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Tea contamination by mycotoxins and azole-resistant mycobiota – The need of a One Health approach to tackle exposures

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ABSTRACT

Despite tea beneficial health effects, there is a substantial risk of tea contamination by harmful pathogens and mycotoxins. A total of 40 tea samples (17 green (raw) tea; 13 black (fermented) tea; 10 herbal infusions or white tea) were purchased from different markets located in Lisbon district during 2020. All products were directly available to consumers either in bulk (13) and or in individual packages (27). Bacterial analysis was performed by inoculating 150 μL of samples extracts in tryptic soy agar (TSA) supplemented with 0.2 % nystatin medium for mesophilic bacteria, and in Violet Red bile agar (VRBA) medium for coliforms (Gram-negative bacteria). Fungal research was performed by spreading 150 μL of samples in malt extract agar (MEA) supplemented with 0.05 % chloramphenicol and in dichloran-glycerol agar (DG18) media. The molecular detection of the *Aspergillus* sections *Fumigati, Nidulantes, Circumdati* and *Flavi* was carried out by Real Time PCR (qPCR). Detection of mycotoxins was performed using high performance liquid chromatograph (HPLC) with a mass spectrometry detector. Azole resistance screening was achieved following the EUCAST guidelines. The highest counts of total bacteria (TSA) were obtained in green raw tea (81.6 %), while for coliform counts (VRBA) were found in samples from black raw tea (96.2 %). The highest fungal counts were obtained in green raw tea (87.7 % MEA; 69.6 % DG18). *Aspergillus* sp. was the most prevalent genus in all samples on MEA (54.3 %) and on DG18 (56.2 %). In the raw tea 23 of the samples (57.5 %) presented contamination by one to five mycotoxins in the same sample. One *Aspergillus* section *Fumigati* isolate from green tea beverage recovered form itraconazole-Sabouraud dextrose agar (SDA) medium, presented itraconazole and posaconazole E-test MICs above MIC90 values. Our findings open further discussion regarding the One-Health approach and the necessary investment in researching biological hazards and azole-resistance associated with the production and consumption of tea (in particular green tea).

1. Introduction

Tea and other herbal infusions are regularly consumed, on a daily basis, by more than half of the Europe population (WHO, 2009). Tea (*Camellia sinensis* (L.) Kuntze) has been consumed worldwide for centuries due to its flavor and beneficial health effects, being one of the most popular and lowest-cost beverage (after water) (EFSA, 2018b; Hilal and Engelhardt, 2007). The most common tea types are green (non-oxidized) and black (fermented, oxidized) tea (Zhao et al., 2010). The largest tea producers in the world are China and India with approximately 43 % and 22 % of world production, respectively (Committee on Commodity Problems, 2018).

The growth in tea consumption, especially of green tea, has been contextualized with the trend of healthy lifestyles in western countries.

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Among the Portuguese population, the following references are the most consumed: plant/herbal infusions (44 %), followed by green (26 %) and black (19 %) tea (Silva, 2014). Herbal teas, prepared with plants other than *Camellia sinensis*, such as lemon balm (*Melissa officinalis* L.) and chamomile (*Chamaemelum nobile* L.), are part of Mediterranean diet and folk medicine (Delgado et al., 2017). A 2012 market study revealed that 'plant and herbal infusions' were the most representative segment of 'teas and infusions' in Portugal, with a weight in sales value of 65.8 % and in sales volume of 66.7 %. 'Green tea' segment represented 16 % and 16.2 % in value and sales volume, respectively, of the total 'teas and infusions', and 'black tea' a share of 11.3 % in value and 11.7 % in volume (Hipersuper, 2012).

Fermentation process is used to avoid tea spoilage by fungal contaminants, besides for improving tea quality and taste (Dugar, 2016; Le, 2016). The health benefits of tea may be compromised by contamination by molds, heavy metals, mycotoxins or pesticide residues above the regulatory limits (Sedova et al., 2018). Of these contaminants, aflatoxins, ochratoxin A and fumonisins, already identified in black tea (Malir et al., 2014; Martins et al., 2001) are described as the most problematic, with mycotoxins' transfer from raw tea to tea beverage reported for several water-soluble mycotoxins, including ochratoxin A (Carraturo et al., 2018; Malir et al., 2014; Toman et al., 2017).

Tea leaves are susceptible to fungal and mycotoxins contamination during culture, storage, handling or packing, with several reports of tea contamination by potentially harmful pathogens (Dayananda et al., 2017; Scientific Committee on Foods, 2016; THIE, 2016; Viegas et al., 2020b). Commonly identified fungal contaminants in tea production environment are *Fusarium* sp. (Wang et al., 2017a), *Aspergillus* sp. (Dutta et al., 2008), including toxigenic species (i.e., fungal species able to produce hazardous mycotoxins) such as *Aspergillus fumigatus, A. niger and A. flavus,* or *Fusarium lactis* (Dutta et al., 2008; Fox et al., 2004; Yang et al., 2011). Regarding mold contamination, a 10^5 CFUs·g^{-1} limit is recommended as fungi total counts in tea (Scientific Committee on Foods, 2016; THIE, 2016; Viegas et al., 2020b). The lack of additional legislation or guidelines on mycobiota diversity leads to insufficient reports of microbiological hazards or food safety studies on tea (Lund et al., 2000; Viegas et al., 2020b).

Mycotoxin production in tea can occur at any stage of its manufacturing, from crop to processing and storage and it is a consequence of poor agricultural practices, improper processing, drying, packaging, storage, and transport conditions that can promote fungal growth and increasing the risk of mycotoxin contamination (Sedova et al., 2018). Nevertheless, mycotoxins in tea are almost not regulated, except for some countries (e.g. Armenia, Belarus) and mainly for aflatoxins (Commission of the Customs Union, 2011). There is a need of research on the occurrence of multiple mycotoxins in tea, as the scarce available data does not allow supporting the implementation of new regulations aiming to prevent consumer's exposure. However, the European Union has recently discussed the expansion of the group of products subject to a maximum level for Ochratoxin A (OTA) to include herbs and herbal teas. This mycotoxin is already regulated in a series of food products, including cereal and grape based foods, spices, coffee and liquorice (Commission Regulation (EC) No 1881/2006).

The use of azole fungicides is a common practice in tea production to control fungal growth, with organic teas (grown without fungicides) being particularly prone to fungal and mycotoxins' contamination. However, azole fungicide use in the agriculture has also been reported as supporting environmental selection for fungal resistance among opportunistic pathogenic fungi, such as *Aspergillus fumigatus,* and a possible cause for the failure of response to medical azoles (Azevedo et al., 2015; Chowdhary et al., 2012; Schoustra et al., 2019; Verweij et al., 2012). Moreover, it is expected that climate change will affect fungal control practices in agriculture and floriculture that will trigger the use of triazole fungicides, such as tebuconazole, hexaconazole, and epoxiconazole, in agriculture (Tibpromma et al., 2021; Wang et al., 2017b; Wang et al., 2019), already associated with the development of fungal resistance in *A. fumigatus* (Azevedo et al., 2015; Schoustra et al., 2019; Verweij et al., 2012), including in tea plantations (Chowdhary et al., 2012). As such, the ECDC technical report of 2012 on risk assessment on the impact of environmental usage of triazoles (Kleinkauf et al., 2013), recommended increased and continuous surveillance of triazole resistance in *A. fumigatus* in each EU Member State. More recently, the environmental azole resistance selection in *A. fumigatus* as a threat to medical therapy, and the need to identify hotspots for azole resistance, have been addressed through a One-Health approach (Kleinkauf et al., 2013).

