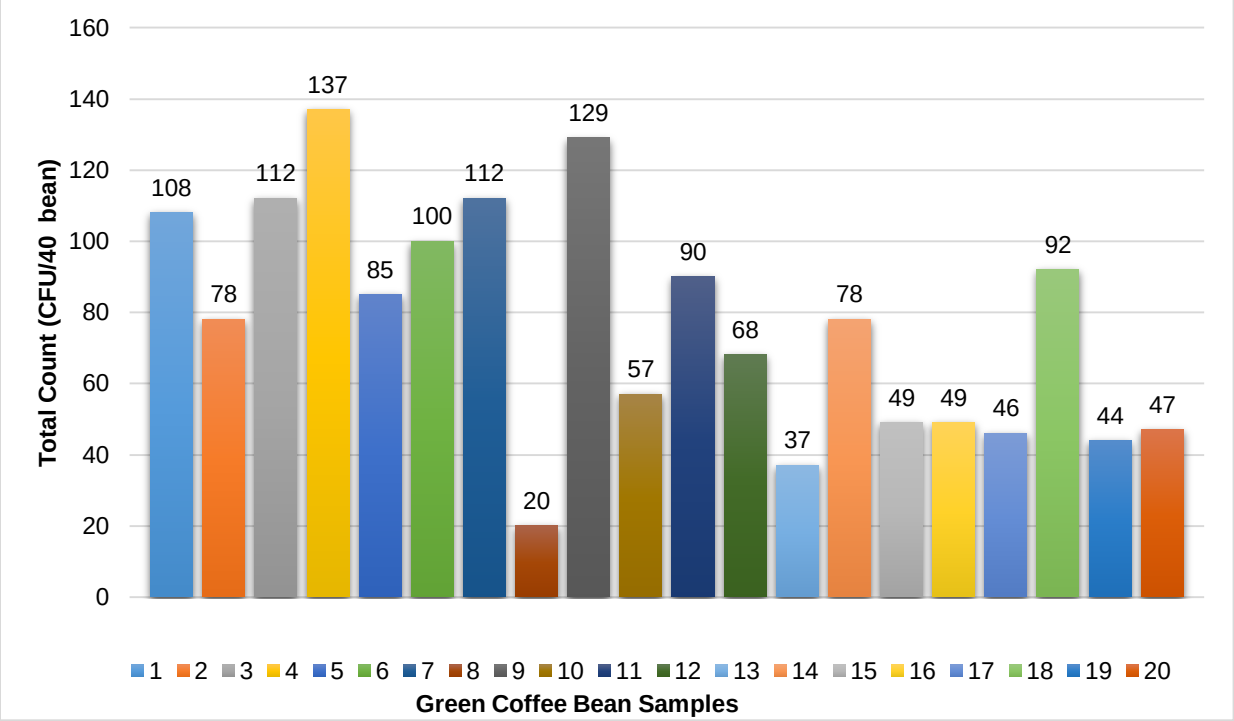

Fig. 1. Total fungal isolates (CFU per 40 beans) from green coffee bean samples.

