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ORIGINAL ARTICLE

A preliminary study on mycobiota and ochratoxin a contamination in commercial palm dates (*Phoenix dactylifera*)

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Abstract

The occurrence of mycotoxins and mycotoxigenic fungi in palm dates has not been thoroughly documented. The aims of the present study were to identify the mycobiota present in commercial date samples (n = 19), to determine the ability of the isolated fungi to produce mycotoxins, and to determine and quantify the presence of OTA in date fruits. The majority of products originated from Tunisia (n = 14) and Algeria (n = 3). The dominant fungal species were *Aspergillus niger*, *Aspergillus tubingensis* and *Aspergillus flavus* which were most frequently found in premium quality and organic produce, produced without chemical preservatives. OTA was found in only one sample at a concentration of 0.75 µg/kg, as determined by HPLC with fluorescence detection. Although this preliminary study did not find elevated levels of OTA, its presence in one out of 19 samples indicates that palm dates for human consumption require continuous and stringent control, in order to prevent contaminated produce from entering the market.

Keywords Dried fruits · Mycotoxins · Food safety · *Aspergillus* · *Penicillium*

Introduction

Dried fruits are currently important foods throughout the world, as they are sources of diverse essential nutrients with potential functional properties. Fruits such as dates, prunes, apricots, figs and raisins are of major significance socially and economically for the Mediterranean populations (Ozer et al. 2012). The fruits of the date palm, in particular, contain a high percentage of sugars (44–88%), protein (2.3–5.6%) and dietary fibre (6.4–11.5%), high diversity of salts and minerals and low fat (0.2–0.5%) (Al-Shahib and Marshall 2003). Dates are also a good source of vitamins, mostly A and group B (Zaid and de Wet 2002). Dates are mainly produced in the desert of Southwest Asia and North Africa, but they are marketed worldwide as a high-value fruit crop, accounting for almost half of the 2 million tonnes of dried fruits traded worldwide (Calcagni 2018). In Europe, the Mediterranean countries France, Italy and Spain are the most important consumers of dried dates, with a yearly intake per capita of 140 to 340 g (FAOSTAT 2021). In Portugal, it is estimated that 17% of the Portuguese population consumes dried dates regularly (Ribeiro and Fernandes 2017), with an estimated intake of around 50 g/capita/year in 2017, but a two-fold increase was observed from 2017 to 2018 (FAOSTAT 2021). Date consumers are no longer limited to traditional ethnic population but are increasing among younger consumers (millennials) that follow healthy living trends (CBI 2020).

Dates mature through three stages during which colour, flavour, texture and microbial profile evolve (Shenasi et al. 2002a): *Kimri*, an inedible green fruit; then *Rutab*, a soft brown stage; and later *Tamr*, with dark brown colouration acquired after harvest and drying (Shenasi et al. 2002a). Throughout these stages, microbial counts also change; they are high at the *Kimri* stage, increase at the *Rutab* stage, and then significantly decrease at the final dried *Tamr* stage. Throughout all the three maturation stages, it is also possible that fungi develop and produce mycotoxins (Shenasi et al. 2002a, b). Given that these fruits are marketed not only as dried fruits for direct consumption but are also intensively incorporated in many food applications (CBI 2020), they could represent an important human dietary source of mycotoxins.

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According to previous surveys, it is noticeable that dried fruits are generally susceptible to fungal contamination and growth, and consequent mycotoxin production.In dried fruits, mycotoxin contamination has been mostly studied on aflatoxins (AFs) in dried figs, and on ochratoxin A (OTA) in dried vine fruits (e.g. Aksoy et al. 2007; Bircan and Koç 2012; Heperkan et al. 2012; Heshmati et al. 2017; Iamanaka et al. 2007), but mycotoxin contamination in dried dates has been poorly studied (Azaiez et al. 2015; Gherbawy et al. 2012; Ozer et al. 2012; Quaglia et al. 2020; Rahimi and Shakerian 2013;). Although the EU regulation 1881/2006 (EC 2006a) does not include OTA maximum admissible levels for dried palm dates, the latest EFSA report on OTA risk assessment reports dried fruits (including dates) among the most important contributors to the chronic dietary exposure of European populations to OTA, mostly in children and toddlers, after meat products, cheese and grain products (EFSA 2020). As a matter of consequence, palm dates should be given extra attention in the global food safety and security context, as they can be regarded not only as an increasing food in healthy diets but also as a good nutrient source able to supply the increased food demand expected for the years to come.