In the present study, different black (n = 13) and green tea (n = 17) [*Camellia sinensis* (L.) Kuntze] and others (n = 10) (*Melissa officinalis* L., *Equisetum hyemale* L., *Peumus boldus* Molina, *Echinacea* sp., white tea) samples were assessed for microbial contamination. In addition to total bacterial and fungal counts, microbiological analysis also included mycotoxins' detection, molecular detection of toxigenic fungal species, and screening of azole resistance, both on raw (i.e., dried shredded leaves or other plant parts in solid state, as present in the tea bags or in bulk) and in brewed tea. Such data can support the identification of critical quality attributes before market distribution of tea, and allow understanding the influence of brewing on tea contamination and consumers exposure.

2. Materials and methods

2.1. Tea sampling

A total of 40 tea samples (*Camellia sinensis* Kuntze and other herbal infusions) of different origins were purchased in supermarkets (23; 57.5 %) and herbal shops (17; 42.5 %) located in the Lisbon district, in January 2020 (Table 1). All products were directly available to consumers in bulk (13; 32.5 %) or in individual packages (27; 67.5 %). Of these, 17 (42.5 %) were green (raw) tea, 13 (32.5 %) were black (fermented) tea, and 10 (25.0 %) were herbal teas or white tea. Some of the samples were flavored teas (7 out of 40). The production type was classified as "organic" if stated as so (or as biologic/natural) in the label, and as "conventional" otherwise (Table 1).

2.2. Sample preparation for laboratorial analysis

The purchased teas were stored at room temperature before each preparation method (raw tea or tea beverage) for subsequent microbiological analyses and mycotoxin detection. For raw tea sample preparation, 4.4 g dry tea sample were washed with 40 mL of 0.9 % sterile NaCl solution with 0.05 % Tween80™ in a 50 mL tube for 30 min at 250 rpm (orbital shaker OHAUS®). Regarding tea beverage, 50 mL of boiled distilled water was poured into each beaker containing 1 g of dry tea sample and then left to stand (5 min) according to ISO3103:1980 (2019) (Viegas et al., 2020b). All samples were prepared in sterilized falcon tubes and clean glassware. After preparation, samples were strained and divided for each research protocol, namely: a) added 2.5 mL of glycerol and kept at −80 °C until inoculation on solid agar media incubated at different temperatures and specific times, based on the microorganism or group of microorganisms researched, as reported below; or b) samples were kept at −20 °C until mycotoxin detection and fungal DNA extraction.

2.3. Microbiological characterization

Bacterial analysis was performed by spreading 150 μL of samples extracts in tryptic soy agar (TSA) supplemented with 0.2 % nystatin medium (Frilabo™) for mesophilic bacteria, and in Violet Red bile agar (VRBA) medium (HIMEDIA™) for coliforms (Gram-negative bacteria). Plates were incubated at 30 °C and 35 °C, respectively, and after 7 days the totality of colonies (colony-forming units, CFUs) was counted and bacterial concentrations (CFUs·g^{-1}) calculated (Viegas et al., 2020b).

Table 1
List of the commercialized tea (*Camellia sinensis* Kuntze) and other herbal infusion products analyzed in this study. Information about the sales network (H = herbalist's shop; S = supermarket), production (organic; conventional), origin (country), packaging (bulk; infusion bag), weight and composition is reported for each product.

Sample code	Sales network	Production	Origin	Packaging	Weight (gr)	Composition (label information)
01	H	Conventional	China	Bulk	50	Green tea
02	H	Organic	Azores	Bulk	200	Black tea
03	H	Organic	Azores	Bulk	100	Green tea
04	H	Organic	Azores	Infusion bag	80	Green tea
05	H	Organic	Austria	Bulk	80	White tea
06	H	Conventional	EU	Infusion bag	120	*Equisetum hyemale* L.
07	H	Conventional	EU	Infusion bag	120	*Melissa officinalis* L.
08	H	Conventional	Chile	Bulk	50	*Peumus boldus* Molina
09	H	Conventional	Poland	Bulk	100	*Echinacea* sp
10	H	Conventional	Portugal	Bulk	100	*Equisetum hyemale* L.
11	H	Conventional	Portugal	Bulk	100	*Melissa officinalis* L.
12	H	Conventional	EU	Infusion bag	60	*Echinacea* sp
13	S	Organic	EU	Bulk	90	Black tea
14	S	Organic	EU	Infusion bag	48	Black tea (99 %), aroma (1 %)
15	S	Conventional	Kenya	Infusion bag	30	Black tea with aroma
16	H	Organic	Sri Lanka	Infusion bag	30	Black tea (97 %), bergamota (3 %) with aroma
17	H	Organic	EU	Infusion bag	48	Green tea
T28	S	Organic	EU	Bulk		Black tea
19	S	Conventional	United Arab Emirates	Infusion bag	50	Black tea
20	S	Conventional	United Arab Emirates	Infusion bag	50	Black tea with aroma
21	S	Conventional	United Arab Emirates	Infusion bag	40	Black tea with aroma
22	S	Conventional	Unknown	Infusion bag	30	Black tea
23	S	Conventional	Unknown	Infusion bag	50	Black tea (97 %), bergamota (3 %) with aroma
24	S	Conventional	Unknown	Infusion bag	45	Green tea
T35	H	Organic	China	Bulk	100	Green tea (100 %)
T36	S	Organic	Unknown	Infusion bag	25	White tea (100 %)
27	S	Organic	EU	Infusion bag	48	Green tea
28	S	Organic	EU	Bulk	90	Green tea
29	S	Organic	Portugal	Bulk	50	Green tea
30	S	Conventional	EU	Infusion bag	50	Green tea
31	H	Organic	Spain	Infusion bag	45	Green tea
32	S	Unknown	Unknown	Infusion bag	60	Green tea leaves powder (5.3 %)
33	S	Conventional	EU	Infusion bag	30	Green tea
34	H	Conventional	Germany	Infusion bag	42	Green tea
HT12	H	Conventional	EU	Infusion bag	60	*Echinacea* sp
HT6	H	Conventional	EU	Infusion bag	120	*Equisetum hyemale* L.
HT10	H	Conventional	Unknown	Infusion bag	39	Green tea "100 % natural"
38	S	Conventional	Unknown	Infusion bag	39	Green tea
39	S	Conventional	Portugal	Infusion bag	45	Green tea (95 %), ginseng (5 %)
40	S	Conventional	Portugal	Infusion bag	45	Green tea (70 %), lemon (20 %), algae (10 %)

Fungal research was performed by spreading 150 μL of samples extracts in malt extract agar (MEA) supplemented with 0.05 % chloramphenicol and in dichloran-glycerol agar (DG18) media (Frilabo™). DG18 was applied to avoid overloaded plates, due to fast growth rates fungi, such as Mucorales order, allowing other fungal species identification (Viegas et al., 2017).

Plates were incubated at 25 °C for 5 to 7 days and the totality of colonies was counted (CFUs and CFUs·g^{-1}) (Pouretedal and Mazaheri, 2013). Different morphology colonies were used for slides preparation using lactophenol cotton blue mount procedures and identified through macro and microscopic characteristics as noted by De Hoog et al. (2016) (Howard et al., 2009). Whenever uncountable colonies were observed in samples, the average count for the respective culture media was considered (Viegas et al., 2020b).

