RESEARCH ARTICLE

Fungal Diversity and Aflatoxin Profile in *Camellia sinensis:* An In-Depth Analysis of Mycological and Mycotoxicological Aspects.

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Abstract

Camellia sinensis tea, is the second most consumed beverage in the world; however, it is contaminated with aflatoxins, resulting in adverse health effects. This study was aimed at determining the fungal diversity and aflatoxins levels in black *C. sinensis* tea retailed in Kericho, Kisii, and Bomet counties in Kenya. A cross-sectional study was conducted and 100 tea samples were collected from informal retail outlets. Fungal contamination was determined and aflatoxin screening was carried out. Aflatoxin analysis was carried out using an ELISA-based assay according to the manufacturer's instructions and read using a TECAN Infinite *f50* ELISA reader. In 311 different isolates, *Aspergillus niger* 34% (106) was the most isolated fungus from black *C. sinensis* (black) tea samples, followed closely by *A. flavus* 26% (81), *A. fumigatus* 16% (50), *A. versicolor* 7% (22), *A. nidulans* 5.79% (18), *Penicillium spp*. 3.5% (11) and *Paeleocimyces spp.* 2.89% (9), *Rhizopus spp*. 2% (7), *Fusarium* 1.6%(5), and *Cladosporium spp*. 0.6%% (2). Pearson correlation showed a significantly negative and insignificant correlation between fungal contamination and aflatoxins levels (r= -.021, *p* =.837). This poses a significant health risk due to the carcinogenic nature of aflatoxins, emphasizing the need for stringent quality control measures in the food chain.

Keywords: Fungal contamination, Aflatoxin, *Camellia sinensis*, Food value chain.

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Introduction

Mycotoxins are secondary metabolites compounds generated by filamentous fungi (Bennett & Klich, 2003; Omotayo *et al*., 2019). These compounds are of varied group forms with diverse chemical properties, which can lead to illness and mortality in humans and animals (Awuchi *et al*., 2021). It is widely recognized that food-borne fungus contamination can pose serious risks to consumer safety (Pandey *et al*., 2023). According to the Food and Agricultural Organization, mycotoxin contamination affects about 25% of the food and animal feed produced worldwide (Ezekiel *et al*., 2019). It is estimated that 500 million individuals are exposed to levels of mycotoxin that increase their vulnerability to illness and death (Wild *et al*., 2015). *Aspergillus flavus* and *Aspergillus parasiticus* are the primary producers of aflatoxins, which are extremely poisonous metabolites and carcinogenic (A. Kumar *et al*., 2021). The International Agency for Research on Cancer (IARC) designates aflatoxins as category 1 carcinogens. It is estimated that about 4.5 billion people in developing countries are at risk of consuming mycotoxin-contaminated food (Smith *et al*., 2015). These mycotoxins are known to cause detrimental health effects ranging from weakened immune systems to the development of cancer (Zhang *et al*., 2022). Studies have shown that type of mycotoxin, duration of exposure, person's age, health, and gender, as well as interacting influences involving genetic variables, dietary conditions, and concurrent exposure to other dangerous substances, have all been shown to have an impact on the effects of mycotoxicosis (Lootens *et al*., 2022). In addition, the severity of mycotoxin-induced toxicity have also been found to be exacerbated by other factors as; vitamin shortages, low calorie intake, alcohol abuse, and viral disorders have also been shown to aggravate the severity of mycotoxin-induced toxicity (Atanda *et al*., 2013). Aflatoxins are often immunotoxic, teratogenic, and can block protein synthesis (Smith *et al*., 2015). In addition, aflatoxins could cause acute liver damage, edema to alteration of digestion (P. Kumar *et al*., 2017; USAID east Africa Regional Mission, 2012). Thus, mitigating these hazards stands as an essential exploit in public health (Okasha *et al*., 2024).