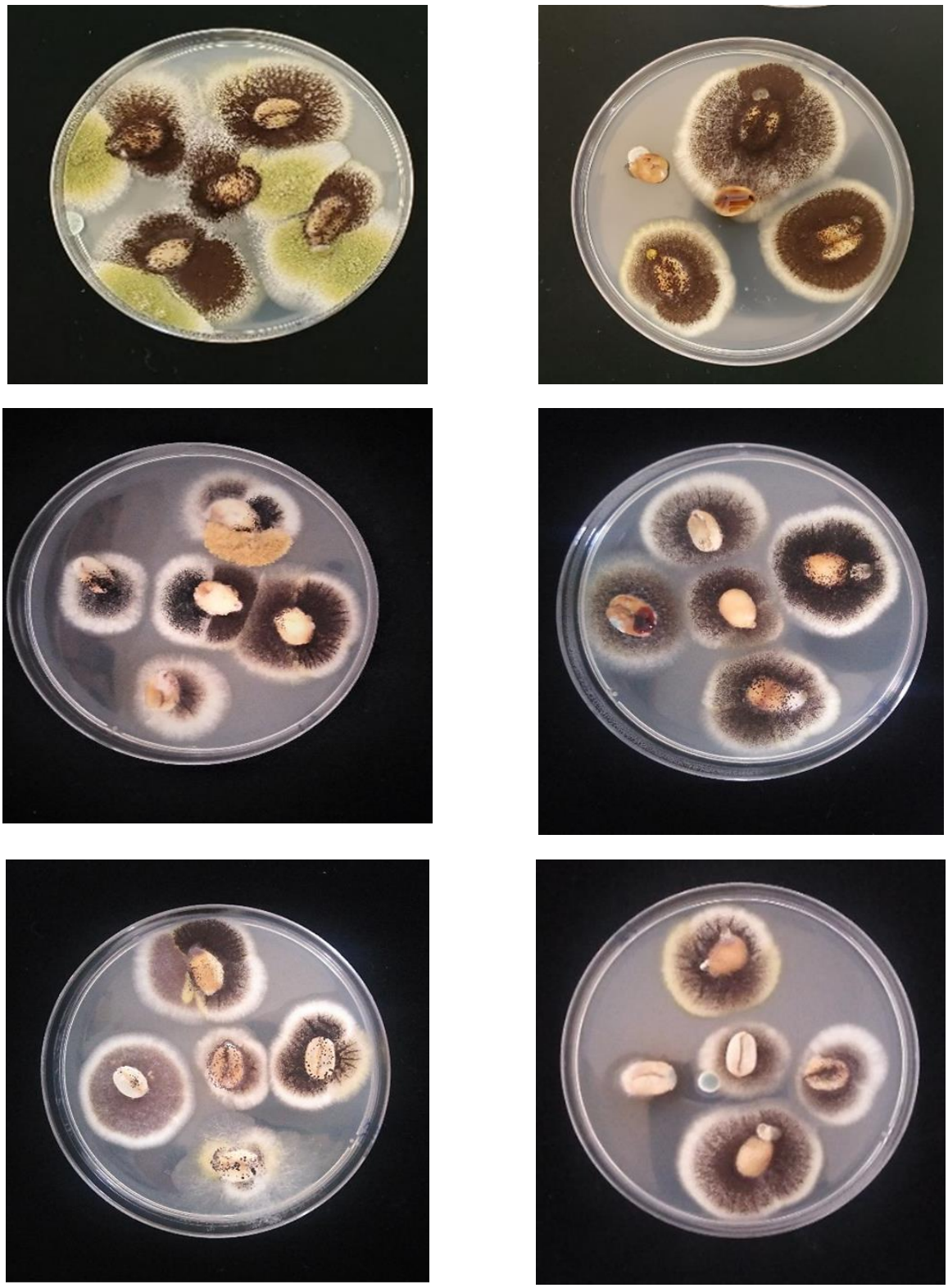

Plate 1. Isolated fungi from the green coffee bean samples.

Microscopic and macroscopic identification revealed three dominant genera: *Aspergillus* (71.84%), *Rhizopus* (18.59%), and *Penicillium* (9.55%) (Figure 2). *Aspergillus* was present in all samples, with a total of 920 CFU/40 beans.

Within this genus, *A. niger* was the most abundant species (518 CFU/40 beans, 33.68%, detected in 95% of samples), followed by *A. melleus* (185 CFU/40 beans, 12.03%, 80% frequency) and *A. aculeatus* (166 CFU/40 beans, 10.79%, 65% frequency) (Table 1). Two varieties of *A. flavus* were also identified: *A. flavus var. flavus* (44 CFU/40 beans, 2.86%, detected in 35% of samples) and *A. flavus var. columnaris* (18 CFU/40 beans, 1.17%, detected in 25%).