Negative controls were performed to ensure the inexistence of background contamination such as in the culture media used.

2.4. Molecular detection of Aspergillus sections

The molecular detection of the *Aspergillus* sections *Fumigati, Nidulantes, Circumdati* and *Flavi* was carried out by Real Time PCR (qPCR) using the same primers as previous published (Pouretedal and Mazaheri, 2013). In summary, fungal DNA extracted from samples (8.8 mL) with ZR Fungal/Bacterial DNA MiniPrep Kit (Zymo Research, Irvine, USA) was analyzed in CFX-Connect PCR System (Bio-Rad). Reactions included 1× iQ Supermix (Bio-Rad, Portugal), 0.5 μM of each primer, and 0.375 μM of TaqMan probe in a total volume of 20 μl. Amplification was done by a three-step PCR, including 40 cycles with denaturation at 95 °C for 30 s, annealing at 52 °C for 30 s, and extension at 72 °C for 30 s. As controls, a non-template control and DNA from a reference as positive control (kindly conceded by the Reference Unit for Parasitic and Fungal Infections, Department of Infectious Diseases, National Health Institute Doutor Ricardo Jorge, IP) were sequenced for ITS, B-tubulin, and Calmodulin.

2.5. Mycotoxins analysis

Although regulated mycotoxin levels are defined in raw material, from a consumer point of view there is also interest in knowing exposure levels through beverage. As such, the analytical procedure for mycotoxin determination focused on both matrixes. A raw tea samples (1.0 g) were shaken for 60 min with 4.0 ml of acetonitrile/water/acetic acid (79:20:1, v/v/v) solution. The sample was then centrifuged for 5 min at 5000 rpm. Raw extract (0.5 ml) was diluted with 0.5 ml of water, mixed and centrifuged again for 30 min at 14,500 rpm. Brewed tea samples were prepared as described in Section 2.2. Then, 100 μl of the brewed tea samples were mixed with 700 μl of the acetonitrile/water/acetic acid mixture (49.5:49.5:1.0; v/v/v), the whole was centrifuged for 20 min at 12,000 rpm, and injected into an LC-MS/MS system.

Detection and quantification of mycotoxins was performed using high performance liquid chromatograph (HPLC) Nexera (Shimadzu, Tokyo, Japan) with a mass spectrometry detector API 4000 (Sciex, Foster City, CA, USA). Chromatographic separation was performed on a Gemini NX-C18 (150 × 4.6 mm, 3 μm) column (Phenomenex, Torrance,

CA, USA). Mobile phases were as follows: eluent A = 1 % acetic acid in water and eluent B = 1 % acetic acid in methanol (both phases contained 5 mmol/L ammonium acetate) with the following gradient: initial time of 2.0 min at 10 % B, 97 % B from 2.0 to 14.0 min, 97 % B up to 16.0 min, then column re-equilibration at 10 % B. The flow rate was set on 0.75 mL/min and the injection volume was 7 μL.

Electrospray ionization (ESI) was performed in positive and negative polarity at 4500 V or −4000 V, respectively. For each sample two separate chromatographic runs were performed in scheduled multiple reaction monitoring (sMRM) detection mode. ESI-source parameters were optimized and set to a collision gas 6 psi, curtain gas 20 psi, ion source gas 1 (nebulizer gas) 50 psi, ion source gas 2 (heater gas) 50 psi, source temperature 500oC. sMRM analysis (detection window 60 s and the target scan time 1 s) had two transitions per compound (except moniliformin). Optimized analyte-dependent MS/MS parameters are given in Table S1 – Supplementary material. Analyst 1.6.2 software was used for data acquisition.

Due to the lack of availability of internal standards for most of the analyzed compounds, the concentrations were calculated using matrix-matched calibration by adding standards to free of mycotoxins samples of tea (mix of different tea types) and brewed tea. Matrix effects (ME) were assessed by comparison of the slopes of calibration curves obtained from analyzing standards of mycotoxins prepared in a pure solvent and in a blank sample extract using the following equation: ME (%) = (slope of calibration in sample matrix / slope of calibration in pure solvent) × 100. Each calibration curve consists of seven points in the working range. The limits of detection (LOD) and quantification (LOQ) obtained for each mycotoxin were calculated from mycotoxin standards spiked into a blank sample extract. The LOD and LOQ values were calculated based on signal to noise (S/N) ratios of 3 and 10, respectively. Recoveries were evaluated in triplicate in two concentration levels by spiking free of mycotoxin samples of tea and brewed tea. Interday precision was calculated by analysis of recoveries samples on three days. Validation parameters are given in Tables S2 and S3 - Supplementary material. The Limits of Detection (LOD) obtained for each mycotoxin with the analytical method used are presented in Table S4 – Supplementary material.

2.6. Azole resistance screening

According to EUCAST guidelines, an aliquot of 150 μL sample was seeded on Sabouraud dextrose agar (SDA) (VWR Chemicals™) supplemented with 4 μg·mL^{-1} itraconazole (ITZ) (99 %, Acros Organics™), 2 μg·mL^{-1} voriconazole (VCZ) (≥98 %, Honeywell™), or 0.5 μg·mL^{-1} posaconazole (PSZ) (≥98 %, Sigma-Aldrich™), then incubated at 25 °C for 3–4 days (Arendrup et al., 2013; EUCAST, 2020). *A. fumigatus* (ATCC 204305) and pan-azole-resistant *A. fumigatus* strains (National Health Institute Doutor Ricardo Jorge, IP) were used as negative and positive controls, respectively. The presence of fungal colonies on azole-supplemented media represents presumptive evidence of azole resistance, further confirmed with additional tests for *Aspergillus* sp.

Briefly, *Aspergillus* sp. colonies from azole-supplemented media were sub-cultured in MEA, as previously described (Viegas et al., 2020a). Then, according to the manufacturer's instructions, 400 μL of the *Aspergillus* sp. inoculum was seeded on 140-mm plate containing solidified RPMI 1640 medium with 2 % dextrose (Liofilchem™). E-test gradient strips of voriconazole, posaconazole, itraconazole, and amphotericin B (AMB) (Liofilchem™) ranging from 0.002 to 32 μg·mL^{-1} were used to determine Minimal Inhibitory Concentration (MIC) after plates incubation at 35 °C for 24 h and 48 h. The E-test MIC was the lowest drug concentration at which the border of the elliptical inhibition intercepted the scale on the antifungal strip.

2.7. Statistical analysis

Data were analyzed using SPSS statistical software for Windows. The results were considered significant at the 5 % significance level. To characterize the sample, frequency analysis (n, %) and graphical representations appropriate to the nature of the data were used. To test the normality of the data, the Shapiro-Wilk test was used. To study the relationship between bacterial contamination, fungal contamination and the concentration of mycotoxins, Spearman's correlation coefficient was used, since the assumption of normality was not verified. To compare two or more independent groups regarding bacterial and fungal contamination and mycotoxin concentration, the Mann-Whitney and Kruskal-Wallis tests were used, respectively, as the assumption of normality was not verified. To compare bacterial and fungal contamination and mycotoxin concentration between the tea leaves and the infusion, the Wilcoxon test was used, since the assumption of normality was not verified. To assess species diversity, the results obtained on MEA (culture media with higher diversity) was used and Simpson and Shannon indices, given by Shannon Index $(H) = -\sum_{i=1}^{s} p_i ln(p_i)$ and Simpson Index $(D) = \frac{1}{\sum_{i=1}^{s} p_i^2}$, were employed, where p_i is the proportion (n_i/n) of individuals of one particular species found (n_i) divided by the total number of individuals found (n).