It is thus necessary to acknowledge the importance of toxigenic fungi in palm dates and their ability to produce mycotoxins, in particular OTA, and the level of contamination of commercial dates. This knowledge will be critical to establish a risk assessment as well as to develop preventive or corrective measures in the date chains, especially during drying and storage.

In this scenario, the main objectives of this study were to identify the mycobiota present in date samples commercialized in Portugal, to determine the ability of the isolated fungi to produce OTA and to determine and quantify the presence of OTA in the samples.

Methods and materials

Sampling plan

Nineteen samples of dried date fruits (200 g to 250 g) representative of all types of dates available in the region were purchased in different markets in Portugal between April and August 2018. Samples characteristics are shown in Table S1.

Fungal isolation from date samples

In order to have a full understanding of the diversity of the native fungal species and to increase the sensitivity of fungi detection and isolation, two different methods of plating were used: serial dilutions and direct plating. Fruits were randomly taken from the package, cut in small pieces (without surface disinfection) and mixed. For the serial dilution method, 10 g were homogenized for 1 min with 90 mL of peptone water and further serially diluted. One hundred microlitres from each dilution were spread (in duplicate) in 9-cm Petri dishes containing 20 mL of Dichloran rose Bengal chloramphenicol (DRBC, Liofilchem). For the direct plating method, fruit pieces (10 g) were placed directly onto the DRBC in 9-cm Petri dishes (in duplicate). Plates were incubated in the dark, at 25 °C, for 5 to 7 days. Monosporic cultures were obtained from representative colonies and used for further testing.

Identification of the isolates

Phenotypic characterization

Moulds were subjected to a preliminary morphological identification. Spores of 7-day-old cultures on malt extract agar (MEA, HiMedia) were inoculated by 3-point inoculation in 9-cm Petri dishes containing MEA and Czapek Yeast Autolysate agar medium (CYA; HiMedia) and were analysed after incubation for 7 days in the dark, at 25 °C for the following characters: colony size and texture, obverse and reverse colony colour, diffusible pigments and exudate production. The isolates were separated into 29 morphologically identical groups (morphotypes), and one representative isolate of each morphotype was used for molecular identification.

Isolates were screened for OTA production in coconut agar medium (CAM) as previously described (Heenan et al. 1998). For the isolates showing fluorescence on CAM, OTA production was confirmed by HLPC with fluorescence detection, following Vipotnik et al. (2017).

Molecular identification

Twenty-two isolates (representative of the morphotypes) selected for molecular identification were identified as follows. DNA was extracted by the SDS extraction protocol as described by Rodrigues et al. (2018). Moulds were identified by the ITS1/58S/ITS2 region of the rRNA gene (ca. 500 bp) using the primers ITS1-F/ITS4 (Gardes and Bruns 1993; White et al. 1990) or by a portion of the calmodulin gene (*cmdA*; comprising exons 1 to 5 and introns 2 to 5; ca. 830 bp) using the primers CL1/CL2A (O'Donnell et al. 2000). PCR products were purified with the commercial kit GF-1 PCR CleanUp Kit (Vivantis) as described by the manufacturer.

Sequence analyses were carried out on an ABI 3730xl DNA Analyzer (Applied Biosystems). PCR products were sequenced in both directions, and a consensus sequence was assembled using the package Sequencher 4.9—Build 4751 (Gene Codes, Ann Arbor Michigan). The consensus sequences were adjusted using the programme BioEdit

sequence alignment editor 7.0.5.1 and then aligned with the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/) using the BLAST algorithm. Sequences were deposited in NCBI GenBank with the accession numbers MW940547 to MW940550 (ITS) and MW962279 to MW962295 (calmodulin) (Table S2).