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Camellia sinensis tea is a widely consumed beverage worldwide and has recognizable health benefits. The plant is cultivated in conducive climates favorable for fungal proliferation and mycotoxin synthesis making *Camellia sinensis* tea vulnerable to fungal contamination. Nevertheless, failure in adequate food value chain protocol and monitoring in retail markets, packaging, storage, and transportation similarly predisposes *Camellia sinensis* tea to fungal and mycotoxin contamination (Zhou *et al*., 2022a). Kenya like the rest of developing countries has a conducive tropical climate that favors tea cultivation, a climate that is similarly optimal for aflatoxin-producing fungus growth (Sedova *et al*., 2018). Through the value food chain for *C. sinesis*, the process is prone to fungal contamination ((Liu *et al*., 2023), posing a public health risk concern (Zhou *et al*., 2022b). The tea quality of black tea (Liu *et al*., 2023) is affected by fungal contamination with a number of mycotoxin-producing fungus being identified. The diverse fungal strains consisting of; *Fusarium spp., Aspergillus spp.,* and *Penicillium spp.,* have contaminated *C. sinesis* before with diverse mycotoxins such as ochratoxin A (OTA), aflatoxins (AFs), zearalenone (ZEN), deoxynivalenol (DON), or citrinin (CIT) (Zhou *et al*., 2022a).

Previous studies have concentrated on the health and nutritional effects of tea use in the last few decades. However, it's important to recognize the safety aspect of tea consumption (Sedova *et al*., 2018). The widespread and high-frequency consumption of tea warrants more attention on its mycotoxicological quality. Thus this study sought to ascertain aflatoxin-producing fungal diversity and levels in *Camellia sinensis*.

Materials and Methods

Sample Collection

A cross-sectional study design was used to randomly collect an estimate of 100 samples of 100 g sachets each of *C. sinensis* tea from informal retail markets in Kisii, Bomet, and Kericho counties chosen based on the geographical location renowned for tea cultivation in Kenya. The outlets were selected using the randomization method and the tea was collected by systematic random sampling. After collection, we ensured proper labeling and documentation of relevant information for each sample to implement quality control measures to maintain accuracy. Packaging was done aseptically to reduce cross-contamination and transported to KEMRI- Mycology laboratories for mycological and aflatoxin analysis.

Fungal isolation and identification

About 2.5 g of *C. sinensis* teas were inoculated onto Sabouraud dextrose agar (SDA) (Pitt & Hocking, 1985) supplemented with chloramphenicol (250 mg/L) (Al-harethi *et al*., 2016) and incubated at ambient temperatures and monitored daily up to 72 hours. Both macroscopic and microscopic culture characteristics were noted and used for fungal identification. In addition, the fungal hyphae and microscopic traits were identified after staining with Lactophenol cotton blue (Prince, 2009).

Detection of total aflatoxins

Detection of aflatoxins was carried out using an aflatoxin ELISA kit according to the manufacturer's instructions. Briefly, 25 mL of 60% methanol was added to 5 g tea and blended vortexed vigorously for 5 minutes, to allow extraction of aflatoxin. The contents were then centrifuged at 2688 xg, for 5 minutes. About 1ml supernatant was extracted and diluted with 4 mL of deionized water and used for ELISA analysis. The ELISA assay protocol was performed according to the manufacturer's instructions. In addition, the results were quality-controlled through HPLC.

Statistical analysis

Statistical analyses were performed using Microsoft Excel and SPSS v.26 software. Multiple regression analysis was performed to determine the effect of fungal contamination on aflatoxin levels. Pearson correlation analysis was conducted to determine the correlation between fungal contamination and the level of aflatoxins. One sample T-test was performed to compare the aflatoxin levels from the regulatory control of 10 ppb. *p-values* of less than 0.05 ($p < 0.05$) were considered statistically significant.

Results

Fungal Contamination in *C. sinensis* Tea

A culture of 2.5 g of sample on SDA was incubated at ambient air for 3 to 7 days and mycotoxigenic fungi were identified. From a total of 100 samples cultured, diverse fungal isolates were isolated. Samples culture showed a positive growth of 67% (67) for fungi while the 33% (33) remaining yielded no fungal growth to insignificant growth. Contamination was evaluated through the colony forms unit (CFU). These were; *Aspergillus niger, A. flavus*, *A. fumigatus, A. nidulans, A. versicolor, Paecilomyces, Rhizopus* and *Cladosporium spp*). Some of the samples yielded no fungal growth even after 7 days (plate C) (Figure 1).