Considering the sample size, the study results obtained are only indicative.

3. Results

3.1. Viable bacterial contamination

Among all the samples collected, the highest counts of total bacteria (TSA) were obtained in green raw tea (4.75 × 10^2 CFUs·g^{-1}), followed by the group of other teas (White; White-scented; Bold leaves; Lemon balm; Horsetail and Echinacea tea - 8.50 × 10^1 CFUs·g^{-1}), and black tea (2.20 × 10^1 CFUs·g^{-1}). The highest coliform counts (VRBA) were found in samples from black raw tea (1.65 × 10^2 CFUs·g^{-1}), followed by green raw tea (6.50 × 10^2 CFUs·g^{-1}). Coliforms were absent in samples from other teas. A lower number of total bacteria counts were found in green tea beverage (1.33 × 10^2 CFUs·g^{-1}). Also, coliforms where only observed in green tea beverage (1 CFUs·g^{-1} VRBA). Higher bacteria counts were reported in beverage samples from other teas (2.70 × 10^2 CFUs·g^{-1}) and black tea (1.07 × 10^2 CFUs·g^{-1}) (Fig. 1).

Between raw and beverage, no statistically significant differences were detected in either TSA (z = −1.461, p = 0.144) or VRBA (z = −1.292, p = 0.196). Among tea types (green, black and others), statistically significant differences were only detected in black raw tea regarding bacterial counts in TSA ($\chi^2_{k\text{-}W}(2) = 14.903$, p = 0.001), black tea presenting the lowest counts. As for beverage, no statistically

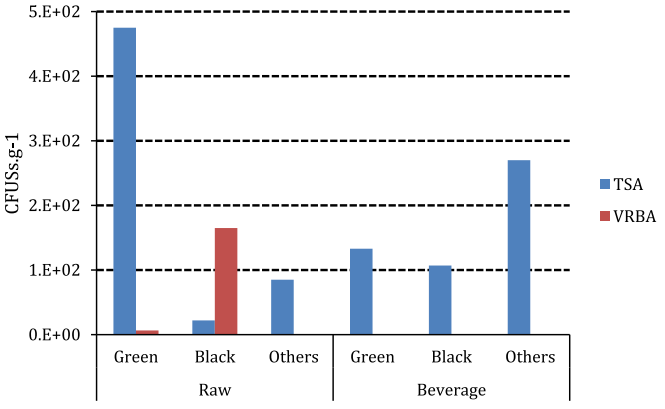

Fig. 1. Bacterial contamination on TSA and VRBA in raw and beverage tea samples. Black tea (n = 13); Green tea (n = 17); Others (White tea/Herbal tea (n = 10). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

significant differences were detected between tea types in terms of bacterial counts in TSA ($\chi^2_{K\text{-}W}(2) = 1.709$, $p = 0.425$) or in relation to coliform counts (VRBA) ($\chi^2_{K\text{-}W}(2) = 2.916$, $p = 0.233$). Also, no statistically significant differences were detected between the type of agricultural production (traditional/organic), either in the raw tea or beverage samples, regarding bacterial counts in TSA or coliform counts (VRBA) (p's > 0.05).

3.2. Viable fungal contamination

The highest fungal counts were obtained in green raw tea (6.63×10^4 CFUs·g^{-1} MEA, 2.90×10^4 CFUs·g^{-1} DG18), followed by black raw tea on MEA (6.0×10^3 CFUs·g^{-1}), and DG18 (7.0×10^3 CFUs·g^{-1}). Concerning the group of others, fungal counts were obtained in raw samples on MEA (3.33×10^3 CFUs·g^{-1}) and DG18 (5.67×10^3 CFUs·g^{-1}). Fungal counts decreased in green tea beverage (4.33×10^3 CFUs·g^{-1} MEA; 3.0×10^3 CFUs·g^{-1} DG18), compared to raw green tea. The highest fungal counts were found in beverage samples from black tea (4.0×10^3 CFUs·g^{-1} MEA; 3.33×10^3 CFUs·g^{-1} DG18) followed by other teas (1.33×10^3 CFUs·g^{-1} MEA; 6.67×10^2 CFUs·g^{-1} DG18) (Fig. 2).

The most common genera on green raw tea was *Aspergillus* sp. (4.50×10^4 CFUs·g^{-1} MEA; 2.73×10^4 CFUs·g^{-1} DG18). The same trend was obtained in raw samples from black tea on DG18 (5.0×10^3 CFUs·g^{-1}), while on MEA, *Penicillium* sp. was the predominant genera (3.3×10^3 CFUs·g^{-1}). *Chaetomium* sp. and *Penicillium* sp. were prevalent among other raw tea samples (1.7×10^3 CFUs·g^{-1} MEA; 5.0×10^3 CFUs·g^{-1} DG18 respectively). The most frequent species in green tea beverage was *Chrysonilia sitophila* (2.7×10^3 CFUs·g^{-1} MEA; 1.3×10^3 CFUs·g^{-1} DG18). Also, similar results regarding the genera were obtained in black tea beverage on MEA (3.7×10^3 CFUs·g^{-1}), while on DG18 *Cladosporium* sp. was the most found (2.7×10^3 CFUs·g^{-1}). Concerning beverage samples from other teas, *Cladosporium* sp. and *Penicillium* sp. were the prevalent genera on MEA (6.7×10^2 CFUs·g^{-1}) and DG18 (3.3×10^2 CFUs·g^{-1}) (Table 2).

Regarding raw tea, three *Aspergillus* sections were identified on DG18, namely sections *Nigri* (1.90×10^4 CFUs·g^{-1}), *Aspergilli* (5.0×10^3 CFUs·g^{-1}), *Circumdati* (2.67×10^3 CFUs·g^{-1}) in green tea, and *Aspergilli* (2.33×10^3 CFUs·g^{-1}), *Flavi* (1.67×10^3 CFUs·g^{-1}) and *Nigri* (67×10^2 CFUs·g^{-1}) in black tea (Fig. 3); on MEA, section *Nigri* was found in green tea (4.50×10^4 CFUs·g^{-1}), and sections *Flavi* (3.33×10^2 CFUs·g^{-1}) and *Circumdati* (3.33×10^2 CFUs·g^{-1}) were identified in other teas. In tea beverage samples, two sections were reported in green tea on MEA only: *Nidulantes* (3.33×10^2 CFUs·g^{-1}) and *Fumigati* (3.33×10^2 CFUs·g^{-1}).

Between raw tea and tea beverage, statistically significant differences were detected in fungal counts in MEA ($z = -3.856$, $p < 0.001$) and DG18 ($z = -4123$, $p < 0.001$), with the lowest counts in tea beverage in both media. In raw tea, statistically significant differences were also detected between tea types (green, black, others) in terms of fungal counts on MEA media ($\chi^2_{K\text{-}W}(2) = 7.232$, $p = 0.027$), with the highest counts in green raw tea. In tea beverage, statistically significant differences were detected between tea types (green, black, others) only in terms of fungal counts on MEA ($\chi^2_{K\text{-}W}(2) = 9.790$, $p = 0.007$), with green and black teas presenting the highest counts. Between tea agricultural production type (traditional/biological), no statistically significant differences were detected in fungal counts on MEA or DG18, in raw tea or tea beverage (p's > 0.05).