Rhizopus spp. were isolated from 11 samples (286 CFU/40 beans, 55% frequency), while *Penicillium* appeared in 17 samples (147 CFU/40 beans, 85% frequency).

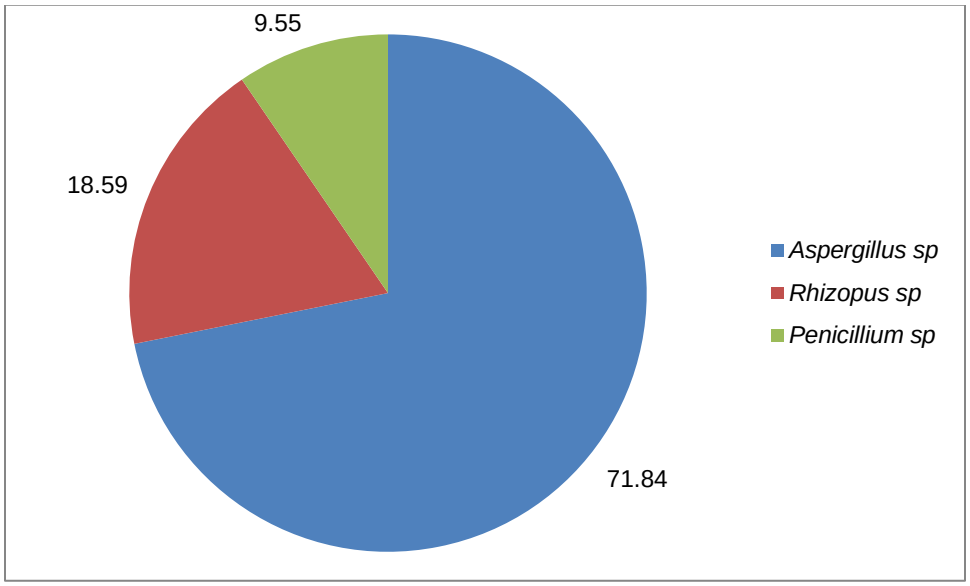

Fig. 2. Distribution of isolated fungi (%) from green coffee bean samples.

Table 1. Total count, percentage %, and frequency of fungal genera and species isolated from green coffee bean samples.

Fungal genera	Fungal species	Total count	Percentage	Frequency
Aspergillus sp		1105	71.84	100
	A. niger	518	33.68	95
	A. aculeatus	166	10.79	80
	A. japonicus	103	6.69	45
	A. flavus var flavus	44	2.86	35
	A. flavus var columnaris	18	1.17	25
	A. melleus	185	12.028	65
	A. tamarii	37	2.4	25
	A. ochraceus	34	2.21	35
Rhizopus sp		286	18.59	55
Penicillium sp		147	9.55	85

Aflatoxins analysis in green coffee bean:

Twenty green Yemeni coffee bean samples were analyzed for total aflatoxins content using the AgraQuant Aflatoxins ELISA Test Kit (validated range: 1–20 ppb, 96 wells). All samples tested positive for aflatoxins (Figure 3). Concentrations varied across samples, with Sample 9 showing the highest level at 23.34 ppb, exceeding the kit's upper quantification limit, while Sample 19 recorded the lowest at 17.61 ppb. Several other samples (2, 1, 17, 6, 4, and 5) also exhibited elevated levels above 20 ppb, indicating widespread contamination beyond the kit's validated range.