Detection and quantification of OTA in dates

Ten grams of dates were homogenized in 20 mL of 70% methanol for 20 min in a magnetic stirrer. Five millilitres of filtered methanolic extract were collected into a 25-mL measuring cylinder, and 20 mL of PBS-T (PBS/0.01% Tween 80) were added and mixed well. The diluted extract was filtered with a microfibre glass filter and 20 mL of this extract (= 1 g sample) were passed through OchraTest™ immunoaffinity column (IAC). The column was then washed with 10 mL of PBS-T, followed by 10 mL ultra-pure water. OTA was eluted with 1.5 mL HPLC-grade methanol with 2% acetic acid (98:2; v:v). OTA was quantified by HPLC with fluorescence detection, as previously described (Vipotnik et al. 2017).

For method validation, recovery rates, limit of detection (LOD), limit of quantification (LOQ) and linearity were determined taking into account the harmonised guidelines for in-house method validation presented in the Commission Regulation (EC) No 401/2006 (EC 2006b). The OTA working solution (C = 1000 ng/mL) was prepared in methanol (MeOH) using commercial standard OTA (Sigma) and stored at − 20 °C until use. Linearity, LOD and LOQ were determined by three series of analyses, using 11 standard solutions with concentrations ranging from 0.05 to 100 ng/mL. LOD and LOQ were calculated according to Taverniers et al. (2004). For recovery tests, the extraction procedure previously described was applied to dates spiked with 10 ng of OTA per gram of sample. Spiked samples were left at 4 °C overnight for toxin incorporation previously to extraction. This procedure was made in triplicate. Recovery rates of OTA were calculated as the ratio of recovered mycotoxin concentration relative to the spiked concentration. Precision was calculated in terms of intra-day repeatability (n = 3).

Statistical analysis

Fungal occurrence was determined as the percentage of samples contaminated with a given species. Fungal frequency was determined as the percentage of isolates of a given species compared with the total number of isolates. The effect of variety, origin, format, packaging and preservatives on the total fungal counts and *Aspergillus* sect. *Nigri* counts was assessed by a generalized linear model following a Poisson distribution and log link function. The analysis was developed using the IBM SPSS Statistics version 22.

Results and discussion

Mycobiota profile of date samples

The total number of fungal isolates was 70, obtained from 10 out of 19 samples. No isolates were obtained from samples S01, S02, S03, S06, S07, S08, S15, S16 and S17. Twenty-one isolates were identified from the 29 morphotype representative isolates (Table S2). The remaining 8 isolates were identified as *Aspergillus niger*/ *Aspergillus tubingensis* but were not confidently discriminated by the calmodulin gene sequence. *Aspergillus niger* aggregate is further used in this study to refer to this group of isolates identified as *A. niger*/*A. tubingensis*.

Table 1 shows the results for total fungal counts, total *Aspergillus* sect. *Nigri* counts and occurrence of each species in the samples. In the present study, only *Aspergillus* and *Penicillium* genera were detected in the samples. *Aspergillus* species consisted of 83% of the total isolates, while *Penicillium* species accounted for 17%. *Aspergillus* section *Nigri* isolates were the most common in the *Aspergillus* genus (70% of total isolated fungi, occurring in 42% of the samples), while *A. flavus* represented 10% of total isolates, with 26% occurrence. *Penicillium citrinum* was the most frequently isolated species from the genus *Penicillium* (10% of total isolates) but was detected in only 2 samples. The remaining species were rarely isolated. Previous studies on fungal contamination of palm dates report similar results (Abdel-Sater and Saber 1999; Azaiez et al. 2015; Gherbawy et al. 2012; Nass 2017; Quaglia et al. 2020). *Aspergillus* section *Nigri* are distributed worldwide and are regarded as common food spoilage fungi (Ismail 2017; Cabañes and Bragulat 2018), and *A. tubingensis* has been frequently reported in Mediterranean countries (Pantelides et al. 2017; Gil-Serna et al. 2019; Quaglia et al. 2020).

As presented in Table 1, most samples showed low or undetectable fungal loads, while three samples (S04, S12 and S13) had a significantly higher frequency of contamination for both fungal counts and *Aspergillus* section *Nigri* ($p = 0.000$). Figure 1 shows the difference in fungal diversity and cumulative number of isolates (average/sample) in samples with and without chemical preservation. As set in the samples' label (Table S1), those three samples were labelled as bioproducts or extra-quality, with no chemical additives, and the remaining was treated with chemical preservatives and stabilisers such as sorbitol (E420ii), sulphites, benzoic acid, citric acid and potassium sorbate. The absence of preservatives significantly influenced the fungal counts, but no significant differences were registered between the different types of preservatives ($p = 0.000$; data not shown).