Regarding species diversity on MEA, raw tea showed greater diversity (Shannon index (H) = 1.393, Simpson index (D) = 2.765) compared to tea beverage. As for tea type (green, black, others), the greatest fungal diversity was observed in "others" (Shannon index (H) = 1.581, Simpson index (D) =3.682). Considering tea production, traditional agriculture led to the greatest fungal diversity (Shannon index (H) = 1.632, Simpson index (D) = 3882), compared to organic (Table S5 – Supplementary material).

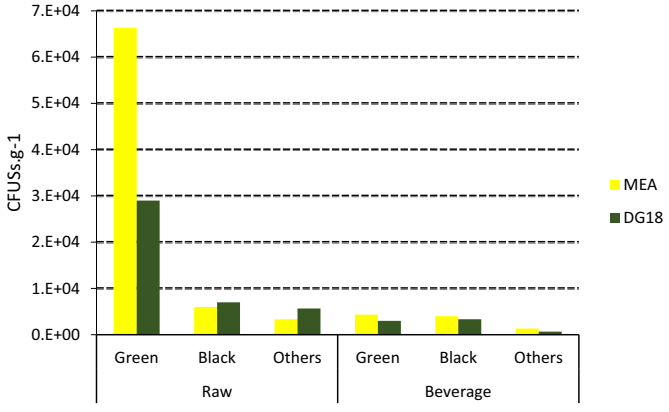

Fig. 2. Fungal contamination on MEA and DG18 in raw and beverage tea samples.
Black tea (n = 13); Green tea (n = 17); Others (White tea/Herbal tea) (n = 10). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Detection of targeted Aspergillus sections by qPCR

Two of the four targeted *Aspergillus* sections were detected: *Aspergillus* section *Fumigati* in raw tea (2 out of 40; 5 %), and *Aspergillus* section *Nidulantes* in beverage (3 out of 40; 7.5 %) and raw tea (1 out of 40; 2.5 %) samples (Table S6 – Supplementary material).

3.4. Mycotoxins detection

In the raw tea 23 of the samples (57.5 %) presented contamination by one to five mycotoxins in the same sample. The green tea presented 11 samples (47 %) contaminated, the black tea 6 samples (26.1 %) and the other types of tea also showed 6 samples (60 %) contaminated with mycotoxins.

The tea beverage, after brewing, presented only one green tea sample with contamination by one single mycotoxin (mycophenolic acid, below LoQ but higher than the LoD of 4 ng/g). The most common mycotoxin reported in raw tea samples was mycophenolic acid (14, 35 %) with values ranging from <LoQ to 66.8 ng/g. Other mycotoxins detected were: fumonisin B2 detected in 7 samples (17.5 %, <LoQ to 98.2 ng/g), sterigmatocystin in 6 samples (15 %, <LoQ to 9.8 ng/g), zearalenon in 4 samples (10 %, <LoQ to 35.4 ng/g) and monoacetoxyscirpenol, fumonisin B1, gliotoxin and ochratoxin A in 2 samples (5 %) and all below the respective LoQs. Diacetoxyscirpenol (18.2 ng/g), fumonisin B3 (<LoQ), griseofulvin (69.0 ng/g), moniliformin (<LoQ) and deoxynivalenol (<LoQ) were only detected in one sample each.

Between the raw tea and the tea beverage, it was only possible to compare the presence of mycophenolic acid, with statistically significant differences being detected ($z = -3.432$, $p = 0.001$) and the tea beverage showing lower values. Between the type of production (traditional/biological), no statistically significant differences were detected (p's > 0.05).

In raw tea, statistically significant differences were detected among tea types, as follows: i) Monoacetoxyscirpenol ($\chi^2_{K\text{-}W}(2) = 7.070$, $p = 0.029$), with lower concentrations in green and black tea; ii) Mycophenolic acid ($\chi^2_{K\text{-}W}(2) = 6.843$, $p = 0.033$), with highest concentrations in green tea and the lowest in black tea; iii) Sterigmatocystin ($\chi^2_{K\text{-}W}(2) = 6.498$, $p = 0.039$), with lower concentrations in green tea and black tea; iv) Zearalenon ($\chi^2_{K\text{-}W}(2) = 14.879$, $p = 0.001$), with lower concentrations in green tea and black tea (Table S7 – Supplementary material). In tea beverage, no statistically significant differences were detected regarding Mycophenolic acid ($\chi^2_{K\text{-}W}(2) = 3444$, $p = 0.179$) (Table S7 - Supplementary material).

Table 2
Fungal distribution per tea type from raw and beverage tea samples. Black tea (n = 13); Green tea (n = 17); Other (White tea/Herbal teas) (n = 10).

	MEA			DG18		
	Fungi	CFUS·g^{-1}	%	Fungi	CFUS·g^{-1}	%
Raw tea						
Green	*A.* section *Nigri*	4.5×10^4	67.8	*A.* section *Nigri*	1.9×10^4	65.5
	C. sitophila	1.7×10^4	261	*A.* section *Aspergilli*	5.0×10^3	17.2
	Penicillium sp.	2.0×10^3	3.0	*A.* section *Circumdati*	2.7×10^3	9.2
	Other species	2.0×10^3	3.0	Other species	2.3×10^3	8.0
Black	*Penicillium* sp.	3.3×10^3	55.6	*A.* section *Aspergilli*	2.3×10^3	33.3
	C. sitophila	2.7×10^3	44.4	*A.* section *Flavi*	1.7×10^3	23.8
				A. section *Nigri*	1.0×10^3	14.3
				Other species	2.0×10^3	28.6
Other	*Chaetomium* sp.	1.7×10^3	50.0	*Penicillium* sp.	5.0E x10^3	88.2
	Cladosporium sp.	1.0×10^3	30.0	*A.* section *Circumdati*	6.7E x10^2	11.8
	A. section *Flavi*	3.3×10^2	10.0			
	A. section *Nigri*	3.3×10^2	10.0			
Beverage tea						
Green	*C. sitophila*	2.7×10^3	61.5	*C. sitophila*	1.3×10^3	44.4
	Cladosporium sp.	1.0×10^3	23.1	*Cladosporium* sp.	1.0×10^3	33.3
	A. section *Nidulantes*	3.3×10^2	7.7	*Penicillium* sp.	6.7×10^2	22.2
	A. section *Fumigati*	3.3×10^2	7.7			
Black	*C. sitophila*	3.7×10^3	91.7	*Cladosporium* sp.	2.7×10^3	80.0
	Cladosporium sp.	3.3×10^2	8.3	*C. sitophila*	6.7×10^2	20.0
Other	*Cladosporium* sp.	6.7×10^2	50.0	*Penicillium* sp.	3.3×10^2	50.0
	Fusarium culmorum	3.3×10^2	25.0	*C. sitophila*	3.3×10^2	50.0
	C. sitophila	3.3×10^2	25.0			