Fungi isolated from *C. sinensis* tea powder

From a total of 100 samples cultured, macroscopic and microscopic identification of fungal were used and a total of 311 isolates were obtained. The fungal isolates included: *A. niger* 34% (106,311) was the most isolated fungi from black *C. sinensis* (black) tea samples, followed closely by *A. flavus* 26% (81,311), *A. fumigatus* 16% (50,311), *A. versicolor* 7% (22,311), *A. nidulans* 5.79% (18,311), *P. spp*.3.5% (11,311) and *Paeleocimyces spp.* 2.89% (9,311)., *Rhizopus spp.* 2% (7,311)), *Fusarium* 1.6% (5,311), and *Cladosporium spp*. 0.6%% (2,311) (Table 1 and Figure 2).

Aflatoxin levels in ng/g

In a total of 100 samples that were collected from the sampled counties, 15% of the samples had less than the permissible level of aflatoxins while most them 85% had aflatoxins above the permissible levels with the average mean of 32.26 in all sampled counties. Individual mean however, Bomet had 19.90 which was the highest followed by Kisii County 7.87 and 4.58 for Kericho County (Table 2). Pearson correlation analysis showed a significantly negative and insignificant correlation between fungal contamination and the level of aflatoxins ($r = -.021$, $p = .837$) and One Sample T-test revealed a significant difference in aflatoxin levels from the regulatory control ($t(99) = 17.921$, $p = 0.00$).

Multiple Regression analysis

Multiple regression analysis was conducted to determine the effect of fungal contamination on aflatoxin levels. From the analysis the overall, results showed that contamination significantly influenced levels of aflatoxins $F(1, 99), = 4.493$, $R^2 = .021$). For data visualization, the histogram shows that our data follows a normal distribution pattern, without skewness.

Discussion

Fungal contamination in *C. sinensis*

Fungal infestations and mycotoxin contamination in tea pose a multifaceted challenge that intersects agricultural methods, processing techniques, and health concerns (Sedova *et al*., 2018). Various studies have identified mycotoxigenic fungi such as; *A. flavus* and *A. parasiticus* in tea cultivated areas and its soils (Nji

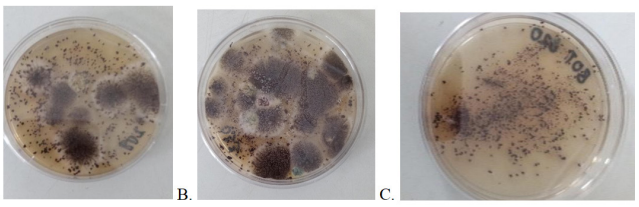

Figure 1: Primary culture plates showing fungal growth on SDA after 5 days of incubation period at ambient temperature: Plate A: 7 CFUs; Plate B: 16 CFUs; Plate C: No growth

Table 1: Frequency of fungal contamination of *C. sinensis* tea powder

Isolate (n = 311)	*Frequency n (%)*
A. niger	106(34.08)
A. flavus	81(26.05)
A. fumigatus	50(16.08)
A. versicolor	22(7.07)
A. nidulans	18(5.79)
Penicillium spp.	11(3.54)
Paeleocimyces spp.	9(2.89)
Rhizopus spp.	7(2.25)
Fusarium spp.	5(1.61)
Cladosporium spp.	2(0.64)
TOTAL	311

Table 2: Mean aflatoxin levels of tea samples analyzed

Sample region	*Samples (n)*	*<10(%)*	*≥10(%)*	*G. Mean*
Bomet	45	12(26.7)	33(73.3)	19.9
Kericho	22	1(4.6)	21(95.5)	4.58
Kisii	33	2(6.1)	31(93.9)	7.87
Total	100	15	85	32.36