The relatively low contamination levels reported in the analysed samples are probably due to the strict regulations

Table 1 Compilation of results for fungi in 10 samples which were found to be positive for at least one species

Sample	Total number of fungi (CFU/g)	Total number of black *Aspergillus* (CFU/g)	*Aspergillus*						*Penicillium*				Total number of isolates
			A. niger aggregate	*A. niger*	*A. tubingensis*	*A. flavus*	*A. clavatus*	*A. ochraceus*	*P. glabrum*	*P. digitatum*	*P. chrysogenum*	*P. citrinum*	*P. novaezeelandiae*	
S04	260	260	3	3	13	0	0	0	0	0	0	0	0	19
S05	22	0	0	0	0	0	0	0	1	2	0	0	0	3
S09	49	48	4	2	0	1	0	0	0	0	0	0	0	7
S10	38	32	4	0	0	3	1	0	0	0	1	0	0	9
S11	30	30	1	2	0	0	0	0	0	0	0	0	0	3
S12	350	340	4	1	0	1	0	0	0	0	0	1	0	7
S13	550	440	5	1	0	1	0	0	0	0	0	6	0	13
S14	17	7	2	0	0	0	0	1	0	0	0	0	0	3
S18	20	15	1	0	2	0	0	0	0	0	0	0	1	4
S19	15	7	0	0	1	1	0	0	0	0	0	0	0	2

in the European Union, which prevent highly contaminated samples from entering the markets.

Considering the mycotoxigenic profile of the isolated fungi, from the 46 isolates tested in CAM, only *P. citrinum* and *Aspergillus ochraceus* showed a green fluorescence under UV light. These two isolates as well as seven isolates representative of *A. niger* aggregate (who tested negative for OTA on CAM) were also tested by HPLC to confirm the ochratoxigenic profile. Only *A. ochraceus* was confirmed to produce OTA, whereas all *A. niger* aggregate isolates confirmed to be negative. This result is in agreement with previous studies reporting that *A. tubingensis* is a non-ochratoxigenic species, while only few isolates of *A. niger* are able to produce OTA (Abarca et al. 2004; Gil-Serna et al. 2019; Quaglia et al. 2020). *Aspergillus flavus* were also considered non-aflatoxigenic (only screened on CAM). These results are lower than others previously reported. Gherbawy et al. (2012) reported that 18 out of 80 *A. flavus* isolates (39%) were aflatoxigenic, while nine out of 36 isolates of *A. niger* (25%) were ochratoxigenic. Quaglia et al. (2020) reported that the only *A. flavus* isolated from dried dates was able to produce aflatoxins B1 and B2, while none of the 16 *A. tubingensis* produced OTA.

OTA contamination of date samples

The method of extraction and quantification of OTA in palm dates was validated with high precision ($y = 820002x + 444{,}102$; $r^2 = 0.998$). The recovery rate of OTA in spiked date samples at a concentration of 10 µg/kg was 94.8%, with RSDr of 0.9. LOD and LOQ were calculated as 0.17 ng/mL and 0.58 ng/mL, respectively. From the 19 samples analysed for OTA contamination, only sample S12 (no additives, extra-quality) showed detectable levels of OTA, at a concentration of 0.75 µg/kg. Although no maximum tolerable limit of OTA has been established for dried dates, this value is under the limit set for dried grape fruits (10 µg/kg) set by the European Commission (EC 2006a).

Iamanaka et al. (2005) observed OTA contamination up to 5 µg/kg in 2 out of 20 dried date samples sold in Brazil. Azaiez et al. (2015) reported that OTA was detected in 38% of the Tunisian date samples (mean 1.26 µg/kg), with the highest detected level of 3.34 µg/kg. This study revealed a higher mycotoxin incidence in date samples collected from Tunisia in comparison to those collected from Spain, although more than 80% of the latter were imported from Tunisian markets (Deglet Nour variety). This could be due to the samples purchased in Tunisia having different quality classes, as some of them belonged to low quality categories and were intended for use as feed. Indeed, the contamination profile of the dates belonging to the variety "Deglet Nour" purchased in Tunisia was similar to those purchased in Spain (Azaiez et al. 2015).