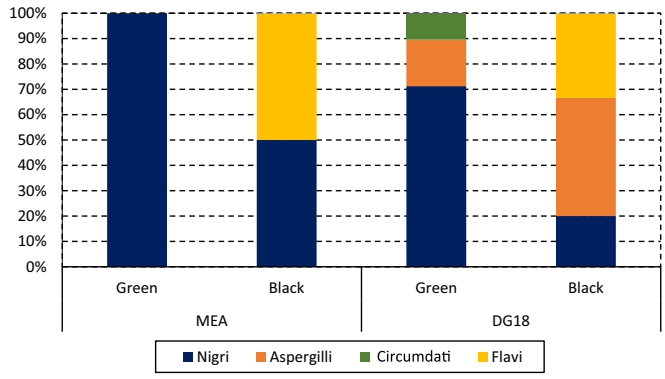

Fig. 3. Aspergillus sections in raw tea (green and black tea samples) on DG18. Black tea (n = 13); Green tea (n = 17). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. Azole-resistant mycobiota

Fungal contamination was lower on azole-supplemented media (compared to MEA and DG18), and lower in tea beverage compared to raw tea, except for green tea in Sabouraud (Fig. S1 – Supplementary material). Fungal diversity on azole-supplemented media is presented in Table S8 (Supplementary material). *Aspergillus* sp. and *Chrysonilia sitophila* were the most prevalent fungi in raw tea, mainly in green tea, whereas *Cladosporium* sp., *C. sitophila* and *Penicillium* sp. were the most prevalent in tea beverage (Table S4 - Supplementary material). *Aspergillus* sp. was present in raw green, black and other teas, and in green tea beverage, being observed on all media except posaconazole. Two *Aspergillus* sections were identified in raw tea (green, black, others), namely section *Nigri* (21.4 % on SDA; 7.5 % on ICZ) and *Circumdati* (52.5 % on ICZ), whereas *Fumigati* section was identified in green beverage tea samples only on ICZ (25.0 %) (Fig. 4).

The evaluation of the in vitro susceptibilities of one *Aspergillus* section *Flavi* isolate recovered from raw tea, and three *Aspergillus* section *Fumigati* isolates recovered from green tea beverage samples, allowed determining itraconazole, voriconazole, posaconazole, and amphotericin B E-test MICs at 24 and 48 h (Table 3).

Statistically significant differences were detected between raw tea

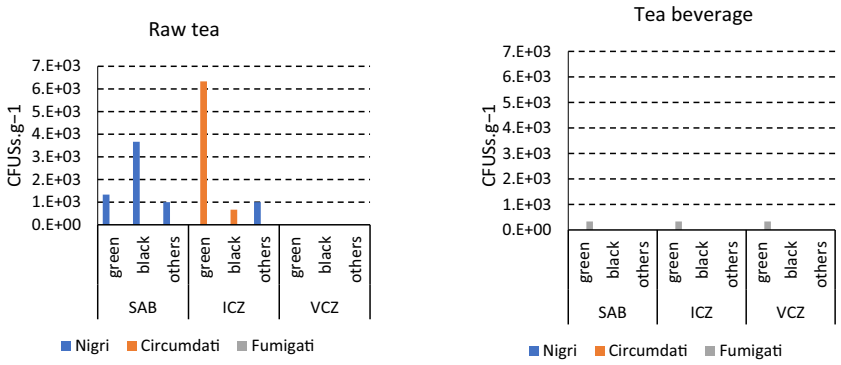

Fig. 4. *Aspergillus* sections distribution on azole-supplemented media, from raw and beverage samples of green (n = 17), black (n = 13) and other (n = 10) teas. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
MIC values (μg·mL^{-1}) for *Aspergillus* sp. isolates by Etest.

Code	Fungi	Tea type	Origin	Sample of origin	24 h				48 h			
					ICZ	VCZ	PSZ	AMB	ICZ	VCZ	PSZ	AMB
E11	*A.* section *Flavi*	Others	Portugal	Raw, SDA	–	–	–	–	–	–	–	–
31	*A.* section *Fumigati*	Green	Spain	Beverage, SDA	–	–	–	–	0.064	0.04	0.032	–
35	*A.* section *Fumigati*	Green	China	Beverage, MEA	–	–	–	–	0.38	0.006	0.08	0.125
35	*A.* section *Fumigati*	Green	China	Beverage, ICZ	–	–	–	–	0.75	0.008	0.125	–

ICZ, itraconazole; VCZ, voriconazole; PSZ, posaconazole; AMB, amphotericin B. MIC90 values (μg·mL^{-1}) for *Aspergillus* section *Fumigati* at 48 h, per antifungal agent: ICZ = 0.5; VCZ = 1.0; PSZ = 0.12; AMB = 2.0 (Espinel-Ingroff et al., 2002). Bold face, E-test MIC values above MIC90. Black tea (n = 13); Green tea (n = 17); Others (n = 10).

and tea beverage in all azole-resistance screening media (p's < 0.05), with lower values in tea beverage (Table S9 – Supplementary material).

Statistically significant differences were detected between tea types during azole resistance screening, with higher values for green raw tea, as follows: raw tea (ITZ ($\chi^2_{K\text{-}W}(2) = 8.269$, $p = 0.016$) and PSZ ($\chi^2_{K\text{-}W}(2) = 12.518$, $p = 0.002$)); tea beverage (SDA ($\chi^2_{K\text{-}W}(2) = 21.890$, $p < 0.001$) (Table S10 – Supplementary material).

3.6. Correlation analysis

In this sample, several correlations were observed in raw tea, namely, between higher microbial counts and per medium. Regarding mycotoxins occurrence in raw tea, higher concentrations of different mycotoxins were also related with microbial counts and types of teas. Considering tea beverage different relations were observed (Table 4). Full text of correlation analyses – Supplementary material).

4. Discussion

The Sustainable Development Goals suggested by World Health Organization (WHO), and more specifically the goal 2, emphasizes the need to end hunger, achieve food security and improved nutrition and promote sustainable agriculture (https://sdgs.un.org/goals). The long-term goal to achieve developing sustainable food systems is also considered a priority for different intergovernmental organizations (Bebber et al., 2013; Fisher et al., 2012). However, crops such as tea are at risk due to climate changes that intensely promote fungal pathogens. In fact, fungal diseases have increased together with climate change, and it is progressively recognized as a worldwide threat to important crops (Bebber et al., 2013; Fisher et al., 2012).

As expected the tea beverage presented lower bacterial contamination (TSA and VRBA) than raw, following the trend of a previous study performed in green tea samples available in Portuguese markets (Viegas et al., 2020c). However, beverage revealed that there is a possibility of having viable bacterial spores, even at the water boiling temperature, and thus present at the time of beverage consumption (Dayananda et al., 2017; Viegas et al., 2020b). Following also the trend of a previous publication that compared green with black tea samples, raw green tea samples were the ones presenting higher bacterial and fungal contamination (Carraturo et al., 2018), leading also to a higher fungal diversity. However, none of the analyzed tea samples (raw and beverage) exceeded the suggested limit of 10^5 CFUs·g^{-1} for filamentous fungi (Scientific Committee on Foods, 2016; THIE, 2016).

Nutrients competition between fungi and bacteria can be the reason why higher bacterial counts in raw are related with lower fungal counts (Mille-Lindblom et al., 2016). However, a wide range of environmental and genetic factors contribute for the difficulty to address the complexity of a microbial community and the competition among different species and/or strains (Bauer et al., 2018).