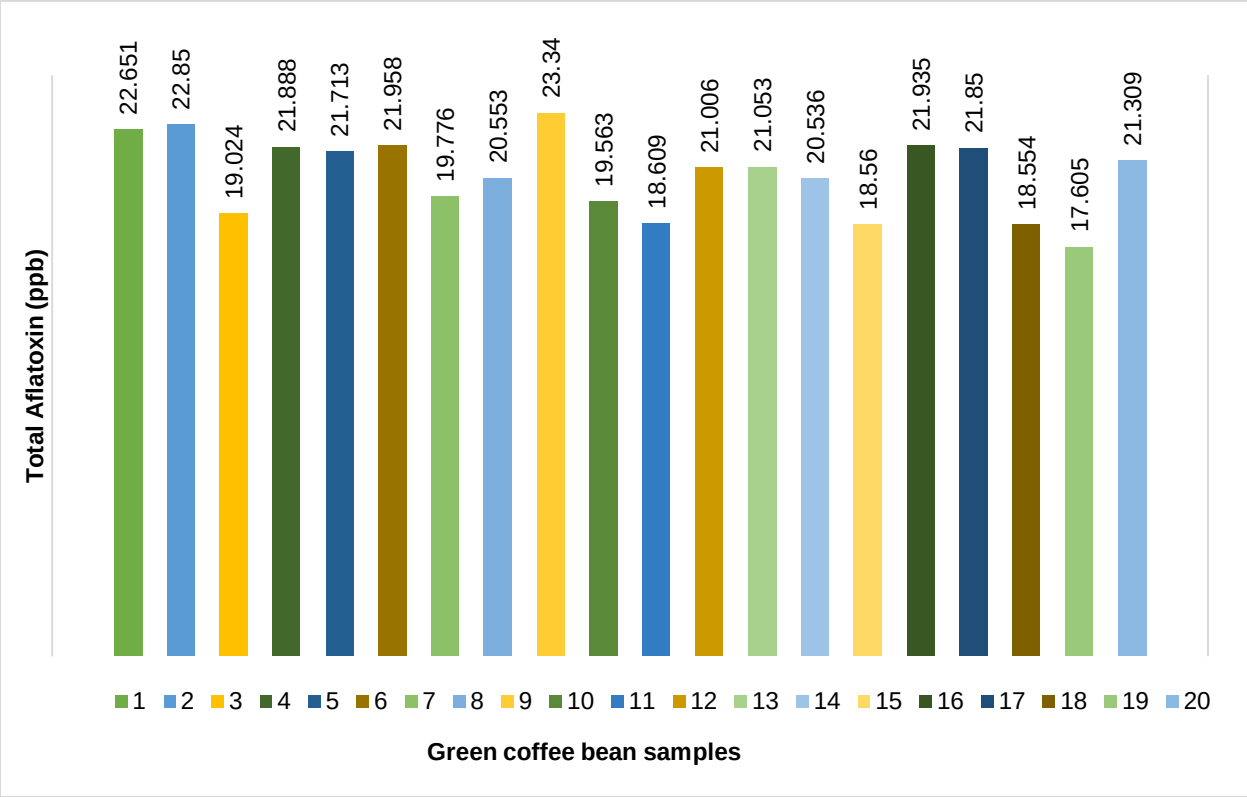

Fig. 3. The total aflatoxin of the green coffee bean samples (ppb).

DISCUSSION

Coffee is among the most widely consumed beverages worldwide, yet its beans remain vulnerable to microbial contamination, particularly by filamentous fungi, at multiple stages of production, including cultivation, harvesting, transport, and storage. Consistent with earlier microbiological investigations, the present study confirmed that all analyzed green coffee bean samples were contaminated with filamentous fungi. Notably, *Aspergillus* was the predominant genus, representing 71.84% of isolates, followed by *Rhizopus* (18.59%) and *Penicillium* (9.55%).

The dominance of *Aspergillus* in our samples corroborates previous reports from Nogaim and Gowri (2013), Barboráková *et al.* (2025), and Hlebová *et al.* (2022), who also identified *Aspergillus* and *Penicillium* as the most frequent contaminants of green coffee beans. However, the proportion observed in our study was considerably higher, suggesting that local environmental or storage conditions may favor

the proliferation of *Aspergillus*. This interpretation is supported by Viegas *et al.* (2017), who highlighted the genus's ability to penetrate bean structures and thrive under elevated temperatures.