et al., 2023). This emphasizes a potential pathway for mycotoxin contamination throughout the tea food value chain with negative implications in various tea products. A study conducted in Turkey showed elevated levels of aflatoxin and fumonisin in locally consumed tea highlighting the widespread occurrence of mycotoxin contamination across diverse geographic regions and tea types (Sedova *et al*., 2018). In the present study, there was a significant incidence and diversity of fungal contaminations of *C. sinensis* (black) tea from retail outlets. *A. niger* was the most predominant fungal contaminant (34%) followed closely by *A. flavus* (26%), and *A. fumigatus* (16%) with *A. versicolor* and *A. nidulans* 7% and 6%, respectively. It is worth noting that these are potential mycotoxigenic fungi responsible for aflatoxin and fumonisin production. Studies have continued to show that *A. niger* remains the most predominant microorganism throughout the *Puerh* tea manufacturing process (Elshafie *et al*., 1999; Mo *et al*., 2005; Sano *et al*., 1986; Stockmann-Juvala & Savolainen, 2008; Xu *et al*., 2005). The present study aligns with prior studies, that have demonstrated a high prevalence of *Aspergillus spp*. as a prominent contaminant. These species include; identified *A. ovori*, *A. acidus*, *A. niger*, and *A. tubingensis* in 16 out of 22 black tea tested (Storari *et al*., 2012). Similarly, a study conducted in Denmark isolated *A. acidus* and *A. niger* (Mogensen *et al*., 2009). One study revealed that 73% of fungal contaminants were with *Aspergillus* and *Penicillium* species (Kazemi *et al*., 2009).

The current findings are also consistent with a survey on the mycoflora of diverse black teas in Poland indicating the presence of *A. fumigatus* and *A. niger*, with *A. niger* being the most prevalent (Elshafie *et al*., 1999). Additionally, other fungal contaminants were detected, although at lower percentages (Elshafie *et al*., 1999). In South Africa, yeasts and filamentous fungi were identified at every

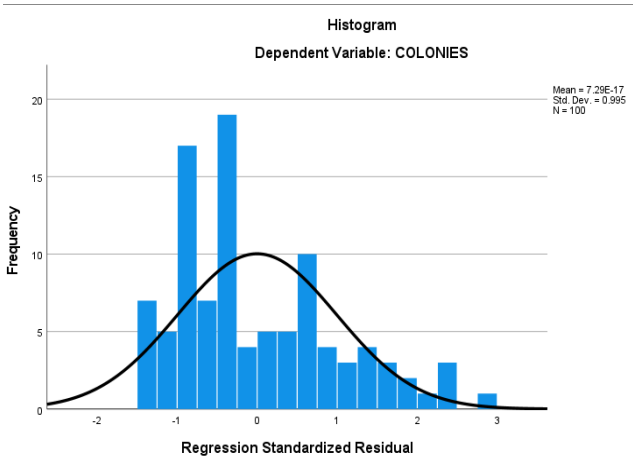

Figure 2: Histogram showing the uniform distribution of data

stage of black tea manufacturing processing extending through the heating process. This is an indication of the potential contamination of the final product during subsequent phases such as; sorting, packaging, transport, and storage.

Aflatoxigenic fungi among them *A. pseudotamarii* have been isolated from the soils of tea fields (Frisvad *et al*., 2019). Moreover, distinct fungal isolates like *A. flavus* and *A. parasiticus* have been recovered from various environments within tea factories, including the phyllosphere and soil (Dutta *et al*., 2012). Tea like any other plant material is susceptible to fungal and mycotoxin contamination just like maize or any other food commodity.

Furthermore, there exists a correlation between fungal infestations and mycotoxin contamination intricately influenced by; processing conditions and the presence of specific fungal species (Pandey *et al*., 2023). Samples collected in informal markets in the three counties showed that only 15% of the sample had less than the permissible level of aflatoxins of 10 ppb. Most of the tea samples (85%) sold in informal markets were contaminated with aflatoxins above the permissible levels. Among the Counties sampled, tea sold in Bomet markets had the highest (19.9%) rate of contamination with Kericho (4.58) being the least. This could be justifiable as Bomet county experiences relatively warmer temperatures compared to the other two counties hence provides ambient temperatures for fungal proliferation (Bore *et al*., 2016). In addition to unique warm temperatures in Bomet, the recent erratic climatic patterns in these tea growing areas has led to increased fungal contamination, hence resulting in high aflatoxin levels (Temba *et al*., 2021). While some samples may manifest fungal contamination without detectable mycotoxins, others may harbor toxins despite lower levels of visible contamination. Moreover, the array of fungal genera identified across different studies implies a clear distinction between regional disparities and environmental elements affecting mycotoxin occurrence in various beverages. A comparison of fungal growth and mycotoxin production revealed instances where mycotoxins were undetectable despite fungal growth, and vice versa. Under the mentioned conditions, samples with lower fungal contamination but elevated toxin levels indicated prior mycotoxin contamination during processing. Additionally, certain strains of *A. flavus*, categorized as nontoxigenic, do not produce mycotoxins after growth. Conversely, if conditions are unfavorable, fungi may be eradicated, yet any toxins already produced are likely to persist. As ongoing research continues to illuminate these dynamics, endeavors to combat mycotoxin contamination in tea production