Although the fungal counts were higher in black tea beverage (ready for consumption), *Aspergillus* sp. was only present in green tea beverage and was the most common found also in raw green tea. Similar results were previously obtained regarding *Aspergillus* genera dominance in green tea samples (Carraturo et al., 2018; Viegas et al., 2020b).

Different agricultural management systems may have an impact on the sustainability of food systems, since they can affect human and animal health, food security and safety and environmental sustainability (Mie et al., 2017). Sustainable agriculture systems are based on small, cost-effective farms that incorporate animal and plant production, maintain the biodiversity (including the microbial diversity), highlight technologies that are appropriate to the scale of production, and ensure the transition to renewable forms of energy (Horrigan et al., 2002). However, in this study tea with conventional production presented higher fungal diversity than the organic. This can be due to several other environmental variables that influence the fungal growth, such as unsuitable storage, handling and packaging conditions (Dayananda et al., 2017; Sedova et al., 2018; Viegas et al., 2020b).

In our study, 57.5 % of the raw tea samples showed mycotoxins contamination. Previously, a similar study developed in Morocco and dedicated to green tea also observed 56 % of the samples contaminated with at least one mycotoxin, although with a different pattern of mycotoxins (Jai et al., 2021). In our study the most reported mycotoxin was the mycophenolic acid (in 35 % of the samples) but the highest concentration measured was obtained for fumonisin B2 (98.2 ng/g). The frequent contamination found by mycophenolic acid is explained by the fact that *Penicillium* sp. was one of the most predominant genera in the raw samples. This was the case in a previous study also developed in Portugal (Assunção et al., 2021), that considered 20 samples of commercialized green tea (10 bulk samples and 10 bag samples) and where mycophenolic acid was also the most reported mycotoxin, together with sterigmatocystin (Assunção et al., 2021). This potentially immunosuppressive mycotoxin can origin immune dysregulation that in the long term may be related to increased oncologic morbidity and susceptibility to infections (Vaali et al., 2022).

Concerning fumonisin B2 different fungal genera are considered to be producers, being *Fusarium* the most reported (EFSA, 2018a). This mycotoxin brings some concerns due to the various and serious adverse health outcomes such as cancer and birth defects (WHO, 2018).

In our study, 2 samples of raw tea presented contamination by OTA, the mycotoxin that is already regulated in several food commodities and that is expected to be regulated also for teas (Assunção et al., 2021). This mycotoxin is produced by several fungal species including *Aspergillus ochraceus, A. carbonarius, A. niger* and *Penicillium verrucosum* and causes nephrotoxicity and renal tumors in a variety of animal species. However, human health effects are less well-characterized and future epidemiological studies to better establish the causal link between exposure and health effects are needed (Bui-Klimke and Wu, 2015).

Nevertheless, we should consider other factors that influence mycotoxins contamination in tea, besides the presence of the natural producers such as temperature and humidity. Therefore, the climate change scenario is already, in some parts of world, or will have an important role in the mycotoxin's contamination and, consequently, in human exposure (Assunção et al., 2018; Martins et al., 2019). Human health effects related with this climate change scenario can also emerge due to the changes in mycotoxins exposure pattern (Assunção et al., 2018; Martins et al., 2019).

Table 4

- Bivariate descriptive statistics: Study of the relationship between bacterial, fungal and mycotoxin contamination in raw tea and tea beverage. Results of Spearman's correlation coefficient.

			Leaves																			Infusion							
			Bacteria	Fungi		Azole resistance screening				Mycotoxins												Bacteria	Fungi		Azole resistance screening				Mycotoxins
			VRBA	MEA	DG18	DAS	ITZ	VCZ	PSZ	1	2	3	4	5	6	7	8	9	10	11	12	TSA	VRBA	MEA	DG18	DAS	ITZ	VCZ	PSZ	8
Leaves	Bacteria	TSA	0.237	-0.085	-0.014	-0.223	-0.339	-0.350	-0.626	-0.130	-0.091	-0.293	-0.473	-0.081	-0.091	-0.293	-0.301	-0.022	-0.005	-0.091	-0.189	-0.113	-0.373	-0.548	-0.231	-0.011	-0.035	-0.092	-0.284	-0.091
		VRBA		-0.245	-0.352	-0.080	-0.016	-0.138	-0.018	-0.096	-0.067	-0.096	-0.192	-0.264	-0.335	-0.096	-0.324	-0.011	-0.096	-0.335	-0.085	-0.159	-0.123	-0.040	-0.009	-0.012	-0.087	-0.018	-0.010	-0.067
	Fungi	MEA			-0.689	-0.742	-0.550	-0.270	-0.519	-0.045	-0.134	-0.015	-0.178	-0.152	-0.198	-0.015	-0.251	-0.030	-0.283	-0.198	-0.247	-0.196	-0.104	-0.278	-0.107	-0.309	-0.323	-0.097	-0.373	-0.198
		DG18				-0.558	-0.395	-0.289	-0.366	-0.265	-0.185	-0.025	-0.081	-0.249	-0.185	-0.025	-0.326	-0.155	-0.066	-0.185	-0.384	-0.070	-0.134	-0.268	-0.001	-0.084	-0.266	-0.066	-0.124	-0.277
	Azole resistance screening	DAS					-0.549	-0.383	-0.497	-0.027	-0.196	-0.162	-0.204	-0.184	-0.234	-0.162	-0.005	-0.122	-.335	-0.234	-0.274	-0.030	-0.360	-0.277	-0.034	-0.031	-0.259	-0.052	-0.223	-0.234
		ITZ						-0.475	-0.737	-0.221	-0.154	-0.287	-0.422	-0.022	-0.154	-0.287	-0.211	-0.112	-0.221	-0.154	-0.320*	-0.011	-0.320	-0.550	-0.165	-0.339	-0.337	-0.322	-0.388	-0.154
		VCZ							-0.442	-0.148	-0.103	-0.074	-0.301	-0.142	-0.103	-0.074	-0.019	-0.161	-0.148	-0.103	-0.215	-0.020	-0.215	-0.281	-0.244	-0.050	-0.367	-0.276	-0.419	-0.103
		PSZ								-0.290	-0.203	-0.163	-0.412	-0.081	-0.203	-0.163	-0.150	-0.118	-0.290	-0.203	-0.421	-0.071	-0.245	-0.667	-0.282	-0.369*	-0.342	-0.297	-0.397	-0.203
	Mycotoxins	1									-0.698	-0.053	-0.105	-0.053	-0.037	-0.053	-0.037	-0.451	-0.053	-0.037	-0.678	-0.390	-0.076	-0.330*	-0.041	-0.265	-0.076	-0.096	-0.096	-0.037
		2										-0.037	-0.073	-0.037	-0.026	-0.037	-0.120	-0.315	-0.037	-0.026	-0.453	-0.280	-0.053	-0.230	-0.130	-0.185	-0.053	-0.067	-0.067	-0.026
		3											-0.571	-0.053	-0.037	-1.000	-0.172	-0.105	-0.053	-0.037	-0.076	-0.262	-0.076	-0.022	-0.186	-0.064	-0.076	-0.096	-0.096	-0.037
		4												-0.210	-0.073	-0.571	-0.092	-0.126	-0.105	-0.073	-0.153	-0.242	-0.153	-0.238	-0.107	-0.188	-0.076	-0.006	-0.354	-0.073
		5													-0.037	-0.126	-0.210	-0.053	-0.037	-0.076	-0.180	-0.076	-0.214	-0.041	-0.101	-0.306	-0.096	-0.265	-0.037
		6														-0.032	-0.388	-0.037	-1.000	-0.520	-0.151	-0.053	-0.015	-0.130	-0.185	-0.053	-0.067	-0.067	-0.026
		7															-0.172	-0.105	-0.053	-0.037	-0.076	-0.262	-0.076	-0.022	-0.186	-0.064	-0.076	-0.096	-0.096	-0.037
		8																-0.260	-0.103	-0.232	-0.129	-0.049	-0.327	-0.012	-0.119	-0.126	-0.262	-0.092	-0.026	-0.264
		9																	-0.105	-0.388	-0.741	-0.269	-0.092	-0.235	-0.132	-0.257	-0.076	-0.006	-0.189	-0.073
		10																		-0.037	-0.076	-0.308	-0.076	-0.154	-0.186	-0.265	-0.076	-0.096	-0.096	-0.698
		11																			-0.520	-0.151	-0.053	-0.015	-0.130	-0.185	-0.053	-0.067	-0.067	-0.026
		12																				-0.195	-0.146	-0.341	-0.101	-0.384	-0.111	-0.139	-0.140	-0.053
Infusion	Bacteria	TSA																					-0.076	-0.018	-0.192	-0.092	-0.116	-0.175	-0.009	-0.244
		VRBA																						-0.342	-0.270	-0.028	-0.111	-0.139	-0.140	-0.053
	Fungi	MEA																							-0.213	-0.285	-0.312	-0.237	-0.463	0.015
		DG18																								-0.142	-0.059	-0.419	-0.199	-0.130
	Azole resistance screening	DAS																									-0.092	-0.021	-0.255	-0.185
		ITZ																										-0.303	-0.577	-0.053
		VCZ																											-0.214	-0.067
		PSZ																												-0.067