At the species level, *A. niger* was the most prevalent, followed by *A. melleus* and *A. aculeatus*. The predominance of the Nigri section is consistent with findings from Barboráková *et al.* (2025) and Bessaire *et al.* (2019), reinforcing the view that this group is particularly well adapted to colonize coffee beans. Importantly, the isolation of two distinct varieties of *A. flavus var. flavus* and *A. flavus var. columnaris* further underscores the diversity of toxigenic fungi present in green coffee beans. The occurrence of *A. flavus* aligns with earlier studies (Nogaim and Gowri, 2013; Barboráková *et al.,* 2025; Al Attiya *et al.,* 2021), but its detection in multiple varieties in our samples may indicate a broader ecological niche or adaptation to local storage practices.

Aflatoxins are recognized as some of the most hazardous mycotoxins due to their potent toxicological effects, including carcinogenicity, teratogenicity, hepatotoxicity, and mutagenicity (Pariza, 1996; Chu, 1997). In acknowledgment of these risks, the International Agency for Research on Cancer (IARC) has classified aflatoxins as Group 1 carcinogens, confirming their carcinogenicity in humans (IARC, 2002). In the present study, all twenty Yemeni green coffee bean samples analyzed using the AgraQuant Aflatoxins ELISA Test Kit were contaminated with aflatoxins. Concentrations ranged from 17.605 ppb in sample 19 to 23.34 ppb in sample 9, with several other samples exceeding 20 ppb.

The detection of aflatoxins in every sample highlights the widespread contamination risk in Yemeni coffee beans. It aligns with findings by Humaid et al. (2019), who reported aflatoxins levels ranging from 14.694 to 27.176 ppb in green coffee beans.

While aflatoxins contamination in coffee has been documented previously (Batista *et al.,* 2003; Wanita *et al.,* 2024; Carbonell-Rozas, 2025), data specifically addressing green coffee beans remain limited, underscoring the importance of our results.

The concentrations observed in this study are particularly concerning when compared with international safety standards. The U.S. Food and Drug Administration (FDA) sets a maximum allowable limit of 20 ng/g for aflatoxins in food products, while the European Commission (EC) imposes stricter limits, including a maximum of 5 ppb for aflatoxin B_1 in roasted coffee (Whitaker and Slate, 2006; EC, 2006).

Several of our samples exceeded the FDA threshold, suggesting potential health risks for consumers and highlighting the urgent need for improved monitoring and control measures. In addition, the finding of 100% aflatoxins positivity indicates universal contamination, meaning all tested samples carried detectable toxin levels. This prevalence underscores systemic issues in post-harvest handling and storage, where environmental conditions consistently favored fungal growth and aflatoxins biosynthesis. Although concentrations varied, the absolute positivity highlights that no sample can be considered safe, reinforcing the urgent need for improved monitoring and preventive measures.

Green coffee beans are frequently colonized by fungi capable of producing aflatoxins. The relationship between fungal contamination and aflatoxins levels is generally positive but influenced by specific conditions. Greater fungal presence raises the risk of aflatoxins accumulation, yet the actual toxin concentration depends on factors such as fungal species, moisture content, and storage environment (Al Attiya *et al.,* 2021; Viegas *et al.,* 2017).

Beyond chemical contamination, insect infestation further exacerbates the risk of mycotoxin accumulation. Insects not only damage the physical and sensory quality of coffee beans but also act as vectors for toxigenic fungi. The coffee berry borer (*Hypothenemus hampei*), in particular, has been identified as a disseminator of mycotoxin-producing fungi within

coffee plantations (Kulandaivelu, 2010). This dual threat, direct fungal contamination and insect-mediated dissemination, represents a significant challenge for coffee safety and quality management.

Once fungal pathogens penetrate the coffee cherry, their spores can persist within insect-created cavities on the beans after harvest and processing. Suboptimal drying practices and inadequate storage conditions further facilitate spore germination, ultimately leading to elevated concentrations of mycotoxins in the beans (Zhang *et al.,* 2015).