necessitate a holistic approach. This involves integrating agricultural best practices, processing standards, transport, and storage plus stringent quality control measures to safeguard consumer health.

Aflatoxins are potent carcinogens and can cause adverse health effects even at low concentrations. Furthermore, we sought to establish the correlation between fungal contamination levels and the levels of aflatoxins in *C. sinensis* (black tea). Consequently, correlation analysis showed a significantly negative and insignificant correlation between fungal contamination and the level of aflatoxins (r= -.021, p = .837). In contrast, several studies have confirmed the direct relationship between fungal communities in food and feed and the levels of mycotoxin (Mahato *et al*., 2019; Sedova *et al*., 2018; Takim & Aydemir, 2021). For instance, studies on black and green tea samples from the Czech Republic and Italy revealed extensive fungal contamination (Carraturo *et al*., 2018; Řezáčová *et al*., 2005). Unfortunately, in most countries, the acceptable levels of aflatoxins in tea and plant-based beverages are not established as that of maize 10 and 15 µg/kg for peanut butter (Massomo, 2020). The Food and Agriculture Administration (FAO) however recommends permissible levels of 1-20 µg/kg in food (Gong *et al*., 2015; Ismail *et al*., 2020). Herein, a t-test comparison of aflatoxin levels from an umbrella regulatory standard of 10µg/kg in food revealed a significant difference in aflatoxin levels from the regulatory control (t(99) = 17.921, p = 0.00). This implies that the aflatoxin levels in most of the *C. sinensis* tea samples in the present study are above the regulatory standards. Despite acknowledging their carcinogenicity, aflatoxins in non-alcoholic beverages including tea are not regulated in most countries, hence presenting a significant health threat to the populace (Ismail *et al*., 2020). Regardless of their small quantities, mycotoxins accumulate in the body damaging the kidneys (Pakshir *et al*., 2020). Mycotoxins can be minimized by utilizing biological control agents, implementing strategic crop rotation, and good hygienic practices along food value chains. In essence, the adoption of these multifaceted strategies ensures the quality, safety, and sustainability of mycotoxin control safeguarding consumer health and well-being. Awareness and educational campaigns in the food industry emphasize hygiene practices and quality control adherence, to mitigate fungal contamination and mycotoxin risks. Despite the achieved findings, this study was faced with some limitations. Throughout the food value chain, this study will not be certain of a potential source of contamination. The study had no longitudinal data and was limited in how temporal data could be evaluated.

Conclusion

This study highlights the risk of mycotoxin and fungal contaminants in *C. sinensis* black tea. *A. niger*, *A. flavus*, and *A. fumigatus* as the most frequent isolates with *A. niger* being the most predominant contaminant. In addition, the detection of high levels of fungal contamination and the concentration of aflatoxins above permissible levels emphasizes the critical importance of vigilant monitoring and control measures to mitigate the risk of fungal proliferation and aflatoxins that would affect the quality of *C. sinensis* black tea. This calls for comprehensive monitoring and mitigation strategies to uphold tea safety.

Data Availability

Data generated from this stud y are controlled by regulations set by Kenya Medical Research Institute and can only be made available upon reasonable request following stipulated guidelines.

Conflict of Interest

The authors declare no conflict of interest

Author's Contribution

Sally Loronyokie and Anthony Kebira Nyamache contributed to protocol development, Vincent Kiprop and Hannah in Laboratory practices and Christine Bii assisted in the implementation of the protocol. Sally Loronyokie prepared the first draft of the manuscript and all the authors read and approved the manuscript.

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