1. Monoacetoxyscirpenol. 2. Diacetoxyscirpenol. 3. Fumonisin B1. 4. Fumonisin B2. 5. Gliotoxin. 6. Griseofulvin. 7. Ochratoxin A. 8. Mycophenolic acid. 9. Sterigmatocystin. 10. Moniliformin. 11. Deoxynivalenol. 12. Zearalenon.

Note: Negative correlations are marked with a green graduation. Positive correlations are marked with a red graduation. The stronger the intensity of the relationship, the more intense the colour.

As it was possible to observe, raw tea samples presented higher mycotoxins contamination than the samples of tea after being brewed. As mentioned by Sedova et al. (2018), a number of parameters impact mycotoxin concentration in tea beverages such as raw tea contamination level, mycotoxin thermal stability, and its ability to transfer from the matrix into aqueous infusions. The brewing does not degrade mycotoxins in a significant manner but the rate of transfer from the raw tea to the beverage will depend of the type of mycotoxin (Kabak, 2009; Sedova et al., 2018). Indeed, different rates are described in the literature depending of the mycotoxin studied (Sedova et al., 2018). Of note is also the fact of several raw tea samples showed the presence of multiple mycotoxins. The same feature was already reported in the study

developed by (Jai et al., 2021). In fact, this is a common feature in the case of mycotoxins contamination that brings challenges concerning possible interactions between mycotoxins and the risk that they can pose to human health when exposure happens to several mycotoxins simultaneously (Assunção et al., 2016; Viegas et al., 2018). Therefore, besides the lack of regulation for the mycotoxins found in raw tea samples, the common presence of a mixture should be considered when regulating and defining limits for the mycotoxin's presence in tea. Moreover, these results also emphasize the possible impact that raw tea contamination can have in the workplaces where it is being handled for processing, and where workers can also be exposed. Therefore, besides the need of considering consumer exposure that, fortunately, the brewing process seems to contribute to reduce contamination, future research work should be developed with the aim of assessing workers' exposure to mycotoxins in the workplaces where tea is processed and prepared to be distributed for selling (Viegas et al., 2020c).

In light of our results regarding fungal contamination mycotoxin's contamination were expected in the samples analyzed. Indeed, it was possible to detect, by culture dependent and independent methods, in raw and beverage, fungal contamination with toxigenic potential. In addition, with molecular tools it was possible to reveal fungal contamination that was not observed through culture-based methods. Thus, as stated in previous studies dealing with tea (Viegas et al., 2017, 2020b) samples, culture dependent and independent methods should be used side by side to obtain a more precise fungal contamination characterization.

Finally, azole resistance regarding *Aspergillus* section *Fumigati* is an emerging health problem in Europe due to the increased probability of treatment failure of fungal infections with currently available azole drugs (Howard et al., 2009; Van der Linden et al., 2011; Verweij et al., 2012). As Europe leads the worldwide usage of agricultural azole fungicides (40 %), followed by Japan and Latin America (Dehne et al., 2007), concerns on crossed-resistance to azoles have been raised and extensively reported in recent years (Verweij et al., 2016; Fisher et al., 2018). Because the number of serious infections caused by *Aspergillus* spp. has been increasing, especially among immunocompromised individuals, and resistance to established agents has been vastly reported (Denning et al., 1997; Espinel-Ingroff, 1998; Lass-Florl et al., 1998), it is necessary to determine the in vitro susceptibilities of *Aspergillus* isolates to therapeutic agents. In that behalf, the present study wanted to screen for azole resistance among commercially available teas in Portugal, for an exploratory estimate of exposure risk to fungi more adapted, hence, potentially less responsive to current clinically available azoles.

The results obtained in this study revealed that the risk due to the current exposure to azole-resistant fungi through tea beverage consumption in Portugal is reduced. Although *Aspergillus* sp. was the most prevalent species in raw teas, its prevalence was greatly reduced after tea beverage preparation, due to exposure to high temperatures of boiling water. Nevertheless, green tea seems to represent a higher risk as higher loads of *Aspergillus* sp. were found both in raw green tea and green tea beverage on itraconazole and voriconazole, including *Aspergillus* section *Fumigati* in green tea beverage on itraconazole.

In our study, the solidified RPMI 1640 medium supported growth of three of four *Aspergillus* isolates at 48 h (the exception was isolate of *A.* section *Flavi*), with no growth observed at 24 h. Trailing growth (presence of growth above the MIC) was not a major problem for most of the antifungal agents tested. The exception was one *Aspergillus section Fumigati* isolate from green tea beverage recovered form itraconazole-SDA medium, presenting itraconazole and posaconazole E-test MICs above MIC90 values defined for *Aspergillus fumigatus* at 48 h (Espinel-Ingroff and Rezusta, 2002). Noteworthy, this sample was from biological origin, i.e., allegedly produced with no azole fungicides.

5. Conclusions

This study claim attention to the necessary investment in researching biological hazards and azole-resistance associated with the production and consumption of tea (in particular green tea) and other food commodities (such as coffee) in Portugal. Despite the uncertainties regarding risk characterization in the present study, due to constraints such as the low number of analyzed samples and knowledge on the related health effects, our findings open further discussion regarding tea contamination and the possible exposure scenarios. In fact, from production to consumption exposure prevention of workers and consumers need a holistic approach such as the One Health approach. Additionally, the climate change scenario might influence the contamination pattern of tea, presenting a new challenge that is important to be tackled through the One Health approach.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

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