In addition to insect-related damage, external defects in coffee beans—such as soil, stones, sticks, and huskscan provide pathways for soil-borne fungi to infiltrate the beans. These extrinsic defects increase the likelihood of fungal contamination and compromise bean quality (Biru and Tassew, 2019).

Contamination of green coffee beans with aflatoxins presents both health and economic challenges. Aflatoxins, particularly aflatoxin B_1, are toxic secondary metabolites produced by *A. flavus* and *A. parasiticus*. The International Agency for Research on Cancer (IARC, 2002) has classified aflatoxin B_1 as a Group 1 carcinogen, confirming its carcinogenicity in humans.

Chronic exposure to aflatoxins through contaminated coffee may result in hepatotoxicity, immunosuppression, and an increased risk of hepatocellular carcinoma (Wild and Gong, 2010). Although roasting can reduce aflatoxins levels, these compounds are heat-stable and may persist in the final beverage, thereby posing a continued risk to consumers (Humaid *et al.,* 2019).

Economically, aflatoxins contamination can lead to the rejection of coffee shipments by importing countries with strict regulatory limits, such as the European Union (Whitaker and Slate, 2006). Such rejections result in significant financial losses for producers and exporters and may damage the reputation of coffee brands in international markets.

In addition, the requirement for routine screening and quality assurance increases operational costs. These challenges are particularly burdensome in regions with limited infrastructure, where producers may lack access to advanced testing facilities or effective monitoring systems.

From a public health perspective, the presence of aflatoxins in coffee is highly concerning, especially in countries with limited regulatory oversight and low consumer awareness. Preventive measures including proper drying, controlled storage conditions, and regular mycotoxin testing are essential to mitigate contamination and ensure product safety (Al Attiya *et al.,* 2021).

The widespread occurrence of aflatoxins in Yemen's food supply underscores the urgent need for improved post-harvest handling, better storage practices, and stronger regulatory oversight. Strengthening these measures is critical to safeguard consumer health and maintain crop quality. Notably, only a few studies have investigated aflatoxins contamination in different crops in Yemen (Alghalibi *et al.,* 2008; Shatar *et al.,* 2009; Safwan *et al.,* 2019), highlighting a gap in knowledge that warrants further research.

CONCLUSION

This study highlights the widespread presence of micromycetes in green coffee beans, with *Aspergillus* emerging as the predominant genus. The ability of certain *Aspergillus* strains to produce aflatoxins presents serious health risks, reinforcing the need for rigorous quality control throughout the coffee production and storage process. The detection of aflatoxins in all analyzed samples underscores the importance of continuous monitoring, as even low-level, chronic exposure may lead to bioaccumulation and long-term health consequences.

Yemeni coffee, globally recognized for its historical legacy and distinctive flavor profiles, plays a vital role in supporting rural livelihoods and contributing to agricultural exports. However, the sector faces persistent challenges, including limited production capacity, inadequate infrastructure, and the absence of certification systems, political instability, and environmental stressors. Addressing these barriers through cooperative development, enhanced quality assurance, and sustained international support could significantly strengthen Yemen's position in the global specialty coffee market.

In response to these challenges, Yemeni authorities working in collaboration with international partners are implementing initiatives aimed at improving coffee production through sustainable agricultural practices and targeted value chain development. Simultaneously, efforts are underway to reduce contamination risks by promoting better post-harvest handling techniques and increasing awareness of the health hazards associated with mycotoxins, particularly aflatoxins. These integrated strategies are essential for safeguarding consumer health and enhancing the competitiveness of Yemeni coffee in international markets.

This study provides essential baseline data for producers, exporters, and regulatory authorities. Such information is crucial for developing effective post-harvest handling practices, establishing monitoring systems, and ensuring compliance with international safety standards. Ultimately, these findings contribute to safeguarding consumer health, protecting the reputation of Yemeni coffee, and strengthening its competitiveness in global markets.

CONFLICT OF INTEREST

There is no conflict of interest among the authors regarding this publication.

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