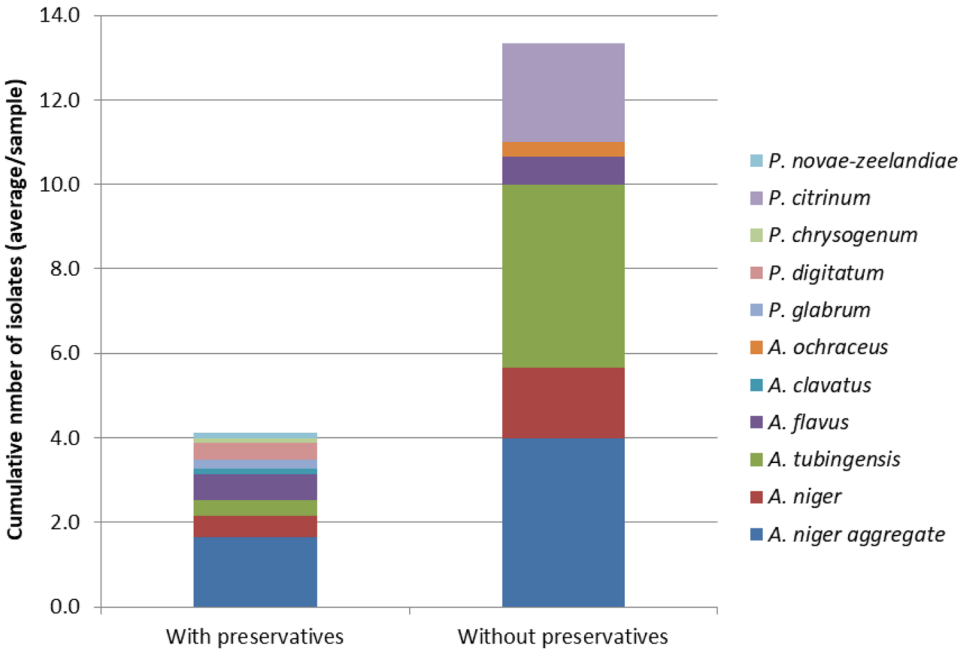

Fig. 1 Cumulative number of isolates of each fungal species, in average, in dried dates with and without preservatives

Han et al. (2016) revealed that 22.5% of date samples were contaminated with OTA with levels as high as 213 µg/kg, while Abdallah et al. (2018) detected OTA in 3 samples (11%) with a contamination ranging from as low as 1.5 µg/kg to the extremely high level of 6070 µg/kg, suggesting that the OTA-producing fungi, in particular *Aspergillus* species, are able to produce high amounts of OTA in these dried fruits. The low percentage and level of OTA contamination in date samples observed in the current study may be explained with the low contamination rate with mycotoxigenic fungi or can be a result of the use of chemical preservatives.

Recently, EFSA estimated that the mean daily dietary exposure (ng/kg bw per day) of the European populations to OTA varies between 1.88 and 5.72 ng/kg bw per day (lower bound) and between 3.81 and 12.33 ng/kg bw per day (upper bound) EFSA 2020). Dried fruits are among the groups of foods with the highest contribution to this exposure, and dates are third in the list, only after dried figs and vine fruits (EFSA 2020). Considering the low OTA contamination levels (in both occurrence and amount) of the analysed samples, and the apparently low consumption of this fruit by the Portuguese population (FAOSTAT 2021; Ribeiro and Fernandes 2017), the exposure to OTA seems to be negligible. Even so, the importation and consumption of dried dates are steadily increasing.in Portugal (FAOSTAT 2021).

In conclusion, commercial dried date fruits from Portuguese markets with different origins and brands seem to be safe in terms of ochratoxigenic fungi and OTA contamination. These results can be due to the strict regulations set for the EU markets, which reduce the probability of highly contaminated samples entering the markets. The highest incidence of fungal contamination was observed for extra-quality or biological products, without chemical preservatives. Even though the high fungal contamination did not generally reflect in detectable OTA contamination, this can be due to the fact that only one OTA-producing fungus was detected. In this respect, it will be necessary to further study the influence of the chemical preservatives on fungal growth and OTA production in dates and to develop biological strategies to reduce the need for these chemical additives.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1007/s12550-021-00432-0.

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